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IDENTIFICATION OF KEY ODORANTS IN FRESH-CUT WATERMELON AROMA AND STRUCTURE-ODOR RELATIONSHIPS OF *CIS,CIS*-3,6-NONADIENAL AND ESTER ANALOGS WITH *CIS,CIS*-3,6-NONADIENE, *CIS*-3-NONENE AND *CIS*-6-NONENE BACKBONE STRUCTURES

BY

ELIZABETH R. GENTHNER

THESIS

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Adviser:

Professor Keith R. Cadwallader

ABSTRACT

The scope of this study involved the identification of key odorants in fresh cut-watermelon, and the synthesis and evaluation of esters with potential watermelon-like aroma attributes. Aroma formation in fresh-cut watermelon is a dynamic enzymatic process, with the characteristic aroma components being formed immediately after cutting. The characteristic fresh-cut aroma is not long lasting due to further enzyme action that modifies the fresh-cut aroma components. The key to identifying the key components responsible for fresh-cut watermelon aroma was the application of a suitable volatile isolation strategy based on static headspace analysis (SHA). In this study, SHA was used to collect the headspace volatiles one minute after initial cutting of the fruit. This enabled a chemical "snap shot" of fresh-cut aroma to be taken. The most potent odorants in the headspace were determined by gas chromatography-olfactometry of decreasing headspace volumes (GCO-H) with confirmation achieved by application of a complimentary method based on GCO and aroma extract dilution analysis (AEDA) of fresh-cut watermelon aroma extracts prepared by solvent-assisted flavor evaporation (SAFE). Eight unsaturated nine-carbon aliphatic aldehydes and one six carbon unsaturated aldehyde were detected by GCO-H. These included *cis*-3-hexenal, *cis,cis*-3,6-nonadienal, *cis*-3-nonenal, *cis*-6-nonenal, *trans*-2-nonenal, *cis*-2-nonenal, *trans,cis*-2,6-nonadienal, *trans,trans*-2,4-nonadienal, and *trans,trans,cis*-2,4,6-nonatrienal. This finding is contrary to previous beliefs that alcohols are the main contributors to fresh-cut watermelon aroma. Most importantly, it was found that *cis,cis*-3,6-nonadienal was not only a potent odorant, but this compound alone possessed an aroma reminiscent of fresh-cut watermelon. Use of sensory evaluation, including ranking test, revealed that the aroma attributes of purified synthetic *cis,cis*-3,6-nonadienal closely matched those of fresh-cut watermelon. This "watermelon aldehyde" can be considered a potent

odorants since its estimated odor detection threshold (in water) is very low (0.2 ppb). Unfortunately, *cis,cis*-3,6-nonadienal is a labile compound and is prone to both *trans* isomerization and oxidation of its aldehyde end group. For this reason, an attempt was made to create an ester with watermelon-like aroma attributes. Esters have been widely used in the food and beverage industries as flavoring agents because they are both stable and possess relatively low thresholds. To develop a watermelon-like ester, alcohol (formate, acetate, propionate, and butyrate) and carboxylic acid (methyl, ethyl, propyl, and butyl) esters with *cis,cis*-3,6-nonadiene backbones were synthesized. To achieve a more thorough understanding of the structure-odor relationship of these esters, the same types of alcohol and carboxylic acid esters were created with *cis*-3-nonene and *cis*-6-nonene backbones. The general structure/threshold trend was that threshold increased with increasing carbon number. In addition, threshold was also dependent on the number and position of the *cis* double bond. Descriptive sensory analysis was used to evaluate the effect of structure on the odor properties of the selected (low threshold) esters; however, no clear trend was found. Finally, further sensory analysis by ranking test was employed to determine, which, if any of these esters might be a suitable replacement for *cis,cis*-3,6-nonadienal for use as a watermelon flavoring. Although all esters had a "fruity" element to their aroma descriptions, none were significantly close in terms of their aroma attributes to *cis,cis*-3,6-nonadienal.

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CHAPTER ONE:

Introduction

The importance of watermelon dates back to prehistoric times with earliest evidence existing as far back as 20th century B.C. to the ancient Egyptian era. During ancient times, wild watermelons were primarily used as "botanical canteens", that is, as water source in the arid regions of the Sahara. However there is evidence of their use as a food due to their presence in many traditional African dishes where the flesh and the seeds (oil and protein source) are used. Watermelons were spread around the world via trade routes and reached a point of cultivation in India and Asia. They still predominate in Asian countries; China is the largest worldwide producer. Moorish invaders brought watermelon to Europe around the 10th century A.D., but the cultivation of this fruit was slow to develop due to the mild continental summers. Watermelons reached the Americas through Spanish settlers in Florida where they were being cultivated by 1576. Watermelon's predominance in the U.S has created a cultural significance. "The true southern watermelon is a boon apart and not to be mentioned with commoner things. It is chief of this world's luxuries, king by the grace of God over all the fruits of the earth. When one has tasted it, he knows what the angels eat." (Twain 1894).

Today, watermelons are mainly enjoyed fresh cut due to their sweet and refreshing taste. There are roughly 1,200 watermelon varieties in existence. In 2009 watermelon production in the U.S. reached 4.0 billion pounds, worth 461 million dollars, with consumption mostly in the form of fresh fruit (Anon, 2010).

The flavor of watermelon has been the subject of much debate. The root of the problem is due to the fact that watermelon aroma is formed via a dynamic enzyme system, thus it is

constantly changing. It is hypothesized that watermelon fruits have no aroma until they are cut open. The introduction of oxygen and the release of enzymes through tissue disruption create a cascade of biochemical events resulting in the formation and release of volatile aroma compounds. The presence of other enzymes further alters the volatiles initially formed.

In previous studies on watermelon aroma, the techniques used for volatile compound isolation was a major influence on which compounds were identified and reported in the volatile profile of watermelon. Techniques can be harsh and disruptive to compounds initially present in freshly cut watermelon, thus biasing the results towards the secondary compounds formed. Currently, it is believed that the most important aroma compound in watermelon is the C_9 double unsaturated alcohol *cis,cis*-3,6-nonadien-1-ol, since it has most often been reported an abundant volatile component and because it has an odor reminiscent of watermelon (Yajima et al. 1985). However, looking at the biochemical reactions that occur in forming the alcohol, a structurally analogous aldehyde must be the precursor (Hatanaka et al. 1975). In the lipoxygenase pathway free fatty acids (i.e. linolenic acid) are subject to enzymatic lipid oxidation which results in the formation of a C_9 or C_{13} hydroperoxides. Hydroperoxide lyase then cleaves the hydroperoxide resulting in an oxo-acid and *cis* unsaturated aldehydes. At this point, other enzymes, such as alcohol dehydrogenase and *cis/trans* isomerase, convert these compounds to the corresponding alcohols and *trans* isomers, respectively.

The central hypothesis of the present study is that by taking an initial "snap-shot" of the volatile constituents of fresh-cut watermelon, that the compounds responsible for the characteristic fresh-cut watermelon aroma can be accurately identified. Static headspace

analysis (SHA) and gas chromatography-olfactometry (GCO) was applied for this purpose. SHA is a technique in which the vapor above a liquid or solid sample is extracted and directly analyzed by GC. The "extract" mimics the aroma compounds a human nose would detect while smelling a food sample. The benefits of this technique are that it is non-destructive and the process of transferring the volatiles from the headspace of the food to the injection part of the GC for analysis is extremely fast. To determine the most important odorant, GCO of decreasing headspace volumes (GCO-H) was performed. In this case, it was hypothesized that unsaturated aldehydes are the most important aromas found in fresh-cut watermelon, with *cis,cis*-3,6-nonadienal, in particular, being the most important based on the compound's odor intensity in the headspace and by applying the odor-activity value concept.

The compound *cis,cis*-3,6-nonadienal is a labile compound. It is prone to both isomerization and oxidation making it difficult, if not impossible, to use it as a flavoring substance. A third hypothesis of the present study is that a suitable structural analogue of this compound can be synthesized which will have a similar watermelon-like aroma based on the structure-odor (function) concept. Specifically, the *cis* bond position and conformation on the nine carbon backbone is believed to be essential for a molecule to have a characteristic watermelon-like odor. It was hypothesized that a watermelon smelling ester could be synthesized by keeping that part of the molecule intact and by substituting a stable terminal ester group in place of the aldehyde moiety. For this compound to serve as a suitable substitute, it should elicit an aroma reminiscent of watermelon and have a low enough threshold for use as a flavoring agent. For this purpose, carboxylic acid and alcohol esters were synthesized in a range from 1 to 4 carbon straight-chain esters with either c*is,cis*-3,6-nonadiene, *cis*-3-nonene or cis-6-nonene backbone structures. The purpose of including *cis*-3 and *cis*-6 analogs in this

investigation was to provide a thorough understanding of the structure-odor effect of the *cis* monounsaturation being at the 3 or 6 position.

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CHAPTER TWO:

Literature Review

2.1 The Watermelon

The watermelon plant (*Citrullus lanatus*), a member of the family Cucurbitaceae, is monoecious and bears fruit annually. Fruits range in size from 1 to 100 kg with the average being between 3 to 13 kg. Watermelon was originally domesticated in central and southern Africa over 6,000 years ago, and has been a significant agricultural crop in Africa and Asia ever since. Originally the fruits were used as a source of water in arid regions where they were referred to as "botanical canteens." The seeds also served as a dietary source for both edible oil and protein. Watermelons reached the Americas through European colonists and African slave ships around the 1600s. They are now primarily consumed for their sweet and juicy fruit (Kiple et al 2000). There are roughly 1,200 varieties grown worldwide and somewhere between 200 and 300 are cultivated in the United States; with Florida, California, Texas, Georgia and Arizona being the top growing regions. Harvested in summer months with the peak falling in July, watermelons have become a part of American culture as one of the favorite summertime treats where they are best enjoyed fresh-cut. By weight, the watermelon is by far the most consumed melon in the U.S (Anon, 2010b). In 2009 watermelon production reached 4.0 billion pounds, valued at 461 million dollars. Most of this consumption was in the form of fresh-cut watermelon (Anon, 2010a).

2.2 Flavor Chemistry of Watermelon

The first major investigation of the volatile components in watermelon was conducted by (Yajima et al. 1985). In that study, volatile compounds were extracted by distillation under reduced pressure (10 mm Hg) at 28 °C and then fractionated based on pH and polarity. Fifty-two compounds were identified. The most abundant compounds were *cis*-3-nonen-1-ol and *cis,cis*-3,6-nonadien-1-ol. Based on its relatively high abundance in the extract, the alcohol *cis,cis*-3,6-nonadien-1-ol was credited with being the most powerful contributor to the aroma of watermelon. Also considered important were *cis*-3-nonenal and *cis,cis*-3,6-nonadienal which were described as having somewhat "watermelon-like" odors. Minor constituents included 2-ethoxy, 2-pentyloxy-, 2-hexyloxy-, 2-octyloxy-, 2-nonyloxy-, 2-[*cis*-3-nonenyloxy]-, 2-[*cis*-2-nonenyloxy]-, 2-[*cis,cis*-3,6-nonadienyloxy]-, 2-benzyloxy-, and 2-phenethyloxy-pentyltetrahydrofurans. The authors deduced that the aliphatic C_9 alcohols and aldehydes (e.g., 1-nonanol, *cis*-3-nonen-1-ol, *cis*-6-nonen-1-ol, *cis,cis*-3,6-nonadien-1-ol, *trans,cis*-2,6-nonadien-1-ol, nonanal, *cis*-3-nonenal, *cis,cis*-3,6-nonadienal, *trans,cis*-2,6-nonadienal) were the most important contributors to the aroma of watermelon. Although *cis*-6-nonen-1-ol was positively identified, there was no mention of the detection of the corresponding aldehyde, *cis*-6-nonenal. However, this could have been due to use of a polar column during gas chromatography-mass spectrometry (GC-MS) for which *cis*-3-nonenal and cis-6-nonenal have almost identical retention indices (RIs). Pino et al. (2003) quantified volatiles in watermelon using GC-MS. These researchers concluded that the compounds most important in watermelon flavor extracts were saturated and unsaturated C_9 aliphatic aldehydes and alcohols, including *cis*-2-nonenal, *trans*-2-nonenal, *trans,cis*-2,6-nonadienal, *cis,cis*-2,6-nonadienal, 1-nonenol, *cis*-3-nonenol, *trans*-6-nonenol, *trans,cis*-3,6-nonadienol, *trans,trans*-3,6-nonadienol and *cis,cis*-3,6-nonadienol. However, the most abundant

compounds were ethyl acetate (23%), acetaldehyde (18%), tetradecanoic acid (14.1%) and methyl acetate (11.4%).

Beaulieu et al. (2006) applied solid-phase microextraction (SPME) coupled with GC-MS to measure watermelon volatiles and concluded that the seedless watermelon may have a different volatile profile than the seeded types. Fifty-nine volatile compounds were identified, including 12 not previously reported in watermelon and 10 which were unidentified. The predominant compounds were C_9 aldehydes and alcohols. Eleven compounds (hexenal, 6-methyl-5-hepten-2-one, *trans*-2-octenal, 4-nonenal, *cis*-6-nonenal, nonanal, *cis*-3-nonen-1-ol, *trans,cis*-2,6-nonadienal, *cis,cis*-3,6-nonadien-1-ol, *trans*-2-nonenal, 1-nonanol) accounted for 77.3 – 81.6% of the total abundance of the volatiles present. Neither *cis*-3-nonenal nor *cis,cis*-3,6-nonadienal were identified despite being considered important aroma components in previous studies. The authors speculated that these two compounds were among the unidentified class, since they lacked the necessary reference standards.

Several studies have investigated the characteristics of specific compounds thought to be among the most important flavor components of watermelon. The compound *cis,cis*-3,6-nonadienal is a commonly occurring compound formed as a result of the enzymatic oxidation of the fatty acid linolenic acid. However *cis,cis*-3,6-nonadienal is difficult to identify because it is unstable in a dynamic food matrix. The compound was first tentatively identified as a potential odorant in cucumber fruits (Kemp et al. 1974a) and its identity was later confirmed by Yajima et al. (1985). It is still debatable as to whether it is the most impacting aroma component of watermelon. Interestingly, *cis,cis*-3,6-nonadienal, is also a

major character impact compound in the aroma of seafood. It has been identified in trout (Milo et al. 1993), salmon and cod (Milo et al. 1996) and oysters (Zhang et al. 2009).

The compound *cis,cis*-3,6-nonadien-1-ol was first believed to be the most important odorant in watermelon because it was isolated in high concentration from watermelon and was described as having a watermelon or watermelon rind-like aroma. Its best estimate odor detection threshold is about 10 ppb in water (Kemp et al. 1974a).

The compound *trans,cis*-2,6-nonadienal is known as the "cucumber aldehyde" due to its odor quality and relatively high abundance in cucumbers. Based on aroma extract dilution analysis (AEDA) of cucumber, its flavor dilution (FD) factor of 4096 was 64 times greater than that of the next potent aroma compound, which had an FD of 64. It is described as having an aroma like fresh cucumbers (Schieberle et al. 1990).

The compounds *cis*-2-nonenal and *trans*-2-nonenal were first noted as important odorants in cucumbers (Schieberle et al. 1990). Both isomers were described as having green/fatty aromas and had the second and third highest FD-factors, respectively, in cucumber aroma extracts.

The compound *cis*-6-nonenal was first identified in muskmelons where its importance to melon flavor was confirmed; not only did it have a distinct melon-like odor quality but its concentration in melons far exceeded its odor detection threshold. Sensory testing revealed that this compound has an odor detection threshold of around 0.02 ppb in water, with panel members describing it as having a "strong melon odor". However, the *trans*-6-nonenal isomer was determined to be an off-flavor in cucumbers as a result of storage (Kemp et al. 1972). The c*is*-6-nonenal isomer was also identified as an important odorant in watermelon. It was found in greater abundance in watermelon than in muskmelon (Kemp et al. 1974b).

The compound *trans,trans,cis*-2,4,6-nontrienal was first identified as a potent odorant in legumes. It was proposed that this compound is formed in legumes by the action of soy lipoxygenase on linolenic acid (Sessa 1979). Schuh et al. (2005) reported *trans,trans,cis*-2,4,6-nontrienal as aroma impacting compound in oat flakes and described it as possessing an intense oatmeal-like and sweet aroma. It was determined to have an extremely low threshold of about 0.0002 ng/L in air. The authors attributed its presence in oat flakes as being the product of autoxidation and not via biosynthesis.

2.3 Aroma Biosynthesis in Watermelon

It has been hypothesized that watermelon aroma is formed enzymatically with the initiation caused by the physical cutting of the tissue. As not much work has been completed on the enzymatic system of watermelons, and as all cucurbits display the same biochemical mechanisms, the present literature review includes information on other cucurbits, like cucumbers, to make an attempt at explaining the aroma formation pathway in watermelons. Free fatty acids were confirmed to be the source of the aroma compounds in cucumbers with linoleic and linolenic acids, in particular, being most important. For confirmation, labeled (C14 isotopes) were added to homogenized cucumber tissue, and the mixture was analyzed for the carbonyl compounds that formed (Grosch et al. 1971). This early study found that hexanal and *trans*-2-nonenal originated from linoleic acid, while *trans*-2-hexenal and *trans,cis*-2,6-nonadienal came from linolenic acid. In the same study, lipid analyses showed linoleic, linolenic and palmitic acids to be the major fatty acids.

Findings from the above mentioned study prompted further research to more clearly establish the enzymatic pathway for formation of these volatile components. Using a

cucumber model, the effects of blending, heating, oxygen and pH on aldehyde formation was studied (Hatanaka et al. 1975). During blending the amounts of linoleic and linolenic acids decreased as the aldehyde content increased. Addition of other fatty acids (arachidoneic acid) had no effect on the production of aldehydes. It was determined that in the presence of oxygen that rapid formation of aldehydes, like *trans*-2-nonenal and *trans,cis*-2,6-nonadienal, occurred. Conversely, the formation of these aldehydes was prevented by blending under an atmosphere of nitrogen or by heating (or blanching) the whole cucumber before blending and dissociating the proteins. These results indicate that aldehydes are formed enzymatically from linoleic and linolenic acid in the presence of oxygen. Some insight was given into the direct precursors of the *trans* aldehydes. It was thought that *cis*-3-unsaturated aldehydes were first formed from linolenic and linoleic acid, and due to the unstable nature these compounds, they were easily isomerized to *trans*-2-aldehydes. If these *cis*-3-aldehydes arose from unsaturated fatty acid, they would accompanied by C_9-oxo acids. This was confirmed through the identification of *cis*-3-nonenal, *cis,cis*-3,6-nonadienal and their respective C_9-oxo acids in volatile extracts prepared from freshly prepared cucumber homogenate (Hatanaka et al. 1975).

According to Tressl et al. (1981), lipid oxidation in fruits and vegetables is due to a lipoxygenase system. This system involves formation of 9 and 13-hydroperoxides which are subsequently cleaved by a bond specific lyase (hydroperoxide lyase) (Gallaird et al. 1976) resulting in C_6 and C_9 aldehydes. These aldehydes can be reduced to their corresponding alcohols or go through an isomerization reaction.

The same enzyme system was confirmed to be responsible for the formation of the aroma compounds in watermelon fruits using specific assays to detect lipoxygenase and

hydroperoxide lyase (Cai 1997). In this study, a demonstration of aroma formation *in vitro* was performed to confirm the importance of these enzymes in the development of watermelon aroma. A substrate solution consisting of linoleic and linolenic acid was incubated along with enzyme isolates of lipoxygenase and hydroperoxide lyase. The resulting volatiles were analyzed by purge-and-trap GC-MS. The highest lipoxygenase activity (81%) was detected in watermelon treated with a 60% saturated ammonium sulfate solution. The *in vitro* experiments showed activity at 8246 and 6728 units/mL of watermelon juice using linoleic and linolenic acids as substrates, respectively. Hydroperoxide lyase activity was not as easy to measure, but was detected after the addition of Triton X-100, which increased activity by 4-fold. Based on these results, it was shown that watermelon aroma is formed via a dynamic biochemical system which is initiated after tissue disruption, with additional enzymatic action occurring after the formation of the character impacting compounds. In this system, lipoxygenase causes the production of both 9- and 13-hydroperoxides from both linoleic and linolenic acids. Hydroperoxide lyase then cleaves the hydroperoxides to form both C_9 aldehydes (e.g., *cis,cis*-3,6-nonadienal and *cis*-3-nonenal) and C_6 aldehydes (e.g., *cis*-3-hexenal and hexanal) (Cai 1997). The general biochemical pathway for the formation of watermelon aroma compounds is shown in **Figure 2.1**.

2.4 Issues Concerning Flavor Stability in Industrial Applications

As discussed previously, the *cis*-aldehydes are known to readily isomerize to the *trans* configuration. In addition, the enzyme alcohol dehydrogenase can convert the *cis* aldehydes to their respective *cis* alcohols (Gardner 1995). These transformations lead to changes in odor thresholds and odor properties. Because of these changes, attempts to produce a good

quality watermelon juice product have not been successful. Reports on juice quality from the consumers' viewpoint have acknowledged that enzymatic degradation of aroma compounds is an issue; however, the results are somewhat contradictory. Sensory evaluation was performed on juices from two cultivars (Silva et al. 1991). Sensory evaluations of flavor, sweetness, appearance and color were performed on unpasteurized, freshly pasteurized and pasteurized-stored juice. The juice from each cultivar was pasteurized at 76.7°C for 17 seconds and was stored for up to 3 months at 2 °C. Results were as expected for the color analysis. The fresh pasteurized juice was rated darkest, likely due to browning during pasteurization; while stored juice was rated the lightest, probably due to the loss of lycopene during storage. A sensory panel was used to rate flavor, sweetness and appearance based on a 7-point hedonic scale. Results contrary to what was expected for the heat-treated and stored watermelon juices were obtained. Panelists rated both types of pasteurized juice, freshly made and after 3 months of storage, higher in flavor quality than the fresh-squeezed unpasteurized juice. Panelists reported a "grassy" off flavor in the fresh juice (Silva et al. 1991). Several things could account for the panelists rating the flavor of the pasteurized/stored juice to be better in flavor than the "fresh" juice. The study did not report how much time elapsed between the juice being squeezed and when the sensory test was performed. If the time before analysis was as little as 10 minutes, the compounds responsible for the fresh watermelon flavor may have been enzymatically altered, leaving behind a less desirable stale/green flavor. On the other hand, the pasteurization process likely disrupted the enzymes, and some of the fresh watermelon flavor compounds may have remained unaltered.

