Characterization and Pathway Investigation of Off-Flavor Formation in Aged Commercial Apple and Orange Juice Products

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Alexander R. Amann

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Devin G. Peterson

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Abstract

During nonrefrigerated storage, fruit juices experience flavor quality loss that leads to decreased flavor acceptability and consumer rejection, partly due to nonenzymatic reaction pathways, such as the Maillard reaction and ascorbic acid degradation. Efforts to limit this problem have focused on the implementation of various processing and ingredient technologies aiming to delay the onset of off-flavor formation. However, in order to more effectively optimize processing parameters and formulations to inhibit loss of flavor quality, an improved understanding of the reaction mechanisms that drive off-flavor formation is essential. Knowledge of mechanistic pathways can lead to tailored ingredient formulations for inhibition of off-flavor generation in fruit juice by targeting the reaction pathways and precursors responsible for flavor quality loss during non-refrigerated storage.

The overall goal of this work was to characterize the off-flavor compounds formed during storage of fruit juice and further examine and investigated the mechanisms of off-flavor formation in juice products by examining the main precursors involved in their formation and their flavor impact. Both apple and orange juice were investigated and off-flavor formation was evaluated after 8 weeks of storage at 35°C. For aroma characterization, fruit juice extraction was carried out using stir-bar sorptive extraction techniques. Gas Chromatography-Mass Spectrometry/Olfactometry (GC-MS/O) was utilized to identify off-flavor markers in aged apple and orange juices. Off-flavor markers were selected based on two criteria; compounds were detected by more than 50% of panelists in aged juice extracts and were detected by less than 50% of panelists in fresh juice extracts. Each off-flavor marker compound was positively identified based on the comparison of LRIs, mass spectra, odor descriptions, and standard addition. Furfural, *p*-vinylguaiacol, 4-hydroxy-2,5-dimethyl-3(2H)-furanone, β-fenchyl alcohol, α-terpinolene, α-terpineol,

terpinen-4-ol, and methional were selected as off-flavor markers in orange juice while furfural, 5-methylfurfural, furfuryl alcohol, and β-damascenone were selected as off-flavor markers in apple juice.

Quantification of off-flavor markers was performed via standard addition techniques. Aroma recombination and sensory difference testing was subsequently employed to characterize the impact of the off-flavor markers on the sensory ratings of the juices. The aroma profile of the recombination model observed by the sensory panel of fresh fruit juice with the spiked off-flavor markers at levels quantified in aged (8 weeks) juices was shifted significantly ($p>0.05$) towards the aroma profile of both aged juices, indicating the importance of the selected marker compounds in off-flavor formation.

Labeled stable isotope mass spectrometry was utilized in order to elucidate off-flavor formation pathways. The known precursors of nonenzymatic browning; glucose, fructose and ascorbic acid, were evaluated *via* isotope-ratio experiments in order to understand the contribution of each precursor in the formation of the selected off-flavors. The observation of isotopically-labeled off flavors after storage establishes a causative relationship between the precursor and the off-flavor compound. The labeled precursors added at a known concentration ratio (2:1) to calculate of the percent contribution each individual precursor to the formation of specific off-flavors. Of the eight off-flavor marker compounds examined in orange juice, furfural and 4-Hydroxy-2, 5-dimethyl-3(2*H*)-furanone (DMHF) were formed from the degradation of either sugars or ascorbic acid. Furfural was generated predominately (93%) from ascorbic acid degradation, while Furaneol was generated via sugar degradation pathways (41% Glucose, 51% Fructose). In apple juice, three of the four markers exhibited carbon labeling, with β-damascenone remaining unlabeled. Only a small percentage of Furfural was generated (1%) via glucose and fructose degradation, indicating that other reaction precursors (not ascorbic

acids, glucose or fructose) are involved in its generation. Furfuryl Alcohol was formed predominately from sugar degradation (100% fructose, 50% glucose). The common non-enzymatic marker, 5-methylfurfural was formed in part due to sugar degradation (14% glucose, 20% fructose).

Lastly, the impact of three reactive amino acids on the generation of off-flavor markers during storage was examined by supplementing the juice with four times the native concentration of the selected amino acid. Glutamine (GLN) and Tryptophan (TRP) in orange juice and Glutamine (GLN) and ɣ-aminobutyric acid (GABA) in apple juice were selected as amino acids of interest, as they have been previously cited as being highly-reactive towards nonenzymatic browning in a juice matrices, and being present in relatively high quantitative amounts by weight. In orange juice, both GLN and TRP accelerated the formation of furfural, *p*-vinylguaiacol, Furaneol, α-terpinolene, α-terpineol, and terpinen-4-ol with TRP having the most considerable effect, resulting in increased concentration off-flavor markers after 8 weeks of storage time. In apple juice, both GLN and GABA resulted in the formation of 5-methylfurfural, furfuryl alcohol, and β-damascenone over an 8-week storage time.

This work afforded an improved insight into off-flavor generation pathways in apple and orange juice products, and as a result, a basis for the development of processing and/or ingredient optimization strategies that yield a final fruit juice product with improved flavor quality and high consumer acceptability. Better understanding the influence of fruit composition on the generation of important off-flavors can aid in the development of reformulation approaches that yield juice products with an improved flavor quality throughout shelf life.

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Chapter 1: Introduction & Study Objectives

The consumption of fruit juice became popular with the domestication of crop plants. Though citrus fruits were domesticated around 1000 BC in Southeast Asia, a substantial import into the West was not observed until the 15th Century [3]. Fruit juices at this time were consumed locally and within days after being produced. It wasn't until the adoption of pasteurization in the 1800's that fruit juices were consumed on a large scale because of how prone the raw product is to spoilage by microbial and enzymatic activity. In 1869, an American dentist by the name of Thomas B. Welch, developed a process based on pasteurization to create the first shelf stable grape juice. This process utilized heat to destroy the microbes and yeasts that lead to spoilage and fermentation. With the extension of shelf life and the utilization of newly-built railways during the Industrial Revolution, the demand for fruit juices increased dramatically and supply reached many regions of the United States. Today, fruit juices are marketed as healthy drink alternatives in part due to their vitamin and nutrient contents. One serving of orange juice contains 100% the recommended daily intake of Vitamin C, a natural antioxidant that has been shown to inhibit the onset of various clinical conditions, such as cancer and heart disease [4]. Apple juice contains many phenolic compounds and carotenoids, which also have been shown to decrease the risk of various chronic pathological conditions [5]. Fruit juices are also good sources of dietary fiber, which has been shown to reduce high cholesterol and control blood sugar levels, as well as improve colon health and regularity [6]. As a result, fruit juices have become a staple in the American household, with three-quarters of Americans reporting that they consume 100% juice on a regular basis [7].

Orange and apple juice are the two most widely consumed juices in the U.S. In 2013, it was estimated that the United States per capita consumption of fresh and processed fruit was 117.2 pounds with 42 pounds of that (or 36% of the total U.S. fruit consumption) being orange and apple juice [8] **(Figure 1.1).**

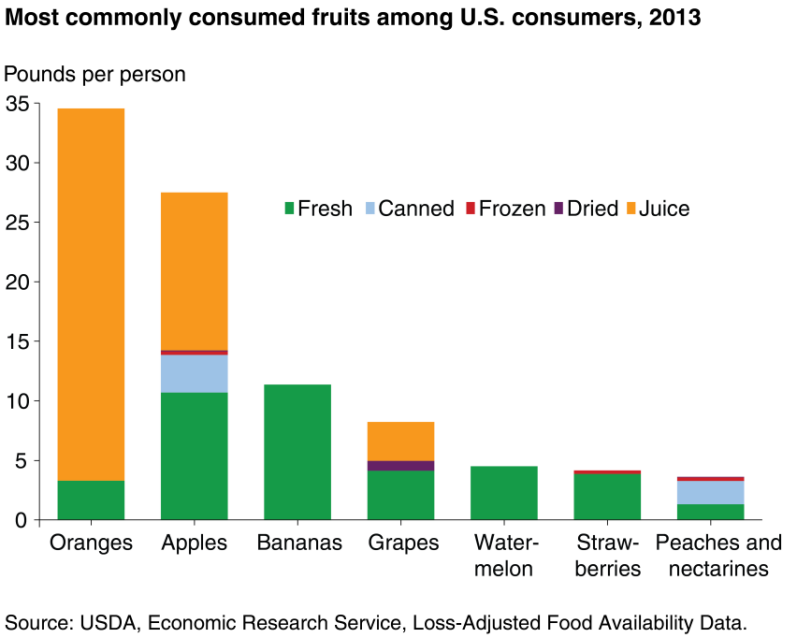


Figure 1.1: Most commonly consumed fruits among U.S. consumers, 2013. Orange and apple juice constitute 36% of total fruit consumed.

Fruit juice presents challenges when attempting to maintain and improve quality because it is highly perishable. Thermal processing is employed to improve stability, but also contributes to the deterioration of flavor quality over storage time. During storage, fruit juice will undergo chemical changes leading to the development of both objectionable flavor and color as well as nutritional loses such as vitamin degradation and the loss of essential amino acids as a result chemical reactions induced via thermal processing [9]. These changes result in a decrease in consumer acceptability, which is known to be a key driving force behind food choice [10].

In order to extend the shelf life of fruit juice, processing technologies are employed to address safety and quality concerns. Processing technologies employ a combination of heat treatments and ingredient additions to inhibit the growth of microorganisms that will spoil the product during storage and include pasteurization, high-pressure treatments, enzyme treatments, filtration, and concentration [11], [12]. In fruit juices, pasteurization combined with aseptic filling is the primary processing technique employed to prolong shelf life. The majority of fruit juices sold commercially in the U.S. are pasteurized because it is the quickest and most cost-effective method to ensure the safety and shelf life extension of a fresh juice product [13]. Without pasteurization, fruit juice quality will rapidly decrease over non-refrigerated storage time due to microbial and enzymatic activity [14]. Aside from microbial load reduction, thermal processing techniques like pasteurization have the added benefit of inactivating enzymes like polyphenoloxidase (PPO), which will oxidize phenolic compounds in the juice to form unfavorable brown-colored quinone compounds over storage [15].

Other processing techniques such as ingredient addition have been developed as a preventative measure to control the non-enzymatic reaction pathways in juice that are associated with quality deterioration. Ascorbic acid is often added to fruit juices to prevent browning, due to its inhibitory effect on PPO. Ascorbic acid reduces the formed quinone compounds back to their original orthophenolic form, resulting in a decrease in color development [16]. It is important to note that the addition of ascorbic acid is known to be a source of off-flavor development during storage [17], which further warrants investigation into off-flavor precursors and routes of off-flavor formation.

While pasteurization and ingredient addition technologies offer advantages of improved shelf life and safety, a change in product quality is still observed due to chemical changes in the juice during storage. Stored fruit juice has often been described as

having “heated” and “cooked” off-flavors, as well as possessing objectionable brown color [18]. These characteristics lead consumers and processors to reject product based on declining quality. In order to control negative quality changes, researchers must consider both the processing conditions employed to manufacture the juice, as well as the reaction pathways that promote the onset of off-flavor generation and color formation. In the past, research has focused on the development of alternative processing technologies to minimize off-flavor generation over storage time; however, the inability to implement these processes cost-effectively presented challenges[14]. As a result, the focus of current research is placed on controlling the reaction mechanisms responsible for flavor quality loss. Since enzymatic activity is eliminated with thermal processing, non-enzymatic reaction pathways are investigated as being mainly responsible for flavor quality loss in fruit juices during storage.

Non-enzymatic browning is considered a key flavor deterioration pathway associated with objectionable changes in thermally processed fruit juice [19]. The ability to limit the non-enzymatic formation of off-flavors during non-refrigerated storage in fruit juices would result in great financial benefit for juice producers as well as consumers by reducing product rejection.

Non-enzymatic browning occurs *via* two main reaction pathways in fruit juice: the Maillard reaction and ascorbic acid degradation [20]. The specific pathways of off-flavor formation *via* non-enzymatic browning remain poorly understood. In the past, research focused on limiting non-enzymatic browning in juice by utilizing two approaches. First, by altering the processing conditions that will minimize color development during non-refrigerated storage, such as high-pressure processing and ion-exchange treatments [19], [21]–[23]. High pressure processing (HPP) has been shown to both inactivate problematic enzymes like PPO, as well as reduce microbial load [12]. Ion-exchange

treatments have been shown to suppress the onset of brown color and off-flavor formation *via* the removal of amino acids, which are key components in non-enzymatic browning reaction pathways that lead to off-flavor generation [19]. Second, research has focused on ingredient additions prior to processing that will influence the extent to which negative reactions progress, such as ascorbic acid and other vitamin/antioxidant addition [24]. However, flavor quality loss is still observed with implementation of these processing steps. In the current state of fruit juice flavor technology, a challenge in improving flavor quality is due to the limited understanding of non-enzymatic reaction pathways and how to control them. Thus, a better understanding of the pathways of quality loss in fruit juices would provide the foundation for more targeted approaches for flavor quality improvement. This work can enable the development of tailored ingredient solutions and a basis for process optimization for improved flavor quality and high consumer acceptability.

Research Objectives

In order to reach this goal, the research strategy has been divided into three main objectives:

1. Identification the off-flavor compounds generated during the accelerated storage of apple and orange juice.
2. Mapping the formation of off-flavor compounds via isotopic labeling of sugars and ascorbic acid and identification of the main precursors involved in generation of these off-flavors.
3. Evaluation of the impact of amino acids on the formation of off-flavor compounds

Chapter 2: Literature Review

2.1 Flavor Development in Fruits

Flavor is a complex multimodal sensation consisting of taste, aroma, and texture, and is the primary driver behind consumer acceptance of foods [25]. In fruit juices, flavor development is attributed to both enzymatic processes during the maturation of the fruit, as well as non-enzymatic processes during processing and storage. During fruit maturation, volatile compounds are formed from the genetically-controlled enzymatic breakdown of secondary metabolites [26]. When the cell walls of a fruit are disrupted during the mechanical production of fruit juices, enzymes that are normally separated from flavor precursors are allowed to interact and form volatile constituents responsible for the flavor perception of fruit [26]. However, if left uncontrolled, enzymatic activity will contribute to the spoilage of juice *via* enzymatic browning. Fruits juices will also spoil during post-harvest storage due to microbial contamination, which is why processing technologies are employed to increase safety and shelf life, while inactivating the enzymes responsible for extensive volatile formation.

However, thermal processing and storage generally result in negative flavor development and acceptability changes by promoting the non-enzymatic reaction pathways that form off-flavors [14]. In order to improve the quality loss resulting from off-flavor formation, the composition of each specific juice must be understood, as off-flavor compounds are degradation products of components present in the juice prior to processing [27].

2.2 Orange and Apple Juice Composition

Orange and apple juices are the most widely consumed fruit juices in the United States, and providing a high quality product after storage is difficult because both juices have a different compositional makeup. Fruit juice is a complex aqueous matrix primarily

consisting of various components such as sugars, acids, pectin, and water. The composition of a specific juice depends on variety, origin and maturity of the fruit, as well as the conditions in which the juice is processed and stored. Along with the major compositional components, fruit juices also contain various components in low concentrations such as amino acids, terpenes, polyphenols, and ascorbic acid that are known to play a role in off-flavor generation during processing and storage [28]–[30]. **Table 2.1** shows a general compositional analysis of apple juice [1].

Table 2.1: Compositional analysis of apple juice [1]

Compound	Concentration (g/l)
Water	860-900
Sugars	100-120
Fructose	46-70
Sucrose	27
Glucose	20
Malic Acid	3-7
Pectin	1-5
Starch	0.5-5
Polyphenols	1
Proteins	0.6
Vitamins (Mainly ascorbic acid)	0.05
Ashes	2

Citrus juices like orange juice are considered a “two phase” system, composed of a heterogeneous mixture of the aqueous serum and the insoluble solids [31]. According to Rega *et al.*, over 90% of hydrocarbon terpenes, like limonene and β-pinene, are associated with the insoluble portion of the juice, and is known from early studies that these compounds impact the aroma character of orange juice [15]-[16]. Regarding composition, orange juice has similar sugar content as apple juice, at around 100g/L, but differs in the major acid makeup and vitamin content [1] and these differences can significantly affect pathways of off-flavor formation during the long-term storage of juice.

2.3 Fruit Juice Aroma

The aroma of freshly squeezed fruit juice is highly desirable, but is also unstable [26], [34]. The aroma attributes of fruit juice depends on many factors; including fruit cultivar, maturity, processing conditions, and storage conditions. The aroma differences between freshly-squeezed juice and thermally treated juice are attributed to both the loss of highly unstable compounds, as well as the generation of off-flavor compounds via non-enzymatic browning, terpene degradation, and other pathways during thermal treatment and subsequent storage [14]. The storage of fruit juice directly translates to a loss in flavor quality [35].

Fresh juice

Over 300 volatile compounds have been reported in fresh orange juice [26]. However, a small fraction (about 5%) of these volatile constituents are thought to be odor-active and contribute to the overall sensory character of the juice [36]. Ketones and terpenes are the main volatile compounds in orange juice on a quantitative basis, making up more than 90% of the total volatiles of orange juice. However, ketones and terpenes have a limited contribution to the aroma of the juice because most are present at concentrations below the sensory threshold [37]. The most influential ketones (1-octen-3-one) and terpenes (limonene, myrcene, etc.) yield fresh, green, floral, and citrus attributes of oranges juice.

Aldehydes and esters have been identified as the major contributors of fresh orange juice aroma [34]. For example, it has been shown that hexanal is an important contributor of green and grassy notes, and acetaldehyde is important contributor of green, pungent notes [38]. Twelve other odor-active aldehydes have been identified in orange juice by at least two independent studies [18], [39]. The combination of these

aldehydes in specific proportions was reported to provide a typical fresh character to the orange juice. Consequently, a change in the balance of aroma compounds can lead to a modification in the juice aroma.

Esters are another class of compounds recognized as important contributors to the fruity, sweet, and floral aroma of orange juice [34]. Total ester concentration has been used in the past to measure overall flavor quality of orange juice [40]. Other studies have reported seven odor-active esters; methyl butanoate, ethyl acetate, ethyl butanoate. ethyl-2-methylpropanoate, thyl-2-methylbutanoate, ethyl octanoate, and ethyl hexanoate, as being important to orange juice aroma by imparting fruity and floral notes [26]. **Table 2.2** shows the compositional distribution of known key odor-active volatiles in orange juices from four different varieties [26].

Table 2.2: Area distribution (%GC/FID) of odor-active compounds present in juices across four orange cultivars.

		Blood Cultivars		**Blond Cultivars**	
LRI	**Odor-Active Volatiles**	**Moro**	**Torocco**	**Washington Navel**	**Valencia Late**
993	Methyl butanoate	1.528	3.783	0.578	0.850
1030	α-pinene	0.669	0.282	0.242	0.442
1049	Ethyl butanoate	0.317	1.227	0.192	0.106
1063	Ethyl-2-methyl butanoate	0.018	0.047	0.010	0.019
1099	Hexanal	-----	0.510	0.221	0.017
1118	β-pinene	0.018	0.043	0.010	0.019
1153	Z-3-hexenal	-----	0.044	-----	-----
1175	β-myrcene	4.828	2.395	0.801	3.651
1235	Limonene	91.91	90.12	97.36	94.35
1246	Ethyl hexanoate	0.477	0.674	0.389	0.377
1319	α-terpinolene	0.005	0.293	0.016	0.018
1366	Hexanol	0.023	0.206	0.040	0.009
1411	Nonanal	0.007	0.018	0.024	0.004
1448	Ethyl octanoate	0.032	0.049	0.012	0.037
1501	Decanal	-----	0.016	0.015	-----
1560	Linalool	0.032	-----	0.003	0.006

The aroma of fresh apple juice has also been well researched for over five decades [21] and its characteristic profile, contrary to orange juice, is attributed mainly to esters (78-92%) formed as secondary metabolites in enzymatic reaction pathways [21]. The general aroma profile of apple juice products is determined by both the apple cultivar and the processing conditions employed to produce the juice. Until the 1970's, most of the research on apple aroma was focused on the volatile compound formation during ripening and maturation [41]. Over 300 volatile compounds have been identified in the aroma profile of apples, and like orange juice, a small fraction of these volatiles are considered character impact compounds [42]. Apple volatiles have a range of aroma thresholds, with around 20 compounds cited as odor active and contributors to the overall flavor perception of apples and apple juices [42]. **Table 2.3** shows the aroma threshold values of the 20 most important volatile compounds in apples, adopted from Dixon & Hewitt [10].

Table 2.3: Aroma threshold values of the 20 important volatile compounds in apples [10]

Compound	Threshold (mL/L)
Aldehydes	
acetaldehyde	0.015-0.12
hexanal	0.005
trans-2-hexenal	0.001-0.017
Alcohols	
ethanol	100-900
propan-1-ol	40-9
butan-1-ol	0.5
hexan-1-ol	0.15-5
2-methyl-butan-1-ol	0.25
Esters	
ethyl acetate	13.5-0.005
propyl acetate	2.0
butyl acetate	0.066
ethyl butanoate	0.001
ethyl-2-methyl butanoate	0.0001
ethyl propionate	0.01
ethyl hexanoate	0.001
propyl butanoate	0.018
2-methyl butyl acetate	0.011-0.005

pentyl acetate	0.043-0.005
hexyl acetate	0.115-0.002
ethyl pentanoate	0.0015

Although a good understanding of the volatiles important for the fresh aroma profile of orange and apple juice exists, loss of freshness and off-flavor development during processing and storage is still poorly understood and controlled.

2.4 Off-Flavor Development in Juice

2.4.1 Storage Implications

The aroma of aged fruit juices is noticeably different than that of freshly squeezed juice [43]. Cooked and heated type notes are typical of juices exposed to processing and long-term unrefrigerated storage [44]. The loss of “fresh” and “green” notes is also characteristic in processed and aged juices as the volatiles that contribute to these attributes are greatly reduced [45]. The loss of key flavor-active volatiles in fresh juice shifts the sensory profile towards an unfavorable aged juice aroma, and has been researched previously.**Table 2.4** shows the quantitative decrease of selected aroma compounds in orange juice over 8 weeks of storage time at 35°C [22]. These compounds are suggested by the authors to be contributors of “freshness” in orange juice and are considered favorable flavor characteristics of the juice. Understanding the development off off-flavor in aged juices should be focused on both the loss of key volatiles, as well as the generation of unfavorable compounds over storage time.

