EXAMINATION OF AROMA VOLATILES FORMED FROM THERMAL PROCESSING OF FLORIDA RECONSTITUTED GRAPEFRUIT JUICE

By

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A THESIS PRESENTED TO THE GRADUATE SCHOOL
OF THE UNIVERSITY OF FLORIDA IN PARTIAL FULFILLMENT
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To my Grandparents, with love.

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Over fifty percent of Florida's grapefruit supply goes into juice production. Grapefruit juice is heated after reconstitution with water to minimize spoilage microorganisms. However, heating induces chemical changes that degrade juice flavor. Fruit juices are traditionally heated using stainless steel surface plates or tubes. Stainless steel contains appreciable amounts of transition metals such as nickel and chromium that are known to catalyze chemical reactions. Acidic (pH 2.9-3.5) juice may also promote leaching of these metals. Glass should be more inert than stainless steel and may offer a means to produce higher quality juice. This study was undertaken to examine changes in aroma volatiles in reconstituted grapefruit juice when heated on stainless steel and glass surfaces.

Sensory analysis of grapefruit juices heated at 100ºC for 10 minutes indicated that both heated samples exhibited a heated, pineapple, metallic, and cooked off-flavor. Unheated reconstituted juice had a fresh grapefruit juice character. Using data from a ten

point rating system, the "Difference from Control" test indicated that significant differences ($p<0.05$) existed between the unheated juice and the two heat treatments.

Analysis of heated juices with GC-O showed appreciable reductions in aroma intensity of compounds that are responsible for fresh grapefruit juice character. Intensities of α-pinene, myrcene, and β-sinensal were at least 45% lower in the heated juices compared to the unheated juice. There was also a corresponding increase in aroma intensity of compounds associated with flavor degradation such as 2,5-dimethyl-4-hydroxy-3(2H) furanone (Furaneol) and methional in heated samples. Two other Maillard reaction products, sotolone and 3-methyl-2(5H)-furanone, were detected by GC-O. Both high processing temperatures and extended times are required to produce cooked or heated off-flavors in grapefruit juice. Increases in 5-HMF concentration were observed with heating, suggesting that Maillard reactions were involved.

Sensory experiments designed to induce cooked, heated off-flavor in unheated reconstituted juice indicated that a combination of Furaneol, homofuraneol and bis (2-methyl-3-furyl) disulfide, a thermal degradation product of thiamine (vitamin B1), could produce a cooked off-flavor similar to that observed in the excessively heated juices.

CHAPTER 1
INTRODUCTION

Grapefruit is the second largest citrus commodity in the State of Florida. Florida supplies over 30% of the world's grapefruit production. Over 55% of Florida grapefruit goes into juice production while over 35% is used for fresh fruit. Grapefruit sales have been declining in recent years due to several factors ranging from uneven flavor quality to interactions with certain drugs. Grapefruit juice has a unique citrus flavor, but low sugar to acid ratios and high bitterness can overwhelm the pleasant flavor aspects. Red grapefruit is used mainly for fresh fruit or for not-from concentrate juice (NFC), while white grapefruit juice is generally converted to "from concentrate" (FCGJ) juice.

Thermal processing alters the overall flavor of grapefruit juice but is necessary to reduce populations of viable spoilage microorganisms, inactivate pectinesterase and, ultimately to increase shelf life. Formation of off-flavors due to processing is highly undesirable to consumers; therefore research is necessary to identify critical factors that cause significant flavor alterations. Cooked and heated off-flavor was observed in both NFC and FCOJ and had profoundly negative impacts on perceived flavor quality.

Traditional processing of grapefruit juice is achieved through the use of stainless steel tubes or plates. Transition metals can catalyze chemical reactions known to cause off-flavors. Glass is an inert material and should not catalyze such reactions.

Previous studies have identified several compounds that contribute negatively to citrus juice flavor. One such class of compounds is the furans that are formed as a result of sugar degradation. These furans impart a caramel aroma depending on the

concentration. Use of 5-HMF and furfural as markers of thermal abuse, have been suggested by several authors. Levels of these compounds in grapefruit juice typically do not approach their aroma threshold but as the level of these compounds increase, juice sensory quality declines. Furaneol, 2,5-dimethyl 4 hydroxy-3(2H) furanone, imparts an aged, pineapple like aroma to citrus juice at levels exceeding 50 µg/L. In citrus based soft drinks, sotolone imparts a burnt spicy note at levels ranging from 1.38ng/mL to 209ng/mL.

The objectives of this study were to determine if heating grapefruit juice in contact with glass or stainless steel resulted in distinguishable sensory flavor differences as well determining there were differences in individual aroma volatiles. A secondary objective was to determine what compounds were responsible for heated or cooked off flavor in grapefruit juice.

CHAPTER 2
LITERATURE REVIEW

Grapefruit

Grapefruit is the second largest citrus commodity in the State of Florida, accounting for over 30% of the world's grapefruit production. The entire United States of America produces 40% of the world grapefruit production. For the 2002-2003 season, 36% of the grapefruit was used for fresh fruit, 16% for the chilled juice sector and over 40% to frozen concentrate products. With the exception of 1999-2000 season, there has been a continuous decline in grapefruit production. Sales have been declining due to factors ranging from poor flavor quality to medical precautions. Significant changes in the overall flavor of processed grapefruit are of concern to the grapefruit processors as well as the consumers.

Thermal Processing

Citrus juice generally undergoes heat treatment in order to achieve reductions in viable spoilage organisms, to inactivate pectic enzymes, and ultimately to extend the shelf life.

Inactivation of pectinesterase is important since it prevents the cloud separation and associated reactions that may alter the flavor profile. Temperatures of at least 70°C to 90°C with hold times varying from a few seconds to 1 minute are necessary for inactivation of most pectic enzymes. Pectin methyl esterase is a heat resistant enzyme found in grapefruit juice and, is inactivated at temperatures exceeding 90°C (*1*). Most processors pasteurize not from concentrate (NFC) or chilled juice at temperatures

exceeding 90°C and majority of the time juice is being heated twice. Grapefruit juice from concentrate (FCGJ) is subjected to at least two heat treatments. The first occurs during the evaporator concentration process where water is removed with a multi effect evaporator. The second heat treatment occurs after the reconstitution of the juice and the add-back of important flavor volatiles.

Possible Interactions with Stainless Steel Pasteurization Tubes

Pasteurization and concentration processes involve heating the juice in stainless steel tubes or plates. Transition metals in food grade stainless steel tubes may catalyze reactions throughout the heating processes. Food grade stainless steel, 316, typically has 16-18% Cr, 10-14% Ni and 2-3% Mo. Since juice contacts steel directly, leaching of metals may be enhanced by factors such as low pH, high temperatures, and contact time. Studies on effect of pH and temperature on chromium release from stainless steel showed that after heating fruit juices with pH ranging from 2.5-3.0 at 95°C for 1hr, 31-50μg/L chromium was released (*2*). Residence times in tubes for NFC pasteurized juices are typically in the range of 6-30s. The thermally accelerated short time evaporator (TASTE) developed by Cook Machinery (Dunedin FL) is generally used to concentrate citrus juices. This evaporator utilizes high temperature with residence times of less than one minute (*3*). Although residence time is not long, high processing temperatures, combined with the low pH may be more damaging than either factor alone.

High temperature pasteurization induces chemical changes that may affect the quality of the juice immediately or will initiate a chain of chemical reactions that will ultimately produce flavor degrading compounds.

Off-flavor

High heating temperatures along with prolonged storage have been shown to be contributing factors to off-flavor development (*4;5;6*). Studies into heated/cooked off flavor found in orange juice was carried out by heating orange juice from concentrate at various time-temperature combinations and then subjecting the samples to varying storage times. Sensory evaluation revealed that orange juice heated at 100°C for 10 min produced a heated, cooked and over processed flavor but was not predominant. Samples were rated either "dislike slightly" or lower by majority of the panelists (*7*).

Consumer testing of commercial orange juices revealed that that several had a cooked flavor. Juices with this cooked flavor were given the lowest quality ratings. Both chilled (NFC) and from concentrate products were evaluated (*8*).

Maillard Reaction Products

Off flavor formation in citrus due to thermal degradation of sugars is of high importance. Non-enzymatic browning products may be directly or indirectly contributory to the flavor.

Furans

In citrus juices, furans are important indicators of thermal abuse. Furfural and 5-hydroxy methyl furfural (5-HMF) have been reported in citrus juices that have undergone heat treatment and high storage temperatures (*4;6*).

Commercial canned and glass packed orange juice samples were stored at temperatures ranging from 5°C to 30°C over a period of 16 weeks. Results showed that storage temperatures above 16°C increased the rate of furfural formation more rapidly than at lower temperatures. Concentrations of furfural observed in canned orange juices stored at 30°C increased 28 times compared to juices stored at 5°C. For every 5°C

increase in storage temperature, the level of furfural doubled, thus demonstrating a hight correlation between storage temperatures and furfural accumulation. Glass packed orange juice samples showed a more rapid increase in furfural formation. In fact, a 72-fold increase in furfural levels were noted in samples ranging from 5°C – 30°C storage temperatures (*6*).

A similar study utilizing commercially canned grapefruit juice revealed that storage temperatures from 10°C to 40°C were enough to induce a 390-fold increase in furfural levels over a 15 week storage time. Panelists were able to detect significant flavor changes in juices in which furfural levels exceeded 0.175 ug/ml. Since its taste threshold in orange juice was 80 μg/mL and thus not directly flavor active, furfural can only be used as an indicator for off flavor (*9*).

Amino acids can increase the rate of ascorbic acid degradation. Furfural is formed through acid catalysis of ascorbic acid (*6*). Ascorbic acid is degraded more rapidly in single strength juice than in concentrated juices (*10*).

Levels of 5-hydroxy methyl furfural (5-HMF) in grapefruit juice samples increased up to 3000 times with a 40°C rise in temperature over 15 week storage study (*9*). Formation of 5-HMF increased slightly between 10°C and 20°C but rose exponentially above 30°C. Under acidic conditions, 5-HMF is formed by the 1,2 enolization and dehydration of hexose sugars (fig.2-1). The R group in figure 2-1 is replaced by a methyl group. It has a high threshold of 100 μg/mL (*11*) and therefore is not flavor active (*12*). In fact, sensory results did not correlate with 5-HMF concentration (*9*). Thus other compounds must be responsible for the observed off-flavor.

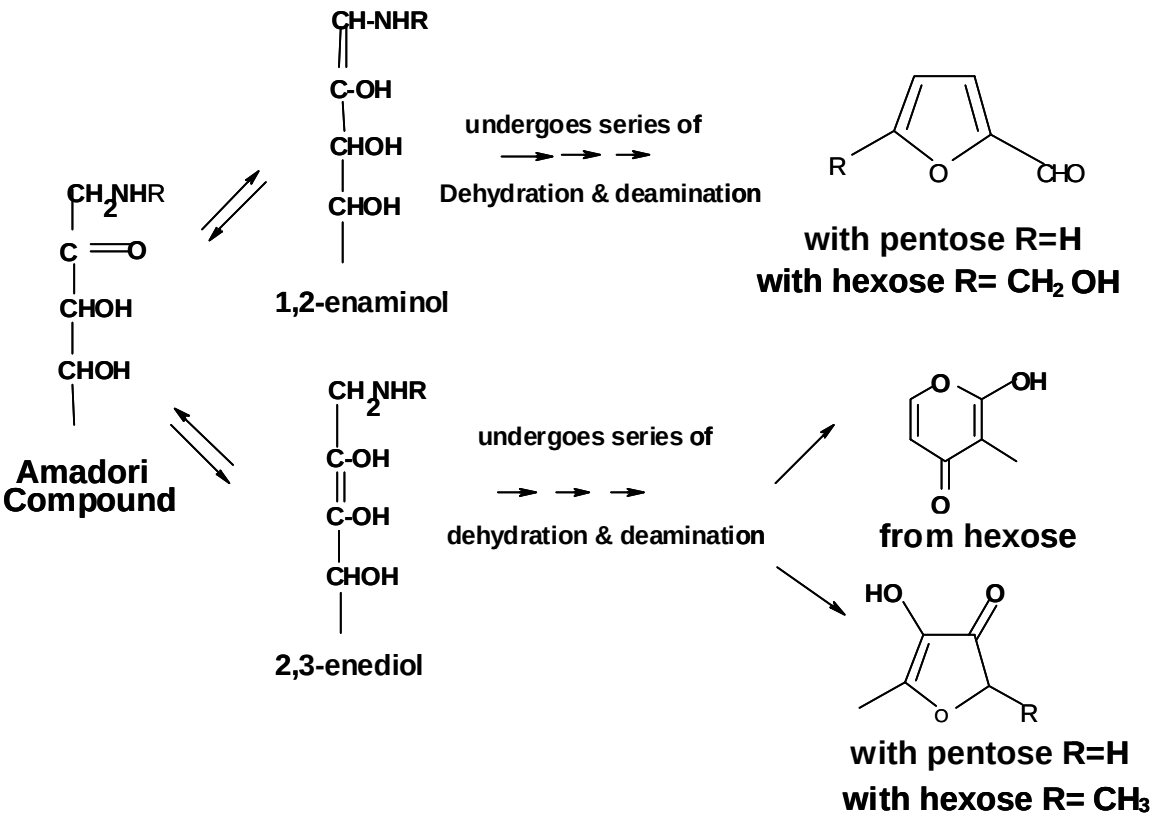


Figure 2-1 Formation of furans by either the acidic pathway (1,2- enaminol) or by the basic pathway (2,3-enediol)

Furanones

Furanones are important flavor compounds that are found both naturally and as a result of thermal processing in foods and beverages. Two important furanones are Furaneol and sotolone.

2,5 dimethyl 4-hydroxy- 3(2H) furanone (Furaneol)

Furaneol gives a caramel-like, burnt pineapple aroma in high concentrations and a sweet, strawberry like aroma at low concentrations. It is one of the major "character impact" compounds in pineapple (*13*) and muscadine grapes (*14*). Furaneol can exist either free or glycosidically bound in other fruits such as mango, grapes, tomato (*15*), and strawberry (*16;17*). It is found in thermally processed foods such as beef broth (*18*), and fruits such as pineapple. This furanone has been shown to mask the flavor of orange

juice, giving an aged, cooked aroma when present at 0.05 μg/mL or greater (*4*). In grapefruit juice, a pineapple-like aged flavor develops with high levels of Furaneol (*19*). It has a threshold of 0.03 μg/L in water (*20*). Orange juice samples stored at 40°C had 5 times more Furaneol compared to samples stored at 4°C (*21*). Grapefruit juice samples stored at 50°C for 15 weeks showed an increase up to 28 times that of samples stored at 20°C (*19*). Furaneol formation in citrus juices occurs as a result of a Maillard sugar degradation reaction.

Studies using model solutions of citrus juice showed that Furaneol is formed over a range of pH values in the presence of rhamnose. At higher pH values Furaneol is formed through the 2,3-enolization pathway of the Amadori compound (fig. 2-1) followed by dehydration and molecular rearrangement (*22*). The amino acid arginine is also a necessary substrate for its formation under acidic conditions. Rhamnose is a 6-deoxy hexose sugar formed from enzymatic degradation of pectin during processing and storage. Flavanone glycosides such as hesperidin and naringin can also degrade to form free rhamnose (*23*). Acid-catalyzed fructose degradation is also a possible formation pathway for Furaneol. In heated solutions it can decompose to smaller molecules (*24*) or it can react with other groups such as thiols to produce flavorful compounds.

Like most Maillard reactions, this reaction requires only a small amount of substrate produce potent flavors; thus; it is hard to measure the depletion of these compounds.

4,5 Dimethyl 3-Hydroxy- 2(5H) Furanone (Sotolone)

Sotolone is a powerful aromatic compound that produces a curry-like aroma in high concentrations, however, at low levels imparts a burnt, spicy aroma (*25*). Sotolone has an aroma threshold of 0.02 μg/L in air (*26*) and is found in wines (*25*), sherry and roasted

coffee (*26*). Recently, it has been reported in orange essence oil (*27*) and was found to be a source of off-flavor in citrus soft drinks (*28*) producing a spicy, burnt aroma. Its formation was monitored in model soft drinks both with and without ascorbic acid. It was postulated that sotolone was formed from ascorbic acid or dehydroascorbic acid in the presence of ethanol. Oxygen and metal ions are also necessary for its formation. Small amounts of ethanol are naturally found in citrus juices.

Other Degradation Products

Methional and 2-methyl-3-furanthiol are possible off-flavor contributors formed from strecker and thiamine thermal degradation, respectively. Methional has a cooked potato aroma while 2-methyl-3-furanthiol has a cooked, meaty aroma. Another thiamine degradation product, bis (2-methyl3-furyl) disulfide, a dimer of 2-methyl-3-furanthiol (MFT), which also produces a meaty aroma. Both MFT and its dimer have been reported in grapefruit juice from concentrate (*29*). Thermal processing can also degrade β-carotene; producing norisoprenoids such as β-damascenone, α-ionone and β-ionone.

