ROLE OF HYDROXYCINNAMIC ACIDS ON THE GENERATION OF
MAILLARD-TYPE AROMA COMPOUNDS IN WHOLE GRAIN WHEAT BREAD

A Dissertation in
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by
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Abstract

The non-enzymatic reaction between sugars and amino acids, also known as the Maillard reaction, affects many aspects of food quality, including color, taste and aroma formation, nutritional value, and toxicity. While many parameters such as pH, temperature, and water activity/content can affect compounds formed by the Maillard reaction, the effect of phenolic compounds is a relatively recent discovery. Specifically, one class of phenolic compounds, hydroxycinnamic acids (HCAs), the predominant type of phenolic compounds in wheat, were further studied to determine their effects on aroma production in various bread model systems using gas chromatography-olfactometry-mass spectrometry (GC-O-MS), liquid chromatography-tandem mass spectrometry (LC-MS/MS), and sensory techniques.

Simple aqueous Maillard model systems were conducted to study the reactivity of HCAs on aroma development. The addition of HCAs to carbonyl-amine Maillard reaction systems showed a suppression in the formation of several key Maillard aroma compounds, such as various pyrazines (pyrazine, methylpyrazine, 2,5-dimethylpyrazine), 2,3-butanedione (diacetyl), and 2-acetyl-1-pyrroline. The mechanisms of this reaction were investigated using isotopically labeled glucose and glycine. In these models, HCAs were found to form adducts with transient Maillard reaction flavor precursors, such as sugar fragments, as well as amino acids and amino acid reaction products.

To further study the effect of HCAs on aroma development, the influence of whole wheat flour, in comparison to refined wheat flour, on bread crust aroma was investigated. Differences in the aroma profile between the two breads were characterized by both comparative gas chromatography-olfactometry-aroma extract dilution analysis (GC-O-
AEDA) and quantitative gas chromatography-mass spectrometry-chemical ionization (GC-MS-CI) analysis utilizing stable isotope surrogate standards. For refined bread crust (versus the whole wheat crust) five compounds were reported to be higher in concentration, 2-acetyl-1-pyrroline, 4-hydroxy-2,5-dimethyl-3(2H)-furanone, 2-phenylethanol, 2-acetyl-2-thiazoline and 2,4-dihydroxy-2,5-dimethyl-3(2H)-furanone by 4.0, 3.0, 2.1, 1.7, and 1.5-fold, respectively; whereas three compounds were at lower concentrations, 2-ethyl-3,5-dimethylpyrazine, (E,E)-2,4-decadienal, and (E)-2-nonenal by 6.1-, 2.1-, and 1.8-fold, respectively. Evaluation of the bread crust by a trained sensory panel reported the perceived aroma intensity of the characteristic ‘fresh refined bread crust’ aroma was significantly higher in the refined bread sample in comparison to the whole wheat sample; however when the five aroma compounds, that were higher in the refined bread crust, were added to the whole wheat crust at equivalent concentrations, no significant differences in the aroma intensity were observed.

Liberation of HCAs from the predominantly insoluble conjugate (bound) form in wheat bran, and their effect on aroma development in bread was further studied through the use of $^{13}$C$_6$-benzene ring labeled ferulic acid. The addition of free ferulic acid to a refined wheat bread system, comparable to the amount released in whole wheat bread after fermentation and baking, resulted in an aroma profile similar to whole wheat bread crust, in that there was a reduction in the generation of five aroma compounds (2-acetyl-1-pyrroline, 2,4-dihydroxy-2,5-dimethyl-3(2H)-furanone, 2-acetyl-2-thiazoline, 2-phenylethanol and 4-hydroxy-2,5-dimethyl-3(2H)-furanone), similar to that observed in whole wheat bread. However, the concentrations of lipid oxidation products ((E)-2-nonenal and (E,E)-2,4-decadienal) remained consistent in concentration to that found in refined bread.
In conclusion, the addition of HCAs to both simple and complex model systems affects the aroma generation in thermally-treated systems. HCAs appear to trap transient sugar fragments and amino acid reaction products, with the largest effect observed for the key bread aroma compound 2-acetyl-1-pyrroline. In addition to HCAs altering aroma development in bread, a second mechanism of aroma generation, related to lipid oxidation, was also found to lead to differences in aroma observed between the two types of bread. This suggests a dual effect of the presence of the entire wheat seed on aroma generation in bread, which can provide new modes to control flavor development in whole wheat bread.
Chapter 1: Literature Review

1.1. Factors that Affect Flavor Perception

1.1.1. Flavor Perception and its Relation to Consumer Acceptability

1.1.2. Olfactory System

1.1.3. Determining Odor Active Compounds

1.1.4. Texture Effects

1.1.5. Taste (Non-Volatile) Effects

1.1.6. Aroma-Aroma Interactions

1.1.7. Phenolic Compound Effects

1.2. Browning Reactions

1.2.1. Importance of Browning Reactions on Product Acceptability

1.2.2. Caramelization

1.2.3. Maillard Reaction

1.2.3.1. Stage One

1.2.3.2. Stage Two

1.2.3.2.1. Parameters Affecting Maillard Reaction Product Production
1.2.3.2.1.1. pH .......................................................... 16
1.2.3.2.1.2. Temperature ........................................ 18
1.2.3.2.1.3. Water .................................................. 19
1.2.3.2.1.4. Reactant Type ..................................... 21
1.2.3.2.1.5. Phenolic Compounds ............................. 22
1.2.3.3. Stage Three .................................................. 25
1.2.3.4. Consequences of the Maillard Reaction on Nutrition ............................... 27
1.2.3.5. Advanced Glycation End-products ............................... 28
1.2.3.6. Creation of Toxic Compounds .............................. 30
1.3. Important Aroma Compounds in Various Types and Forms of Bread .............. 32
1.3.1. Volatile Classes of Aroma Compounds in Bread ..................................... 32
1.3.2. Determining Odor-Active Compounds ........................................ 33
1.3.3. Important Aroma Compounds Formed in Bread during Baking .................. 36
1.3.3.1. Wheat Bread ................................................ 36
1.3.3.2. Rye Bread .................................................. 39
1.3.4. Other Influences on Bread Aroma ........................................ 41
1.3.4.1. Ingredients .................................................. 41
1.3.4.1.1. Raw Flour .............................................. 41
1.3.4.1.2. Yeast ..................................................... 43
1.3.4.2. Flavor Changes after Baking ................................ 44
1.3.4.2.1. Staling ..................................................... 44
1.3.4.2.2. Toasting ................................................ 47
1.4. 2-Acetyl-1-pyrroline: Key Bread Aroma Compound .................................. 47
<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.4.1. Sources of 2-Acetyl-1-pyrroline</td>
<td>47</td>
</tr>
<tr>
<td>1.4.2. 2-Acetyl-1-pyrroline Formation</td>
<td>49</td>
</tr>
<tr>
<td>1.5. Hypothesis and Objectives</td>
<td>53</td>
</tr>
<tr>
<td>1.6. References</td>
<td>53</td>
</tr>
<tr>
<td>CHAPTER 2: HYDROXYCINNAMIC ACID – MAillard REACTIONS IN SIMPLE AQUEOUS MODEL SYSTEMS</td>
<td>67</td>
</tr>
<tr>
<td>2.1. Abstract</td>
<td>67</td>
</tr>
<tr>
<td>2.2. Introduction</td>
<td>67</td>
</tr>
<tr>
<td>2.3. Materials and Methods</td>
<td>68</td>
</tr>
<tr>
<td>2.3.1. Chemicals</td>
<td>68</td>
</tr>
<tr>
<td>2.3.2. Model Reaction Systems</td>
<td>69</td>
</tr>
<tr>
<td>2.3.3. Gas Chromatography/Mass Spectrometry-Electron Impact Analysis (GC/MS-EI)</td>
<td>69</td>
</tr>
<tr>
<td>2.3.4. Liquid Chromatography/Mass Spectrometry-Electrospray Ionization Analysis (LC/MS-ESI)</td>
<td>70</td>
</tr>
<tr>
<td>2.4. Results and Discussion</td>
<td>71</td>
</tr>
<tr>
<td>2.5. References</td>
<td>80</td>
</tr>
<tr>
<td>CHAPTER 3: INFLUENCE OF ENDOGENOUS FERULIC ACID IN WHOLE WHEAT FLOUR ON BREAD CRUST AROMA</td>
<td>82</td>
</tr>
<tr>
<td>3.1. Abstract</td>
<td>82</td>
</tr>
<tr>
<td>3.2. Introduction</td>
<td>83</td>
</tr>
</tbody>
</table>
3.3. Materials and Methods

3.3.1. Chemicals
3.3.2. Wheat Samples
3.3.3. Bread Making
3.3.4. Preparation of Bread Crust Extracts
3.3.5. Gas Chromatography-Olfactometry-Mass Spectrometry (GC-O-MS): Aroma Extract Dilution Analysis (AEDA)
3.3.6. Hydroxycinnamic Acid Analysis
3.3.7. Quantification of Ferulic Acid (FA) Liberated in Whole Wheat Bread Crust during Manufacture
3.3.8. Refined (RWF) Bread Made with Flour Spiked with FA
3.3.9. Quantification of Aroma Compounds
3.3.10. Sensory Evaluation

3.4. Results and Discussion

3.5. Acknowledgements

3.6. References

CHAPTER 4: CONCLUSIONS

4.1. Conclusions from Simple Model Systems and Isotopic Labeling Experiments
4.2. Conclusions on the Effect of Hydroxycinnamic Acids Present in Whole Wheat Flour on Bread Crust Aroma
4.3. Conclusions on Ferulic Acid Reactivity on Aroma Generation in Bread
CHAPTER 5: SUGGESTED FUTURE WORK ................................................................. 113

5.1. Enhancing Stability of 2-Acetyl-1-pyrroline ............................................. 113
5.2. Effect and Modulation of Lipid Oxidation Products ..................................... 114
5.3. Effect of Aroma Compounds with Low Flavor Dilution Values .................... 115
5.4. Production Changes to Bread to Improve Flavor ........................................ 115
5.5 References .................................................................................................. 116

APPENDIX A: EFFECT OF DIGESTION AND PHENOLIC COMPOUNDS ON
ACRYLAMIDE AVAILABILITY AND FORMATION ............................................. 117

A.1. Abstract ..................................................................................................... 117
A.2. Introduction .............................................................................................. 118
A.3. Materials and Methods ............................................................................ 119
    A.3.1. Chemicals .......................................................................................... 119
    A.3.2. Extraction Procedures ..................................................................... 120
    A.3.3. Model Systems ............................................................................... 121
    A.3.4. Liquid Chromatography-Tandem Mass Spectrometry (LC-MS/MS) ... 121
A.4. Results and Discussion ............................................................................. 122
A.5. References ................................................................................................ 129

APPENDIX B: ADDITIONAL METHODS FOR AROMA CHARACTERIZATION OF
REFINED AND WHOLE WHEAT BREAD CRUST ........................................... 133

B.1. Overview of Procedure Optimization ....................................................... 133
B.2. Aroma Extraction Methods ...................................................................... 133
B.2.1. Solvent Extraction ........................................................................................................... 134

B.2.1.1. Soxhlet Extraction (continuous) ................................................................. 136

B.2.1.1.1. Long extraction time ................................................................................. 137

B.2.1.1.2. Short extraction time ............................................................................... 137

B.2.1.1.3. Effect of liquid nitrogen .......................................................................... 137

B.2.1.2. Simple Extraction ......................................................................................... 138

B.2.1.2.1. Water (ether extractions) .......................................................................... 138

B.2.1.2.2. No water (dichloromethane extractions) ..................................................... 139

B.2.2. Headspace sampling ......................................................................................... 139

B.2.2.1. Static Headspace Sampling (SHS) ............................................................... 140

B.2.2.1.1. Direct air injection .................................................................................... 141

B.2.2.1.2. Solid Phase MicroExtraction (SPME) ......................................................... 141

B.2.2.1.3. Stir Bar Sorptive Extractions (SBSE, Twister®) .......................................... 141

B.2.2.2. Dynamic Headspace Sampling (DHS): Purge and Trap ................................ 142

B.2.2.2.1. Flow sampling pump ................................................................................ 142

B.2.2.2.2. Simple purge and flow device ................................................................... 142

B.2.2.2.3. Dynamic thermal stripper system ............................................................. 143

B.2.2.2.4. Direct crust sampling ............................................................................... 143

B.3. Adjustments to Formulations .................................................................................. 144

B.3.1. Yeast .................................................................................................................... 144

B.3.2. Moisture effects ................................................................................................. 144

B.3.3. Fat effects ........................................................................................................... 144

B.4. References .............................................................................................................. 145
List of Figures

Figure 1-1. Cascade of events leading to aroma detection. OBP = olfactory binding protein; OR = odor receptor; GDP = guanosine diphosphate (reproduced from (2)).

Figure 1-2. Examples of sugar degradation (adapted from (38)).

Figure 1-3. Generalized cascade of events of the Maillard Reaction (reproduced from (39)).

Figure 1-4. Amino acids and their corresponding Strecker aldehydes.

Figure 1-5. Decomposition of compounds in the Maillard reaction as a function of pH (adapted from (40)).

Figure 1-6. Chemical structures of common hydroxycinnamic acids; ferulic: R₁=OCH₃, R₂=OH, R₃=H; cinnamic: R₁= R₂=R₃=H; p-coumaric: R₁=R₂=H, R₃=OH; caffeic: R₁= R₂=OH, R₃=H; sinapic: R₁= R₂=R₃=OCH₃, R₂=OH.

Figure 1-7. Structure of colored compound 5(S)-(2-carboxy-1-pyrrolidinyl)-2-hydroxy-(E,E)-2,4-pentadienal-(S)-(2-carboxypyrrolidine)imine (adapted from (83)).

Figure 1-8. Gas chromatography-olfactometry (GCO) schematic (adapted from (113)).

Figure 1-9. Key bread aroma compounds.

Figure 1-10. 2-Acetyl-1-pyrroline formation (adapted from (40)).

Figure 2-1. Chemical structures of select hydroxycinnamic acids; ferulic: R₁=OCH₃, R₂=OH, R₃=H; cinnamic: R₁= R₂=R₃=H; p-coumaric: R₁=R₂=H, R₃=OH; caffeic: R₁= R₂=OH, R₃=H.

Figure 2-2. Concentrations of key volatiles generated in aqueous model systems, 125 °C, pH 7; left to right, Model A (control): glucose + glycine, Model B (treatment): glucose + glycine + phenolic acid (ferulic , cinnamic , p-coumaric , caffeic acid , respectively); Phenolic acid treatments marked with an asterisk are significantly different from the control (α = 0.05).

Figure 2-3. LC/MS-ESI (-ve) Spectrum of select analyte m/z 209[M-1]⁻ generated in aqueous model systems, 125 °C, pH 7: (i) Model A, glucose + glycine, (ii) Model B, glucose + glycine + ferulic acid, (iii) Model C, CAMOLA: ¹³C₆, ¹⁵N, ¹²C₆ glucose + glycine + ferulic acid, (iv) Model D, AAMOLA: glucose + ¹³C₂, ¹⁵N, ¹²C₂, ¹⁴N glycine + ferulic acid; all at equivalent retention time.

Figure 2-4. LC/MS-ESI (-ve) chromatogram of a 4-vinylguaiacol-glycolaldehyde adduct (m/z 209[M-1]⁻) generated in aqueous model systems, 125 °C, pH 7: (i) Model F, glyoxal +
glycine + ferulic acid; (ii) Model F: glycolaldehyde + glycine + ferulic acid; (iii) Model B. glucose + glycine + ferulic acid.

Figure 2-5. Proposed formation mechanism of the analyte identified in Figures 2-3 and 2-4 with the predicted molecular weight of 210 Da.

Figure 2-6. LC/MS-ESI (+ve) Spectrum of select analyte m/z 428[M+1]^+ generated in aqueous model systems, 125 °C, pH 7: (i) Model A. glucose + glycine, (ii) Model B. glucose + glycine + ferulic acid, (iii) Model C. CAMOLA: $^{13}$C$_6$: $^{12}$C$_6$ glucose + glycine + ferulic acid, (iv) Model D. AAMOLA: glucose + $^{13}$C$_2$, $^{15}$N : $^{12}$C$_2$, $^{14}$N glycine + ferulic acid; all at equivalent retention time.

Figure 2-7. LC/MS-ESI (+ve) chromatogram of a 4-vinylguaiacol-methylglyoxal adduct (m/z 428[M+1]^+) generated in aqueous model systems, 125 °C, pH 7: (i) Model F. hydroxyacetone + glycine + ferulic acid; (ii) Model F. methylglyoxal + glycine + ferulic acid; (iii) Model B. glucose + glycine + ferulic acid.

Figure 3-1. Structures of labeled aroma compounds (● / d: deuterium; ■ / c: carbon-13); 2-methylpropanal (2MP, d-1); 2-acetyl-1-pyrroline (2AP, d-2); 2-ethyl-3,5-dimethylpyrazine (EDMP, d-3); methional (d-4); (E)-2-nonenal (d-5); 2,4-dihydroxy-2,5-dimethyl-3(2H)-furanone (DHDMA, c-6); 2-acetyl-2-thiazoline (2A2T, d-7); (E,E)-2,4-decadienal (d-8); 2-phenylethanol (2PE, d-9); 4-hydroxy-2,5-dimethyl-3(2H)-furanone (HDMF, d-10).

Figure 3-2. Mean intensity rating scores for “fresh refined bread crust” aroma in three bread crusts: (1) refined wheat (RWF), (2) whole wheat (WWF) with aroma compounds (2-acetyl-1-pyrroline (2AP), 2,4-dihydroxy-2,5-dimethyl-3(2H)-furanone (DHDMA), 2-acetyl-2-thiazoline (2A2T), 2-phenylethanol (2PE), 4-hydroxy-2,5-dimethyl-3(2H)-furanone (HDMF)), and (3) whole wheat (WWF); a Samples with the same letter are not significantly different (α = 0.05).

Figure A-1. LC-MS/MS-ESI (+ve ion mode) chromatogram of acrylamide and $^{13}$C$_3$-acrylamide (internal standard) detected in potato chips by FDA method.

Figure A-2. LC-MS/MS-ESI (+ve ion mode) chromatogram of acrylamide, $^{13}$C$_3$-acrylamide (internal standard), and 3-aminopropionamide (3-APA) detected in potato chips by digestion method.

Figure A-3. LC-MS/MS-ESI (+ve ion mode) chromatogram of acrylamide and $^{13}$C$_3$-acrylamide (internal standard), and 3-aminopropionamide (3-APA) detected in potato chips by partial digestion method (no enzymes).

Figure A-4. LC-MS/MS-ESI (+ve ion mode) chromatogram of acrylamide and $^{13}$C$_3$-acrylamide (internal standard) detected in potato chips by partial digestion method (no enzymes, no pH modifications).
Figure A-5. LC-MS/MS-ESI (+ve ion mode) chromatogram of unlabeled acrylamide, $^{13}$C$_3$-acrylamide (internal standard), and $^{13}$C$_3$,$^{15}$N-acrylamide detected in a $^{13}$C$_6$-glucose + unlabeled asparagine + ferulic acid model reaction (30min at 170 °C, pH 7, 10mM/reactant)

Figure A-6. LC-MS/MS-ESI (+ve ion mode) chromatogram of unlabeled acrylamide, $^{15}$N-acrylamide, $^{13}$C$_3$-acrylamide (internal standard), and $^{13}$C$_3$,$^{15}$N-acrylamide detected in an unlabeled glucose + $^{13}$C$_4$,$^{15}$N$_2$-asparagine + ferulic acid model reaction (30min at 170 °C, pH 7, 10mM/reactant)

Figure B-1. Solvent Assisted Flavor Evaporation apparatus (SAFE; adapted from (1))

Figure B-2. Soxhlet apparatus (adapted from (2))

Figure B-3. Simple cryofocusing system (adapted from (8))

Figure B-4. Water-jacketed purge and trap container (adapted from (9))
List of Tables

Table 1-1. Sources of 2-acetyl-1-pyrroline (adapted from source (135)) ........................................ 48

Table 2-1. Model system compositions .................................................................................................................. 69

Table 3-1. GC/MS-CI quantification parameters for select aroma compounds .......................................................... 91

Table 3-2. Odorants with FD-factor ≥ 16 and difference ≥ 2 between refined (RWF) and whole wheat (WWF) bread crust ................................................................................................................................. 98

Table 3-3. Quantification of select aroma compounds in refined (RWF), whole wheat (WWF), and refined (RWF) with spiked ferulic acid bread crust samples ............................................................................ 99

Table 3-4. Aroma recombination model: Whole wheat (WWF) bread crust with equivalent aroma concentration of five select compounds in comparison to refined wheat (RWF) bread crust ........................................................................................................................................................ 101

Table 3-5. Concentration of bound, soluble conjugate, and free ferulic acid (FA) in refined (RWF) and whole wheat (WWF) flour ......................................................................................................................... 104

Table A-1. Influence of digestion on detected acrylamide concentration in potato chips ......................................................................................................................................................................................... 123

Table A-2. Acrylamide generation in model systems .................................................................................................. 127
### List of Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>2AP</td>
<td>2-Acetyl-1-pyrroline</td>
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<td>3-APA</td>
<td>3-Aminopropionamide</td>
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</tr>
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<td>2-Phenylethanol</td>
</tr>
<tr>
<td>AAMOLA</td>
<td>Amino Acid Module Labeling</td>
</tr>
<tr>
<td>AEDA</td>
<td>Aroma Extract Dilution Analysis</td>
</tr>
<tr>
<td>AGEs</td>
<td>Advanced Glycation End-products</td>
</tr>
<tr>
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<td>6-Acetyl tetrahydropyridine (isomers of 6-acetyl-1,2,3,4-tetrahydropyridine and 6-acetyl-2,3,4,5-tetrahydropyridine)</td>
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<tr>
<td>A_w</td>
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</tr>
<tr>
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</tr>
<tr>
<td>--------------</td>
<td>-------------</td>
</tr>
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</tr>
<tr>
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<tr>
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</tr>
<tr>
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<td>Heterocyclic Aromatic Amine</td>
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<td>Hydroxycinnamic Acid(s)</td>
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<td>4-Hydroxy-2,5-dimethyl-3(2H)-furanone (furaneol)</td>
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<td>Lactic Acid Bacteria</td>
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<td>LC</td>
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</tr>
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<td>Multiple Reaction Monitoring</td>
</tr>
<tr>
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</tr>
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<td>Tandem Mass Spectrometry</td>
</tr>
<tr>
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</tr>
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</tr>
<tr>
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</tr>
<tr>
<td>--------------</td>
<td>------------------------------------</td>
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</tr>
<tr>
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</tr>
<tr>
<td>WWF</td>
<td>Whole Wheat Flour</td>
</tr>
</tbody>
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Acknowledgements

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CHAPTER 1: LITERATURE REVIEW

1.1. Factors that Affect Flavor Perception

1.1.1. Flavor Perception and its Relation to Consumer Acceptability

Flavor is a key quality that determines acceptance of foodstuffs by consumers. Flavor typically refers to the combined response to taste (non-volatile) and aroma (volatile) compounds that a person has to a food, and is one of the most important determinants in which foods a consumer will purchase. However all foodstuffs are dynamic systems, and the perception of food items is ultimately a result of the integration of numerous sensory inputs. The taste and aroma of a food can be affected by several factors, not only through cooking and storage, but also based on how a food looks, feels and even sounds as one chews. These factors can all play a role in how flavor is perceived, and therefore affect consumer acceptability. Bread is certainly no exception. By simply changing which part of the wheat seed is included in the formulation, it can affect color (white vs. brown), texture (level of chewiness), taste (sweet vs. bitter) and aroma (bready, sweet vs. earthy, grassy). Understanding how all these inputs can ultimately affect the aroma of a food is important to producing products that consumers will want to purchase.