Table 2.4: Concentrations (µg/ml) of various components of orange juice over 8 weeks of storage time at 35°C measured by GC/MS[22]**.**

Component	Time 1[a]	Time 8
Octanol	2.35±0.47[b]	0.87±1.67
3-Hexen-1-ol	2.00±0.81	0.65±1.49

1-Hexanol	4.32±3.43	2.67±2.93
Hydroxy-ethyl-hexanoate	22.74±0.89	18.16±0.94
Valencene	66.27±6.3	43.75±6.1
Octanal	0.52±0.62	0
Decanal	1.99±1.12	0
α-Pinene	5.44±0.83	4.39±0.36
β-Myrcene	20.56±1.95	12.99±2.63
Limonene	1773±227	1278±172

[a]One month after processing
[b]Mean ± standard error of mean

Storage of fruit juices greatly affects their flavor profile and must be controlled in order to limit non-enzymatic reaction pathways. Storage at low temperatures is the recommended method of preserving flavor quality of fruit juices. However, due to costs, complex supply chains, refrigerated storage is not always an option. Some pasteurized juices can be stored in commercial warehouses and trucks for several months before being shipped to retailers, exposing the product to uncontrolled temperature abuse and leading to reduced product acceptability.

The critical parameters considered to impact flavor quality loss during storage are temperature and time, both of which have a profound effect on the extent of volatile formation in aged juices [14]. Other factors that contribute to flavor deterioration are oxygen content, light, and packaging exposure [24].

Mashonas and Shaw stated that temperature is the parameter of storage that has the greatest impact the quality of juice [39]. The aroma of fresh orange juice was not reported to change significantly over time at lower storage temperatures (4°C), but was significantly changed at higher storage temperatures (35°C) after 10 weeks of storage [39].

Studies investigating the storage degradation of fruit juices have long attributed ascorbic acid losses and oxygen content as markers for quality degradation, but have not been directly linked to flavor profile changes [23], [46]. Subsequent studies have focused on using chemical markers such as furfural as an indicator for juice quality due to its role in non-enzymatic browning and off-flavor generation [47], [48]. Investigation into the most important reaction pathways occurring during fruit juice storage is needed to develop a universal method to limit quality loss over storage time. Non-enzymatic browning, terpene degradation, and the hydrolytic breakdown of secondary fruit metabolites are all reaction pathways known to be favored during nonrefrigerated storage of fruit juice, and will be responsible for generating problematic off-flavor volatiles.

2.5 Non-enzymatic browning

Non-enzymatic browning is one of the most important chemical reaction pathways responsible for the quality deterioration of juice products during storage [49]. This chemical process results in the production of brown colors and objectionable flavors without the activity of enzymes. In general, degradation products of sugars, ascorbic acid, and other carbonyl compounds are involved in this reaction and react with nitrogen-containing compounds to produce off-flavors and colors.

The three pathways of non-enzymatic browning are: 1) The Maillard reaction, which occurs when a reducing sugar interacts with an amine compound (i.e. Amino acids) during thermal processing and storage [50], 2) Ascorbic acid degradation, and 3) Caramelization, which only involves reducing sugars and is favored at high temperatures (>150°C). A brief schematic of the three routes of non-enzymatic browning and how they overlap is shown in **Figure 2.1.** The Maillard reaction, ascorbic acid degradation, and caramelization all result in the formation of reactive carbonyl intermediates, which react

with components in the juice such as amino acids to form brown pigments and off-flavors.

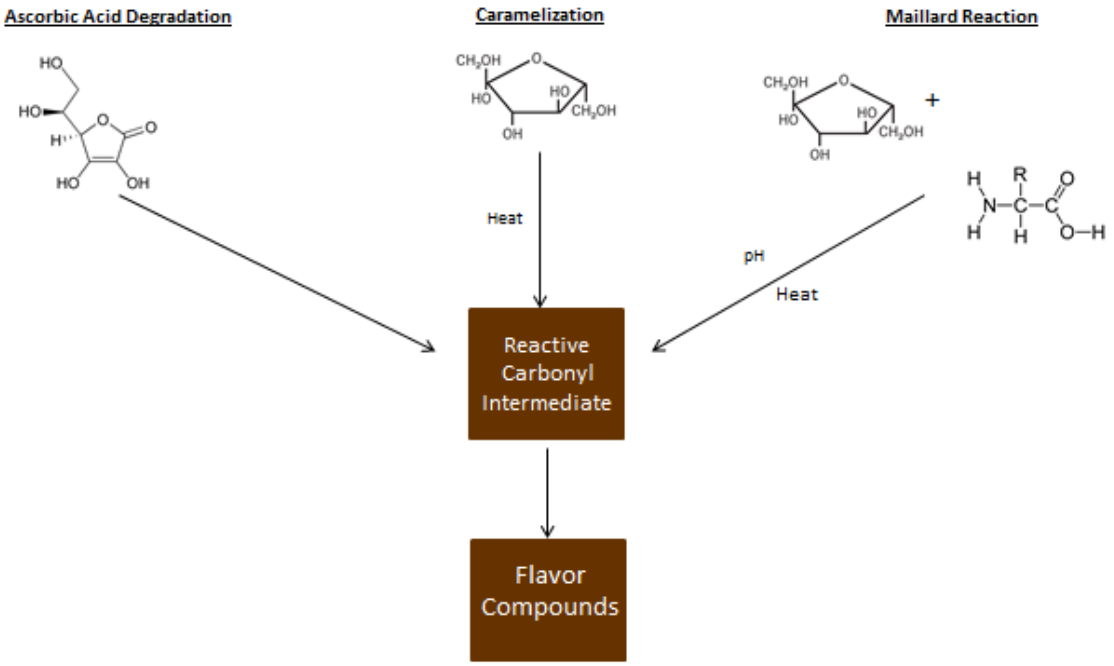


Figure 2.1: The three routes of non-enzymatic browning: Ascorbic acid degradation, Caramelization, and the Maillard Reaction.

2.5.1 Maillard Reaction

In many food systems, non-enzymatic browning via the Maillard reaction is desirable; coffee and baked goods are among the products that are improved by brown, roasted, or cooked flavors. However, non-enzymatic browning is identified as one of the most important reaction pathways that leads to off-flavor formation in aged juice [49].

The Maillard reaction occurs when the α-carbonyl functional group of a reducing sugar interacts with a free amino group of an amino acid or protein to form brown pigments and flavors. An overall reaction scheme of the Maillard reaction adopted from Hodge is presented in **Figure 2.2** [2]. The Maillard reaction is divided into three stages: 1) The initial stage involving the reaction of a carbonyl and amine group, 2) An Amadori

rearrangement involving the formation of flavor compounds, and 3) The advanced stage resulting in the generation of brown pigments and color formation.

The Maillard reaction is most noted for color and flavor generation; however, it is also responsible for nutritional changes within a food product such as loss of essential amino acids, vitamin degradation and decrease in bioavailability of trace elements [9].

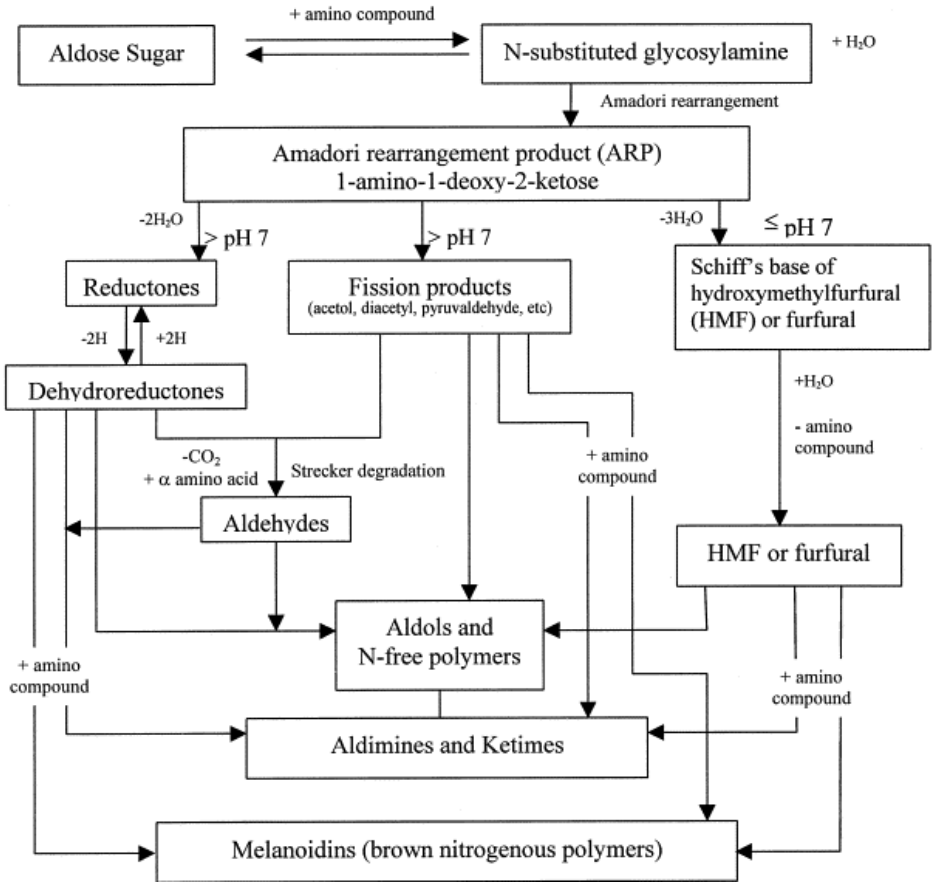


Figure 2.2: General Maillard Reaction scheme, adopted from Hodge[2]

Reducing sugars are a key component in the Maillard reaction. In orange and apple juice, the simple sugars glucose, fructose, and sucrose are the main contributors by

weight to the sugar content, in addition to trace amounts of other sugars like rhamnose [1]. Fructose and glucose are reducing sugars, possessing an active carbonyl group that can participate in non-enzymatic browning and lead to the flavor and color deterioration of fruit juice [20]. Sucrose is not directly involved in Maillard browning, but is hydrolyzed to form glucose and fructose under acidic conditions. It was also demonstrated by Haleva-Toledo *et. al*. that the rhamnose content of orange juice after storage is unchanged when compared to fresh juice, hinting at the importance of other more abundant reducing sugars in the formation of deteriorative off-flavors [51].

However, formation of off-flavors in any food system is multi-dimensional, and can be the result of a combination of reaction pathways. Furaneol, or 2,5-dimethyl-4-hydroxy-3-(2H)-furanone, is an off-flavor compound documented to have sensory significance in aged orange juice, and is responsible for a cooked sugar or caramel odor associated with many processed products. Furaneol production is heat-induced, and has been documented to be formed and potentiated *via* the Maillard reaction under acidic conditions, as well as *via* the direct pyrolysis of fructose[52].

This was demonstrated by Mills, showing that certain pyranones can undergo a ring contraction to form Furaneol and be perceived in the product [53]. **Figure 2.3** depicts glucose and fructose undergoing a pyrolysis and ring contraction to form Furaneol [19].

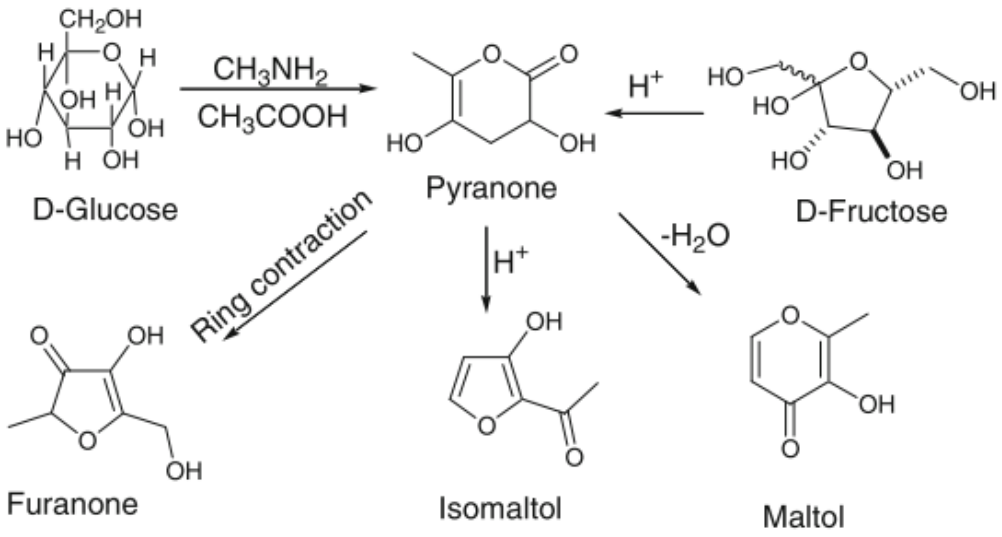


Fig 2.3: A reaction mechanism depicting typical sugar degradation in a food system to form furaneol [19]

Methional is a potentially important off-flavor compound in orange juice, as mentioned by Bezman [54]. The production of this very pungent odor compound can follow the same route as ascorbic acid degradation. The amine compound methionine can undergo oxidation under the right conditions, and yield methional via Strecker degradation [55]. Methional can be detected by humans at sub-ppb levels, so even if the reaction is unfavored, the slightest production of methional can result in a sensory change in juice. Methional and other sulfur-containing volatiles are often overlooked because they are challenging to measure. Since most of these compounds possess both a concentration and odor detection limit lower than many instruments capabilities, the reporting of sulfur-containing volatiles have been sparse in fruit juice products. It is most likely that these compounds are thermally generated, and then potentiated throughout storage time; similar to other off-flavor compounds like *p*-vinyl guaiacol.

2.5.2 Ascorbic Acid Degradation

Ascorbic acid is recognized as playing an important role in the flavor deterioration of aged fruit juices over time [56]. Ascorbic acid falls into the same class of compounds as glucose and fructose, in that it is a highly reactive reducing agent. Therefore, it can interact similarly with amine-containing compounds and form end products analogous to the Maillard reaction. Ascorbic acid degradation is favored at low pH, and will occur at milder heat conditions than the Maillard reaction [56]. Orange juice is acidic, with an average pH of 4.0, which is why most research on the storage implications of citrus juices cites ascorbic acid degradation as the pathway responsible for sensory deterioration [20]. A sample reaction mechanism for ascorbic acid degradation is shown in **Figure 2.4** [19]. Native ascorbic acid is readily oxidized to form dehydroascorbic acid (DHA) in an acidic environment, which is highly unstable and reactive in aqueous solutions [50]. DHA will then further react with sugars under anaerobic conditions to form furfural and its derivatives, which are important quality markers in fruit juices [47], [56].

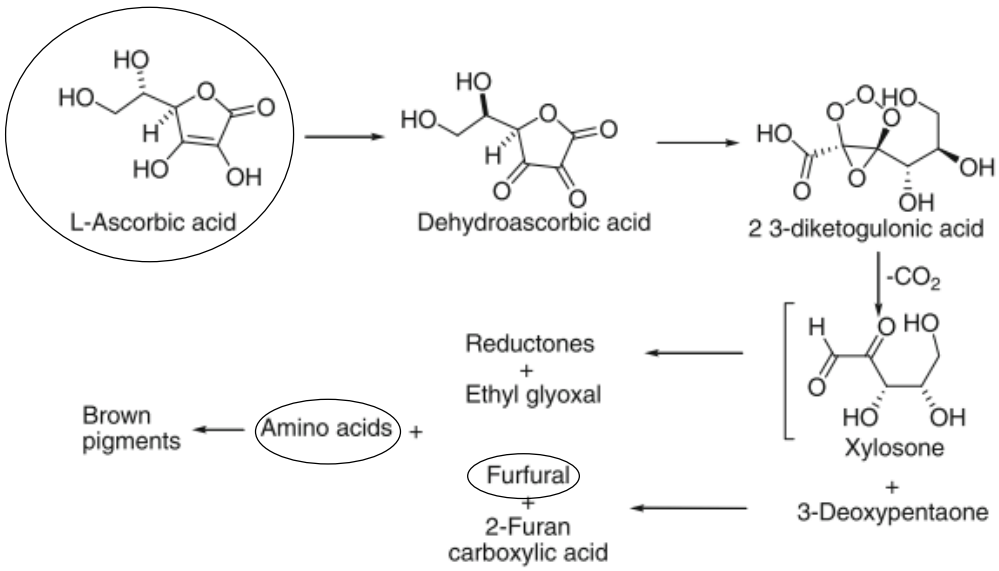


Fig 2.4: A reaction mechanism depicting typical ascorbic acid degradation in a food system, with compounds of interest circled[19]

It should be noted that ascorbic acid is also used commercially to prevent browning because of its ability to reduce pH and act as a radical scavenger. Ascorbic acid is known to prevent enzymatic browning in fruits and other products due to its inhibition of polyphenol oxidase [24]. However, when ascorbic acid is used to prevent the formation of potential degradative compounds, it is oxidized to DHA, and therefore promoting browning/flavor formation pathways. During prolonged storage, the addition of ascorbic acid to the juice results in increased color development, as evidenced by multiple storage studies [20], [48].

Furfural has been extensively researched as a marker of both off-flavor and off-color formation [47]. Ascorbic acid can produce furfural upon the acid-catalyzed breakdown of sugars, and further react with constituents in the juice to form compounds like 5-methyl furfural, furfuryl alcohol, and 5-hydroxymethyl furfural. Koca reported that ascorbic acid degradation is the sole reaction mechanism responsible for the formation of furfural and 5-HMF, and that sugars play a minimal role in their formation [57].

In apple juice, ascorbic acid degradation is of less concern because of the relatively low amounts of ascorbic acid present. By comparison, apple juice contains 4 µg/mL of ascorbic acid whereas orange juice contains 1233 µg/mL [58].

2.5.3 Caramelization

The direct degradation of sugars favored at high temperatures is known as caramelization. The main difference between caramelization and other forms of non-enzymatic browning is that caramelization does not involve nitrogen-containing compounds in the formation of colors and flavors. Caramelization and the Maillard reaction can often result in many of the same end products; however, the Maillard reaction is more often cited as the source of brown flavors and colors in food products

[59]. In fruit juice, caramelization is often discounted as being the cause of off-flavor formation because the conditions present in fruit juices do not favor this pathway [19]. Therefore, the breakdown of sugars in fruit juice is most likely the result from either acid-catalyzed (ascorbic acid) or amine-catalyzed (Maillard reaction) non-enzymatic pathways.

2.6 Role of Amino Acids

Amino acids serve as important precursors for the Maillard Reaction, as well as participate in degradation pathways that form potent oxygen-containing aroma compounds in aged fruit juices [60]. In this way, amino acids can be recognized as “flavor potentiators”, referring to the fact that they affect the flavor of a food product via reaction pathways favoring the formation of odor-active compounds [25].

When examining the effect of amino acids on off-flavor development, two important parameters must be considered. First, the concentration of the amino acid in solution may play a role in enhancing the formation of off-flavors and colors. Second, the reactivity of each individual amino acid must be considered because of the effect on both the rate and pathway of flavor deterioration in fruit juices [61]. Each fruit cultivar has varying proportions of amino acids, and as a result, will behave differently when participating in the reactions responsible for off-flavor generation.

Ashoor and Zent reported that there are three distinct categories of amino acids regarding reactivity towards Maillard reaction pathways, namely high, intermediate and low [28]. This study reported lysine, glycine, tryptophan, and tyrosine to have the highest reactivity, while cysteine, histidine, arginine, aspartic acid, glutamic acid, serine, and threonine possessed very low reactivity towards browning [28]. Along with the reactivity, the concentration of amino acids also had an effect on the onset of flavor deterioration.

Kacem *et. al*. reported that the greatest loss of ascorbic acid occurred in a juice system containing the highest concentration of amino acids (1.26%), suggesting amino acid concentration has an effect on ascorbic acid degradation [62].

Clegg also showed that the removal of amino acids from a juice system reduced or halted the formation of non-enzymatic browning products during storage [50]. Studies have examined the removal of amino acids as a way to increase resistance to sensory deterioration in fruit juices via ion-exchange treatment; however, this approach will cause a decrease in the nutritional composition and increase the price of the product [19].

Nine free amino acids are reported to occur unbound in orange juice, of which arginine and ɣ-aminobutyric acid are the most abundant [63]. However, these amino acids may not be the most reactive, and their role in the formation of off-flavor still needs to be investigated.

Early studies conducted on apple juice identified asparagine, aspartic acid, and glutamic acid to be the major amino acid constituents by weight [64]. Work has been conducted on the compositional characteristics of many apple varieties [65]. Babsky has published work on the relative decrease of amino acids in clarified apple juice during storage, and showed that there was only a 13.2% retention of free amino acids in clarified apple juice after 111 days of non-refrigerated storage [66]. This result indicates that amino acids are utilized in degradation and off-flavor formation pathways during storage, and play a role in the non-enzymatic flavor quality loss of apple juice. However, there are hundreds of apple varieties and cultivars used in the production of apple juice, each containing a unique amino acid makeup, resulting in the inability to establish a standardized value that works for every juice [65].

2.7 Terpene Degradation

Acidic conditions (pH~4), such as those present in orange juice, favor the degradation of hydrocarbon terpenes via hydration, dehydration, cyclation, and ester hydrolysis to form alcohols such as a-terpineol and terpinen-4-ol [67]. A reaction scheme for the acid-catalyzed degradation of the major terpenes in orange juice can be observed in **Figure 2.5 [19].**

One of the major off-flavor compounds generated via terpene degradation pathways is α-terpineol, which is currently used as a marker for fruit juice quality [68]. In early Gas Chromatography-Olfactometry (GC-O) studies, α-terpineol was discovered to contribute a musty and stale off-flavor to orange juice [68]. It is recognized that this compound is formed as a result of non-oxidative terpene degradation, typically from the precursors limonene and linalool. Durr reported a linear increase in α-terpineol with increasing storage time as a result of limonene deterioration, lending some insight into the favored formation pathways of this compound [69]. Limonene, the most predominant terpene in orange juice, undergoes degradation to form α-terpineol. Linalool, an important terpene alcohol which is considered a character impact compound of fresh orange juice can also degrade to form a-terpineol. Thus, the loss of linalool, combined with the formation a-terpineol, results in a shift of the overall sensory perception of the juice [26].