Instrumental Analysis

GC Techniques

Gas chromatography can separate complex mixtures of volatiles using high resolution capillary columns. GC works on the principle that volatiles are sequentially eluted in the general order of boiling point. Thus they are thermally desorbed from the capillary column as the column (oven) temperature increases. Separation efficiency also depends on polarity of both the compound and the stationary phase, which lines the column. On a polar column such as DB-wax, polar compounds will be retained by the stationary phase more than they would on a non-polar DB-5 column.

Gas chromatography-olfactometry (GC-O) is a powerful analytical technique that combines an instrumental detector as well as human response. It offers the ability to define aroma active volatiles in terms of odor and intensity (*30*). The human nose has an odor detection threshold of 10^{-19} moles (*31*) compared to 10^{-12}grams for a mass spectrometer (*32*). Isolation of the compounds of interest from the food matrix is very important (*31*). GCO analysis can involve flavor dilution techniques such as Charm or aroma extraction dilution analysis. These techniques are useful in determining flavor threshold of important aroma active compounds. Another technique, OSME or time-intensity, is based on evaluating intensities of aroma over the course of the GC analysis and in most cases is not as time consuming as the flavor dilution techniques. Drawbacks to sniffing include fatigue, saturation and adaptation (*31*) therefore assessors should limit repetitions that are done in a single day and also lengthen the time between sniffs.

HPLC Methods

HPLC is an excellent alternative method of analysis. It is a non-thermal chromatographic technique, which allows the separation of both volatiles and non-volatiles. Reversed phase chromatography has been one of the choice methods for furan analysis in citrus products (*19;21;28;33*). Grapefruit juice is a complex mixture and efficient separation of its components lies in sample preparation and chromatographic conditions. There have been several sample preparation procedures for furan analysis in citrus juices. Use of the Carrez clarifying reagent was found to be effective in removing pulp, fat, protein and carotenoids that may co-elute with the furans (*34*). The same author (*9*) employed a simplified method of centrifuging the juice to remove the pulp layer. In both cases passing the remaining solution through a C-18 cartridge to selectively remove the desired polar compounds (*34*). Previous studies used mobile phases of consisting of

acetonitrile-water (85:15), (*34*), acetonitrile-glacial acetic acid-water (*9*) to effectively isolate 5-HMF and furfural. Methanol and sodium acetate buffer were found to satisfactorily isolate Furaneol. Initial efforts to quantify Furaneol in grapefruit juice using aqueous methanol (30%) or phosphate buffer with 30% methanol were unsuccessful due to co-elution with another compound (*19*). A linear gradient and mobile phase consisting of methanol, acetonitrile and pH4 acetate buffer provided good separation of Furaneol (*21*). However, in citrus juices another compound will co-elute with Furaneol. The technique of creating difference chromatograms by subtraction of two detection wavelengths (*35*) has been employed to isolate Furaneol. Furaneol has minimal absorbance at 335nm and appreciable absorbance at 292nm whereas the interfering compound had an absorbance maximum at 335 nm. Thus, the difference chromatogram should provide a peak which is due almost entirely to Furaneol.

Sotolone has previously been isolated using both reversed and normal phase chromatography. Reversed phase chromatography using a mobile phase consisting of acetonitrile and water adjusted to pH 2.5 with sulfuric acid was used to quantify sotolone in citrus soft drinks (*28*). This compound was monitored at 235nm, a wavelength close to its maximum absorbance. Normal phase chromatography has been the predominant method of isolating or analyzing sotolone. The sotolone content in wines was determined with a detection limit of 10µg/L using a diol column and hexane/ dichloromethane as the mobile phase (*25*). HPLC can also be used as a preparative step by collecting fractions of sotolone and then using GC-MS to identify and quantify sotolone (*36*).

CHAPTER 3
MATERIALS AND METHOD

Reagents and Standards

Pentane, an organic solvent used in the gas chromatographic analysis was obtained from Fisher Scientific (Pittsburgh, PA). Acros Organics (New Jersey) supplied diethyl ether used for solvent extraction, and the methanol used in HPLC solvents and for preparation of standards. Standards of 3-methyl-2(5H) furanone, 4-hydroxy-5-methyl-3(2H) furanone, 2,5-dimethyl-4-hydroxy-3(2H) furanone, 2,5-dimethyl-4-methoxy-3(2H) furanone, 2-ethyl-4-hydroxy-5-methyl-3(2H) furanone, 5-hydroxy methyl furfural, 4,5-dimethyl-3-hydroxy-5(2H) furanone, E-2-decenal, E,E-2,4-decadienal, methional, E,Z-2,4-decadienal, E,E-2,4-nonadienal, E,Z-2,6-nonadienal, 1-octen-3-one, maltol and 2-methyl-3-furanthiol were obtained from Aldrich Chemical Co. (Milwaukee, WI). Vanillin was obtained from Sigma Chemical Co (St. Louis, MO). Linalool, octanal, decanal, nootkatone, β-sinensal, α-terpineol, myrcene, and α-pinene Sodium phosphate was obtained from Sigma while the phosphoric acid from Fisher Scientific.

Apparatus Setup

Figure 3-1 illustrates the heating apparatus setup. An oil bath consisting of light mineral oil (Fisher Scientific) was heated using a hot plate. A condenser with chilled (-5°C) 50:50, ethylene glycol: water, was attached to the round bottom flask to allow for refluxing of volatiles through condensation. The mouth of the condenser was also covered with aluminum foil. For each heating in the stainless steel and the glass containers, temperatures of both the oil bath and the juice samples were monitored.

Sample Preparation

Mid Season White Grapefruit Concentrate was obtained from a local processor. The concentrate was reconstituted to 10°Brix using deionized water. 300ml of the reconstituted grapefruit juice was placed in a 1000ml stainless steel round bottom flask. This was then submerged into a pre-heated oil bath. A hold time of 10 min was maintained once the sample reached a temperature of 100ºC. After heating, samples were placed in a pre-sterilized 10oz glass flint bottle (All American Container, Tampa, FL), and immediately cooled on ice. The heating was repeated using a glass round bottom flask.

Sample preparation for GC analysis was carried out using liquid-liquid extraction. 20 ml of grapefruit juice were added to 10 ml of 1:1 pentane and diethyl ether for extraction. A Mixxor-like apparatus consisting of two 50 ml syringes connected via a stainless steel luer-lock connector was used to extract the volatiles. Extraction was facilitated by moving syringes in a back and forth motion twenty times. The mixture was then centrifuged at 4000 rpm for 10 minutes to break the emulsion. The solvent layer was removed and retained, while the aqueous portion was extracted a second time. After the second extraction, both solvent fractions were combined. The extract was dried with sodium sulfate.

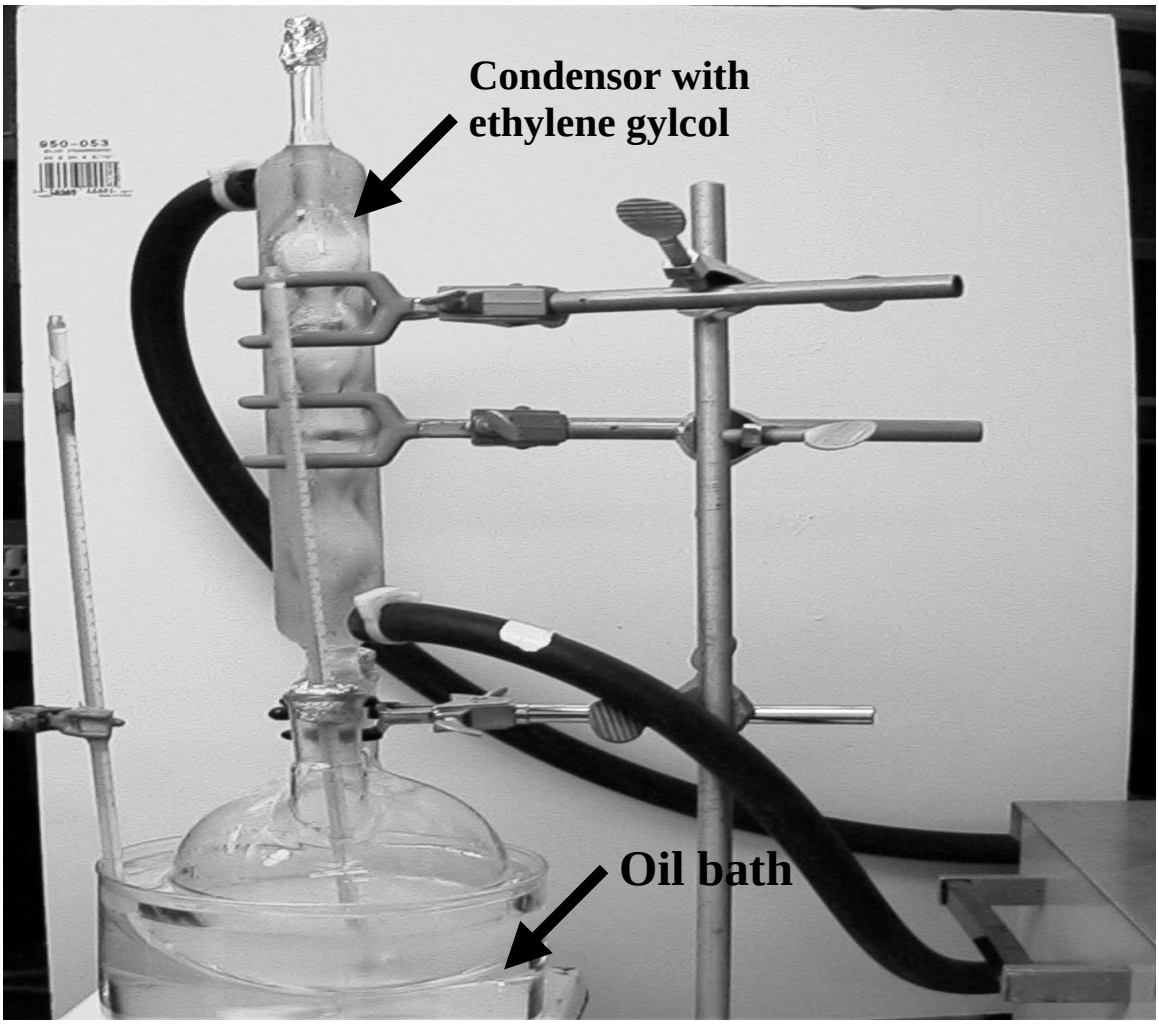


Figure 3-1 Heating apparatus setup

A 50 µl volume of 2000 µg/mL ethyl valerate and 2-heptadecanone were added as internal standards. The solvent layer was concentrated to approximately 100µl using a stream of high purity nitrogen and placed in a 100µl limited volume insert, placed in a 1.8 mL sampling vial and sealed with a screw top lid. Each sample was refrigerated until GC-O/GC-FID (GC) and GC-MS analysis. Each sample was analyzed four times for GC-O analysis and twice for GC-MS.

Sensory Analysis–Difference from Control Test

Six batches of grapefruit juice were heated in stainless steel and glass containers. The batches were then combined so as to present a uniform mixture to panelists.

Twenty-one to twenty-five untrained panelists from the Citrus Research and Education Center were recruited for the study. A control and three samples, each coded with a randomly selected 3-digit number were presented to the panelists. Order of presentation randomized for each panelists based on six orders of presentation (ABC, ACB, BCA, BAC, CBA, BAC). Panelists were then asked to taste the control then to taste the sample and to rate the degree of difference between them. They were also told that a hidden control was present among the samples. A sample ballot is shown in figure 3-2. The control was unheated reconstituted grapefruit juice.

Panelists were also asked to cleanse their palettes by eating a piece of unsalted cracker and drinking a sip of water between samples to prevent any carryover from the previous sample. A numerical ten-point scale similar to the hedonic scale was used, with scale ranging from no difference to extreme difference. Color changes during the heating process prompted the use of red lighting so as to mask the color difference. The above procedure was repeated two additional times yielding a total of the three sensory evaluation sessions.

DIFFERENCE FROM CONTROL TEST

Name: _____ Date: _____

Today you will be tasting grapefruit juice.
Please eat a piece of cracker and drink a sip of water in between each sample to cleanse your palette.

Three samples and one control are presented to you. Please circle the degree of difference against the control for each sample.

It is possible that there may be no difference between the control and one of the samples.

Control vs. #	**Control vs. #**	**Control vs. #**
0 =No difference	0 =No difference	0 =No difference
1= slight difference	1= slight difference	1= slight difference
2	2	2
3 = mod difference	3 = mod difference	3 = mod difference
4	4	4
5 = large difference	5 = large difference	5 = large difference
6	6	6
7 = very large difference	7 = very large difference	7 = very large difference
8	8	8
9 = extreme difference	9 = extreme difference	9 = extreme difference

Comments

Figure 3-2 Sample ballot for the grapefruit juice difference from control test using a ten-point rating scale

Instrumental Methods

Gas Chromatography-Flame Ionization Detector (GC-FID)

Volatiles from grapefruit juice extracts were separated using an HP 5890 gas chromatograph (Hewlett Packard, Palo Alto, Ca, USA) equipped with a flame ionization detector. One-half micro liter sample was injected onto a ZB-5 or DB-Wax capillary column (30m x 0.32mm I.D., 0.5µm film thickness) (J&W Scientific, Folsom, CA). Oven temperature was programmed with an initial temperature of 40°C and ramped up to 240°C for wax column and 265°C for ZB-5 column, at 7°/min with a final hold time of 5 minutes. Samples were injected using splitless mode with an injector port temperature of 220°C and the detector at 250ºC. The effluent was split 1:2 between a flame ionization detector and a sniff port (Datu, Geneva, NY). Data were collected and integrated using the ChromPerfect v. 5.0software (Justice Innovations, Denville, NJ).

Gas Chromatography-Olfactometry (GC-O)

A sniff port was attached to the GC in order to facilitate the identification of aroma active volatiles. The GC temperature program was the same as that for the GC-FID.

Two assessors were used to evaluate the aroma quality of the samples. Each assessor analyzed the samples in duplicate accounting for four sniffs per sample. Only those odors that were detected in 50% of the sniffs were considered to be aroma active. Sniffing began after the elution of the solvent peak, approximately three minutes into each run. A sliding scale ranging from none to extreme was used to indicate the aroma intensity. Assessor's responses were captured using a variable potentiometer, which was collected on the ChromPerfect software. Sniffing time for each run was 25 and 29 minutes for the ZB-5 and DB-wax column respectively. Aroma quality of each sample was recorded.

Gas Chromatography-Mass Spectrometry (GC-MS)

Data were collected using a Finnigan GCQ Plus GC-MS system (Thermo Electron, San Jose, CA) using a 99.999% pure helium as the carrier gas. A DB-5 capillary column (60m x 0.25 mm id, 0.25µm film thickness column (J&W Scientific) was used. Oven temperature was programmed from 40°C to 250°C at a rate of 7°C/min. Samples were injected using split less mode with an injector port temperature of 220°C. Electron impact (EI) mode was used with ionization energy of 70eV. Scans were obtained after 7 minutes to avoid the solvent peak and were measured from 40-300 m/z. Data were analyzed with XCalibur v.1.3 software (Thermo Electron, San Jose, CA).

HPLC Analysis of the Furans

Sample Preparation

Solid Phase Extraction using a method modified by Walsh was employed (*21*). 10ml of grapefruit juice were centrifuged for 5 min at 3000 rpm. Three milliliters supernatant were passed thru a C-18 cartridge (J & W Scientific, SPE Accubond 500mg/6ml, Folsom, CA) that had been pre-conditioned with 2.5 ml methanol and washed with 6 ml water. The cartridge was then washed with 2 ml water to remove the sugars and eluted with 2 ml methanol at a rate of 1 drop per 10s. Samples were filtered thru a 0.45µ nylon filter (Fisher Scientific) and stored in a 1.8 ml amber vial until injected into the HPLC.

HPLC Instrumentation

Separation was achieved by injecting 20 µl of the juice extract onto a Phenomenex 5µ C-18 column (4.6mm id x 250mm) (Phenomenex, Torrance, CA). A 10ul injection volume was used for standards. Column temperature was kept at 25ºC. Chromatographic analysis was carried out using Surveyor HPLC equipped with a PDA

detector. Versatility of the PDA detector enabled the monitoring of three individual wavelengths: 235nm, 290nm and 335nm, while obtaining a spectral scan 220-380nm. Data was collected and analyzed using Atlas software v.1.0 at an acquisition rate of 10Hz (Thermo Electron) over a 40 minute run time.

The mobile phase consisted of methanol and phosphate buffer. Phosphate buffer (pH4.1 ±1) was made using a 0.05M sodium phosphate solution and phosphoric acid. Linear gradient conditions are summarized in table 3-1 while figure 3-3 shows the graphical gradient conditions. A flow rate of 1ml/min was maintained and a re-equilibration time of 10 min was applied.