1.1.2. Olfactory System

An organism’s sense of smell allows for the detection of a large diversity of odorants. Aroma, or volatile, detection can come from two sources, orthonasal and retronasal methods, the former coming from when a food is smelled by the nose, prior to chewing in the mouth, which results in the latter. Aroma detection is structured so that an odorant must first bind to an olfactory (odorant) binding protein (OBP) in order to result in
an electrical impulse that can be interpreted by the brain. After binding to the OBP, the odorant must reach the olfactory receptor (OR), a G-protein-coupled receptor (1,2). Upon activation, an enzymatically-catalyzed cascade of events occurs which leads to an influx of calcium ions that depolarizes the neuron and produces an electrical nerve impulse that is relayed through the olfactory bulb and, then, onto to the brain for processing (Figure 1-1) (2). Drugs that act as calcium-channel blockers (calcium antagonists), typically used to control high blood pressure, have the additional side-effect of blocking aroma detection in the nose (3). Although there is variety among the OR proteins, odor identification most likely comes from a unique pattern of OR activation, as well as binding strength, as ORs seem to be able to recognize multiple “elements” of an odorant molecule (4).

Figure 1-1. Cascade of events leading to aroma detection. OBP = olfactory binding protein; OR = odor receptor; GDP = guanosine diphosphate (reproduced from (2))
1.1.3. Determining Odor Active Compounds

Foods contain tens to hundreds of volatile compounds (5,6). The sheer volume of compounds can be daunting to study in a food, so a variety of methods have historically been employed that can identify the compounds that have the most influence on the overall aroma of a food. Though compounds are present at various quantities, it is the strength of that odor (i.e. how intensely it is perceived by a person), rather than its absolute amount, that is considered to be the most important to the overall aroma (7). Even if a particular compound is present in large amounts, if it is not present above its sensory threshold, it is not expected to be as important to the overall aroma. The higher the amount a compound is present above its sensory threshold, the more it is considered to contribute to the overall aroma of a food. These methods are discussed further in Section 1.3.2.

However, some have noticed that even those compounds with low odor activity values (OAVs) seem to affect the overall aroma of different foods. OAVs are based on the ratio of odor concentration to that compound’s threshold. Escudero et al., (8) found that while studying wine aroma, omitting single compounds from a model containing aromas with the highest OAVs did not lead to any compound as having an important effect on the overall aroma. The authors found that including an additional compound, 4-methyl-4-mercaptopentan-2-one, which had a low OAV, changed the odor quality of the model to make it even more like the original wine. The addition of this compound resulted in a fruitier-type model that was less chemical-like, even though the compound on its own is described as “box tree” in aroma.
1.1.4. Texture Effects

Matrix interactions in foods may change the perception of an aroma. Texture relates to more physical descriptors used in foods such as hardness, cohesiveness, viscosity (flow), and springiness, as well as refer to particle size and shape, in addition to moisture and fat content (9). By simply changing the viscosity of a food, one can affect the strength of the aroma perceived while eating, even if the concentration of volatiles remains the same.

Visschers et al., (10) found that the intensity of a strawberry aroma decreased upon ingestion of a firmer food, such as a gel, as compared to plain water. Similar results are found in model beverages that differ in viscosity (11).

The effect of a food’s texture on perceived aroma has been studied in dairy products; however, some of the observations are not as apparent as in the aforementioned model studies (12). Aroma release in dairy foods is dependent on two main factors, as interaction with proteins, and especially fat, can decrease aroma release (13), and increased viscosity can physically hinder the perception of aroma compounds (10,14). Some studies found that yogurt thickness did not affect flavor intensity (15,16), and the thicker yogurt was preferred for two out of three flavors (15). The opposite result was found in low-fat yogurt, where increased thickness correlated to decreased perception of aroma (14,17). Milk proteins can lead to hydrophobic interactions with aroma compounds, therefore dairy products with higher protein levels were found to retain more aroma compounds (13).

1.1.5. Taste (Non-Volatile) Effects

Taste is known to affect aroma perception. Non-volatile compounds have been shown to enhance or suppress the perceived intensity of different aroma compounds (18).
Roberts et al., (11) found that in sucrose-water beverages, volatility depended on flavor polarity, in that, as sucrose concentration increased, volatility of non-polar compounds (α-pinene (woody), 1,8-cineole (camphor-like), ethyl 2-methylbutyrate (fruity, green)) decreased. Bonnans and Noble (19) showed that sweet (both natural and artificial sweeteners) and sour solutions enhanced fruity aroma perception in beverages. Davidson et al., (20) found that perceived mint flavor in gum followed sucrose, rather than menthone (mint aroma compound), release. Even though levels of menthone remained high, perceived mint flavor tapered off the longer the gum was chewed, as all the sucrose was spent. Additionally, studies have been conducted in the opposite direction, looking at the effect of certain aromas on enhancing taste properties. Stevenson et al., (21) found that sweet-smelling aroma compounds, such as caramel and strawberry, enhanced sweetness ratings, and suppressed sourness ratings. Increasing salt can lead to a commonly referred-to phenomenon known as “salting out” which results in increased volatility of aroma compounds. This has been demonstrated in wine, in that when a variety of salts were added to a wine, the amount of volatile components increased, with increasing salt concentration (22).

Taste and odor have been shown to interact with one another to result in different perceived intensity than when presented alone. Delwiche and Heffelfinger (23) showed that mixing subthreshold levels of taste and odor compounds resulted in panelists detecting both components of the mixture. The authors noted that this occurred for taste-odor pairings that were typical (sweet-fruity) and atypical (savory-fruity). Labbe et al., (24) showed similar results when looking at the interaction of ethyl butyrate and maltol on the sweetness-enhancing effects on a sucrose solution. The two compounds were chosen due to their
significant differences in the ability to enhance sweetness perception, with the former having the larger effect. When mixed at subthreshold levels, only ethyl butyrate significantly increased the sweetness intensity as compared to maltol.

1.1.6. Aroma-Aroma Interactions

Most aroma research has focused on looking at individual compounds, with very few studies looking at the interaction between aroma compounds (25). Escudero et al., (26) noticed that norisoprenoids and dimethyl sulfide enhanced fruity notes in different red wines. Gillan (27) found that odor-odor mixtures of citral (lemon) and anethole (licorice) suppressed the perceived intensity of both compounds, but when sugar or salt was added to the mix, it enhanced perception. However, it was noted that the perceived intensity of any of these mixtures was still less than when the compounds were evaluated individually. Results have been mixed, but generally it has been found that suprathreshold mixtures of aroma compounds appear to suppress the intensity of both aroma compounds, but perithreshold mixtures can have both additive and subadditive results (28,29), and may depend on how similar the structures of the aroma compounds are (30).

1.1.7. Phenolic Compound Effects

Phenolic compounds have also been shown to affect the volatility of different aroma compounds. In wine, this is a particular issue, due to the relatively high concentration of polyphenols, such as flavanols and the pigmented anthocyanins, as well as interactions with wood lignins in oak barrel-aged wines. Catechin, a polyphenol, has been shown to retain isoamyl acetate, ethyl hexanoate and benzaldehyde, as compared to the more polar
compound limonene in model systems \((31)\). NMR spectroscopy indicated that polyphenols and aroma compounds were forming a complex, most likely through hydrophobic interactions \((31)\). Jung et al., \((32)\) also used NMR to demonstrate a similar interaction between phenolic compounds (gallic acid and naringin) with aroma compounds (2-methylpyrazine, vanillin, and ethyl benzoate). They found that structure greatly influenced binding abilities, showing that gallic acid interacted more strongly with the aromatic compounds compared to naringin, and 2-methylpyrazine and vanillin interacted more strongly than ethyl benzoate. Jung et al., \((32)\) attributed this interaction due mainly to the structural characteristics of the compounds, which could affect the formation of hydrogen bonds between the phenolic and aromatic compounds. Fulcrand et al., \((33)\) also found that tannins could form a covalent complex with acetaldehyde, a common oxidation product of ethanol that is also formed as a yeast byproduct during fermentation. The binding of acetaldehyde by tannin led to a decrease in astringency in wine.

1.2. Browning Reactions

1.2.1. Importance of Browning Reactions on Product Acceptability

In addition to how aroma compounds interact with other compounds present in a food, how a food is handled or processed can greatly influence the ultimate acceptability of a food product by a consumer. Such processing techniques most commonly involve enzymatic or thermal changes to the food product. Of particular importance are browning reactions, due to their occurrence in just about every food item, and can have direct effects on consumer acceptability of foods. Browning reactions result from either enzymatic or non-enzymatic means. Enzymatic browning, occurring in damaged plant material, is the
product of polyphenol oxidase (PPO) which oxidizes phenolic compounds to their respective quinones. These quinones can then polymerize to form brown pigments and can lead to loss of product quality, resulting in lower acceptability of such products by consumers (34,35).

Non-enzymatic browning can result in both acceptable and unacceptable flavor development, and is typified by three types of reactions: the Maillard reaction, caramelization, and ascorbic acid browning. The latter process is typically associated only with formation of off-flavor compounds, especially in citrus juices (36). The control of these reactions is crucial to producing products that consumers will want to purchase. For example, Maillard reaction products are responsible for the production of the appealing roasty and savory aroma development in thermally treated foods such as bread, coffee, chocolate, and meat, as well as the unappealing stale or gluey aromas in dehydrated foods, intended to have long shelf-lives, such as skim milk and egg powders (6,37). Additionally, the brown colors formed by the reaction can be both desirable in heat-treated foods, but also lead to rejection in dehydrated products. Understanding the conditions that can affect reaction rates is important to maintaining foodstuffs that are satisfactory to consumers, with the longest shelf life possible.

1.2.2. Caramelization

Caramelization is a reaction that occurs only among sugars, and requires more forcing conditions, such as higher temperature and more acidic or basic pH, as compared to the Maillard reaction, which occurs with amino acids, under most conditions, albeit with widely varying reaction rates. Such an extreme environment involves temperatures over 120°C and pH values of either less than three or greater than nine (38). Foods that are baked
and roasted are the most likely places for this type of reaction to occur, especially in foods containing fruit juice or preserves, or other sweets.

As the name implies, one would expect caramel-like aromas and brown colors to be produced as a result of caramelization. Colors formed by this reaction have been used to color many foods and beverages. The dominant types of aroma compounds include furans (e.g. furfural, hydroxymethylfurfural), furanones (e.g. furaneol), pyrones (e.g. maltol, hydroxymaltol) and carbocyclics (e.g. cyclotene). Flavors produced can range from mild and sweet-type caramel aromas, to more burning and bitter flavors with increased reaction time and greater extremes in temperature and pH during cooking. Acidic pH tends to favor dehydration and cyclization reactions, whereas alkaline conditions favor cleavage of the carbon chain (38). Formation of these compounds requires the initial degradation of the sugar molecule by enolisation (1,2-enolisation at low pH, and 2,3-enolisation at high pH), via the de Bruijn van Eckenstein rearrangement, followed by dehydration, further degradation and reactions (see Figure 1-2 from source (38)). Sugar degradation products, such as α-dicarbonyls, are extremely reactive, and can go on to further catalyze the formation of aroma and colored compounds.
Figure 1-2. Examples of sugar degradation (adapted from (38))
1.2.3. Maillard Reaction

The Maillard reaction is widely regarded as one of the most important reactions leading to flavor and aroma generation in thermally-processed foods. However, the reaction’s effect is more far-reaching than in just food. The Maillard reaction is known to proceed under a wide range of conditions. The initial reactants for the reaction require protein and sugars, or more specifically, amine and carbonyl compounds, the latter typically from reducing sugars, substances that are ubiquitous in all living things. Reaction rates and types of products formed vary considerably in foods, with temperature, pH, water activity and water content, all affecting how fast, and to what degree, the reaction proceeds. As such, even though the Maillard reaction is discussed as a single reaction, it truly resembles more of a cascade pathway with any number of outcomes dependent on a wide variety of reaction conditions.

The Maillard reaction (Figure 1-3) can be divided into three general stages. The first includes condensation of the initial reactants. The second involves forming Amadori or Heyns compounds which can undergo a wide variety of reactions to form sugar fragmentation products, heterocyclic compounds, and reactive α-dicarbonyl compounds that result in the formation of flavor compounds, for which the reaction is well-known. The third stage involves polymerization of these products into higher molecular weight melanoidins that result in the brown color associated with the reaction.
Figure 1-3. Generalized cascade of events of the Maillard Reaction (reproduced from (39))
1.2.3.1. Stage One

The first of three stages in the Maillard reaction involves condensation of the reactants, resulting in the Schiff base, $N$-substituted glycosylamine (or $N$-fructosylamine) structures. This new compound can either revert back to the original reactants, or can irreversibly rearrange to form extremely reactive Amadori (1-amino-1-deoxy-2-ketose) or Heyns (1-amino-2-deoxy-2-aldose) compounds (5,40). The mechanisms of formation are well understood and do not result in aroma or color production at this stage.

1.2.3.2. Stage Two

The second stage of the Maillard reaction refers to the further reaction of Amadori or Heyns compounds. One such pathway would lead to the loss of an amino group and dehydration to form furfural, from pentoses, and hydroxymethylfurfural (HMF), from hexoses (5). Additionally, Amadori and Heyns compounds can undergo further rearrangement, leading to a complex cascade of flavor development involving sugar degradation and fragmentation (fission), primarily by retroaldol reactions, to form furans, furanones, pyranones, and $\alpha$-dicarbonyls (41). Other reactions that occur include amino acid degradation, condensation, dehydration, enolisation, and Strecker degradation, to form numerous small and reactive molecules.

Aroma compounds are some of the major products of this stage of the reaction, and they typically fall into three categories: sugar degradation products, amino acid degradation products, and compounds formed from the reaction between those two groups (42). One class of compounds formed during this stage includes highly reactive $\alpha$-dicarbonyl compounds such as 2,3-butanedione (diacetyl), glyoxal, and 2-oxopropanal (methylglyoxal),
which are important odorants (i.e. diacetyl) in their own right, and/or can help catalyze the formation of other flavor compounds \((5,43)\). For example, they can help initiate the formation of Strecker aldehydes, which contain one fewer carbon atom than the corresponding amino acid. Some common Strecker aldehydes found in foods as aroma compounds and their corresponding amino acids are shown in Figure 1-4. The amino acids proline and ornithine form the unstable Strecker aldehyde 4-aminobutanal, that rapidly cyclizes and dehydrates to 1-pyrroline. The latter compound tends to form other volatiles (e.g. 2-acetyl-1-pyrroline), rather than be present itself in foods, unlike other Strecker aldehydes \((44)\). Additionally, \(\alpha\)-dicarbonyl compounds can interact with reaction products of Strecker degradation, such as ammonia and hydrogen sulfide to produce other flavor compounds \((5,40)\).
<table>
<thead>
<tr>
<th>Amino Acid</th>
<th>Corresponding Strecker Aldehyde</th>
</tr>
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<tbody>
<tr>
<td>Valine</td>
<td>2-methylpropanal</td>
</tr>
<tr>
<td>Leucine</td>
<td>3-methylbutanal</td>
</tr>
<tr>
<td>Isoleucine</td>
<td>2-methylbutanal</td>
</tr>
<tr>
<td>Methionine</td>
<td>Methional</td>
</tr>
<tr>
<td>Proline</td>
<td>4-aminobutanal/1-pyrroline</td>
</tr>
<tr>
<td>Phenylalanine</td>
<td>Phenylacetaldehyde</td>
</tr>
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</table>

**Figure 1-4.** Amino acids and their corresponding Strecker aldehydes
1.2.3.2.1. Parameters Affecting Maillard Reaction Product Production

1.2.3.2.1.1. pH

The pH of a system can affect the reaction rate of the Maillard reaction, with faster reaction rates occurring at higher pH (45). Amino groups are not protonated at pH values above their pKa, which makes them more reactive as nucleophiles (46,47). Leahy and Reineccius (45), as well as Shibamoto and Bernhard (48), demonstrated that higher pH correlates with increased pyrazine formation.

The degradation of Amadori compounds is also extremely pH-dependent (Figure 1-5). Under acidic conditions (pH < 7), 1,2-enolisation is favored, and after the loss of the amine group, 3-deoxyosones (3-deoxy-1,2-dicarbonyls) are formed, which further undergo dehydration and cyclization to form furfural (from pentoses) and hydroxymethylfurfural (HMF; from hexoses) (47). At higher pH, 2,3-enolisation occurs, resulting in the formation of 1-deoxyosone (1-deoxy-2,3-dicarbonyl), after loss of the amine group. Cyclization of this compound results in the formation of furanone and pyranone compounds, which can fragment further via retro-aldol reactions to form reactive α-dicarbonyl compounds (40).

As higher pH correlates with increased reaction rates, there also appears to be higher rates of aroma formation in model systems. Blank et al. (49) observed increased formation of 2-acetyl-1-pyrroline (2AP) at pH 7 and 8, compared to pH 6. Additionally, formation of Amadori compounds proceeded at a faster rate with increased pH (49), as well as degrading more rapidly (49,50).
Figure 1-5. Decomposition of compounds in the Maillard reaction as a function of pH (adapted from (40))
1.2.3.2.1.2. Temperature

An increase in temperature increases the rate of the Maillard reaction (46). The rate of color development also increases with temperature. Similar effects are observed with volatile development (51), for example, the rate of pyrazine formation increased with temperature (45). Additionally, in bread, the outer crust portion, which is subjected to higher temperatures, forms more aroma compounds, and in higher amounts, compared to the comparatively cooler, inner crumb portion (52–54).

Tressl et al. (55) investigated the effect of temperature on proline-glucose model systems. Proline is an important amino acid for the production of flavor compounds in bread and beer. It was noted that upon reacting proline with reducing sugars at 100°C, very few compounds were formed, even after an extended period of two hours. Malt production typically uses higher temperatures (140°C) to form desirable aroma compounds, and there were only two compounds detected at the lower temperature that were also found in the beer extractions (55). The lower temperature favored the formation of products that included six carbon atoms, assumed to be derived from the same molecule of glucose, as fragmentation of sugars was not favored under those conditions. Increasing the reaction temperature further to 150°C increased the concentration of all compounds. Sugar fragmentation can lead to the formation of α-dicarbonyl compounds, which were noted to be more reactive than monosaccharides, resulting in a thousand-fold increase in the concentration of products (55).

Even with the same starting materials, temperature can change the aroma profile produced. Lane and Nursten (56) noticed that aroma in model systems started to become aromatically detectable at 100°C, and only became more intense and eventually harsh and
unpleasant (due to development of burnt characteristics), as temperature increased, especially above 180°C. In addition, certain aroma descriptors, such as crusty/bread-like (proline, lysine systems), meaty/beefy (cysteine, threonine systems), and cocoa (serine, tyrosine systems), attributed to the respective reaction mixtures tended to be consistent throughout that range of 100-180°C, though additional descriptors to the system emerged with increased temperature. For example, in a glucose-cysteine system, the reaction mixture began with an aroma resembling puffed wheat, after which it was described as crusty and burnt and evolving to burnt bread and over-roasted meat.

Stahl and Parliment (57) performed time course experiments on proline-glucose model systems, using high temperature (160-220°C), short time (0.25-5 min) experiments. They calculated activation energy to measure the kinetics of formation for three compounds, 6-acetyltetrahydropyridine (ATHP), maltoxazine, and 5-acetyl-2,3-dihydro-1(H)-pyrrolizine (5-ADP). Low concentrations for the first two compounds were observed, and their activation energies were calculated at about 6-15 kcal/mole. In contrast, 5-ADP was found to increase more rapidly with increasing temperature, with an activation energy at about 45 kcal/mole. These observations were in line with Leahy and Reineccius (45), who found that the higher the activation energy for the formation of a compound, the more its formation is favored at higher temperatures, as well as conversely the favored formation of compounds with low activation energies at lower temperatures.

1.2.3.2.1.3. Water

Water activity (A_w) and content can also affect the rate of the Maillard reaction. The higher the water activity, typically the lower the reaction rate will be, due to dilution of
reactants. However, if the system’s water activity is extremely low, the reactants cannot efficiently mix. The optimum $A_w$ has been listed in the range of 0.65-0.75 (40) or 0.5-0.8 (43). Several steps in the Maillard reaction result in the formation of water, which can further dilute reactants, and may explain why slightly concentrating the initial reactants leads to higher reaction rates.

Simple amino acid-sugar (fragment) model systems have been run predominantly at opposite extremes, where water makes up most (aqueous systems), or very little (dry or roast systems) of the reaction medium. The aqueous systems allow for studying the Maillard reaction at different temperatures, with the use of pressure, for example through the use of autoclaves (41,55) or smaller scale pressure reactors (58). The studies that limited the amount of water in the system to low levels have been predominantly studied at elevated temperatures, mimicking roast conditions. However, dry systems have also been useful in studying the Maillard reaction under storage conditions, at much lower temperatures as compared to roasting (59).

Shu and Ho (60) looked at the effect of moisture, using a cysteine-furaneol model system. This system produced cooked meat flavors and when the moisture was changed, they found the best, most “balanced” cooked meat aromas at 75% moisture. Too low moisture led to mostly “biting” smells, whereas the opposite lead to not only cooked meat aromas, but also burnt aromas. They also measured the amount of total volatiles produced, and found the highest amounts at 75% moisture levels. However water content affects different compounds differently; thiazole concentration decreased with increasing moisture, whereas most of the other compounds (trithiolanes and thiophenones) analyzed showed highest concentration at 75% moisture.
Additional studies have found more aroma generation at intermediate moisture levels. Adams et al., (61) looked at the formation of ATHP under both dry/roast and more dilute conditions. The authors reacted proline with 1,3-dihydroxyacetone, the latter a sugar degradation product. The drier conditions led to the formation of ATHP, and although present in low yield, there were very few side products formed. Running the same experiment, but under aqueous conditions, led again to low ATHP formation, but this time, a greater formation of additional products occurred. Lu et al., (62) studied the effect of water concentration on the reaction of glucose with glycine, diglycine and triglycine, using glycerol as a dilution medium. Volatile generation increased as water decreased, however the highest formation was not at 0% moisture, probably due to the highly viscous environment. However, when Hwang et al., (63) looked at pyrazine formation in dry and aqueous systems between the reaction of glucose and glutamine, pyrazine yields were higher in the drier system than aqueous, which demonstrates the wide variety of outcomes that can occur in the Maillard reaction, depending on what products are being studied.

1.2.3.2.1.4. Reactant Type

Although the Maillard reaction is typified by the initial reaction of proteins with sugars, this truly encompasses a wide variety of compounds. Although one half of the starting materials usually denotes sugars, in reality, it is the presence of any compound that contains a carbonyl structure, which most often involves sugars, but can also comprise sugar degradation products, low molecular weight aldehydes and ketones, as well as lipid oxidation products and ascorbic acid (34). The other part of the reaction involves amino acids, however any compound that includes an amine group, such as free amino acids,
peptides, proteins, nucleic acids, and even ammonia, can participate in the reaction. Typically the lower the molecular weight of the reactant, the faster the reaction rate (46). Open chain forms of sugars and the unprotonated forms of amino acids are the more reactive types, which are favored at higher pH (47,64). Additionally, basic amino acids (e.g. lysine) react faster than acidic ones (e.g. glutamic acid) (65).