An examination of flavor compound retention throughout cold storage of watermelon juice was recently done using two processing techniques (Aguilo-Aguayo et al. 2010). One was a

basic pasteurization technique. The second was a high-intensity pulsed electric field (HIPEF) technique. HIPEF is a processing system consisting of a high voltage source that induces an electric potential difference across membranes. It is capable of killing microorganisms and inactivating enzymes (Barbosa-Canovas et al. 1998). Aguilo-Aguayo et al. (2010) used hexanal, *trans*-2-nonenal, *cis*-6-nonenal and nonanal as markers of flavor retention. All of these compounds, except for *cis*-6-nonenal, were enhanced during HIPEF, with initial increases from 17% to 27%. Either no change or a reduction in levels of the marker compounds occurred with pasteurization. This could be due to some loss of aroma compounds by volatilization during the heating process. However, after the first day of storage, flavor compounds declined drastically. The highest rate occurred between 0.05 and 0.085 days, i.e. very quickly after initial measurements of retention. In contrast, the alcohol compounds, 1-nonanol and *cis*-3-nonen-1-ol showed no significant change in retention during storage. In fact, the alcohols were retained quite well up to 54 days. This was expected, as alcohol dehydrogenase is one of the major enzymes responsible for altering the "fresh-cut" aldehyde aroma compounds in watermelon.

2.5 Aroma Analysis Techniques

Snow et al. (2002) defines headspace analysis as "a vapor-phase extraction, involving the partitioning of analytes between a non-volatile liquid or solid phase and the vapor phase above the liquid or solid sample…and that this mixture is transferred to a GC or other instrument for analysis." Static headspace analysis (SHA) involves letting the vapor above the sample come into equilibrium before extracting it for analysis. SHA has been used for decades for flavor and fragrances analysis as well as in the environmental and pharmaceutical

sciences. Today SHA remains the most validated among the headspace techniques. There are many advantages of headspace analysis. It is fast and simple. It is reproducible, non-destructive and nearly un-biased in contrast to solvent extraction techniques. It also eliminates solvent peaks and non-volatile contaminants during analysis (Rouseff et al. 2001). Essentially, SHA captures those same volatile compounds that the human nose would detect coming from a sample, thus enabling one to get a "true" chemical image of what humans detect in a sniff or smell event. The only real disadvantage of SHA is a lack of sensitivity. However, applying a cryogenic trapping technique can help correct this deficiency. Cryogenic condensing is technique in which the volatile compounds are captured by condensation in a trap by using liquid nitrogen to lower the temperature of the gas chromatographic injector. This can be done using a programmable temperature vaporizer (PTV) inlet - a universal injection system with the capability of handling large sample injections – which is perfect for headspace analysis (Heiden et al. 2001).

To determine the most important volatiles in a headspace sample the concept of odor activity value (OAV) is used. OAV is the concentration of the odorant in the food as compared to its odor detection threshold, generally expressed as a ratio of concentration of a compound divided by its odor detection threshold. One way to put this concept into practice is by application of gas chromatography-olfactometry of decreasing headspace volumes (GCO-H). This technique was first attempted while investigating the "freshness" aroma of roasted coffee. It was noted, "Major progress was achieved when GC/sniffing was applied, because ADA allowed differentiation between sensorily important, less or unimportant peaks and enable estimation of the contribution of single volatiles to the overall perceptible aroma freshness" (Holsher et al. 1992). In this study, there were far fewer odorants in the headspace than in the stream distilled extract, facilitating the aroma analysis.

As the headspace volume was reduced, fewer and fewer compounds were detected by GCO until only the most intense/important odorants were left. GCO-H has been applied to many foods for aroma analysis, particularly those foods having dynamic aroma systems (Milo et al. 1997; Triqui et al. 1997; Roberts et al. 1996; Rychlik et al. 2001; Lee et al. 2001; Zhou et al. 2002; Buckling et al. 2002; Cadwallader et al. 1998; etc.).

2.6 Esters

The importance of esters in melon flavor was first demonstrated by (Buttery et al. 1982) in a study aimed at the identification of aroma components of honeydew melon. They reported *cis*-6-nonenyl acetate, *cis*-3-nonenyl actetate, and *cis,cis*-3,6-nonadienyl acetate as key aroma compounds. All were described as having pleasant honeydew melon-like aromas. The investigators synthesized *cis*-6-nonenyl acetate and determined it to have an approximate odor detection threshold of 2 ppb in water. They did not synthesize *cis*-3-nonenyl acetate and *cis,cis*-3,6-nonadienyl acetate, so the threshold of these two compounds was not determined. In the food industry, volatile esters are common ingredients in fruit flavored products. This is due to their unique fruity aroma characters, chemical stabilities and generally low odor detection thresholds. There is always a general demand for new flavors (Liaquat et al. 2000).

2.7 Structure-Odor Relationships

Even though various types of structure-function relationships have been well studied, the relationship between molecular structure and odor quality still remains mostly theoretical

(Chastrette 1997. Further complications arise when dealing with human subjects. The pathway of perception is intricate. It involves airborne molecules entering the olfactory system through either the nostrils (orthonasally) or the back of the throat to the nose (retronasally). The molecules make contact with the mucus overlaying the olfactory epithelium then interact with receptor proteins located in the membrane of the olfactory cells. These receptor sites are part of a G coupled protein with seven transmembrane domains. A secondary messenger is formed which sends the signal to the brain. The molecular features (shape, size, functional groups and volume) of each compound are important to the perception of both odor and intensity. Intensity, measured by odor detection threshold has some limitations depending on methodology and person to person variability (Chastrette 1997). The best approach in determining thresholds requires hundreds of comparisons among hundreds of trained panelists. However, there are acceptable procedures to determine mean values that can be extrapolated to the general public (Meilgaard et al. 1991). Forced choice ascending concentration series method of limits (3-AFC) is a reasonably good method (ASTM 1992). The odor detection threshold is a valuable piece of knowledge especially for industry to know how much of a substance must be added to a food or beverage product to be at detectable levels.

Attempts have been made to define trends in ester thresholds. Although there is no consistent overall trend, some general observations have been made. Among the low molecular weight esters, those with butanoate and pentanoate end groups have the lowest thresholds as opposed to the methyl and ethyl esters. Methyl esters have a higher threshold than ethyl esters. Branched ester groups have a lower threshold than their straight chain counterparts. Extending the carbon backbone chain by one carbon has a dramatic effect on increasing the threshold (Takeoka et al. 1995). Studies where carbon chain length was varied

while keeping the ester group intact have shown that a pentyl backbone has the lowest threshold when compared to propyl, butyl, hexyl, heptyl, octyl and nonyl acetates (Takeoka et al. 1996). Double bond position and configuration is also influential. Adding a double bond to the second position (closest to the end group) increased threshold values, with *trans* having a greater effect than *cis* and the *cis* isomer having a lower threshold than the *trans*. Moving the double bond position further away from the ester end group lowered the threshold to a level closer to the saturated ester. Exceptions were noticed with higher molecular weight esters, where addition of a double bond decreased the threshold as compared to the saturated ester (Takeoka et al. 1998).

Many theories revolve around structure-odor relationships (SORs). The only conclusive information is known for general SORs and whole molecules rather than for specific structural features. However, when addressing small structural variations one observes a range in diverse odors (Chastrette 1997). One example is defining SORs of *n*-hexen-1-ols and *n*-hexenals (Hatanaka et al. 1992). With each compound, panelists were asked to rank 10 descriptive terms (leafy green, grassy, insect green, vegetable green, fruity, sweet, fresh, spicy, oily, and herbal) on an intensity scale from 0-5. Placing a double bond at the C-2 position of the aldehdyde created a very fruity/sweet aroma, whereas the corresponding alcohol gave a less fresh and more grassy aroma. The compound *cis*-3-hexenol displayed high green and fresh notes while *cis*-3-hexenal gave a more spicy/grassy aroma. The compound *trans*-3-hexenal had much less of a spicy grassy green aroma. The compound *cis*-4-hexenal was found to have a more vegetable green smell and 5-hexenal/ol was found to have an oily-fatty, insect green aroma. It was noted that the position of the double bond influenced the first principle component (fresh, fruity, sweet) and the functional group influenced the second principle component (spicy, green). A closer look at the effect of double bond

position alone, using *n*-nonen-1-ols (by keeping the end group the same), showed once again how influential double bond position is on the odor property (Sakoda et al. 1995). Here eight descriptors (grassy, vegetable-like, fruity, sweet, fresh, spicy, oily, and herbal) were given to panelists and they were asked to rank the intensity from 0-3, with 0 indicating no detection and 3 very intense. It was noted that by moving the double bond position away from the ω-carbon resulted in less and less oily notes with 5 and 6 bond positions having the lowest ranking, but then slightly increasing when in the 7 and 8 positions. Compounds with C-3 and C-4 double bonds had the strongest vegetable-like odor and *cis*-6-nonen-1-ol was the most different having a highly fruity, sweet and fresh aroma. Switching isomeric configuration always changed the odor but did not change it in a consistent way. The comparison of *cis,cis*-3,6-nonadien-1-ol to *trans,cis*-2,6-nondien-1-ol had a complete horizontal and vertical switch on the spectrum. The *trans,cis* isomer was described as heavily oily, herbal, grassy, and vegetable-like whereas the *cis,cis* isomer was described as fresh, fruity side with some grassy notes. It is interesting to note that the *cis*-6-, *cis,cis*-3.6- and *trans,cis*-2,6-C_9 alcohols differed greatly in the principle odors from the nonen-1-ols not containing a double bond at the 6^{th} position. This may be due to their existence in nature, such that humans would more easily recognize them, or the fact that they all have a *cis* bond in the ω-3 position and being present in that position has a specific interaction with the olfactory system.

cis-3-nonenal-1-ol

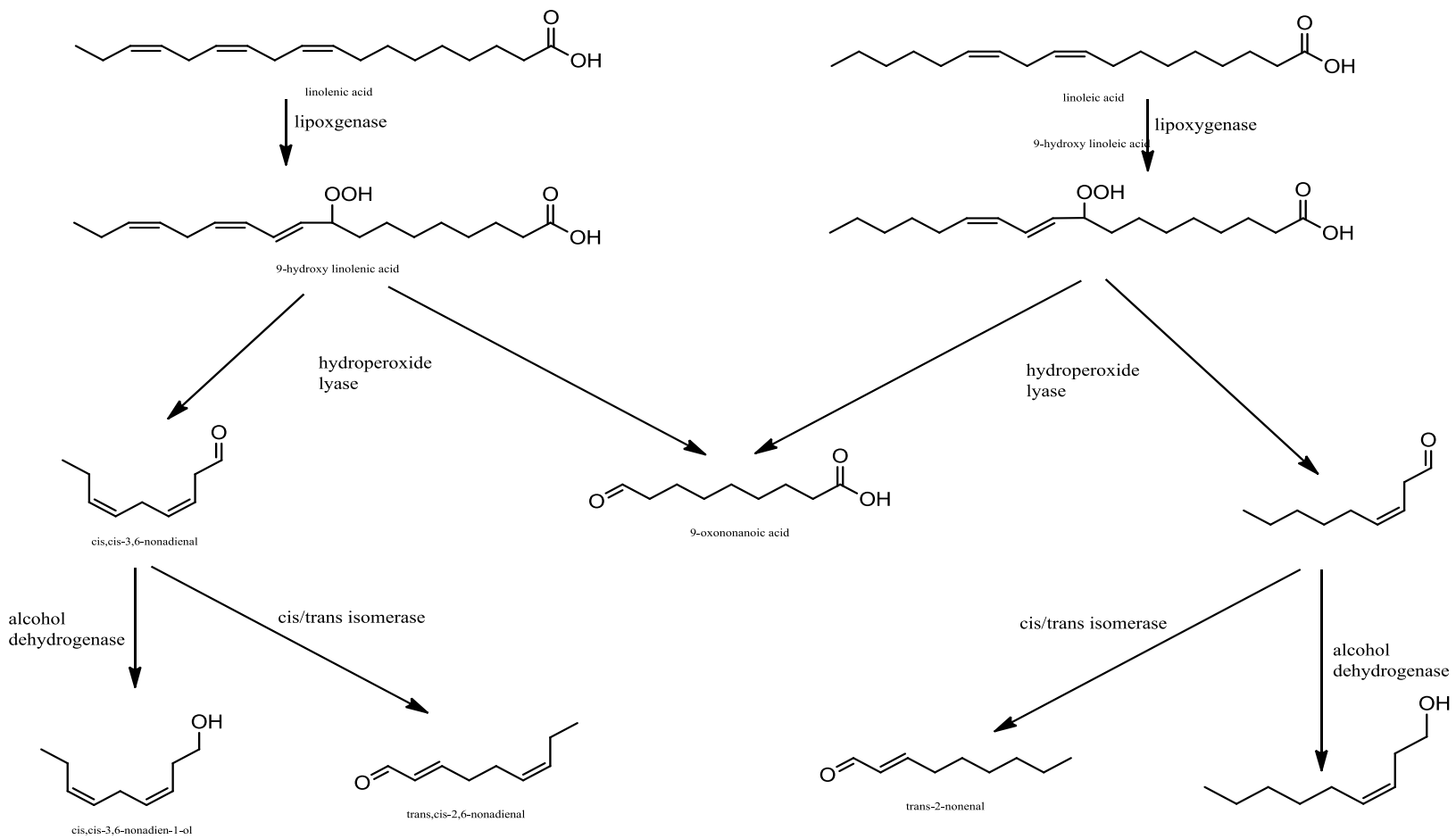

Figure 2.1. Aroma formation cascade in watermelon

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CHAPTER THREE:

Identification of Compounds Responsible for the Characteristic Aroma of Fresh-Cut Watermelon

3.1 ABSTRACT

A "snapshot" of the aroma profile of fresh-cut watermelon, formed as a result of a highly dynamic enzymatic system, was determined using gas chromatography-olfactometry of decreasing headspace samples (GCO-H) of watermelon juice within one minute of its preparation. GCO-H results were confirmed by GCO and aroma extract dilution analysis (AEDA) of an aroma extract prepared by solvent-assisted flavor evaporation (SAFE) of freshly squeezed watermelon juice treated with saturated ammonium chloride solution. All nine predominant odorants detected by GCO-H were also detected by AEDA. These included *cis*-3-hexenal, *cis*-3-nonenal, *cis,cis*-3,6-nonadienal, *cis*-6-nonenal, *cis*-2-nonenal, *trans,cis*-2,6-nonadienal, *trans*-2-nonenal, *trans,trans*-2,4-nonadienal and *trans,trans,cis*-2,4,6-nonatrienal. The most interesting compound among these was *cis,cis*-3,6-nonadienal, which was not only was found among the predominant odorants in fresh watermelon juice, but was also the only compound described as having a characteristic "fresh, watermelon-like" aroma note.

3.2 INTRODUCTION

The history of consuming watermelons dates back thousands of years. It was first used as a source of water in the arid regions of Africa, where, as in present times it is consumed throughout the world mainly because of their sweet taste and characteristic refreshing aroma (Kipple et al. 2000). In 2009 watermelon production in the United States reached 4.0 billion pounds, worth 461 million dollars (Anon, 2010).

The characteristic aroma of fresh-cut watermelon is a result of the enzyme-catalyzed oxidation of free fatty acids, where enzymes released by tissue disruption (e.g., by cutting), in concert with the oxygen entering the system, react with the available free fatty acids, in particular linoleic and linolenic acids, to produce the aroma-impact aroma compounds (Grosch et al. 1971). Lipoxygenase creates C-9 and C-13 hydroperoxides (Tressl et al. 1981) and these hydroperoxides are subsequently cleaved into C_6 and C_9 saturated and *cis* unsaturated aldehydes (Galliard et al. 1976). The C_6 and C_9 aldehydes are then reduced to C_6 and C_9 alcohols by alcohol dehydrogenase or the *cis* unsaturated bonds are isomerized to *trans* by *cis/trans* isomerase . This scheme in watermelon was confirmed by Cai (1997).

The aroma-impact compounds of watermelon have been a debated subject. Past studies gave credit to *cis,cis*-3,6-nonadien-1-ol as the most important aroma compound in watermelon (Yajima et al. 1985; Kemp et al. (1974); Pino et al. 2003; Beaulieu et al. 2006). However, according to the lipoxygenase scheme, the aldehyde *cis,cis*-3,6-nonadienal must be formed prior to the formation of the corresponding alcohol. It is likely that previous researchers, who used prolonged periods for volatile isolation, were unable to isolate the aldehyde because it had already been reduced to the corresponding alcohol by alcohol dehydrogenase action (Cai 1997). This, along with the knowledge that, almost always,

alcohols have appreciably higher odor detection thresholds than aldehydes (Hatanaka et al. 1992), leads us to hypothesize that the unsaturated aldehydes, and in particular, *cis,cis*-3,6-nonadienal, are the predominant odorants in fresh-cut watermelon.

The technique of static headspace analysis (SHA) is ideal for taking a chemical "snapshot" of fresh-cut watermelon aroma. In SHA the vapor (aroma compounds) above a sample is allowed to come into equilibrium and then a portion of it is collected for analysis by gas chromatography (GC). It is fast and simple, reproducible, non-destructive and un-biased in contrast to solvent extraction. SHA also eliminates solvent peaks and non-volatile contaminants during analysis (Rouseff et al. 2001). The concept of odor activity value (OAV) can be applied through SHA using a technique called gas chromatography-olfactometry of decreasing headspace volumes (GCO-H) (Holsher et al. 1992). OAV is the concentration of an odorant in food divided by its odor detection threshold. This eliminates the idea of "more is stronger" and gives a truer analysis of the most potent odorants. Aroma extract dilution analysis (AEDA) can be used complimentary to GCO-H. AEDA is a quantitative GCO procedure for determining the potency of odorants in food aroma extracts (Grosch 1993). In the case of watermelon, the aroma extract will be obtained by solvent-assisted flavor evaporation (SAFE). SAFE allows the isolation of volatiles from solvent extracts, aqueous foods, aqueous food suspensions such as fruit pulps, or even matrices with a high oil content (Engel et al. 1999).

In the present study, GCO-H and AEDA were used to acquire a chemical snapshot of the predominant aroma-active components of fresh-cut watermelon.

3.3 MATERIALS AND METHODS

Materials

Ripe watermelons were purchased at local grocery stores or farmers' markets (Champaign-Urbana, IL) with origins from Illinois, Indiana, Texas and other unspecified states in the U.S. and kept in a cool, dry storage area until extractions were performed.

Static Headspace Analysis of Freshly Squeezed Watermelon Juice

At room temperature (~22-23 ºC) a mid cross-sectional slice of (~ 4 cm thick) was cut from the center of the melon. The rind was removed by slicing off the edges and the remaining "meat" was cut into quarters. The meat quarters were placed in a nylon mesh paint strainer bag (Trimaco Co; Durham, NC) and 100 mg of juice was squeezed into a 500 mL flask, which was then immediately sealed with a silicon septum stopper. Exactly one minute after the initial watermelon cut, a gas tight syringe (SGE Analytical Science Pty Ltd; Ringwood, Australia) was used to draw headspace samples for analysis by gas chromatography-olfactometry (GCO). A freshly prepared sample was used for each analysis.

Gas Chromatography-Olfactometry of Decreasing Headspace Volumes

The GCO system consisted of an HP-6890 GC (Agilent Technologies Inc., Santa Clara, CA) equipped with a flame ionization detector (FID) and a sniff port (ODP2, Gerstel, Germany). Headspace volumes of 10 mL, 2.5 mL, 0.625 mL, 0.156 mL were analyzed. A CIS4 programmable temperature vaporizer (PTV) inlet (Gerstel) was used to cryofocus the headspace volatiles prior to injection. Initial inlet temperature was programmed as follows: initial temperature, -120°C (0.1 min hold); ramp rate, 10 °C/sec; final temperature, 260 °C (10 min hold). Separations were performed using a RTX®-Wax column (15 m length x 0.53

mm i.d. x 1.0 μm film thickness; Resteck; Bellefonte, PA). Helium was used as the carrier gas at a constant flow of 5 mL/minute. FID temperature was 250°C. Oven temperature was programmed as follows: initial temperature, 40°C (5 min hold), ramp rate, 10 °C/min, final temperature, 225 °C (10 min hold). To aid in compound identification, analysis of 20 mL, 10 mL, 2.5 mL, 0.625 mL, and 0.156 mL headspace volumes were also conducted using a RTX®-5MS column (15 m length x 0.53 mm i.d. x 1.5 μm film thickness; Resteck; Bellefonte, PA). Conditions were same as above except GC oven temperature was programmed as follows: initial temperature, 40 °C (2 min hold); ramp rate, 6°C/min; final temperature, 225°C (15 min hold).

Volatile Isolation by Solvent-Assisted Flavor Evaporation

At room temperature (~22-23 ºC) a mid cross-sectional slice (~4 cm thick) was cut from the center of the melon. The rind was removed by slicing off the edges and the remaining "meat" was cut into quarters. The meat quarters were placed in a nylon mesh paint strainer bag (Trimaco Co; Durham, NC) and 100 mg of juice was squeezed into a 1 L beaker. Approximately 1 minute after the initial cut, 100 mL of chilled saturated calcium chloride solution was added to the juice and thoroughly mixed by hand.

The SAFE apparatus was prepared beforehand so that distillation could be started immediately after juice preparation. The apparatus and general operating procedures have been previously described (Engel et al. 1999), with modifications described by Rotsatchakul et al. (2008). SAFE feed time was 30 minutes followed by 2.5 h of distillation. After 3 h total run time, 50 mL of diethyl ether was added to the cryogenic trap containing the watermelon juice volatiles. After thawing of the trap and recovery of the ether layer, the aqueous phase was re-extracted (2 x 50 mL) with ether. The combined ether extract was

dried over anhydrous sodium sulfate and then the volume was reduced to 200 μL under a gentle flow of nitrogen gas. Extract was stored in a 1.5 mL septum-capped Target DP vial (National Scientific, Rockwood, TN) at -70ºC until GC-MS/GCO analysis.

Gas Chromatography – Mass Spectrometry (GC-MS)

An HP 6890 GC-HP 5973N mass selective detector (Agilent Technologies Inc.) was used for GC-MS analysis. One μL of SAFE extract was injected into a cool on-column inlet (+3°C oven tracking mode). Separations were performed using a RTX®-5MS column (30.0 m length x 0.25 mm i.d. x 0.25 μm film thickness; Restek; Bellefonte, PA) or RTX®-Wax column (30.0 m length x 0.25 mm i.d. x 0.25 μm film thickness; Restek) . Helium was used as the carrier gas a constant flow of 1.0 mL/minute. MS transfer line temperature was 280°C. Oven temperature was programmed as follows: initial temperature, 35°C (5 min hold), ramp rate, 4 °C/min, final temperature, 240 °C (20.0 min hold). The MSD conditions were as follows: capillary direct interface temperature, 280 °C; ionization energy, 70 eV; mass range, 35 to 300 amu; electron multiplier voltage (Autotune + 200 V); scan rate, 5.27 scans/s.