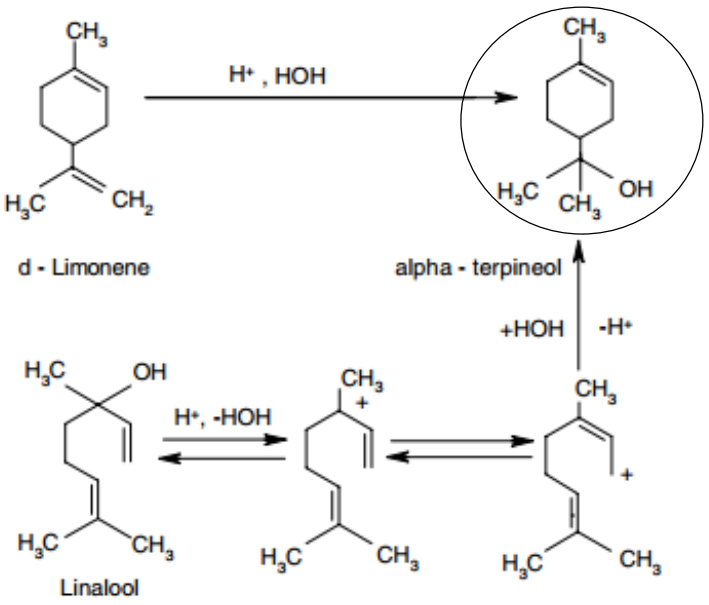


Fig 2.5: Reaction mechanism showing the formation of α-terpineol from limonene and linalool[19]

Terpene degradation in apple juice is of little concern due to of the lack of terpene compounds contained within the product. High terpene concentration is associated with citrus fruits and juices, and less so with deciduous fruits like apples and pears.

2.8 Other Off-Flavors in Apple and Orange Juice

p-Vinyl guaiacol (cooked, nutty) is cited as the most potent aroma compound generated during the storage of heat-abused orange juice [70]. There has been work looking into the formation and precursors of this compound [71]. It is a result of the decarboxylation of cinnamic acids; in particular free ferulic acid, present in orange juice. Under mild acidic conditions, cinnamic acids are decarboxylated to form vinyl phenols [72]. Although orange juice has a small amount of free ferulic acid, it has been proposed that this is more than enough to generate *p*-vinyl guaiacol concentrations above the sensory threshold [70]. **Figure 2.6** depicts the decarboxylation of ferulic acid to *p*-vinyl guaiacol [52].

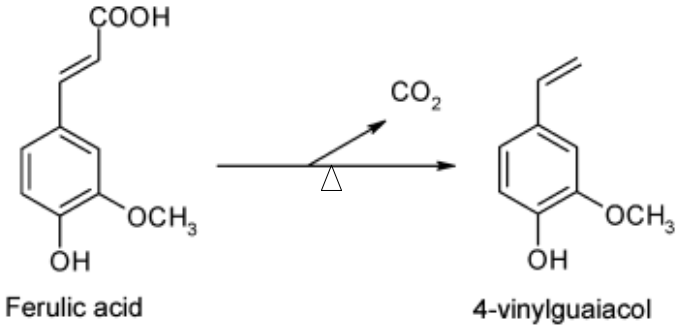


Figure 2.6: Heat-catalyzed degradation of ferulic acid to *p*-vinylguaiacol [52]

β-damascenone is an important compound in the odor perception of apples, and increases in concentration over storage time [73]. The formation pathways of this compound are varied and heavily researched, due to the importance of β-damascenone in the flavor and fragrance industry. One suggested pathway is the hydrolytic breakdown of secondary metabolites that are derived from carotenoids [74]. It is worthy to note that carotenoid breakdown can be caused by ascorbic acid deterioration, opening up a reaction pathway for the formation of β-damascenone over time.

2.9 Flavor Extraction & Analysis

Over the years, an immense amount of work has been carried out analyzing food flavor and perception. Volatile compounds have been the focus of many of these studies, as it is understood that aroma compounds are a key part of flavor perception [25]. Taste and nonvolatile analysis has served as an “emerging” field of study in flavor science, because of the lack of understanding and published work on the topic of the role of nonvolatile components in flavor perception [25].

The study of aroma compounds can be difficult because they are often present in foods in trace amounts, making the detection and quantification of aromatic components analytically challenging. Food matrices contain sugars, carbohydrates, lipids, and

proteins which all increase the complexity of flavor isolation. Aroma isolation methods have been developed for years, all of which work for certain applications and present drawbacks as well as advantages. Aroma isolation and extraction methods are based on two physical properties of compounds; volatility and solubility [25]. The most common methods of flavor extraction and isolation from aqueous matrices are headspace (dynamic and static) [75], distillation [76], solvent [77], and sorptive extraction[78]. In the case of aqueous samples like fruit juice, methods like sorptive extraction and headspace are most commonly used [79].

2.9.1 Stir Bar Sorptive Extraction

Sorption-based methodologies have been employed for trace compound analyses, because they allow for the direct extraction of volatiles from a wide array of matrices, while being relatively solvent–free and clean.

Sorption-based extraction methodologies, such as those used in solid phase micro extraction (SPME) are based on the principle of adsorption, wherein analytes are transferred from the sample matrix to an extraction phase based on the physiochemical properties of each analyte, with polarity being the driving force behind absorption[80].

Introduced in 1999, stir bar sorptive extraction (SBSE) is a relatively new technique based on the adsorption principle of SPME [81]. SBSE is used to analyze trace amounts of volatile constituents in an aqueous matrix more efficiently than SPME.

The theory that SBSE is more efficient than SPME is based on the assumption that the partitioning coefficients between the extraction phase and water ($K_{PDMS/W}$) are proportional to the octanol-water coefficients ($K_{O/W}$), as shown below[81].

$$K_{O/W} \approx K_{PDMS/W} = \frac{C_{SBSE}}{C_W} = \frac{m_{SBSE}}{m_W} \times \frac{V_w}{V_{SBSE}} \quad (1)$$

C_w and C_{SBSE} are the analyte concentrations in the water and SBSE phase, respectively, m_{SBSE} and m_W are the masses of analytes in each phase, and V_{SBSE} and V_W are the volumes of each phase. Rearranging equation (1) and replacing V_W/V_{SBSE} with a phase ratio, β, the extraction efficiency of SBSE can be viewed in equation (2) below:

$$\frac{m_{SBSE}}{m_0} = \frac{(\frac{K_{O/W}}{\beta})}{1+(\frac{K_{O/W}}{\beta})} \quad (2)$$

From this equation, it can be understood that the only dependent variable determining extraction efficiency is the ratio of the partitioning constant ($K_{O/W}$) to the phase ratio β [81]. Therefore, an increase in extraction phase will result in increased extraction efficiency. Traditionally, 0.5 µL of PDMS is used in a SPME extraction, and SBSE allows for the use of up to 100 µL of sorbent for volatile extraction. A 200-fold increase in extraction volume, along with the speed, simplicity, and sensitivity of this method make stir bar sorptive extraction an attractive alternative to traditional sample extraction methods when dealing with an aqueous, low-fat, and low-alcohol matrix [82].

When carrying out SBSE extraction, a small stir bar about 10 mm in length is coated with nonpolar polydimethylsiloxane (PDMS) phase. The stir bars are introduced into the sample matrix, and allowed to stir for a defined amount of time at a defined temperature and rotational speed. After extraction, the stir-bars are rinsed, dried, and thermally desorbed. **Figure 2.7** shows a typical stir bar coated with PDMS.

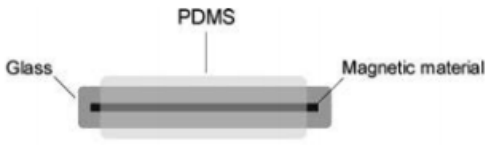


Figure 2.7: A stir bar coated with PDMS for use in SBSE

Unfortunately, PDMS is effective for extracting non-polar components out of a sample matrix, but can prove difficult when targeting polar compounds. There have been advances in developing polar phases for this type of application, including EG-Silicone [83].

SBSE has been used in the past to detect trace amounts of compounds in a wide array of analytical applications. SBSE has been used in environmental applications to detect residual pesticides and other organic contaminants [69]-[70] as well as in the pharmaceutical industry to analyze drug absorption in the blood [83]. In food applications, SBSE has been used in the wine and spirits industry for flavor and compositional analyses [86] as well as in fruit juices to study grape juice volatiles [76].

2.9.2 GC/MS-Olfactometry

The evaluation of aroma-active compounds can be accomplished through analytical and sensory techniques. Analytically, the characterization of aroma-active components in foods is accomplished through the use of capillary gas chromatography. Gas Chromatography is the most common method used for volatile compound separation, analysis and quantification [87]. Modern gas chromatography techniques utilize capillary columns coated with a thin layer of stationary phase, which functions to help separate volatile compounds based on their interaction with the phase [25]. Volatile compound separation is based on both the volatility of the analyte, as well as its affinity to the stationary phase which is mainly driven by polarity. A wide array of chemical detectors are available in order to interpret separation data into signals that can be used for quantification and identification of compounds, including Flame Ionization detectors (FID), Thermal Conductivity detectors (TCD), and Mass Selective detectors (MS) [25].

A mass-selective detector, or mass spectrometer, is often paired with Gas Chromatography to assist with the identification of volatile compounds in foods [88]. A MS detector will collect molecular data of unknown compounds *via* the ionization fragmentations of each compound, lending information into the identity of the analyte being examined [89]. It is worth noting that MS information alone does not warrant the confirmed identification of each compound. Many compounds have similar structures, warranting the need for secondary confirmation method. Methods using a second detector or the utilizing linear retention indices are most commonly employed today[90].

Gas Chromatography-Mass Spectrometry/Olfactometry utilizes the human nose as a supplementary GC detector with the goal of lending sensory value to analytical data [25]. In GC-MS/O studies, the effluent is split between a MS detector and an olfactometry port, and a trained panelist is used to assign odor information to the compounds eluting from the column during an analytical run. In addition to assigning peaks an odor, GC-MS/O methods can aid in compound identification, as odor is a piece of information that can be used to distinguish compounds with similar retention times and mass spectra [25].

Methods have been developed for GC-MS/O techniques in order to discriminate impactful aroma compounds from those with little to no sensory significance [91]. These methods are aimed at objectifying the obtained data to estimate the sensory significance of each individual odor-active component in a flavor extract. Dilution techniques and time-intensity measurements are the two major GC-O methods aimed at accomplishing this type of discrimination. Dilution techniques evaluate the odor-activity of individual compounds by sniffing a GC effluent in a series of dilutions of the original aroma extract [91]. Official dilution methods have been developed by Acree [92] and Grosch [36]. Time-intensity measurement methods such as OSME have been developed by

McDaniel et al [93], and aim to measure the perceived odor intensity of each compound in a GC effluent. These methods are subjective, and a panel of well-trained assessors is required to obtain consistent and reliable data.

2.9.3 Sensory Evaluation and Aroma Recombination

Although important odorants can be identified by GC-MS/O techniques, aroma recombination experiments are necessary in order to confirm the impact of selected odor-active compounds in the food matrix of interest [36], [94]. Recombination techniques are part of the state of the art process in food aroma research, as it can correlate analytical and quantitative data with a sensory impact and validate selection of important markers [91]. In this technique, quantitative amounts of odor compounds are introduced into a control sample matrix, and then evaluated by traditional sensory evaluation techniques, such as difference-from-control test or descriptive analysis (DA) test [38], [44]

In beer, recombination studies have been successfully used to determine character impact odorants in a wide variety of beer types [94]. Similar experiments have been conducted in strawberries, snack chips, and orange juice [95]–[97]. Recently, complex aroma recombination models have been used to evaluate the most intense odor active compounds in orange juice, of which 23 volatiles were selected to be added to a sunflower oil matrix and compared to a freshly squeezed orange juice. Sensory recombination was proven effective at demonstrating the compounds important for fresh orange juice aroma. However, this study did not evaluate heated or aged juice to determine how closely the model relates to thermally abused and aged juices [97]. Therefore, sensory recombination experiments utilizing odor active compounds present

in aged fruit juice is warranted to investigate and better understand off-flavor formation pathways.

Chapter 3: Characterization of Off-Flavor Compounds in Aged Fruit Juices via Gas Chromatography-Olfactometry and Aroma Recombination Techniques

Summary

Off-flavor compounds formed during the long-term storage of apple and orange juice were identified and quantified via Gas Chromatography-Mass Spectrometry/ Olfactometry (GC-MS/O). Off-flavor markers were selected based on two criteria; compounds were detected by more than 50% of panelists in aged juice extracts and were absent in fresh juice extracts. Each off-flavor marker compound was positively identified based on the comparison of LRIs, mass spectra, odor descriptions, and standard addition.

In orange juice, furfural, *p*-vinylguaiacol, Furaneol, β-fenchyl alcohol, α-terpinolene, α-terpineol, terpinen-4-ol, and methional were chosen as off-flavor markers based on selection criteria. In apple juice, furfural, 5-methylfurfural, furfuryl alcohol, and β-damascenone were chosen as off-flavor markers based on selection criteria. Quantification of each compound was conducted by standard addition technique in the fresh and aged samples. Sensory difference testing of the aroma recombination models indicated the aroma profile of aged juice model (fresh juice with off-flavor compounds) shifted significantly ($p>0.05$) towards the profile of aged juices (versus fresh juice) for both orange and apple juice, indicating the importance of the selected off-flavor compounds for the flavor quality changes noted in the aged samples. This work afforded insight into off-flavor markers and thus a basis to investigate important pathways of flavor generation in juice products, which could yield targeted processing and/or ingredient optimization strategies to limit the flavor quality loss in juice during storage.

Introduction

Orange and apple juice make up about 36% of total U.S. fresh or processed fruit consumption, [8]. The size of this market is the driver behind delivering a product to the consumer with an aroma that is as close to freshly squeezed juice as possible. However, given the complex supply chains and large-scale production centers, it is becoming increasingly difficult to provide high-flavor quality products to consumers. Fruit juice aroma is extremely unstable due to a number of reaction pathways involved in flavor deterioration, both enzymatic and non-enzymatic. In most commercially available juices, enzymatic and microbiological flavor generation pathways are limited due to the use of thermal treatment during processing. Thermal treatment increases the safety and shelf life of the product. However, thermal processing additionally induces deterioration of flavor quality in juices over time via both the loss of highly volatile compounds that contribute to the fresh juice aroma, as well as the generation of undesirable compounds over storage time via nonenzymatic reaction pathways [19]. In order to provide solutions to control nonenzymatic browning in juices, the off- flavor compounds need to be characterized.

The goal of this study was to characterize and identify contributing off-flavor aroma compounds generated during the long term (9-12 months ambient) non-refrigerated storage of apple and orange juice products via GC/MS-O, and then evaluate the impact of selected off-flavor compounds on the sensory profile of the juice via aroma recombination studies. This information can help establish a basis for targeted reaction pathway studies on off-flavor formation during storage, ultimately to help suggest processing and/or ingredient optimization strategies that yield a final fruit juice product with improved flavor quality and higher consumer acceptability.

Materials & Methods

Orange Juice/Apple Juice

Commercial aseptically processed reconstituted orange (10.8°Brix) and apple (11.4°Brix) juice purchased from a local grocer (Tropicana, St. Paul, MN) was use for this study. The fresh aseptically processed juice was frozen at -20°C and unfrozen after 8 weeks. The aged juice was placed in an incubator for 8 weeks at 35°C. Samples were kept frozen at -20°C from the end of storage time until extraction and further analysis.

Volatile Compound Extraction Procedure

Extraction of volatile compounds was carried out using Stir-Bar Sorptive Extraction (SBSE) with a 10mm x 0.5mm (length x thickness) PolyDiMethyl Siloxane (PDMS) extraction phase, supplied by Gerstel (Mulheim a/d Ruhr, Germany). Fruit juice (10g) was weighed in a 20mL headspace vial and spiked with 2-methyl-3-heptanone as an internal standard, at 2 and 0.2 (mg/kg) for orange juice and apple juice, respectively. Samples were extracted at 1000rpm for one hour at room temperature. Thermal conditioning of the stir bars was carried out prior to extraction with adherence to the manufacturer's specifications. The stir bars were then removed from the sample, washed with NanoPure water, gently dried using a paper tissue, and inserted into a clean glass tube for thermal desorption and gas chromatographic analysis.

Gas Chromatography/Mass Spectrometry – Olfactometry

Gas chromatographic analyses were carried out on an Agilent 6890 Gas Chromatograph (Agilent Technologies , Santa Clara, CA) equipped with a Thermal Desorption System installed on a programmed Cold Injection System (TDS-2 and CIS-4, Gerstel, Germany). GC was coupled with a DB-WAX 30m x 250µm x 0.25µm (length x thickness x internal

diameter) column (Agilent Technologies, Santa Clara, CA) and an Agilent 5973 Mass Selective Detector (Agilent Technologies, Santa Clara, CA).

The extracted stir bars were thermally desorbed in the TDS unit using a temperature starting at 40°C and ramping at 50 °C/min to a final temperature of 250°C. The cryofocusing temperature was set at -40°C, and the desorption flow rate was set at 50ml/min.

The GC oven temperature program was as follows: 40°C for 2min, 40-250°C at 5°C/min, 250°C for 1min. The injection was carried out in splitless mode, and transfer line temperature was maintained at 250°C. The carrier gas was helium and the column flow was 1.7mL/min. Detection was carried out using EI mode with a scan range of 33-350 m/z, and a characteristic parent ion for each compound was used to obtain the peak area for quantification.

The column outlet was split (flow as 1:1) between the MSD and olfactory detection port (Hillesheim, heated transfer line with nose cone, Waghausel, DE).

Olfactometry analysis was conducted using six experienced panelists. Panelists evaluated the GC effluent for both fresh and aged juices and recorded the detected aroma attributes from 0.5min to 30min. Descriptions of the odors were based on each sniffer's subjective response. A systematic approach was used to examine the differences in odor active compounds between the fresh and aged juices by criteria discrimination. Off-flavor markers were chosen based on two criteria: (1) the odor-active regions perceived by >50% of panelists detected the odor in one of the juices, and (2) the odor-active regions were detected by >50% of panelists in the aged juice, and <50% of panelists in the fresh juice. The resulting aromagrams of the aged juice samples were compared to those of fresh juice.

Identification and Quantification

Identification of off-flavor markers was based on comparing linear retention indices (LRI) with published values, mass spectra fragmentation libraries and published odor attributes. Confirmation of identification was completed by comparison and matching to authentic compound standards under the identical experimental conditions.

Quantification of markers was carried out using standard addition methodology. A five-point standard calibration curve was generated in triplicate with concentrations in a linear range ($r^2 > 0.95$). Five different concentrations of a known compound standard were added to a juice, extracted, and a linear curve ($r^2 > 0.95$) was developed based on the peak area response for each compound.

Sensory Analysis

Recombination juice systems were created and sensory evaluation was conducted to assess the impact of the identified off-flavor markers. A difference-from-control test was was conducted according to methods outlined in Meilgaard [98]. The difference in aroma character between the aged juice (control), the fresh juice and the recombination model juice sample was evaluated. Apple and orange juices were purchased from a local retail market (St. Paul, MN). The juice used as "fresh" was purchased just before sensory testing. The aged juice samples were held for 8 weeks at 35°C. An aroma recombinant model was created using the fresh juice samples spiked with quantified amounts of each identified off-flavor marker in aged orange and apple juice.

Twenty-two panelists (10 male, 12 female, ages 22-72 from the Food Science & Nutrition Dept. at University of Minnesota) participated in two difference-from-control tests (1 apple juice, 1 orange juice) occurring over the period of 1 day. During each test, panelists were presented with the three pairs of samples described above. Briefly, 100 mL of fresh juice, aged juice, and recombination model were presented to panelists in

coded 300 mL plastic bottles and paired with the same volume of a control (aged juice). The following pairs were evaluated and their presentation was randomized:

- Control vs. Recombination Model
- Control vs. Fresh Juice
- Control vs. Blind Control

Panelists were asked to first evaluate the aroma of the control and then the coded sample, and rate the size of the difference in aroma character between the samples on a seven-point scale (0=No difference, 1 = slight difference, 3 = moderate difference, 5 = large difference, 7= Very large difference). Data was collected using Compusense Cloud v7.2 software (Compusense Inc., Guelph, Ontario, Canada.)

Statistical Analysis

Mean scores for each sample and the blind control were calculated and the data was analyzed using 2-way ANOVA followed by a Dunnett's test for multiple comparisons with a control. Dunnett's test was utilized to determine if the sample means of the fresh juice and recombinant model were significantly different than the blind control. Additionally, Fischer's LSD was used to determine if significant differences exist between the test samples. All analyses were performed with Compusense Cloud v7.2 software (Compusense Inc., Guelph, Ontario, Canada).

Results & Discussion

Identification of aroma differences between control and aged juice

The flavor profiles of apple and orange juices were analyzed for changes over storage time. GC/MS-O revealed 42 odor-active compounds in orange juice, and 26 odor-active compounds in apple juice were detected by more than 50% of panelists in either the

fresh or aged sample. All identified aroma active compounds in orange and apple juice along with panelist detection frequencies in both aged and fresh juice are presented in **Tables 3.1 and 3.2.** The compounds identified were overall consistent with the literature and have been previously cited in fruit juice [26], [42] thus, validating the use of SBSE as an extraction method for aroma active region determination by GC-O [68].

Comparison of the odorants detected in the fresh versus aged samples revealed15 compounds in orange juice and 11 compounds in apple juice were observed to decrease in panel detection frequency; whereas 8 compounds in orange juice and 4 compounds in apple juice were observed to increase in panel detection frequency from fresh to aged juice samples. The odor-active compounds that were shown to increase in panel detection frequency between the fresh and aged juice were targeted for further evaluation as the focus of this study, because they were generated during storage and thus present the basis for investigation of off-flavor generation after processing and during shelf-life. The compounds that decrease may influence flavor quality loss as shown by Sadecka [13], however investigating the mechanisms of this loss was outside of the scope of this study.

Based on these findings, α-terpinolene, α-terpineol, furfural, methional, terpinene-4-ol, β-fenchyl alcohol, Furaneol, and p-vinylguaiacol were selected as off-flavor markers generated in orange juice while, furfural, 5-methylfurfural, furfuryl alcohol, and β-damascenone were selected as important off-flavor markers in apple juice.

Orange Juice

Table 3.1: Aroma-active compounds detected *via* GC-MS/O of orange juice, with linear retention indices (LRI), perceived odor characteristics, and panelist detection frequencies in both fresh and aged (8 week at 35°C) juices.