Different combinations of reactants also affect the kinds of compounds that are produced. For instance, model systems involving ribose and cysteine result in meaty flavors (56), whereas model systems with proline and glucose result in roasty, cracker-like aromas (66). Serine and tyrosine reactions with glucose resemble chocolate aromas (56) and floral notes have been observed upon reaction with phenylalanine.

Lipid oxidation products can also indirectly form aroma compounds, as the carbonyl-containing structures can combine with amines to participate in the Maillard reaction (40,67). This effect is particularly pronounced in the formation of meat flavors, as the removal of phospholipids from beef muscle results in a marked decrease in typical meat flavor upon cooking (68).

1.2.3.2.1.5. Phenolic Compounds

Phenolic compounds have also been shown to directly alter the profile of compounds formed via the Maillard reaction by forming adducts with reactive intermediates (58,69–71). Found in plants, phenolic compounds are secondary metabolites that protect the plant from environmental stressors such as oxidants, microbial infection, and physical damage (35). Increasing epidemiological data has found that consumption of whole grains and other plant-based foods can reduce the risk of several chronic diseases (e.g. cardiovascular disease,
diabetes, cancer), the mechanism of which may be related to phenolic compounds found in those foods (72).

Phenolic compounds encompass a wide variety of chemicals that consist of an aromatic ring with at least one hydroxyl group constituent. Phenolic compounds are further subdivided into different classes, with some of the more common ones as follows: simple phenols, phenolic acids (hydroxybenzoic and hydroxycinnamic acid derivatives), flavonoids, lignins, and tannins. Hydroxycinnamic acids (HCAs) are found primarily associated with cereal grains (73), but are found in many different plant materials (74,75). HCAs consist of a benzene ring with a propenoic (acrylic) acid side chain (Figure 1-6). Differences in the functional groups attached to the benzene ring can greatly affect reactivity, with caffeic acid as the most reactive followed by ferulic acid (76). However, ferulic acid is by far the most predominant HCA found in cereal grains, in particular wheat (73). In nature, HCAs are found in different forms, bound to polysaccharides (soluble conjugates) or more commonly bound to cell wall lignins (insoluble conjugates), forming crosslinks between arabinoxylan chains (73).

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**Figure 1-6.** Chemical structures of common hydroxycinnamic acids; ferulic: \( R_1=OCH_3, \ R_2=OH, \ R_3=H \); cinnamic: \( R_1=R_2=R_3=H \); p-coumaric: \( R_1=R_3=H, \ R_2=OH \); caffeic: \( R_1=R_2=OH, \ R_3=H \); sinapic: \( R_1=R_3=OCH_3, \ R_2=OH \)
Phenolics have been known to reduce the formation of reactive carbonyl compounds produced by lipid oxidation and thermal degradation of sugars. Phenolic compounds were originally studied as antioxidants, and are thought to react via a free radical mechanism (77). Recently, attention has been focused on the ability of phenolics to react with two- and three-carbon, carbonyl-containing sugar fragments (e.g. glyoxal, glycolaldehyde, methylglyoxal, and hydroxyacetone) by ionic mechanisms, forming adducts. Such action would eliminate the ability of these reactive intermediates to react with other compounds to form volatiles and colors. Totlani and Peterson (71) found that the addition of epicatechin to glucose-glycine model systems altered aroma generation. Use of carbon-13 labeled glucose and carbon-13 and nitrogen-15 labeled glycine indicated the formation of new compounds that consisted of epicatechin with either two- and three-carbon sugar fragments, results verified with NMR analysis (78). Analogous results were observed with the addition of HCAs to similarly conducted model systems (58, 69). Unique to HCAs was the ability to also trap amino acid reaction products (58, 69), which differs from flavanols which only trap sugar fragments (71).

Others have shown that the addition of phenolic compounds can affect the aroma profiles of different food systems. Wang (79) reported that the presence of HCAs, such as caffeic, ferulic, and chlorogenic acids, dramatically inhibited formation of aroma compounds in a roast coffee model system. (-)-Epicatechin addition to ultra-high temperature (UHT) treated milk (80) and low-heat skim milk powder (81) changed the aroma profile such that the systems with the added epicatechin lead to statistically decreased cooked and stale flavor intensities respectively. Though phenolic compounds can taste bitter
on their own, when added at these levels, no differences in bitterness intensity were found (80,81).

These findings indicate that the addition of phenolic compounds to processed foods may have an impact on the consumer acceptability of food products. Though the addition can have positive effects on acceptability (80,81), the presence of phenolic compounds may also negatively affect consumer perception of products. For example, the wheat seed consists of an outer protective bran layer that contains phenolic compounds, which is only included in whole grain wheat (bread) formulations. Model systems performed with ferulic acid show changes in the aroma profile (58,69). Consumer tests have shown that consumers will prefer breads made without bran (82). Therefore, it is reasonable to infer that the presence of these phenolic acids in a whole grain wheat bread could be a potential explanation of the changes in the aroma profile compared to that of a refined wheat bread, however this mechanism has not yet been explored in the literature.

Due to the relatively nascent study of phenolic compounds and their effect on the Maillard reaction, especially in more complex food systems, the reactivity of HCAs is explored in greater detail and is the major focus of this thesis. The effect of HCAs on aroma generation in simple aqueous model systems is discussed further in Chapter 2. How HCAs react in a more complex bread system, such as when they are added in the form of whole wheat flour used to make bread, is studied in Chapter 3.

1.2.3.3. Stage Three

The carbonyl and heterocyclic compounds formed during the second stage of the Maillard reaction are reactive and can further combine and polymerize in the reaction’s third
stage, to form the characteristic brown colored compounds, melanoidins (47). However, due to the complex nature of these compounds, there has been little progress on their characterization in the literature.

Browning is considered to be desirable in certain products, such as bread, meat, and coffee, but needs to be controlled in items stored over long periods, such as dehydrated products like nonfat dry milk, eggs, and fruit. Lysine leads to the most color formation, as opposed to cysteine, which results in the least. Foods that contain high concentrations of lysine, such as milk products, are more susceptible to browning, which can lead to shorter shelf-lives (46).

In order to elucidate the structures of the colored compounds, and determine mechanisms of formation, some have followed specific carbohydrate degradation products in forming brown pigments. Hofmann (83) studied the reaction between furan-2-carboxaldehyde and various amino acids in order to identify colored compounds produced upon heating. One compound (5(S)-(2-carboxy-1-pyrroldinyl)-2-hydroxy-(E,E)-2,4-pentadienal-(S)-(2-carboxypyrrolidine)imine; see Figure 1-7) was identified upon reaction with proline. The proposed structure was validated by forming analogous compounds with the structurally-related amine-containing compounds, pyrrolidine and piperidine, leading to the formation of a chromophore consisting of four linked rings with an amino acid moiety. Tressl et al., (84) studied N-substituted pyrroles and 2-formylpyrroles, and observed that polycondensation of these sugar degradation products led to the formation of an oligomer with as many as 15-30 methine-bridged N-substituted pyrroles.
Hofmann (85) used $^{13}$C-labelling experiments to elucidate formation pathways of the pyrano[2,3-b]pyranone chromophore, which was identified as one of the most intense colorants formed during pentose degradation. Synthetic carbohydrate-derived intermediates were also used to determine the ability to form specific chromophores, as determined by quantification experiments. Hydroxyacetaldehyde led to the highest amounts of the pyrano[2,3-b]pyranone chromophore, as compared to other Maillard reaction intermediates used in the study, acetaldehyde and glyoxal. $^{13}$C-labelling experiments also pointed to furan-2-aldehyde and 3-deoxy-2-pentosulose as precursors to this particular chromophore.

1.2.3.4. Consequences of the Maillard Reaction on Nutrition

Due to the nature of the Maillard reaction (i.e. the reaction of carbohydrates and amino acids), the nutritional quality of a food can be affected as certain essential nutrients are consumed during the reaction (86). One well-studied example is the essential amino acid lysine, which is one of the most reactive amino acids in the Maillard reaction due to its ε-amino side chain. Lysine is a limiting amino acid in wheat, so its loss in foods is even
more important. Lysine is present in higher quantities in milk, which is an issue for the storage of dehydrated milk powder (59), as the amino acid can become tightly bound with lactose present in casein to form lactuloselysine, an Amadori product, an effect that is more pronounced with increasing temperatures (34). Similar results can occur in dehydrated egg powder, as lysine can react with glucose, resulting in denaturation and conformational changes in the protein that can also lead it to be resistant to digestion (59). Alonso and Zapico (87) showed that the decrease in lysine in baby food was affected by temperature, but more so based on storage time.

Additional reactions affecting human nutrition can occur in foods as a result of the Maillard reaction. The formation of different types of enzymatic-resistant glycated proteins leads to decreases in protein bioavailability, causing lower protein efficiency rates (PER), and therefore amino acid deficiencies (59). Ascorbic acid (Vitamin C), another essential nutrient, can also participate in the Maillard reaction due to the presence of its carbonyl moiety, and therefore become unavailable for use within the body. All of these changes are especially important in infant formula and for people on restricted diets, as it greatly impacts the quality of the finished food (34).

1.2.3.5. Advanced Glycation End-products

Just as the Maillard reaction can proceed at lower temperatures in foods, the Maillard reaction can also proceed at the lower relative temperatures in the body. Louis Maillard, after which the reaction is named, originally studied the reaction during biological peptide synthesis using different sugars that naturally occur in the human body. Owing to the involvement of amino acids in the Maillard reaction, reaction products can form at the
terminal ends of proteins, leading to alterations. This protein modification via glycation results in compounds known as advanced glycation end-products (AGEs), which can undergo further changes, such as cross-linking, impacting their original function (34,88).

Protein cross-linking is an especially important issue in “long-lived” proteins. Most proteins in the body are replaced at regular intervals, however, “long-lived” proteins are those that are not regenerated, and must last over the lifespan of the organism. Two such examples include collagen and lens crystallins (89,90). The cross-linking of proteins in collagen causes structural changes, decreasing elasticity and increasing the formation of physical barriers that prevents passage of immune cells and nutrients. Similar reactions in lens crystallins result in the formation of yellow chromophores leading to the development of cataracts. Other AGEs, such as carboxymethyllysine, do not form crosslinks, but are believed to also increase lens opacity (91). As a result, these compounds have been used as a marker for the degeneration of proteins, and also as a measure of the cumulative exposure of proteins to glucose in the body (91). However, one limitation in using this compound as a marker is that carboxymethyllysine is also present in foods, and can be absorbed by the body, affecting such measurements (34).

AGEs are related to complications in not only aging, but also diabetes, exacerbated due to the presence of high blood sugar (92). In order to control blood sugar levels, the body produces insulin which helps cells take in sugar. If the body cannot make insulin (Type I), or cells are resistant to insulin (Type II), the sugar stays in the blood. The longer sugar stays in the blood, the more likely it will react with proteins present. Proteins most susceptible to reaction would include non-renewable long-lived proteins, as well as proteins present in the blood, such as hemoglobin, which would affect oxygen transport (88).
1.2.3.6. Creation of Toxic Compounds

Along the lines of the mal-effects the Maillard reaction can have in stored foods, or in the body, toxic or mutagenic compounds can also be formed in cooked foods as a result of the reaction. One example includes acrylamide, which is most notably found in high heat-treated starchy foods, which contain higher amounts of the amino acid asparagine, such as in French fries and potato chips (40,93). Additionally, the Maillard reaction can lead to the formation of heterocyclic aromatic amines (HAA), or imidazoles, which are found in cooked (>150°C) fish and meat products (34,59,86,94). HAAs are only found in animal products, as they require creatine for formation, which is not found in plant foods (95). Though acrylamide and HAAs have long been shown to be potent carcinogens, it has only been relatively recently that acrylamide was found to be present in foods, as opposed to only environmental sources (96).

Detrimental effects due to acrylamide are attributed to its ability to modify proteins. Acrylamide reacts preferentially with SH groups of cysteine, as well as ε-NH₂ group of lysine side chains, but can also react with the N-terminal valine of hemoglobin, which can then act as a biomarker for human exposure to acrylamide (93,97). Acrylamide reacts with tryptophan and tyrosine, leading to the inactivation of enzymes (98,99), as well as non-covalently binding to DNA (100). The amount of acrylamide in the body is controlled by conjugation to glutathione, catalyzed by the enzyme glutathione-S-transferase, so that it can be excreted. If the consumption of sulfur-containing amino acids is low, glutathione concentrations in the body will likewise be low and cannot adequately keep acrylamide levels in check (101). The depletion of glutathione puts cell membranes at risk for other
toxic molecules that may be present. Additionally acrylamide can affect the body neurologically, starting off as tingling or numbness in the hands or feet, and can progress to cerebellar dysfunction through longer exposure (102).

The toxicology of acrylamide is a serious issue for human health, and as a result, much attention has focused on mechanisms of formation as well as determination of acrylamide levels in foods (93). Other extensions of acrylamide research, such as its fate during digestion and ways to prevent its formation through the use of phenolic compounds, have yielded conflicting results in the literature. Both matters are addressed in the study detailed in Appendix A.

Reactive intermediates of the Maillard reaction can also be toxic to humans (103). Methylglyoxal is one of the more heavily studied compounds, as not only is it an intermediate of the Maillard reaction, but it is also involved in glycolysis. Some of the effects methylglyoxal can have in organisms is related to its participation in the formation of AGEs, by reacting with lysine and arginine residues in proteins (34,88,92). Methylglyoxal can change protein structure in different organs, such as kidneys, which can affect their function (104), as well as suppress various enzymes, typically ones related to liver function, especially glutathione-S-transferase. Glutathione has been shown to protect prokaryotic cells from methylglyoxal by forming adducts such as S-D-lactoylglutathione (105).

In addition the role of methylglyoxal in promoting cancer has also been studied; however, there have been conflicting results. Methylglyoxal has been shown to stimulate DNA synthesis (106), which is a marker of tumor promoting activities, but it can also lead to limits of tumor size as too much methylglyoxal leads to apoptosis (103). This discrepancy
has been attributed to the fact that methylglyoxal appears to react only with single stranded DNA, competing for hydrogen bonding with other nucleotides (103).

1.3. Important Aroma Compounds in Various Types and Forms of Bread

1.3.1. Volatile Classes of Aroma Compounds in Bread

Owing to the wide range of reaction pathways within the Maillard reaction, it is not surprising that many thermally processed foods are made up of tens, if not hundreds, of volatile compounds. However, not all volatile compounds are necessarily important to the character of the particular food from which they are found. The use of gas chromatography (GC) helped to tremendously advance aroma research in the 1960s (5,6). Some of the early papers discussing bread aroma listed hundreds of compounds, typically classified by their chemical structure (107–110). The categories containing larger amounts of aroma compounds found in bread include pyrazines, pyrrolines, aldehydes, ketones, alcohols, and furans, and to a lesser extent, esters and sulfur-containing compounds. However, it became clear that identifying all volatile compounds in a food was misleading. For example, early papers that looked at the difference in fresh and stale bread would find little difference in the GC chromatograms. Therefore it was likely that some of the most important aroma compounds were probably present in small amounts, even below limits of detection of the analytical equipment (111). As a result, more sophisticated techniques have been developed to elucidate which compounds have greater impact on flavor.
1.3.2. Determining Odor-Active Compounds

Gas chromatography-olfactometry (GCO, Figure 1-8) has been used to narrow down the long list of volatile components identified in various foods to those that actually have a high character impact to the overall aroma in that food (7,112). A sample extract is injected, and separated into individual compounds on a GC column. The resulting effluent can then be identified using various types of detectors including, but not limited to, mass spectrometry (MS) and flame ionization detection (FID) as the most common detector types. In GCO, the effluent is typically split between a detector and a heated arm, with an opening, where a panelist can situate oneself. The effluent is continuously analyzed, orthonasally, by a panelist, to determine when a response is elicited.

![Figure 1-8. Gas chromatography-olfactometry (GCO) schematic (adapted from (113))](image)

Although this technique indicates which compounds are odor-active, it does not then elucidate which compounds contribute the most (or least) to the overall aroma. Both the
concentration of a compound, and its threshold level in that particular food, are needed to determine relative importance of each aroma compound to that food (114). These two components contribute to an aroma’s impact, or an aroma’s odor activity value (OAV). This concept is also referred to as “aroma value,” “flavour unit,” “odour unit,” and “odour value” in the literature (7,114). The lower the threshold needed to detect a compound, the lower amount of that compound is necessary to elicit a response by a panelist. Oftentimes, the most important compounds are the ones that are present in very low amounts, and are not easily quantified using traditional detectors. However, Frijters (115) noted that the calculation of OAVs cannot be regarded as an absolute measure of importance. Inherent to the concept would be that each compound’s contribution is linearly proportional to the overall aroma. If true, this would assume that the change in intensity of every aroma compound is the same such that equal changes in concentration will result in similar changes in odor intensity for all compounds. Despite this flaw, OAV calculations remain a relevant technique for guiding the direction of further aroma research by identifying those compounds with the potential to affect the aroma profile most.

Some of the earliest procedures published in the literature required the panelist to estimate the intensity of each compound detected during every GCO run. Psychophysical techniques, like cross-modal matching and magnitude estimation, required extensive training in order for panelists to be able to both identify odor-active regions, and at the same time, estimate intensity for a wide variety of compounds in a single run (116). In order improve on the method, two major techniques for elucidating the most odor-active compounds in foods were introduced, including Aroma Extract Dilution Analysis (AEDA) developed by
Ullrich and Grosch \((117)\), and CHARM, developed by Acree et al., \((116)\). The former technique has been cited more frequently in the literature to date.

In AEDA, an extract is serially diluted, often by half, with each dilution being evaluated until no more volatile compounds can be detected by a panelist. The highest dilution at which a compound can still be detected is expressed as the flavor dilution (FD) value or factor. Those compounds with the highest FD values are considered to be more potent in the overall aroma. This calculation is based on the specific sample analyzed, as the value can change depending on the amount of food that is sampled from and how concentrated the original sample extract is to start.

The CHARM technique uses the same concept, but attempts to combine the steps so that overall the technique is less time intensive. However, the technique also requires more coordination, in that once an aroma is detected, the panelist needs to not only be able to describe the odor quality, but also indicate the length of time the aroma is detected. This latter part may be difficult as aromas typically only last for a few seconds. The name of the technique was based on the meaning of the word “charm,” which is “a feature in something or someone that attracts or delights people” \((116)\). The name CHARM is also based on the acronym formed from the description: “combined hedonic response measurement” \((118)\). Responses from multiple runs are summed, and the more often a compound is detected, the higher the overall response. This method is intended to relieve the panelist of having to make magnitude estimates while analyzing each sample, as well as eliminating the need to serially dilute samples.
1.3.3. Important Aroma Compounds Formed in Bread during Baking

1.3.3.1. Wheat Bread

The flavor of fresh baked bread comes mostly from nonenzymatic browning reactions. For bread, these consist of compounds formed predominantly from the Maillard reaction, and to some extent, caramelization \((109,119)\). The crust is the major source of potent aroma compounds observed in bread, and is subjected to higher heat and lower moisture conditions during baking. The inner part of the bread, known as the crumb, is subject to much lower temperatures. As a result, flavors in the crumb are more likely to come from the compounds produced during dough fermentation \((109,120)\). Several compounds have become associated as some of the more potent compounds in wheat bread through GCO analysis (Figure 1-9). Examples (with alternate name and/or abbreviation if applicable, and odor descriptor in parentheses) encompass a wide variety of odor characteristics, such as 2-acetyl-1-pyrroline (2AP; roasty, cracker-like), 2-methylpropanal (2MP; malty), 2-phenylethanol (2PE; flowery), 3-(methylthio)propionaldehyde (methional; potato-like), 4-hydroxy-2,5-dimethyl-3(2H)-furanone (furaneol; caramel-like), \((E)\)-2-nonenal (green), and 2,3-butanedione (diacetyl; buttery) \((121–123)\).
Figure 1-9. Key bread aroma compounds

2AP is widely regarded as the most important compound in bread aroma, particularly refined wheat bread, but also to some extent in whole wheat bread (54,121,122). Other N-heterocyclic compounds have similar roasty, cracker-like odor characteristics and have also been identified as being odor-active in bread. Such compounds include 2-acetyl-2-thiazoline and the isomers 6-acetyl-1,2,3,4-tetrahydropyridine and 6-acetyl-2,3,4,5-tetrahydropyridine, both of which are most commonly referred to as 6-acetyltetrahydropyridine (ATHP) (53). However, these compounds, in comparison to 2AP, are not expected to contribute as strongly to the overall aroma of wheat bread, due to their higher respective odor thresholds (53).
Several other classes of aroma compounds have been determined to be crucial to the overall aroma in bread. These include the malty smelling Strecker degradation products, 2-methylpropanal and 2-3-methylbutanal, which form from the amino acids valine, isoleucine and leucine respectively (Figure 1-4). In addition, the buttery smelling compounds diacetyl and 2,3-pentanedione, as well as the lipid oxidation products, (E)-2-nonenal (green) and (E,E)-2,4-decadienal (fatty), are found to have strong perceived intensity in bread (121). Other important compounds include 2-phenylethanol (2PE; flowery), 3-methylbutanol (malty), 2-/3-methylbutanoic acid (sweaty), as well as various sugar fragmentation products, such as the oxygen-containing furanone compounds: 4-hydroxy-2,5-dimethyl-3(2H)-furanone (furaneol; caramel-like), 3-hydroxy-4,5-dimethyl-2(5H)-furanone (sotolon; spicy/savory), and 5-ethyl-3-hydroxy-4-methyl-2(5H)-furanone (caramel-like).

Pyrazines are characteristic hallmarks of the Maillard reaction, however they typically have high odor thresholds and must be present in large amounts to contribute to flavor. As a result, few pyrazines have been shown to have a large impact on wheat bread aroma. One compound, however, 2-ethyl-3,5-dimethylpyrazine (EDMP; earthy), does show an important impact in wheat bread (121).

Compared to the crust, the wheat bread crumb contains fewer potent aroma compounds (53). Of those identified, (Z)- and (E)-2-nonenal, (E,E)-2,4-decadienal, (E)-4,5-epoxy-(E)-2-decenal, and 1-octen-3-one, had the highest FD factors, lending green, fatty, metallic and earthy aroma attributes respectively to the crumb (53). Increasing fermentation time from one to three hours significantly increased the concentration of 2PE and 3-methylbutanol, which were not originally identified as potent odorants of the crumb during “short-fermentation” experiments. Since those two compounds are alcohols, the authors
hypothesized that their increased potency was most likely due to yeast metabolism during fermentation. Others have looked at the effect of using a pre-ferment on the final flavor on French-type bread crumb (52). Pre-ferments, also known as fermentation starters, are made up of mixtures of yeast, flour, and water that are typically quite liquid in nature. Pre-ferments are commonly used in the production of sourdough bread. After pre-determined fermentation times, as decided upon by the individual baker, the other ingredients for a bread dough formulation (more flour, salt, sugar, fat, etc.) are then added, after which further fermentation occurs. The use of pre-ferments involves increased fermentation times, and is used due to their association with improved aroma profiles in bread. The resultant increased fermentation time found similar results to the aforementioned studies, with higher levels of the alcohols 2PE and 3-methylbutanol in the crumb.