Aroma Extract Dilution Analysis (AEDA)

The GCO system used for analysis of SAFE extracts consisted of an HP6890 GC (Agilent Technologies Inc.) equipped with an FID and sniff port (DATU, Geneva, NY). Separations were performed using a Stabilwax®-DA column (15 m length x 0.32 mm i.d. x 0.5 μm film thickness; Resteck; Bellefonte, PA). Helium was used as the carrier gas at 9.6 mL/minute. FID temperature was 250ºC. Oven temperature was programmed as follows: initial temperature, 40ºC (5 min hold), ramp rate 10ºC/min, final temperature, 225 ºC (30 min

hold). Starting with 200 μL extract, AEDA was performed using a 1:3 dilution series. For this, 50 μL was diluted into 100 μL of diethyl either serially to obtain 1:3, 1:9, 1:27, 1:81 and 1:243 dilution ratios. Each dilution was kept in a 1.5 mL septum-capped Target DP vial (National Scientific, Rockwood, TN) at -70ºC. To aid in identification, analysis was also conducted using a RTX®-5MS column (15 m length x 0.32 mm i.d. x 0.5 μm film thickness; Resteck; Bellefonte, PA). Evaluations were performed by three experienced panelists. Results are based on the 2 out of 3 panelists' consensus scores.

Compound Identification

Compound identifications were confirmed by retention indices (RI), odor properties and comparison to authentic standards. Tentative identifications were based on matching retention indices of unknowns with those of authentic standard compounds.

Chemical Standards

Standards used to confirm compound identifications were either purchased or synthesized. The following compounds were obtained from Sigma-Aldrich Co. (St. Louis, MO): *trans,cis*-2,6-nonadienal, *trans,trans*-2,4-nonadienal, *trans*-2-nonenal, heptanal and *trans*-2-octenal. The compound *cis*-3-hexenal was purchased from Bedoukian Research Inc. (Danbury, CT).

The following chemicals used for syntheses were purchased from Sigma-Aldrich Co. (St. Louis, MO): *cis*-3-nonen-1-ol, *cis*-6-nonen-1-ol, *cis*-2-nonen-1-ol, Dess-Martin periodinane 0.3M in CH2Cl2, ethyl magnesium bromide (3.0M in diethyl ether), vinylmagnesium bromide (1.0M in THF), 2-(3-butynyloxy)tetrahydropyran, copper (I) bromide, 1-bromo-2-pentyne, p-toluenesulfonic acid, quinoline, tris[2-(2-methoxyethoxy)ethyl]amine, methyl-4-

(triphenylphosphorium)crotonate bromide, trans-2-pentenal, lithium aluminum hydride, diethyl ether, tetrahydrofuran.

The following chemicals used for synthesis were purchased from Fisher Scientific Co. (Fair Lawn, NJ): dicholormethane, diethyl ether, ammonium chloride, methanol, sodium bicarbonate, sodium carbonate, sodium chloride, hydrochloric acid, pentane, sulfuric acid. sodium sulfate (Arcos Organics, Morris Plains, NJ), Lindlar catalyst (Fluka, licensed to Sigma-Aldrich).

Synthesis of *cis*-3-Nonenal, *cis*-6-Nonenal, and c*is*-2-Nonenal

Syntheses were performed via oxidation of the corresponding alcohols using Dess-Martin periodinane (DMP) as described by Meyer et al. (1994). Briefly, water saturated dichloromethane was added to a stirring mixture of the corresponding alcohol and DMP (0.3M in dicholormethane) (1 : 1.5 molar equivalent). This was left to react until all of the alcohol was oxidized to the aldehyde (~5 h).

Synthesis of *cis*-1,5-Octadien-3-one

The synthesis of *cis*-1,5-octadien-3-one was accomplished by following closely the procedure described by Swoboda et al. (2006). Briefly, the Grignard reaction between *cis*-3-hexenal and vinyl magnesium bromide was used to make *cis*-1,5-octadien-3-ol, which was subsequently oxidized to the corresponding ketone using DMP as described above.

***trans,trans,cis*-2,4,6-Nonatrienal synthesis**

This synthesis was performed as described by Schuh et al. (2005). A modified Wittig reaction with tris[2-(2-methoxyethoxy)ethyl]amine, methyl-4-

(triphenylphosphorium)crotonate bromide, and *trans*-2-pentenal were combined then hydrolyzed overnight with hydrochloric acid. The resulting methyl ester was reduced to the corresponding alcohol using lithium aluminum hydride to create *trans,trans,cis*-2,4,6-nonatrien-1-ol. The alcohol was then oxidized into the aldehyde via the DMP method described above.

***cis,cis*-3,6-Nonadienal synthesis**

The synthesis of 3,6-nonadiyn-1-ol was performed as described by Milo et al. (1993). A solution of ethyl magnesium chloride (1.5 mol equivalent), 2-(3'-butynyl-1-oxy) tetrahydropyran (1.5 mol eq.) was stirred in dry tetrahydrofuran (10 mL) for 2 h at 60°C after which copper (I) bromide (60 mg) was added. Following an additional 15 min of incubation, 1-bromo-2-pentyne (1 mol eq.) was mixed into the solution which was then allowed to stir for 18 h at 40 °C. The product, a pyranyl ether (3,6-nonadiynyl -1-oxy-THP), was hydrolyzed with *p*-toluenesulfonic acid resulting in the formation of the alcohol, 3,6-nonadiyn-1-ol. Stereospecific semi-hydrogenation of the triple bonds to create *cis,cis*-3,6-nonadien-1-ol was performed using a Lindlar catalyst tainted with quinoline as described by Bendall et al. (2001) where 3,6-nonadiyn-1-ol in methanol, quinoline (500 mg), and Lindlar catalyst (200 mg) were stirred under 20 psi hydrogen gas for 1.5 h. Oxidation of the alcohol was accomplished using the DMP procedure as described in the previous paragraph. Column purification was performed after synthesis both 3,6-nonadiyn THP and 3,6-nonadiyn-1-ol. The sample was applied to a column (2.54 cm diameter) packed with 50 g of activated silica (100-200 mesh, grade 923, Sigma Aldrich Co). Stepwise elution was performed with 100 mL of pentane:ether (90:10) followed by 250 mL of pentane:ether

(70:30). 3,6-nondiynyl-1-oxy-THP appeared in the elution range 70-150 mL. The 3,6-nonadiyn-1-ol appeared in the elution range 230-320 mL.

A detailed synthesis scheme is presented in the Appendix pages (87-89).

3.4 RESULTS

Odor-Active Components of Fresh-Cut Watermelon

A combined total of 19 odor-active compounds were detected in the static headspace and solvent (aroma) extracts of fresh-cut watermelon by gas chromatography-olfactometry (GCO). Compound identities, odor descriptions (characteristics) and GC retention indices on two different polarity columns are given in **Table 3.1.**

Gas Chromatography-Olfactometry of Decreasing Headspace Volumes (GCO-H)

The most notable result is that all nine compounds detected in the headspace of fresh-cut watermelon were aldehydes (**Table 3.2**). The compounds *cis*-2-nonenal (no. **12**; *stale/hay*), *trans*-2-nonenal (no. **14**; *stale hay*) and *trans,cis*-2,6-nonadienal (no. **13**) *cucumber*) were the most potent odorants, being detected at the highest dilution (FD) factor of 64 (or the lowest headspace volume of 0.156 mL). The next most potent odorant was *trans,trans,cis*-2,4,6-nonatrienal (no. **17**; *sweet/oats)*, with a an FD factor of 16 (0.625 mL). This compound has not previously been identified in watermelons. Other compounds with FD factors of 16 were *cis*-3-nonenal (no. **9**; *melon rind*), *cis,cis*-3,6-nonadienal (no. **10**; *fresh/watermelon*), *cis*-6-nonenal (no. **11**; *melon*) and *trans,trans*-2,4-nonadienal (no. **15**; *fatty/fried*). Lastly, *cis*-3-hexenal (*green/cut-leaf*) was detected at the highest headspace volume tested (10 mL; FD factor = 1).

Among the above mentioned compounds, only *cis,cis*-3,6-nonadienal (no. **10**) was described as having a odor reminiscent of fresh-cut watermelon.

Table 3.1. Odor Descriptions and Retention Indices for Compounds Detected by Gas Chromatography-Olfactometry Analysis of the Static Headspace and Solvent Extracts of Fresh-Cut Watermelon

No.[a]	Compound	Odor Description[b]	RI[c]	
			RTX-Wax	RTX-5MS
1	3-methylbutanal[d]	chocolate, malty	- -[e]	661
2	*cis*-3-hexenal	green, cut-leaf	1147	801
3	heptanal	stale, citrus	1194	901
4	methional[d]	potato	1453	911
5	1-octen-3-one[d]	mushroom, earth	- -	974
6	*cis*-1,5-octadien-3-one[d]	metalic	1371	984
7	nonanal[d]	pungent, green	1396	- -
8	*trans*-2-octenal	stale, raw peanut	1432	1065
9	*cis*-3-nonenal	melon rind	1442	1095
10	*cis,cis*-3,6-nonadienal	fresh, watermelon	1495	1097
11	*cis*-6-nonenal	melon	1442	1103
12	*cis*-2-nonenal	stale, hay	1504	1152
13	*trans,cis*-2,6-nonadienal	cucumber	1583	1157
14	*trans*-2-nonenal	stale, hay	1527	1165
15	*trans,trans*-2,4-nonadienal	fatty, fried	1697	1223
16	3-methylnonane-2,4-dione[d]	creamy, fatty	1725	1252
17	*trans,trans,cis*-2,4-6-	sweet, oats	1876	1279
18	trans,trans-2,4-decadienal[d]	fatty, fried	1818	1325
19	*trans*-4,5-epoxy-*trans*-2-decenal[d]	metallic, unripe	- -	1377

[a]Numbers correspond to those in **figure 3.1** and **Tables 3.2** and **3.3**. [b]Odor description as perceived during GCO. [c]Retention indices on polar (RTX-Wax) and non-polar (RTX-5MS) columns. [d]Compound was tentatively identified by comparison of its retention indices with those of authentic standard or against literature data.

Table 3.2. Potent Odorants in Fresh-Cut Watermelon Determined by Gas Chromatography-Olfactometry of Decreasing Headspace Volumes (GCO-H)

No.[a]	Compound	Odor Description[b]	FD-Factor[c]
2	*cis*-3-hexenal	green, cut-leaf	1
9	*cs*-3-nonenal	melon rind	16
10	*cis,cis*-3,6-nonadienal	fresh, watermelon	16
11	*cis*-6-nonenal	melon	16
12	*cis*-2-nonenal	stale, hay	64
13	*trans, cis*-2,6-nonadienal	cucumber	64
14	*trans*-2-nonenal	stale hay	64
15	*trans,trans*-2,4-nonadienal	fatty, fried	16
17	*trans,trans,cis*-2,4,6-nonatrienal	sweet, oats	16

[a]Numbers correspond to those in **Figure 3.1** and **Tables 3.1 and 3.3**. [a]Odor description as perceived during GCO. [b]Flavor dilution factor = the highest headspace volume tested (10 mL) divided by lowest headspace volume at which a compound was detected by GCO.

Aroma Extract Dilution Analysis (AEDA): Confirmation of GCO-H Results

A total ion chromatogram (TIC) of the GC-MS analysis of the aroma extract prepared by SAFE is shown in **Figure 1**. Nineteen compounds were detected by AEDA. As mentioned previously, nine of these compounds were also identified in the static headspace of fresh-cut watermelon by GCO-H, including compounds nos. **2**, **9**, **10**-**15**, **17**. An additional ten compounds were detected, including other aldehydes (3-methylbutanal, heptanal, methional, nonanal, *trans*-2-octenal, *trans,trans*-2,4-decadienal and *trans*-4,5-epoxy-*trans*-2-decenal) and ketones (1-octen-3-one, *cis*-1,5-octadien-3-one and 2-methylnonane-2,4-dione).

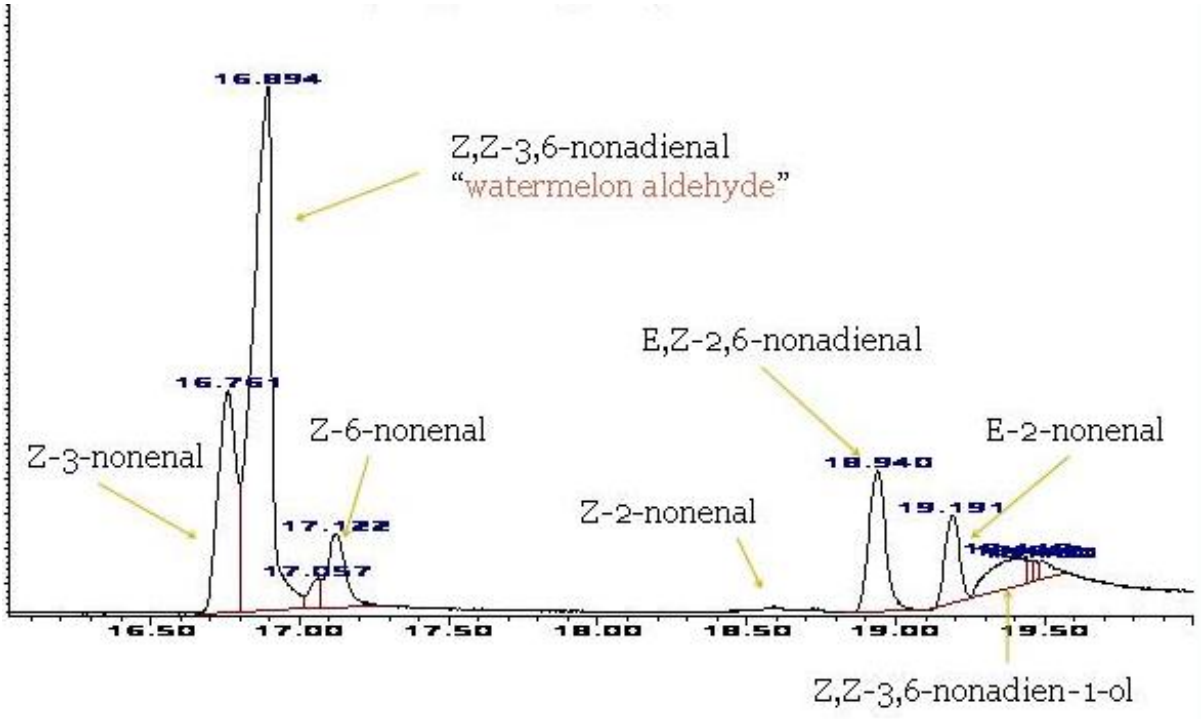

Figure 3.1: **Total Ion Chromatogram of a Fresh-cut Watermelon Volatile Constituents Isolated by Solvent-Assisted Flavor Evaporation**.

Results of AEDA revealed the most important odorants in the aroma extracts prepared by SAFE from fresh watermelon juice. Numerous C_9 and one C_6 aldehydes were the predominant odorants based on their high FD factors (>27).

These included *cis*-3-hexenal (no. **2**; *green/cut-leaf*), *cis*-3-nonenal (no. **9**; *melon rind*), *cis,cis*-3,6-nonadienal (no. **10**; *fresh/watermelon*), *cis*-6-nonenal (no. **11**; *melon*), *cis*-2-nonenal (no. **12**; *stale/hay*), *trans,cis*-2,6-nonadienal (no. **13**; *cucumber*), *trans*-2-nonenal (no. **14**; *stale rind*) and *trans,tran,cis*-2,4-6-nonatrienal (no. **17**; *sweet/oats*). The compounds *cis*-1,5-octadien-3-one (no. **6**; *metallic*) and nonanal (no. **7**; *pungent/green*) had intermediate FD factors (=9).

Table 3.3. Results of Aroma Extract Dilution Analysis of Fresh-Cut Watermelon

No.[a]	Compound[b]	FD-Factor[c]	
		RTX-Wax	RTX-5
1	3-methylbutanal[d]		
2	*cis*-3-hexenal	27	3
3	heptanal	3	3
4	methional[d]	3	3
5	1-octen-3-one[d]		3
6	*cis*-1,5-octadien-3-one[d]	9	
7	nonanal[d]	9	
8	*trans*-2-octenal		
9	*cis*-3-nonenal	27	9
10	*cis,cis*-3,6-nonadienal	81	9
11	*cis*-6-nonenal	27	27
12	*cis*-2-nonenal	81	9
13	*trans,cis*-2,6-nonadienal	243	27
14	*trans*-2-nonenal	81	27
15	*trans,trans*-2,4-nonadienal	3	
16	3-methylnonane-2,4-dione[d]		
17	*trans,trans,cis*-2,4-6-nonatrienal	81	9
18	trans,trans-2,4-decadienal[d]	3	3
19	*trans*-4,5-epoxy-*trans*-2-decenal[d]		

[a]Numbers correspond to those in **figure 3.1** and **Tables 3.1 and 3.3**. [b]Odor description as perceived during GCO. [c]Flavor dilution factor. [d]Compound was tentatively identified by comparison of its retention indices with those of authentic standard or against literature data.

The least potent odorants (FD-factor = 3) were heptanal (no. **3**; *stale/citrus*), methional (no. **4**; *potato*), *trans,trans*-2,4-nonadienal (no. **15**; *fatty/fried*) and *trans,trans*-2,4-decadienal (no. **18**; *fatty/fried*). The compounds 3-methylbutanal, *trans*-2-octenal, 3-methylnonane-2,4-dione, and *trans*-2,5-epoxy-*trans*-decenal were not detected in the dilution analysis.

3.5 DISCUSSION

Results from the GCO-H were confirmed by the AEDA analysis. The only difference was that *trans,tran*-2,4-nonadienal (no. 15; *fatty/fried*) was not a predominant odorant by AEDA (FD=3). Both GCO-H and AEDA confirmed that C_9 aldehydes were the most important odorants in fresh-cut watermelon, whereas contribution by C_9 alcohols was negligible. This is a new observation, as there are no previous reports in the literature on watermelon aroma that made this conclusion. Kemp et al. (1974a) identified *cis,cis*-3,6-nonadien-1-ol to be the most important odorant in watermelon because it was extracted in a high concentration and described as watermelon or watermelon rind-like. However these same authors state that the C_9 alcohols were most likely formed by reduction of the corresponding aldehydes. In the present study, *cis,cis*-3,6-nonadien-1-ol was identified by GC-MS in the aroma extract prepared by SAFE, but it was not detected by GCO in either the static headspace or the SAFE extract.

Of the 19 compounds identified in the SAFE extract, three were described as having odors that were melon-like, including *cis*-3-nonenal (no. **9**; *melon rind*), *cis*-6-nonenal (no. **11**; *melon*) and *cis,cis*-3,6-nonadienal (no. **10**; *fresh/watermelon*). Although these were not detected in the highest dilutions, they were still the most important as a result of their odor descriptions and being detected in only 0.625 mL of headspace (FD=16), especially *cis,cis*-3,6-nonadienal, which was consistently described as having an odor reminiscent of fresh-cut watermelon. Interestingly, these three compounds were difficult to identify in previously studies. Pino et al. (2003) reported the reduced alcohol and *trans* isomers, but not the original aldehydes. This could have been due to the 90 min simultaneous steam distillation/solvent extraction technique they used, which is enough of a time lapse for reduction of the aldehydes to

alcohols and for *cis* to *trans* isomerization. Beaulieu et al. (2006), in a study using solid-phase microextraction (SPME) on seedless watermelon, positively identified *cis*-6-nonenal, but not *cis*-3-nonenal or *cis,cis*-3,6-nonadienal. However, they recognized the possibility of the other two *cis* unsaturated aldehyes being present and they speculated that these compounds might be among the compounds they classified as an "unknowns" in their paper. In a study to determine the volatile components of watermelon, Yajima et al. (1985) positively identified both *cis*-3-nonenal and *cis,cis*-3,6-nonadienal, but not *cis*-6-nonenal. These authors suggested that *cis*-3- and *cis,cis*-3,6-C_9 aldehydes were important to the overall aroma, but they still concluded that *cis,cis*-3,6-nonadien-1-ol was the most powerful contributor, once again, based on the relatively high abundance of this compound obtained in the extract.

The compounds *cis*-3-hexenal (no. **2**; *green/cut-leaf*), *cis*-2-nonenal (no. **12**; *stale/hay*), *trans*-2-nonenal (no. **14**; *stale/hay*), *trans,cis*-2,6-nonadienal (no. **13**; *cucumber*), *trans,trans*-2,4-nonadienal (no. **15**; *fatty/fried*) have all been positively identified, and have been deemed important in one or more watermelon studies (Cai 1997; Pino et al. 2003; Beulieu et al. 2006; Aguilo-Aguayo et al. 2010) as well as other studies on other cucurbit (Schieberle et al. 1990; Palma et al. 2001; Kemp et al. 1974b; Kourkoutas et al. 2006).

The compound *cis,cis,trans*-2,4,6-nonatrienal (no. **17**; sweet/oats) has never before been reported as an odorant in watermelon or any cucurbit. It had been previously identified as a character impact compound in oat flakes (Schuh et al. 2005). As an enzymatic pathway has been proposed, but not confirmed (Sessa 1979). Schuh et al. (2005) demonstrated a proposed formation via autoxidation. However, presence of this compound in watermelon suggests that its formation probably occurs via an enzymatic process.

Of all the compounds identified as important to the flavor of fresh-cut watermelon, *cis,cis*-3,6-nonadienal stands out as probably the most essential. Its prominence in the static headspace of watermelon juice (FD = 16) and in the SAFE extract (FD_{Wax} = 81) demonstrates its potency. Furthermore, it is also the only compound consistently described as having fresh/watermelon-like aroma note. Further sensory studies involving concept matching are needed to confirm its odor quality is indeed most similar to that of fresh-cut watermelon.

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CHAPTER FOUR:

***cis,cis*-3,6-Nonadienal: The Watermelon Aldehyde**

4.1 ABSTRACT

Five of the most potent odorants in watermelon were subjected to sensory evaluation using a rank test to determine their potential importance in contributing to the characteristic aroma of fresh-cut watermelon. Three compounds; *cis,cis*-3,6-nonadienal, *cis*-3-nonenal, and *cis*-6-nonenal were selected due to their melon-like aroma descriptions during GCO analysis. The other two compounds, *trans*-2-nonenal and *trans,cis*-2,6-nonadienal, were included because they are the respective *trans* isomers of the first two compounds, and, furthermore, both compounds were indicated as potentially important odorants based on results of AEDA and GCO-H (Chapter 3). The rank test revealed that among the five aldehydes tested *cis,cis*-3,6-nonadienal had an aroma that was most like the reference "fresh-cut watermelon". Based on these findings, it can be concluded that *cis,cis*-3,6-nonadienal best mimics the aroma of fresh-cut watermelon.