Table 3-1 HPLC gradient conditions with flow rate at 1mL/min

Time	% Methanol	% phosphate
0	5	95
10	15	85
27	30	70
33	40	60
40	5	95
50	5	95

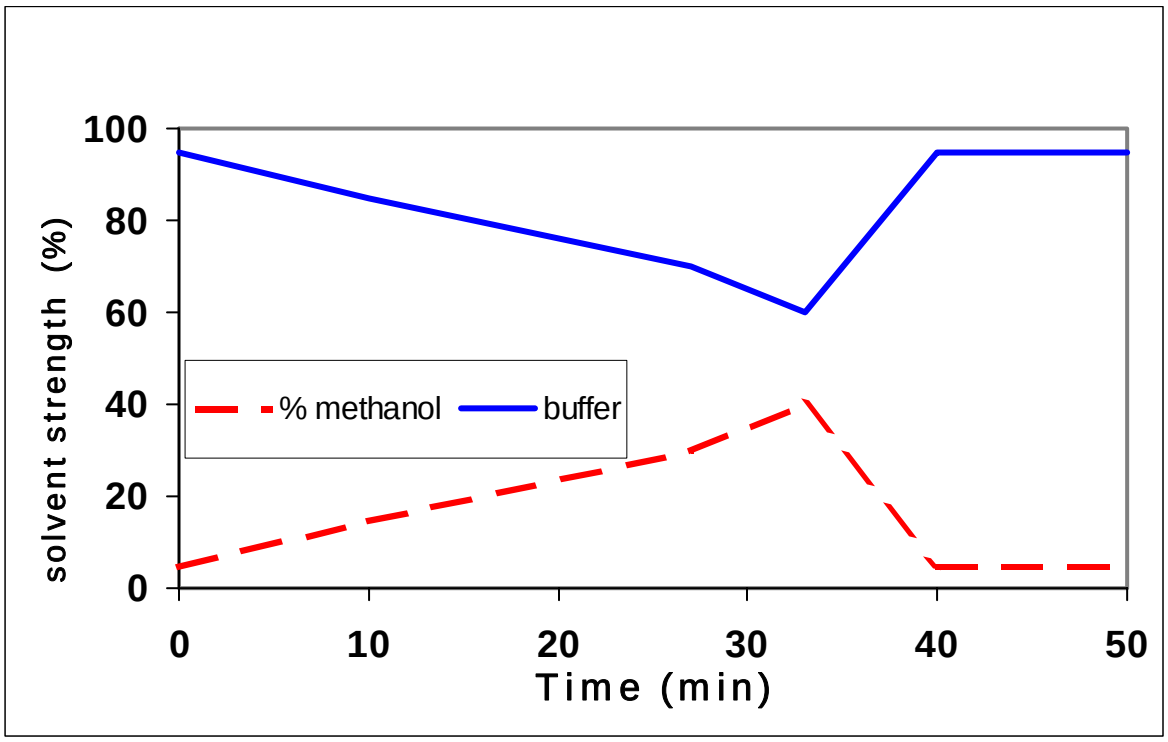


Figure 3-3 Graphical representation of the HPLC gradient conditions

Identification and Quantification

Identification of aroma active components was achieved by comparing linear retention indices and aroma descriptors with those from a database or published values. Confirmation was achieved by matching fragmentation pattern of the sample with that of standards or library. A series of alkanes (C_5-C_{25}) analyzed under the same GC conditions

were used to calculate linear retention indices (*37*). A scatter plot of retention time along with retention indices of alkane standards were graphed on Excel. Retention indices for the standards were obtained by multiplying the carbon number by a factor 100. A polynomial was obtained from a regression. This equation was then used to calculate linear retention indices for individual components in grapefruit juice. Alkane standards were analyzed for both DB-wax and ZB-5 columns, as well as for GC-MS.

Components from HPLC analysis were identified using a combination of comparing retention times and spectra of standards with that of sample. Quantification was done using external standard method. Sets of standards with known concentrations were analyzed in triplicate. Areas of those standards were plotted against the amounts and a linear regression was obtained along with a linear equation. Responses from unknown components were then substituted in the linear equation in order to find the amounts and ultimately the concentration of components in juice samples.

CHAPTER 4
SENSORY ANALYSIS RESULTS AND DISCUSSION

There are several sensory analysis tools that are used in difference testing of foods. "Difference from Control" test was used in this study as it enabled the evaluation of three treatments in one sitting. There are two objectives of the difference test. First, it determines if a difference exists between samples and the control. Secondly, it estimates the magnitude of the difference (*38*).

The three samples that were evaluated were

Unheated – unheated reconstituted grapefruit juice (as well as hidden control)

Glass - FCGJ heated 10 min. on glass surface

Metal – FCGJ heated 10 min. on stainless steel surface

The sensory evaluation data for each replicate are shown in tables 4-1 and 4-2. Except for one (panelist #12, table 4-2 rep 3), all panelists were able to identify the hidden control, as having little or no difference from the control. Although overall scores (table 4-2 rep3) ranged from no difference (0) to very large or extreme (8) difference, only 68% of the panelists were able to distinguish differences between the control and the juice heated in metal. A difference score of three and greater was considered to show difference, difference scores of less than three was not considered to be different from the control. Scores for juice heated in glass ranged from moderate difference to very large difference (7) for over 86% of the panelists. Seven of the twenty-two panelists rated the juice heated in metal with a score of two or less compared to only three for the juice heated in glass. Perhaps, these panelists could not distinguish the differences due to their

low bitterness threshold and hence high sensitivity. This sensitivity incapacitates panelists from being able to distinguish flavor differences among samples. An example of this is panelist number 15 (table 4-1 rep 1) and panelist # 4 9table 4-2 rep 2) who was unable to detect much difference and commented that all three samples had high bitterness levels. Also they could be anosmic or unable to detect heated off-flavors. Although panelists' scores improved for the second and third evaluations, some panelists were still unable to detect flavor differences in the juices. Average difference score for the unheated reconstituted juice ranged from 1.96 in rep 1 to 0.45 in rep 3 suggesting an improvement in identifying the hidden control. Most panelists had difficulty detecting differences between the two heated samples e.g. panelists #1, 2, 5, 6, 8 & 10 from table 4-1 rep1 indicated at most, a one-point difference between the glass and metal samples. Although there may have been a difference in overall flavor between the two heated sample types, the severe heating produced such a strong off flavor that it was difficult for panelists judge the degree of difference. Both samples had very strong heated/ cooked off-flavors.

Statistical Tests for Significant Differences

Statistical differences between samples were determined by using a two-way analysis of variance (ANOVA) test, at the 95% confidence level, as it allows for the comparisons of more than two samples (table 4-3). The advantage of using a two-way ANOVA is that any error due to the panelists can be distinguished.

Table 4-1 Difference from control sensory data from 22 panelists between a control and three samples, a hidden control (unheated reconstituted), grapefruit juice heated in glass, and juice heated in metal using a 10-point scale.

	Rep 1		
Panelists	**Unheated reconstituted**	**Glass**	**Metal**
1	1	5	5
2	0	6	5
3	4	5	1
4	0	5	3
5	5	8	7
6	2	6	5
7	2	3	5
8	1	6	7
9	0	3	3
10	0	5	4
11	0	3	5
12	1	0	3
13	4	6	4
14	1	2	5
15	0	0	0
16	4	3	2
17	3	5	3
18	4	7	2
19	3	5	1
20	2	2	4
21	2	5	7
22	1	7	7
23	5	7	7
24	3	9	1
25	1	1	3
Average	1.96	4.56	3.96
Median	2	5	4
St Dev	1.62	2.46	2.09
Min	0	0	0
Max	5	9	7

Table 4-2 Difference from control sensory data for the second (rep 2) and third replication (rep 3).

Panelist No.	Rep 2			Rep 3		
	Unheated reconstituted	**Glass**	**Metal**	**Unheated reconstituted**	**Glass**	**Metal**
1	0	5	3	0	5	5
2	4	9	6	0	7	3
3	1	3	1	0	3	1
4	1	0	0	1	1	3
5	1	4	6	0	3	3
6	0	3	5	1	6	0
7	1	3	3	1	3	1
8	0	5	8	0	4	5
9	0	7	5	1	4	7
10	1	3	5	0	1	2
11	3	5	7	1	7	7
12	2	1	3	2	7	8
13	1	3	3	1	5	8
14	1	6	5	0	3	4
15	0	5	5	1	5	5
16	0	5	3	0	5	1
17	1	3	0	0	5	6
18	1	3	1	0	5	0
19	1	0	5	0	7	3
20	3	5	1	0	5	5
21	3	3	3	0	3	1
22				1	1	3
Average	1.19	3.86	3.71	0.45	4.32	3.68
Median	1	3	3	0	5	3
St Dev	1.14	2.10	2.21	0.58	1.87	2.46
Min	0	0	0	0	1	0
Max	4	9	8	2	7	8

Table 4-3 Results of the analysis of variance for each of the three replications. Rep1-table 4-1, reps 2 and 3 data are shown in table 4-2.

Rep1

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	F_{crit}
Panelists	158.08	24	6.59	2.08	0.015	1.75
Treatments	92.67	2	46.33	**14.63**	**1.09E-05**	**3.19**
Error	152	48	3.17			
Total	402.747	74				

Rep2

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Panelists	107.94	20	5.40	1.89	0.043	1.84
Treatments	94.51	2	47.25	**16.56**	**5.77E-06**	**3.23**
Error	114.16	40	2.85			
Total	316.603	62				

Rep3

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Panelists	105.15	21	5.01	1.88	0.040	1.81
Treatments	188.82	2	94.41	**35.45**	**9.58E-10**	**3.22**
Error	111.85	42	2.66			
Total	405.82	65				

The F test is a statistical technique that was utilized to compare multiple means and to test for variability among the means for the three samples (treatments) against the variability of the observations (error). In this study the null hypothesis assumed that the treatment means were the same, however, if the mean for the treatments were different then the null hypothesis would be rejected. The maximum ratio between variations among the treatments was measured using the F_{crit} value. If the calculated F value were greater than the F_{crit} then there would be significant variations, which would lead to differences among the sample means.

From table 4-3 it can be seen that the F_{calc} value is greater than the $F_{crit,}$ in all three replicates, therefore we reject the null hypothesis and conclude that at least one of the treatments means is different. Critical F values were approximately 3 compared to calculated F values of 15, 17 and 35 respectively for reps 1, 2, & 3.

Since the analysis of variance test only suggests that a difference exist among the populations, a multiple comparison test was used to rank the means and identify the means that were different. Fisher LSD multiple comparison test was applied to the means because it is the least conservative test in comparison to the Tukey and Duncans test and should produce the most difference. The LSD test is defined as " any observed difference necessary to declare the corresponding population means different"(*39*). The following equation was used to calculate the LSD:

$$LSD = t_{\alpha/2}\sqrt{(2*EMS)/n}$$

where $t_{\alpha/2}$ was found at the degree of freedom of the error and an alpha level of 0.025 from a t-table, EMS, error mean square was taken from the ANOVA results and n was the number of panelists.

Means for each population were subtracted from each other. Those with a difference greater than the LSD value, were considered to be significantly different, while those that were similar or less than the LSD value were not significantly different. From the data in table 4-4 rep3, the difference between the glass and the metal was only 0.64 while the difference between the unheated reconstituted juice and glass was 3.87. The calculated LSD value was 0.99.

Table 4-4 LSD comparisons of the means for the three samples

	Unheated reconstituted	**Glass**	**Metal**	**LSD**
Means Rep 1	1.96a	4.56b	3.96b	1.02
Means Rep 2	1.19a	3.86b	3.71b	0.50
Means Rep 3	0.45a	4.32b	3.68b	0.99

Since the difference between the unheated reconstituted juice and two heat treatments in all three replications were greater than LSD values, it can be concluded that a significant difference existed between unheated reconstituted and heated samples. However the difference of means for two heated samples were less than the LSD value, therefore they were not statistically different.

Cooked, heated, processed, pineapple flavor were some comments recorded by panelists for heat-treated samples while the unheated reconstituted sample was identified as having good grapefruit flavor. Panelists who had difficulty recognizing differences between the control and two heat-treated samples recorded comments such a high bitterness, acidity.

Producing Heated/Cooked Flavor

Attempts to reproduce the heated cooked off-flavor were carried out using three furans known to produce a caramel aroma, Furaneol, sotolone, homofuraneol. In addition, bis (2-methyl-3-furyl) disulfide, a thiamine degradation product sometimes found in thermally abused citrus juices, was also added. Table 4-5 shows the combination of the compounds used as well as their sensorial characteristics when added to grapefruit juice based on a 6-member panel. It has been previously reported that

concentration greater than 0.05 µg/mL Furaneol masked the flavor of grapefruit juice. This was seen in the combinations that had 1µg/mL Furaneol. Little or no grapefruit juice character detected by the panelists. However, in the combination containing 0.5 µg/mL, a slight grapefruit juice character was noticed, but caramel and sweet flavor impression were more prominent. The combination of homofuraneol (1µg/mL), Furaneol (1µg/mL) and bis (2-methyl3-furyl) disulfide (2µg/mL) produced a cooked off-flavor.

Extremely low concentrations of sotolone (0.01µg/mL) also contributed to over-processed flavor. Sotolone is a very potent aroma active compound and would not require a high concentration to produce a pronounced off-flavor.

Table 4-5 Combination used in off-flavor duplication along with their descriptors

Combination No.	Concentration	Flavor Descriptor
1	1µg/mL homofuraneol + 1µg/mL Furaneol + 1µg/L MFT-MFT	Sl cooked fl, caramel aroma, no gfj character, sweet, less acid
2	1µg/mL homofuraneol +1µg/mL Furaneol + 2µg/mL MFT-MFT	More cooked/caramel aroma, less caramel aroma, less tart, sl gfj character
3	0.5µg/mL Furaneol + 1µg/mL homofuraneol +2µg/L MFT-MFT	Less cooked aroma, more gfj character, more sweet
4	0.5µg/mL sotolone + combination #1	Spicy, strong caramel, spoiled juice, no gfj character
5	0.01µg/mL sotolone	Processed, spicy aroma

CHAPTER 5
GAS CHROMATOGRAPHIC RESULTS AND DISCUSSION

Gas Chromatography is a useful method of separating volatiles based on several factors including polarity and boiling point. In this study, juice volatiles were separated using high-resolution capillary gas chromatography. The use of at least two dissimilar chromatographic phases is advantageous in that compounds that are not well separated on a polar phase column can often be better separated on a non-polar column and the reverse. Polar compounds tend to remain on a polar column longer and thus would be better separated. Since GC is a separation technique, these components cannot be identified without additional information. From the overlay of the FID chromatograms (fig. 5-1) obtained from the three sample types, it was seen that some peaks were diminished due to heating while others increased.

Peaks I, II and III showed a higher FID response for the unheated reconstituted juice compared to that of the two heated treatments. In contrast, peak VII had a higher response in the two unheated reconstituted samples. Table 5-1 lists some of the volatiles that were tentatively identified using the linear retention indices on two columns.

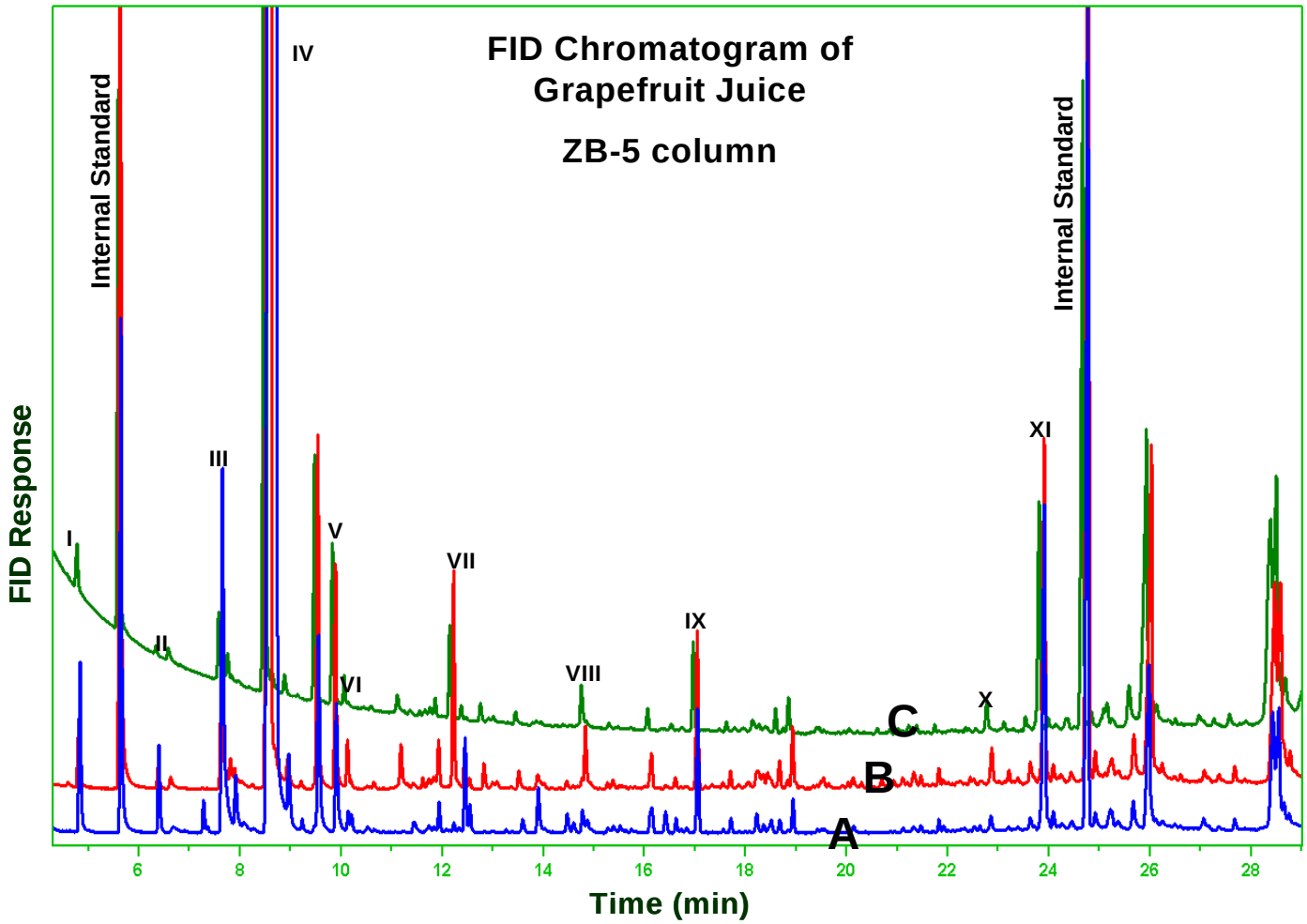


Figure 5-1 Overlay of partial grapefruit juice FID chromatogram on a ZB-5 column. A-unheated reconstituted juice, B-juice heated in glass, C-juice heated in metal.