Overall, the most potent compounds in the crumb are found to be mostly lipid oxidation products, indicating that the Maillard reaction is not significantly involved with flavor formation in the crumb, as it is with the crust. This is supported with the finding that although 2AP was found in the crumb, it was at significantly lower amounts (52,53,121). Although yeast cells produce the precursors to form 2AP, it is the higher temperature that the outer portion of the bread is subjected to during baking, that is necessary for its formation. This may be due to the necessity for yeast cell lysis, as grinding yeast cells prior to heating with sugar is essential in the formation of 2AP (124).

1.3.3.2. Rye Bread

Rye bread is another type of bread that has been studied in great detail in the literature, due to its popularity in European countries. In addition to using a different cereal
grain for the basis of the flour, rye bread is typically made via a sourdough fermentation, which is not always used to make wheat bread (52,119). Sourdough fermentation introduces other microorganisms that influence the final flavor of the product. A small bit of starter culture is allowed to pre-ferment, before being added to the dough, allowing for the growth of not only yeast, but lactic acid bacteria (LAB) (119).

In comparing the aroma profiles of wheat and rye bread, rye is more complex, as a result of the presence of more aroma compounds with high FD values (54,122). Several of the important odor-active compounds, such as 3-methylbutanal, (E)-2-nonenal, (E,E)-2,4-decadienal, and methional, are the strongest in both rye and wheat breads (54,122,123).

Upon comparing the strength of these compounds, in terms of perceived odor, between the two types of bread, methional, (E)-2-nonenal, and (E,E)-2,4-decadienal were found to be stronger in rye bread crust (54). These compounds have earthy, green, and fatty smells respectively. These descriptors are also used to describe the overall aroma of rye bread, as opposed to the more roasty and buttery smells associated with wheat bread. This difference is supported by the fact that 2AP has been consistently shown to be present in lower amounts in rye bread, and is therefore less important to the overall profile, even though it is still present (54,122,123,125). Finally, some compounds have been found to only be strongly odor-active in rye bread, including 2-acetylpipridine (corn chip-like), 5-methyl-6,7-dihydro-5H-cyclopenta(b)pyrazine (nutty), and 2,6-dimethyl-3-ethyl-pyrazine (cocoa) (122,125).

Schieberle and Grosch (54) looked closer at the differences between the two types of bread. Calculating the OAV for 2AP supports the observation that wheat bread has a more roasted aroma, as 2AP has a much higher OAV in wheat bread crust than it does in rye bread.
crust (950 vs. 40). Additionally, the OAV calculated for methional found a higher value in rye bread (4800 vs. 510), when compared to wheat bread, leading the authors to believe that these two compounds lead to the major differences between these two types of breads.

As is the case with wheat bread, rye bread crust and crumb have different odor profiles, mostly due to temperature differentials during baking (54). Schieberle and Grosch (54) compared odor active compounds in both rye bread crust and inner crumb, and found there to be fewer odor-active compounds in the crumb. Some of the most odor-active compounds in the crumb were the same as the crust: phenylacetaldehyde (honey-like), (E)-2-nonenal, and (E,E)-2,4-decadienal, but were found to be even stronger in the crumb. Overall, compounds that have more of a fatty quality to them were found to be more odor active in the crumb, compared to the crust, such as hexanal (grassy), (E)-2-octenal (green), (Z)-2-nonenal, (E,Z)-2,6-nonadienal (green) (54). Comparatively, compounds that have earthy and nutty characteristics were found to be higher in the crust compared to the crumb including: 1-octen-3-one (mushroom-like), (Z)-1,5-octadien-3-one (mushroom-like), 2-ethyl-3,5(or 6)-dimethylpyrazine, 2,3-diethyl-5-methylpyrazine (earthy). Those malty, earthy notes were found in significantly lower amounts in the crumb versus the crust.

1.3.4. Other Influences on Bread Aroma

1.3.4.1. Ingredients

1.3.4.1.1. Raw Flour

It is widely regarded that the major aroma compounds that are found in baked bread are a result of the baking process, and to a lesser extent, from fermentation (109). Although flour makes up a major portion of every bread recipe, and contains its own aroma
compounds, its role in the final aroma of baked bread is to thought to provide starch which imparts the necessary reactants for both yeast growth and thermally generated aromas that are produced during baking.

In order to study if aroma compounds natively found in the flour truly do not have an effect on the final aroma of bread, Czerny and Schieberle (126) worked to identify the most important odor compounds in wheat flour. These compounds include lipid oxidation products such as (E)-2-nonenal, (E,Z)- and (E,E)-2,4-decadienal, and (E)-4,5-epoxy-(E)-2-decenal. Other compounds such as methional, 3-hydroxy-4,5-dimethyl-2(5H)-furanone (sotolon), and vanillin were also found to have high OAVs in raw flour. Comparing whole grain and refined flours did reveal a few differences, as three of the more potent compounds, vanillin, (E,E)-2,4-decadienal, and methional, were higher in the whole grain flour when compared to refined flour. All of the compounds identified were present before and after fermentation. The only change was from the difference in concentration. Unsaturated aldehydes decreased after fermentation. Some compounds that did not have a strong presence in raw flour increased after fermentation. These include acids and amino acid degradation products, more specifically, acetic acid, 2-/3-methylbutanal and 2-/3-methylbutanoic acid.

Kirchhoff and Schieberle (127) also studied odor-active compounds in raw flour, but this time in rye. Compiling data from flavor dilutions and odor thresholds indicated that methional, (E)-2-nonenal, and hexanal were the most important odor-active contributors to rye flour aroma. This same flour, after undergoing fermentation, showed 3-methylbutanal, vanillin, 3-methylbutanoic acid, methional, (E,E)-2,4-decadienal, diacetyl, and acetic acid as the most important odorants. 3-Methylbutanol, acetic acid, and diacetyl increased after
fermentation, whereas (E,E)-2,4-decadienal and 2-methylbutanal decreased, similar to the findings by Czerny and Schieberle (126) in wheat flours.

1.3.4.1.2. Yeast

Yeast play an integral role in making bread taste and smell like bread, both by providing reactants that are present within the yeast cell, and by breaking down starches in the flour to simpler sugars during fermentation. However, their original use was to leaven the bread, through fermentation, which would produce the carbon dioxide needed to make the dough rise.

The presence or absence of yeast can have a profound effect on the aroma of baked cereal products. Upon replacement of yeast with chemical leavening agents, such as baking powder, the loss of the typical bread aroma in these baked loaves was readily apparent (124). Breads baked without yeast were noted as having “day-old” aroma, similar to that of stale bread, and was attributed to the fact that 2AP formation was significantly decreased in breads made without yeast (34 μg/kg vs. 9.6 μg/kg) (124). Alternatively, it has been noted that an aqueous mixture of yeast and sugar, allowed to ferment separately before being added to the other ingredients just prior to baking, results in the typical aroma of fresh bread upon baking, further demonstrating the importance of yeast on proper bread aroma development (128). Heating yeast by itself does not produce very much 2AP (1.5 μg), indicating that the addition of sugar is necessary to yield higher amounts of 2AP (124).

Schieberle (129) determined that yeast provide the main source of the amino acid precursors, proline and ornithine, necessary to generate 2AP. Although proline is one of the more prevalent amino acids in wheat flour (130), by itself, it is not enough to produce the
levels of 2AP needed to generate a proper smelling loaf. Proline was measured at 12 mg/kg in flour, which increased to 32 mg/kg after fermentation (124). The effect of adding proline to the dough to enhance 2AP production was studied, but only resulted in a moderate increase of 2AP. For every 100 mg of proline added, there was only an increase between 4.2 and 6.4 μg of 2AP, up from 34 μg of 2AP formed from the originally calculated 32 mg/kg of proline measured in the fermented dough (124). This indicates that there must be other pathways for forming 2AP in bread. Ornithine, a relatively rare amino acid supplied exclusively by yeast in a bread formulation, was found to be a more potent precursor of 2AP, as adding ornithine to bread dough showed more significant increases in 2AP formation, compared to additions of proline (129). Furthermore, ornithine is present as the third highest concentration of free amino acids in yeast, whereas proline has the 11th-highest concentration (129).

1.3.4.2. Flavor Changes after Baking

1.3.4.2.1. Staling

Staling is one issue of importance in bread, due to its highly perishable nature, which can lead to a decrease in acceptability from consumers. According to Reineccius (131), there are different ways that loss of freshness can occur in food products. One possible way is through the loss of flavor compounds that are described as “fresh.” However very few compounds have been expressed as such, and typically the attribute “fresh” is just one of several descriptors associated with a compound, making this pathway unlikely to have significant contributions to stale flavor development.
Two major methods of staling occur in most foods. One occurs with the loss of desirable aroma compounds, specifically those that are the strongest and most associated with the overall aroma. This loss can occur from chemical reactions, such as the compound reacting with other compounds in the food, or through degradation. Other methods of aroma loss can involve evaporation of the compound, or conversely, the binding of the compound to the food matrix, thus making the compound of interest unavailable to being sensed. The second method is through the formation of off-flavors that can mask these more desirable aroma compounds. How these two options interact, or which one is more significant, depends on the food in order to lead it to be described as stale.

For wheat bread, the most important aroma compounds are Maillard reaction products, which primarily produce smells with roasty and toasty odor notes. Schieberle and Grosch (132) looked at the difference between fresh baked refined wheat bread, and similar bread stored for 96 hours. In the fresh bread, they found the Maillard reaction products 2AP, 3-methylbutanal, and diacetyl were the strongest compounds, and are described as roasty/cracker-like, malty, and buttery, respectively. After storage, these same compounds had significantly lower FD values, with differences between 4- and 8-fold. Other compounds, 1-octen-3-one, 2-ethyl-3,5-dimethylpyrazine, and (E)-2-nonenal, had similar FD values both before and after storage. This latter set of compounds lend oxidized, earthy, and green odor to the samples, which when coupled with the loss of the roasty, malty, and buttery compounds found in fresh bread, adds together to become a noticeable off-odor in stored bread. Though the latter set of compounds did not change with time, they became more prominent with the loss of the stronger compounds more associated with fresh bread.
aroma. In this case, with short-term storage, the staling appears to be due to the loss of important odor-active compounds, and not by the formation of off-flavor compounds.

Zehentbauer and Grosch (133) showed similar results to that of Schieberle and Grosch (132). Their paper dealt with looking at the differences between baguettes made by a traditional method and those made through a shorter process, typically used in commercial samples. Those made with the traditional method, which is a more time intensive process, kept a fresher aroma for longer, most likely due to the fact that there was a higher initial amount of the characteristic roasty, malty, and buttery aroma compounds.

Lorenz and Maga (111) never identified key aroma compounds important to the overall aroma of fresh bread, but rather studied the change in total carbonyl content in the aroma with time. They stated that carbonyl compounds appear to comprise the most important aroma compounds in bread. Subsequent authors that were able to better identify these compounds essentially agree with this, as most of the compounds that have been identified as key aroma compounds in bread do tend to include a carbonyl moiety. However, not being able to distinguish between those carbonyl compounds with a pleasant aroma (such as 2AP and 3-methylbutanal) and those that do not (such as the lipid oxidation products (E)-2-nonenal and (E,E)-2,4-decadienal) lead to the conclusion that sensory liking scores decreased with increasing carbonyl content with stored bread. However, as bread was stored longer, the carbonyl content was found to decrease over the course of the first three days, after which it began to increase, even to levels higher than that originally found in the bread. This led the authors to decide that it is not carbonyl content alone which influences a panelists’ liking score.
1.3.4.2.2. Toasting

Toasting is one method that can result in re-forming highly reactive compounds that lend themselves to the aroma of freshly baked bread; however the process cannot mimic the smell completely. Toasting provides high heat to the outer portion of the bread, almost mimicking the formation of crust during baking. As noted by Rychlik and Grosch (134), toasting wheat bread led to similar aroma characteristics of fresh baked wheat crust. This aroma was described as roasty, caramel-like, malty, and buttery. Sensory and quantification experiments performed on toast showed the strongest presence by 2AP, which would lend the roasty character to toasted bread. 4-Hydroxy-2,5-dimethyl-3(2H)-furanone (furaneol), diacetyl, (E)-2-nonenal, 3-methylbutyric acid, and methional were also important aromas to the toasted bread. The first two compounds can explain the caramel and buttery notes that were observed. However, the malty character could not be explained by an individual compound’s contribution in the aforementioned list. Two compounds, methylpropanal and 3-methylbutanal, both are described as malty. Although their OAVs were not as high as the other compounds, since they have similar odor characteristics, their contributions were considered additive to lead to the presence of the malty character to the overall aroma.

1.4. 2-Acetyl-1-pyrroline: Key Bread Aroma Compound

1.4.1. Sources of 2-Acetyl-1-pyrroline

2AP is found in a wide variety of foods, most notably cereal-type products (Table 1-1). Its extremely low odor threshold, and presence in a wide variety of foods, has established it as one of the most important Maillard reaction products (135). Though the odor threshold varies with the medium, it has been calculated to be as high as 0.1 μg/L (or
ppb) in water (136) and as low as 0.0073 μg/kg (or ppb) in starch (134) and 0.02 ng/L (or ppt) in air (137). 2AP is highly reactive and degrades rapidly upon storage (135). In fact, it is not readily commercially available, and in order to measure it, it either must be synthesized, or extracted from foods that contain it in large amounts, such as pandan leaves (Pandanus amaryllifolius Roxb.) (80,135).

Table 1-1. Sources of 2-acetyl-1-pyrroline (adapted from source (135))

<table>
<thead>
<tr>
<th>Origin</th>
<th>Sources</th>
</tr>
</thead>
<tbody>
<tr>
<td>Plant materials</td>
<td>Cooked rice&lt;br&gt;Fragrant-type (Jasmine, Basmati)&lt;br&gt;Non-fragrant-type (Texas Long Grain, Calrose)&lt;br&gt;Wheat and Rye bread (crust and toast)&lt;br&gt;Corn products (popcorn, tortillas, canned corn, extruded flour)&lt;br&gt;Leaves (Pandanus amaryllifolius, dried green tea, tobacco leaves)&lt;br&gt;Bread flower (Vallaris giabra)&lt;br&gt;Boiled potatoes&lt;br&gt;Roasted seeds (sesame, mango, peanut and pumpkin seed oils)&lt;br&gt;Pearl millet&lt;br&gt;Fruit (Chempedak, jackfruit, Myabi muskmelon)&lt;br&gt;Theobroma grandiflorum pulp (thermally treated)&lt;br&gt;Pale lager beer&lt;br&gt;Mushrooms</td>
</tr>
<tr>
<td>Animal materials</td>
<td>Honey&lt;br&gt;Milk (fresh, dried powder, ultra-high temperature processed)&lt;br&gt;Cheese (Camembert, Swiss Gruyere)&lt;br&gt;Cheese related products (rennet casein, liquid Cheddar whey)&lt;br&gt;Cooked meats (beef, salami, dry-cured ham, Mediterranean dried sausages)&lt;br&gt;Seafood products (boiled carp fillet, salmon, cod, trout, prawns; cooked blue crab claw meat; cooked lobster tail meat; ripened anchovy; crayfish-processing byproducts; tuna sauce; stored sardines; enzyme-hydrolyzed oyster cooker effluent; American lobster)&lt;br&gt;Tiger urine*</td>
</tr>
</tbody>
</table>

*Though not necessarily used for human consumption, its presence demonstrates the ubiquity of 2AP.
Although 2AP has predominantly been characterized in heated foods, its presence has also been detected in products kept at temperatures much lower than those that occur with cooking, such as raw plant materials as well as products of bacterial synthesis (135). Although raw plant materials have been used in a positive way to flavor non-fragrant varieties of rice (138), bacterial synthesis of 2AP is typically associated with the development of off-flavors. For example, mousy off-flavor of wine is associated with the presence of 2AP, produced by wine lactic acid bacterium Lactobacillus hilgardii DSM 20176 (139). Additionally, during the fermentation of cocoa beans, development of roasty, popcorn-like aromas was attributed to synthesis of 2AP by Bacillus cereus (140). A possible explanation on how 2AP is formed at near ambient temperatures is that arginine can be enzymatically converted to ornithine, a potent precursor of 2AP, in Lactobacillus sanfranciscensis (141).

1.4.2. 2-Acetyl-1-pyrroline Formation

Reacting amino acids singly with glucose produces solutions that vary in aroma characteristics. It has been noted that reactions between proline and glucose results in solutions that smell roasty, with corn chip-like aromas (55). Therefore it is not surprising that 2AP was found to be an important aroma compound in these reactions, along with other compounds with similar odor characteristics including 2-acetyl-2-thiazoline and the tautomeric compounds 6-acetyl-1,2,3,4-tetrahydropyridine and 6-acetyl-2,3,4,5-tetrahydropyridine (ATHP). In fact it was deduced that these α-acetyl-Ν-heterocycles tend to have similar roasty, and cracker-like odor characteristics thought to stem from the basic arrangement of an unsaturated ring structure containing adjacent N and C atoms, forming
either α-iminoketone or α-enamine structures with an acetyl group attached to the C atom in the ring (135,142–144).

Carbon-13 labeled reactants are commonly used to elucidate formation pathways of Maillard products (145). Schieberle (124) utilized fully carbon-13 labeled glucose, and upon reaction with proline, resulted in the formation of 2AP whose molecular weight increased by two mass units (111 vs. 113). Reacting 1-13C-proline (label at the carboxyl carbon), with unlabeled glucose resulted in no significant formation of 2AP that included a 13C-labeled carbon. Additionally, reacting 1-13C-glucose with proline, resulted in the methyl carbon of 2AP being labeled after reaction (146). These results indicate that proline undergoes decarboxylation, due to a lack of integration of the carboxyl carbon atom into the 2AP structure (Figure 1-10). The pyrrolidine ring rearranges to a pyrroline ring after which the addition of a two-carbon fragment from the sugar source is incorporated into the acetyl group of 2AP.
Figure 1-10. 2-Acetyl-1-pyrroline formation (adapted from (40))

Lending support to the fact that sugar fragmentation products are necessary in forming 2AP, use of specific sugar fragmentation products, in place of glucose or other sugars, have shown to result in even higher generation of 2AP in model systems. Cleavage of fructose forms dihydroxyacetone, an unstable product that forms 2-oxopropanal, via dehydration, almost immediately upon heating (124,128). 2-Oxopropanal (also known as methylglyoxal or pyruvaldehyde) was found to be a more potent precursor of 2AP than
dihydroxyacetone, fructose, or glucose (124,128). Additionally, 2AP analogues have been reported, using compounds structurally similar to 2-oxopropanal. 2-Oxobutanal and α-oxophenylacetaldehyde were found to form 2-propionyl-1-pyrroline, an important compound in popcorn aroma (147), and 2-benzoyl-1-pyrroline (128), respectively.

2-Oxopropanal, in addition to being an important precursor in 2AP formation, is also a glycolytic product found in yeast (124). Therefore, it was reasonable to find that without the presence of phosphate ions, no 2AP was formed in model systems carried out in distilled water. But providing fructose 1,6-diphosphate, instead of fructose, showed significant increases in 2AP formation (124). This is also supported by the use of phosphate buffer, instead of malonate buffer, the former which showed significant increases in 2AP formation (129,148). Phosphate groups are thought to stimulate the generation of 2-oxopropanal which can then combine with 1-pyrroline, which itself forms from the Strecker degradation of proline.

Another amino acid source present in yeast cells, ornithine, can also serve as a precursor of 2AP (129). Upon reaction with 2-oxopropanal, 2AP is formed, and in similar amounts, compared to the amount formed upon reaction with proline (43 and 41 μg, respectively) (129). Looking more closely at the mechanism, Schieberle (129) hypothesized that the Strecker degradation of ornithine leads to the formation of 4-aminobutyraldehyde (4-aminobutanal) as an intermediate, which reacts with 2-oxopropanal, perhaps after its immediate cyclization to 1-pyrroline. Using 4-aminobutyraldehyde as the amine source resulted in the formation of 2AP, but in a smaller amount compared to using 1-pyrroline (125 vs. 562 μg). De Angelis et al., (141) showed that arginine can be converted to ornithine in certain bacteria, and although the structure of arginine is similar to 4-
aminobutyraldehyde, arginine did not form 2AP in model systems (129). Additionally, although 4-aminobutyric acid is also found in yeast, it does not produce any 2AP (129).

1.5. Hypothesis and Objectives

I hypothesize that hydroxycinnamic acids (HCAs) can trap reactive intermediates of Maillard reaction products, thereby altering the generation of volatile Maillard reaction compounds. The hypothesis will be tested in a series of simple aqueous solutions and more complex bread systems with the following objectives:

1) To investigate the effect of HCAs on aroma generation in aqueous model Maillard systems, in addition to identifying Maillard-phenolic adducts via labeling experiments.

2) To investigate the effect of wheat flour-type (refined vs. whole wheat) on aroma generation in wheat bread crust, by identifying and quantifying those compounds with high flavor dilution values that also demonstrate differences between the two bread types. A focus will be placed on defining the reactivity of ferulic acid on modulating flavor development in whole wheat bread.

3) To conduct sensory tests based on the results from objective 2, investigating if adding back certain aroma compounds that are deficient in one bread crust (e.g. whole wheat) can result in an overall aroma closer to the other crust (e.g. refined wheat).

1.6. References


(2) Pernollet, J.-C.; Briand, L. Structural Recognition between Odorants, Olfactory-Binding Proteins and Olfactory Receptors - First Events in Odour Coding. In Flavor


(54) Schieberle, P.; Grosch, W. Potent odorants of rye bread crust-differences from the crumb and from wheat bread crust. Z. Lebensm. Unters. Forsch. 1994, 198, 292-296.


CHAPTER 2: HYDROXYCINNAMIC ACID – MAILLARD REACTIONS IN SIMPLE AQUEOUS MODEL SYSTEMS


2.1. Abstract

Hydroxycinnamic acids (HCAs) in foods have been suggested to contribute to flavor properties by multiple mechanisms. In this chapter, the reactivity of HCAs in simple aqueous Maillard model systems on flavor development is investigated. Using isotope labeling techniques, HCAs were observed to form adducts with transient Maillard reaction flavor precursors, hexose fragments, as well as with amino acids and/or amino acid reaction products. The generation of Maillard-type volatile compounds, such as pyrazines, cyclotene, diacetyl, 2-acetyl-1-pyrroline, in the model systems, was also suppressed by addition of HCAs. In summary, the HCAs were reported to alter the mechanisms of Maillard flavor development in these aqueous model systems.

2.2. Introduction

The Maillard reaction is a well-known mechanism of flavor development in food products. The generation of Maillard-type flavor compounds is desirable for many foods such as bread, chocolate, and coffee (1), but can also produce unpleasant attributes in other
products, such as in ultra-high temperature (UHT) milk (2). The basic reactants for the Maillard reaction include a carbonyl and an amine, typically provided by reducing sugars and amino acids or peptides in foods (1). The types of carbonyls and amines in foods, as well as the reaction conditions, such as water content/activity, temperature, and pH, are widely reported to influence the products formed via this reaction (1). In addition to the precursors that define the reaction (carbonyl and amine), food molecules such as phenolic compounds and aldehydic lipid oxidation products, have also been reported to influence the mechanisms of the Maillard reaction (3–5).