Compound	LRI	Odor description	% Detected in	% Detected

			Fresh	in Aged
Acetaldehyde	1063	green, fruit	100	0
2-butanone	1066	butter	100	100
ND	1076	fruity, alcohol, citrus	100	33
butanoic acid	1092	green grass, fruit	66	33
hexanal	1145	green, metallic	100	0
β-pinene	1198	green	100	100
Limonene	1301	cleaner, lemon, mint	100	100
β-myrcene	1315	sweet, green	100	66
Octanal	1350	popcorn, baked	100	33
α-terpinolene	1381	old orange, green	33	66
nonanal	1404	off, cooked pasta	83	17
ethyl octanoate	1446	peanut butter	83	66
furfural	1501	roasted, peanut	0	66
methional	1512	bad, green, potato	0	100
acetic acid	1482	bread, fermented	100	100
decanal	1520	citrus, aldehyde	100	0
linalool	1556	cleaner, fruity, candy	33	100
octanol	1569	pine, green, seed	100	0
fenchyl alcohol	1626	cleaner, soil	0	83
terpinen-4-ol	1638	butter, sweat	0	66
α-terpineol	1653	spices, green	33	100
NI	1656	cooked meat	33	50
NI	1660	petal, floral, sweet	50	50
4-methylbenzaldehyde	1670	chemical, fruity	100	100
hexanoic Acid	1675	licorice, cleaner, peanuts	66	66
valencene	1764	green, old orange	100	66
nerol	1798	caramel, sweet	100	66
carveol	1834	fruity, cleaner	100	33
geraneol	1854	green, mushroom	100	66
β-ionone	1977	floral, woody	50	50
NI	2036	sweet, red	100	66
NI	2063	mushroom, cooked	50	50
4-Hydroxy-2,5-dimethyl-3(2*H*)-furanone	2078	heated fruit	0	100
guaiacol	2082	cinnamon	66	66
p-vinylguaiacol	2134	smokey, burnt	0	83
NI	2241	fireplace, BBQ	66	100
NI	2265	smokey, brown	0	33
nootkatone	2570	Citrus, woody	100	66

NI=not identified

In orange juice, 4-Hydroxy-2,5-dimethyl-3(2*H*)-furanone (DMHF), *p*-vinyl guaiacol, and α-terpineol have been identified in early studies on quality loss of heated juice [70]. Three additional terpene degradation compounds: terpinen-4-ol, α-terpinolene and fenchyl

alcohol; and two additional nonenzymatic browning compounds: furfural and methional exhibited differences between fresh and aged orange juice. All of these compounds have been documented in the past to be related to storage of juices [47], [54], [67], [99]. Terpinen-4-ol and α-terpinolene are two terpenoid compounds similar in structure and odor to α-terpineol. It is likely their formation pathways are very similar, and α-terpineol may be the favored degradation compound of hydrocarbon terpenes, as outlined in Schieberle & Grosch [100].

Fenchyl alcohol is a less commonly identified aroma-active terpene degradation compound, but its formation has been cited to follow the degradation pathway of β-pinene, which is known odor active terpene in orange juice, as suggested in Kimura et al. [101].

The non-enzymatic browning compounds furfural and methional were both detected in the aged orange juice, and not in the fresh juice. Furfural is a characteristic compound formed during nonenzymatic browning, and is used as a marker compound for storage and temperature abuse in fruit juices [19]. Furfural is present in many food products, but is not usually reported as an odor-active volatile due to its relatively high odor threshold of 0.25-5ppm in water [102]. [71]. The compound *p*-vinylguaiacol is a result of the decarboxylation of cinnamic acids; in particular free ferulic acid, present in orange juice[71], and has been cited as the most sensory-significant off-flavor compound in aged orange juice [52]

Methional has been suggested as an off-flavor compound in aged orange juice in previous research [54], but was present in levels below the detection limits of the MSD. All six panelists detected methional-like odors in aged juice, while no panelists detected methional-like odor in the fresh juice. An authentic methional standard confirmed the

presence of the compound, as the retention time and odor descriptor were consistent with the GC-O results. Methional was detected by the MSD and thus was not quantified due to the low affinity of the PDMS phase of the sorptive stir bar towards polar sulfur compounds. The use of a more polar phase of sorptive extraction, Ethylene glycol-Silicone (EG-SIL), combined with a decrease in transfer line temperature to 200°C (TDS unit) in an attempt to prevent methional degradation, helped to improve extraction efficacy and suggest the detection of methional by MS spectra parent ion. However, its concentration remained below linear region for quantification due to obscurity in the ion fragmentation.

Apple Juice

Table 3.2: Odor-active compounds detected *via* GC-MS/O of apple juice, with panelist detection frequencies in both fresh and aged (8 week 35°C) juices.

Compound Name	LRI	Odor	% Detected in Fresh	% Detected in Stored
Acetaldehyde	942	apple	100	66
NI	985	butter	100	100
Ethyl Butyrate	1032	fruity, pleasant	100	83
Hexanal	1070	green grass	100	33
δ-carene	1182	fruity	100	66
trans-2-hexenal	1239	green, rancid	100	66
Limonene	1286	citrus, cleaner	100	100
Octanal	1304	Green, metallic, sharp	100	33
NI	1341	Cracker, corn chip	100	100
NI	1354	green, metallic, fruit	100	100
NI	1378	pungent	50	83
Nonanal	1402	citrus, cleaner	100	33
acetic acid	1455	Vinegar, butter	100	100
methional	1475	Seed, potato	0	100
furfural	1504	nutty, peanut butter	33	100
decanal	1512	fruity, floral	100	33
NI	1559	roasted, nutty	50	50
5-methylfurfural	1579	yeasty	0	66
methyl benzoate	1627	citrus, sweet	100	66
4-methylbenzaldehyde	1653	sweet, licorice	66	50
furfuryl alcohol	1676	oil, cooked	0	83
β-damascenone	1836	roasted, fruity, apple	50	100

NI	1868	sweet, vanilla, roasted	33	50
NI	2077	burnt, pie crust	33	50
Decanoic Acid	2286	cooked, oil	83	50

Of the 26 observed odor-active compounds in apple juice, only four were shown to increase during storage. It is interesting to note that ethyl butyrate, hexanal, octanal, nonanal, methyl benzoate, and acetaldehyde, compounds cited to contribute to fresh apple aroma, were all observed to decrease over storage time [11], [42], [65], [103].

Three of the four markers shown to increase in panel detection frequency during storage belong to the same chemical family of substituted heterocyclic furans and are known nonenzymatic browning products in apple juice, namely, furfural, 5-methylfurfural, and furfuryl alcohol [19]. However, little work exists relating the presence of these compounds to off-flavor perception in apple juice. The fourth off-flavor compound was β-damascenone (roasted fruit) which has been identified as a decomposition product of the secondary metabolites of carotenoids [74]. The compound β-damascenone has been previously cited as a contributor to apple juice aroma [73], and as an off-flavor in other heated fruit juice studies [54].

Quantification of Markers

Quantification of off-flavor markers in both fresh and aged juice was carried out by standard addition methodology. Individual compound quantification curves can be found in the appendix. Concentrations of off-flavor marker compounds for fresh and aged orange & apple juice are presented in **Tables 3.3 and 3.4** respectively, along with odor thresholds for each compound reported in water. These findings were used for sensory recombination juice, and were the basis for isotopic precursor contribution calculations discussed in Chapter 4.

Table 3.3: Concentrations of off-flavor markers in fresh and aged orange juice presented as mean concentrations and standard error (SE) calculated from triplicate analysis of each sample, with odor thresholds in water included

Compound	Mean Concentration Fresh, mg/kg ± SE	Mean Concentration Aged, mg/kg ± SE	Odor Threshold in Water*, mg/L
Furfural	0.135 ± 0.067	0.317 ± 0.074	$2.5x10^{-1}$
p-vinyl guaiacol	0.050 ± 0.032	1.180 ± 0.040	$3.0x10^{-3}$
β-fenchyl alcohol	0.001 ± 0.001	0.018 ± 0.001	$2.1x10^{-3}$
α-terpinolene	0.039 ± 0.016	0.088 ± 0.018	$2.0x10^{-1}$
α-terpineol	0.762 ± 0.560	2.900 ± 0.400	$3.0x10^{-1}$
Terpinen-4-ol	0.021 ± 0.018	0.072 ± 0.011	$3.0x10^{-1}$
Methional	ND	ND	$5.0x10^{-6}$
4-Hydroxy-2,5-dimethyl-3(2*H*)-furanone	0.002 ± 0.004	0.762 ± 0.450	$4.0x10^{-5}$

ND= Not Determined

*Source: Leffingwell & Associates

Table 3.4: Concentrations of off-flavor markers in fresh and aged apple juice presented as mean concentrations and standard error (SE) calculated from triplicate analysis of each sample, with odor thresholds in water included.

Compound	Mean Concentration Fresh, mg/kg ± SE	Mean Concentration Aged, mg/kg ± SE	Odor Threshold in Water*, mg/L
Furfural	0.373 ± 0.139	0.997 ± 0.027	$2.5x10^{-1}$
5-Methyl Furfural	0.001 ± 0.0002	0.002 ± 0.0002	20
Furfuryl Alcohol	0.947 ± 0.073	1.820 ± 0.560	6-8
β-damascenone	0.006 ± 0.005	0.212 ± 0.052	$2.0x10^{-9}$
Methional	ND	ND	$5.0x10^{-6}$

*Source: Leffingwell & Associates

Aroma Recombination and Sensory Analysis

In order to demonstrate the impact of off-flavor markers on the aroma of apple and orange juices, recombination models were prepared containing the selected off-flavor markers generated during storage at the concentration quantified in the aged juice.

A difference-from-control test was used for the sensory evaluation of recombination models according to methods outlined in Meilgaard [98] in order to evaluate the if there is significant difference between fresh, recombination and aged juice samples, and the size of the difference perceived between the samples. Evaluating both the fresh juice and the recombinant model compared to the control (aged juice) allows the confirmation that the aroma compounds detected contribute to the aroma character of the aged juice.

Orange Juice

The results of the 2-way ANOVA for orange juice are shown in **Table 3.5,** and show that the sample treatments are significantly different from each other. A Dunnett's test for multiple comparisons with a control **(Table 3.6)** was applied to the sample means and revealed that both the fresh juice and recombinant model were significantly different than the blind control. It can also be concluded that recombinant models was significantly less different than the control (aged juice) than the fresh juice based on a Fisher's LSD multiple comparison (Table 3.7).

Table 3.5: 2-Way ANOVA summary table of Orange Juice Samples

Source	Degree of Freedom	Sum of Squares	Mean of Squares	F value	p-value
Samples	2	153.36	76.68	35.27	0.00
Judges	21	126.79	6.04	2.78	0.00
Error	42	91.30	2.17		
Total	65	371.45	5.71		
Standard Error	0.31				

Table 3.6: Dunnett's t-test results for Orange Juice

Orange Juice	Mean Difference From Control* (n=22)	Significantly different from Control ($p<0.05$)
Recombinant Model (Fresh + Markers)	2.68	yes
Fresh Juice	3.59	yes

*Dunnett's t= 1.01 (Significance level p<0.05)

Table 3.7: Fisher's LSD results for Orange Juice

Sample	Sample Name	Mean Difference from Control*	Significantly Different from Sample (p<0.05%)
A	Blind Control (Aged)	1.10	B,C
B	Recombinant Model (Fresh + Markers)	3.60	A,C
C	Fresh Juice	4.50	A,B

*Fisher's LSD = 0.67 (Significant level p<0.05%); Different letters indicate a significant difference

These results suggested that the addition of off-flavor markers shifted the aroma character of the fresh orange juice closer to that of the aged orange juice supporting the important sensory impact of the off-flavor compounds identified to increase in the aged sample **(Table 3.6).**

The noted difference between the aroma profile between the recombination model of the aged sample and the control (aged) of orange juice (**Table 3.6**) could be attributed to the noted change in the aroma compounds in the aged juice that were not adjusted for in the recombinant model system. GC-O results **(Table 3.1)** indicated that 15 compounds in orange juice were decreased during storage, all of which have been cited in the literature to be involved in the flavor perception of fresh orange juice, lending fresh, fruity, and green attributes to the aroma profile [26], [68]. Because the recombinant sample was made from the fresh juice (plus 4 odorants that increased during storage, Table X) it would also contain higher levels of these 15 compounds, and therefore the noted

different between the recombinant and aged was expected. However the addition of these off-flavor markers were shown to statistically change the sensory profile of the orange juice towards a characteristic aged orange juice aroma, however compounds contributing to a characteristic fresh orange juice flavor present in the recombination model likely lead to a significant difference of the recombination model with the aged juice sample. Therefore, it can be suggested that loss of "fresh" aroma compounds was also influential in flavor deterioration of orange juice.

Apple Juice

The sensory results of the 2-way ANOVA for apple juice samples (fresh, aged and recombinant aged) are presented in **Table 3.8**, and show that the sample treatments are significantly different from each other. A Dunnett's test for multiple comparisons with a control (aged sample) was applied to the sample treatment means. The fresh juice was shown to be significantly ($P<0.05$) different from the blind control, while no significant difference was observed between the recombinant model and the blind control, as shown in **Table 3.9.** These results support the major contribution of the selected off-flavor markers **(Table 3.9)** to the flavor profile of aged apple juice and unlike the orange juice samples, suggested the loss of compounds from the fresh sample play a minor role in the sensory depreciation of apple juice.

Table 3.8: 2-Way ANOVA summary table for Apple Juice Samples

Source	Degree of Freedom	Sum of Squares	Mean of Squares	F value	p-value
Samples	2	138.09	69.05	22.44	0.00
Judges	21	52.62	2.51	0.81	0.69
Error	42	129.24	3.08		
Total	65	319.95	4.92		
Standard Error	0.37				

Table 3.9: Dunnett's t-test results for Apple Juice

Apple Juice	Mean Difference From Control* (n=22)	Significantly different from Control (p<0.05)
Recombinant Model	0.5	no
Fresh Juice	3.09	yes

*Dunnett's t=1.21(Significance level p<0.05)

Table 3.10: Fisher's LSD results for Apple Juice

Sample	Sample Name	Mean Difference from Control*	Significantly Different from Sample (p<0.05%)
A	Blind Control	1.55	C
B	Recombinant Model	1.59	C
C	Fresh Juice	4.64	A,B

*Fisher's LSD =0.75 (p<0.05%); Different letters indicate a significant difference

The off-flavor markers demonstrated a causative relationship between a concentration increase and a sensory effect, provided insight into the possible important pathways of off-flavor generation which can serve as targets for mitigation strategies. Elss [21] has shown that furfural is the major component of apple juice concentrates, however, single strength apple juices like the one examined in this study typically contain much lower amounts of volatile constituents, and little work has been done relating concentrations to off-flavor impact in aged apple juice [104]. Only β-damascenone and furfural were present at concentrations above reported odor thresholds, suggesting that they are c more involved in off-flavor perception than 5-MF or furfuryl alcohol. However, competitive effects by 5-MF or furfuryl alcohol could still play a role in the sensory perception.

Off-flavors in apple juice formed from microbial origin has been reported [104], [105]. Up to fifteen odorants have been identified; however none of them have been cited to form from a non-microbial source. The lack of research on non-microbial storage-related off-

flavor generation pathways in apple juice further evidences the importance of this study towards the elucidation of off-flavor development pathways in apple juice.

Although different approaches have been employed by producers in the past to prevent off-flavor development and loss of freshness in fruit juices during storage, an effective and universal solution has not been adequately developed. Approaches targeting the reaction pathways responsible for off-flavor formation are needed, to allow processor to optimize fruit blending strategies and juice formulations in order to ensure a product of the highest quality over shelf life. Further insight into mechanistic pathways of off-flavor formation is needed. Understanding the precursors that take part in these reactions, as well as the effect juice composition has on the extent of odorant formation is required.

Overall, through identification of important off odorants in both orange and apple juice this study afforded a better understanding regarding the mechanistic pathways involved in off-flavor formation during processing and storage. Non-enzymatic browning, terpene degradation, ferulic acid decarboxylation, and the hydrolytic breakdown of secondary metabolites were identified as important pathways and provided a basis for targeted flavor optimization procedures such as processing, ingredient/formulation and blending approaches.

In the next chapter, approaches to understand off-flavor formation via reaction pathway mapping will be presented. Isotope labeling experiments were utilized to identify the main precursors involved in the off-flavor markers identified in the current chapter. Additionally, the influence of select amino acids on the formation of the off-flavors was investigated.

Chapter 4: Investigation of non-enzymatic browning off-flavor formation pathways in aged fruit juice and the impact of amino acid composition

Summary

Major precursors of non-enzymatic browning in juice; glucose, fructose and ascorbic acid, were evaluated *via* isotope-ratio mass spectrometry experiments to better understand their role in the formation of off-flavor markers in orange and apple juice during storage. Of the eight off-flavor marker compounds previously identified in orange juice (α-terpinolene, α-terpineol, furfural, methional, terpinene-4-ol, β-fenchyl alcohol, 4-Hydroxy-2,5-dimethyl-3(2*H*)-furanone(DMHF), and p-vinylguaiacol), only furfural and DMHF exhibited carbon labeling, and thus generated from the precursor under investigation. Furfural was generated predominately (93%) via ascorbic acid degradation, while DMHF was generated mainly via Maillard chemistries (41% Glucose, 51% Fructose). In apple juice, three of the four previously identified off-flavor markers (furfural, 5-methylfurfural, and furfuryl alcohol) exhibited carbon labeling. Only a small percentage of Furfural was generated (1%) via glucose and fructose degradation, indicating that alternate pathways are involved in its generation. Both furfuryl Alcohol and 5-methylfurfual were formed via sugar degradation pathways with 100% fructose, 50% glucose and 14% glucose, 20% fructose, respectively.

The impact of the amino acids, Glutamine (GLN) and Tryptophan (TRP) in orange juice, and Glutamine (GLN) and ɣ-aminobutyric acid (GABA) in apple juice, on off-flavor marker formation during storage were also examined. In orange juice, both GLN and TRP increased the formation of DMHF, α-terpinolene, α-terpineol, and terpinen-4-ol after 8 weeks of storage. In apple juice, both GLN and GABA accelerated the formation of 5-methylfurfural, furfuryl alcohol, and β-damascenone over an 8-week storage time.

These findings provided insight into the pathways involved in off-flavor generation in aged apple and orange juice products, and a basis for the development of process and ingredient flavor optimization strategies for juice products.

Introduction

Off-flavor generation in fruit juices results in a negative impact on the aroma and thus decreases consumer acceptability. Although enzymatic browning can be inhibited via enzyme inactivation during thermal processing or ascorbic acid addition, non-enzymatic browning remains a challenge for the juice processor as it promotes both negative color and flavor changes. Non-enzymatic browning in has been well-studied due to its detrimental influence on high-quality flavor in fruit juices [75]. However, the chemical pathways responsible for off-flavor formation in fruit juices are not adequately understood.

The precursors of non-enzymatic browning reaction pathways include reducing sugars and amino acids [106]. Thus, in order to better understand and ultimately develop strategies to control nonenzymatic browning in fruit juices, identifying the contribution of these precursors to the formation of off-flavor compounds would be beneficial.

In the previous chapter, important off-flavor markers contributing to the negative aroma attributes associated with aged fruit juice were identified and their impact verified by sensory recombination experiments. The focus herein is to understand the pathways of formation of selected off-flavor markers using juice model systems spiked with isotope labeled precursors and select amino acids.

Materials & Methods

Volatile Compound Extraction Procedure

Extraction of volatile compounds was carried out using Stir-Bar Sorptive Extraction (SBSE) with a 10mm x 0.5mm (length x thickness) PolyDiMethyl Siloxane (PDMS) extraction phase, supplied by Gerstel (Mulheim a/d Ruhr, Germany). Fruit juice (10g) was weighed in a 20mL headspace vial and spiked with 2-methyl-3-heptanone as an internal standard, at 2 (mg/kg) and 0.2 (mg/kg) for orange juice and apple juice, respectively. Samples were extracted at 1000rpm for one hour at room temperature. Thermal conditioning of the stir bars was carried out prior to extraction with adherence to the manufacturer's specifications. The stir bars were then removed from the sample, washed with NanoPure water, gently dried using a paper tissue, and inserted into a clean glass tube for thermal desorption and gas chromatographic analysis.

Gas Chromatography/Mass Spectrometry

Gas chromatographic analyses were carried out on an Agilent 7890A Gas Chromatograph (Agilent Technologies, Santa Clara, CA) equipped with a Thermal Desorption Unit installed on a programmed Cold Injection System (TDU and CIS-4, Gerstel, Germany). GC was also coupled with a LECO Pegasus 4D GCxGC TOF Mass Spectrometer (LECO, St. Joseph, MI) equipped with DB-WAX 60m x 250µm x 0.25µm column (Agilent Technologies, Santa Clara, CA).

The extracted stir bars were thermally desorbed in the TDU unit using a temperature starting at 40°C and ramping at 50 °C/min to a final temperature of 250°C. The cryofocusing temperature was set at -40°C, and the desorption flow rate was set at 50ml/min.

The GC oven temperature program was as follows: 40°C for 1min, 40-250°C at 5°C/min, 250°C for 10min. The injection was carried out in splitless mode, and transfer line temperature was maintained at 250°C. The carrier gas was helium and the column flow

was 1.7mL/min. Detection was carried out with a scan range of 33-350 m/z, and a characteristic parent ion for each compound was used to obtain the peak area for quantification.

Identification & Quantification

Identification of compounds was carried out using relative retention indices, parent ion peaks, and mass spectrum fragmentation patterns sourced from the database NIST. Quantification of markers was carried out using standard addition methodology. A five-point standard calibration curve was generated in triplicate with concentrations in a linear range ($r^2 > 0.95$) from 0.01 to 2ppm.