4.2 INTRODUCTION

Fresh-cut watermelon is considered a favorite summertime treat in the U.S. Consumption reached 4.0 billion pounds in 2009 with an estimated value of 461 million dollars (armrc.org 2010). The identity of the compounds responsible for the characteristic aroma of watermelon has been the subject of debate. Previous studies speculated that *cis,cis*-3,6-

nonadien-1-ol was the most important contributor to the flavor of watermelon based on its relatively high abundance and watermelon-like aroma quality (Yajima et al. 1985; Kemp et al. 1974, Pino et al. 2003; Beaulieu et al. 2006). More recent studies investigating the formation of watermelon aroma have demonstrated that in the specific biochemical cascade involved in aroma formation that the aldehyde, *cis,cis*-3,6-nonadienal, was formed prior to the alcohol, *cis,cis*-3,6-nonadien-1-ol (Cai 1997). Therefore, it is clear that watermelon aroma formation is a rapid and dynamic process in which enzymes catalyze the formation of the aroma compounds characteristic of fresh-cut watermelon, and then which subsequently alter them. In the previous chapter (Chapter 3), an attempt to take a chemical "snapshot" of fresh cut watermelon aroma was performed by analyzing the static headspace of watermelon 1 minute after cutting. Our data indicated that only aldehydes, including *cis*-3-nonenal, *cis*-6-nonenal, *cis*-2-nonenal, *cis,cis*-3,6-nonadienal, *trans*-2-nonenal, *trans,cis*-2,6-nonadienal and *trans,trans,cis*-2,4,6-nonatrienal, had an impact on watermelon aroma, with the aroma of *cis,cis*-3,6-nonadienal was consistently described as fresh/watermelon-like. Thus, we now believe that *cis,cis*-3,6-nonadienal contributes the most to the flavor of fresh cut watermelon. The purpose of the present study was to further demonstrate the importance of *cis,cis*-3,6-nonadienal in the characteristic aroma of fresh-cut watermelon using sensory analysis techniques.

4.3 MATERIALS AND METHODS

Materials

The compounds *trans*-2-nonenal and *trans,cis*-2,6-nonadienal were purchased from Sigma-Aldrich Co. (St. Louis, MO). The compounds *cis*-3-nonenal, *cis*-6-nonenal and *cis,cis*-3,6-

nonadienal were synthesized using the materials and methods described in Chapter 3. Compound purities were determined by gas chromatography with flame ionization detection. Odor purities were determined by gas chromatography-olfactometry.

Rank Test

Test solutions of the aldehydes were formulated to be of about equal odor intensities by a sensory panel of nine participants (6 males and 3 females age ranged from 21-47). Stock aroma solutions (1.0 mg/mL) were prepared in methanol. The methanolic solution was then added in 10 μL aliquots to 10 mL of odor-free water in sniff bottles [125-mL Nalgene PTFE wash bottles (Fisher, Pittsburgh, PA) with siphon tubes removed from the caps]. The concentrations for the various aldehyde solutions were: 0.75 mg/L of *cis,cis*-3,6-nonadienal (87% purity); 1 mg/L for *trans,cis*-2,6-nonadienal (97% purity); 15 mg/L for *cis*-3-nonenal (96% purity); 2 mg/L for *trans*-2-nonenal (95% purity); and 0.5 mg/L for *cis*-6-nonenal (95% purity).

A rank test was assembled with solutions of each of the five aldehydes in separate sniff bottles coded with 3-digit random numbers. Panel consisted of 11 females and 8 males ranging in age from 21 to 47 years. Panelists were first asked if they could recognize the aroma of fresh-cut watermelon aroma. If a panelist answered "no", they were taken into a separate room where a watermelon was cut open for them to smell so they would become familiar with the odor. For the sensory evaluation, panelists were presented the samples at room temperature (~ 23 °C) and asked to sniff the odor emitted from each sniff bottle using short "bunny" sniffs and then rank each odor from "1" to "5", with "1" being the most similar to fresh-cut watermelon aroma and "5" being least similar to fresh-cut watermelon aroma. Data were analyzed by Friedman-type statistics of ranked sums analysis with

multiple comparison procedure of least significant difference (LSD) to determine if the samples differ significantly (Meilgaard et al. 1991). Exact instructions given to the panelists are presented in the Appendix (page 103).

Determination of Odor Detection Threshold for *cis,cis*-3,6-Nonadienal

ASTM procedure E679-91 was used to determined the orthonasal odor detection threshold in odor-free water for *cis,cis*-3,6-nonadienal (87.0 % purity by GC). The stock solution (1.0 μg/mL) was prepared in methanol. Aliquots of the stock solution were dissolved in the odor-free water (10 mL) and presented to panelists in sniff bottles as previously described (Guadagni et al. 1978). Panelists (10 females and 8 males, ages 21 to 47 years) were given each concentration (1:3 dilution series) along with two matrix blanks containing the same volume of methanol and water used in preparing the sample solutions. A group of six series was tested in ascending order. The individual best estimate threshold was calculated as the geometric mean of the last concentration with an incorrect response and the first concentration with a correct response using the criteria previously described (ASTM 1992). The group best estimate threshold (BET) was calculated as the geometric mean of the individual BETs. Exact instructions given to the panelists are presented in the Appendix (page 102).

4.4 RESULTS

Rank Test

The rank test revealed that among the five aldehydes tested *cis,cis*-3,6-nonadienal had an aroma that was most like the reference "fresh-cut watermelon" (**Table 4.1**). The compound

cis,cis-3,6-nonadienal was ranked number "1" (i.e., most similar to fresh cut watermelon, 15 of 19 times). On the other hand *trans,cis*-2,6-nonadienal was ranked in the "1" position only 2 of 19 times, while *cis*-3-nonenal and *cis*-6-nonenal were each selected only 1 of 19 times as most similar to "fresh-cut watermelon". The compound *cis*-6-nonenal was ranked in the "2" position most often (11 of 19 times). By rank sums calculation and multiple comparison procedure, two compounds stand out to be considered significantly different from the rest with *cis*-6-nonenal in second place and *cis,cis*-3,6-nonadienal in first place.

Table 4.1. Results from the Rank Test of the Aroma Quality Similarity of Various Aldehydes to that of Fresh-Cut Watermelon

code	compound	1	2	3	4	5	Ranked sum[a]
738	*cis,cis*-3,6-nonadienal	15	0	3	1	0	**28 (A)**
159	*cis*-6-nonenal	1	11	4	1	2	49 (B)
615	*cis*-3-nonenal	1	5	4	3	6	65 (C)
846	*trans,cis*-2,6-nonadienal	2	0	6	7	4	68 (C)
327	*trans*-2-nonenal	0	3	2	7	7	75 (C)

[a] Sum of ranks (n = 19). Values with different letters are significantly different [LSD_{rank} = 12.184 (at $\alpha = 0.05$)].

Odor Detection Threshold of *cis,cis*-3,6-Nonadienal

The best estimate group threshold for *cis,cis*-3,6-nonadienal in water was found to be 0.2 μg/L (ppb).

4.5 DISCUSSION

The odor detection threshold for *cis,cis*-3.6-nonadienal was previously reported as 0.05 ppb in water using a panel of three trained judges (Milo et al. 1993). The threshold reported here, 0.2 ppb, is about 10 times higher than that value. The previous researchers did not report on the chemical purity of the compound. It is possible that their value is lower due to the possible presence of the *trans,cis*- isomer, which may have caused a decrease in the threshold. Further more, using a panel of trained versus untrained judges has shown that a trained panel will have a lower threshold result than an untrained panel (Guadagni et al. 1978). *Trans* isomerization is an unavoidable bi-product of hydrogenation and purification by normal phase flash chromatography (silica gel) could not be performed due to the unstable nature of *cis,cis*-3,6-nonadienal. To assure that no bias or influence was introduced during threshold determination by the *trans, cis*- isomer, the odor purity of the *cis,cis*-3,6-nonadienal was confirmed by GCO analysis of the highest dilution (1 ppb) tested. The determined threshold for *cis,cis*-3,6-nonadienal lies within that reported for *cis*-3-nonenal, 0.25 ppb, (Schieberle et al. 2001) and *cis*-6-nonenal, 0.02 ppb, (Kemp et al. 1972). The threshold in water of 0.2 ppb for *cis,cis*-3,6-nonadienal when compared to the threshold of *cis,cis*-3,6-nonadien-1-ol, 10 ppb (Kemp et al. 1974), suggests that, although there may be a greater amount of the alcohol present in watermelon, the alcohol may not be detectable due to its higher threshold value. This further supports the conclusion that *cis,cis*-3,6-nonadienal has the greatest impact on the aroma of fresh-cut watermelon.

Calculation of the rank sums followed by the multiple comparison test of least significant difference (LSD) indicated that *cis,cis*-3,6-nonadienal was perceived among the five compound tested to be the most similar to fresh-cut watermelon (**Table 4.1**). The

compound *cis*-6-nonenal was the second most similar by the individual sensory evaluations based on its rank sum. This is an understandable result as *cis*-6-nonenal has been previously reported as the most potent odorant in honeydew melon (Kemp et al. 1972) due to its obvious melon-like aroma. The compound *cis*-3-nonenal was grouped with *trans*-2-nonenal and *trans,cis*-2,6-nonadienal, meaning that none of the compounds had an aroma that was similar to fresh-cut watermelon. As previously stated, *cis,cis*-3,6-nonadien-1-ol was thought to be the most impacting odorant in watermelon (Yajima et al. 1985; Kemp et al. 1974; Pino et al. 2003; Beaulieu et al. 2006). However, researchers based their conclusion on the relatively high abundance of the alcohol in the volatile extract of watermelon and upon the aroma descriptions of the individual aroma compounds. Neither threshold values nor sensory panels were employed. Furthermore, *cis,cis*-3,6-nonadienal was not included as a major odorant in that study. From conclusions made in the previous chapter (Chapter 3) and considering the additional information provided by threshold determination and results from the rank test, our findings clearly indicate that *cis,cis*-3,6-nonadienal has an aroma that is the most reminiscent of fresh-cut watermelon among a the most potent odorants identified in fresh-cut watermelon.

4.6 REFERENCES

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CHAPTER FIVE:

Structure-Odor Relationships of Ester Analogs with *cis,cis*-3,6-Nonadiene, *cis*-3-Nonene and *cis*-6-Nonene Backbone Structures.

5.1 ABSTRACT

The watermelon aldehyde, *cis,cis*-3,6-nonadienal, is a labile compound, and thus, it would not perform satisfactorily as a flavoring agent in the food and beverage industry. More stable alcohol (methyl, ethyl, propyl and butyl) and carboxylic acid (formate, acetate, propanoate and butanoate) esters based on the *cis,cis*-3,6-nonadiene skeleton were synthesized and subjected to sensory evaluation. This included determination of odor detection thresholds, descriptive analysis and rank tests to determine if any were suitable as a replacement for the watermelon aldehyde. In addition, *cis*-3-nonene and *cis*-6-nonene esters were also evaluated to provide a full spectrum of the *cis* unsaturated nine carbon compounds responsible for the overall aroma of watermelon. Some relative threshold trends were established. The *cis*-6-nonenyl esters had the lowest thresholds compared to the respective *cis*-3 and *cis,cis*-3,6 esters. Thresholds increased with increasing carbon length of the ester end group. Carboxylic acid esters had consistently higher thresholds than the alcohol esters of the same molecular weight. Structure-odor relationships were more difficult to identify. The formate esters and ethyl esters were the only two that demonstrated correlations with each other. The formate esters all had general odor descriptions of "green apple", "sweet", and "green". The ethyl esters all had "off" or unpleasant odors as the main descriptions. The rank test to identify the most watermelon-like ester was inconclusive, showing that no one ester clearly stood out as a potential candidate for use as a watermelon flavoring.

5.2 INTRODUCTION

The compound *cis,cis*-3,6-nonadienal was synthesized in the present study (Chapter 3) and confirmed to be the most important component of the characteristic aroma of watermelon (Chapter 4). However, this compound is unstable and within the watermelon it is subjected to *trans*-isomerization resulting in *trans,cis*-2,6-nonadienal or subsequent reduction to *cis,cis*-3,6-nonadien-1-ol (Hatanaka et al. 1975; Cai 1997). In solution as a pure compound *cis,cis*-3,6-nonadienal is still labile as it isomerizes readily to *trans,cis*-2,6-nonadienal and oxidation of the exposed aldehyde group is possible resulting formation of *cis,cis*-3,6-nonadienoic acid.

Esters are common flavoring materials used in the food and beverage industry. In general, they are very stable and typically have low odor detection thresholds. Owing to these properties, novel esters are in constant demand as new flavors and ingredients in the ever-changing food industry (Liaqaut et al.2000).

Structure-odor relationships continue to be a subject of interest within the flavor industry. Thus, efforts to determine relationships between structure and odor threshold, as well as between structure and aroma description are goals of the present investigation. The working hypothesis was that the *cis,cis*-3,6-nonadienyl ester would have odor properties resembling fresh-cut water and thus be suitable and stable replacements for the labile *cis,cis*-3,6-nonadienal. Esters based on *cis*-3 and *cis*-6-nonenyl moieties were also evaluated for comparison purposes.

5.3 MATERIALS AND METHODS

Ester synthesis

The following chemicals used for the syntheses were purchased from Sigma-Aldrich Co. (St. Louis, MO): *cis*-3-nonen-1-ol, *cis*-6-nonen-1-ol, acetyl chloride, triethylamine, propionyl chloride, butyryl chloride, chromium (VI) oxide, ethyl magnesium bromide, 2-(3-butynyloxy)tetrahydropyran, copper (I) bromide, and 1-bromo-2-pentyne**,** quinoline and diethyl ether. Chemicals purchased from Fisher Scientific Co. (Fair Lawn, NJ) included formic acid, sodium chloride, pentane, sodium bicarbonate, methylene chloride, sulfuric acid, acetone, sodium hydroxide, hydrochloric acid, methanol, ethanol, 1-propanol, 1-butanol, ammonium chloride and sodium bicarbonate. Lindlar catalyst was purchased from Fluka (licensed to Sigma-Aldrich Co.). Sodium sulfate was purchase from Arcos Organics (Morris Plains, NJ).

Synthesis of *cis,cis*-3,6-Nonadien-1-ol

The synthesis of 3,6-nonadien-1-ol was performed as described in Chapter 3.

Synthesis of Formate Esters (*cis*-3-Nonenyl formate; *cis*-6-Nonenyl formate; *cis,cis*-3,6-Nonadienyl formate)

Formate esters were synthesized using an acid catalyzed esterification between formic acid and either *cis*-3-nonen-1-ol, *cis*-6-nonen-1-ol, or *cis,cis*-3,6-nonadien-1-ol following the method of Vogel (1989a).

In a one step reaction, a mixture of formic acid and the C_9 alcohol (3:1 molar ratio) contained in an amber vial was stirred for 24 hours in the dark at room temperature. The reaction was terminated by the addition of 50 mL of an aqueous saturated NaCl solution and the target compound extracted with 50:50 mixture of pentane:ether (3 x 10 mL). The

organic extract was washed with saturated aqueous sodium bicarbonate (2 x 10 mL). The step-by-step synthesis is described in the Appendix (page 80).

Synthesis of Acetate Esters (*cis*-3-Nonenyl Acetate, *cis*-6-Nonenyl Acetate and *cis,cis*-3,6-Nonadienyl Acetate)

Acetate esters were synthesized in a one step acylation reaction using acetyl chloride, triethylamine and either *cis*-3-nonen-1-ol, *cis*-6-nonen-1-ol or *cis,cis*-3,6-nonadien-1-ol following the general acylation procedure described by Vogel (1989b).

The synthesis was performed in a dry, nitrogen purged, round bottom flask in which the C_9 alcohol (1.07 mmol) and triethylamine (1.3 mmol) were dissolved in 10 mL of methylene chloride. Acetyl chloride (1.3 mmol) was then added drop-wise while stirring at 0 °C then left to stir for 6 hours. Reaction was quenched with water and the organic layer collected. The organic layer was washed with 10% aqueous sulfuric acid (2 x 10 mL) and then with aqueous saturated sodium bicarbonate (2 x 10 mL). The step-by-step synthesis is described in the Appendix (pages 80-81).

Synthesis of Propionate Esters (*cis*-3-Nonenyl Propionate; *cis*-6-Nonenyl Propionate and *cis,cis*-3,6-Nondienyl propionate)

Propionate esters were synthesized in a one step acylation reaction using propionyl chloride, triethylamine, and either *cis*-3-nonen-1-ol, *cis*-6-nonen-1-ol or *cis,cis*-3,6-nonadien-1-ol following the general acylation procedure described by Vogel (1989b).

The synthesis was performed in a dry, nitrogen purged, round bottom flask, in which the C_9 alcohol (1.07 mmol) and triethylamine (1.3 mmol) were dissolved in 10 mL of methylene

chloride. Propionyl chloride (1.3 mmol) was added drop-wise while stirring at 0 °C then left to stir for 6 hours. Reaction was quenched with water and the organic layer collected. Organic layer was washed with 10% aqueous sulfuric acid (2 x 10 mL) and aqueous saturated sodium bicarbonate (2 x 10ml). The step-by-step synthesis is described in the Appendix (pages 81-82).

Synthesis of Butyrate Esters (*cis*-3-Nonenyl Butyrate, *cis*-6-Nonenyl Butyrate and *cis,cis*-3,6-Nonadienyl Butyrate)

Synthesis of the butyrate esters was accomplished in a one step acylation reaction using butyryl chloride, triethylamine and either *cis*-3-nonen-1-ol, *cis*-6-nonen-1-ol or *cis,cis*-3,6-nonadien-1-ol following the general acylation procedure described by Vogel (1989b).

The synthesis was performed in a dry, nitrogen purged, round bottom flask in which the C_9 alcohol (1.07 mmol) and triethylamine (1.3 mmol) were dissolved in 10 mL of methylene chloride. Butyryl chloride (1.3 mmol) was added drop-wise while stirring at 0 °C and left to stir for 6 hours. Reaction was quenched with water and the organic layer collected. Organic layer was washed with 10% aqueous sulfuric acid (2 x 10 mL) and aqueous saturated sodium bicarbonate (2 x 10 mL). The step-by-step synthesis is described in the Appendix (pages 82-83).

Synthesis of Carboxylic Acids (*cis*-3-Nonenoic acid, *cis*-6-Nonenoic acid and *cis,cis*-3,6-Nonadienoic acid)

This synthesis was accomplished by oxidation of the corresponding alcohol using a Jones Oxidation Reagent described by Achilefu et al. (1995).

The Jones Reagent was prepared by mixing chromium (VI) oxide (25g, 0.25 mol), water (70 ml) and concentrated sulfuric acid (25 mL) in a sealable jar.

A round bottom flask equipped with a stir bar and placed in 0° C ice bath was used for the oxidation reaction. In a cooled flask, acetone (20 mL) and ether (10mL) were added to 2.5 g of the alcohol (*cis*-3-nonen-1-ol, *cis*-6-nonen-1-ol or *cis,cis*-3,6-nonadien-1-ol). The Jones reagent was then added drop-wise until a persistent red-brown color formed. The reaction was stopped by the addition of aqueous saturated sodium chloride followed by backwashing with 1M NaOH to isolate the acid fraction. The step-by-step synthesis is described in the Appendix (pages 83-85).

Synthesis of Methyl Esters (Methyl *cis*-3-Nonenoate, Methyl *cis*-6-Nonenoate, Methyl *cis,cis*-3,6-Nonadienoate)

Methyl esters were synthesized in a one step acid-catalyzed esterification using methanol, sulfuric acid and the corresponding C_9 carboxylic acids by Vogel (1989c).

A large molar excess (1:100) of methanol was added to a carboxylic acid (*cis*-3-nonenoic acid, *cis*-6-nonenoic acid or *cis,cis*-3,6-nonadienoic acid) in a vial followed by the addition of a few drops of sulfuric acid. The vial was capped and incubated at 60 °C for 3 h. The reaction was quenched with water (50 mL) followed by extraction of the target ester with diethyl ether (3 x 30 mL). The step-by-step synthesis is described in the Appendix (page 85).

Synthesis of Ethyl Esters (Ethyl *cis*-3-Nonenoate, Ethyl *cis*-6-Nonenoate and Ethyl *cis,cis*-3,6-Nonadienoate)

Ethyl esters were synthesized in a one step acid-catalyzed esterification using ethanol, sulfuric acid and the corresponding C9 carboxylic acids by Vogel (1989c).

A large molar excess (1:100) of ethanol was added to a carboxylic acid (*cis*-3-nonenoic acid, *cis*-6-nonenoic acid, or *cis*,*cis*-3,6-nonadienoic acid) in a vial followed by the addition of a few drops of sulfuric acid. The vial was capped and incubated at 60 °C for 3 h. The reaction was quenched with water (50 mL) followed by extract with diethyl ether (3 x 30 mL). Detailed synthesis is shown in Appendix (pages 85-86).

Synthesis of Propyl Esters (Propyl *cis*-3-Nonenoate, Propyl *cis*-6-Nonenoate and Propyl *cis,cis*-3,6-Nonadienoate)

Propyl esters were synthesized in a one step acid-catalyzed esterification using 1-propanol, sulfuric acid and the corresponding C9 carboxylic acids by Vogel (1989c).

A large molar excess (1:100) of 1-propanol was added to a carboxylic acid (*cis*-3-nonenoic acid, *cis*-6-nonenoic acid, or *cis*,*cis*-3,6-nonadienoic acid) in a vial followed by the addition of a few drops of sulfuric acid. The vial was capped and incubated at 60 °C for 3 h. The reaction was quenched with water (50 mL) followed by extract with diethyl ether (3 x 30 mL). Detailed synthesis is shown in Appendix pages (86-87).

Synthesis of Butyl Esters (Butyl *cis*-3-Nonenoate, Butyl *cis*-6-Nonenoate and Butyl *cis,cis*-3,6-Nonadienoate)

Butyl esters were synthesized in a one step acid-catalyzed esterification using 1-butanol, sulfuric acid and the corresponding C9 carboxylic acids by Vogel (1989c).

A large molar excess (1:100) of 1-butanol was added to a carboxylic acid (*cis*-3-nonenoic acid, *cis*-6-nonenoic acid, or *cis*,*cis*-3,6-nonadienoic acid) in a vial followed by the addition of a few drops of sulfuric acid. The vial was capped and incubated at 60 °C for 3 h. The reaction was quenched with water (50 mL) followed by extract with diethyl ether (3 x 30 mL). Detailed synthesis is shown in Appendix (page 87).