Hydroxycinnamic acids (HCAs) are ubiquitous in plant material and are the predominant phenolic compounds in products such as grain and coffee beans. The HCAs have been reported to alter the flavor properties of related foods by multiple mechanisms, including Maillard-type flavor development (6). The objective of this chapter was to investigate the reactivity of HCAs on flavor development in simple aqueous Maillard model reactions. It has previously reported on the reactivity of HCAs in low moisture glucose-glycine models (7). Based on preliminary experiments in aqueous models, the reactivity of the HCAs was found to be different in comparison to low moisture model systems and therefore was further investigated.

2.3. Materials and Methods

2.3.1. Chemicals

Glucose, glycine, proline, ferulic acid, cinnamic acid, caffeic acid, p-coumaric acid, sodium phosphate monobasic, sodium phosphate dibasic, hydrochloric acid, sodium hydroxide, n-dodecane, pyrazine, methylpyrazine, 2,5-dimethylpyrazine, 2,3-butanedione,
3-hydroxy-2-butanone, furfuryl alcohol, cyclotene, glyoxal, glycolaldehyde, methylglyoxal, and hydroxyacetone were purchased from Sigma Aldrich (St. Louis, MO). Anhydrous sodium sulfate, diethyl ether, methanol, and ammonium acetate were purchased from EMD Chemicals (Gibbstown, NJ). $^{13}$C$_6$-glucose and $^{13}$C$_2$, $^{15}$N-glycine was purchased from Cambridge Isotope Laboratories (Andover, MA).

2.3.2. Model Reaction Systems

Equimolar concentrations (10 mM) of reactants (see Table 2-1) were reacted in 100 mL of a 0.1 M, pH 7.0 phosphate buffer solution at 125°C for 30 min in a 600 mL Parr Reactor (model 4563, Parr Instrument Co., Moline, IL) under constant stirring and immediately cooled to approximately 30°C and prepared for further analysis.

<table>
<thead>
<tr>
<th>Reactants $^a$</th>
<th>Reactant Concentration (mM)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Model A</td>
</tr>
<tr>
<td>Glucose</td>
<td>10</td>
</tr>
<tr>
<td>Amino acid $^b$</td>
<td>10</td>
</tr>
<tr>
<td>Phenolic acid $^c$</td>
<td>10</td>
</tr>
<tr>
<td>$[^{13}\text{C}_6]$-glucose</td>
<td>5</td>
</tr>
<tr>
<td>$[^{13}\text{C}_2,^{15}\text{N}]$-glycine</td>
<td>5</td>
</tr>
<tr>
<td>Sugar fragment $^d$</td>
<td></td>
</tr>
</tbody>
</table>

$^a$ Reactants were dissolved in 0.1 M phosphate buffer, reacted at 125°C for 30 min (does not include reactor heating time to 125°C) and cooled down to 30°C prior to sample preparation. $^b$ amino acid; glycine or proline. $^c$ phenolic acid; ferulic, cinnamic, p-coumaric, or caffeic acid. $^d$ sugar fragment; glyoxal, glycolaldehyde, methylglyoxal, or hydroxyacetone.

2.3.3. Gas Chromatography/Mass Spectrometry-Electron Impact Analysis (GC/MS-EI)

Ninety mL of A, B, E, and F model reaction mixtures were extracted three times with 25 mL of diethyl ether containing 0.8 ppm n-dodecane as the internal standard. The
organic extract was dried with anhydrous sodium sulfate, filtered, and concentrated down to 0.5 mL using a spinning band distillation apparatus (model 800, B/R Instruments, Easton, MD). Samples were prepared from models conducted in triplicate for further analysis. Distillates were subsequently analyzed with a 6890 Agilent GC equipped with a flame ionization detector and 5973 Mass Selective Detector (Agilent Technologies, Inc.; Santa Clara, CA), using a DB-Wax capillary column (30 m x 0.25 mm ID with 0.25 μm film thickness, Agilent Technologies, Inc.; Santa Clara, CA). One μL of sample was injected in split mode, with inlet temperature set to 200°C and detector temperature of 250°C. MS analysis was done with Electron Impact Ionization, scanning between 35 and 250 m/z. The oven/time program was as follows: 30°C for 2 minutes, ramped by 5°C/min to 180 °C, ramped by 35°C/min to 230°C, and then held for 6 min.

2.3.4. Liquid Chromatography/Mass Spectrometry-Electrospray Ionization Analysis (LC/MS-ESI)

Nine mL of A-F model reaction mixtures were fractioned on 0.5 g C18 Sep-pak cartridge (Supelclean™ LC-18; Supelco, Bellefonte, PA), preconditioned with 5 mL each of methanol, then reverse osmosis water. Ten μL of an internal standard (300 ppm butylparaben in methanol) was added to the sample prior to SPE clean-up. The cartridge was washed with 5 mL of reverse osmosis water and then eluted with 2 mL of HPLC grade methanol under vacuum at a rate of 1 mL/min. The eluent was concentrated to approximately 1 mL under a stream of nitrogen and filtered through 0.45 μm PTFE tip filter. The concentrate was subsequently analyzed with a Shimadzu HPLC system (Shimadzu, Columbia, MD), consisting of two pumps (LC-10ADvp), an autosampler (SIL-10vp) and
on-line degasser (DGU-14A). The system was interfaced to a Waters Micromass Quattro Micro mass spectrometer (Waters, Milford, MA). The samples were analyzed using an Ultra Aqueous C18 column (250 x 2.1 mm I.D.; 5 μm particle size; Restek; Bellefonte, PA) kept at 28°C using a column heater (model TCM; Waters, Milford, MA). The flow rate was 0.2 mL/min, with a linear concentration gradient of two mobile phase solvents A (10 mM ammonium acetate, pH 7.0) and B (methanol). The program began at 10% B in A (0 – 2 min) and then increasing B to 99% over 28 min (2 – 30 min) and held for 4 min (30 – 34 min) before decreasing to 10% (34 – 35) and held for 8 min (35 – 43 min). MS data was collected over the scan range of 120 to 500 m/z with a scan time of 1.0 s in either negative or positive ion mode.

2.4. Results and Discussion

The influence of four common food hydroxycinnamic acids (shown in Figure 2-1) on the generation of Maillard-type aroma compounds in a glucose/glycine model system is illustrated in Figure 2-2. In general, the addition of hydroxycinnamic acids (HCAs) significantly reduced the generation of six of the seven main aroma compounds detected in these Maillard models. Differences in the level of suppression were also noted among the four structurally unique HCAs analyzed; caffeic acid was the most reactive, and therefore the most effective in suppressing aroma development.
Figure 2-1. Chemical structures of select hydroxycinnamic acids, ferulic: $R_1=OCH_3$, $R_2=OH$, $R_3=H$; cinnamic: $R_1=R_2=R_3=H$; p-coumaric: $R_1=R_3=H$, $R_2=OH$; caffeic: $R_1=R_2=OH$, $R_3=H$

Figure 2-2. Concentrations of key volatiles generated in aqueous model systems, 125 °C, pH 7; left to right, Model A (control): glucose + glycine ■, Model B (treatment): glucose + glycine + phenolic acid (ferulic ■, cinnamic ■, p-coumaric ■, caffeic acid ■, respectively); Phenolic acid treatments marked with an asterisk are significantly different from the control ($\alpha = 0.05$)
To investigate the reactivity of HCAs on mechanisms of Maillard product generation in these models, two isotopic labeling techniques, CAMOLA (Carbohydrate MOdule LAbeling; model C) (8) and AAMOLA (Amino Acid MOdule LAbeling; model D) (9), were utilized as previously reported (7). Since ferulic acid is the most abundant HCA found in wheat (10), it was selected for further analysis.

When ferulic acid was reacted with glucose and glycine (model B), an analyte with the predicted molecular weight of 210 Da (m/z 209 [M-1]) was detected (Figure 2-3ii); which was not reported in the control glucose/glycine reaction (Figure 2-3i, model A).

For the CAMOLA reaction (Figure 2-3iii, model C), the plus 2 isotopomer (m/z 211) indicated this analyte consisted of an intact C2 glucose fragment, whereas for the AAMOLA technique, no isotopomer was detected (Figure 2-3iv, model D) or glycine was not part of this reaction product. Consequently this analyte consisted of a ferulic acid moiety and a two-carbon glucose fragment. Two common two-carbon sugar fragments of hexose sugars, glyoxal and glycolaldehyde, were reacted with ferulic acid and glycine (no glucose) and compared to the ferulic acid/glucose/glycine reaction for the generation of this phenolic-sugar fragment adduct (see Figure 2-4). Based on the chromatograms in Figure 2-4, glycolaldehyde generated a similar analyte ‘fingerprint’ as was generated in the glucose/glycine/ferulic acid model (Figure 2-4iii) and thus was identified as the sugar fragment moiety of this ferulic acid-Maillard reaction product.
Figure 2-3. LC/MS-ESI (-ve) Spectrum of select analyte m/z 209[M-1] generated in aqueous model systems, 125 °C, pH 7: (i) Model A. glucose + glycine, (ii) Model B. glucose + glycine + ferulic acid, (iii) Model C. CAMOLA: $^{13}$C$_6$ : $^{12}$C$_6$ glucose + glycine + ferulic acid, (iv) Model D. AAMOLA: glucose + $^{13}$C$_2$, $^{15}$N : $^{12}$C$_2$, $^{14}$N glycine + ferulic acid; all at equivalent retention time.
Figure 2-4. LC/MS-ESI (-ve) chromatogram of a 4-vinylguaiaol-glycolaldehyde adduct (m/z 209[M-1]) generated in aqueous model systems, 125 °C, pH 7: (i) Model F: glyoxal + glycine + ferulic acid; (ii) Model F: glycolaldehyde + glycine + ferulic acid; (iii) Model B: glucose + glycine + ferulic acid
Based on the preceding information, the mechanism of formation of this particular compound is depicted in Figure 2-5. Upon heating, ferulic acid would undergo decarboxylation to form 4-vinylguaiacol, after which the two-carbon glucose fragment, glycolaldehyde, could then attach to the more reactive benzene ring resulting in a product with the predicted molecular weight of 210 Da.

Figure 2-5. Proposed formation mechanism of the analyte identified in Figures 2-3 and 2-4 with the predicted molecular weight of 210 Da
Ferulic acid-Maillard reaction products consisting of both a glucose fragment and a glycine moiety were also detected in these models. Figure 2-6 illustrates an analyte detected in model B (Figure 2-6ii) but not in model A (Figure 2-6i) with the predicted molecular weight of 427 Da based on the \( m/z \) 428 [M+1]+ ion. This analyte consisted of an intact three-carbon glucose moiety (plus 3 isotopomer detected with \( m/z \) 431 ion, Figure 2-6iii) as well as an intact C and N skeleton of glycine (plus 3 isotopomer detected with \( m/z \) 431 ion, Figure 6iv). Similarly, to identify the source of the three-carbon fragment incorporated into the analyte observed in Figure 2-6ii, common three-carbon hexose fragments, hydroxyacetone (Figure 2-7i) and methylglyoxal (Figure 2-7ii), were reacted with glycine and ferulic acid. Based on the single peak generated in the chromatogram (Figure 2-7ii and 2-7iii), methylglyoxal and glycine were identified as the reactants for this phenolic reaction product.
Figure 2-6. LC/MS-ESI (+ve) Spectrum of select analyte m/z 428[M+1]$^+$ generated in aqueous model systems, 125 °C, pH 7: (i) Model A. glucose + glycine, (ii) Model B. glucose + glycine + ferulic acid, (iii) Model C. CAMOLA: $^{13}$C$_6$: $^{12}$C$_6$ glucose + glycine + ferulic acid, (iv) Model D. AAMOLA: glucose + $^{13}$C$_2$, $^{15}$N: $^{12}$C$_2$, $^{14}$N glycine + ferulic acid; all at equivalent retention time
Figure 2-7. LC/MS-ESI (+ve) chromatogram of a 4-vinylguaiacol-methylglyoxal adduct (m/z 428[M+H]+) generated in aqueous model systems, 125 °C, pH 7: (i) Model F. hydroxyacetone + glycine + ferulic acid, (ii) Model F. methylglyoxal + glycine + ferulic acid, (iii) Model B. glucose + glycine + ferulic acid
The observed reactivity of HCAs with sugar fragments or sugar fragment/amino acid products, which are known precursors of Maillard-type aroma compounds, provided a mechanism to define how HCAs altered Maillard chemistry and aroma development in these model systems. Considering HCAs are abundant in grains, additional model systems were analyzed for the influence of ferulic acid on the generation of 2-acetyl-1-pyrroline, an important aroma compound found in cereal products, such as bread (11). Methylglyoxal is a known precursor of 2-acetyl-1-pyrroline (12). Two model systems (Models E-F, Table 2-1) consisted of proline, as the amino acid source, and methylglyoxal with and without ferulic acid. The addition of ferulic acid to this model system resulted in a 39% decrease in the amount of 2-acetyl-1-pyrroline generated.

In summary, HCAs were reported to trap precursors of Maillard-type aroma compounds, such as two- and three-carbon sugar fragments and amino acid moieties, and consequently suppressed aroma development in these aqueous model systems. On-going research in our laboratory is focused on translating the findings of these model systems to flavor development in whole grain foods.

2.5. References


CHAPTER 3: INFLUENCE OF ENDOGENOUS FERULIC ACID IN WHOLE WHEAT FLOUR ON BREAD CRUST AROMA


3.1. Abstract

The influence of wheat flour-type (refined (RWF) vs. whole (WWF)) on bread crust aroma was investigated. Differences were characterized by aroma extract dilution analysis and quantified utilizing stable isotope surrogate standards. For RWF breads, five aroma compounds were higher in concentration: 2-acetyl-1-pyrroline, 4-hydroxy-2,5-dimethyl-3(2H)-furanone, 2-phenylethanol, 2-acetyl-2-thiazoline, and 2,4-dihydroxy-2,5-dimethyl-3(2H)-furanone by 4.0-, 3.0-, 2.1-, 1.7-, and 1.5-fold, respectively, whereas three compounds were lower: 2-ethyl-3,5-dimethylpyrazine, (E,E)-2,4-decadienal, and (E)-2-nonenal by 6.1-, 2.1-, and 1.8-fold, respectively. A trained sensory panel reported the perceived aroma intensity of the characteristic “fresh refined bread crust” aroma was significantly higher in RWF compared to WWF crust samples. Addition of 2-acetyl-1-pyrroline, 4-hydroxy-2,5-dimethyl-3(2H)-furanone, 2-phenylethanol, 2-acetyl-2-thiazoline, 2,4-dihydroxy-2,5-dimethyl-3(2H)-furanone to the WWF crust (at equivalent concentrations as the RWF crust) increased the intensity of the “fresh refined bread crust” aroma attribute; no significant difference was reported when compared to RWF crust. The liberation of ferulic acid from WWF during baking was related to the observed reduction in these five...
aroma compounds and provides novel insight into the mechanisms of flavor development in WWF bread.

3.2. Introduction

With health and nutrition becoming increasingly important to consumers, whole grain foods have been touted as a key component of a healthy diet. Recommendations made by the USDA advocate that at least three servings of whole grain foods be consumed daily, which should be substituted for refined grain foods (1). Whole grain foods provide fiber and important phytochemicals, both of which are linked, in an increasing number of epidemiological studies, to the reduced risk of several chronic diseases such as cardiovascular disease, diabetes, cancer, and obesity (2).

The consumption of whole grain food, in comparison to refined grain formulated products, can be challenged by observed lower product consumer acceptability ratings (3). This is, in part, attributable to changes in the flavor profile and intensity of whole grain foods, as well as other color and textural changes. The negative flavor attributes imparted by whole wheat flour (WWF) would be further exacerbated in reduced sugar or salt formulated products, which have their own complications regarding flavor and acceptability by consumers.

Most of the aroma research conducted on bread has focused on either refined wheat flour (RWF)-formulated breads, or whole grain rye breads (4–7). In RWF bread crust, the main compounds reported to contribute to aroma are products of two major sources of aroma generation, the Maillard reaction and lipid oxidation, with a third, albeit minor pathway, resulting from yeast fermentation. Major aroma active products of the Maillard
reaction in bread include 6-acetyl tetrahydropyridine, 2-acetyl-1-pyrroline (2AP), 3-methylbutanal, methional, 2-ethyl-3,5-dimethylpyrazine (EDMP), 4-hydroxy-2,5-dimethyl-3(2H)-furanone (HDMF), 3-hydroxy-4,5-dimethyl-2(5H)-furanone, and 5-ethyl-3-hydroxy-4-methyl-2(5H)-furanone, with lipid oxidation resulting in the formation of (E)-2-nonenal and (E,E)-2,4-decadienal (8). Yeast metabolism has been reported to generate the two odor-active alcohols, 2-phenylethanol (2PE) and 3-methylbutanol, in yeast-leavened breads (8).

The compositional differences between RWF and WWF are thought to influence flavor development. The wheat seed is made up of three major components: the starchy endosperm, the phenolic-rich bran, and the lipid-rich germ. RWF consists of only the endosperm, whereas WWF includes all three seed components, which could explain the variation in flavor and aroma between the two types of bread, especially with all other ingredients being equal. For example, the lipid (germ) fraction of WWF is oxidatively labile and, thus, could yield aromas characteristic of lipid oxidation reactions. Jensen et al., (9) reported that the hydroperoxide content in margarine (main lipid source) used in a bread formulation was related to the generation of lipid oxidation products in the bread. Similar results were observed in puff pastry, wherein those made with margarine had a higher degree of lipid oxidation compared to those made with butter, due to a higher level of linoleic acid in margarine (8). Though these studies only investigated the generation of lipid oxidation products from a major ingredient (margarine), these findings are applicable to the lipid material in WWF.

An additional distinction between RWF and WWF bread is the bran content. Bran is the major source of phenolic compounds, specifically the hydroxycinnamic acids (HCAs), and the concentrations of these compounds are negligible in RWF in comparison to WWF.
HCAs have been reported in model food systems to alter Maillard chemistry and related flavor generation by binding and forming adducts with key transient Maillard intermediates. Additionally, other phenolic compounds, such as flavonoids, have been shown to modify aroma development in model systems by also forming adducts with intermediates. The mechanism by which this modulation occurs is thought to involve carbonyl trapping, rather than free radical mechanisms, which has been supported by structure identification performed with NMR spectroscopy.

With respect to bread flavor, ferulic acid (FA) was shown to suppress the generation of 2AP, a well-known and critical aroma active compound in bread, by reacting with an important precursor of 2AP, methylglyoxal, in a methylglyoxal/proline model system. In wheat, particularly in the bran layer of the seed, the predominant HCA is FA, which is present in three different forms: free, soluble conjugate, and insoluble conjugate. Typically, less than 1% of the phenolic content in wheat is in the free form, which was considered the reactive form related to altering Maillard chemistry and product generation. However, little is known about the release of HCAs from the more prevalent bonded form (insoluble conjugate) in products during baking or fermentation, and how the related chemistry impacts flavor development in whole wheat bread.

The overall objective of this chapter was to investigate the influence of wheat flour-type (RWF versus WWF) on the aroma profile of wheat bread crust. A focus was placed on defining the reactivity of FA on modulating flavor development in whole wheat bread.
3.3. Materials and Methods

3.3.1. Chemicals

2-acetyl-2-thiazoline (2A2T), butylated hydroxytoluene (BHT), (E,E)-2,4-decadienal, deuterium oxide, dimethyl fumarate, 2,6-dimethylpyrazine, 2-ethyl-3,5-dimethylpyrazine (EDMP), ferulic acid (FA), glucose, hydrochloric acid, 4-hydroxy-2,5-dimethyl-3(2H)-furanone (HDMF), lithium slivers, magnesium turnings, manganese oxide, methional, methylglyoxal (2-oxopropanal), 2-methyl-3-heptanone, 2-methylpropanal (2MP), (E)-2-nonenal, 2-phenylacetic acid chloride, 2-phenylethanol (2PE), phosphoric acid, potassium cyanide, proline, selenium dioxide, sodium bicarbonate, sodium bisulfite, sodium chloride, sodium hydroxide, sodium phosphate, anhydrous sodium sulfate, sulfuric acid, tricaprylin, triethyl orthoformate, 1-vinyl-2-pyrrolidinone, $^{13}$C$_2$-acetone, $^2$H$_5$-ethylbromide, $^2$H$_7$-isopropyl bromide, and LiAl$^2$H$_4$ were obtained from the Sigma Aldrich Company (St. Louis, MO). 2-Nonyn-1-ol was obtained from Alfa Aesar (Ward Hill, MA). The solvents dichloromethane, diethyl ether, pentane, tetrahydrofuran, and hexane were obtained from Fisher Scientific (Fairlawn, NJ). Diethyl ether was distilled once before use for synthesis experiments. 2-Acetyl-1-pyrrole (2AP) (11) and 2,4-dihydroxy-2,5-dimethyl-3(2H)-furanone (DHDMF) (21) were synthesized as previously described. $^2$H$_3$-2-Acetyl-1-pyrrole, $^3$H$_3$-methional, $^2$H$_2$-(E,E)-2,4-decadienal, and $^2$H$_6$-4-hydroxy-2,5-dimethyl-3(2H)-furanone were gifts from Dr. Gary Reineccius.

3.3.2. Wheat Samples

Milled whole wheat (WWF) and refined wheat (RWF) flour from the same hard red spring wheat kernels were obtained from ConAgra (Omaha, NE). Prior to receiving the
flour, whole hard red spring wheat kernels were tempered and then crushed and flattened with cast iron rollers, and sifted through a series of sieves in order to remove the bran and germ from RWF. The samples arrived already milled and ready for use in baking, and were stored under argon in brown glass vessels with Teflon lined lids at -20 °C.

3.3.3. Bread Making

Bread samples were prepared according to AACC method 10-10B (22). Briefly, two liquid solutions were prepared, one consisting of salt (24 g) and sugar (96 g), and the other consisting of yeast (32 g; Fleischmann’s Active Dry), with each dissolved in 200 g of distilled water. Flour samples were removed from the freezer 1 h prior to mixing to bring to room temperature. Flour (1600 g; RWF or WWF) was added to a mixing bowl and a well was made in the center for addition of all liquids, including bringing the total amount of distilled water used in the recipe to 1032 g or 1164.8 g for RWF and WWF breads, respectively. A farinograph (Brabender GmbH & Co. KG, Duisburg, Germany) was used to determine the optimal amount of water and time needed to form both breads by measuring the dough viscosity. A separate sample of flour and water were initially placed in a mixing chamber where rotating blades measured the resistance of the dough in order to determine the amount of water needed for peak gluten formation. The time to arrive at peak gluten formation (prior to breakdown) was used for all mixing/kneading times. Vegetable shortening (48 g) was melted prior to addition at 60 ºC and added to the liquids. The dough was mixed for 2 min on the lowest speed, followed by 2 min each at the next two highest speeds, finished with one minute at the next highest speed in a standing ten-speed mixer (KitchenAid, KSM75W, St. Joseph, MI). Batches of dough were taken based on 100 g flour
basis (177 g dough for RWF, 185.3 g for WWF) and were placed in a proofing oven (National MFG Co., Lincoln, NE) held at 30 °C and 85% relative humidity. First punch occurred at 52 min, second punch 25 min after that, and the final punch 13 min after that. The dough was then panned and replaced in the proofing cabinet for a final 33 min. The loaves were placed in a rotary oven (Despatch Oven Company, Minneapolis, MN), preheated to 215 °C, primed with beaker filled with 1 L of water, and were baked for 17 min. The loaves were removed from the pans and immediately prepped for extraction as outlined in the following section.