Isotope Ratio Mass Spectrometry

Glucose-$^{13}C_6$, Fructose-$^{13}C_6$, and L-Ascorbic acid-$^{13}C_6$ were used to prepare juice samples and investigate the precursors impact on the generation of off-flavor markers. Quantification of glucose, fructose, and ascorbic acid was conducted by Paravisini (X).Three orange juice and two apple juice samples were spiked one-half of the amounts of glucose, fructose, or ascorbic acid naturally present in the juice, resulting in a ~2:1 ratio of native component to isotope in orange juice **(Table 4.1)** and in apple juice **(Table 4.2),** similar to methods described by Totlani and Peterson [107]. In brevity, the results can be observed in **Table 4.1.**

Table 4.1: Concentrations of Ascorbic Acid (AscAcid), Glucose (GLU), and Fructose (FRU) natively present in orange juice, as well as concentration of each added precursor resulting in a ~2:1 ratio of precursor to isotope

Samples	Endogenous Precursor Concentration (g/L)			Added ^{13}C Precursor Concentration (g/L)			**Ratio Unlabeled: Labeled**
	AscAcid	GLU	FRU	AscAcid	GLU	FRU	
OJ+^{13}C AscAcid	0.2	28.3	31.8	0.1	-	-	**2.0**
OJ + ^{13}C FRU	0.2	28.3	31.8	-	-	15.3	**2.1**
OJ + ^{13}C GLU	0.2	28.3	31.8	-	15.2	-	**1.9**

Table 4.2: Concentrations of Glucose (GLU), and Fructose (FRU) natively present in orange juice, as well as concentration of added precursor in each model resulting in a ~2:1 ratio of precursor to isotope

Samples	Endogenous Precursor Concentration (g/L)		Added ^{13}C Precursor Concentration (g/L)		**Ratio Unlabeled: Labeled**
	GLU	FRU	GLU	FRU	
OJ + ^{13}C FRU	37	85.1	0.0	39.7	**2.1**
OJ + ^{13}C GLU	37	85.1	13.5	0.0	**2.7**

Juices were aseptically processed prior to storage in order to prevent enzymatic and microbial reactions by using a small-scale bench top process outlined in Kokkinidou [108]. In brevity aseptic processing was performed on a Gerstel Multipurpose Sampler equipped with heated agitator and cooled sample tray (MPS II, Gerstel, Germany). Briefly, a 10g of sample was measured in a 10mL headspace vial, and was placed in a heated agitator at 200°C, held for 75 seconds, and then placed on a tray and cooled at 10°C. This process was optimized to simulate commercial juice pasteurization time-temperature profile (90°C for 30 sec). No microbial growth was visible after storage.

Amino Acid Modeling

Two amino acids were chosen for each juice based on their expected reactivity towards non-enzymatic browning in order to investigate compositional impact on off-flavor formation during storage. They were also selected based on previous experiments conducted by Paravisini (X), wherein the amino acids chosen constituted the highest percent loss over time in aged apple and orange juice.

Orange juice was spiked with four times the native concentration of Glutamine (GLN) and Tryptophan (TRP), and placed into 10mL headspace vials for simulated aseptic processing and non-refrigerated storage for 2, 4, and 8 weeks at 35°C alongside a control sample. A 4x concentration was used facility investigating an observable effect of each amino acid on off-flavor formation. The same procedure was applied to apple juice spiked with ɣ-aminobutyric acid (GABA) and Glutamine (GLN). Each model was developed in triplicate. After storage, each sample was frozen at -20°C until extraction and analysis. A table showing model sample amino acid concentrations can be observed in **Table 4.3**. The initial concentrations for each amino acid were previously determined by LC-MS by Paravisini (X), based on methods outlined in Guo [109].

Table 4.3: Concentrations of amino acids in model juice samples present at 4X the native concentration in orange and apple juice

Juice Type	Glutamine (mg/L)	Tryptophan (mg/L)	ɣ-Aminobutyric Acid (mg/L)
Orange	209.6	30.4	**-**
Apple	7.2	-	76.4

Results and Discussion

4.3 Investigation of off-flavor precursors via stable isotope analysis

In order to identify the precursors involved in the formation of off-flavors in aged orange and apple juice, isotopically-labeled reducing sugars and ascorbic acid were used to

track the pathways of formation. If the labeled precursor is involved in the formation of an off-flavor marker during storage, a partially labeled compound will be observed in the mass spectrum. The isotope-labeled precursor is similar to the native precursors in terms of both physical and chemical properties and will exhibit the same behavior over storage time as the endogenous precursor compounds [110].

Often reaction pathways in foods are investigated in simplified model systems to control every aspect of the sample, such as the composition and processing conditions. Even though model systems can provide basic knowledge of chemical pathways, they are limited in scope as they reduce the effect of the food matrix. In this study, an original approach was carried out by supplementing a real juice matrix with a defined ratio of labeled glucose-$^{13}C_6$, fructose-$^{13}C_6$ and ascorbic acid-$^{13}C_6$. This approach is comparable to the CArbon MOdule LAbeling (CAMOLA) method often used to identify formation pathways of volatile compounds in model systems [111].

After storage, the contribution of each precursor to the formation of the off-flavor markers was calculated based on the ratio of parent ion (M) to fully labeled ion (M+X), and then compared to the initial concentration of each marker in the control juice. Consequently, the quantification of volatile reaction product isotopomers *via* GC/MS from a 2:1 mixture of unlabeled to ubiquitously labeled glucose, fructose, and ascorbic acid model establishes a causative relationship between each precursor and off-flavor marker. Theoretically, if an off-flavor compound is formed from a single precursor in a model system, a 100% contribution would be observed; meaning that the quantity of $^{13}C_6$-labeled precursor is shown to exhibit half of the relative abundance of the parent ion (base on the original ratio of 2:1, unlabeled: labeled). Additionally a complete intact $^{13}C_6$-label may not be observed in the off-flavor compound spectra, due to a breakdown of the precursor compound into precursor fragments that take part in the formation of the off-

flavor compound [110]. If precursor fragmentation is observed, off-flavor molecules present in the mass spectra that will exhibit different isotopomeric distributions. Schieberle has discussed the conditions that favor precursor fragmentation in Maillard-type reactions, such as pH and pressure [110]. The current study was used to investigate the fate of chemical precursors (glucose, fructose and ascorbic acid) in a juice system, and lend insight into the chemical pathways of deterioration that each precursor follows during nonrefrigerated storage. An example of the mass spectrum off-flavor marker after 8-weeks of storage from a carbon labeling experiment for DMHF supplemented with Glucose-$^{13}C_6$ is shown in **figure 4.1.**

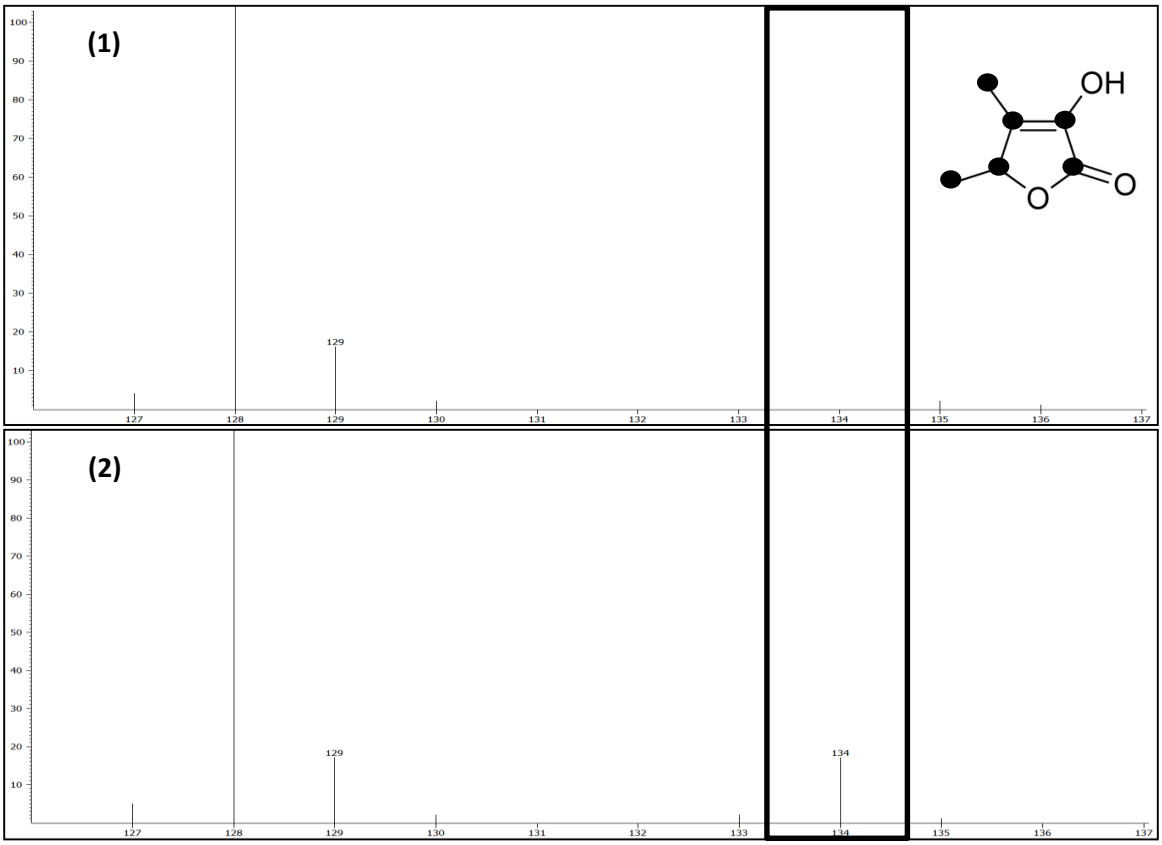


Figure 4.1: Mass spectrum of DMHF (127-137) obtained from isotope ratio experiments: (1) =Unlabeled and (2) (●) carbon-13 label from [U-$^{13}C_6$]-D-glucose

Orange Juice

The contribution of the selected precursor of nonenzymatic browning on the previously reported off-flavor markers (chapter 3) was investigated. Of the seven odorant compounds namely furfural, 4-Hydroxy-2,5-dimethyl-3(2*H*)-furanone (DMHF), *p*-vinylguaiacol, fenchyl alcohol, α-terpineol, terpinen-4-ol, and α-terpinolene, only furfural and 4-Hydroxy-2,5-dimethyl-3(2*H*)-furanone (DMHF) exhibited carbon labeling, indicating that the remaining five compound markers do not originate from the selected labeled precursors (Table 4.3). This was expected, considering the formation pathways of all terpene degradation compounds such as α-terpineol, α-terpinolene, fenchyl alcohol, and terpinen-4-ol, as well as *p*-vinylguaiacol, is not thought to involve the breakdown of sugars and/or ascorbic acid.

DMHF exhibited 41% and 51% contribution **(Table 4.4)** from glucose and fructose degradation, respectively, while showing a barely detectable contribution from ascorbic acid. These results suggest that DMHF is produced predominately from the degradation of reducing sugars *via* Maillard Reaction pathways. Tressl et al. [112] investigated the isotopomeric contribution of fructose and glucose to the formation of DMHF in a sugar-amino acid model system and concluded that DMHF forms in 90% from fructose degradation (10% from another source, possibly an amino acid), and 100% from glucose degradation. This is consistent with the presented results in Table 4.4, as DMHF is formed in about a 1:1 ratio from glucose and fructose, meaning that they possess similar reactivity potentials for the formation of DMHF. However, these results contradict those presented in previous experiments by Rouseff et al. [51], which suggest that DMHF is not produced in aged acidic model orange juice lacking rhamnose content, and that the formation of DMHF from fructose and glucose is pH dependent, forming only at basic pH. It should be noted that DMHF is a 6-carbon molecule, meaning its formation pathways most likely involve C3 Maillard intermediates like methylglyoxal in a retro Aldol

reaction at low pH, as suggested by Schieberle [110]. Fully labeled DMHF is most likely formed via hydroxycarbonyls which originate from the fragmentation of either labeled fructose or glucose. The specific carbohydrate fragmentation patterns that lead to the formation of DMHF were beyond the scope of this study.

It is possible that the sum of each precursor contribution can exceed 100%, because each model being evaluated contains added concentration of each precursor (fructose, glucose or ascorbic acid). Additionally sucrose, a disaccharide that can hydrolyze in solution to form glucose and fructose was not evaluated, and could have led to an underestimation of the contribution of each sugar to the formation of off-flavor markers.

Furfural was predominately formed from the degradation of ascorbic acid **(Table 4.4.** Indeed, furfural was shown to exhibit a 15% contribution from glucose breakdown product and a 5% contribution from fructose breakdown product, while showing more than 90% contribution from ascorbic acid degradation. These results were consistent with hypotheses concerning non-enzymatic browning product formation in orange juice [47], [48], [57]. As shown in **Figure 2.2** and **Figure 2.4**, furfural is a precursor to the formation of brown compounds in both the Maillard reaction and ascorbic acid degradation pathways. The results of the isotopomeric analysis (**Table 4.4)** support the notion that ascorbic acid is the major precursor of furfural in orange juice, as well as supports the use of this compound as a marker of off-flavor formation. A proposed reaction pathway for the formation of furfural follows that shown in Figure 2.4, in that under anaerobic conditions like those present in aseptically packaged fruit juice, ascorbic acid will degrade *via* the cleavage of the ring and the addition of a water molecule, and then undergo a decarboxylation and intermolecular rearrangement, followed by a dehydration to form furfural, as demonstrated by Yuan [56]. It is worth noting that the Maillard reaction contributes to the formation of furfural but to a lesser

extent. The degradation of glucose and fructose via the Maillard reaction produces a small amount of furfural, with glucose having a 15% h contribution to furfural formation, and fructose demonstrating a 5% contribution. This reaction pathway can be observed in the Maillard reaction under acidic conditions in **Figure 2.2**, in that the Amadori products will undergo a dehydration and amination to form furfural and hydroxymethylfurfural (HMF).

Although not a known off-flavor in orange juice, it is interesting to note that the formation of 5-methylfurfural, a compound that is extremely similar to furfural in structure and reactivity, follows a different reaction pathway than furfural. The compound 5-MF was predominantly formed via glucose and fructose degradation at 67% and 43%, respectively, while showing a barely detectable contribution from ascorbic acid. The observed differences regarding favored reaction pathways may be driven by pH, as a low pH has been suggested to favor the direct oxidation of Maillard reaction intermediates, such as 3-deyoxyhexosomes, to the formation of 5-methylfurfural [113]. Also, 5-MF can be a direct degradation product of 5-hydroxymethylfurfural (HMF), *via* condensation, which is a compound present in very high concentration after the storage of both citrus and non-citrus juice[114].

Table 4.4: Results of Isotope-Labeled Mass Spectrometry for Orange Juice. Furfural, DMHF, and 5-MF are presented along with their three precursor treatments, parent ion mass, percentage of labeled ion, and C_6 contribution percentage to the formation of the off-flavor marker.

Compound	Treatment	Parent (M)	Labeled (M+X)	% Contribution
Furfural	Glucose	96	3.07%	**15%**
Furfural	Fructose	96	1.06%	**5%**
Furfural	Ascorbic Acid	96	17.65%	**93%**

DMHF	Glucose	128	9.49%	**41%**
DMHF	Fructose	128	11.74%	**51%**
DMHF	Ascorbic Acid	128	0.19%	**1%**
5- MF	Glucose	110	17.53%	**67%**
5-MF	Fructose	110	11.13%	**43%**
5- MF	Ascorbic Acid	110	0.251%	**1%**

These findings in orange juice further the understanding of favored reaction pathways responsible for the formation of off-flavors during storage time, and suggest that the Maillard reaction is more impactful on off-flavor formation than has been previously suggested. Although off-flavor in citrus juices is a result of many different reaction pathways, non-enzymatic browning products are important in altering the aroma profile of juice during storage, as shown in Chapter 3. With an increased understanding of non-enzymatic browning precursors, new processing and fruit blending/selection techniques can be suggested to minimize the formation of nonenzymatic browning products during storage. The formation pathways for the selected off-flavors are pH driven; at a low pH both Maillard and ascorbic acid mechanisms of non-enzymatic browning occur. Potentially the development of a processing method to protect the precursors from acid-catalyzed degradation, such as by linking the sugar moieties with a protein, would be of extreme value to the citrus juice industry.

It is interesting to note that this is the first time that ascorbic acid has been shown to directly result in the formation of furfural in orange juice. Past studies focusing on non-enzymatic browning in fruit juices have assumed that ascorbic acid degradation results in the formation of furfural based on a correlation between ascorbic acid loss and furfural formation[19]. The isotopic labeling study could be expanded upon to include ^{13}C

limonene, linalool, or cinnamic acid, in order to investigate the formation pathways of α-terpineol, α-terpinolene, fenchyl alcohol, and terpinen-4-ol, as well as *p*-vinylguaiacol from ferulic acid degradation.

Apple Juice

The four off-flavor marker compounds in apple juice, namely furfural, 5-methyl furfural, furfuryl alcohol, and β-damascenone, were evaluated for their formation contribution from glucose and fructose. Three of the four markers (furfural, furfuryl alcohol, and 5-methylfurfural (5-MF) in the aged samples, exhibited a ^{13}C label, as shown in **Table 4.4.**

Furfural showed little to no formation contribution from either glucose or fructose. Alternate reaction pathways have been proposed for the formation of furfural at low pH in apple juice such as via degradation of rhamnose [28], galactouronic acid [115], or potentially from trace quantities of ascorbic acid. However, rhamnose was not examined in this study and the endogenous amount of ascorbic acid was not taken into consideration as a major precursor because it was unable to be detected by previous experiments in apple juice

However because ascorbic acid is present in trace amounts, it was assumed furfural was most likely formed *via* the Maillard reaction using reducing carbonyls other than glucose or fructose, such as rhamnose or galactouronic acid. This is further evidenced by Clegg, who suggested that furfural formed in a model lemon juice system containing sugars, amino acids, and ascorbic acid was not equal to the extent of furfural formation in a native lemon juice aged over the same time period [116]. If furfural formation in a model system containing known major precursors does not replicate the furfural formation in a real juice matrix, it is likely other components must be involved in its formation.

This hypothesis could be extended to the formation of 5-MF. 5-methylfurfural showed contributions from both fructose and glucose, at 20% and 14%, respectively, equating to about 34% of the total precursor contribution. This result suggests that other precursors are involved in the formation of 5-MF in apple juice as well. A suggested reaction pathway for the glucose and fructose related formation of 5-MF in apple juice can be similar to the oxidation of Maillard intermediates suggested in orange juice above [117]. Further work is needed in order to investigate the contribution of these alternate precursors on off-flavor formation in juice during storage.

Furfuryl alcohol exhibited a 50% contribution from glucose, as well as a 100% contribution from fructose. Furfuryl alcohol is a Maillard reaction product, and has been shown to form in glucose-amino acid model systems at neutral pH [118], and fructose-amino acid model studies in orange juice at low pH (3.5)[119]. The fructose-$^{13}C_6$ model demonstrated a 100% contribution of fructose to the formation of furfuryl alcohol; meaning under these experimental conditions, furfuryl alcohol was formed almost exclusively from fructose degradation. The glucose-$^{13}C_6$ model exhibited a 50% contribution to the formation of furfuryl alcohol, demonstrating that fructose was the preferred reducing sugar for the formation of this compound. This was in agreement with findings by Isbell, in that fructose is more prone to the formation of Maillard products due to its potential to be enolized faster than glucose [120]. Furfuryl alcohol is cited as an acid-catalyzed reduction product of furfural; a reaction pathway that possesses a low activation energy at low pH [121]. Spillman has also cited the potential for furfural to reduce to furfuryl alcohol during storage in barrel-aged wines, citing a correlation between furfural loss and furfuryl alcohol formation [117]. This could also help to explain the lack of glucose and fructose contribution to the formation of furfural. If furfural is formed as a degradation product of either glucose or fructose, it could immediately reduce to form furfuryl alcohol, given the acidic nature of the juice. Therefore, furfural

could be considered an intermediate compound in the formation pathway of furfuryl alcohol from glucose and fructose [117].

Table 4.4: Results of Isotope-Labeled Mass Spectrometry for Apple Juice. Furfural, Furfuryl Alcohol, and 5-MF are presented along with their three precursor treatments, parent ion mass, percentage of labeled ion, and C_6 contribution percentage to the formation of the off-flavor marker.

Compound	Treatment	Parent (M)	Labeled (M+X)	% Contribution
Furfural	Glucose	95	0.57%	**2.41%**
Furfural	Fructose	95	0.006%	**0.03%**
Furfuryl Alcohol	Glucose	98	14.22%	**50.5%**
Furfuryl Alcohol	Fructose	98	31.12%	**100%**
5- MF	Glucose	110	1.61%	**14.5%**
5-MF	Fructose	110	2.26%	**20.4%**

Based on the observations of the current study, the Maillard reaction can be considered a key reaction pathway for off-flavor formation in apple juice. This hypothesis mimics color formation, as the Maillard reaction is considered the most important cause of browning in apple juice [122]. As a result, subsequent research aimed at limiting the Maillard reaction by interfering with the production of Maillard-related off-flavor precursors, like Amadori compounds or reactive carbonyls, in apple juice may also have a significant effect on off-flavor formation, and thus could result in the improved acceptability of apple juice products.

4.4 Compositional Effects of Reactive Amino Acids

Previously, Ashoor & Zent [28] compared the non-enzymatic browning potentials of each free α-amino acid in heated model sugar solutions, demonstrating that each amino acid

possesses a different reactivity towards the production of nonenzymatic browning products at a defined pH. The amino acids were classified into three groups: high, intermediate, and low reactivity towards nonenzymatic browning. The present study examined the impact of high-reactivity amino acids on the formation of off-flavor volatiles in aged fruit juice. Two amino acids were selected in each juice according to their known reactivity towards nonenzymatic browning in an acidic matrix. In orange juice, Glutamine (GLN) and Tryptophan (TRP) were selected. TRP is listed as a “high potential” for non-enzymatic browning in the Ashoor & Zent study [63] while GLN has been noted in other Maillard studies to be the most reactive amine-containing compound that predominately contributes to color and aroma formation, which supports its selection for investigation in both apple and orange juice [123]. In apple juice, Glutamine (GLN) and ɣ-aminobutyric acid (GABA) were selected as the high-reactivity amino acids. GABA has been reported as an indicator of non-enzymatic browning due to its potential to form a 2-furoylmethyl derivative, which is an Amadori compound representative of the Maillard reaction pathways in juice [19]. GLN, TRP, and GABA have all been shown to decrease in concentration over time in fruit juice, warranting the need to investigate their role in the formation of storage-related degradation products [66]. The structures of the three highly-reactive amino acids used are presented in **Figure 4.2** below.