Spectral Data (Appx 91-101)/Retention Indices/Yields/Purities of Synthesized Esters

cis,*cis*-3,6-Nonadienyl formate, m/z mass ion (percentage): 79(100), 93(83), 41(65), 67(62), 40(52), 80(48), 77(37), 55(31), 53(21), 107(20), 122(20), 81(19), 94(18), 68(17), 65(15), 66(15); $RI_{RTX\text{-}Wax}$ =1626; Yield = 70%; Purity = 87%.

cis-3-Nonenyl formate, m/z mass ion (percentage): 54(100), 41(84), 67(70), 55(49), 68(49), 39(41), 81(40), 82(30), 95(21), 69(20), 124(19), 42(16), 96(16), 43(14), 79(10).; $RI_{RTX\text{-}Wax}$ =1562; Yield = 87%; Purity = 99%.

cis-6-Nonenyl formate, m/z mass ion (percentage): 41(100), 67(92), 54(52), 68(50), 55(47), 95(46), 82(44), 43(38), 81(37), 96(23), 124(21), 42(21), 69(17), 53(16), 56(14), 45(11), 57(11), 65(11), 48(10); $RI_{RTX\text{-}Wax}$ =1576; Yield = 85%; Purity = 97%.

cis,*cis*-3,6-Nonadienyl acetate, m/z mass ion (percentage): 43(100), 79(61), 93(54), 41(26), 80(24), 67(23), 39(22), 91(18), 77(16), 122(14), 55(11), 81(11), 107(11); $RI_{RTX\text{-}Wax}$ =1672; Yield = 76%; Purity = 91%.

cis-3-Nonenyl acetate, m/z mass ion (percentage): 43(100), 54(50), 67(33), 41(31), 68(27), 81(21), 82(20), 55(18), 39(15), 95(12), 96(10), 124(10); $RI_{RTX\text{-}Wax}$ =1605; Yield = 92%; Purity = 99%.

cis-6-Nonenyl acetate, m/z mass ion (percentage): 43(100), 67(65),41(56), 95(47), 68(40), 82(40), 55(33), 81(33), 39(25), 54(21), 124(16), 69(14), 53(12), 96(12); RI $_{RTX\text{-}Wax}$ =1630; Yield = 70%; Purity = 99%.

cis,cis-3,6-Nonadienyl propionate, m/z mass ion (percentage): 57(100), 79(94), 93(82), 80(40), 41(39), 39(27), 91(26), 122(23), 67(23), 77(20), 81(20), 55(19), 107(16), 68(16), 94(14), 53(13), 66(13); RI $_{RTX\text{-}Wax}$ =1739; Yield = 77%; Purity = 56%.

cis-3-Nonenyl propionate, m/z mass ion (percentage): 57(100), 54(68), 67(42), 41(41), 68(39), 81(30), 82(32), 55(26), 39(19), 95(19), 96(16), 124(16), 69(13); RI $_{RTX\text{-}Wax}$ =1680; Yield = 88%; Purity = 96%.

cis-6-Nonenyl propionate, m/z mass ion (percentage): 57(100), 67(89), 95(78), 41(72), 82(62), 68(57), 55(46), 81(42), 54(30), 39(28), 69(25), 124(20), 53(16), 96(16), 42(13), 83(13); RI $_{RTX\text{-}Wax}$ =1697; Yield = 90%; Purity = 98%.

cis,cis-3,6-Nonadienyl butyrate m/z mass ion (percentage): 79(100), 93(87), 43(84), 71(66), 41(57), 80(46), 39(29), 67(29), 81(23), 91(23), 122(23), 77(21), 107(21), 55(20), 94(16), 53(15), 66(15), 68(13); RI $_{RTX\text{-}Wax}$ =1811; Yield = 88%; Purity = 52%.

cis-3-Nonenyl butyrate, m/z mass ion (percentage): 54(100), 43(96), 71(89), 41(84), 67(68), 68(68), 82(57), 81(49), 55(43), 95(37), 39(32), 96(32), 124(30), 42(24), 69(22), 83(11); RI $_{RTX\text{-}Wax}$ =1760; Yield = 98%; Purity = 94%.

cis-6-Nonenyl butyrate, m/z mass ion (percentage): 41(100), 67(94), 95(86), 43(77), 82(74), 71(68), 68(62), 55(50), 81(47), 69(30), 39(29), 54(29), 42(23), 124(23), 96(22), 53(14), 83(14), 89(11); RI $_{RTX\text{-}Wax}$ =1770; Yield = 81%; Purity = 98%.

Methyl cis,cis-3,6-Nonadienoate, m/z mass ion (percentage): 79(100), 67(72), 94(71), 41(46), 39(42), 93(30), 59(29), 77(27), 55(24), 108(22), 95(21), 53(20), 91(17), 80(13), 51(12), 65(12), 68(11), 136(11), 42(10); RI $_{RTX\text{-}Wax}$ =1630; Yield = 90%; Purity = 91%.

Methyl cis-3-Nonenoate, m/z mass ion (percentage): 41(100), 55(84), 74(83), 96(75), 59(66), 54(62), 39(61), 69(46), 137(36), 43(35), 67(34), 81(33), 53(27), 68(24), 42(23), 85(22), 84(20), 97(18), 71(18), 82(17), 87(14), 110(12), 56(12), 57(11), 111(11) $RI_{RTX\text{-}Wax}$=1550; Yield = 86%; Purity = 97%.

Methyl cis-6-Nonenoate, m/z mass ion (percentage): 39(44), 41(100), 42(20), 43(42), 53(18), 54(26), 55(70), 59(40), 67(32), 68(14), 69(25), 74(84), 79(13), 81(30), 82(12), 84(18), 87(25), 94(10), 95(20), 96(46), 97(15), 138(31), 139(12); $RI_{RTX\text{-}Wax}$=1546; Yield = 90%; Purity = 97%.

Ethyl cis,cis-3,6-Nonadienoate, m/z mass ion (percentages): 79(100), 67(79), 93(62), 41(54), 39(48), 94(48), 108(42), 55(38), 53(24), 43(23), 95(19), 80(17), 81(16), 65(16), 107(15), 77(14), 54(12), 68(12), 109(12), 136(12), 91(11), 51(11); $RI_{RTX\text{-}Wax}$=1648; Yield = 92%; Purity = 88.6%.

Ethyl cis-3-Nonenoate, m/z mass ion (percentage): 41(100), 55(83), 69(76), 54(53), 39(49), 88(40), 43(35), 67(29), 70(27), 138(27), 95(26), 53(25), 81(22), 57(20), 42(18), 56(16), 71(15), 61(14), 97(14), 97(14), 110(11), 111(11), 60(11), 84(11); $RI_{RTX\text{-}Wax}$=1590; Yield = 95%; Purity = 98%.

Ethyl cis-6-Nonenoate, m/z mass ion (percentage): 41(100), 55(70), 96(56), 88(40), 39(37), 67(35), 138(35), 69(33),43(32), 95(32), 70(28), 54(27), 60(27), 81(27), 61(22), 73(22), 42(21), 84(19), 53(17), 139(17), 45(15), 68(15), 97(15), 101(15), 79(12), 82(12), 94(12), 56(11), 71(11), 109(11), 83(10), 110(10); $RI_{RTX\text{-}Wax}$=1593; Yield = 95%; Purity = 97%.

Propyl *cis,cis*-3,6-Nonadienoate, m/z mass ion (percentage): 41(100), 94(86), 79(80), 43(79), 93(74), 67(70), 39(54), 55(40), 108(38), 95(30), 81(23), 107(23), 77(22), 54(21), 80(19), 136(19), 53(17), 109(17), 137(17), 68(16), 40(15), 65(11), 91(11), 138(11); $RI_{RTX\text{-}Wax}$=1724; Yield = 92%; Purity = 57%.

Propyl *cis*-3-Nonenoate, m/z mass ion (percentages): 43(100), 41(93), 96(56), 55(50), 69(50), 39(38), 54(38), 138(28), 95(26), 61(17), 67(17), 81(17), 53(16), 68(15), 84(14), 56(13), 57(13), 97(12), 71(11), 139(11), 111(10); RI $_{RTX\text{-}Wax}$ =1672; Yield = 93%; Purity = 90%.

Propyl *cis*-6-Nonenoate, m/z mass ion (percentages): 41(100), 55(63), 43(56), 96(50), 95(38), 138(38), 39(35), 69(32), 139(31), 67(28), 42(26), 81(24), 54(23), 59(23), 61(23), 84(21), 97(16), 68(14), 73(14), 94(14), 53(13), 109(13), 71(12), 79(11), 83(11), 56(10); RI $_{RTX\text{-}Wax}$ =1679; Yield = 95%; Purity = 95%.

Butyl *cis,cis*-3,6-Nonadienoate, m/z mass ion (percentages): 94(100), 41(94), 79(68), 93(53), 67(50), 39(35), 55(35), 57(30), 108(32), 95(27), 107(24), 77(17), 81(15), 91(14), 53(13), 136(12), 68(11), 69(10), 137(10); RI $_{RTX\text{-}Wax}$ =1830; Yield = 92%; Purity = 57%.

Butyl *cis*-3-Nonenoate, m/z mass ion (percentages): 41(100), 57(51), 96(51), 55(45), 69(39), 138(35), 39(29), 54(26), 56(25), 95(21), 43(19), 84(16), 97(15), 81(14), 67(13), 42(12), 53(12), 68(11), 139(11); RI $_{RTX\text{-}Wax}$ =1764; Yield = 95%; Purity = 95%.

Butyl *cis*-6-Nonenoate, m/z mass ion (percentages): 41(100), 55(61), 96(51), 138(37), 95(35), 39(29), 56(28), 57(27), 69(26), 67(23), 43(22), 84(22), 139(22), 81(21), 54(20), 97(17), 42(16), 94(15), 60(13), 109(13), 53(12), 68(12), 71(11), 73(10), 83(10); RI $_{RTX\text{-}Wax}$ =1780; Yield = 95%; Purity = 95%.

Determination of Relative Odor Detection Thresholds by Gas Chromatography-Olfactometry (GCO)

Concentrated stock solutions (1,000 mg/L or ppm) of each ester (*cis,cis*-3,6-nonadienyl formate, *cis,cis*-3.6-nonadienyl acetate, *cis,cis*-3,6-nonadienyl propionate, *cis,cis*-3,6-nonadienyl butyrate, methyl *cis,cis*-3,6-nonadienoate, ethyl *cis,cis*-3,6-nonadienoate, propyl *cis,cis*-3,6-nonadienoate, butyl *cis,cis*-3,6-nonadienoate, *cis*-3-nonenyl formate, *cis*-3-nonenyl acetate, *cis*-

3-nonenyl propionate, *cis*-3-nonenyl butyrate, methyl *cis*-3-nonenoate, ethyl *cis*-3-nonenoate, propyl *cis*-3-nonenoate, butyl *cis*-3-nonenoate, *cis*-6-nonenyl formate, *cis*-6-nonenyl acetate, *cis*-6-nonenyl propionate, *cis*-6-nonenyl butyrate, methyl *cis*-6-nonenoate, ethyl *cis*-6-nonenoate, propyl *cis*-6-nonenoate and butyl *cis*-6-nonenoate) were prepared by adding 10 mg of the ester to 10 mL of dichloromethane. A 100 ppm working stock solution was prepared by making a 1/10 (v/v) dilution with dichloromethane. This working stock solution was then serially diluted 1:3, 1:9, 1:27, 1:81, 1:243 and 1:729 in dichloromethane. The compound ethyl hexanoate, reported to have an odor detection threshold in air of 0.00182 μl/L (0.00159 mg/L) (Komthong et al. 2006) was used as the internal standard prepared in the same serial dilution manner as the esters. The relative odor detection threshold determined by gas chromatography-olfactometry (GCO) of each ester was calculated based on the average results from three test subjects.

Each of the above dilutions was injected into a GCO and the esters were either recorded as "detected" or "not detected" at each concentration. The GCO system consisted of an HP-6890 GC (Agilent Technologies, Inc.) equipped with a RTX®-Wax column (15 m length x 0.53 mm i.d. x 1.0 μm film thickness), a flame ionization detector (FID), and a sniff port (OD2, Gerstel, Germany). Each dilution (1 μL) was injected by into a CIS4 PTV inlet (Gerstel) in the on-column injection mode. The carrier gas (helium) was at a constant flow of 18 mL/minute. The detector temperature was 250°C. The oven temperature was programmed as follows: 40 °C (held for 5 minutes), ramped at 10°C/minute to 225°C (held 10 minutes). The odor detection threshold in air via GC-O was determined relative to the dilution at which the internal standard, ethyl hexanoate, (0.00182 μl/L; 0.00159 mg/L) was recorded as "not detected".

Term Generation for Aroma Description for Esters

The lower molecular weight esters with threshold values below 0.2 mg/L, including *cis,cis*-3,6-nonadienyl formate, *cis,cis*-3,6-nonadienyl acetate, methyl *cis,cis*-3,6-nonadienoate, ethyl *cis,cis*-3,6-nonadienoate, *cis*-3-nonenyl formate, *cis*-3-nonenyl acetate, methyl *cis*-3-nonenoate, ethyl *cis*-3-nonenoate, *cis*-6-nonenyl formate, *cis*-6-nonenyl acetate, methyl *cis*-6-nonenoate, and ethyl *cis*-6-nonenoate, were used for term generation. Thresholds for the other esters - these were the higher molecular weight esters - were deemed to be too high and were not analyzed further. A trained panel consisting of 3 females and 6 males, ranging in age from 21 to 47 years, was used for term generation. During the first three sessions, panelists were individually introduced to each ester in a sniff bottle [125-mL Nalgene PTFE wash bottles (Fisher, Pittsburgh, PA) with siphon tubes removed] (recorded concentrations: *cis,cis*-3,6-nonadienyl formate (4.25 ppm), *cis,cis*-3,6-nonadienyl acetate (5.1 ppm), methyl *cis,cis*-3,6-nonadienoate (5.1 ppm), and ethyl *cis,cis*-3,6-nonadienoate (6.4 ppm) were in test 1. The compounds *cis*-3-nonenyl formate (2 ppm), *cis*-3-nonenyl acetate (3 ppm), methyl *cis*-3-nonenoate (3 ppm), and ethyl *cis*-3-nonenoate (4 ppm) were in test 2. The compounds *cis*-6-nonenyl formate (1 ppm), *cis*-6-nonenyl acetate (3 ppm), methyl *cis*-6-nonenoate (3 ppm), and ethyl *cis*-6-nonenoate (4 ppm) were in test 3) and instructed to write down odor terms that could be used to describe the aroma attributes. Later, the panel met as a group to discuss the terms that were generated during the first three sessions and came to a consensus on the key aroma terms to describe each ester.

Rank Test to Determine Aroma Similarity to the Watermelon Aldehyde

Three separate rank tests were performed, each with 4 esters, in which *cis,cis*-3,6-nonadienal (watermelon aldehyde) was used as the reference (R). Three different tests were conducted

with *cis*-3 esters in test 1, *cis*-6 esters in test 2, and *cis,cis*-3,6 esters in test 3. Test solutions of each ester were prepared to achieve the concentrations (ppm) indicated below by adding appropriate aliquots of individual esters in a methanol solution (1,000 ppm) to 10 mL of deodorized water in sniff bottles. Intensities were calibrated to equal that of n-butanol (150 ppm). Test 1 used *cis,cis*-3,6-nonadienyl formate (4.25 ppm), *cis,cis*-3,6-nonadienyl acetate (5.1 ppm), methyl *cis,cis*-3,6-nonadienoate (5.1 ppm), and ethyl *cis,cis*-3,6-nonadienoate (6.4 ppm); while *cis*-3-nonenyl formate (2 ppm), *cis*-3-nonenyl acetate (3 ppm), methyl *cis*-3-nonenoate (3 ppm), and ethyl *cis*-3-nonenoate (4 ppm) were in test 2; and *cis*-6-nonenyl formate (1 ppm), *cis*-6-nonenyl acetate (3 ppm), methyl *cis*-6-nonenoate (3 ppm) and ethyl *cis*-6-nonenoate (4 ppm) in test 3. A panel consisting of 9 males and 9 females (aged ranged 21 to 47 years) participated. For each test, panelists were asked to first sniff the bottle labeled "R" as a reference smell. Next they were instructed to smell each bottle and rank them in order from "1" to "4", with "1" being most similar to the reference and "4" being least similar. Data were analyzed by Friedman-type statistics of ranked sums analysis with multiple comparison procedure of least significant difference (LSD) to determine if the samples differ significantly (Meilgaard et al. 1991). The specific instruction given to the panelists are shown in the Appendix (page 104).

5.4 RESULTS

Relative Threshold

Table 5.1 shows the calculated relative threshold in air of the thirty-two esters as compared to the known in-air threshold of ethyl hexanotate (internal standard). The compound, c*is*-6-

nonenyl formate had the lowest threshold of the esters at 0.000191 mg/L (0.191 ppb), while butyl-*cis*-6-nonenoate had the highest threshold at 1.09 mg/L (1.09 ppm). There was a 4-odor of magnitude between the highest and the lowest thresholds, and all other ester thresholds fell within this range. A previously reported threshold in water for *cis*-6-noneyl acetate was found to be 2 ppb in water (Buttery et al. 1982).

Term Generation

Table 5.2 lists all terms generated by panelists after smelling the individual esters; *cis,cis*-3,6-nonadienyl formate, methyl *cis,cis*-3,6-nonadienoate, *cis,cis*-3,6-nonadienyl acetate, and ethyl *cis,cis*-3,6-nonadienoate. The more specific terms were grouped under the general aroma term categories of fresh, fruity, green, off or sweet.

Table 5.3 lists all terms generated by panelists after smelling the individual esters: *cis*-3-nonenl formate, methyl *cis*-3-nonenoate, *cis*-3-nonenyl acetate and ethyl *cis*-3-nonenoate. The more specific terms were grouped under the general aroma term categories of fruity, green, off or sweet.

Table 5.4 lists all terms generated by panelists after smelling the individual esters: *cis*-6-nonenyl formate, methyl *cis*-6-nonenoate, *cis*-6-nonenyl acetate, and ethyl *cis*-6-nonenoate. The more specific terms were grouped under the general aroma term categories of fresh, fruity, green, off, sweet, or floral.

Table 5. 1. Relative Thresholds for Esters

Compound	Relative Threhold in air[c]		RI[d]
	μmol/L	mg/L	RTX-wax
methyl *cis,cis*-3,6-nonadienoate	0.0529	0.00889	1612
ethyl *cis,cis*-3,6-nonadienoate	0.143	0.0259	1648
propyl *cis,cis*-3,6-nonadienoate	1.10	0.216	1724
butyl *cis,cis*-3,6-nonadienoate	2.66	0.558	1830
cis,cis-3,6-nonadienyl formate	0.00537	0.000902	1626
cis,cis-3,6-nonadienyl acetate	0.0446	0.00812	1672
cis,cis-3,6-nonadienyl propionate	0.376	0.0738	1739
cis,cis-3,6-nonadienyl butyrate	1.04	0.219	1811
methyl *cis*-3-nonenoate	0.0339	0.00576	1550
ethyl *cis*-3-nonenoate	0.725	0.133	1590
propyl *cis*-3- nonenoate	1.83	0.363	1672
butyl *cis*-3-nonenoate	4.60	0.975	1764
cis-3-nonenyl formate	0.00944	0.00160	1562
cis-3-nonenyl acetate	0.233	0.0429	1605
cis-3-nonenyl propionate	1.82	0.360	1680
cis-3-nonenyl butyrate	1.53	0.325	1760
methyl *cis*-6-nonenoate	0.0126	0.00213	1546
ethyl *cis*-6-nonenoate	0.248	0.0456	1593
propyl *cis*-6-nonenoate	1.60	0.316	1679
butyl *cis*-6-nonenoate	5.14	1.09[a]	1780
cis-6-nonenyl formate	0.00112	0.000191[b]	1576
cis-6-nonenyl acetate	0.0107	0.00198	1630
cis-6-nonenyl propionate	0.0814	0.0161	1697
cis-6-nonenyl butyrate	1.57	0.334	1770

[a] highest threshold. [b] lowest threshold. [c]Calculated relative threshold in air as compare to ethyl hexanoate (0.00159 mg/L). [d]Retention Index

.

Table 5.2. Terms Generated by Individual Panelists for *cis,cis*-3,6-Nonadiene Esters

Formate		Methyl		Acetate		Ethyl	
CT [a]	ST [b]	CT [a]	ST [b]	CT [a]	ST [b]	CT [a]	ST [b]
Fresh		Fresh	clean	Fresh	dew	Fresh	chlorine
			refreshing				
			cool				
Fruity	melon	Fruity	melon	Fruity	melon	Fruity	melon
	watermelon		berry		pineapple		watermelon
	green apple		pineapple				green apple
	strawberry		watermelon				pear
							pineapple
Green	leaf	Green	unripe	Green	cucumber	Green	
	cucumber		cucumber		rindy		
	rind				beany		
Off	bug	Off	waxy	Off	plastic	Off	plastic
	soapy		minerally		waxy		wet socks
	musty		soapy		buttery		stale
			plastic		stale		
					fatty		oily
					musty		rancid
					rubbery		waxy
Sweet	sugary	Sweet	candy	Sweet	caramel	Sweet	
	candy				brown		

[a]Category odor terms. [b]Specific example odor terms

Table 5.3. Terms Generated by Individual Panelists for *cis*-3-Nonene Esters

Formate		Methyl		Acetate		Ethyl	
CT[a]	ST[b]	CT[a]	ST[b]	CT[a]	ST[b]	CT[a]	ST[b]
Fruity	berry	Fruity	strawberry	Fruity	green apple	Fruity	pineapple
	melon		pineapple		orange peel		apple
	green apple		green apple		watermelon		tropical
	watermelon		watermelon		tropical		melon
			canteloupe		melon		grapefruit
			melon		pear		cherry
							watermelon
							grape
							cocount
Green	rindy	Green		Green	unripe	Green	
	fresh grass				leaf		
	unripe						
	cucumber						
Off	bug	Off	chemical	Off	alcholish	Off	fermented
	plastic		play-doh		plastic		vinager
			waxy		painty		waxy
			plastic				plastic
			creamy				sour
			plastic				

[a]Category odor terms. [b]Specific example odor terms

Table 5.4. Terms Generated by Individual Panelists for *cis*-6-Nonene Esters

Formate		Methyl		Acetate		Ethyl	
CT[a]	ST[b]	CT[a]	ST[b]	CT[a]	ST[b]	CT[a]	ST[b]
Fresh	spring water	Fresh	cool	Fresh	airy	Fresh	
			river water		sea breeze		
Fruity	melon	Fruity	melon	Fruity	melon	Fruity	melon
	canteloupe		watermelon		watermelon		berry
	watermelon		honeydew		honeydew		cherry
	green apple		cherry		strawberry		
			strawberry				
Green	rind	Green	cucumber	Green	rindy	Green	cucumber
	cucumber				cilantro		
Off	buttery	Off	waxy	Off	chemical	Off	plastic
	fatty		fatty		fatty		vanilla
	rancid				waxy		waxy
							soapy
							chemical
							acrid
Sweet	candy	Sweet	cotton candy	Sweet	candy	Sweet	
	sugary						
				Floral	perfume	Floral	

[a]Category odor terms. [b]Specific example odor terms

Table 5.5 lists the final key aroma terms developed by the panel for the individual esters. The panel had come to a consensus on final terms using the lexicon developed from the individual smell test.