Table 5-1 Preliminary identification of major grapefruit juice components based on standardized retention times using FID responses from both polar (wax) and non-polar (ZB-5) columns.

Peak	RT (min)	LRI (ZB-5)	Lit LRI (ZB-5)	Preliminary Identification	LRI (wax)	Lit LRI (wax)
i	4.61	847	846	Ethyl 2-methyl	1022	1041
ii	6.39	939	939	α-pinene	978	1011
iii	7.49	989	989	myrcene	1172	1168
iv	8.5	1032	1025	limonene	1214	1205
v	9.82	1087	1074	cis-linalool oxide	1480	1448
vi	10.16	1100	1100	linalool	1552	1552
vii	12.37	1192	1207	α-terpineol	1713	1713
viii	14.75	1315	1376	α-copaene	1480	1472
ix	16.97	1432	1418	β-caryophyllene	1644	1624
x	21.38	1677	1706	β-sinensal	2254	2251
xi	23.81	1834	1814	nootkatone	2587	2595

GC-O

Since the invention of GC-O as a tool to evaluate flavor volatiles, it has been adapted by the food, environmental, and flavor industry as a major tool used to identify those compounds that are aroma active. The selectivity and sensitivity of the human nose combined with the resolving power of the GC provides useful information about those volatiles that produce any aroma sensation. This is a particularly useful tool for those potent aroma compounds that have extremely low thresholds. It can be seen that little or no FID peaks were detected for the two peaks, 1-octen-3-one and 1-octen-3-ol, in the region of 7-8 minutes (fig. 5-2). Their analytical concentrations were below detection levels of the FID detector, yet their concentrations exceeded their aroma thresholds so they were aroma active.

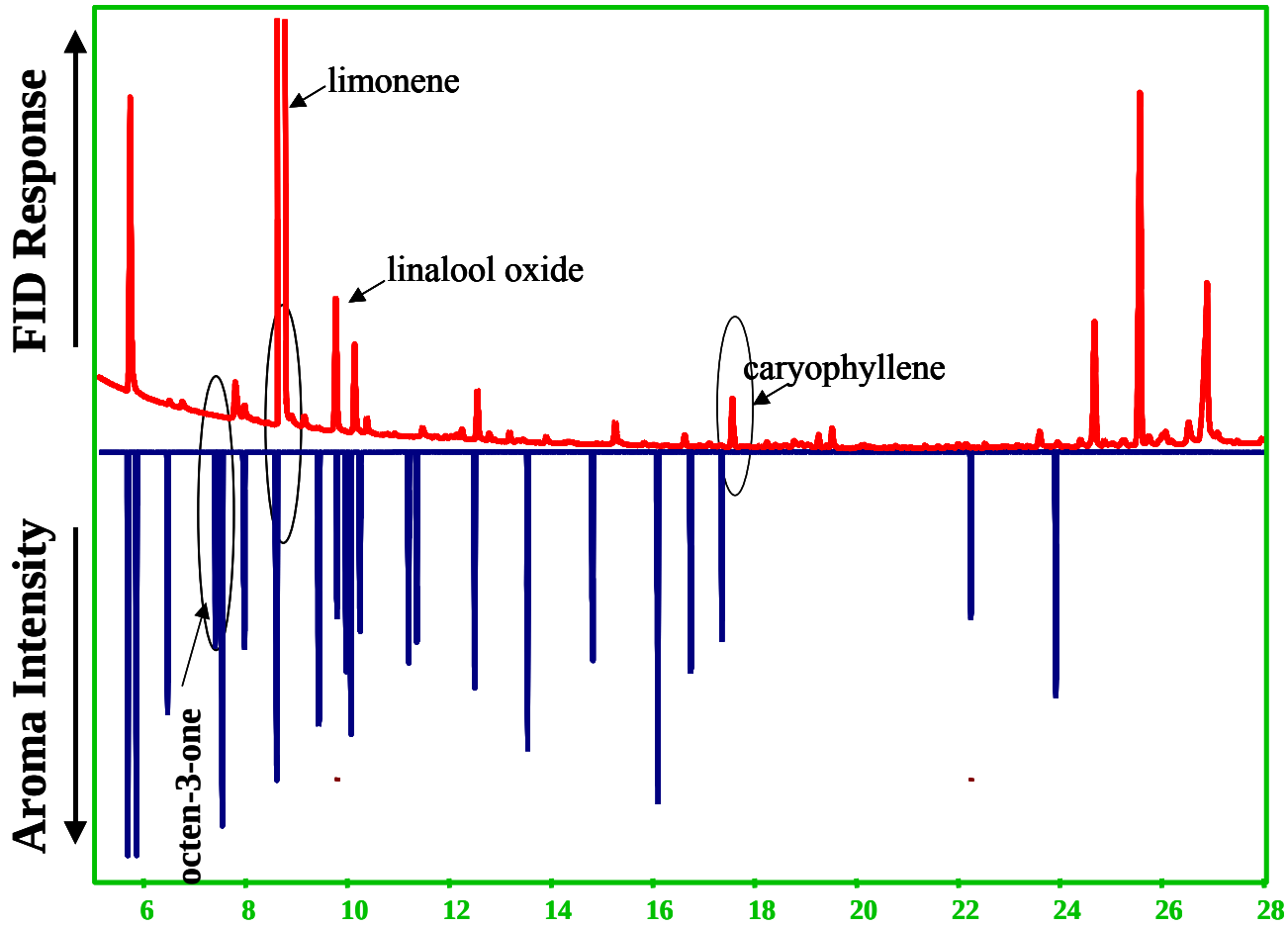


Figure 5-2 Partial FID (top) and aromagram (bottom) overlay of grapefruit juice analyzed on a ZB-5 column.

Similarly, not all volatiles found in relatively high concentrations or large FID peaks are aroma active. Large FID peaks such as limonene and β-caryophyllene shown in fig. 5-2 had little to no aroma activity. In the region between 24-28 min there were several prominent FID peaks that did not produce detectable aroma activity.

Aroma Active Compounds in Grapefruit Juice

GC-O is particularly useful in explaining sensory data. However, not everyone has the same sensitivity to all aroma compounds and there will be variability between individuals in assessing these aromas. To compensate for individual sensory variability, each sample was sniffed at least four times, twice per assessor. A compound was considered aroma active only if it was detected in 50% of the sniff runs. By using two panelists, effects of individual hypersensitivities and specific anosmias are minimized. If one assessor is anosmic to a certain class of compounds and the other isn't, then there is a better chance of detecting these compounds in the averaged assessor results.

GC-FID and GC-O were carried out on two dissimilar columns. The advantage to this method is that volatiles are eluted in a different order with each column type. Tentative identification is based upon cross-referencing calculated retention indices with those from a library, published results or with standards.

Over forty aroma active grapefruit juice volatiles were detected using both wax and ZB-5 columns. Shown in table 5-2 are twenty-six aroma active compounds that were identified by matching retention indices along with aroma descriptors of components on both columns. Standards were run under similar conditions to confirm retention values and odorant descriptors.

Aroma intensities were normalized for each assessor to minimize differences between assessor uses of the intensity scale. Aroma intensities detected on wax column were normalized based on vanillin, which had the highest peak height among all the volatiles (see table 5-3 and fig. 5-3).

Table 5-2 Aroma active peaks with tentative identification based on aroma attributes and retention characteristics for grapefruit juice samples as detected by GC-O on polar and non-polar columns.

DB-Wax	ZB-5	Component Name	Descriptor
978	939	α-pinene	piney
1001	800	ethyl butyrate	Sweet, fruity
1022	847	ethyl-2-methyl butyrate	fruity
1172	989	myrcene	green, earthy, metallic
1214	1032	limonene impurity	minty, licorice
1306	1003	octanal	green, fresh, lemony
1315	978	1-octen-3-one	mushroom
1390	943	4-mercapto-4-methyl-pentanone	cat litter
1470	906	methional	Cooked potato
1509	1206	decanal	green, fatty, lemony
1514	1095	Z-2-nonenal	metallic, green
1544	1544	Z-4-decenal	fresh, floral, fatty
1552	1103	linalool	floral, orange, greenish
1596	1194	E,Z-2,6-nonadienal	faint cucumber, green
1633	820	butanoic acid	skunky, rotten, fresh
1773	1232	citronellol	sweet, roasted
1828	1321	E,E-2,4-decadienal	fatty, sl orange
1840	1393	β-damascenone	tobacco(faint), piney
1861	1264	geraniol	rose, fresh, lemon pledge
2017	1383	4,5-epoxy-(E)-2-decenal	metallic, cooked, burnt
2043	1062	Furaneol	caramel
2201	1325	4-vinyl guaiacol	spice
2254	1677	β -sinensal	grapefruit, citrusy
2259	1470	wine lactone	nutty, musty
2587	1834	nootkatone	tropical, sour
2608	1418	vanillin	vanillin

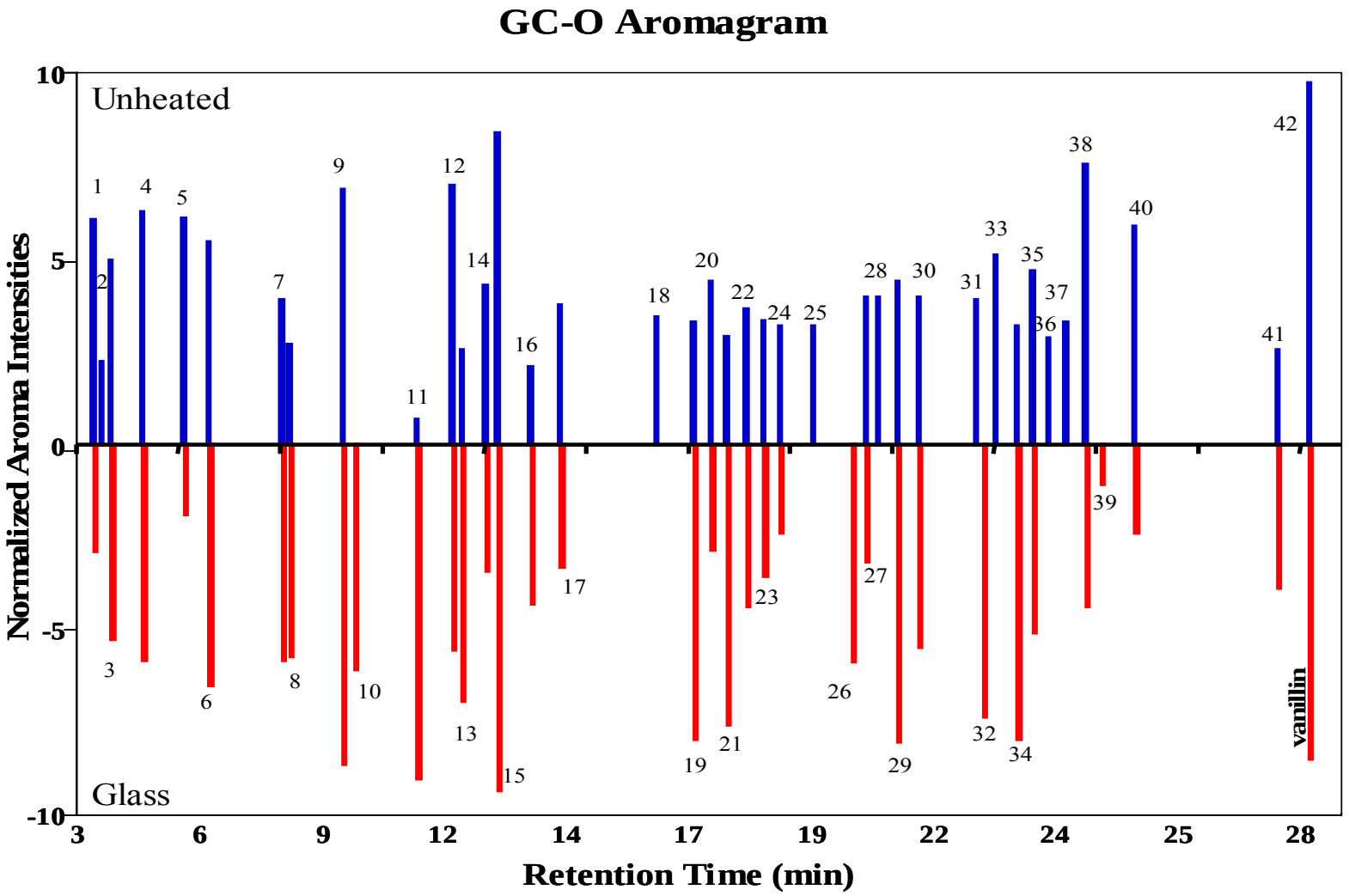


Figure 5-3 Aroma intensities for the unheated reconstituted juice and glass-heated samples analyzed on wax column. Intensities were normalized peak height based on vanillin, which had the highest peak height. Peak numbers correspond to those listed in table 3.

Table 5-3 Aroma intensities based on normalized peak heights of samples analyzed using wax column. Descriptors are listed in ascending order of LRI.

			Normalized Aroma Intensities		
Component No	**Calculated LRI**	**Descriptors**	**Unheated reconstituted**	**Glass**	**Metal**
1	978	piney	**6.06**	**2.91**	**2.36**
2	1001	sweet, fruity	**2.24**		
3	1022	fruity	**4.94**	**5.22**	**6.88**
4	1098	sulfury, stale	**6.26**	**5.81**	
5	1172	green, earthy, metallic	**6.09**	**1.88**	**0.83**
6	1214	minty, licorice	**5.45**	**6.52**	**3.83**
7	1306	green, fresh, lemony	**3.91**	**5.84**	**5.38**
8	1315	mushroom	**2.70**	**5.74**	**5.65**
9	1381	green, grassy, piney	**6.85**	**8.66**	**8.40**
10	1390	cat litter		**6.11**	
11	1470	cooked potato	**0.65**	**9.01**	**8.12**
12	1509	green, fatty, lemony	**6.98**	**5.58**	**7.17**
13	1514	metallic, green	**2.53**	**6.91**	**2.81**
14	1544	fresh, floral, fatty	**4.24**	**3.41**	**4.06**
15	1552	floral, orange, greenish	**8.37**	**9.32**	**4.09**
16	1596	faint cucumber, green, sweet	**2.06**	**4.30**	**3.59**
17	1633	skunky, rotten, fresh	**3.72**	**3.31**	**3.06**
18	1773	sweet, roasted	**3.43**		
19	1828	fatty, sl orange	**3.27**	**7.95**	**6.55**
20	1840	tobacco(faint), piney	**4.37**	**2.87**	**1.53**
21	1861	rose, fresh, lemon pledge	**2.88**	**7.52**	**6.30**
22	1868	bug spray,spice	**3.64**	**4.36**	**5.92**
23	*1875*	*caramel, sweet, fresh*	***3.34***	***3.57***	***3.90***
24	1891	fresh cucumber,floral, fresh	**3.18**	**2.36**	
25	1940	metallic	**3.16**		
26	2000	burnt, spicy		**5.85**	**2.72**
27	2017	metallic,cooked, burnt	**3.95**	**3.16**	**3.30**
28	2038	spice	**3.92**		
29	***2043***	***caramel***	***4.35***	***8.00***	***8.61***
30	*2059*	*spicy(strong), curry*	***3.93***	***5.48***	
31	***2154***	***sweet caramel***	***3.90***		***1.48***
32	*2163*	*sweet, caramel*		***7.35***	***7.84***
33	2179	charred, sweet	**5.07**		
34	2188	grapefruit, spice(lingering)	**3.14**	**7.94**	**5.82**
35	2201	spice	**4.68**	**5.11**	**8.78**
36	***2207***	***sweet, burnt wood***	***2.85***		
37	2243	rancid fruit	**3.27**		
38	2254	grapefruit, citrusy	**7.55**	**4.37**	**4.87**
39	2259	nutty, musty, crayons		**1.05**	
40	2298	grapefruit	**5.86**	**2.37**	
41	2587	tropical, sour	**5.20**		**2.89**
42	2608	vanillin	**9.73**	**4.26**	**10.00**

Aroma Active Compounds Lost during Heating

Many of the components listed in table 2 have been previously reported in fresh grapefruit juice (*40*). However, their concentrations have probably been altered due to the heating process. During the evaporation process when making concentrate, most volatiles are lost along with water. Although it has been reported that over 95% of the volatiles in the fresh juice are lost in the process of concentrating grapefruit juice, over 57% of the total aroma activity remains (*29*). Typically, fresh juice flavor characteristics are restored to the concentrated juice by the addition of juice essence oil, aqueous essence and peel oil. The juice concentrate used in this study had 0.012% cold pressed grapefruit oil added to it. This oil was the source for most of the volatiles and many aroma active volatiles. Since essence oil and aqueous essence are obtained from the condensate of the evaporation process, they will contain volatile heat reaction products and thus were not used for this study. Therefore, any heated, cooked, caramel aromas were produced during the specific heating conditions in this study. Normalized intensities of aroma active volatiles (table 5-3) present in both the unheated reconstituted juice as well as the two heat-treated juice samples were compared in order to identify aroma differences due to heat treatment and contact surface.