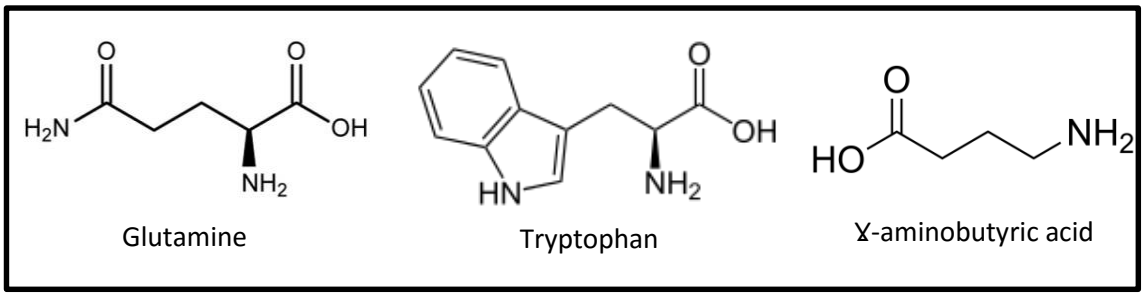
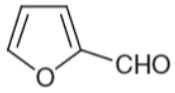
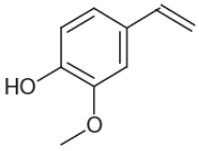
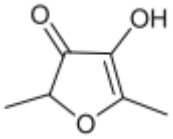
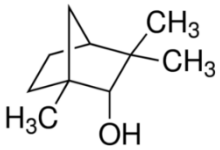
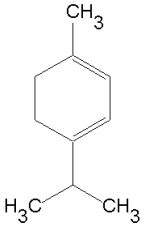


Figure 4.2: Structures of glutamine (GLN), Tryptophan (TRP) and ɣ-aminobutyric acid (GABA)

The impact of amino acids on the formation of off-flavor markers during nonrefrigerated storage was evaluated by spiking with amino acids in the juice samples and measuring the concentration of off-flavor markers in juices after 2, 4, and 8 weeks of high temperature (35°C) storage. The off-flavor markers of interest were identified in Chapter 3, and are presented in **Tables 4.6 & 4.7** along with their odor descriptors and structures.

Table 4.6: Off-Flavor Markers identified in Orange Juice

Marker Compound	Odor	Structure
furfural	Roasted	
p-vinyl guaiacol	smokey, burnt	
4-Hydroxy-2,5-dimethyl-3(2*H*)-furanone (DMHF)	heated sugar, caramel	
β-fenchyl alcohol	cleaner, soil	
α-terpinolene	old orange	

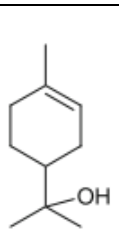
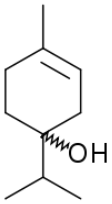
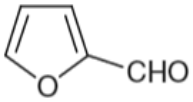
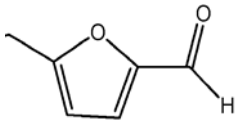
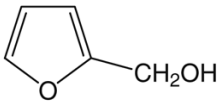
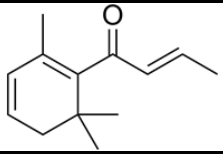
α-terpineol	musty, spicy	
terpinen-4-ol	sweaty, citrus	

Table 4.7: Off-Flavor Markers identified in Apple Juice

Marker Compound	Odor	Structure
furfural	roasted, seed	
5-methyl furfural (5-MF)	yeasty, cooked	
furfuryl alcohol	oil, cooked	
β-damascenone	roasted fruit, apple	

Orange Juice

An interesting pattern can be observed with furfural, a common nonenzymatic browning marker in orange juice [47]. **Figure 4.3** shows the concentration changes of furfural during storage of the three orange juice models over 8 weeks. GLN promoted the highest concentration of furfural after 2 weeks of storage, but resulted in a lower overall concentration of furfural than the control or TRP model after 8 weeks of storage

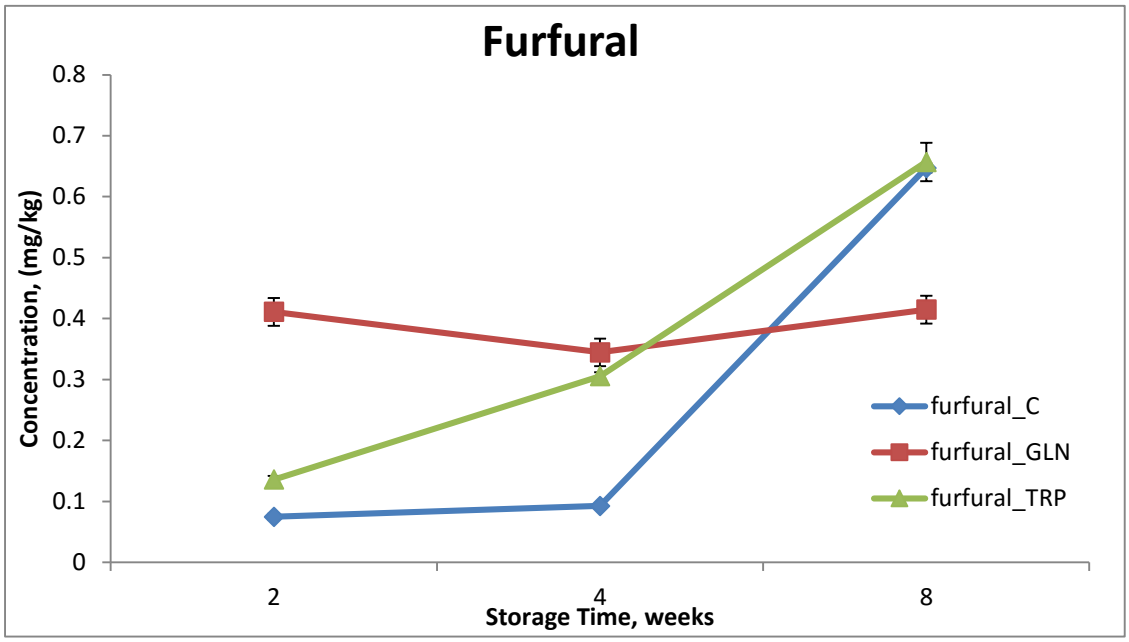


Figure 4.3: Furfural time-concentration relationship over 8-weeks of 35°C storage in orange juice for Tryptophan (TRP) and Glutamine (GLN) models compared to a control (C)

A similar kinetic relationship can be observed in a study conducted on a glutamine/sugar relationship in grape juice, where glutamine was shown to accelerate the formation of furfural more quickly than other amino acids like arginine after only one week of storage [106]. This observation suggests that GLN is likely to be involved in the initial formation of furfural during the storage orange juice, as well as impacts the rate of the formation of furfural over time. A reason as to why furfural concentration after 8 weeks of storage is not observed to be increased with the addition of reactive amino acids could be that, as shown in the isotope study, ascorbic acid degradation is responsible for the production of a majority of furfural in orange juice. Ascorbic acid degradation does not require amino acids to proceed, and therefore explains why the concentration of furfural remains unchanged between the TRP treatment and the control over 8 weeks. The lower concentration of furfural observed in the GLN model after 8 weeks may be attributed to the potential of GLN to alter the reaction chemistry of non-enzymatic browning

pathways. Niquet [123] has suggested that GLN is the most reactive (and unstable) amine in a heated Maillard reaction system, citing its potential to release reactive ammonia following an acid-catalyzed hydrolysis of the amide bond. Ammonia can react with reducing sugars and Maillard intermediates like methylglyoxal, and alter the chemistry leading to the formation of furfural. It is possible that when exposed to a high concentration of GLN, the carbonyl species involved in furfural production during ascorbic acid degradation, such as the xylosones in **Figure 2.4**, will react with free ammonia and limit the formation of furfural during storage. The ammonia-carbonyl interaction can, however, promote Maillard reaction pathways and generate other Maillard derived products [123]. Izzo and Ho demonstrated this in a gluten-glucose model system at pH 6. The deamination of GLN and release of ammonia can participate in the Maillard reaction and form heterocyclic amine-contain compounds like pyrazines under high temperature conditions [124]. When examining the two amino acids used in this study for potential to form ammonia, GLN releases 100% of ammonia on a molar basis when heated compared to TRP releasing about 10%, which could explain the lack of furfural inhibition in the TRP model [125]. Therefore, although furfural production may be limited in orange juice with the exposure to highly-reactive GLN, it will still be formed in some concentrations along with other Maillard products in a GLN-rich system, and remain fairly unchanged in a TRP-rich system.

The addition of either amino acid exhibited no significant difference on the formation of fenchyl alcohol, as shown in **Figure 4.4.**

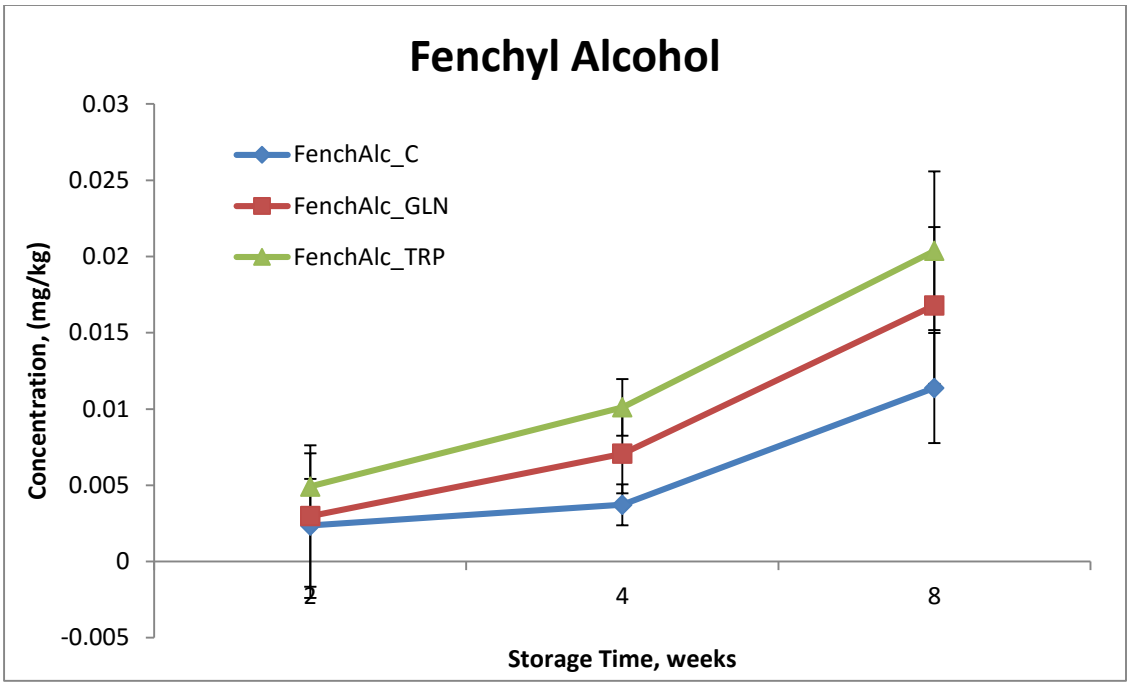


Figure 4.4: Fenchyl Alcohol formation over 8-week 35°C storage time for Tryptophan (TRP) and Glutamine (GLN) models compared to a control (C)

In addition to being a known microbial contaminant, fenchyl alcohol can be a formed as an acid-catalyzed degradation product of β-pinene, as proposed by Kimura et al [101]. β-pinene is considered a character impact compound in fresh juices, and lends a characteristic green and citrus-like aroma [68]. It is likely fenchyl alcohol is unaffected by amino acid concentration because it is formed from a reaction pathway that does not involve the degradation by amino acids.

4-Hydroxy-2,5-dimethyl-3(2*H*)-furanone, a well-recognized off-flavor compound in orange juice [52], was observed to increase in concentration with the addition of TRP and GLN after 8 weeks of storage time, as well as after 4 weeks with the addition of TRP. However, the rates of formation differ for each amino acid. TRP demonstrated the most rapid rate of generation, as shown in **Figure 4.5.**

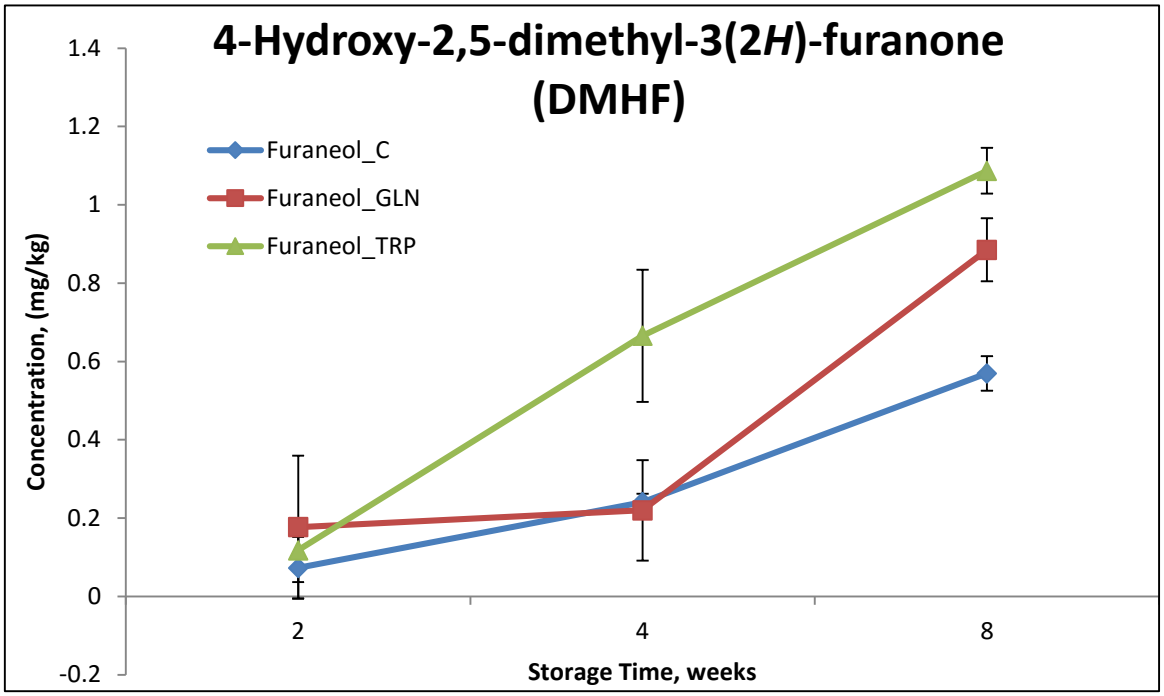


Figure 4.5: DMHF formation over 8-week 35°C storage time for Tryptophan (TRP) and Glutamine (GLN) models compared to a control (C)

However, after 8 weeks, both the GLN and TRP models resulted in the formation of significantly higher concentration of 4-Hydroxy-2,5-dimethyl-3(2*H*)-furanone (DMHF) when compared to the control with TRP and GLN spiked systems having 36 and 48% more DMHF respectively. This further evidences the impact of the Maillard reaction on DMHF formation, as sugar degradation *via* the Maillard reaction was shown to contribute 100% of DMHF formation in the isotope study. In previous studies, DMHF was shown to form *via* the Maillard reaction from the reducing sugar rhamnose and the amino acid arginine, and not from other reducing sugars or amine compounds in an acidic environment [51]. However, these findings do not take into account the possible precursors of DMHF like C3 hexose fragments or Strecker degradation products, which may be favored to form *via* Maillard reaction pathways utilizing glucose and fructose, as well as GLN and TRP [29], [110]. Hexose fragmentation, as discussed earlier, can

significantly impact the reaction routes of the Maillard reaction [107]. Therefore, a proposed reaction pathway for the formation of DMHF from the Maillard reaction utilizing TRP or GLN follows a reduction of acetylformoin-type sugar fragments formed from the chain-elongation of Strecker aldehydes, as suggested by Blank [126], and further evidenced by Schieberle [110]. A mechanism depicting the formation of DMHF utilizing these reactive carbonyl sugar fragments is shown in **Figure 4.6** below [110]. Hydroxyacetone, an established sugar degradation product, may tautomerize into an endiol, react with another sugar fragment (in this case, 2-oxopropanol), undergo a cyclization, and lose water to form DMHF. This carbonyl fragment formation of Furaneol, therefore, can be impacted by amino acids like TRP and GLN in orange juice, and can explain the observations in **Figure 4.5.**

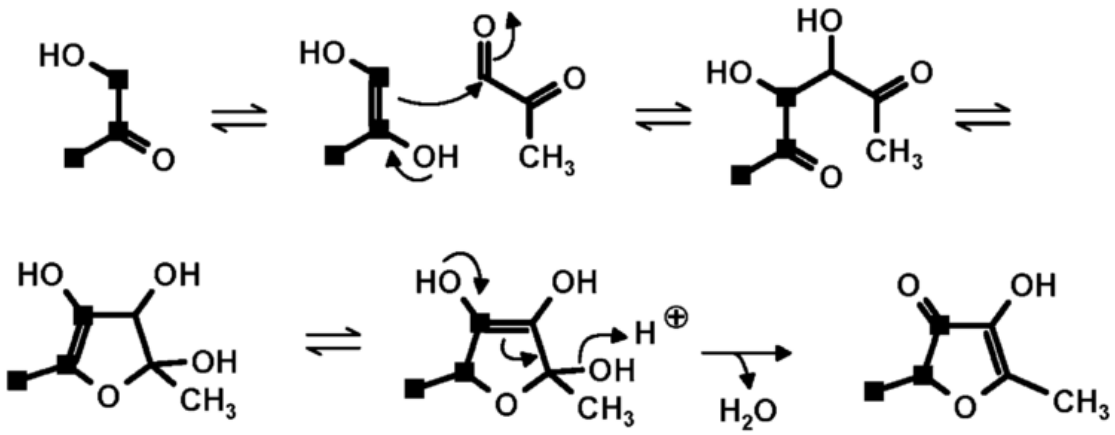


Figure 4.6: Mechanism depicting the formation of Furaneol from the transient Maillard intermediated hydroxyacetone and 2-oxopropanal [110]

Both TRP and GLN contain two amino moieties, making them both highly reactive towards non-enzymatic browning [59]. Niquet and Tessier showed that GLN is one of the most impactful amino acids in the Maillard reaction, due to its high concentration in many foods and its potential for degradation when heated [123]. However, Ajandouz has

demonstrated that TRP is one of the most reactive amino acids towards the Maillard reaction in a glucose/amino acid model system based on percent loss and formation of Maillard products [59]. Thus, when an increased concentration of TRP is present, it may promote the formation of Maillard reaction intermediates more than that of GLN.

The addition of TRP and GLN also promoted formation of p-vinylguaiacol more rapidly than the control, as shown In **Figure 4.7;** however the final concentration of *p*-vinylguaiacol in the amino acid models was lower than that of the control. The maximum concentration of about 0.4 mg/kg was achieved after 4 weeks of storage in the amino acid models, whereas a concentration of about 0.55 mg/kg of *p*-vinylguaiacol is reached after 8 weeks in the control.