Table 5.5. Consensus Terms Generated for Esters by Panelists

***cis*,*cis*-3-6 esters**	***cis*-3 esters**	***cis*-6 esters**
cis,*cis*-3,6-Nonadienyl formate	*cis*-3-Nonenyl formate	*cis*-6-Nonenyl formate
Fruity	Green apple	Fruity
Watermelon	Cilantro	Melon
Green apple	Parsley	Green apple
Strawberry	Slighly sweet	Strawberry
Leaf green	Slightly berry	Fatty
Sweet		Sweet
		Cucumber
Methyl *cis*,*cis*-3,6-Nonadienoate	Methyl *cis*-3-Nonenoate	Methyl *cis*-6-Nonenoate
Fruity	Fruity	Fruity
Fruit punch	Pineapple	Cherry
Cool refreshing	Sweet	Melon
Unripe	Creamy	Cucumber
Sweet	Slightly unripe	Sweet
Minerally		
cis,*cis*-3,6-nonadienyl acetate	*cis*-3-Nonenyl acetate	*cis*-6-Nonenyl acetate
Pineapple	Fruity	Melon
Buttery	Orange peel	Honeydew
Musty	Pear	Sweet
Caramel	Green	Waxy
brown butter	Plastic	Rindy
	Painty	Floral
		Fresh breeze
Ethyl *cis*,*cis*-3,6-Nonadienoate	Ethyl *cis*-3-Nonenoate	Ethyl *cis*-6-Nonenoate
Chlorine clean	Sour	Soapy
Pear	Plastic	Acrid
Stale	Coconut milk	Plastic
Wet clothes	Grapefruit	Slightly sweet
Slightly rancid		Slightly berry

Rank Test

Results from the rank test are shown in **Table 5.6**. The number of times each ester was ranked at either position 1, 2, 3 or 4 is presented along with the calculated rank sums and letter assigned grouping. The compounds c*is*-3-nonenyl acetate was ranked "1" most often, "1" being most similar to the reference (*cis,cis*-3,6-nonadienal). The compound ethyl *cis,cis*-3,6-nonadienoate was never ranked in the position "1"; its ranking was nearly evenly distributed among 2, 3 and 4. The compounds methyl *cis*-3-nonenoate and ethyl *cis*-3-nonenoate were each ranked only once in the "1" position. The compound c*is*-6-nonenyl formate was ranked least similar most ("4" position) often (11 times) among the odorants in its set. The compound ethyl *cis*-6-nonenoate was never rated least similar.

Table 5.6. Results From Rank Test on Esters' likeness to *cis,cis*-3,6-Nonadienal

Compound	Rank[a]				Ranked sum[b]
	1	2	3	4	
test 1					
cis,*cis*-3,6-nonadienyl formate	7	4	4	3	40 (A)
methyl *cis*,*cis*-3,6-nonadienoate	6	5	4	3	40 (A)
cis,*cis*-3,6-nonadienyl acetate	5	3	4	6	47 (A)
ethyl *cis*,*cis*-3,6-nonadienoate	0	6	6	6	54 (A)
test 2					
cis-3-nonenyl acetate	10	1	4	3	36 (AA)
cis-3-nonenyl formate	6	4	3	5	43 (AA)
methyl *cis*-3-nonenoate	1	9	5	3	46 (AA, BB)
ethyl *cis*-3-nonenoate	1	4	6	7	55 (BB)
test 3					
cis-6-nonenyl acetate	4	5	5	4	35 (AAA)
methyl *cis*-6-nonenoate	7	5	3	3	38 (AAA)
ethyl cis-6-nonenoate	4	7	7	0	39 (AAA)
cis-6-nonenyl formate	3	1	3	11	58 (BBB)

[a] number of times ranked from most similar, i.e. 1, to least similar, i.e. 4.
[b] Sum of ranks (n = 18). Values with different letters are significantly different [LSD_{rank} = 10.767 (at $\alpha = 0.05$)]

5.5 DISCUSSION

Relative Threshold

The relative threshold testing revealed many trends in structure-odor relationships among the aroma compounds tested. In all cases, regardless of the *cis* bond location in the carbon backbone, the carboxylic acid esters (methyl, ethyl, propyl, and butyl) showed a trend of the threshold value increasing with increase in carbon chain length. In the present study, methyl esters had consistently lower thresholds than ethyl esters. Ethyl esters in turn had lower thresholds than propyl esters, and butyl esters had the highest thresholds. The relationship between threshold and carbon length does not always hold true, as many times ethyl esters have lower thresholds than methyl esters. This mainly occurs with lower molecular weight aroma compounds, e.g., methyl butanoate (60-76 ppb) which has a higher threshold than ethyl butanoate (1ppb) (Takeoka et al. 1989). In the example shown in this current study it may be that the overall molecular weight of the ester has greater influence on the threshold than the types of ester end group.

In the present study, this trend was also observed with alcohol esters. i.e. the threshold increased with number of carbons. Specifically, the lowest threshold was recorded for formate esters, followed acetate esters, and then propionate esters. As expected, butyrate esters had the highest thresholds of all the alcohol esters. An exception was observed with *cis*-3-nonenyl propionate (0.3600 ppm) and *cis*-3-nonenyl butyrate (0.3249 ppm), however, as there was only a slight difference of 0.04 units between them. A second interesting observation was that alcohol esters had consistently lower thresholds than carboxylic acid esters. Despite having the same molecular weight, thresholds of alcohol esters and carboxylic acid esters differed by a factor of 10, which suggests that the position of the

carbonyl group has a large influence on odor detection. The location of the *cis* double bonds in the carbon backbone had a clear influence on the structure-odor relationship. In general, the *cis*-6 esters had lower thresholds than *cis*-3 esters. The only exception was the butyrate and butyl esters, where *cis*-3-nonenyl butyrate (t = 0.325 ppm) and *cis*-6-nonenyl butyrate (t = 0.334 ppm) had nearly the same threshold. Likewise, butyl *cis*-3-nonenoate (t =0.9747 ppm) and butyl cis-6-nonenaote (t = 1.089 ppm) also had similar thresholds. This suggests that for large esters the size (molecular weight) overrides any other structural influence.

This same influence of *cis* double bond location on the carbon backbone can be seen in the threshold values of *cis*-6-nonenal and *cis*-3-nonenal. The compound c*is*-6-nonenal was reported to have a threshold of 0.02 ppb (Kemp et al. 1972), while *cis*-3-nonenal had a threshold of 0.3 ppb (Kemp et al. 1974) indicating that the *cis* bond location has a definite influence on odor threshold. There is a similar, but less significant trend with *cis,cis*-3,6 esters. The carboxylic acid esters of *cis,cis*-3,6 have a lower threshold than both *cis*-3 and *cis*-6 carboxylic acid esters. Furthermore, the thresholds for the *cis,cis*-3,6 alcohol esters fall in between the *cis*-3 and *cis*-6 esters with the exception of cis,cis-3,6-nonadienyl butyrate which is lower value than both *cis*-3 and *cis*-6 butyrate. It can be speculated that depending upon the ester end group, one of the double bonds can have a greater influence than the other on threshold value.

Term Generation for Odor Descriptions

The purpose for term generation was to determine if any of the esters possessed an odor description of "watermelon". The individual sessions were used as a training tool to get panelists accustomed to smelling the esters and devising appropriate terms to describe them. Although many specific terms were generated, most could be placed into six different

categories: fresh, fruity, green, off, sweet, and floral, but not all esters required all six categories for description.

The group panel session was used to consolidate the aroma description terms to come to a consensus about which terms best describe each ester. Slight trends can be noticed for the varying end groups. All formate esters were described as having a "green apple" odor; and were considered "sweet" smelling; and a "green" term was usually used to describe them. The compound c*is*-3-nonenyl formate possesed an "herb green" aroma, like cilantro or parsley. The compounds c*is*-6-nonenyl formate had a "cucumber green" odor, and *cis,cis*-3,6-nonenyl formate was described as having a "leaf green" aroma. All three also had a berry, or strawberry, in their odor description. The compound c*is*-6-nonenyl formate had the additional term of "melon" and "fatty". Interestingly, *cis,cis*-3,6-nonadienyl formate was the only one for which the description of "watermelon" was applied, however, it was perceived as an "artificial watermelon" aroma. The methyl esters did not exhibit any specific trend. The compound methyl *cis*-3-nonenoate was described as "pineapple" and "melon" fruity, while methyl *cis*-6-nonenoate was "cherry" and "melon" fruity, and methyl *cis,cis*-3,6-nonadienoate was determined to be a general "fruit punch" fruity. All three were described as "sweet" with a green aroma. The compounds methyl *cis*-3 and methyl *cis,cis*-3,6 were both "unripe" green, whereas methyl *cis*-6 was "cucumber green". The compounds methyl *cis*-3 and methyl *cis,cis*-3,6 both had off notes of "creamy" and "minerally", respectively. Lastly, methyl *cis*,*cis*-3,6-nonadienoate also had the term "cool refreshing" assigned to it. The acetate esters were the only compounds, among the esters tested, that had previously been identified as natural volatile component of honeydew melon (Buttery et al. 1982). The only compound reminiscent of melon in the current study was *cis*-6-nonenyl acetate which was very specifically described as "melon, honeydew, sweet, waxy, rindy, fresh and slightly

floral". In the study by Buttery et al. (1982), a model using the most potent odorants in honeydew was created and compared to real honeydew melon. Only *cis*-6-nonenyl acetate out of the acetate esters was included in the model, and the model was deemed easily confused with the actual extract. This means *cis*-6-nonenyl acetate has an aroma necessary for creating the honeydew melon aroma. This makes sense as the other two esters were described very differently and were not included in this model. The compound c*is*-3-nonenyl acetate was described as having a odor of "orange peel, pear, green, plastic and painty", while *cis,cis*-3,6-nonenyl acetate was described as "pineapple, buttery, musty, caramel and brown butter". Neither was similar to the *cis*-6-nonenyl acetate nor to each other. The ethyl esters were consistent in having off odors dominate. The compound ethyl *cis*-3-nonenoate was mainly "sour" and "plastic" with "coconut" and "grapefruit", while ethyl *cis*-6-nonenoate was termed mostly "soapy", "acrid", and "plastic" with a hint of "sweetness" and "berry" lastly. The compound ethyl *cis,cis*-3,6-nonadienoate was described as "chlorine fresh", "stale", and "rancid" with a slight "pear" aroma.

Within groups of compounds having the same carbon backbone there was no solid evidence of a trend. It has always been a struggle to develop specific structure-odor relationships for aroma compounds. This study is no exception. The only trend in structure-odor trend that was evident was among the formate and ethyl esters with both having similar characteristics. Formate esters, in general, were described as "green", "green apple", "sweet" and "berry", while ethyl esters, in general, elicited more of an "off" odor.

Interestingly*, cis,cis*-3,6-nonadienyl formate was the only ester in the present study to be described as having a watermelon aroma, although is was specified to be "artificial" smelling.

Rank Test

Despite finding only one ester described as having a watermelon-like aroma, a rank test was performed to determine if any of the compounds were significantly closer in aroma to the watermelon aldehyde (*cis,cis*-3,6-nonadienal) than the others. As shown in **Table 5.6**, the rank sums indicated that none of the esters are significantly different from each other in likeness to *cis,cis*-3,6-nonadienal. The one ester described as "artificial watermelon" by the descriptive panel (*cis*,*cis*-3,6-nonadienyl formate), although chosen as "1" most often in its set, was not significantly different from other esters within its set. The compound c*is*-3-nonenyl acetate had the highest number of "1" rankings, this could be due to the aroma of the other *cis*-3 esters being so dissimilar to that of watermelon, or even a melon aroma for that matter. It was, however, not significantly different enough from two other esters within its group to be considered similar to *cis,cis*-3,6-nonadienal. The only conclusive result is that *cis*-6-nonenyl formate is the most dissimilar from *cis,cis*-3,6-nonadienal. From these findings we have to conclude that none of the alcohol and carboxylic acid esters tested would be a suitable watermelon flavoring.

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CHAPTER SIX:

Summary and Conclusions

Aroma-impact compounds of fresh-cut watermelon were determined in this study. Results from gas chromatography-olfactometry of decreasing headspace volumes (GCO-H) and aroma extract dilution analysis (AEDA) revealed that seven C_9 aldehydes (*cis*-3-nonenal, *cis*-6-nonenal, *cis,cis*-3,6-nonadienal, *cis*-2-nonenal, *trans*-2-nonenal, *trans,cis*-2,6-nonadienal and *trans,trans,cis*-2,4,6-nonatrienal) and one C_6 aldehyde (*cis*-3-hexenal) make the greatest impact contribution to the overall aroma of fresh-cut watermelon. These results settle the debate of whether aldehydes of alcohols (enzymatically derived from aldehydes in watermelon) are the main contributors to overall watermelon aroma. Previous reports erroneously indicated *cis,cis*-3,6-nonadien-1-ol as the most important odorant (Kemp et al. 1974), but it is now clear that aldehydes, and in particular *cis,cis*-3,6-nonadienal, are the predominant aroma components of fresh-cut watermelon.

Sensory tests were conducted to establish the odor characteristics and odor detection threshold of *cis,cis*-3,6-nonadienal. The low threshold for the aldehyde (t = 0.2 ppb), means that it is a more potent odorant than its respective alcohol (*cis,cis*-3,6-nonadien-1-ol; t = 10 ppb; Kemp et al. 1974). Results of a rank test showed that the aroma attributes of *cis,cis*-3,6-nonadienal are most similar to those of fresh-cut watermelon. A sum of ranks Friedman-type statistical analysis was performed resulting with *cis,cis*-3,6-nonadienal standing out alone ranked as the closest to the reference of fresh-cut watermelon.

Because *cis,cis*-3,6-nonadienal is a labile compound, issues arise when considering its use as a flavoring agent by the food and beverage industry. Stable ester moieties with the *cis,cis*-3,6-nondiene backbone were synthesized in an attempt to create an alternative compound with

the same odor property (low threshold and fresh-cut watermelon aroma description) as the aldehyde. Alcohol ester (formate, acetate, propionate, butyrate) and carboxylic acid esters (methyl, ethyl, propyl, butyl) were subjected to sensory testing to determine their relative thresholds in air and develop a lexicon to describe their aroma attributes. In order to gain a full spectrum of structure odor-relationships (SOR) of *cis* unsaturated C_9 esters, additional esters with *cis*-3-nonene and *cis*-6-nonene backbone structures were also synthesized and tested. A rank test was also conducted to determine if any of the esters had an odor that was similar to that of *cis,cis*-3,6-nonadienal; however, this data was inconclusive. Relative threshold testing revealed some SOR trends. Alcohol esters are consistently lower in threshold than carboxylic esters despite having the same molecular weight. The *cis*-6 esters were consistently lower in threshold than the *cis*-3 esters. Thresholds increased with increasing number of carbons. Trends in structure-odor description were more difficult to identify. The only obvious trend were with formate esters which were consistently described as "green apple", "berry", "sweet", and "green" and the ethyl esters which were most often described with having "off" or unpleasant odors.

In addition to the above studies more in depth experiments would be useful. This could include researching the instability of *cis,cis*-3,6-nonadienal. Knowing the rate at which this compound isomerizes or oxidizes in different media could help in development of effective ways to stabilize it. Although esters are commonly known to be stable compounds, research on the stability of the *cis,cis*-3,6-nonadiene, *cis*-3-nonene, and *cis*-6-nonene esters could confirm the efficacy of ester end groups on stabilizing labile compounds. Lastly *cis,cis*-3,6-nonadienal was confirmed to be the most important odorant in watermelon; however, more research could be performed on *cis,cis*-3,6-nonadien-1-ol including stability and sensory tests to determine if it could be used as a suitable replacement for the aldehyde.

6.1 REFERENCES

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APPENDIX

SYNTHESES

cis-3-Nonenyl formate

1) In a amber vial (40ml) equipped with a cap and stir bar, add formic acid (0.486 g; 10.056 mmol) and *cis*-3-nonen-1-ol (0.5g; 3.52 mmol)

2) Leave stirring for 24 hours at room temperature.

3) Quench with saturate sodium chloride (50ml) and extract with pentane:ether (50:50) 3 x 15ml. Wash extract with saturated sodium bicarbonate (3 x 10ml). Dry over extract over sodium sulfate and subject to HVT.

cis,cis-3,6-Nonadienyl formate

1) In an amber vial (40ml) equipped with a cap and stir bar, add formic acid (0.148 g; 3.21mmol) and *cis,cis*-3,6-nonadien-1-ol (0.15g; 1.07mmol).

2) Leave stirring for 24 hours at room temperature.

3) Quench with saturate sodium chloride (50ml) and extract with pentane:ether (50:50) 3 x 15ml. Wash extract with saturated sodium bicarbonate (3 x 10ml). Dry over extract over sodium sulfate and subjected to HVT.

cis-6-Nonenyl formate

1) In an amber vial (40ml) equipped with a cap and stir bar, add formic acid (0.2g; 4.2mmol) and *cis*-6-nonen-1-ol (0.20g; 1.4mmol).

2) Leave stirring for 24 hours at room temperature.

3) Quench with saturate sodium chloride (50ml) and extract with pentane:ether (50:50) 3 x 15ml. Wash extract with saturated sodium bicarbonate (3 x 10ml). Dry over extract over sodium sulfate and subjected to HVT.

cis-3-Nonenyl acetate

1) Set up a dry 100ml round bottom flask with stir bar and purge with nitrogen gas.

2) Dissolve *cis*-3-nonen-1-ol (0.5g; 3.52mmol) and triethylamine (0.38g; 3.75mmol) in methylene chloride (20ml).

3) Cool flask to 0ºC in ice bath

4) Add drop-wise acetyl chloride (0.3g; 3.75mmol) in methylene chloride (5ml) to the stirring solution.

5) Leave stirring at 0ºC for 6 hours.

6) Quench reaction with water (20ml) and collect the methylene chloride layer.

7) Reextract with ether (2 x 25ml) and wash extract with 10% sulfuric acid (2 x 10ml) and saturated sodium bicarbonate (2 x 10ml). Dry over sodium sulfate and subject to HVT.

cis,cis-3,6-Nonadienyl acetate

1) Set up a dry 100ml round bottom flask with stir bar and purge with nitrogen gas.

2) Dissolve *cis,cis*-3,6-nonadien-1-ol (0.15g; 1.07mmol) and triethylamine (0.132g; 1.3mmol) in methylene chloride (20ml).

3) Cool flask to 0ºC in ice bath

4) Add drop-wise acetyl chloride (0.102g; 1.3mmol) in methylene chloride (5ml) to the stirring solution.

5) Leave stirring at 0ºC for 6 hours.

6) Quench reaction with water (20ml) and collect the methylene chloride layer.

7) Reextract with ether (2 x 25ml) and wash extract with 10% sulfuric acid (2 x 10ml) and saturated sodium bicarbonate (2 x 10ml). Dry over sodium sulfate and subject to HVT.

cis-3-Nonenyl propionate

1) Set up a dry 100ml round bottom flask with stir bar and purge with nitrogen gas.

2) Dissolve *cis*-3-nonen-1-ol (0.5g; 3.52mmol) and triethylamine (0.38g; 3.75mmol) in methylene chloride (20ml).

3) Cool flask to 0ºC in ice bath

4) Add drop-wise propionyl chloride (0.488g; 3.75mmol) in methylene chloride (5ml) to the stirring solution.

5) Leave stirring at 0ºC for 6 hours.

6) Quench reaction with water (20ml) and collect the methylene chloride layer.

7) Reextract with ether (2 x 25ml) and wash extract with 10% sulfuric acid (2 x 10ml) and saturated sodium bicarbonate (2 x 10ml). Dry over sodium sulfate and subject to HVT.

cis,cis-3,6-Nonadienyl propionate

1) Set up a dry 100ml round bottom flask with stir bar and purge with nitrogen gas.

2) Dissolve *cis,cis*-3,6-nonadien-1-ol (0.15g; 1.07mmol) and triethylamine (0.132g; 1.3mmol) in methylene chloride (20ml).

3) Cool flask to 0ºC in ice bath

4) Add drop-wise propionyl chloride (0.12g; 1.3mmol) in methylene chloride (5ml) to the stirring solution.

5) Leave stirring at 0ºC for 6 hours.

6) Quench reaction with water (20ml) and collect the methylene chloride layer.

7) Reextract with ether (2 x 25ml) and wash extract with 10% sulfuric acid (2 x 10ml) and saturated sodium bicarbonate (2 x 10ml). Dry over sodium sulfate and subject to HVT.

cis-6-Nonenyl propionate

1) Set up a dry 100ml round bottom flask with stir bar and purge with nitrogen gas.

2) Dissolve *cis*-6-nonen-1-ol (0.2g; 1.4mmol) and triethylamine (0.152g; 1.5mmol) in methylene chloride (20ml).

3) Cool flask to 0ºC in ice bath

4) Add drop-wise propionyl chloride (0.14g; 1.5mmol) in methylene chloride (5ml) to the stirring solution.

5) Leave stirring at 0ºC for 6 hours.

6) Quench reaction with water (20ml) and collect the methylene chloride layer.

7) Reextract with ether (2 x 25ml) and wash extract with 10% sulfuric acid (2 x 10ml) and saturated sodium bicarbonate (2 x 10ml). Dry over sodium sulfate and subject to HVT.

cis-3-Nonenyl butyrate

1) Set up a dry 100ml round bottom flask with stir bar and purge with nitrogen gas.

2) Dissolve *cis*-3-nonen-1-ol (0.5g; 3.52mmol) and triethylamine (0.38g; 3.75mmol) in methylene chloride (20ml).

3) Cool flask to 0ºC in ice bath

4) Add drop-wise butyryl chloride (0.4g; 3.75mmol) in methylene chloride (5ml) to the stirring solution.

5) Leave stirring at 0ºC for 6 hours.

6) Quench reaction with water (20ml) and collect the methylene chloride layer.

7) Reextract with ether (2 x 25ml) and wash extract with 10% sulfuric acid (2 x 10ml) and saturated sodium bicarbonate (2 x 10ml). Dry over sodium sulfate and subject to HVT.

cis,cis-3,6-Nonadienyl butyrate

1) Set up a dry 100ml round bottom flask with stir bar and purge with nitrogen gas.
2) Dissolve *cis,cis*-3,6-nonadien-1-ol (0.15g; 1.07mmol) and triethylamine (0.132g; 1.3mmol) in methylene chloride (20ml).
3) Cool flask to 0ºC in ice bath
4) Add drop-wise butyryl chloride (0.139g; 1.3mmol) in methylene chloride (5ml) to the stirring solution.
5) Leave stirring at 0ºC for 6 hours.
6) Quench reaction with water (20ml) and collect the methylene chloride layer.
7) Reextract with ether (2 x 25ml) and wash extract with 10% sulfuric acid (2 x 10ml) and saturated sodium bicarbonate (2 x 10ml). Dry over sodium sulfate and subject to HVT.

cis-6-Nonenyl butyrate

1) Set up a dry 100ml round bottom flask with stir bar and purge with nitrogen gas.
2) Dissolve *cis*-6-nonen-1-ol (0.2g; 1.4mmol) and triethylamine (0.152g; 1.5mmol) in methylene chloride (20ml).
3) Cool flask to 0ºC in ice bath
4) Add drop-wise butyryl chloride (0.16g; 1.5mmol) in methylene chloride (5ml) to the stirring solution.
5) Leave stirring at 0ºC for 6 hours.
6) Quench reaction with water (20ml) and collect the methylene chloride layer.
7) Reextract with ether (2 x 25ml) and wash extract with 10% sulfuric acid (2 x 10ml) and saturated sodium bicarbonate (2 x 10ml). Dry over sodium sulfate and subject to HVT.

cis-3-Nonenoic acid

1) Prepared Jones Reagent by mixing together chromium (VI) oxide (25g; 0.25mol), water (70ml) and sulfuric acid (25ml).
2) Cool a round bottom flask equipped with a stir bar in a ice water bath
3) Add and stir together *cis*-3-nonen-1-ol (2.5g; 18mmol), acetone (20ml) and diethyl ether (10ml).
4) Add dropwise to the stirring solution the Jones reagent. Color will change from deep green/blue to brown/red color

5) When brown/red color is achieved quench reaction with saturate sodium chloride solution (50ml) and extract with diethyl ether (30ml).