Three aroma active compounds, α-pinene (1), myrcene (5) and β-sinensal (38), exhibited greater aroma intensities in the unheated reconstituted juice compared to that of the two heated juice samples (fig. 5-4). Another aroma active compound, ethyl butyrate (2), was detected only in the unheated reconstituted juice with a normalized intensity of 2.24 on a 10-pt scale. Ethyl butyrate imparts a sweet, fruity aroma and is considered to be a positive contributor to grapefruit juice aroma (*40*). It is a low

molecular weight compound and is highly volatile, thus it likely to be degraded during extended heating times.

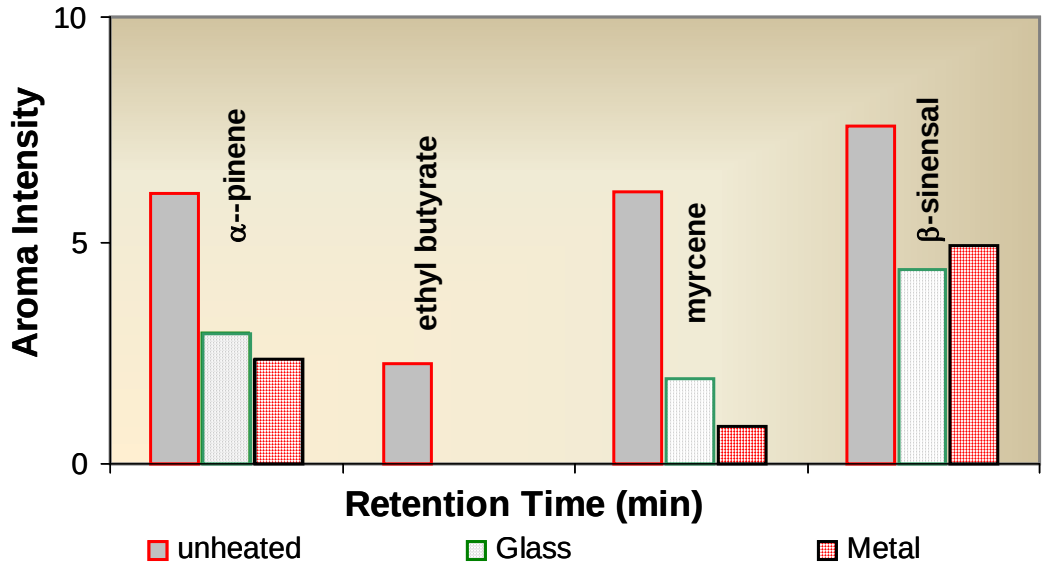


Figure 5-4 Aroma active volatiles whose intensity diminished due to heating

As seen in table 5-3, alpha-pinene (1) and myrcene (5) are two early eluting, low boiling point compounds, which had higher aroma intensities in the unheated reconstituted juice compared to the two heated juice treatments. In both heated samples, the response for α-pinene was less than 45% that of the unheated reconstituted juice while that for myrcene was less than one-third of the intensity in the unheated reconstituted juice. A piney aroma was elicited by α-pinene while myrcene imparted a green, metallic, earthy aroma. Both these compounds are positively correlated with citrus juice flavor (*41*). They are responsible for the green notes in citrus juices.

Beta sinensal (38) is a rather high boiling point volatile, which was reduced through heating. The aroma intensity of this aliphatic aldehyde was 42% higher in the unheated reconstituted juice compared to that of the juice heated in glass and 36% higher than the juice heated in metal. Beta sinensal possesses a grapefruity, citrusy

aroma and is one of the important compounds responsible for the fresh grapefruit juice flavor.

Aroma Active Compounds That Intensified after Heating

Eight aroma active compounds (1-octen-3-one (8), methional (11), (E,Z)-2,4-nonadienal (16), (E,E)-2,4-decadienal (19), Furaneol (29), and 4-vinyl guiaicol (35) and two unknowns (26 & 32) were present in intensities greater than 50% in comparison to the unheated reconstituted juice (fig 5-5). Both Furaneol (29)(discussed later) and methional (11) are thermally generated. Furaneol is formed from the reaction of rhamnose and an amino acid in the presence of heat. Methional is believed to be a Strecker degradation product of methionine in the presence of ascorbic acid. Its normalized intensity in the two heated samples was 8 and 10 compared to 0.65 in the unheated reconstituted juice. (E,Z)-2, 6-nonadienal (16) and (E,E)-2,4-decadienal (19) are unsaturated aldehydes and are likely to be formed from fatty acid degradation (*42*). Both of these aldehydes are essential flavor components of citrus oils. A faint cucumber, green aroma was detected from (E,Z) 2,6 nonadienal while the aroma of (E,E)-2,4-decadienal was described as fatty with a slight orange character. The two unknown compounds (26 & 32) have a spicy and sweet, caramel aroma respectively. Neither of the two unknowns was detected in the unheated reconstituted juice. However the intensity of unknown # 26 in the juice heated in glass was 54% higher than the juice heated in the metal. Unknown # 32 had similar aroma intensity in both the heated samples. Their linear retention index values on wax were 2000 and 2163 respectively. Maltol, a Maillard reaction product elutes at an LRI close to 2000 possesses a caramel aroma and could be a possible identity. Identification of this compound would be satisfied through the use of GC-MS and by matching the spectra of

the compound alongside that of the pure standard. However, maltol does not have a unique mass spectrum and may be present in concentrations lower than the detection threshold of the GC-FID or GC-MS, but not of the human nose.

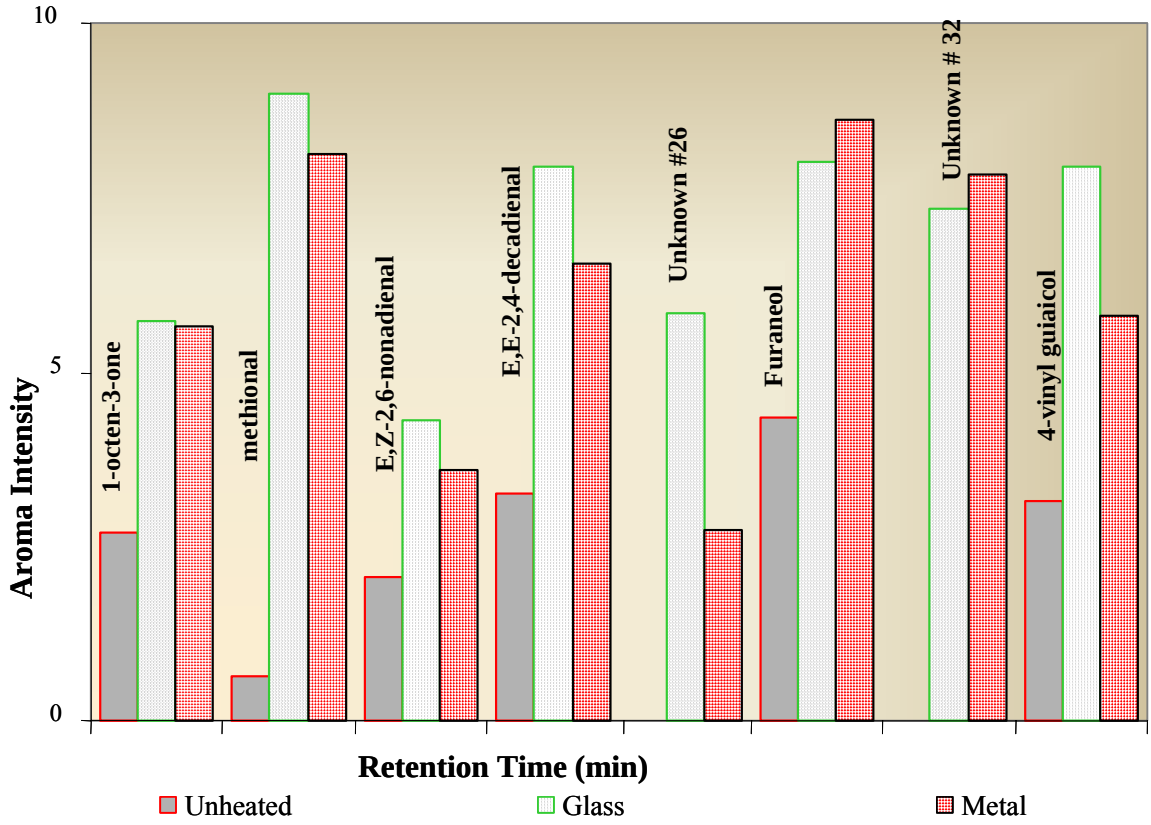


Figure 5-5 Aroma active volatiles (peak numbers 8, 11, 16, 19, 29, 26, 32, & 35) whose intensity increased after heat treatment was applied.

Caramel Aroma Compounds Formed from Sugar Degradation Reactions

From table 5-3, it can be noted that caramel burnt aroma was detected several times throughout the sniff run. In some cases the volatiles lingered for up to 20 sec. In order to identify these compounds, several standards that are known to impart caramel aromas were analyzed under similar conditions on both the wax and ZB-5 columns. Table 5-4 lists the furans and a pyranone standard that were examined to identify the unknown compounds responsible for the caramel aroma.

Table 5-4 LRI for ten sugar degradation products possessing caramel aroma analyzed by GC on both polar (wax) and non-polar (ZB-5) columns.

Compounds	**LRI (ZB-5)**	**Present (ZB-5)**	**Present (Wax)**	**LRI (DB-Wax)**
Furfural	850	+	-	1215
5-methyl furfural	967	-	-	1560
3-methyl- 2(5H) furanone[1]	989	+	+	2153
Furaneol[1]	1064	+	+	2096
Norfuraneol	1054	-	-	2124
Mesifurane	1064	-	-	1602
Maltol	1114	-	-	2002
Sotolone[1]	1118	-	+	2203
Homofuraneol	1145	-	-	2045
5-HMF[1]	1236	+	-	2526[1]

[1] Identified in all three samples

[2] found in unheated reconstituted only

All the furans listed are oxygenated. Furaneol, mesifurane and norfuraneol elute in close proximity on the ZB-5 column. Both Furaneol and mesifurane had identical retention indexes values (1064) while norfuraneol had a linear retention index value of 1054. The ease of volatilization of a compound is one of several factors influencing GC separation. Most of the furans are structurally similar and, would be expected to volatilize easily. Also, column chemistry plays a key role in separation. Polar compounds elute more quickly on a non-polar column but will be better retained on a polar column. The rule "like attracts like" applies. On the ZB-5 column it was seen that the 3(2H) furanones elute in close proximity to each other e.g. Furaneol and mesifurane eluted at 1064, and norfuraneol eluted at 1054. The structures of these compounds are shown below (fig. 5-6).

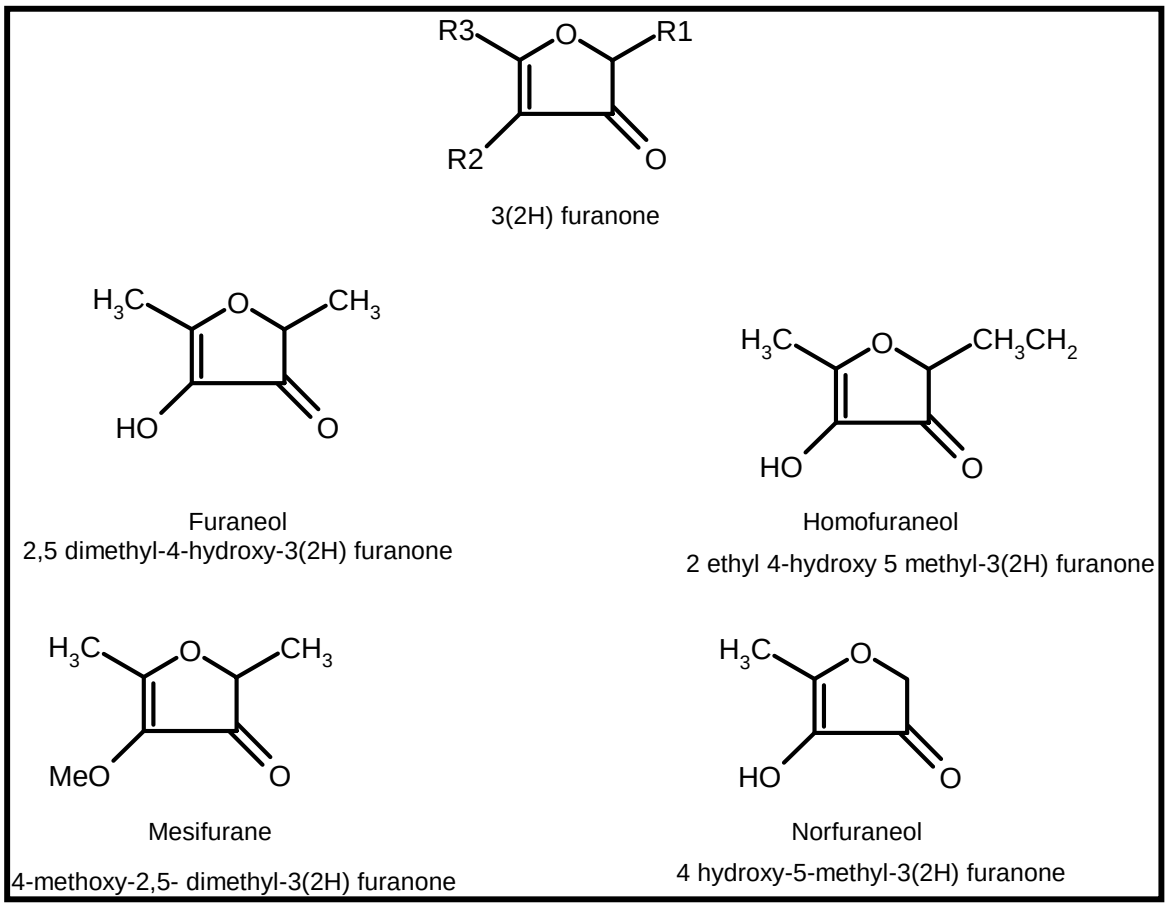
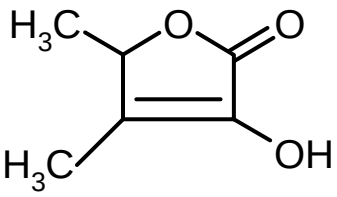


Figure 5-6 Structure of 3(2H) furanones

Homofuraneol has an ethyl group as one of the side groups and this additional increase in molecular weight and boiling point is probably the reason for its later elution compared to the other furanones.

Sotolone (fig.5-7), a 2(5H) furanone, also has a molecular weight of 128 and combined with the different position of the R groups could be responsible for the decrease in its volatility.



Sotolone

Figure 5-7 Structure of sotolone, a 5(2H) furanone

The use of a dissimilar column such as the wax column is an excellent choice in situations like these, as the order of elution will be different. By applying the "like attracts like" principle, polar compounds would be retained for a longer time on the polar wax column.

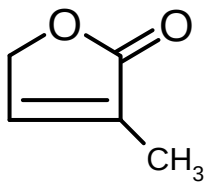
Caramel Compounds Found in Grapefruit Juice

Furaneol and sotolone were detected by GC-O while 5-HMF was detected by GC-FID. Furaneol has previously been reported in grapefruit juice and has been known to mask the flavor of orange juice (*4;11*). Its intensity was over 50% higher in the two heat treated samples as opposed to the unheated reconstituted juice (table 5-3). Furaneol is formed from rhamnose through the 2,3-enolization pathway of the Amadori compound followed by dehydration and molecular rearrangement (*22*). The amino acid arginine has been shown to be a necessary substrate for its formation under acidic conditions. Sotolone has recently been reported as an off-flavor to citrus sodas containing ethanol and ascorbic acid (*28*). Sotolone imparted a caramel, cooked flavor to grapefruit juice. The 5-HMF has been reported in citrus juices and has been suggested as a marker for thermal abuse. This compound is formed from the 1,2-enolization pathway and can be polymerized to form colored pigments or other flavor compounds. Unlike the other furanones, 5-HMF has a high threshold and consequently does not have a direct aroma impact. However, it produced substantial FID response.