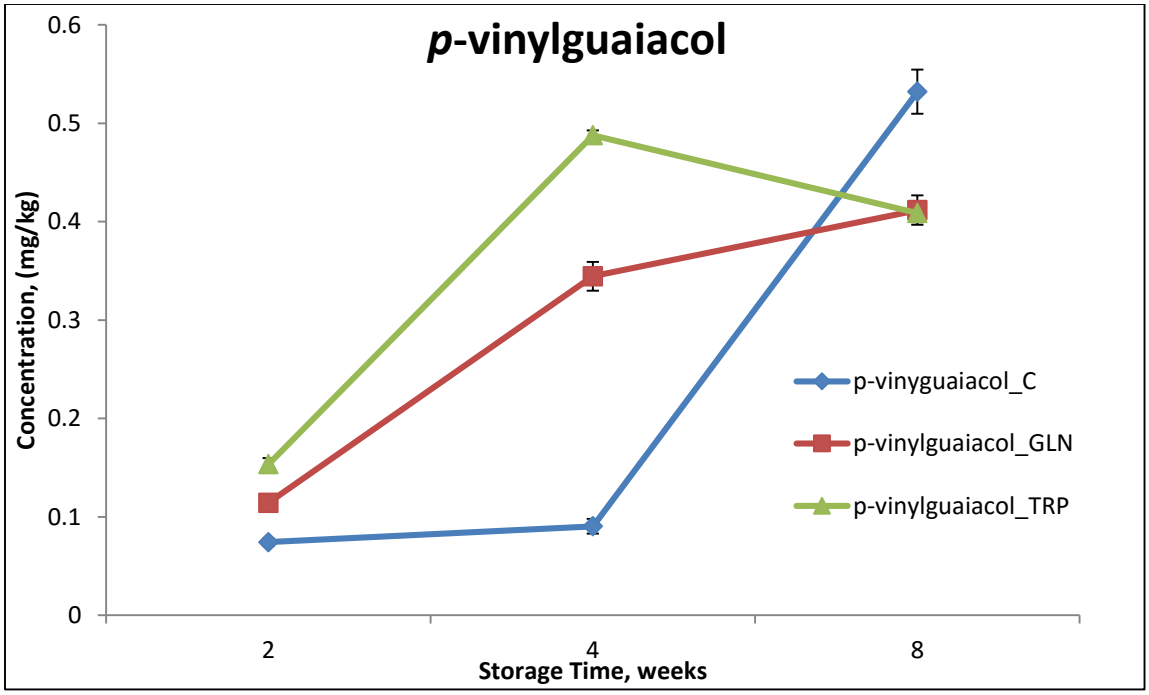


Figure 4.7: p-vinylguaiacol formation over 8-week 35°C storage time for Tryptophan (TRP) and Glutamine (GLN) models compared to a control (C)

The compound *p*-vinylguaiacol is considered the most sensory-significant off-flavor compound in aged orange juice [52], and is not considered a direct nonenzymatic

browning product in prior research [14], [52]. Decarboxylation of free ferulic acid is cited as the primary route of formation for *p*-vinylguaiacol[14]. An acidic environment and exposure to heat during thermal processing is thought to impact the extent of decarboxylation during nonrefrigerated storage [127]. However, in the presence of high concentrations of highly-reactive amino acids like TRP and GLN, the concentration of *p*-vinylguaiacol is shown to be greater than the control after 4 weeks of storage, but 20% less than the control after 8 weeks of storage time. This data suggests an accelerated pathway for the generation of p-vinylguaiacol in the presence of GLN and TRP during the first four weeks of storage, but limitation of the extent of total *p*-vinylguaiacol formation after 8 weeks of storage. This can be explained by the potential of Maillard chemistry to be involved in hydroxycinnamic acid breakdown reactions as suggested by Jiang & Peterson [128]. In the presence of high concentrations of GLN and TRP, the Maillard intermediates that are favored to form could be different than the 3-deoxy-2-hexocelluloses which go on to react with p-vinylguaiacol and form a phenolic-Maillard adduct. This phenolic-Maillard adduct is proposed to limit the extensive decarboxylation that allows free *p*-vinylguaiacol to exist at high levels in fruit juice, and can explain the lower concentration of *p*-vinylguaiacol after 8 weeks of storage when compared to the control. However, with different sugar fragmentation products, the interaction between *p*-vinylguaiacol and the reactive carbonyl species could be limited, and allow free *p*-vinylguaiacol to be present in the juice, explaining the increase in concentration of *p*-vinylguaiacol in the amino acid models during the first four weeks of storage. **Figure 4.8** outlines the proposed formation of a phenolic-Maillard adduct, which can be limited by the addition of GLN and TRP, and explains the observations in **Figure 4.7.**

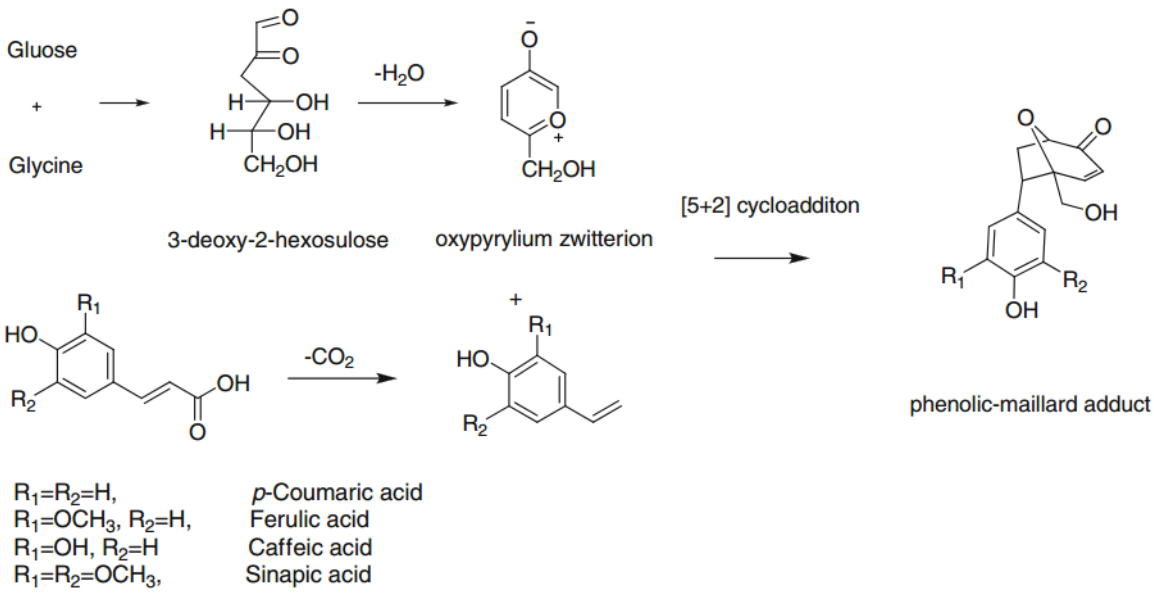


Figure 4.8: Reactivity of hydroxycinnamic acids via Maillard chemistry in low moisture baking model systems; adapted from Jiang and Peterson[128]

Mixtures of ferulic acid and amino acids have been cited in the past to act as natural preservatives that can prevent browning [129]. Hurrell as cited that a ferulic acid-TRP complex can prevent the polymerization of quinones that lead to the formation of brown compounds during non-enzymatic browning [130]. Further, Jiang and Peterson also indicated that the addition of ferulic acid to cereal will reduce the generation of Maillard-like aroma compounds like furfural[128]. This result further evidences the interconnecting role of the Maillard reaction in off-flavor development in juices during storage, as well as highlights the importance of individual amino acids that drive the formation of different reactive Maillard intermediates. A hypothesis for this observation is the formation of a phenolic-Maillard adduct is limited in the presence of higher amounts of TRP and GLN, influenced by the selectivity of Maillard intermediate formation.

The compound α-terpineol was present at the highest concentration among the off-flavor markers in this study, and its concentration showed a positive correlation with the amino

acid concentration **(Figure 4.9).** Both TRP and GLN accelerated the formation of α-terpineol, as well as resulted in an overall higher concentration of the compound than the control after the 8 week storage time.

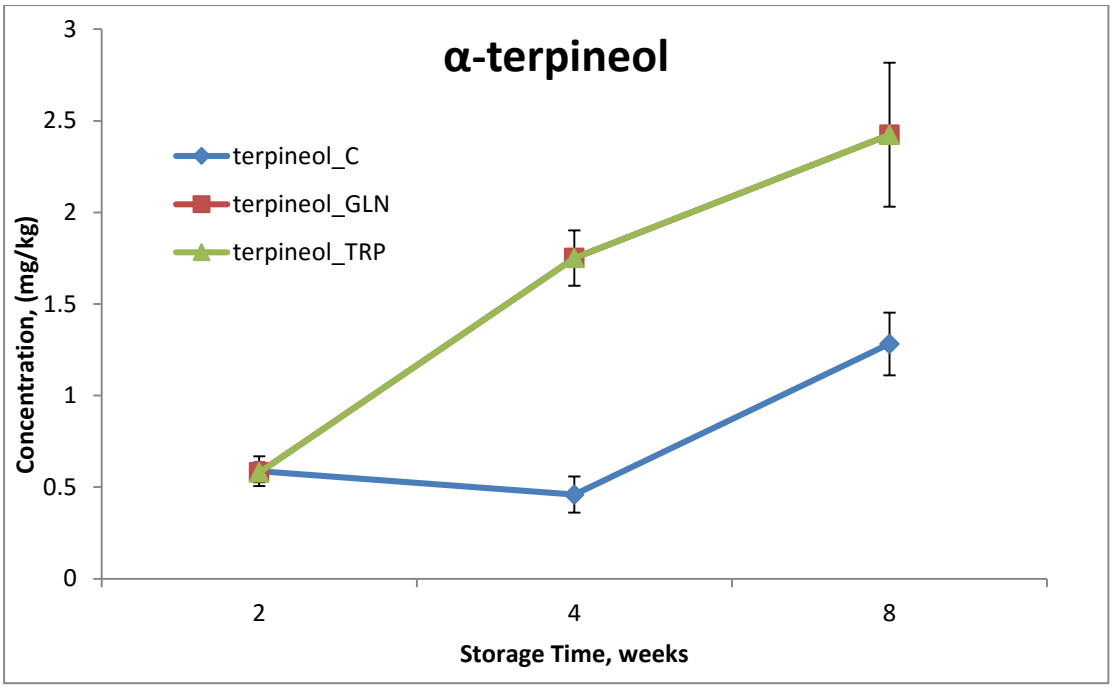


Figure 4.9: α-terpineol formation over 8-week 35°C storage time for Tryptophan (TRP) and Glutamine (GLN) models compared to a control (C)

The precursors of α-terpineol, namely, linalool and limonene, are the most abundant terpenoid compounds present in fresh citrus juice. The degradation of limonene and linalool to form α-terpineol is not uncommon, and has been established as a way to monitor citrus juice quality when exposed to different processing and storage conditions [67]. Terpene degradation has been thought to be unrelated to non-enzymatic reaction pathways. However, the observed effect of amino acids on the formation of α-terpineol indicated that the presence of highly reactive amino acids plays a role in the degradation of monoterpenes in citrus juice. An exact mechanism for this phenomenon is not well-documented; however, it is possible that terpene degradation is accelerated by the addition of amino acids *via* Strecker-like degradation pathways, as suggested by Rizzi

[29]. In both non-aqueous and aqueous systems, Strecker-like reactions have been observed between α-olefinic carbonyls and α-amino acids, similar to the precursors present in the orange juice matrices. Naturally occurring terpenoids can act as unsaturated carbonyl species in this reaction pathway, and react with α-amino acids to form volatile flavor compounds [29]. A proposed reaction mechanism can be viewed in **Figure 4.10** below [29]. With an increase in the concentration of highly-reactive amino acids, Strecker-like reactions could be promoted between abundant terpene compounds (1) and the reactive α-amino acids (2), leading to a decarboxylation of the amino acids and formation of odor-active volatile products like α-terpineol from an unstable amino-alcohol complex (4).

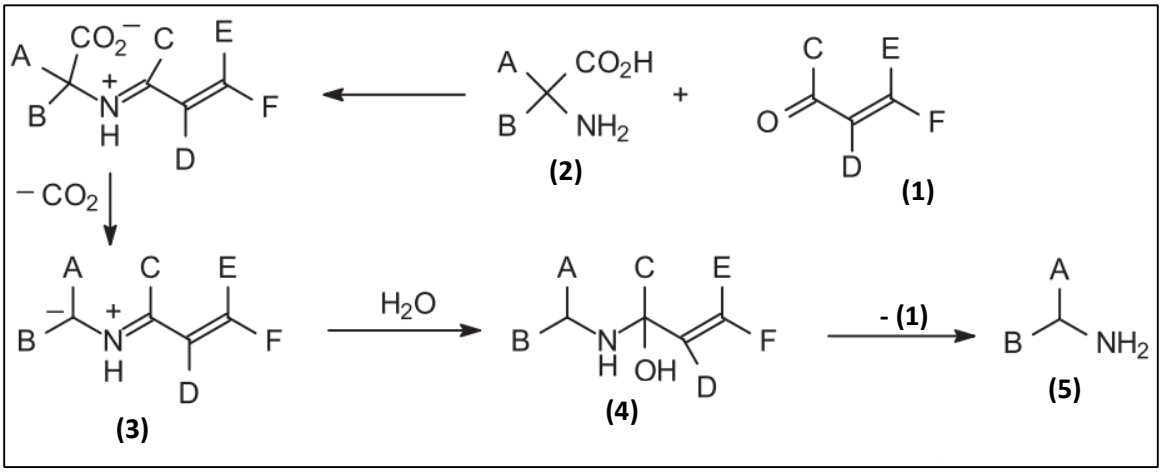


Figure 4.10: A proposed reaction scheme for α-amino acids reacting with unsaturated carbonyls [29]. (1)=unsaturated carbonyl (terpenoid), (2) = α-amino acid, (3) =Schiff base, (4) =Unstable amino alcohol, (5) = saturated amine derived from amino acid reactant and regenerated carbonyl reactant.

This hypothesized mechanisms, however, must be researched further, as another study conducted by Zhang and Ho concluded that there was no evidence of Strecker aldehydes derived from this type of chemistry in an aqueous system at neutral pH [131]. The current study however was conducted in an acidic matrix (juice), and was aged.

Further investigation on Strecker and Strecker-like reactions between amino acids and terpenoid compounds as potential sources of novel flavor compounds is warranted.

Although terpinein-4-ol was at much lower concentrations than α-terpineol, it also was affected by the addition of GLN and TRP. Terpinen-4-ol was observed to have a more rapid formation, as well as a significantly higher overall concentration than the control for both the GLN and TRP models, suggesting that the reaction pathway responsible for its formation is similar to that of α-terpineol.

Terpinen-4-ol **(Figure 4.11)** has been cited as an off-flavor compound in citrus juices in the past [67], and is used as a quality marker in juices that are thermally processed and aged.

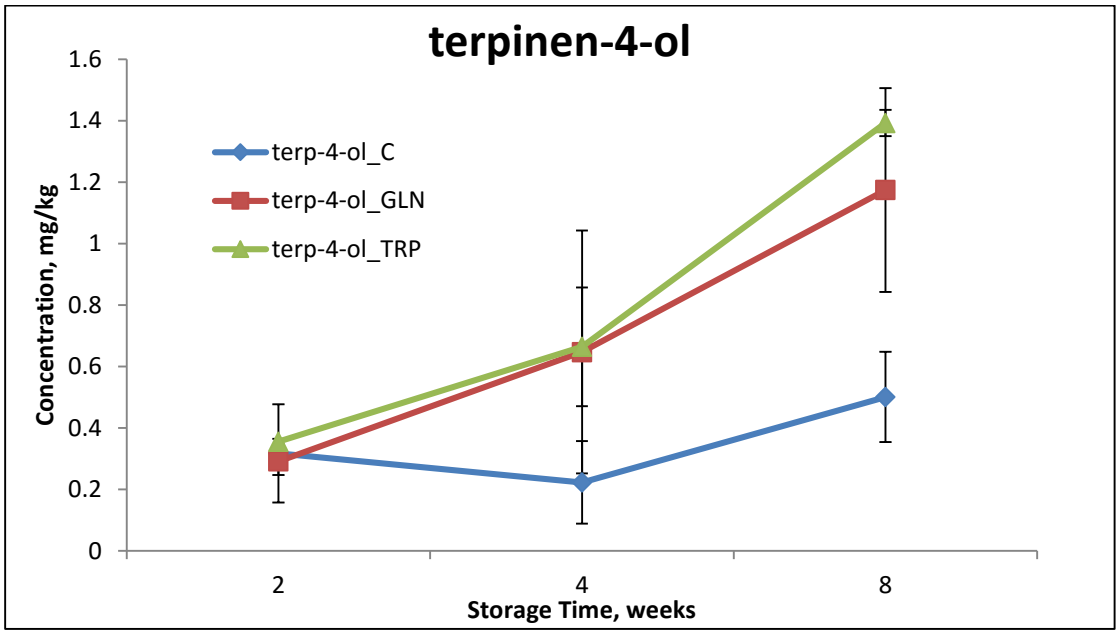


Figure 4.11: Terpinen-4-ol formation over 8-week 35°C storage time for Tryptophan (TRP) and Glutamine (GLN) models compared to a control (C)

A hypothesis can be made that the reaction pathway responsible for terpinen-4-ol formation mirrors α-terpineol, because both terpinen-4-ol and α-terpineol are terpene

alcohols that can be formed from the acid-catalyzed breakdown of limonene or linalool [67]. However, like α-terpineol, amino acids are not involved in this type of terpene degradation, suggesting that alternate reaction pathways such as Strecker-like degradation described above are involved in the generation of terpinen-4-ol.

Terpinolene was only affected by the addition of GLN, as shown in **Figure 4.12.** This compound differs from α-terpineol and terpinen-4-ol by the presence of an alcohol (-OH) group, a structural difference that could play a role in the favored reaction pathway and products formed.

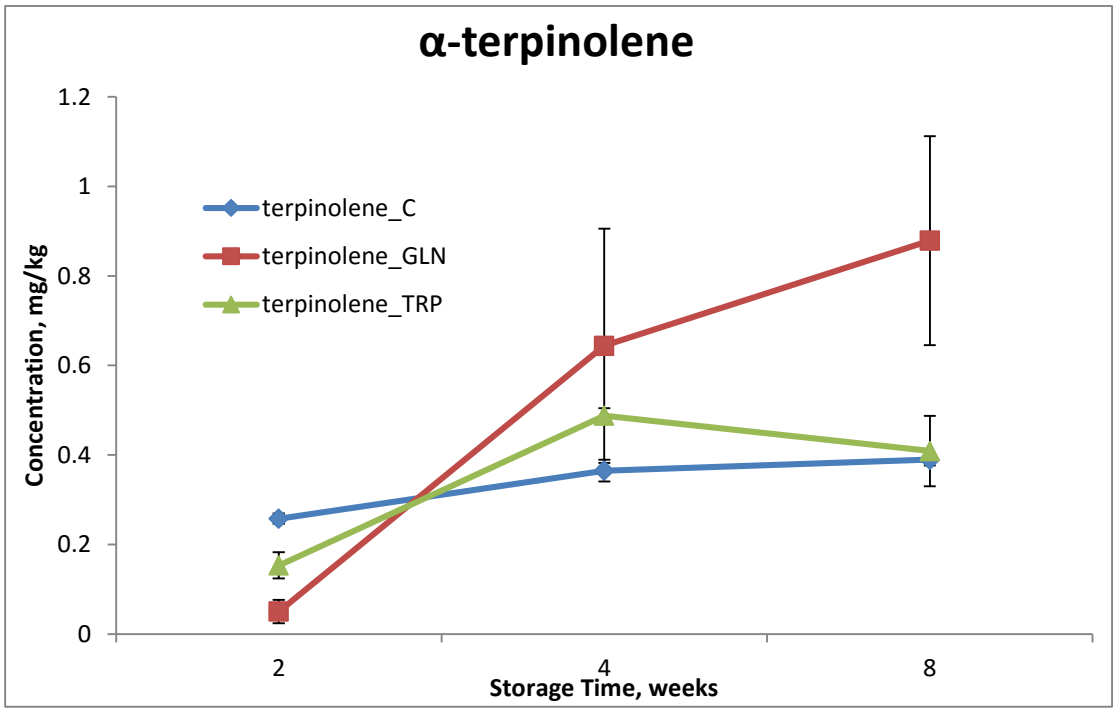


Figure 4.12: α-terpinolene formation over 8-week 35°C storage time for Tryptophan (TRP) and Glutamine (GLN) models compared to a control (C)

Terpene degradation is considered an independent reaction pathway from nonenzymatic browning, and normally occurs due to the oxidative rearrangement under thermal conditions [30]. However, the results of this study clearly indicate the addition of highly-

reactive amino acids play a role in the formation of terpene degradation compounds. The Maillard reaction also seems to interconnect with terpene degradation, as they may share the same initial reaction pathways of Strecker degradation [29]. It is unlikely that the amino acids play a direct role in the formation of terpene degradation compounds, as the reaction pathway is most commonly a direct rearrangement of monoterpenes via oxidation due to heat, or acid-catalyzed breakdown of monoterpenes due to the low pH of citrus juices [30] [132]. Amino acids play a more diverse role in the formation of off-flavors than previously thought; and further research can be focused on evaluating the extent of this impact in order to begin developing flavor optimization procedures to prevent off-flavor formation in orange juices during non-refrigerated long term storage.

Apple Juice

The effect of the addition of GLN and GABA on the formation furfural, 5-MF, furfuryl alcohol, and β-damascenone in an apple juice matrix was examined. Furfural exhibited no significant difference in formation between the control and either amino acid model, while 5-MF and furfuryl alcohol both showed a positive correlation with increasing concentration of amino acids (**Figure 4.13 & 4.14).** The last marker, β-damascenone, also demonstrated an increase in concentration and in the rate of formation for both the GABA and GLN models when compared to the control (**Figure 4.16).**

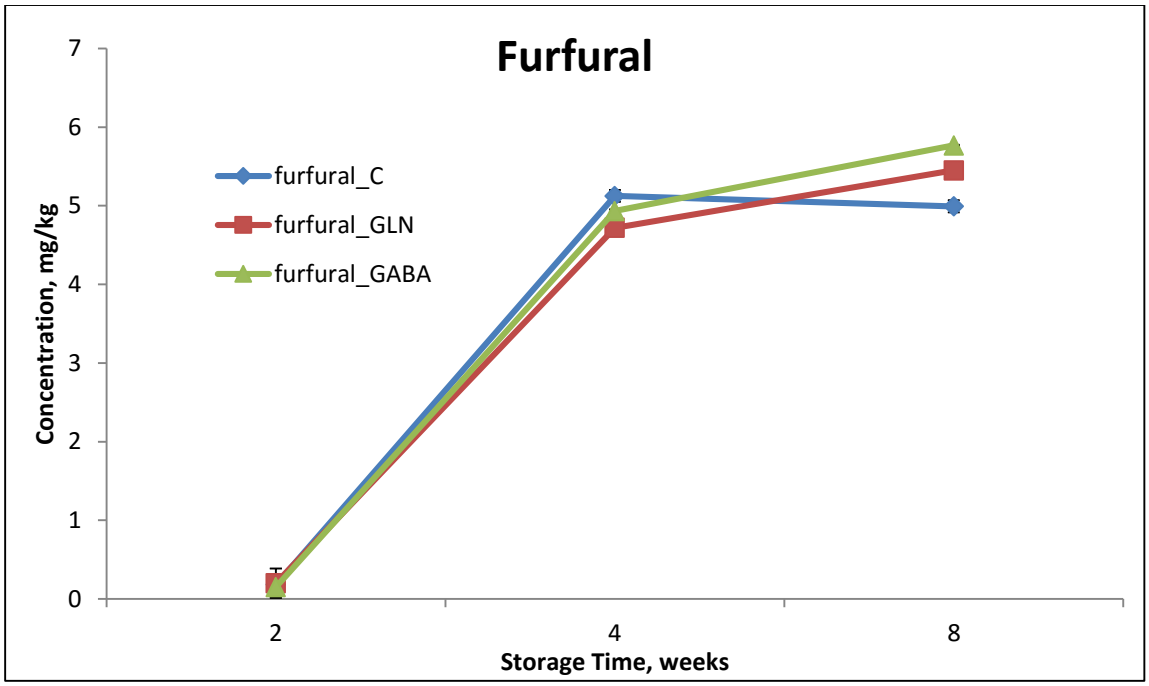
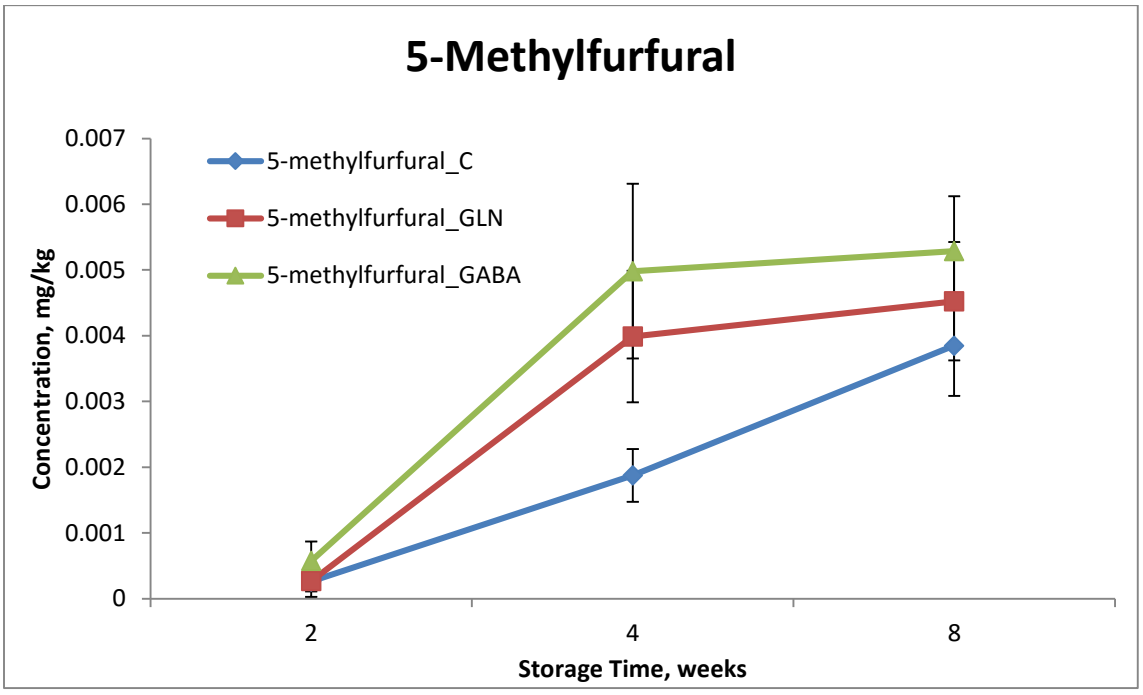