6) Backwash ether fraction with 1M sodium hydroxide (2 x 5ml). Keep aqueous layer.

7) While keeping in an ice bath, acidify aqueous layer with 4N HCl and extract with diethyl ether (3 x 30ml).

8) Dry over sodium sulfate and subject ether extract to HVT

cis,cis-3,6-Nonadienoic acid

1) Prepared Jones Reagent by mixing together chromium (VI) oxide (25g; 0.25mol), water (70ml) and sulfuric acid (25ml).

2) Cool a round bottom flask equipped with a stir bar in a ice water bath

3) Add and stir together *cis,cis*-3,6-nonadien-1-ol (1.0g; 7.14mmol), acetone (12ml) and diethyl ether (4ml).

4) Add dropwise to the stirring solution the Jones reagent. Color will change from deep green/blue to brown/red color

5) When brown/red color is achieved quench reaction with saturate sodium chloride solution (50ml) and extract with diethyl ether (30ml).

6) Backwash ether fraction with 1M sodium hydroxide (2 x 5ml). Keep aqueous layer.

7) While keeping in an ice bath, acidify aqueous layer with 4N HCl and extract with diethyl ether (3 x 30ml).

8) Dry over sodium sulfate and subject ether extract to HVT

cis-6-Nonenoic acid

1) Prepared Jones Reagent by mixing together chromium (VI) oxide (25g; 0.25mol), water (70ml) and sulfuric acid (25ml).

2) Cool a round bottom flask equipped with a stir bar in a ice water bath

3) Add and stir together *cis*-6-nonen-1-ol (1.75g; 12.3mmol), acetone (20ml) and diethyl ether (10ml).

4) Add dropwise to the stirring solution the Jones reagent. Color will change from deep green/blue to brown/red color

5) When brown/red color is achieved quench reaction with saturate sodium chloride solution (50ml) and extract with diethyl ether (30ml).

6) Backwash ether fraction with 1M sodium hydroxide (2 x 5ml). Keep aqueous layer.

7) While keeping in an ice bath, acidify aqueous layer with 4N HCl and extract with diethyl ether (3 x 30ml).

8) Dry over sodium sulfate and subject ether extract to HVT.

Methyl *cis*-3-Nonenoate

1) In an amber vial (40ml) add *cis*-3-nonenoic aid (0.2g; 1.28mmol), methanol (5ml) and three drops of sulfuric acid.

2) Bake at 60ºC for 3 hours.

3) Quench reaction with water (50ml) and extract with diethyl ether (3 x 30).

4) Dry over sodium sulfate and subject to HVT.

Methyl *cis,cis*-3,6-Nonadienoate

1) In an amber vial (40ml) add *cis,cis*-3,6-nondienoic aid (0.1g; 0.65mmol), methanol (5ml) and three drops of sulfuric acid.

2) Bake at 60ºC for 3 hours.

3) Quench reaction with water (50ml) and extract with diethyl ether (3 x 30).

4) Dry over sodium sulfate and subject to HVT.

Methyl *cis*-6-Nonenoate

1) In an amber vial (40ml) add *cis*-6-nonenoic aid (0.2g; 1.28mmol), methanol (5ml) and three drops of sulfuric acid.

2) Bake at 60ºC for 3 hours.

3) Quench reaction with water (50ml) and extract with diethyl ether (3 x 30).

4) Dry over sodium sulfate and subject to HVT.

Ethyl *cis*-3-Nonenoate

1) In an amber vial (40ml) add *cis*-3-nonenoic aid (0.2g; 1.28mmol), ethanol (5ml) and three drops of sulfuric acid.

2) Bake at 60ºC for 3 hours.

3) Quench reaction with water (50ml) and extract with diethyl ether (3 x 30).

4) Dry over sodium sulfate and subject to HVT.

Ethyl *cis,cis*-3,6-Nonadienoate

1) In an amber vial (40ml) add *cis,cis*-3,6-nonadienoic aid (0.1g; 0.65mmol), ethanol (5ml) and three drops of sulfuric acid.

2) Bake at 60ºC for 3 hours.

3) Quench reaction with water (50ml) and extract with diethyl ether (3 x 30).

4) Dry over sodium sulfate and subject to HVT.

Ethyl *cis*-6-Nonenoate

1) In an amber vial (40ml) add *cis*-6-nonenoic aid (0.2g; 1.28mmol), ethanol (5ml) and three drops of sulfuric acid.

2) Bake at 60ºC for 3 hours.

3) Quench reaction with water (50ml) and extract with diethyl ether (3 x 30).

4) Dry over sodium sulfate and subject to HVT.

Propyl *cis*-3-Nonenoate

1) In an amber vial (40ml) add *cis*-3-nonenoic aid (0.2g; 1.28mmol), 1-propanol (5ml) and three drops of sulfuric acid.

2) Bake at 60ºC for 3 hours.

3) Quench reaction with water (50ml) and extract with diethyl ether (3 x 30).

4) Dry over sodium sulfate and subject to HVT.

Propyl *cis,cis*-3,6-Nonadienoate

1) In an amber vial (40ml) add *cis,cis*-3,6-nonadienoic aid (0.1g; 0.65mmol), 1-propanol (5ml) and three drops of sulfuric acid.

2) Bake at 60ºC for 3 hours.

3) Quench reaction with water (50ml) and extract with diethyl ether (3 x 30).

4) Dry over sodium sulfate and subject to HVT.

Propyl *cis*-6-Nonenoate

1) In an amber vial (40ml) add *cis*-6-nonenoic aid (0.2g; 1.28mmol), 1-propanol (5ml) and three drops of sulfuric acid.

2) Bake at 60ºC for 3 hours.

3) Quench reaction with water (50ml) and extract with diethyl ether (3 x 30).

4) Dry over sodium sulfate and subject to HVT.

Butyl *cis*-3-Nonenoate

5) In an amber vial (40ml) add *cis*-3-nonenoic aid (0.2g; 1.28mmol), 1-butanol (5ml) and three drops of sulfuric acid.

6) Bake at 60ºC for 3 hours.

7) Quench reaction with water (50ml) and extract with diethyl ether (3 x 30).

8) Dry over sodium sulfate and subject to HVT.

Butyl *cis,cis*-3,6-Nonadienoate

9) In an amber vial (40ml) add *cis,cis*-3,6-nonadienoic aid (0.1g; 0.65mmol), 1-butanol (5ml) and three drops of sulfuric acid.

10) Bake at 60ºC for 3 hours.

11) Quench reaction with water (50ml) and extract with diethyl ether (3 x 30).

12) Dry over sodium sulfate and subject to HVT.

Butyl *cis*-6-Nonenoate

13) In an amber vial (40ml) add *cis*-6-nonenoic aid (0.2g; 1.28mmol), 1-butanol (5ml) and three drops of sulfuric acid.

14) Bake at 60ºC for 3 hours.

15) Quench reaction with water (50ml) and extract with diethyl ether (3 x 30).

16) Dry over sodium sulfate and subject to HVT.

cis,cis-3,6-Nonadienal

3,6-Nonadiynyl-1-oxy-THP

1) In a completely dry atmosphere, purge with nitrogen gas a round bottom flask (100ml) equipped with a stir bar.

2) Add ethyl magnesium bromide (3.4ml of 3.0M solution; 10.036 mmol), 2-(3-butynyloxy) tetrahydropyran (1.55g; 10.036mmol) and dry tetrahydrofuran (10ml).

3) Leave stirring for 2 hours at 60ºC.

4) Cool reaction to room temperature and add copper (I) bromide (60mg).

5) After 15 minutes of stirring add 1-bromo-2-pentyne (0.98g; 6.69mmol).

6) Leave stirring for 1 hour at room temperature.

7) Heat to 40ºC and leave stirring for 18 hours.

8) Quench with saturate ammonium chloride (10ml) and extract with diethyl ether (3 x 15ml).

9) 3,6-nonadiynyl THP was purified by gravity column. Pack 50g of silica (100-200 mesh) in a column (2.54cm diameter). Perform stepwise elution with pentane:ether 90:10 (100ml) followed by pentane:ether 70:30 (300ml). 3,6-nonadiynyl THP appeared in elution range 70-150ml.

3,6-Nonadiyn-1-ol

1) In a amber vial (40ml) add the purified 3,6-nonadiynyl THP (1.3g; 6.3mmol) in methanol (5ml) and p-toluenesulfonic acid (100mg) in methanol (10ml).

2) Leave stirring at 60ºC for 2 hours.

3) Cool reaction to room temperature and quench with diethyl ether (30ml).

4) Ether was wash with 0.5mol/L sodium carbonate (2 x 5ml) and saturated sodium chloride (3 x 5ml).

5) 3,6-nonadiyn-1-ol was purified by gravity column. Pack 50g of silica (100-200 mesh) in a column (2.54cm diameter). Perform stepwise elution with pentane:ether 90:10 (100ml) followed by pentane:ether 70:30 (300ml). 3,6-nonadiyn-1-ol appeared in elution range 230-320ml.

cis,cis-3,6-Nonadien-1-ol

1) In a high pressure vial (20ml) add 3,6-nonadiyn-1-ol (0.5g; 4.41mmol), Lindlar catalyst (200mg), quinoline (500mg) and methanol (10ml).

2) Flush vial with hydrogen gas then leave stirring under 20psi hydrogen.

3) Check every hour for progression until completion.

4) Centrifuge and collect methanol layer. Add 10ml of diethyl ether and re-centrifuge (x2).

5) Wash extract with 1M HCl (2 x 10ml) to remove quinoline. Dry over sodium sulfate.

cis,cis-3,6-Nonadienal

1) In a dry round bottom flask (100ml) add *cis,cis*-3,6-nonadien-1-ol (0.1g; 0.736mmol) and Dess-Martin periodinane (3.68ml; 3.0 M solution in methylene chloride).

2) Slowly add wet methylene chloride (50μL water in 25ml methylene chloride).

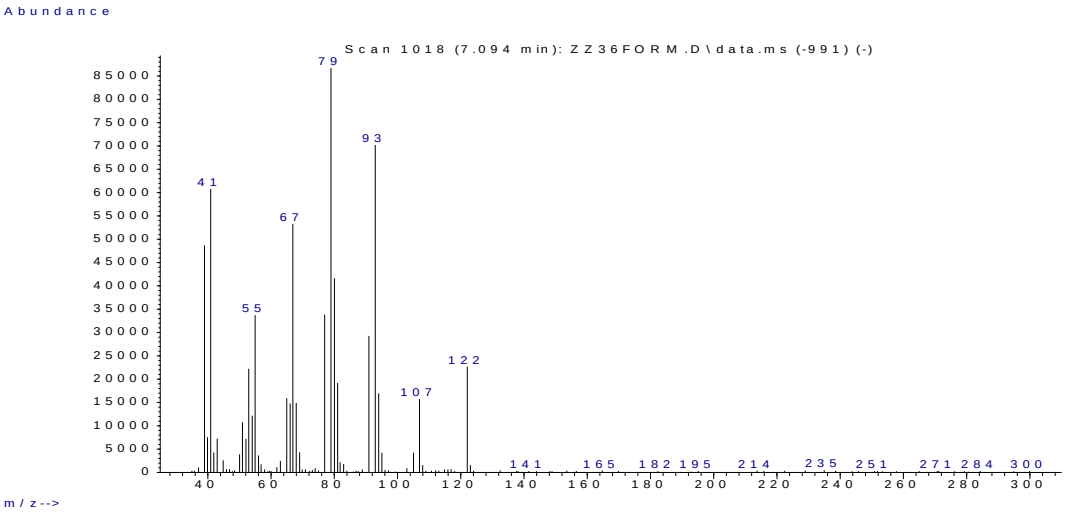
3) Leave stirring over night and check progression.

4) Evaporate off solvent with nitrogen gas and add diethyl ether (50ml) with 10% sodium thiosulfate (25ml) and saturated sodium bicarbonate (25ml).

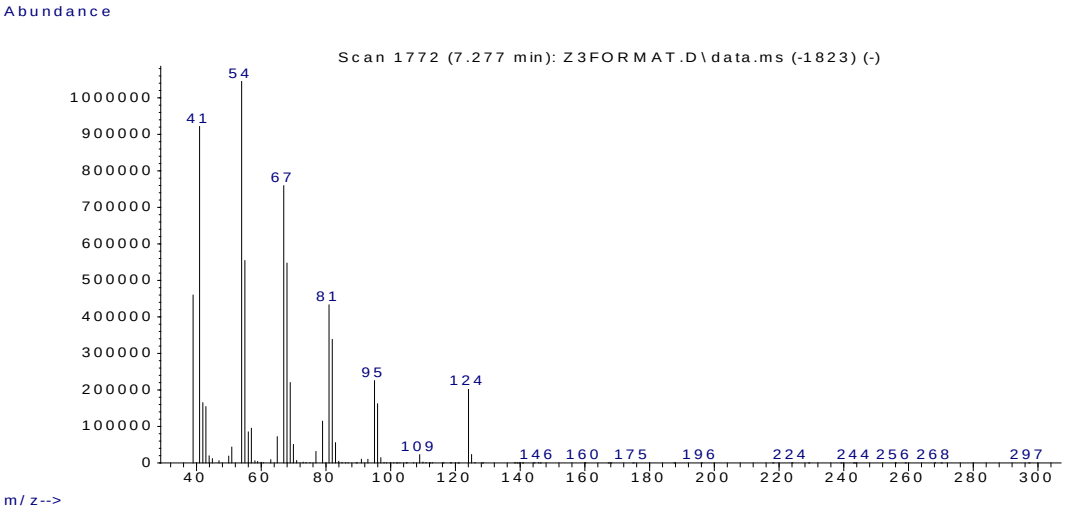
5) Transfer to a seperatory funnel and collect ether layer. Reextract with 20ml ether (x2).

6) Wash the combined ether layers with saturated sodium bicarbonate (2 x 25ml) and saturated sodium chloride (2 x 25ml). Dry over sodium sulfate.

MASS SPECTRA GRAPHS



cis,cis-3,6-Nonadienyl formate



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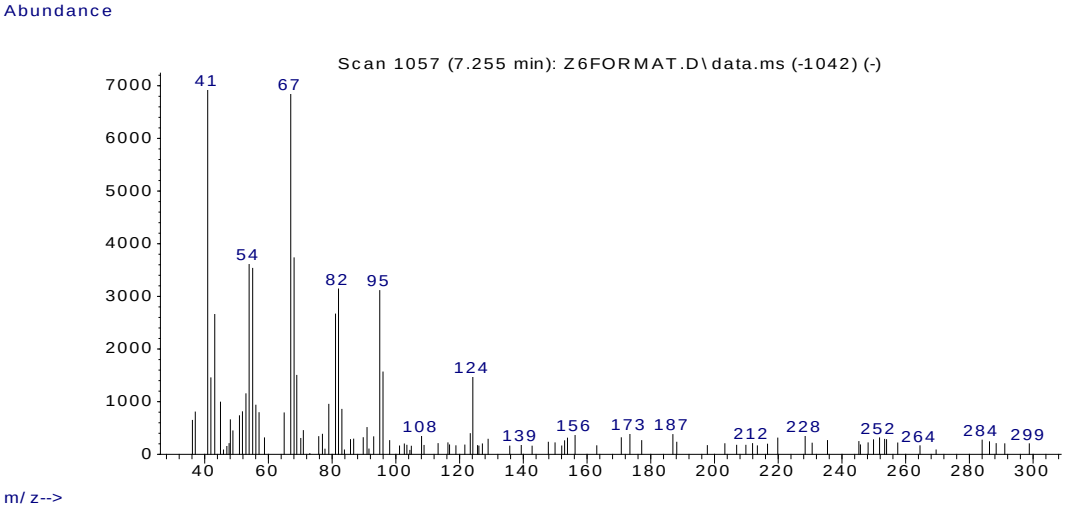

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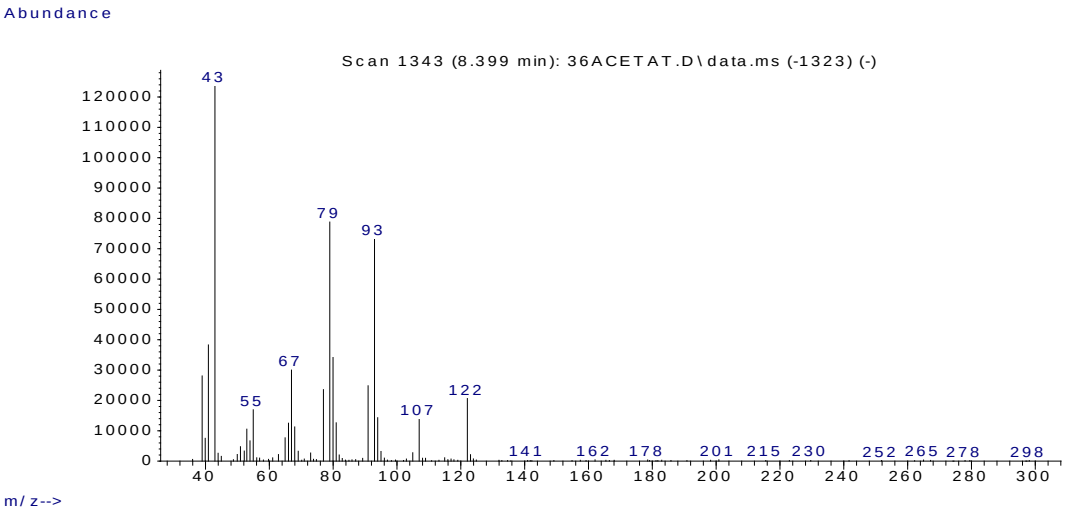
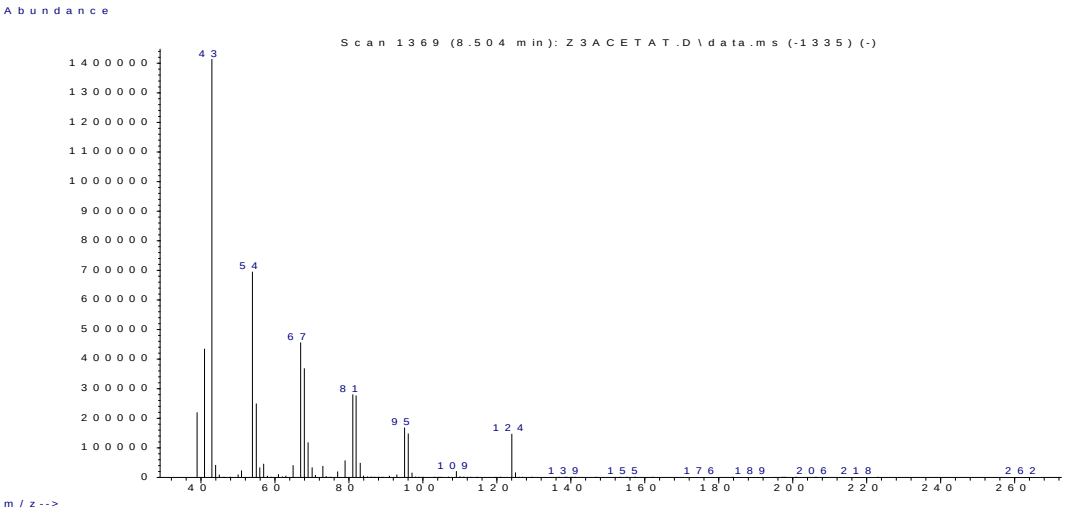
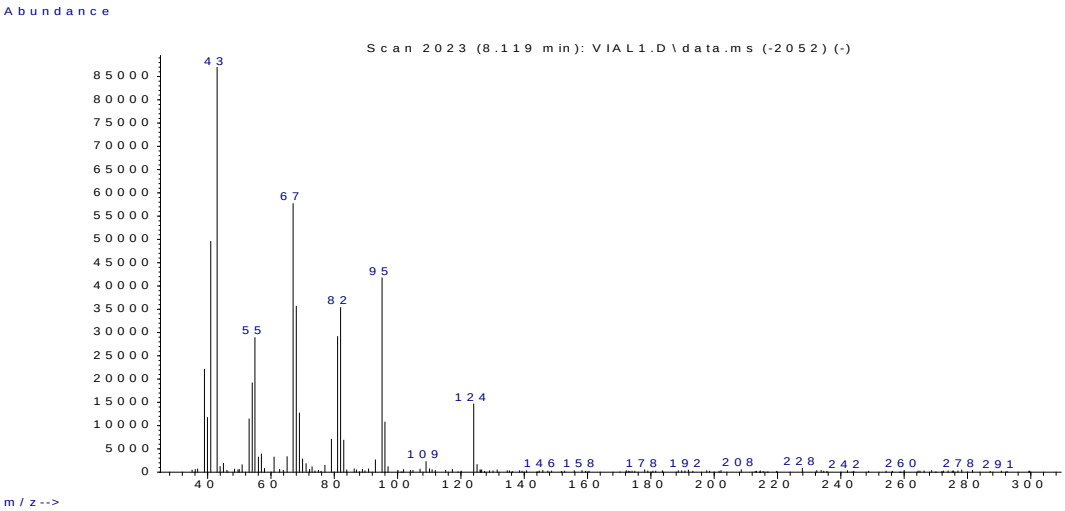