3.3.4. Preparation of Bread Crust Extracts

The crust was separated from the bread immediately after baking, frozen in liquid nitrogen, and ground to a powder using a mortar and pestle. The crust (1250 g) was extracted with 2.5 L of dichloromethane spiked with 2-methyl-3-heptanone (8 μg) as an internal standard, and BHT (8 μg) as an antioxidant. The extraction was conducted under a blanket of argon gas and agitation overnight, at room temperature, for 15 h. The solvent was removed, and the residue was extracted again with another 2.5 L of the dichloromethane solution, with agitation under argon for an additional 3 h. The solvent was removed, pooled together, and dried over anhydrous sodium sulfate. The extract was filtered and concentrated to ca. 100 mL using a Vigreux distillation column (60 cm) and further fractionated by Solvent Assisted Flavor Evaporation (SAFE) (23). The volatile isolate was neutralized by washing 3 times with a 1:1 volume of 0.5 M sodium bicarbonate and once with a half volume equivalent of saturated sodium chloride (15). Anhydrous sodium sulfate
was added and the solution was frozen prior to a final distillation to 1 mL. The isolate was frozen (-20 °C) prior to further analysis.

### 3.3.5. Gas Chromatography-Olfactometry-Mass Spectrometry (GC-O-MS): Aroma Extract Dilution Analysis (AEDA)

GC-O analysis was performed on a GC (Agilent Technologies, model 6890N, Santa Clara, CA) equipped with a DB-5ms column (30m x 0.25mm ID x 0.25μm film thickness; J&W Scientific, Folsom, CA) coupled with a MS (Agilent Technologies, model 5973, Santa Clara, CA, operated in EI mode) and a Gerstel sniffing port (Gerstel, Inc, model ODP 2, Linthicum, MD). The effluent was split 1:1 after separation, between the MS and ODP 2 port. Purified air was bubbled through distilled water and purged the end of the sniffing arm at a rate of 20 mL/min. The GC conditions were as follows: the sample (0.5 μL) was injected using a cold on-column injection technique, with the helium carrier gas set to a constant flow of 1 mL/min. The oven program was 40 °C for 2 min and then the temperature was increased at a rate of 3 °C/min until 150 °C and then raised by 30 °C/min to 250 °C and held for 5 min. Each sample was serially diluted by half volume in dichloromethane until no further aromas were detected. The largest dilution at which each aroma was detected was defined as the flavor dilution (FD) value. Each dilution was analyzed in duplicate by two panelists (1 female, 1 male).

Positive compound identifications were based on comparison with mass spectra, odor, and LRI of the authentic compound. LRI values were calculated using an n-alkane ladder. Nine of the 10 aroma compounds (d-1, d-3-10; Table 3-1) were positively identified on the same GC/MS system described above with the slight modification that the effluent
flow was stopped to the olfactometry port. All samples were run on two columns of different polarities; DB-5ms (30m x 0.25mm x 0.25μm film thickness; J&W Scientific, Folsom, CA) and DB-Wax (30m x 0.25mm x 0.25μm film thickness; J&W Scientific, Folsom, CA). GC conditions for DB-5ms were same as above whereas those for DB-Wax were as follows: 40 °C for 2 min, ramped at 5 °C/min to 230 °C and held for 5 min. The MSD conditions were as follows: capillary direct interface temperature at 250 °C, source temperature at 150 °C, mass range 35-250 amu at 6.35 cycles/min. The positive identification of the remaining compound, 2AP (d-2, Table 3-1), was conducted on a GC (Agilent Technologies, model 6890, Santa Clara, CA) coupled to a Varian MS-Ion Trap (Saturn 3, Agilent Technologies, Santa Clara, CA) equipped with a DB-5ms column (30m x 0.25mm x 0.5μm film thickness; J&W Scientific, Folsom, CA). The GC conditions were the same as above with the exception 1 μL was injected in splitless mode at 200 °C. The MSD conditions were as follows: transfer line temperature at 250 °C, ion trap temperature at 150 °C, mass range 35-150 amu.
Table 3-1. GC/MS-CI quantification parameters for select aroma compounds

<table>
<thead>
<tr>
<th>Compound a</th>
<th>Code</th>
<th>Molecular weight</th>
<th>CI ion (unlabeled)b</th>
<th>CI ion (labeled)b</th>
<th>Calibration factor</th>
</tr>
</thead>
<tbody>
<tr>
<td>2-methylpropanal</td>
<td>d-1</td>
<td>72</td>
<td>73</td>
<td>80</td>
<td>1.00</td>
</tr>
<tr>
<td>2-acetyl-1-pyrroline</td>
<td>d-2</td>
<td>111</td>
<td>112</td>
<td>115</td>
<td>0.95</td>
</tr>
<tr>
<td>2-ethyl-3,5-dimethylpyrazine</td>
<td>d-3</td>
<td>136</td>
<td>137</td>
<td>142</td>
<td>0.87</td>
</tr>
<tr>
<td>methional</td>
<td>d-4</td>
<td>104</td>
<td>105</td>
<td>108</td>
<td>0.96</td>
</tr>
<tr>
<td>(E)-2-nonenal</td>
<td>d-5</td>
<td>140</td>
<td>141</td>
<td>143</td>
<td>0.84</td>
</tr>
<tr>
<td>2,4-dihydroxy-2,5-dimethyl-3(2H)-furanone</td>
<td>c-6</td>
<td>144</td>
<td>145</td>
<td>149</td>
<td>0.69</td>
</tr>
<tr>
<td>2-acetyl-2-thiazoline</td>
<td>d-7</td>
<td>129</td>
<td>130</td>
<td>134</td>
<td>1.00</td>
</tr>
<tr>
<td>(E,E)-2,4-decadienal</td>
<td>d-8</td>
<td>152</td>
<td>153</td>
<td>155</td>
<td>0.87</td>
</tr>
<tr>
<td>2-phenylethanol</td>
<td>d-9</td>
<td>122</td>
<td>105</td>
<td>107</td>
<td>1.00</td>
</tr>
<tr>
<td>4-hydroxy-2,5-dimethyl-3(2H)-furanone</td>
<td>d-10</td>
<td>128</td>
<td>129</td>
<td>135</td>
<td>1.00</td>
</tr>
</tbody>
</table>

a Compound positively identified (LRI, MS, and authentic compound), b Select ion monitored

3.3.6. Hydroxycinnamic Acid Analysis

Phenolic acids were characterized based on previous methods reported by Krygier et al., (24) and Sosulski et al., (25). Briefly, 10 g of the starting material (RWF or WWF) was rinsed twice with hexane (25 mL). The solid material was washed six times (50 mL) with a solution of acetone-methanol-water (35-35-30). The extract was pooled and solvent removed in vacuo. The pH was adjusted to 2 (HCl) and then extracted with hexane (1:1; 5 times) to ensure removal of all lipids. This resulting aqueous layer containing free and soluble conjugate phenolics was then extracted 6 times, with 1:1 volume of diethyl ether-ethyl acetate, to separate the free phenolic acids. The remaining aqueous material was digested for 4 h with 2 N sodium hydroxide (200 mL), with shaking, under an atmosphere of argon. The pH of the resulting digest was decreased to pH 5 and was then extracted 6 times with a 1:1 volume of diethyl ether-ethyl acetate. The original meal was also digested for 4 h
with 2N sodium hydroxide (200 mL), under a blanket of argon with shaking. Following digestion, the meal was defatted with hexane (1:1; 5 times), and the phenolics were extracted 6 times with a 1:1 volume of diethyl ether-ethyl acetate. All diethyl ether-ethyl acetate extracts were evaporated in vacuo to dryness and then re-dissolved in methanol. Extracts were frozen (-20 °C) prior to LC-MS analysis.

3.3.7. Quantification of Ferulic Acid (FA) Liberated in Whole Wheat Bread Crust during Manufacture

Only the quantification of FA from WWF bread crust, outlined in this subsection, and not the subsequent aroma analysis, was performed by Q. Bin and was her contribution to the submitted paper.

The release of FA from its bound form during bread production was quantified by utilizing carbon-13 labeled FA as an internal standard that was applied on the top of the dough (crust region) prior to baking. \(^{13}\)C\(_6\)-benzene ring labeled FA was synthesized according to (26). Immediately prior to baking, a 2.5 mL aqueous solution (606 mg labeled FA/L) was sprayed evenly onto the surface of the bread dough. Immediately after baking, the bread was allowed to cool to room temperature, the top portion of bread crust was removed, ground, and a 5 g subsample was extracted with 20% ethanol aqueous solution (50 mL) for 16 h. The extract was then centrifuged at 4000 rpm at 5 °C for 20 min (Beckman Coulter, model Allegra X-30, Brea, CA) and filtered (cellulose fiber papers, grade P5, Fisher Scientific, Fairlawn, NJ), and the permeate was pooled. The ethanol was removed under vacuum and subsequently freeze-dried. The freeze-dried material was dissolved in 25 mL of 10% methanol in water solution, after which 5 mL was loaded on a 500 mg preconditioned
C18 cartridge (Supelco, Bellefonte, PA) and eluted with 5 mL of methanol. The methanol from the eluent was removed under vacuum and the concentrate was filtered over a 0.20 μm Nylon syringe filter (Millex, Billerica, CA) and analyzed by liquid chromatography-mass spectrometry (LC-MS).

LC-MS analysis was conducted using a Waters ACQUITY UPLC system interfaced with a Quattro Premier XE™ mass spectrometer (Waters, Milford, MA). A sample (2 μL) was separated on an ACQUITY UPLC BEH C18 1.7 μm column (2.1mm × 50mm) (Waters, Milford, MA) at 25 °C. The mobile phase was maintained at a flow rate of 388 μL/min using a 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), was held at 100% B (8-9 min), decreased to 10% B (9-10 min), and was held at 10% B (10-20 min). The MS operation parameters were as follows: positive electrospray ionization (ESI+), source temperature, 110 °C; desolvation temperature, 350 °C; capillary voltage, 3.0kV. The mass analyzer was set for multiple reaction monitoring (MRM) mode. The ion transitions for FA along with the cone voltages and collision energies were as follows: 195.29 > 177 for unlabeled FA; 201.31 > 183.02 for labeled FA; cone 20V; collision 10V. Samples were analyzed in duplicate.

3.3.8. Refined (RWF) Bread Made with Flour Spiked with FA

Breads formulated with RWF spiked with FA were made as described previously with the exception that 68 mg FA/kg flour was blended with the flour prior to addition of the other ingredients. The baking and extraction protocol was as described above in Sections 3.3.3. and 3.3.4.
3.3.9. Quantification of Aroma Compounds

The selected compounds (Table 3-1) were quantified by stable isotope surrogate standards consisting of deuterium or carbon-13 labeled analogs (Figure 3-1). Those isotopically labeled compounds needed for this study, that were not provided as previously mentioned, were synthesized as previously reported: \([^2\text{H}_7]-\text{d}-1\) (27), \([^3\text{H}_5]-\text{d}-3\) and \([^4\text{H}_4]-\text{d}-7\) (28), \([^2\text{H}_2]-\text{d}-5\) (29), \([^{13}\text{C}_4]-\text{c}-6\) (21), and \([^2\text{H}_2]-\text{d}-9\) (30).

Bread samples were prepared as previously described (see Sections 3.3.3. and 3.3.4.) with the exception that the labeled standard compounds were added to the initial extraction.
solvent. The amounts of labeled standards added to extraction solvent for RWF bread were as follows (reference corresponding compound names in Table 3-1): d-1 (2450 μg); d-2 (6.5 μg); d-3 (2 μg); d-4 (60 μg); d-5 (6 μg); c-6 (330 μg); d-7 (25 μg); d-8 (5 μg); d-9 (1200 μg); d-10 (7000 μg); whereas for the WWF bread were as follows, d-1 (2830 μg); d-2 (1.5 μg); d-3 (4 μg); d-4 (220 μg); d-5 (10 μg); c-6 (220 μg); d-7 (25 μg); d-8 (20 μg); d-9 (600 μg); d-10 (5750 μg); while for the RWF bread spiked with FA were as follows, d-1 (2830 μg); d-2 (1.5 μg); d-3 (4 μg); d-4 (220 μg); d-5 (10 μg); c-6 (220 μg); d-7 (25 μg); d-8 (20 μg); d-9 (600 μg); d-10 (5750 μg).

Analysis was performed on a Hewlett Packard GC (Agilent Technologies, model 5890plus, Santa Clara, CA) equipped with a DB-Wax column (30m x 0.25mm ID x 0.5μm film thickness; J&W Scientific, Folsom, CA) that was coupled with a mass detector (Agilent, model 5972) using Chemical Ionization (CI) with isobutane as the reagent gas, under Selective Ion Monitoring (SIM) mode for maximum sensitivity. The ions monitored are listed in Table 3-1. The GC conditions were as follows: constant flow rate 1 mL/min (helium), 1 μL of the sample was injected under splitless mode, inlet temperature was 200 °C, oven program was 40 °C and held for 2 min, followed by an increase of 15 °C/min to 115 °C and was held for 10 min. The temperature was then increased 5 °C/min to 165 °C and then increased 25 °C/min to 230 °C and held for 5 min. Quantification was based on five point calibration curves (r² > 0.97 for all compounds).

3.3.10. Sensory Evaluation

Descriptive sensory analysis was conducted on three bread samples: RWF bread crust, WWF bread crust, and WWF bread crust with added flavor compounds (see Table 3-
The panel consisted of six females and four males, ages 22 to 46, and was trained on the “fresh refined bread crust” aroma attribute over 12 x 1 h sessions. This attribute was chosen prior to the start of the training to investigate if a quantitative deficiency in Maillard-type aroma compounds in WWF bread, as a result of the presence of HCAs, results in a significantly appreciable aromatic difference to a trained sensory panel, which relates to the major objective of this chapter. Alternatively, a triangle test could be used to measure global (overall) differences among the three samples. Should all three samples be found to be statistically different from one another in a triangle test, this would indicate that changes in Maillard reaction products is only one explanation for the difference in aroma between RWF and WWF breads; however this test would not be able isolate if the difference in concentration of specifically the Maillard reaction products results in significant aroma differences detected by a trained panel. All samples and standards were presented, under red lights, in amber bottles (60 mL), fitted with conical polyethylene lined lids, which were filled with 10 g samples for analysis. The intensity of the “fresh refined bread crust” aroma was based on the intra-modality n-butanol scale in water (31). Three standard references of 4, 6, and 8 on a 12 cm line scale were used, and were determined an appropriate scale for the bread crust samples. Six test sessions were completed to familiarize the panelists with the butanol scale; each panelist could identify unknown reference samples within a unit of 1.

RWF and WWF bread samples for sensory evaluation were prepared as previously described (see Section 3.3.3.). Immediately after baking, the crust was separated from the bread, frozen in liquid nitrogen, and finely ground. The crust of each sample was then stored in glass jars with Teflon-lined lids at -80 °C under a blanket of argon and used within 24 h after baking. Two h prior to analysis the samples were removed from the freezer to
come to room temperature. For the additional WWF bread sample with added flavor compounds, the five compounds in **Table 3-4** were added in a 10 μL ethanol solution to 10 g WWF bread crust 30 min prior to sensory evaluation, and mixed gently, via rotation, until analysis.

Products were evaluated in duplicate over two sessions in one day, with replicates of samples included at both sessions. The samples were presented to the panelists in coded amber bottles in random order. The data were analyzed by a general linear model analysis of variance test with sample, panelist, and their interaction as variation factors. Statistical differences between samples were analyzed by Tukey’s pairwise comparison (Statistix 9.0, Tallahassee, FL).

### 3.4. Results and Discussion

Differences in the aroma composition of bread crust formulated with refined wheat (RWF) versus whole wheat (WWF) flour were characterized by comparing the AEDA FD-values for each sample. Compounds that had a FD-value ≥ 16 with at least a 2-fold difference between the two samples were selected (shown in **Table 3-2**) and were subsequently quantified (**Table 3-3**). In the RWF bread crust, 2AP, HDMF, 2PE, 2A2T, and DHDMF, were reported to be higher in concentration, by 4.0, 3.0, 2.1, 1.7, and 1.5-fold, respectively; whereas EDMP, (E,E)-2,4-decadienal, and (E)-2-nonenal were at present at lower concentrations by 6.1-, 2.1-, and 1.8-fold, respectively. No significant differences between 2MP and methional were observed between RWF and WWF samples (which are within expected error of FD values).
Table 3-2. Odorants with FD-factor ≥ 16 and difference ≥ 2 between refined (RWF) and whole wheat (WWF) bread crust

<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>2-methylpropanal</td>
<td>malty</td>
<td>&lt;800</td>
<td>594</td>
<td>128</td>
<td>512</td>
<td>0.25</td>
</tr>
<tr>
<td>2-acetyl-1-pyrroline</td>
<td>corn chip</td>
<td>1335</td>
<td>920</td>
<td>128</td>
<td>32</td>
<td>4</td>
</tr>
<tr>
<td>2-ethyl-3,5-dimethylpyrazine</td>
<td>earthy</td>
<td>1457</td>
<td>1080</td>
<td>32</td>
<td>64</td>
<td>0.5</td>
</tr>
<tr>
<td>methional</td>
<td>potato</td>
<td>1461</td>
<td>907</td>
<td>128</td>
<td>64</td>
<td>2</td>
</tr>
<tr>
<td>(E)-2-nonenal</td>
<td>cucumber</td>
<td>1534</td>
<td>1159</td>
<td>32</td>
<td>64</td>
<td>0.5</td>
</tr>
<tr>
<td>2,4-dihydroxy-2,5-dimethyl-3(2H)-furanone</td>
<td>caramel</td>
<td>1540</td>
<td>979</td>
<td>64</td>
<td>16</td>
<td>4</td>
</tr>
<tr>
<td>2-acetyl-2-thiazoline</td>
<td>corn chip</td>
<td>1772</td>
<td>1102</td>
<td>128</td>
<td>32</td>
<td>4</td>
</tr>
<tr>
<td>(E,E)-2,4-decadialen</td>
<td>fatty</td>
<td>1815</td>
<td>1317</td>
<td>32</td>
<td>128</td>
<td>0.25</td>
</tr>
<tr>
<td>2-phenylethanol</td>
<td>flowery</td>
<td>1916</td>
<td>1112</td>
<td>128</td>
<td>64</td>
<td>2</td>
</tr>
<tr>
<td>4-hydroxy-2,5-dimethyl-3(2H)-furanone</td>
<td>caramel</td>
<td>2043</td>
<td>1063</td>
<td>128</td>
<td>64</td>
<td>2</td>
</tr>
</tbody>
</table>

\(^a\)Compound positively identified (LRI, MS, and authentic), \(^b\)Odor described at the GC sniffing port during GC-O, \(^c\)Flavor Dilution (FD) factors based on the average from two panelists
Table 3-3. Quantification of select aroma compounds in refined (RWF), whole wheat (WWF), and refined (RWF) with spiked ferulic acid bread crust samples

<table>
<thead>
<tr>
<th>Compound</th>
<th>Concentration in RWF Bread Crust (μg/kg crust)</th>
<th>Concentration in WWF Bread Crust (μg/kg crust)</th>
<th>Concentration in RWF Bread with 68mg ferulic acid/kg flour a (μg/kg crust)</th>
<th>Concentration Ratio (RWF/WWF Bread Crust)</th>
</tr>
</thead>
<tbody>
<tr>
<td>2-methylpropanal</td>
<td>4868</td>
<td>5517</td>
<td>ND</td>
<td>0.9</td>
</tr>
<tr>
<td>2-acetyl-1-pyrroline</td>
<td>10.4</td>
<td>2.6</td>
<td>0.94</td>
<td>4.0</td>
</tr>
<tr>
<td>2-ethyl-3,5-dimethylpyrazine</td>
<td>0.07</td>
<td>0.43</td>
<td>0.21</td>
<td>0.2</td>
</tr>
<tr>
<td>methional</td>
<td>109.3</td>
<td>124.6</td>
<td>ND</td>
<td>0.9</td>
</tr>
<tr>
<td>(E)-2-nonenal</td>
<td>1.2</td>
<td>2.2</td>
<td>1.1</td>
<td>0.5</td>
</tr>
<tr>
<td>2,4-dihydroxy-2,5-dimethyl-3(2H)-furanone</td>
<td>640</td>
<td>436</td>
<td>213</td>
<td>1.5</td>
</tr>
<tr>
<td>2-acetyl-2-thiazoline</td>
<td>30.7</td>
<td>18.1</td>
<td>7.4</td>
<td>1.7</td>
</tr>
<tr>
<td>(E,E)-2,4-decadienal</td>
<td>0.7</td>
<td>1.5</td>
<td>0.7</td>
<td>0.5</td>
</tr>
<tr>
<td>2-phenylethanol</td>
<td>311</td>
<td>149</td>
<td>93</td>
<td>2.1</td>
</tr>
<tr>
<td>4-hydroxy-2,5-dimethyl-3(2H)-furanone</td>
<td>3417</td>
<td>1149</td>
<td>713</td>
<td>3.0</td>
</tr>
</tbody>
</table>

a ND = not determined

Many of the compounds reported in Table 3-2 have been previously identified as important wheat bread odorants (e.g. 2AP, EDMP, methional, (E)-2-nonenal, (E,E)-2,4-decadienal, 2PE, and HDMF) (8). Notably, 2AP, the compound with the largest concentration reduction in WWF bread (in comparison to RWF bread, Table 3-3), is considered to be one of the most important bread aroma compounds (32). The perception of this compound is facilitated by an extremely low odor threshold (0.0073 μg/kg in starch) (33) and in addition to degrading rapidly after baking, is thought to also play a major role in stale bread flavor (34).

In general, wheat bread formulated with RWF generated more Maillard-type products with desirable corn chip, caramel, and floral notes (Table 3-2 & 3-3). 2PE can be
produced by the reduction of the Strecker aldehyde, phenylacetaldehyde, as well as by fermentation pathways. Wheat bread with WWF produced more compounds with cucumber and fatty notes ((E)-2-nonenal and (E,E)-2,4-decadienal respectively) which are typically generated through lipid oxidation (35). WWF bread crust also had a higher concentration of a select pyrazine, EDMP, with earthy notes. Between the decreased quantity of 2AP and the generation of lipid oxidation compounds during bread storage, WWF bread crust shows many similarities in aroma to stale bread flavor (34). The increase in lipid oxidation products in WWF bread (Table 3-3) can be related to the additional lipid material (germ) and more particularly the hydroperoxide content of said material (9).

The observed differences between RWF and WWF bread samples, with respect to chemical composition, were also in agreement with the descriptive sensory analysis. A trained sensory panel reported the intensity of “fresh refined bread crust” aroma was significantly lower in the WWF versus the RWF bread (α = 0.05; see Figure 3-2). To further study the importance of the Maillard-type aroma compounds in RWF bread aroma, an aroma recombination study was conducted. The compounds reported to be higher in concentration in RWF bread (2AP, DHDMF, 2A2T, 2PE, and HDMF) were added at equivalent concentrations (compared to the RWF bread) to the WWF sample (see Table 3-4). Adding these compounds back to the WWF bread crust increased the “fresh refined bread crust” aroma and resulted in no statistical differences in the intensity of this aroma character when compared to the RWF sample (Figure 3-2). This indicated that the lower concentration of Maillard reaction compounds is a major factor in the observed difference in aroma between the two types of bread (Table 3-4). These results further relate back to
model systems where ferulic acid (FA) was shown to inhibit the formation of aroma compounds upon heating (11,12).