Figure 4.13: Furfural formation over an 8-week 35°C storage time for ɣ-aminobutyric acid (GABA) and Glutamine (GLN) models compared to a control (C)



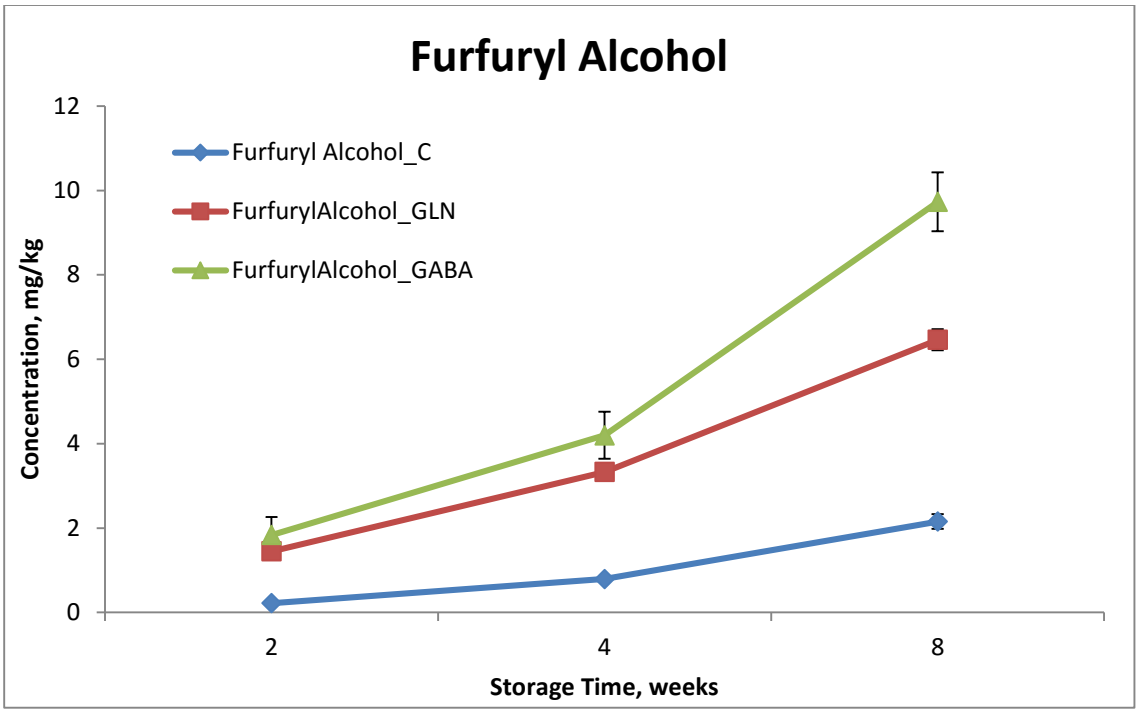


Figure 4.14: 5-methylfurfural and Furfuryl Alcohol formation over an 8-week 35°C storage time for ɣ-aminobutyric acid (GABA) and Glutamine (GLN) models compared to a control (C)

It can be inferred that furfural follows a different formation pathway than both 5-MF and furfuryl alcohol. The isotope labelling experiments conducted previously support this as well; 5-MF and furfuryl alcohol exhibited prominent formation contributions from reducing sugars, whereas furfural is not formed in large amounts via degradation of glucose or fructose. Amino acid concentration did not impact the formation of furfural in apple juice in the same way as it did in orange juice. This could be due to the concentration of ascorbic acid; furfural is formed primarily from ascorbic acid degradation in orange juice, and therefore can be impacted by reactive amino acids and prevent the formation of furfural as discussed above. Ascorbic acid levels in apple juice are very low, and present little opportunity for amino acids to impact the formation of furfural *via* ascorbic acid degradation. Both 5-MF and furfuryl alcohol, on the other hand, were more rapidly formed with the addition of both GABA and GLN. This observation

can most likely be attributed to the Maillard reaction, were in agreement with the results of the isotope study.

The compound 5-methylfurfural, as discussed in the isotope section, is proposed to form from either the direct oxidation of Maillard reaction intermediates, such as 3-deyoxyhexosomes [113], or the direct degradation of 5-hydroxymethylfurfural (HMF) *via* condensation [114], as shown in **Figure 4.15.** Both of these proposed formation pathways are favored at an acidic pH [117], as well as can be influenced by amino acid composition [119]. Tressl has published work on the formation of GABA-specific Maillard products from glucose and fructose, which include the formation of 5-methylfurfural, from the oxidation of 3-deoxyaldoketose intermediates [112]. It could be hypothesized, then, that GABA and GLN drive the Strecker degradation of α-dicarbonyls to form Maillard reaction intermediates more prone to oxidation, and thus, form 5-methylfurfural in higher concentrations than the control.

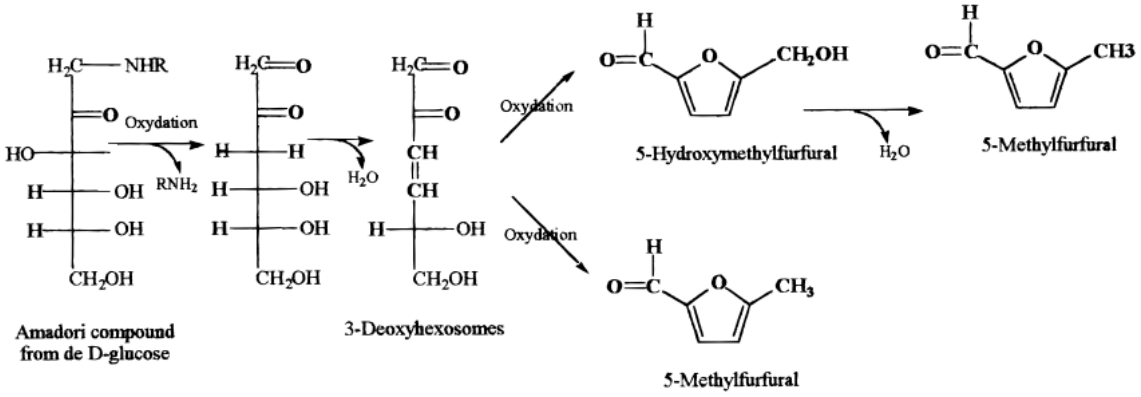


Figure 4.15: Possible formation of 5-methylfurfural from the degradation of Maillard intermediates, as well as the condensation of 5-hydroxymethylfurfural (HMF)[114]

Furfuryl alcohol exhibited a similar formation relationship to 5-methylfurfural, in that the final concentration after 8 weeks of 35°C storage was significantly higher than that of the control. However, furfuryl alcohol is not cited as an oxidation product of Maillard

intermediates. As discussed in the isotope section, furfuryl alcohol is known as an acid-catalyzed reduction product of furfural at low pH [121]. Furfural concentration, however, was not altered by the addition of highly reactive amino acids. Further, Spillman as suggested that furfuryl alcohol can form from a decomposition of 5-methylfurfuryl alcohol in barrel-aged wines [117]. Five-methyl furfuryl alcohol can form from 5-methylfurfural via the reduction of the aldehyde under acidic conditions in the presence of water [133]. Therefore, a proposed reaction pathway for the formation of furfuryl alcohol in apple juice would include formation of 5-methylfurfural *via* the oxidation of Maillard reaction intermediates as described earlier, reduced to 5-methylfurfuryl alcohol *via* acid catalysis, and then finally decomposed to furfuryl alcohol, as outlined in Spillman [117]. As a result, amino acids like GABA and GLN will be able to impact the Maillard intermediate formation just as discussed with 5-methylfurfural, and thus form furfuryl alcohol in higher concentrations than the control. This proposed reaction pathway can also help to explain why the concentration of 5-MF was very low in comparison to furfuryl alcohol, in that potentially 5-MF was an intermediate to the formation of furfuryl alcohol. It is interesting to note that the reduction of 5-methylfurfural to 5-methyl furfuryl alcohol *via* nucleophilic addition is most readily induced with a hydride reagent, which in this case could be free ammonia released from the acid-catalyzed breakdown of GLN or GABA, as discussed earlier. Also, GABA is more reactive in non-enzymatic browning reactions utilizing hexose sugars than other naturally occurring α-amino acids like GLN in heated sugar/amino acid model systems, as concluded by Lamberts [134]. This could help explain why the GABA amino acid models showed higher concentrations of 5-MF and furfuryl alcohol after 8 weeks of storage.

Finally, β-Damascenone concentration during storage time was shown to increase with the addition of TRP and GLN. This compound is thought to be formed from the hydrolytic

breakdown of secondary metabolites of fruits, such as carotenoids [74]. Chevance and co-workers have investigated the formation of β-damascenone in aged beers, and has suggested that the acidic nature of beer promotes the acid hydrolysis of β-damascenone precursors during aging [135]. Potential precursors for this reaction include various allene triols and acetylene diols that arise from the degradation of neoxanthins present in the beer as observed in wines [135]. Chevance [135] has also suggested that glycosides containing β-damascenone precursors constitute an important source of off-flavors related to aging of beer and wine. These degradation reactions are entirely pH-dependent, as a lower pH will result in the formation of a higher concentration of β-damascenone over storage time [136]. It can be hypothesized, that the addition of highly-reactive amino acids promotes the release of β-damascenone precursors more rapidly over storage time. The exact mechanism for this remains unknown, and can be the subject of further research.

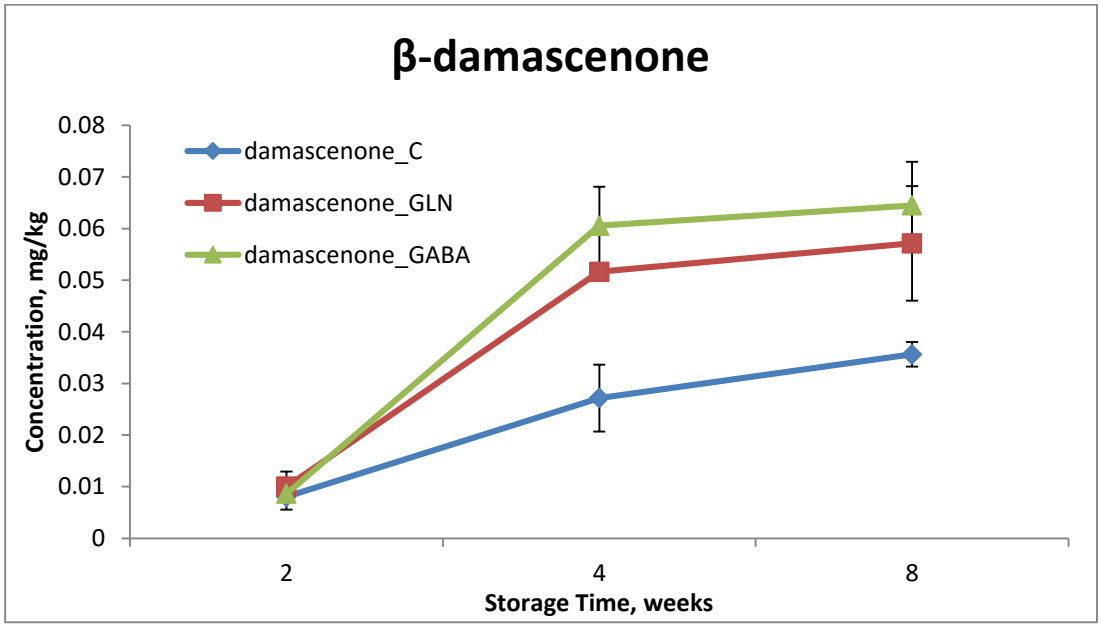


Figure 4.16: β-damascenone formation over an 8-week 35°C storage time for ɣ-aminobutyric acid (GABA) and Glutamine (GLN) models compared to a control (C)

4.4 *Conclusion*

In fruit juice, the production of off-flavors during nonrefrigerated storage can be attributed to several different reaction pathways; including ascorbic acid degradation, the Maillard reaction, terpene degradation, and ferulic acid decarboxylation. The results of this study clearly highlight the impact of Maillard reaction pathways on the formation of off-flavors during the storage of apple and orange juices, and lend new insights into the mechanisms that can be controlled to limit quality loss over storage time.

In both apple and orange juice, Maillard-like reaction pathways are suggested to have the highest impact on the formation of off-flavor, as well as on the reaction environment, over long-term storage. As discussed above, the interaction of amino acids with α-carbonyls and α-dicarbonyls can help explain the reaction pathways that form furfural, Furaneol, α-terpineol, terpinen-4-ol, terpinolene in orange juice, as well as the formation of furfural, 5-methylfurfural, and furfuryl alcohol in apple juice. The remaining off-flavor markers *p*-vinylguaiacol and β-damascenone were influenced by amino acid concentration, even though the formation mechanisms suggesting the interplay between Maillard reaction pathways and their generation. The information obtained from this study can be used in targeted reaction pathway studies that focus on limiting the impact that amino acids have on the production of off-flavor compounds. Inhibiting the reactivity of amino acids either by removal, competition, or ingredient addition will result in a direct inhibition and/or control of formation of off-flavor over time.

With this information, amino acids with the highest off-flavor formation potential could be used as markers when developing apple and orange blending strategies during the harvesting and processing of commercial fruit juice varieties. However, further research must be conducted carrying out these classifications, because the complexity and

interdependence of amino acids on reaction pathways mases the assigning of specific roles to any given amino acid very challenging.

Overall, the results of this study demonstrated that the Maillard reaction is more involved in the production of off-flavors during storage than previously considered. Further flavor optimization of aged orange and apple juice products could be accomplished with the addition of ingredients to the juice, such as other amino acids or carbonyl-scavengers that will disrupt the reaction chemistries responsible for the production of off-flavor from Maillard reaction intermediates. For example, the addition an amino acid in large amounts known to be less reactive towards non-enzymatic browning, like valine [59], could help in the mitigation of the reaction pathways responsible for off-flavor formation during storage. Fruit blending varieties can also be developed in order to limit the amount of highly reactive amino acids or other Maillard precursors in the fruit in order to limit the formation of off-flavors over time. It also may be beneficial to alter the pH of juice prior to storage, because a majority of the off-flavor compounds described in this study is formed as a result of a low pH favoring their formation pathways. Further, early research on amino acid deactivation *via* UV treatment has been suggested as a way to limit the activity of amino acids in model systems [137]. Flavor optimization techniques could lead to the improved acceptability of aged orange and apple juices, and provide the basis for flavor improvement in other typed of fruit juices aged over long periods of time.

Chapter 5: Suggested Future Work

Overall, this study has provided new insights into the aroma characterization of off-flavor formation during the long term non-refrigerated storage of commercial apple and orange juice products, as well as provided the opportunity for future research on off-flavor formation pathway investigation. State of the art techniques such as SBSE and isotope ratio mass spectrometry were employed to aid in this characterization, and have provided value for researchers and industry professionals attempting to advance their understanding of off-flavor formation in fruit juice during storage.

In addition to elucidating off-flavor generation, expanding our understanding of the mechanisms of loss of desirable flavors is equally important. As an expansion of the GC-O section, the 15 compounds in orange juice observed to decrease over storage time should be further investigated for impacts on flavor quality loss. Recent research has focused on identifying the major shelf-life volatile changes in orange juice, which includes the examination of the important chemical classes in orange juice—terpene alcohols, aldehydes, and esters, and their decrease over time [138]. By using untargeted methodologies like the ones presented in Widow [138], the kinetic modeling of both increasing and decreasing volatile compounds can be utilized for characterizing a chemical fingerprint of the volatile changes that occur during long-term storage of citrus juices.

Further work focused on understanding off-flavor precursors is also warranted in order to understand the changes that place during the long term storage of fruit juice. Investigation of alternate precursors like rhamnose, galactouronic acid, or lysine in carbon labeling studies could help to explain the favored reaction pathways for the complete formation each off-flavor constituent. Understanding the precursors involved in the formation of off-flavor compounds, as well as how they relate to the processing and

storage of fruit juice will allow researchers to develop methodologies to increase the flavor quality of fruit juice exposed to non-refrigerated storage.

Lastly, future research concerning off-flavor formation in fruit juice products *via* non-enzymatic browning should consider the impact of reactive carbonyl species. As highlighted earlier, non-enzymatic browning produces reactive carbonyl intermediates such as 1- and 3- deoxyosones and sugar fragmentation products that will interact with constituents in the juice to produce off-flavors and brown colors [119]. Clegg has stated that build-up of carbonyl compounds following the Maillard reaction and ascorbic acid degradation are responsible for the oxidation and fragmentation of molecules that lead to off-flavor and color formation in lemon juice[116]. It is possible that amino acids, among other juice constituents, promote the formation of these reactive carbonyl species that lead to quality loss in fruit juice products. In order to help solve this problem, development of a means to trap reactive carbonyl intermediates with naturally occurring scavengers, or a deeper understanding of the reaction mechanisms that form reactive carbonyl species is warranted.

Compounds such as polyphenols naturally contained within the juice could be utilized to inhibit browning and off-flavor development [108]. It is suggested that the addition of polyphenolic compounds before, during, or after processing can work to limit the formation of browning in juices over time *via* carbonyl trapping [15]. A large body of work has investigated trapping of carbonyl species by phenolic compounds and their respective mechanisms. Totlani and Peterson have published work on the mechanisms of carbonyl-trapping reactions in aqueous Maillard systems [139], and Kokkinidou has applied these mechanisms in UHT milk as an approach for flavor optimization [108]. The peels of fruits like oranges contain a large concentration of naturally-occurring

polyphenols, so a waste stream from juice production could be utilized as an ingredient source for off-flavor suppressors in the future.

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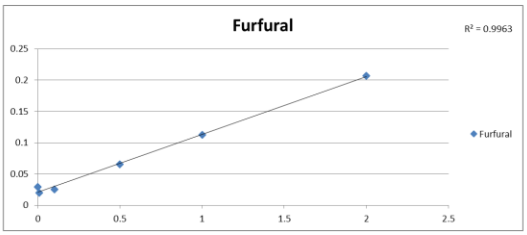
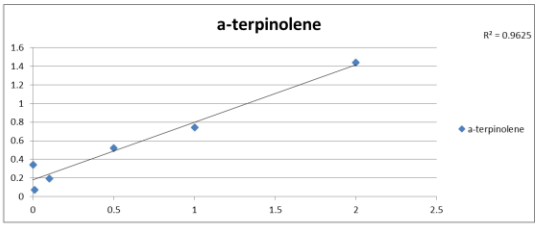
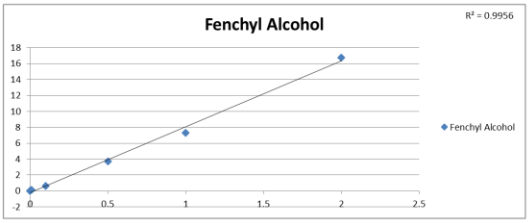
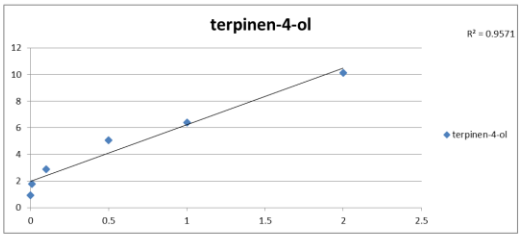
Appendix 1

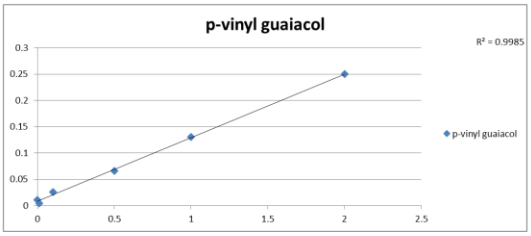
Orange Juice Standard Curves

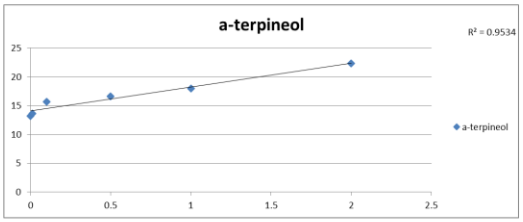


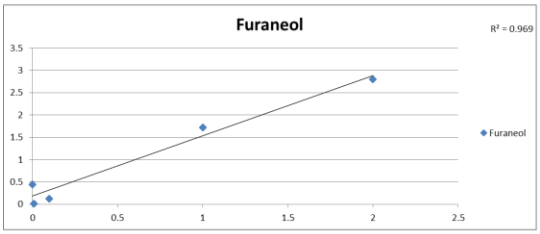


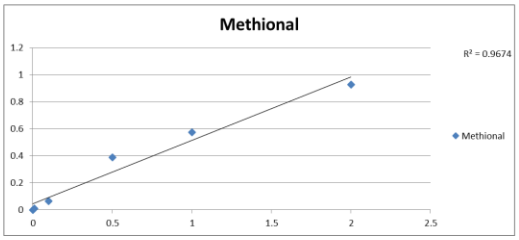












Apple Juice Standard Curves