Furfural was detected by GC-MS. It has a unique fragmentation pattern with the major ion found at mass 95, indicating the cleavage of a hydrogen atom. By using this knowledge as well as selecting mass peaks 96 and 95, the spectra was deduced and then matched with that of the standard at the expected retention time.

3-Methyl 2(5H) Furanone

A maltol degradation product, 3-methyl-2(5H) furanone (fig. 5-8), (*43*) not previously reported in grapefruit juice was tentatively identified on both polar and non-polar columns. Its LRI on a the non-polar column was 982 on and 2153 on the polar column. It had a weak-moderate caramel aroma. It has been reported in mammee apples (*44*).



3-methy 2(5H) furanone

Figure 5-8 Structure of the 3-methyl-2(5H) furanone

When the standard was analyzed on GC-MS, the retention time and linear retention index suggested that it eluted with peak # II (fig. 5-9).

Commercial FCGJ undergoes at least two heat treatments, once during the evaporation step and second after reconstitution. However, the untreated juice, which was reconstituted grapefruit juice, underwent only the first heat treatment, which involves heating temperatures as high as 93ºC. A high temperature such as this is sufficient to induce the formation of these furanones. The heating of the juice in both the glass and stainless steel containers may have increased the formation or

concentration of these compounds, but due to their extremely low threshold the aroma remains quite intense.

The Maillard reaction is initiated by high temperature treatment of foods, and its products are increased with extreme heat treatment and long-term storage. The first step involves the reaction of a sugar with an amine and subsequent loss of water. There is only a slight loss of reactants since sugars are relatively plentiful in grapefruit juice.

Identification of Volatiles Using GC-MS

Since MS is an identification tool, it was used to identify the volatiles based on their specific fragmentation spectra as well as their linear retention indices. Most compounds fragment in a unique pattern and by comparing this pattern with standards analyzed under the same conditions or by comparing to library databases with known spectra, compounds can usually be identified. Fig. 5-9 shows the total ion chromatograph of grapefruit juice from 10-33 minutes while table 5-5 lists the identities of a few of the major components in grapefruit juice. The areas were normalized to internal standard #2.

Matching linear retention indices was used for a secondary confirmation. This is particularly important in the detection of terpenes. Terpenes are $C_{10}H_{16}$ hydrocarbons and have a molecular weight of 136. It is very difficult to identify these compounds by solely matching mass spectra as they tend to exhibit similar fragmentation patterns particularly for the major mass peaks.

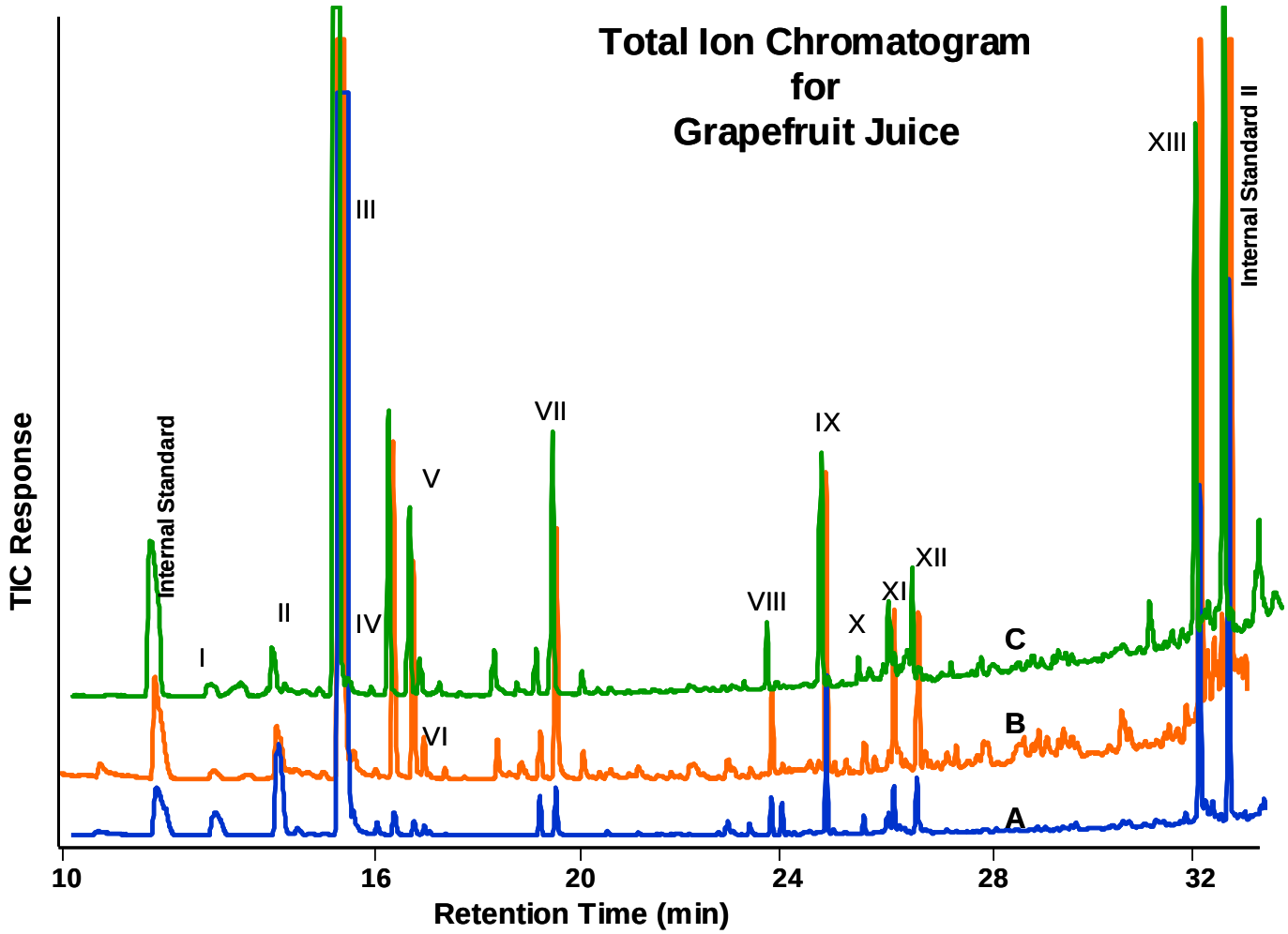


Figure 5-9 Segmented MS total ion chromatogram using ZB-5 column for unheated reconstituted grapefruit juice (A), juice heated in glass (B) and juice heated in stainless steel (C).

Table 5-5 Components identified in grapefruit juice by GC-MS and analyzed on a DB-5 column.

Peak Number	Retention Time (min)	LRI	Component Name	Unheated reconstituted	Glass	Stainless Steel
				%Area	%Area	%Area
I	12.47	941	α-pinene	15.68	2.63	0.74
II	13.68	987	myrcene	45.93	7.52	4.66
III	14.93	1035	limonene	2512.60	611.60	364.36
IV	15.14	1043	E-ocimene	2.25	0.74	0.73
V	15.92	1073	linalool oxide	6.72	27.23	18.83
VI	16.31	1089	linalool	3.79	16.42	11.51
VII	19.05	1201	α-terpineol	10.57	20.67	11.74
VIII	23.19	1385	α-copaene	6.80	0.32	3.39
IX	24.24	1435	β-caryophyllene	30.21	1.73	12.61
X	25.55	1499	α-humulene	12.40	7.43	7.75
XI	26.00	1522	bisabolene	11.22	1.02	6.49
XII	26.14	1529	γ-cadinene	1.43	1.77	0.96
XIII	31.44	1823	nootkatone	80.76	43.69	41.95

An example of this can be seen in peaks number I and III, which were identified as α-pinene and myrcene respectively. Both these compounds are terpenes having piney and musty aroma respectively. From the spectra of these two compounds, fragments 121, 91 and 77 (fig. 5-10) were common to both and appeared to have the same intensity. Mass peaks at 92, 93 and 79 had slightly different intensities and may be used as distinguishing fragments. However, by using the linear retention indices of 939 and 989 respectively, these two compounds could be easily distinguished.

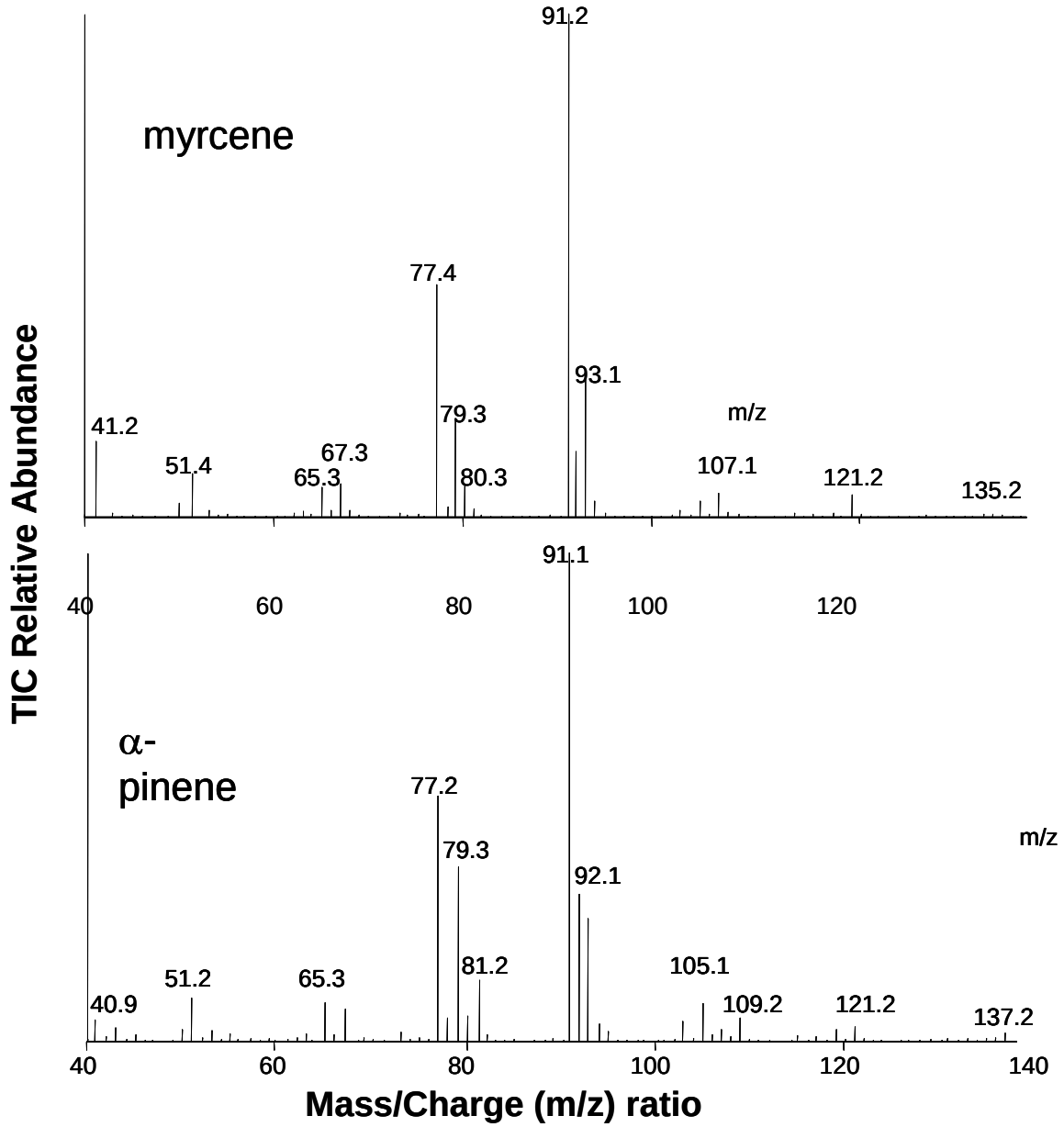


Figure 5-10 Mass spectra of myrcene (top) and α-pinene (bottom) taken from a sample analyzed on a DB-5 column.

Limonene (peak III) was present in the highest amount as expected. Limonene is the most abundant terpene present in citrus juices. Peak VII was identified as α-terpineol. Figure 5-11 is an expanded view of the chromatogram indicating the differences among the three samples. Alpha terpineol content of juice heated in glass was higher than both the stainless steel (metal) and unheated reconstituted from concentrate juice. It 63%

higher than the unheated reconstituted juice and 43% higher than the juice heated in metal.

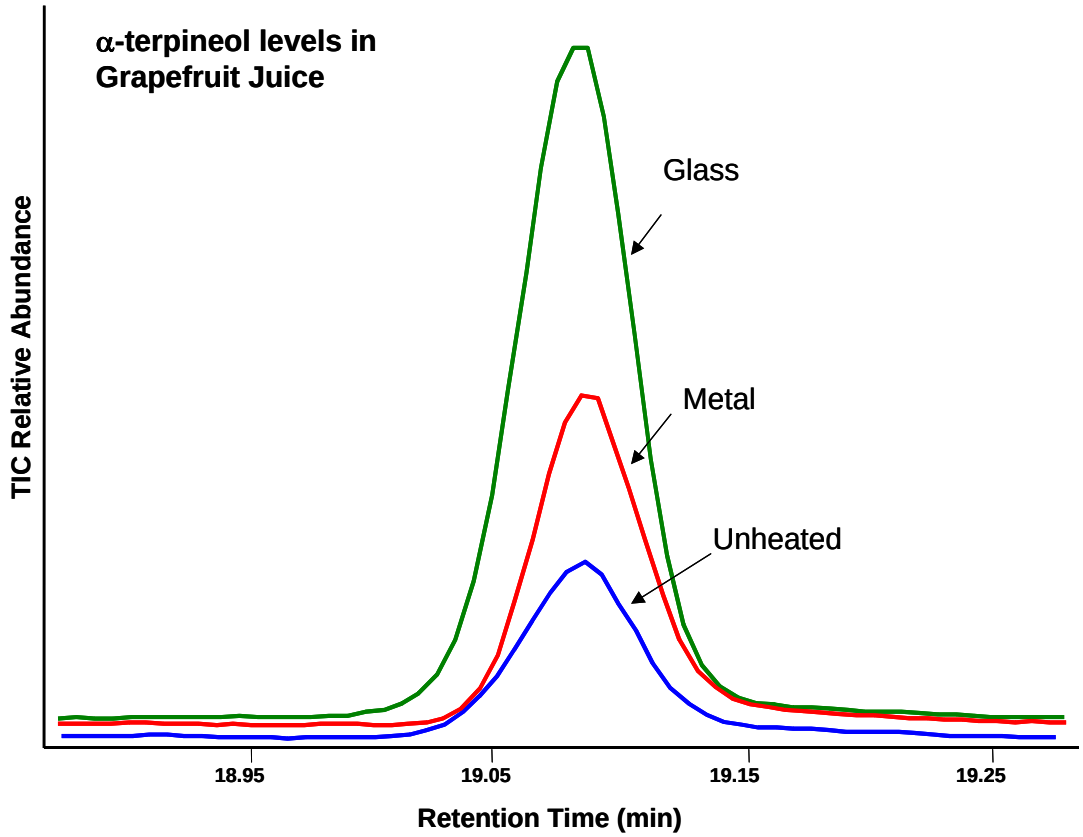


Figure 5-11 Alpha-terpineol levels in the three samples, unheated reconstituted juice, juice heated in glass and juice heated in metal

Alpha-terpineol can be formed from either (or both) the acid-catalyzed reaction of limonene or linalool (fig. 5-12) in the presence of heat (*45*). Alpha terpineol has also been shown to negatively correlate with the flavor of citrus juices (*4*) and has been suggested as an indicator for over-processed or stored orange juices.