**Figure 3-2.** Mean intensity rating scores for “fresh refined bread crust” aroma in three bread crusts: (1) refined wheat (RWF), (2) whole wheat (WWF) with aroma compounds (2-acetyl-1-pyrroline (2AP), 2,4-dihydroxy-2,5-dimethyl-3(2H)-furanone (DHDMF), 2-acetyl-2-thiazoline (2A2T), 2-phenylethanol (2PE), 4-hydroxy-2,5-dimethyl-3(2H)-furanone (HDMF)), and (3) whole wheat (WWF); a Samples with the same letter are not significantly different (α = 0.05)

**Table 3-4.** Aroma recombination model: Whole wheat (WWF) bread crust with equivalent aroma concentration of five select compounds in comparison to refined wheat (RWF) bread crust

<table>
<thead>
<tr>
<th>Compound</th>
<th>Quantity Added in WWF Bread Crust (μg/kg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>2-acetyl-1-pyrroline</td>
<td>8</td>
</tr>
<tr>
<td>2,4-dihydroxy-2,5-dimethyl-3(2H)-furanone</td>
<td>200</td>
</tr>
<tr>
<td>2-acetyl-2-thiazoline</td>
<td>12.5</td>
</tr>
<tr>
<td>2-phenylethanol</td>
<td>160</td>
</tr>
<tr>
<td>4-hydroxy-2,5-dimethyl-3(2H)-furanone</td>
<td>2270</td>
</tr>
</tbody>
</table>
Though the amount of Maillard reaction products were corrected for by adding back aroma compounds to WWF crust, that sample still differed from RWF crust in terms of lipid oxidation concentration. These compounds, (E)-2-nonenal and (E,E)-2,4-decadienal, appear to have a lower effect on “fresh refined bread crust” aroma compared to compounds generated via the Maillard reaction. This indicates that the major mechanism in differences in aroma between the two types of bread is related to modulation of the Maillard reaction during processing.

With all other ingredients being equal, the major differences in aroma between the two types of bread must be due to the inclusion of the additional parts of the wheat seed in WWF bread. The change in flavor related to lipid oxidation appears due to the inclusion of the germ, whereas the alteration of Maillard-type product generation in WWF bread may be related to the phenolic content in the bran portion of WWF. Hydroxcinnamic acid (HCA) phenolic compounds, native to wheat, have been reported to alter Maillard chemistry in model systems by forming new compounds (11,12). One such compound formed in a glucose/glycine/FA model system was proposed as a 4-vinylguaiacol-glycolaldehyde adduct, a compound that was also directly produced after substituting glycolaldehyde for glucose (11). These findings were further substantiated after utilizing the decarboxylated form of FA, 4-vinylguaiacol, in place of FA, and was also found to significantly reduce the generation of Maillard-type aroma compounds (12). Proline/methylglyoxal systems used to mimic bread flavor formation have found similar reduction in aroma compound formation, specifically 2AP, with the addition of FA (11), findings substantiated with time course experiments detailing the significant reduction of methylglyoxal over time (14). Adduct formation with reactive intermediates, such as dicarbonyls, are not unique to HCAs, and can
occur with other phenolic compounds such as flavonoids (15–19), but distinctive of HCAs is also the ability to form adducts with amino acid reaction products (11,12). Though much work has focused on the effect of HCAs to alter aroma compounds generated during the Maillard reaction in simple model systems, to the best of our knowledge they have not been further extrapolated to food, such as wheat bread. The role of HCAs in WWF bread on Maillard-type flavor development was further investigated in the present study.

The concentrations of free, soluble conjugate and insoluble conjugate (bound) FA content were determined in the WWF and RWF utilized (see Table 3-5). FA was found to be present predominantly in the bound form (Table 3-5), which is consistent with the literature; however, enzymatic and fermentation treatments (36), as well as high temperatures (37), such as those processes that happen during bread production, have been shown to increase the bioaccessibility of FA. Because the free form of FA was considered the primary reactive species affecting Maillard chemistry (11,12) and the potential for bound FA to be converted into a free form during fermentation and baking, a method was developed to measure liberated FA. $^{13}$C$_6$-benzene ring-labeled FA was synthesized and a solution was applied to the top of the dough prior to baking to estimate the “free FA” content liberated in the bread crust. LC/MS analysis of the crust revealed approximately 68 mg of FA per kilogram of flour. This value is considered a best estimate, as the calculation is based on the stability of the internal standard (carbon-13 FA). Since it is expected that FA is liberated, in part, during the baking process, the internal standard could potentially undergo more extensive degradation because it was added prior to baking, as a consequence of receiving a higher thermal dose. Nevertheless, this analysis provided a quantitative estimate of the free FA available during bread manufacture. Based on the initial free FA
content in WWF (1.3 mg/kg, **Table 3-5**), approximately 68 mg of free FA/kg flour was
liberated during the manufacturing process (fermentation and baking), which accounted for
27% of the bound phenolic content.

**Table 3-5.** Concentration of bound, soluble conjugate, and free ferulic acid (FA) in refined (RWF) and whole (WWF) wheat flour

<table>
<thead>
<tr>
<th>Type of Phenolic</th>
<th>Type of Flour</th>
<th>FA Concentration (^b) (mg/kg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bound</td>
<td>RWF</td>
<td>ND</td>
</tr>
<tr>
<td></td>
<td>WWF</td>
<td>250</td>
</tr>
<tr>
<td>Soluble Conjugate</td>
<td>RWF</td>
<td>ND</td>
</tr>
<tr>
<td></td>
<td>WWF</td>
<td>2.3</td>
</tr>
<tr>
<td>Free</td>
<td>RWF</td>
<td>ND</td>
</tr>
<tr>
<td></td>
<td>WWF</td>
<td>1.3</td>
</tr>
</tbody>
</table>

\(^a\) All concentrations are based on the same starting weight for each portion, \(^b\) ND = not detected

The influence of the available free FA content in WWF bread on flavor development
was subsequently evaluated by adding an equivalent concentration (68 mg FA/kg flour) to
RWF prior to making bread. The concentrations of select flavor compounds were quantified
and are reported in **Table 3-3**. Overall, the addition of FA to the RWF resulted in a bread
aroma profile similar to the WWF bread sample. When FA was added to RWF bread, the
concentration of five Maillard-type compounds were reduced (2AP, DHDMF, 2A2T, 2PE,
and HDMF) whereas the EDMP content increased. Furthermore, the addition of FA to the
RWF bread did not influence the generation of lipid oxidation products ((E)-2-nonenal and
(E,E)-2,4-decadienal), suggesting that the germ (lipid material) was responsible for the
higher observed levels of these compounds in the WWF sample. Additionally, while FA
can also function as a free radical scavenger capable of inhibiting lipid oxidation reactions (38), no such antioxidant activity was observed.

While there are other differences between breads made with the two types of flour, such as changes in amino acids, and differences in levels of moisture, this was corrected for by adding only free FA to RWF bread. This supports the findings that FA can react with Maillard reaction products, thereby changing the aroma profile of bread. The most notable decrease is that of 2AP. This might indicate that FA plays a major role in quenching the precursors of the compound, supporting previous model system data (11,14). Owing to the fact that 2AP is considered to be the most important wheat bread aroma compound (32), with its absence a major factor in stale flavor (34), and the fact that FA is present in larger amounts in the WWF, could be a predominant reason for the difference in aroma between RWF and WWF bread. Additionally, the amount of reduction of aroma compounds in the RWF bread with added FA model was generally lower than the WWF bread sample. This could be related to the fact that during normal bread baking, FA is gradually released with time, whereas in this model bread system, it was added all at one time.

In summary, these findings indicate the phenolic component of WWF, in part, influences the mechanisms of Maillard-type flavor generation in bread. This leads to a reduction in the development of desirable flavor notes (i.e. 2AP) in WWF compared to RWF formulated products. The observation that FA can be liberated during baking also provides further insight into whole grain food chemistry and related changes in product quality.
3.5. Acknowledgements

This work was supported by the USDA-NIFA program, project number 2009-35503-06066. We thank Smaro Kokkinidou for the synthesis of $[^{2}H_{4}]$-2-acetyl-2-thiazoline, Deshou Jiang for the synthesis of $[^{13}C_{6}]$-ferulic acid, Dr. Gary Reineccius for donating $[^{2}H_{3}]$-2-acetyl-1-pyrroline, $[^{2}H_{3}]$-methional, $[^{2}H_{2}]$-(E,E)-2,4-decadienal, and $[^{2}H_{6}]$-4-hydroxy-2,5-dimethyl-3(2H)-furanone, and ConAgra for donating the flour.

3.6. References


(31) ASTM. Standard practices for referencing suprathreshold odor intensity, E544-10. 2010.


CHAPTER 4: CONCLUSIONS

4.1. Conclusions from Simple Model Systems and Isotopic Labeling Experiments

Overall, through the use of different types of model systems, hydroxycinnamic acids (HCAs) were found to affect the generation of aroma compounds upon heating. In simple aqueous Maillard model systems between glucose and glycine, the addition of various HCAs (ferulic, cinnamic, p-coumaric, and caffeic acid) significantly reduced the generation of several aroma compounds, such as various pyrazines (pyrazine, methylpyrazine, 2,5-dimethylpyrazine) and 2,3-butanedione (diacetyl). Similar suppression was observed with 2-acetyl-1-pyrroline in a methylglyoxal-proline system upon addition of ferulic acid. Differences in the level of suppression were also noted among the HCAs analyzed, with caffeic acid as the most reactive, as it was associated with the greatest suppression in aroma development.

Looking further at the mechanisms of reactivity of these HCAs, model systems were conducted using isotopically labeled glucose and glycine. When ferulic acid was added to the glucose-glycine reaction system, new analytes were detected. Replacing half the glucose with fully labeled $^{13}$C$_6$-glucose, these new analytes were found to incorporate carbon atoms originating from glucose. Analogous experiments conducted with fully labeled $^{13}$C$_2$,$^{15}$N-glycine found that some analytes incorporated either part or the entire glycine molecule into the new structure. Substituting common two- and three-carbon sugar fragments that form during the Maillard reaction, for glucose, lead to the reproduction of these new compounds. This lends further evidence that the addition of HCAs to heated systems can alter aroma
generation by forming adducts with Maillard reaction intermediates, thereby making them unavailable for further reaction.

4.2. Conclusions on the Effect of Hydroxycinnamic Acids Present in Whole Wheat Flour on Bread Crust Aroma

Differences between refined and whole wheat breads were characterized by aroma extract dilution analysis and quantified utilizing stable isotope surrogate standards, with both methods demonstrating similar trends. For refined bread crust, five compounds were reported to be higher in concentration, 2-acetyl-1-pyrroline, 4-hydroxy-2,5-dimethyl-3(2H)-furanone, 2-phenylethanol, 2-acetyl-2-thiazoline and 2,4-dihydroxy-2,5-dimethyl-3(2H)-furanone by 4.0, 3.0, 2.1, 1.7, and 1.5-fold, respectively. These compounds are products of the Maillard reaction except 2-phenylethanol, a product of yeast fermentation. Three compounds were at lower concentration in refined bread crust, 2-ethyl-3,5-dimethylpyrazine, (E,E)-2,4-decadienal, and (E)-2-nonenal by 6.1-, 2.1-, and 1.8-fold, respectively, the latter two compounds resulting from lipid oxidation.

Evaluation of the bread crust by a trained sensory panel reported the perceived aroma intensity of the characteristic “fresh refined bread crust” aroma was significantly higher in the refined sample in comparison to the whole wheat sample. However when the five aroma compounds that were quantified higher in refined bread crust (2-acetyl-1-pyrroline, 4-hydroxy-2,5-dimethyl-3(2H)-furanone, 2-phenylethanol, 2-acetyl-2-thiazoline, and 2,4-dihydroxy-2,5-dimethyl-3(2H)-furanone) were added to the whole wheat crust at equivalent concentrations, no significant differences in the aroma intensity were observed.
4.3. Conclusions on Ferulic Acid Reactivity on Aroma Generation in Bread

Analysis of the wheat flours used in this study determined that the predominant type of HCA present is ferulic acid, which was found mainly as the insoluble conjugate (bound) form, which only occurs in whole wheat flour. The influence of available free ferulic acid in whole wheat bread on aroma development was subsequently evaluated by adding an equivalent concentration (68 mg ferulic acid/kg flour) to refined wheat flour prior to making the bread. This value estimates the amount of ferulic acid released during processing of whole wheat bread. Overall, the addition of ferulic acid to refined wheat bread resulted in an aroma profile similar to whole wheat bread crust. There was a reduction in the generation of five Maillard-type aroma compounds (2-acetyl-1-pyrroline, 2,4-dihydroxy-2,5-dimethyl-3(2H)-furanone, 2-acetyl-2-thiazoline, 2-phenylethanol and 4-hydroxy-2,5-dimethyl-3(2H)-furanone), similar to that observed in whole wheat bread crust. This supports aqueous model system data showing that ferulic acid can quench products of the Maillard reaction, thereby changing the aroma profile of bread, with the most notable decrease for the key bread aroma compound 2-acetyl-1-pyrroline. However, the addition of ferulic acid to the refined wheat flour did not influence the generation of lipid oxidation products ((E)-2-nonenal and (E,E)-2,4-decadienal). This would indicate that in addition to HCAs altering aroma development in bread, a second mechanism of aroma generation, related to lipid oxidation, was also found to lead to differences in aroma observed between the two types of bread.
CHAPTER 5: SUGGESTED FUTURE WORK

5.1. Enhancing Stability of 2-Acetyl-1-pyrroline

Throughout the course of this research, the importance of the roasty and corn chip smelling compound 2-acetyl-1-pyrroline (2AP) has been evident. However, the stability of this compound is notoriously fleeting, with most disappearing within two hours after cooking foods, such as in the case of baking bread (1). It is important to determine the main cause of instability, whether it is due to degradation, further reaction to form other compounds, or diffusion into the environment. Much mechanistic research on 2AP has focused on formation, with little in comparison to study its degradation (1). Investigation of this latter mechanism could result in maintaining higher levels of 2AP in cooked foods, increasing shelf life.

Looking at the effect of compounds, such as hydroxycinnamic acids (HCAs), on model systems can help elucidate reaction mechanisms. For instance, in this study, the addition of the HCA ferulic acid to a refined bread formulation caused a 4-fold decrease in 2AP concentration (see Chapter 3). Although ferulic acid has been shown to form adducts with Maillard reaction intermediates, it is also an antioxidant and can react via free radical based mechanisms (2). Both of these methods of reaction can affect 2AP formation, as the compound has been shown to form via the Maillard reaction upon heating, with part of that mechanism involving oxidation to form the final compound (1).

In addition to studying mechanisms of degradation, further supplementation of appropriate materials could aid in the increase in shelf-life, prolonging the perceived freshness quality that 2AP imparts to bread. For instance, forming stable salts of 2AP with the use of food grade acids has been used to enhance stability in foods, and would be
appropriate in bread as it is already a slightly acidic food (1). Moreover, the use of encapsulation, such as through the use of maltodextrin, gum acacia, and/or β-cyclodextrin, could provide another medium for supplementing levels of 2AP in bread (1).

5.2. Effect and Modulation of Lipid Oxidation Products

Though this work primarily focused on the effect of HCAs on aroma development in various simple and complex (bread) model systems, other methods altering aroma content were shown to occur. Although the presence of HCAs decreased the amount of Maillard reaction products in whole wheat bread, a second cause of aroma modulation was determined, due to a higher amount of lipid oxidation products in whole wheat breads. With all other ingredients in the formulations used in this study being equal, the increase in lipid oxidation was thought to be due to the presence of lipid-rich germ in whole wheat flour. Further work could involve studying the effect of lipid oxidation products. Major focus would be on those compounds that showed in this study to have both a strong intensity and a large difference between the two bread types (i.e. (E)-2-nonenal and (E,E)-2,4-decadienal). Due to their higher presence in whole wheat bread, they could explain part of the lowered acceptability of whole wheat breads (3), and limiting their presence could improve the perceived flavor by consumers.

Additionally, although HCAs have been shown to inhibit lipid oxidation in other systems (2), adding free ferulic acid to refined bread in this study did not affect the generation of lipid oxidation products. The addition of other food phenolics (e.g., green tea- or cocoa-derived compounds) could be studied to determine if reduction of lipid oxidation products occurs, resulting in breads with more a pleasing taste. Furthermore, the
supplementation of such phenolics would result in value-added products that would appeal to consumers, due to the positive perception of green tea and chocolate in the media.

5.3. Effect of Aroma Compounds with Low Flavor Dilution Values

Further study could be performed looking at other modes of aroma modulation in wheat bread. This work only focused on aroma compounds that had high flavor dilution values, as determined by aroma extract dilution analysis (AEDA). Though models, based on quantification of those compounds, mimic the original breads, it is possible that compounds with lower flavor dilution (FD) values could also cause aroma differences between the two types of bread. For instance, although ferulic acid degradation products, such as 4-vinylguaiacol, guaiacol, and vanillin (4), did not have large FD values, their greater presence in whole wheat bread could potentially provide an additional, albeit minor, mechanism in aroma modulation between the two types of bread.

5.4. Production Changes to Bread to Improve Flavor

Further changes in how whole wheat bread is produced may lead to desirable changes in aroma development. For instance, the removal of lipids from whole wheat flour could decrease the production of lipid oxidation products, resulting in a bread with an odor more reminiscent of the oftentimes preferred refined bread. Additionally, increasing the amount of Maillard reaction products, in particular 2AP, such as through supplementation to breads with yeast pre-ferments (5) or amino acid overproducing yeast (6), could also lead to positive aroma development in whole wheat bread. Other roasty-smelling compounds found in bread crust aroma, such as 2-acetyl-2-thiazoline (7,8), could supplement the loss of 2AP
over time. Finally, the use of new wheat varieties in bread production, in particular hard white wheat (versus the traditional hard red wheat) could produce more aesthetically pleasing loaves in terms of a lighter color and a noted more mild taste (9). AEDA experiments could be performed, along with HCA and lipid content analysis, in order to determine what factors have an effect on aroma modulation, and may provide additional mechanisms for control of aroma development in traditional whole (red) wheat breads.

5.5 References


APPENDIX A: EFFECT OF DIGESTION AND PHENOLIC COMPOUNDS ON ACRYLAMIDE AVAILABILITY AND FORMATION

A.1. Abstract

Acrylamide, a possible carcinogen and known neurotoxin, was studied to determine if digestion could have an effect on its bioavailability. Acrylamide concentration in potato chips, as determined by the FDA method, was found to be consistent with an extraction procedure modeled on the human digestion system. However, the digestion simulation did lead to the detection of a key, direct precursor of acrylamide, 3-aminopropionamide (3-APA), which was not observed using the FDA method. The digestion simulation involved the use of enzymes, as well as pH control. Only adjusting pH, according to the digestion simulation, without enzyme addition, was found to also lead to the detection of 3-APA. This suggests that during digestion, acrylamide precursors may be released, or generated, which could have implications on the dietary exposure to acrylamide. If 3-APA could form acrylamide in the body, current acrylamide estimates in foods may be underestimated. Additionally, the influence of the common dietary phenolic compound ferulic acid was studied as to its effect on acrylamide formation. Ferulic acid was found to increase formation of acrylamide in asparagine/glucose model systems. Using fully carbon-13 and nitrogen-15 labeled asparagine indicates that ferulic acid does not directly lead to the formation of acrylamide, but more likely acts to catalyze its formation.
A.2. Introduction

The Maillard reaction can produce aromas and brown colors that are typical in many foods, but can also result in the formation of toxic, or mutagenic, compounds. One example is acrylamide, which is most notably found in high heat-treated starch-containing foods, such as French fries, potato chips and bread (1–6). Acrylamide can have several harmful effects in the body, from reacting with amino acid residues (7,8), to alkylating DNA (9), to causing tingling or numbness in the extremities, and even cerebellar dysfunction with enough exposure (10).

Due to its effects on the body, and its presence in food, significant attention has focused not only on acrylamide content, but also the mechanisms of formation, and methods for reducing its levels in food. Asparagine is the main amino acid precursor of acrylamide, providing the carbon backbone for the compound (11). Asparagine heated by itself can form acrylamide, however the presence of carbohydrates significantly increased the yield of acrylamide from asparagine (4,11). It is thought that carbonyl compounds activate asparagine by forming a Schiff base which can decarboxylate to form 3-aminopropionamide (3-APA), and then with a loss of ammonia, can result in acrylamide formation (12). Model systems using 3-APA formed more acrylamide than with asparagine (12,13). 3-APA has also been found in foods, such as potatoes (13), and with knowing how potent a precursor it is in the formation of acrylamide, may indicate that previous measurements of acrylamide in foods could underestimate the ultimate effect in the body.

Most acrylamide extraction procedures involve the use of water, at neutral pH, as the molecule is extremely soluble in water (14). However, several labs have looked at the effect of digestive enzymes (15,16) and pH (16,17) on acrylamide extraction. Thinking that some
acrylamide might be bound up in starch or protein matrices in foods. Jezussek and Schieberle (15) looked at the effect of using digestive enzymes (α-amylase, protease) on acrylamide extraction, and found no difference compared to control. Eriksson and Karlsson (16) also looked at the effect of digestive enzymes (α-amylase, protease, amyloglucosidase) on acrylamide extraction, however they based their procedure on an established protocol for dietary fiber extraction, which involved coupling the addition of enzymes with changes in pH, varying from as high as 7.5 down to 4.4 for the entire procedure. This protocol resulted in increased levels of extracted acrylamide, however the enzymes were not necessary, as only the changes in pH appeared to result in the increased extraction numbers. Eriksson and Karlsson (16) also looked at the effect of pH over a larger range and found a maximum of measured acrylamide at pH 12, and attributed this result to better extraction from the matrix. However, this last observation is most likely due to the formation of artifacts (17), which are most likely not bioavailable (18).

Based on these studies, the effect that digestion and cooking can have on acrylamide fate in vivo and in simple model systems respectively was further examined. First, the effect of enzymes and pH was studied in a system that mimics human digestion. Additionally, the role of dietary phenolic compounds, naturally found in potatoes and cereal grains, were further studied to determine the effect they could have on acrylamide formation.

A.3. Materials and Methods

A.3.1. Chemicals

The following chemicals were obtained from the Sigma Aldrich Company (St. Louis, MO): acrylamide, formic acid (99%), glacial acetic acid (99%), pepsin, pancreatin, bile
extract, hydrochloric acid, sodium bicarbonate, sodium chloride, sodium hydroxide. Labeled compounds $^{13}$C$_3$-acrylamide, $^{13}$C$_6$-glucose, $^{13}$C$_4$, $^{15}$N$_2$-asparagine were obtained from Cambridge Isotope Laboratory (Andover, MA). The solvents acetonitrile, methanol, 2-propanol were obtained from Fisher Scientific (Fairlawn, NJ). Maxi-Spin filter tubes (0.45μm PVDF) were obtained from Alltech Associates (Deerfield, IL). Oasis HLB 6mL solid phase extraction cartridge, 200mg packing were obtained from Waters Corporation (Milford, MA). Bond Elut – Accucat (mixed mode, C8, SAX, and SCX) 3mL solid phase extraction cartridges with 200mg packing were obtained from Varian, Inc. (Harbor City, CA). Potato chips (Middleswarth Potato Chip Company, Ira Middleswarth and Son, Inc., Middleburg, PA) were bought from a local vendor.