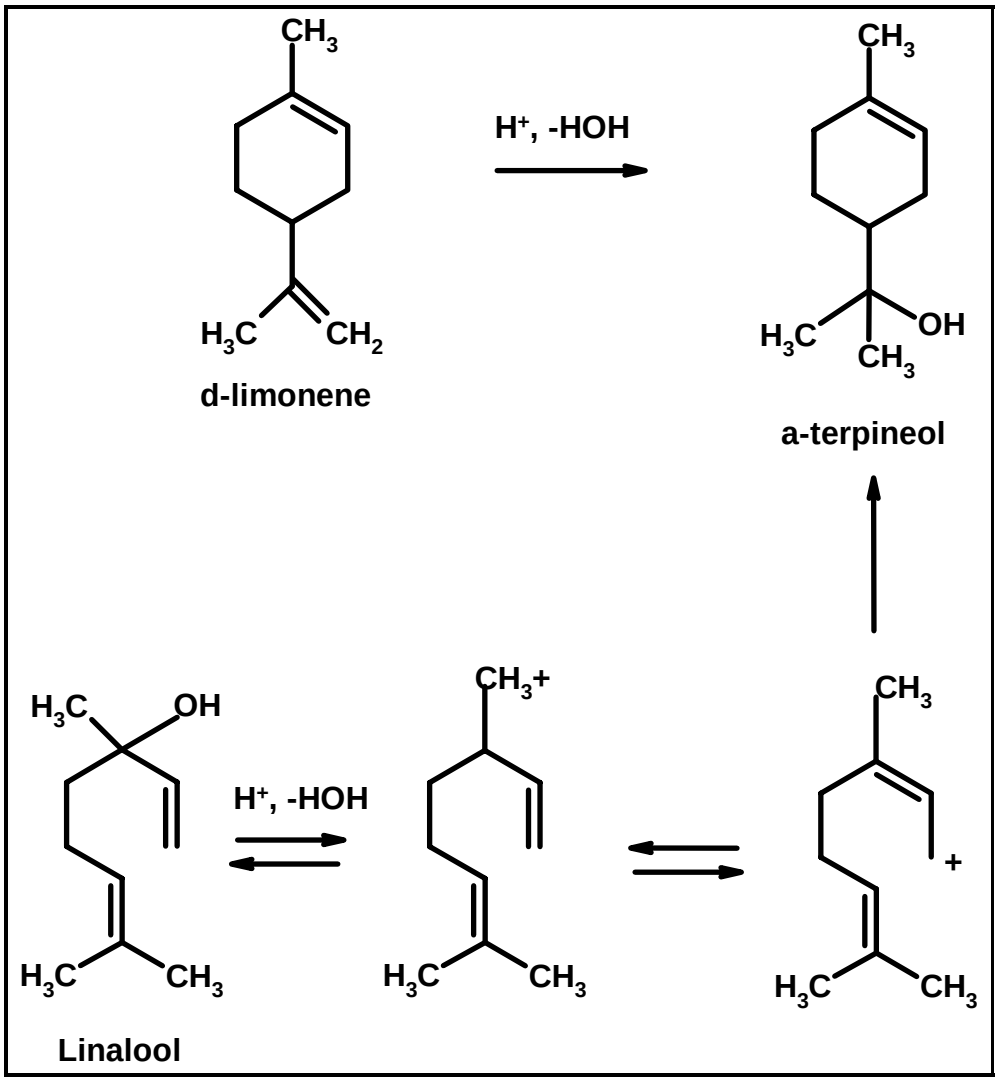


Figure 5-12 Formation pathway for α-terpineol in the presence of limonene and linalool in grapefruit juice

CHAPTER 6
HPLC RESULTS AND DISCUSSION

Unlike GC, HPLC is a non-thermal method of analysis used in the identification of both volatiles and non-volatiles. HPLC is useful in situations where polar volatiles have strong UV chromaphores and are difficult to extract using typical GC extraction procedures. For example, Furaneol in grapefruit juice is usually found at levels below the detection threshold of the flame ionization detector, making detection and quantification difficult. Solid phase extraction employing C-18 chemically bonded silica, can trap polar volatiles onto the modified silica for later analysis on reversed phase HPLC.

Since furans are thought to be responsible for heated off-flavor, HPLC was used to identify and measure furans in grapefruit juice. Over the years reversed phase HPLC has been favored in the isolation of furans in several fruit juices (*19;21;46*). Reversed phase LC is the most popular liquid chromatographic method used today (*47*). Compounds with increasing hydrophobicity or non-polar behavior will be retained on the column more strongly. Water is the weak solvent and a methanol or acetonitrile gradient is employed to selectively elute the materials off a reverse phase column.

In citrus juices several mobile phase compositions have been used ranging from methanol or acetonitrile/water, acetonitrile/methanol/buffer, methanol/acetate buffer. These solvent systems either did not produce a good separation or did not exhibit good reproducibility. Acetate buffer has an absorbance maximum at 235nm, which interferes with the spectra of sotolone whose maximum absorbance is 237nm. Although previous studies (*19*) reported that methanol/phosphate buffer condition could not resolve furfural

and Furaneol, this mobile phase composition was re-examined since the phosphate buffer had a lower UV absorbance background. It had been reported that 30% methanol/phosphate buffer could not resolve furfural and Furaneol. Thus several gradients were evaluated until a suitable separation was between 5-HMF, furfural, and Furaneol was achieved. These three compounds had been previously been identified in grapefruit juice using RP-HPLC (*19*). In order to increase retention time and promote resolution, the amount of modifier, methanol was decreased to 5% with a slow increase up to 30% in 27 minutes. It was found that at approximately 20% methanol Furfural was eluted and Furaneol close to 24% methanol. Additionally, the temperature of the column was kept at 25°C and the sample 24°C. Since the three compounds were completely resolved, other Maillard reaction products, (mesifurane, homofuraneol, sotolone, and maltol) were evaluated under similar conditions and the result shown in fig. 6-1. The small humps at the end of the late eluting peaks were apparently due to a small void at the head of the HPLC column. These standards are all products of the Maillard reaction and possess caramel or burnt sugar aroma that could possibly be formed in grapefruit juice during heating. These seven compounds along with their retention times and maximum absorbance are listed in table 6-1.

Table 6-1 Retention time and maximum absorbance of furans standards on a C-18 column and using a methanol/phosphate buffer at flow rate of 1mL/min

Furan Standards	Retention Time (min)	Max Wavelength (nm)
5-HMF	13.04	283
Furfural	14.95	276
Maltol	17.41	274
Furaneol	18.86	287
Sotolone	20.24	235
Mesifurane	27.63	278
Homofuraneol A	30.40	287
Homofuraneol B	31.80	287

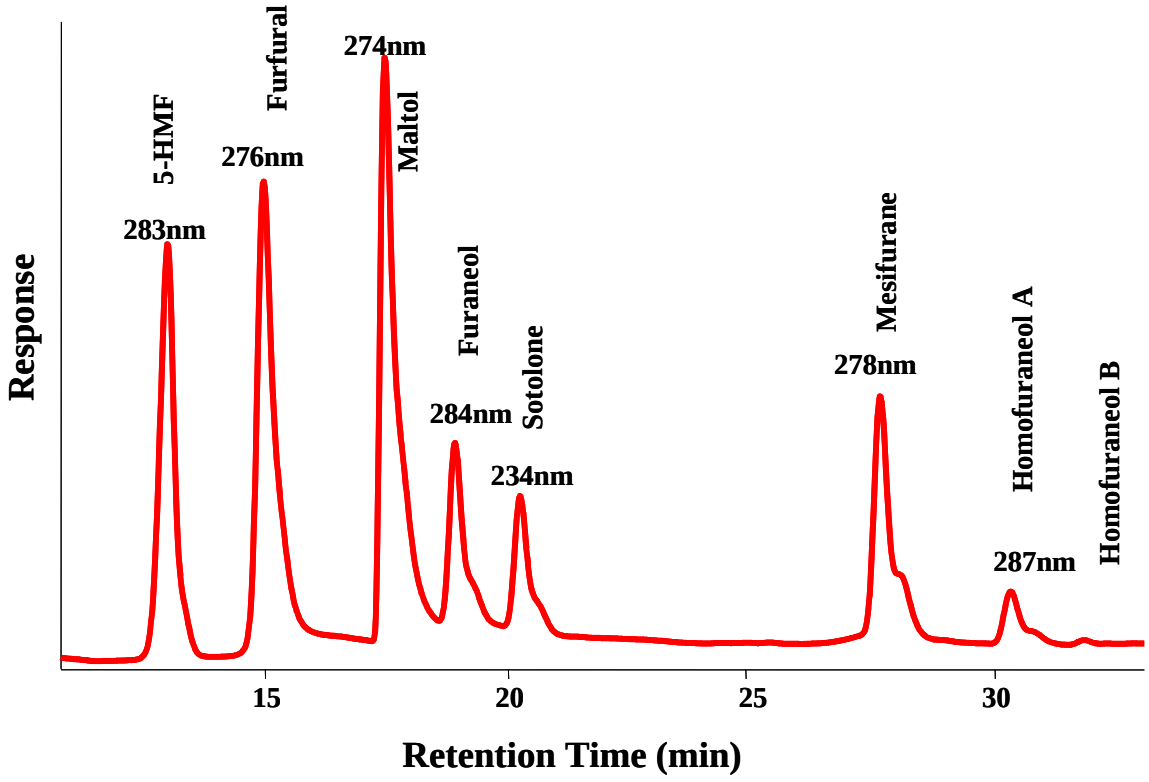


Figure 6-1 HPLC spectra of the seven standards (5-HMF, furfural, Furaneol, sotolone, mesifurane and homofuraneol) monitored from 230-380nm.

Homofuraneol has been known to exist in two tautomeric forms accounting for the presence of an A and B form (*48*) and produces two distinct HPLC peaks. As can be

seen from fig. 6-2, homofuraneol has an ethyl group that can be interchanged with the methyl group so that it can be either in the 2 or 5 position.

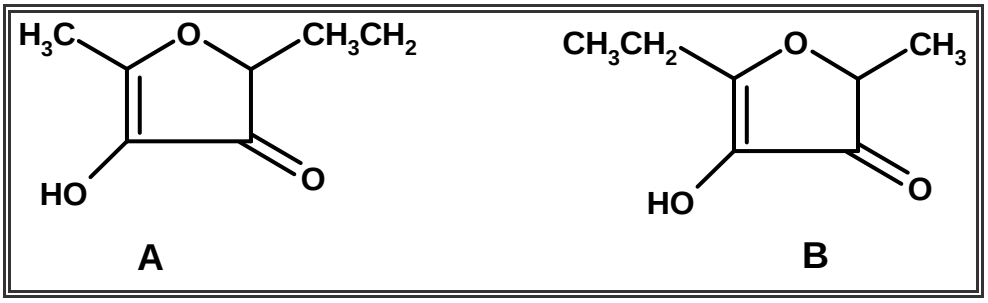


Figure 6-2 Homofuraneol tautomers, **A**- 2-ethyl-4-hydroxy-5-methyl-3(2H) furanone; **B**- 5-ethyl-4-hydroxy-2-methyl-3(2H) furanone

The value of the PDA detector lies in its ability to distinguish compounds based on their spectra as well as retention time. This feature can be useful in identifying unknown peaks and determining if there are co-eluted compounds in a single HPLC peak. With the exception of sotolone, which has a maximum absorbance at 235nm, most furans exhibit maximum wavelength absorbance between 270-290nm. For this reason dual wavelength monitoring at 235nm and 280nm was carried out. Additionally, monitoring was done at 335nm as these furans had minimum absorbance at that wavelength. This would be useful in distinguishing furans from other co-eluting compounds. A comparison of the spectra and the retention times of components in the sample with those of standards, suggest the presence of 5-HMF, furfural, and Furaneol in the grapefruit juice (fig. 6-3). As discussed earlier, these three compounds are heat induced hexose sugar degradation products that have been previously identified in citrus juices.

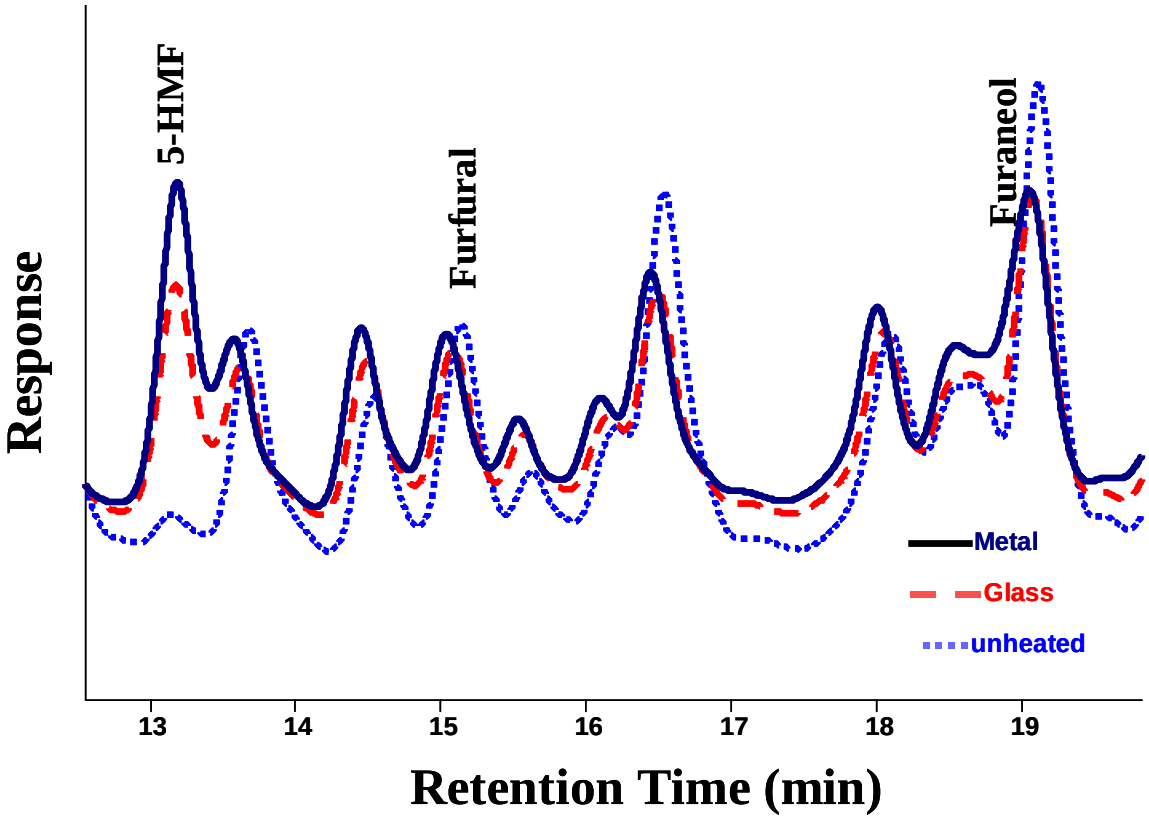


Figure 6-3 Segment of HPLC chromatogram showing overlay of unheated, juice heated in glass and juice heated in metal and monitored at 290nm

By looking at the spectra of each of these compounds in the sample and comparing it with that of the standard, it was found that 5-HMF was the only compound of interest that was well resolved from other grapefruit juice components (fig. 6-4).

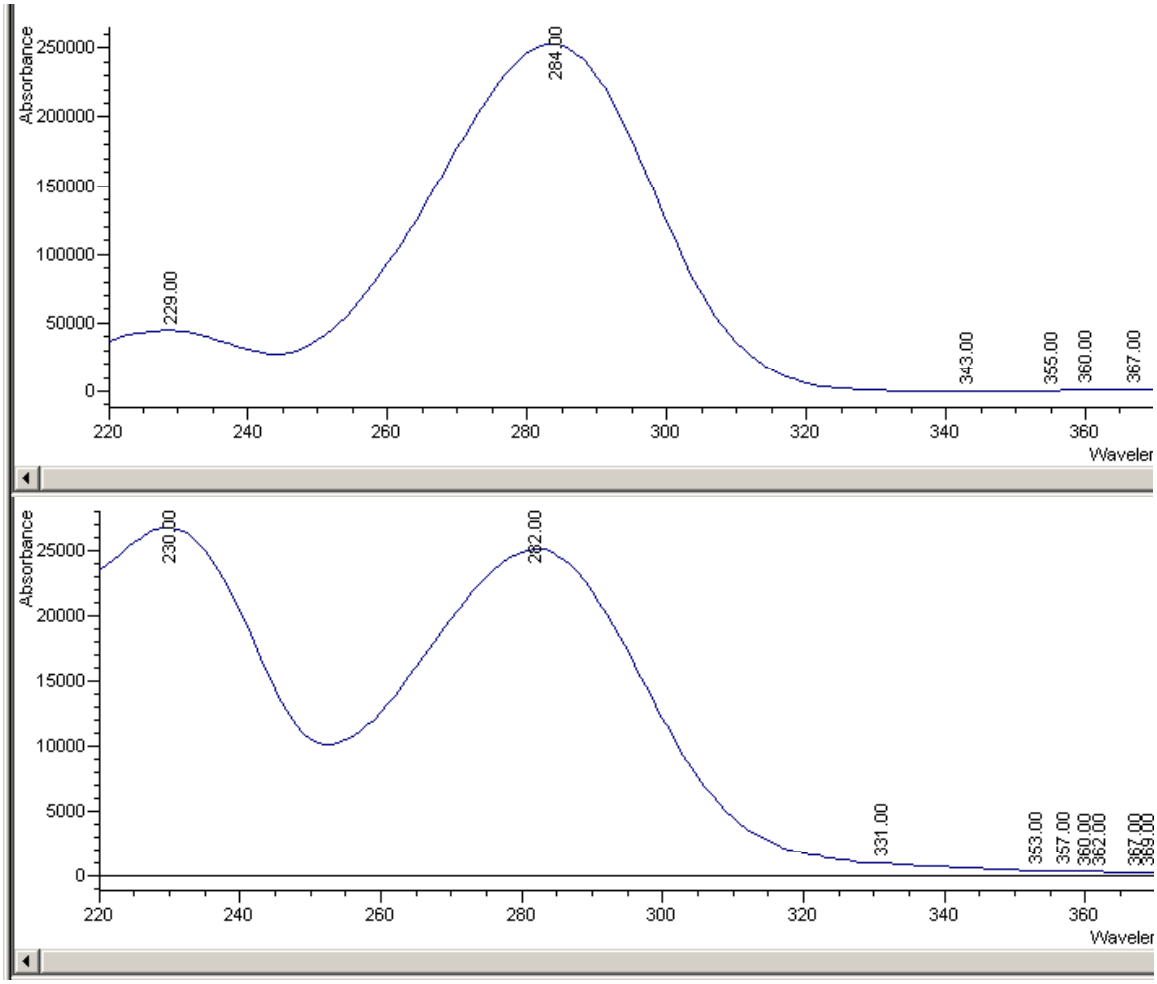


Figure 6-4 Spectra of 5-HMF in standard (top) and in the grapefruit juice sample (bottom) analyzed on C-18 column and monitored at 290nm.