A.3.2. Extraction Procedures

Acrylamide extraction, and subsequent cleanup, was performed as per the FDA protocol, as published by Roach et al., (19), and was performed in triplicate. The resultant extracts were analyzed via LC-MS/MS.

Acrylamide extraction was also performed under digestion conditions, in triplicate, and was adapted from the procedure of Garrett et al., (20). Briefly, 4 g of crushed potato chips (from a freshly opened package of plain Middleswarth brand chips) were placed in an amber bottle. A saline solution (30 mL, 0.85 wt% NaCl) was added, as well as 2 mL of the internal standard, $^{13}$C$_3$-acrylamide (2 μg/mL). The pH was lowered to 2 with 1 M HCl, after which 2 mL porcine pepsin solution (40 mg/mL in 0.1 M HCl) was added. The solution was set in a shaking water bath for one hour (37°C, 95 rpm). The pH was then raised to 5.3 with a 0.9 M NaHCO$_3$ solution, after which 9 mL (in 0.1 M NaHCO$_3$) of a bile extract (12
mg/mL) and pancreatin (2 mg/mL) solution was then added. The pH was then raised to 7.5 with 1 M NaOH and then incubated as before for two hours. After the second digestion step, the sample was centrifuged and filtered and went through the SPE cleanup as described by Roach et al., (19).

A.3.3. Model Systems

Acrylamide model systems (performed in triplicate) were conducted with equimolar (10 mM) concentrations of reactants (glucose + asparagine; glucose + asparagine + ferulic acid) in 100 mL of 0.1 M phosphate buffer (pH 7.0). The solutions were heated in a 600 mL Parr reactor (Parr Instrument Company, Moline, IL) at 170°C for 30 min under constant agitation, after which 1 mL of the reaction mixture was added to 3 mL deionized water and 1 mL of the internal standard, 13C3-acrylamide (200 ng/mL in water) (21). Sample preparation (SPE) was conducted as previously mentioned (19).

A.3.4. Liquid Chromatography-Tandem Mass Spectrometry (LC-MS/MS)

LC-MS/MS analysis was conducted with a Shimadzu HPLC system (Columbia, MD) equipped with two pumps (LC-10ADvp), a degasser (DGU-14A), and autosampler (SIL-10vp) interfaced with a Waters Micromass triple quadrupole mass spectrometer (Milford, MA). A sample (10 μL) was separated on a Restek Ultra Aqueous C18 column (2.1 mm x 250 mm, 5 μm particle size; Bellefonte, PA). The mobile phase was maintained at a flow rate of 200 μL/min using a solution of 0.5% methanol/0.1% formic acid in water, with a post-column addition of 50 μL/min 1% acetic acid in 2-propanol. Each run was 10 min long. Analytes were analyzed via positive electrospray ionization (ESI+) with the following
conditions: source temperature, 100°C; desolvation temperature, 250°C; capillary voltage, 3.0 kV. The mass analyzer was set for multiple reaction monitoring (MRM) mode. The ion transitions for acrylamide and $^{13}$C$_3$-acrylamide were 72$\rightarrow$55 and 75$\rightarrow$58 respectively. Cone voltage was 20V and collision energy was 10V.

A.4. Results and Discussion

Most of the data surveying the amount of acrylamide in food utilizes simple water extractions, such as the method used by the FDA (19). There is relatively little focus on looking at the effect of human digestion on the fate of acrylamide in the body, or quantifying potent precursors (e.g. 3-APA) that could potentially lead to the formation of acrylamide in vivo. Experiments were performed mimicking human digestion, which also involved looking at the effect of changing pH on acrylamide extraction in the absence of enzymes, as compared to the standard FDA procedure. Though other papers have used enzymes to look at the effect on extraction and found no difference (15,16), these papers did not adequately mimic the human digestion system. This digestion method was then tested and compared to that of Roach et al., (19) for acrylamide quantification.

The amount of acrylamide detected in potato chips based on both methods is shown in Table A-1. Overall, no statistical differences were reported in the amount of acrylamide detected by either of these two analytical methods, which is similar to previous studies using enzymes (15,16). However, a unique observation was observed for the digestion protocol in comparison to the FDA method. For only the digestion assay, 3-APA was detected during analysis (Figures A-1, A-2). This is interesting from the standpoint that 3-APA is known as a potent precursor of acrylamide, as it can undergo deamination to directly generate
acrylamide (12). In order to identify if the detection of 3-APA during the digestion assay was related to the enzymatic or pH conditions, two further assays were run, one without enzymes and another without enzymes, as well as no changes in pH (Figures A-3, A-4). The presence of 3-APA was still observed when the enzymes were removed (Figure A-3), however it was not present in the samples without enzymes and pH adjustment, similar to the FDA procedure (Figure A-4). It would indicate that changes in pH led to the detection of this compound, which suggests that during the digestion process, this key precursor of acrylamide was identified, at least in potato chips. This may be important in terms of biological exposure, as it may indicate that amounts of acrylamide are being underestimated. Further studies need to be conducted to determine the potential toxicity of this compound, and the ability to convert to acrylamide in the body.

Table A-1. Influence of digestion on detected acrylamide concentration in potato chips

<table>
<thead>
<tr>
<th>Analytical Detection method</th>
<th>Mean Concentration a (µg/kg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>FDA (water extraction)</td>
<td>320 A</td>
</tr>
<tr>
<td>Digestion Method</td>
<td>318 A</td>
</tr>
</tbody>
</table>

a Determined by carbon-13 internal standard methodology (MRM 72→55 and 75→58 for acrylamide and 13C3-acrylamide, the internal standard, respectively); Means in the same column having the same letter are not significantly different (α = 0.05).
Figure A-1. LC-MS/MS-ESI (+ve ion mode) chromatogram of acrylamide and $^{13}$C$_3$-acrylamide (internal standard) detected in potato chips by FDA method.

Figure A-2. LC-MS/MS-ESI (+ve ion mode) chromatogram of acrylamide, $^{13}$C$_3$-acrylamide (internal standard), and 3-aminopropionamide (3-APA) detected in potato chips by digestion method.
Figure A-3. LC-MS/MS-ESI (+ve ion mode) chromatogram of acrylamide, $^{13}$C$_3$-acrylamide (internal standard), and 3-aminopropionamide (3-APA) detected in potato chips by partial digestion method (no enzymes).

Figure A-4. LC-MS/MS-ESI (+ve ion mode) chromatogram of acrylamide and $^{13}$C$_3$-acrylamide (internal standard) detected in potato chips by partial digestion method (no enzymes, no pH modifications).
Many studies have looked at ways of mitigating acrylamide formation in foods. Such suggestions range from using different varieties of potatoes or grains, to using the enzyme asparaginase to decrease asparagine content, to adding other amino acids such as glycine or cysteine, and to using antioxidants (see (22)). The latter is an attractive option as adding natural antioxidants would be appealing to consumers who would consider this as value-added to food products. Phenolic compounds have been previously shown to bind two- and three-carbon compounds formed via the Maillard reaction (23–26), so it would be reasonable to think that they might have an effect on acrylamide formation. Capuano et al., (6) found that acrylamide content was higher in refined wheat bread, as compared to its whole wheat counterpart, the latter version, which uses flour that contains the phenolic compound ferulic acid. However the possible effect of antioxidants on acrylamide content have had mixed results (1,3,5,27). These studies all used food extracts that happen have antioxidant power, so the conflicting results may be due to confounding variables from other components which may influence the results. To circumvent this problem, other papers have looked at the effect of using pure phenolic compounds, such as ferulic acid or epicatechin, however, these too have had mixed results (28–31). For the studies that found an increase in formation, none looked at possible mechanisms of formation (29,30).

Ferulic acid, a phenolic compound found in wheat, particularly the bran layer, was used to look at its influence on acrylamide generation. Running model systems of asparagine and glucose, the addition of ferulic acid appeared to catalyze the generation of acrylamide by approximately 30% (Table A-2). Simply reacting asparagine and ferulic acid, in the absence of glucose, increased acrylamide generation, compared to asparagine alone. From these results, it is not clear if the additional acrylamide is formed from the C3
side chain from ferulic acid, or if ferulic acid is somehow catalyzing the formation of acrylamide, or possibly protecting intermediates from destruction. If acrylamide could form from ferulic acid, the C3 side chain could react with ammonia in the system to generate acrylamide (1). To study this, two systems were used, reacting $^{13}$C$_6$-glucose with unlabeled asparagine and ferulic acid (Figure A-5), and also unlabeled glucose, $^{13}$C$_4$,$^{15}$N$_2$-asparagine and ferulic acid (Figure A-6). No acrylamide with any labels is present in the system using labeled glucose, which agrees with previously published research that glucose does not donate any atoms to the structure of acrylamide (4,11,12). Results indicate that the only form of labeled acrylamide occurred in the latter model (Figure A-6), with the only significant amount detected as the fully carbon and nitrogen labeled form, which indicates that ferulic acid does not directly generate acrylamide. Although glucose is not incorporated into the structure of acrylamide, its role is most likely due to the formation of carbonyl intermediates that catalyze Strecker aldehyde formation. It is possible that ferulic acid could also react in this manner, if the hydroxyl group on the benzene ring were oxidized to a carbonyl, it could also catalyze the formation of Strecker aldehydes (32).

### Table A-2. Acrylamide generation in model systems

<table>
<thead>
<tr>
<th>Model system</th>
<th>Acrylamide concentration (μg acrylamide/g asparagine)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glucose + Asparagine $^a$</td>
<td>537 ± 27</td>
</tr>
<tr>
<td>Glucose + Asparagine + Ferulic acid $^a$</td>
<td>695 ± 50</td>
</tr>
<tr>
<td>Asparagine</td>
<td>10</td>
</tr>
<tr>
<td>Asparagine + Ferulic acid</td>
<td>27</td>
</tr>
</tbody>
</table>

$^a$ average of triplicate ± 95% Confidence Interval
Figure A-5. LC-MS/MS-ESI (+ve ion mode) chromatogram of unlabeled acrylamide, $^{13}$C$_3$-acrylamide (internal standard), and $^{13}$C$_3$,$^{15}$N-acrylamide detected in a $^{13}$C$_6$-glucose + unlabeled asparagine + ferulic acid model reaction (30min at 170 °C, pH 7, 10mM/reactant).

Figure A-6. LC-MS/MS-ESI (+ve ion mode) chromatogram of unlabeled acrylamide, $^{15}$N-acrylamide, $^{13}$C$_3$-acrylamide (internal standard), and $^{13}$C$_3$,$^{15}$N-acrylamide detected in an unlabeled glucose + $^{13}$C$_4$,$^{15}$N$_2$-asparagine + ferulic acid model reaction (30min at 170 °C, pH 7, 10mM/reactant).
In conclusion, from a bioavailability standpoint, the FDA sample preparation protocol may be too simplistic in determining acrylamide content in foods. Although enzymatic digestion did not affect the amount of quantifiable acrylamide in potato chips, coupling the digestion with pH changes, which naturally occur during human digestion, did lead to the detection of the immediate precursor to acrylamide, 3-APA. If this precursor could be transformed to acrylamide in the body, simple water extractions may underestimate the dietary exposure to this compound. Additionally, research is conflicted as to the ability of antioxidants, or phenolic compounds, to affect acrylamide content in foods. The present study demonstrates that ferulic acid can catalyze the formation of acrylamide in simple aqueous model systems, possibly through catalysis in the formation of Strecker aldehydes, however this effect is complicated with other confounding factors in more complex food systems.

A.5. References


APPENDIX B: ADDITIONAL METHODS FOR AROMA CHARACTERIZATION OF REFINED AND WHOLE WHEAT BREAD CRUST

B.1. Overview of Procedure Optimization

Throughout the course of this work, a wide variety of bread production techniques and extraction methods were used to optimize the final procedure detailed in Chapter 3. This was necessitated due to the difference in availability of ingredients and bread making equipment at Penn State University and the University of Minnesota. Additionally, difficulties with the compound 2-acetyl-1-pyrroline (2AP) required the adoption of different techniques to allow for detection, identification, and quantification of the compound.

B.2. Aroma Extraction Methods

Two main methods of aroma extraction utilized during this study were solvent extraction and headspace sampling. The latter, which will more closely represent the aroma of bread, is quite difficult to utilize for quantification purposes, due to the extremely low quantity of volatile compounds that are collected. Although there may be enough of a particular type of volatile to be detected by the nose, oftentimes these amounts are below the limits of detection for a mass spectrometer or other type of detector. Additionally, odor intensity measurements by aroma extract dilution experiments would necessitate the baking of a fresh bread for each dilution and replication measurement, for every panelist, adding in additional effects due to human error during preparation of the breads.
B.2.1. Solvent Extraction

Unless otherwise noted, bread crust was prepared as follows: immediately after baking, the crust was separated from the bread, frozen in liquid nitrogen, and pulverized, after which solvent (dichloromethane or ether) was immediately added to the sample. In addition to extraction of aroma compounds, fat is also isolated. To prevent damage to GC columns, the solvent extracts went through further purification to separate the fat (non-volatile) from the aroma (volatile). One such technique is Solvent Assisted Flavor Evaporation (SAFE; Figure B-1), a technique developed by Engel et al. (1).

Injections for analysis were originally performed at 200°C under splitless conditions. However, to minimize losses of 2AP, a cold on-column injection technique was subsequently utilized, where the sample was introduced at 40°C, the same as the starting temperature of the gas chromatography oven program.
Figure B-1. Solvent Assisted Flavor Evaporation apparatus (SAFE; adapted from (1))
B.2.1.1. Soxhlet Extraction (continuous)

Continuous extraction allows for more thorough extraction of aroma compounds from (food) matrices. For a Soxhlet extraction (Figure B-2), solvent is boiled with the vapor traveling up to a cooling chamber and then condenses into a compartment containing the product of interest. Once the level of condensed solvent reaches the top of the siphon, the solvent is drained back into the boiling flask and the process is then repeated. Depending on the size of the Soxhlet, each cycle can take between 15 and 60 minutes to complete. Though compounds are never extracted with 100% efficiency, the amount increases with increased number of extractions.

Figure B-2. Soxhlet apparatus (adapted from (2))
B.2.1.1.1. Long extraction time

Bread crust powder was soaked in dichloromethane overnight (15h) at 4°C. The sample was then transferred to a cellulose thimble, and the extraction procedure lasted for another 8h at 50°C.

B.2.1.1.2. Short extraction time

Though long extraction times are more common in the literature (3–5), the use of the procedure in this study led to the inability to detect 2AP during GCO runs. Due to the ease in which 2AP is degraded (6), a short extraction procedure was attempted to mitigate these consequences. In this case, the bread powder was soaked in dichloromethane for 2h at 4°C, after which the sample was transferred to a cellulose thimble with additional extraction time of 2h at 50°C.

B.2.1.1.3. Effect of liquid nitrogen

Freezing bread crust in liquid nitrogen serves two purposes: to limit the evaporative loss of aroma, and to aid in grinding the sample. Although the bread crust and crumb have distinct differences in color and texture, as a result from the subjection to different cooking temperatures during baking, they are difficult to separate due to the continuous nature of the bread loaf. The dry crust is only a few millimeters thick, but it can flake off from the softer, damper crumb using physical methods such as a mortar and pestle. However this can take extended amounts of time, which could result in variable aroma losses prior to extraction. Liquid nitrogen is used to minimize these losses, however its addition causes the entire sample to become frozen solid, eliminating the ability to completely separate the crust and
crumb. Though care can be taken to remove as much of the crumb from the crust prior to liquid nitrogen addition, some will remain.

Soxhlet extraction was also conducted with only the outermost bread crust (brownest part only; no liquid nitrogen used) to eliminate possible “dilution” effects of the crumb, and extraction proceeded as listed above. This resulted in almost no detectable aroma compounds during GCO analysis, so further extraction procedures continued to use liquid nitrogen in bread crust powder preparation.

B.2.1.2. Simple Extraction

Simple extraction involved the use of two or three additions of solvent to the bread crust powder. It is one of the more common types of extraction for aroma research, as it is rapid, does not require special equipment, and does not lead to the formation of artifacts, which can happen with the use of boiling solvents (as occurs with Soxhlet extraction). These extractions utilized magnetic stirrers as orbital shakers did not produce sufficient agitation.

B.2.1.2.1. Water (ether extractions)

Water was added in various ratios to bread crust powder (1:2, 1:1, 2:1) in an attempt to enhance 2AP extraction. Addition of water could mimic retronasal aroma release in the mouth, where the food comes in contact with saliva. Extractions were performed with ether, as the lower density aided in separation from the water/bread mixture.
B.2.1.2.2. No water (dichloromethane extractions)

Extractions without the use of water were used to more closely resemble an orthonasal aroma profile.

B.2.2. Headspace sampling

Though solvents provide more thorough extraction of aroma compounds, this could potentially alter the aroma profile observed in a food before or during consumption, as solvents cause aroma compounds to release differently from a food than saliva or air. Headspace sampling involves very little, if any, sampling preparation, leading to the lowest formation of artifacts (7).

Most headspace sampling techniques involve the use of cryofocusing, which concentrates the volatiles prior to injection onto a GC column for separation and subsequent analysis. Cryofocusing in this study was achieved through the use of a Varian Chrompack system (model CP4020, Agilent Technologies, Santa Clara, CA). An additional method involves simply placing a length of column near the injection site in a dewar filled with liquid nitrogen that was removed after concentration of the volatiles, and prior to separation by GC (Figure B-3). Great care was taken to minimize the trapping of the most common volatile, water, which can block the desorption trap, leading to ineffective concentration of volatiles prior to injection, leading to poor chromatography.
Sampling occurred at different times during and after baking. In a further attempt to identify 2AP during GCO trials, toasting was also implemented due to the fact that it has been found to re-form highly reactive odor-active compounds (5). For this procedure, the bread was removed from the pan immediately after baking and a 2cm thick slice of bread was cut from the center of the loaf and immediately toasted to different browning levels.

B.2.2.1. Static Headspace Sampling (SHS)

SHS is a simple headspace sampling technique that samples the air directly above the item of interest, without further concentration methods. Freshly baked bread crust was placed in a water-jacketed glass container heated to 50°C that was fitted with an inert septum.
B.2.2.1.1. Direct air injection

A large sampling syringe (50mL) was used to collect a sample of air after various equilibration times. The syringe tip was then inserted into a heated inlet (50°C) to minimize condensation of compounds on the needle tip prior to reaching the cryofocusing trap. This method resulted in the first detection of 2AP in bread during GCO runs in this study.

B.2.2.1.2. Solid Phase MicroExtraction (SPME)

A manual needle assembly fitted with Divinylbenzene/Carboxen/Polydimethylsiloxane fibers (DVB/CAR/PDMS; Supelco, St. Louis, MO) was used for analysis. After equilibration, the fiber assembly was inserted into sample chamber, and the fiber was extended and exposed for 30min. Immediately after adsorption, the SPME assembly was removed and inserted into the GC injection port and the fiber was extended and desorbed for 5min at 250°C.

B.2.2.1.3. Stir Bar Sorptive Extractions (SBSE, Twister®)

A polydimethylsiloxane (PDMS) coated magnetic Twister® bar (Gerstel Inc., Linthicum, MD) was placed above the sample on filter paper, to avoid direct contact with the bread crust. Multiple stir bars were used (up to 8), and kept separate from each other during adsorption. Additionally, a variety of extraction times were used. The samples were desorbed for 10min at 250°C.
B.2.2.2. Dynamic Headspace Sampling (DHS): Purge and Trap

To further concentrate volatiles released from freshly baked bread, the absorbent material Tenax TA (20:35 mesh, 100mg sample, Restek, Bellefonte, PA) was placed in a glass tube, flanked by pieces of glass wool. After sampling, the trap was thermally desorbed at 250°C for 10min prior to analysis.

B.2.2.2.1. Flow sampling pump

A Tenax-filled glass tube was placed at a distance of 2cm above the bread oven to allow for maximum collection of volatiles, minimizing diffusion losses in the room, while also keeping water content to a minimum. A gas sampling pump was set at a flow rate of 20mL/min. The trap was also dried prior to desorption and analysis in an attempt to eliminate water, but possibilities of losses of extremely volatile compounds (such as 2AP), as well as compounds not well adsorbed onto the material (low breakthrough), were a concern.

B.2.2.2.2. Simple purge and flow device

A large, water-jacketed glass container with a Teflon lined lid was fitted with two openings, and was filled with a sample of bread (Figure B-4). One opening was attached to a bag that was connected to an external gas tank. Filling the bag would displace air in the container, forcing volatile compounds out through the second opening fitted with a Tenax trap. Sampling occurred at both room temperature and upon heating to 50°C.
B.2.2.2.3. Dynamic thermal stripper system

An additional purge and trap technique used nitrogen as the purging gas through the use of a Dynatherm purge and trap device (model 1000, CDS Analytical, Oxford, PA). Both “dry” and “wet” samples were analyzed, with the latter involving varying amounts of water at ratios of 1:2, 1:1 and 2:1.

B.2.2.2.4. Direct crust sampling

Though the outermost bread crust was found to contain very little aroma after Soxhlet extraction, the idea that toasting can regenerate desirable bread aroma compounds was applied to a purge-and-trap system. The outermost crust was separated from the bread and 100mg (similar to the amount of Tenax TA material used in other experiments) was placed in a glass tube that was heated to 50°C for 10min. The use of bread crumb was not
possible due to the inhibition of gas flow through the glass tube. This procedure led to a large amount of water production, so 10mg of calcium chloride salt was added to trap water from entering the cryofocusing trap, while remaining inert to the aroma compounds (10).

B.3. Adjustments to Formulations

B.3.1. Yeast

Two types of yeast were used throughout this research, the more common freeze-dried yeast and fresh yeast. The latter type of yeast is more commonly used in commercial preparations, as not only is it attributed to producing more desirable aroma profiles, but is highly perishable. Freeze-dried yeast must be proofed prior to use, which involves their activation in warm water, after which the presence of bubbles (carbon dioxide) indicates that the yeast are alive and undergoing fermentation.

B.3.2. Moisture effects

Refined and whole wheat flours utilize different amounts of moisture for optimal mixing. Whole wheat flour requires additional moisture, which could be lead to a dilution of ingredients. However GCO experiments did not find differences in aroma intensity for refined breads baked at both its optimized moisture level, and at higher levels of moisture similar to that used in whole wheat bread.

B.3.3. Fat effects

Due to the importance of lipid oxidation products on the difference in aroma between refined and whole wheat bread, non-endosperm portions of the wheat seed (referred to as
just “bran”) were defatted prior to use. Bran was defatted twice, with double the volume of hexane. Residual hexane was first removed in a heated vacuum oven and then in a freeze dryer. The freeze dryer also removed water, so the amount of moisture eliminated from the bran was sprayed onto the bran prior to mixing with refined flour for the whole wheat bread formulation. During baking, the aroma produced by these breads did resemble refined wheat bread aroma, however results from quantification experiments were inconclusive.

B.4. References


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