Spectral data for both Furaneol and furfural peaks suggested the presence of coeluted interfering compounds. Figure 6-5 shows the spectra of furfural and it can be seen that the peak characteristics are different from the standard. An absorbance maximum of 276nm was seen for the standard while a maximum of 326nm was seen for the sample. Between 260 nm and 300nm, there appears to be another peak indicating that co-elution might have occurred. It was not possible to obtain an absorbance "difference" chromatogram using output from the two wavelengths (326nm and 278nm) at this time. Previous studies had used this technique to identify and characterize Furaneol in a grapefruit juice matrix (*21*).

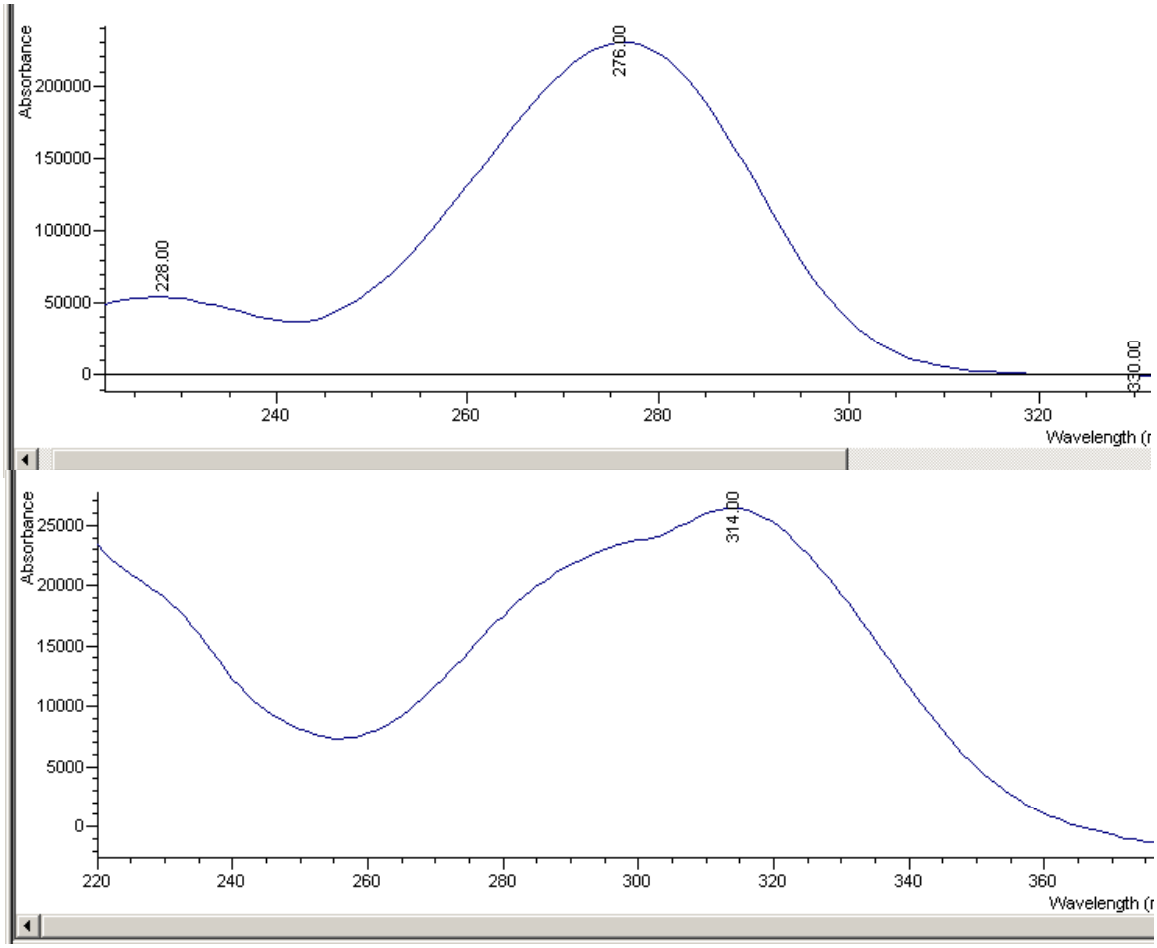


Figure 6-5 Spectra of furfural standard (top) and sample (bottom) showing a difference in the wavelength maxima at 15.15 min

Grapefruit juice furan and furanones were quantified using the external standard method, wherein different concentrations of standards were injected onto the LC in triplicate. Area responses were then plotted against the amounts injected and linear responses were obtained. The 5-HMF amounts ranged from 1.07ng to 21.4ng to ensure that the range found in samples was covered. From calibration plot (fig. 6-6), the r^2 value which shows how well the data fit a linear regression was 0.9993, indicating good linearity.

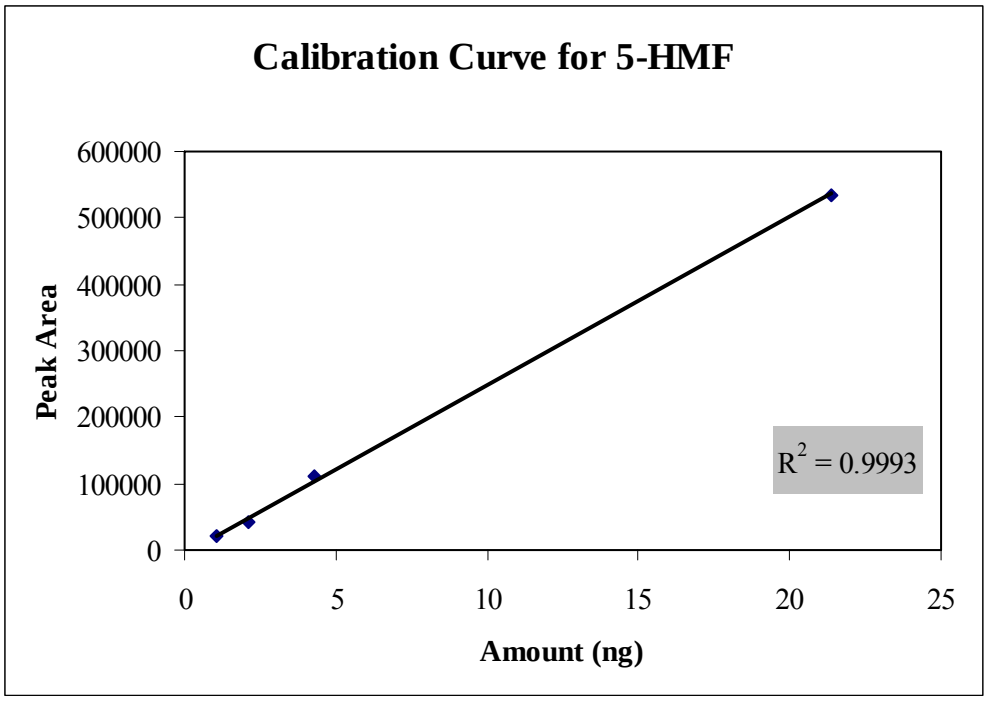


Figure 6-6 Linear calibration curve for 5-HMF analyzed in triplicate.

Based on the linear equation, the amount of the each furan in grapefruit juice samples could be calculated. Juice heated in the presence of stainless steel had a 5-HMF concentration of 0.47ng/ul, while the same juice heated in glass had 0.34ng/ul (table 6-2). Unheated juice contained the least amount of 5-HMF (0.03ng/ul).

Table 6-2 Concentrations of 5-HMF, furfural and Furaneol in the three grapefruit juice samples

	5-HMF (ng/ul)	**Furfural (ng/ul)**	**Furaneol (ng/ul)**
Unheated	0.03	0.40	0.22
Glass	0.34	0.25	0.08
Metal	0.47	0.25	0.08

A similar plot (fig.6- 7) was conducted for Furaneol with amounts ranging between 1.8ng to 18ng and an r^2 value of 0.92 was found. Unheated reconstituted juice had a concentration of 0.22ng/ul while the two heated juice samples had levels of 0.08ng/ul.

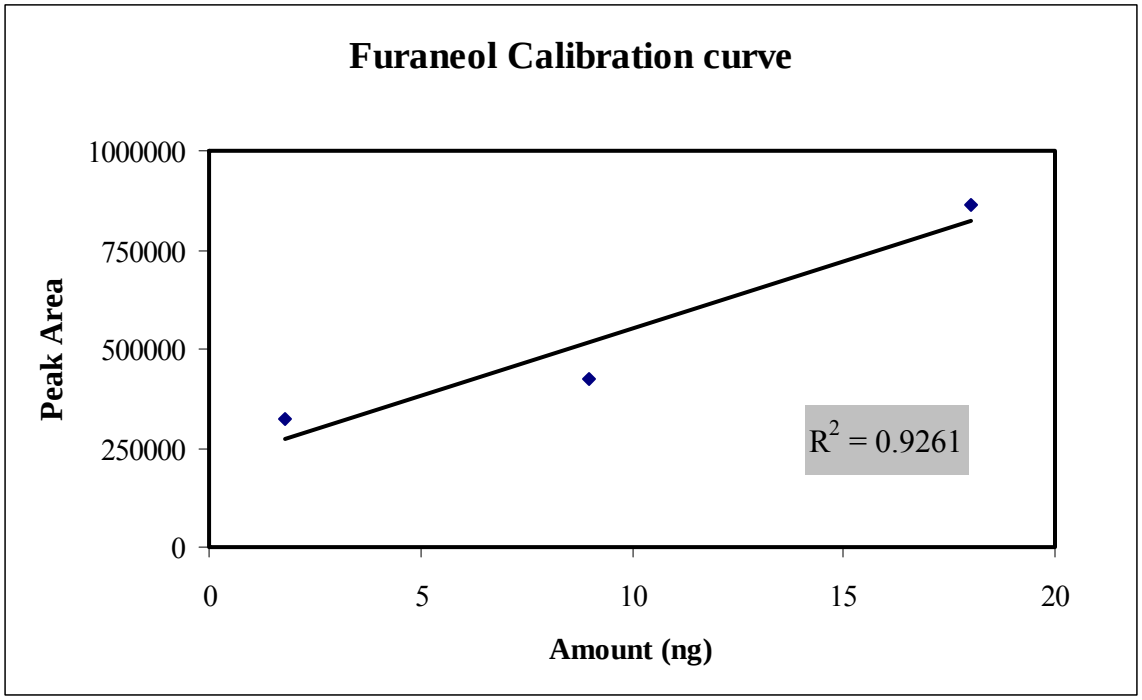


Figure 6-7 Linear calibration curve for Furaneol at 290nm.

The standard curve (fig. 6-8) for furfural also had a good fit with an r^2 value of 0.99 using a range of 2ng to 200ng. Concentration of furfural in the unheated reconstituted juice was 0.40ng/ul while juices heated in glass and metal had 0.25ng/ul.

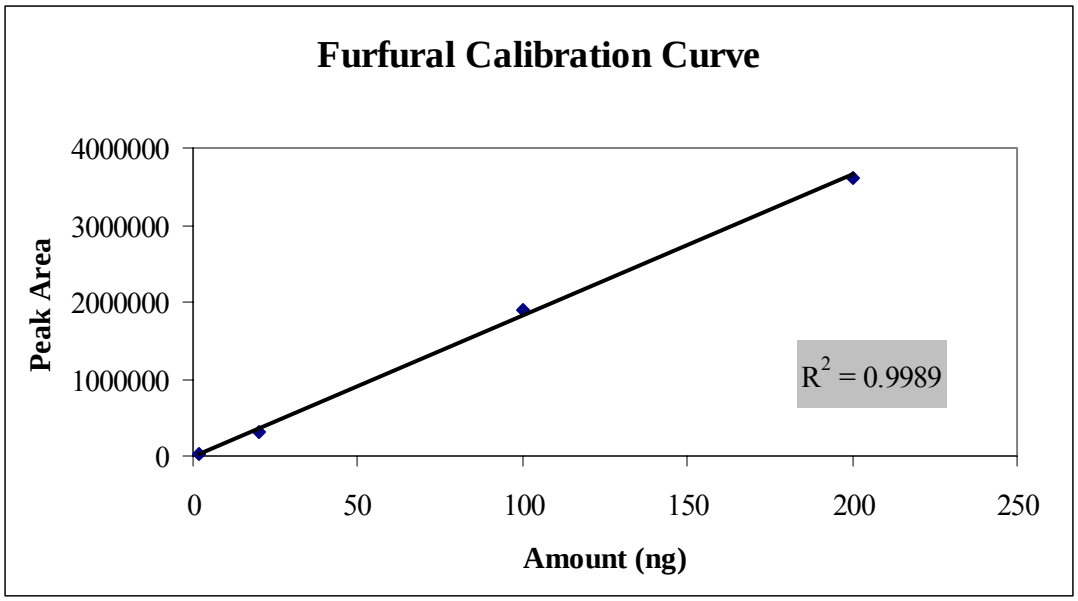


Figure 6-8 Linear calibration curve for furfural standard at 290nm.

All three compounds, 5-HMF, furfural and Furaneol are sugar degradation products. Formation of 5-HMF occurs through the 1,2 enolization at low pH values in the presences of a hexose sugar. The pH of grapefruit juice is usually between 2.9- 3.2, which is highly acidic, and provides an explanation for the increase in the 5-HMF formation in the two heated samples. Studies have shown when grapefruit juice that under similar conditions the rate of formation of 5-HMF was far greater than that of furfural (*9*). Furfural can be formed from acid catalysis of ascorbic acid in the presence of an amine. Since it can be formed from ascorbic acid, which is known to degrade with heating, it was expected that this compound would have higher concentrations in the two heated samples. However, it is a very reactive compound and, in the presence of acids can react with aldehydes, ketones and amino acids (*49*). This could possibly be a reason for the lower levels in the heated samples.

Furaneol is also a sugar degradation product and its concentration was expected to increase as a result of the heating. Its formation is favored at high pH and goes through

the 2,3-enolization pathway, however at low pH values it is formed in the presence of alanine. Studies have shown that Furaneol is unstable and can be degraded at high temperatures (*24*) to form highly reactive dicarbonyls, ketones and alcohols. In equilibrium, Furaneol can exist in its open chain form, thereby facilitating attack of sulfur groups such as thiols on the carbonyl (*50*). It can also react with sulfur compounds, such as cysteine at low pH, and hydrogen sulfide during thermal processing (*51;52*). Consequently, at high temperatures, the concentration of Furaneol would be lower than expected.

CHAPTER 7
CONCLUSION

No significant flavor difference was detected between juices heated 10 min in contact with stainless steel and the same juice heated in glass. However, this excessive heating induced a flavor change that was significantly different from the unheated reconstituted grapefruit juice at the 95% confidence interval. Thermal processing produced heated, cooked, pineapple and metallic off-flavors.

Similar volatiles were detected in both heat-treated samples, suggesting that high processing temperatures and long times appeared to be major contributing factors for cooked off flavor formation. The quality of the juice heated on glass surface was not of superior quality to that of metal surface, therefore, the current practice of using stainless steel is agreeable.

Intensities of compounds such as α-pinene and myrcene that are known to positively contribute to fresh citrus juice character were diminished during the heating process, while aroma intensity of heat induced compounds such a methional and Furaneol increased during heating. Sugar degradation products such as sotolone, and 3-methyl-2 (5H) furanone were detected by GCO. The 3-methyl-2 (5H) furanone was tentatively identified in grapefruit juice for the first time. Concentrations of 5-hydroxy methyl furfural, another sugar degradation product, increased with heating.

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BIOGRAPHICAL SKETCH

Wendy Bell was born in Westmoreland, Jamaica, and graduated from the Wolmer's High School for Girls in Kingston. In 1995, she moved to the United States and embarked upon her undergraduate studies at Barry University, Miami, Florida. She then transferred to the University of South Florida, Tampa, Florida, where she completed her Bachelor of Arts in Chemistry in May 1999. In pursuance of her career, Wendy gained employment at Pasco Beverage Company and Florida Department of Citrus.

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