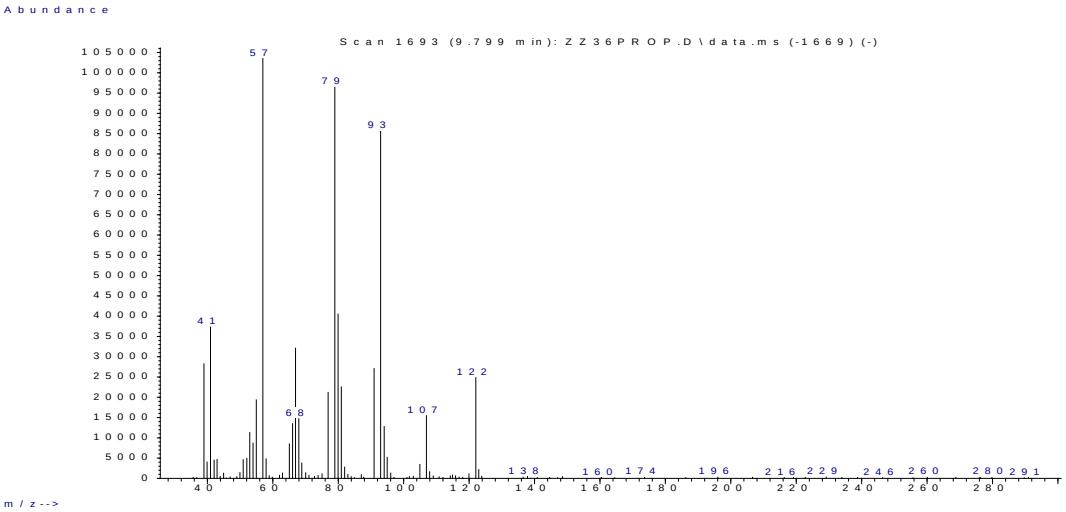
cis,cis-3,6-Nonadienyl acetate



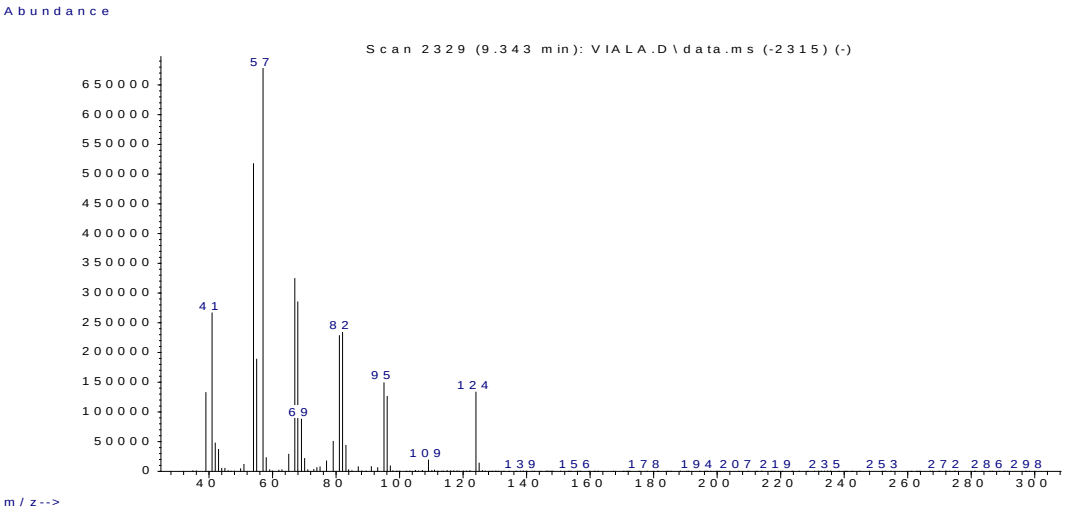
cis-3-Nonenyl acetate



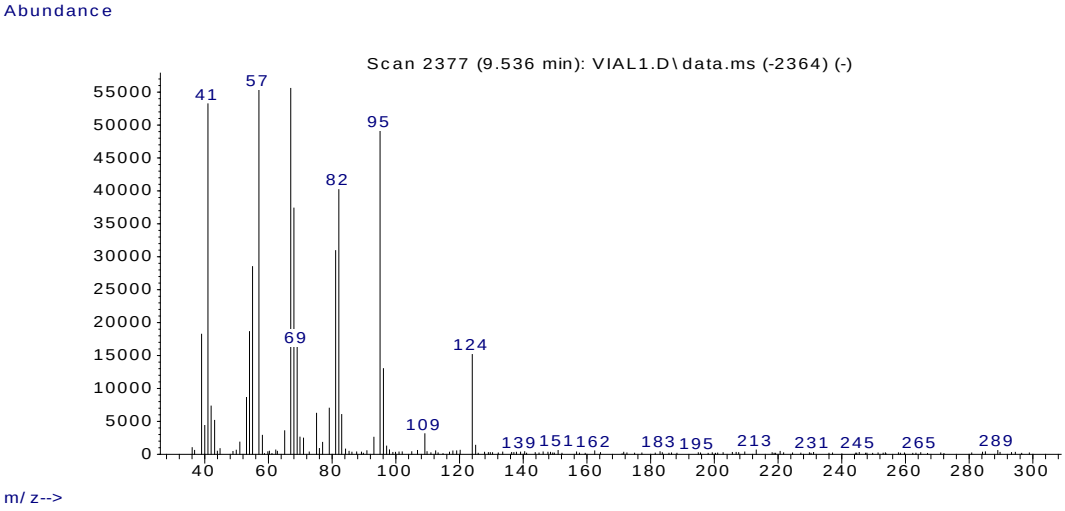
cis-6-Nonenyl acetate



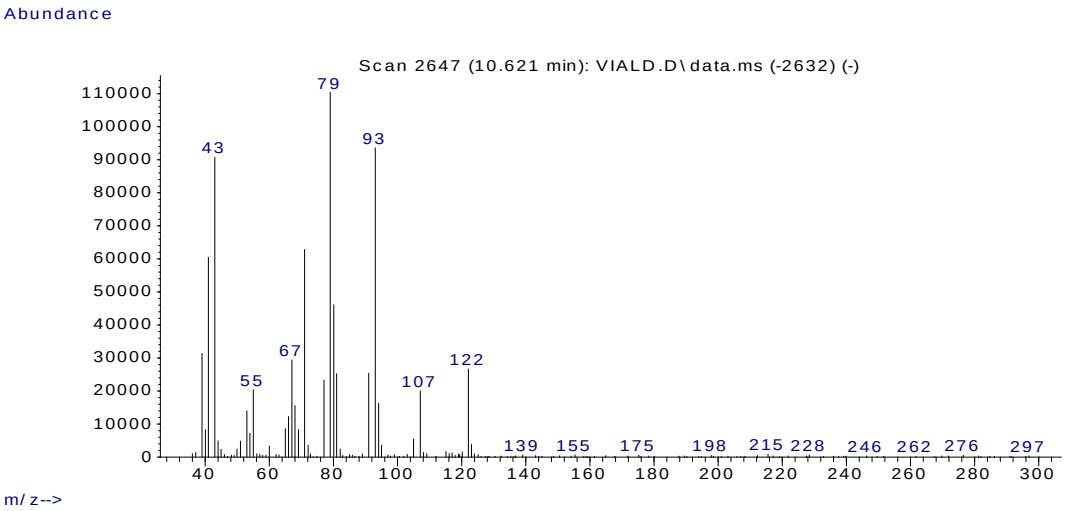
cis,cis-3,6-Nonadienyl propionate



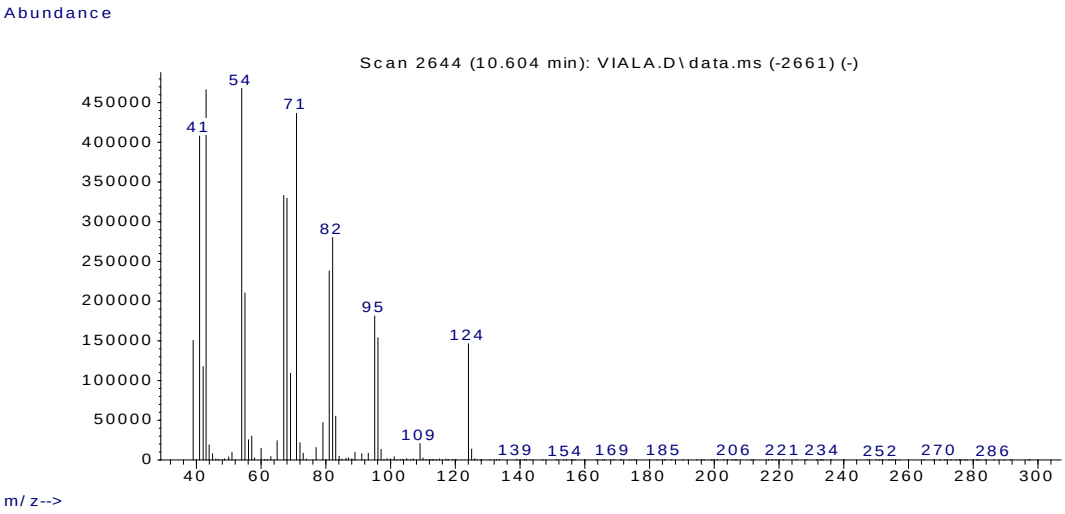
cis-3-Nonenyl propionate



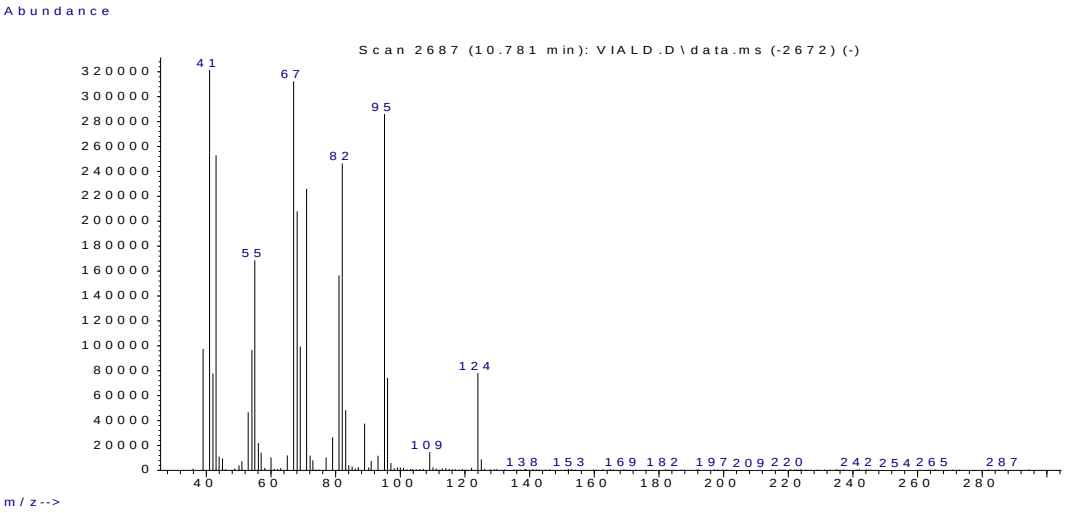
cis-6-Nonenyl propionate



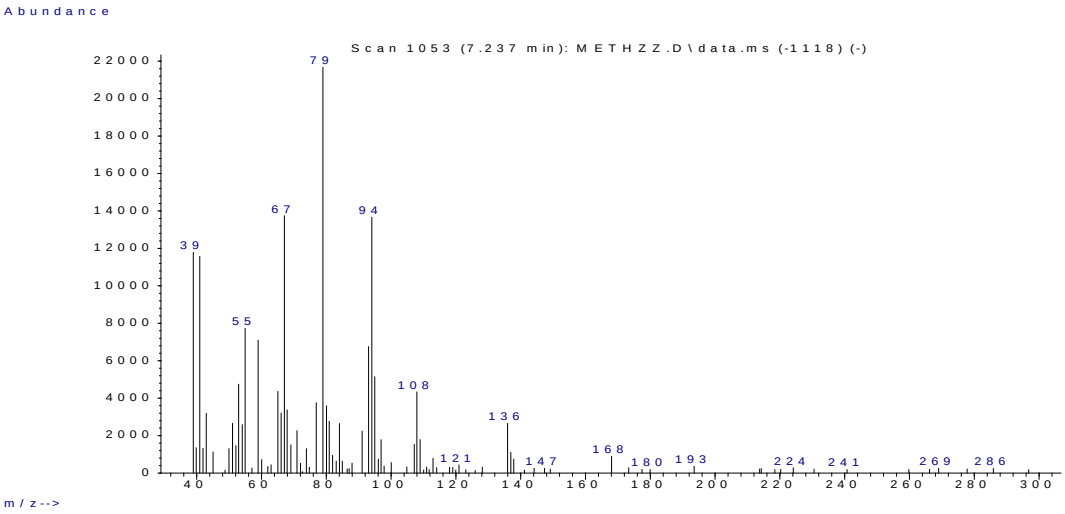
cis,cis-3,6-Nonadienyl butyrate



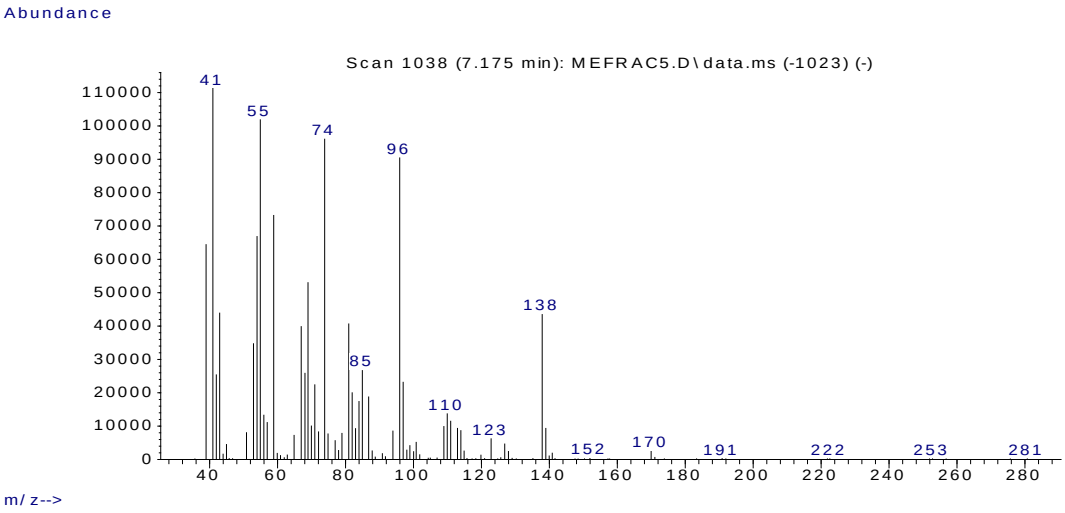
cis-3-Nonenyl butyrate



cis-6-Nonenyl butyrate



Methyl *cis,cis*-3,6-Nonadienoate



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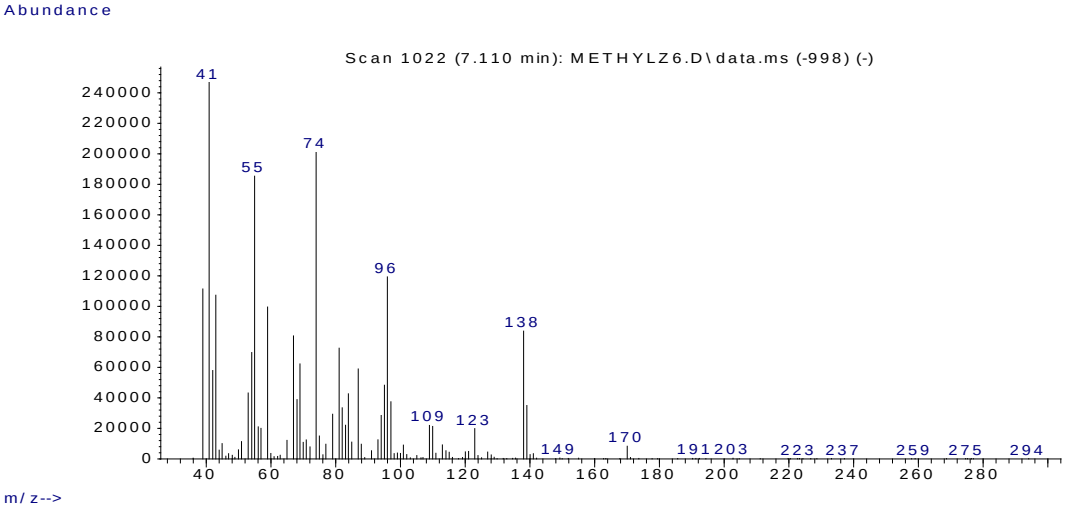

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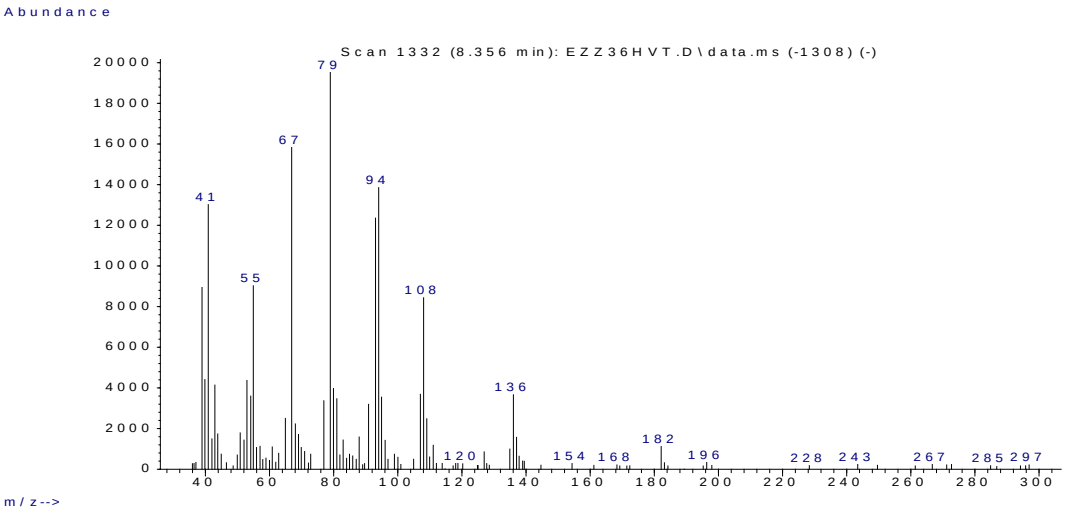

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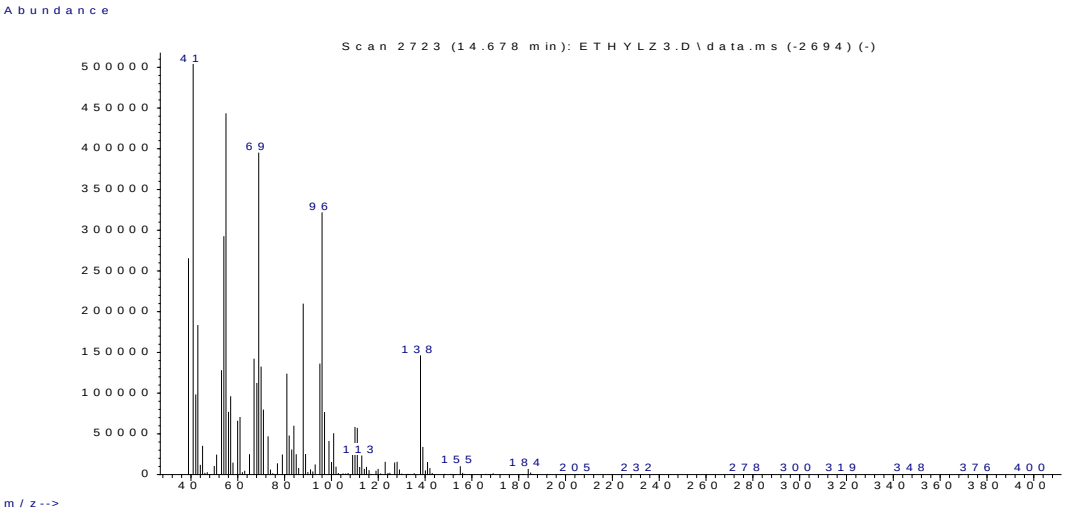
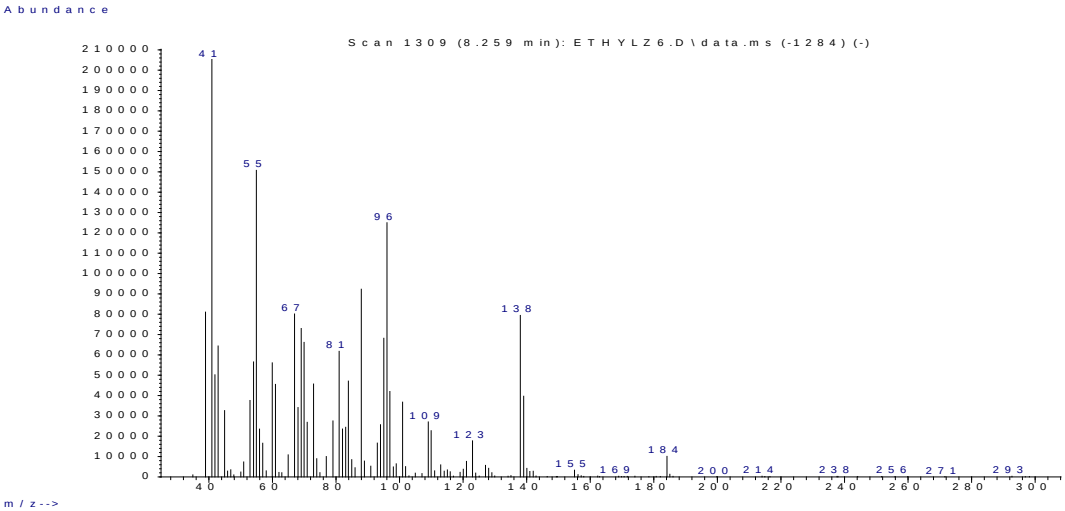


Ethyl *cis*-3-Nonenoate



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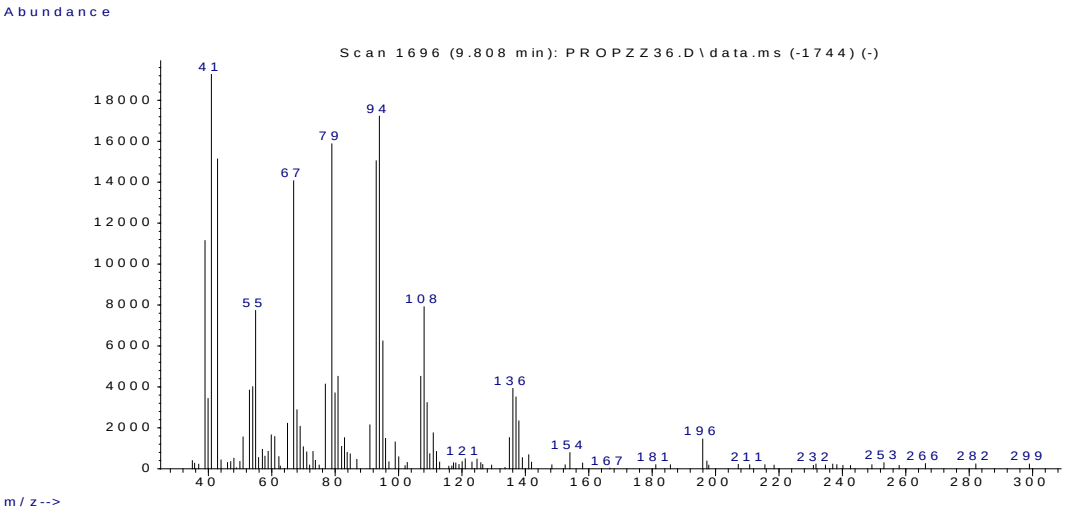
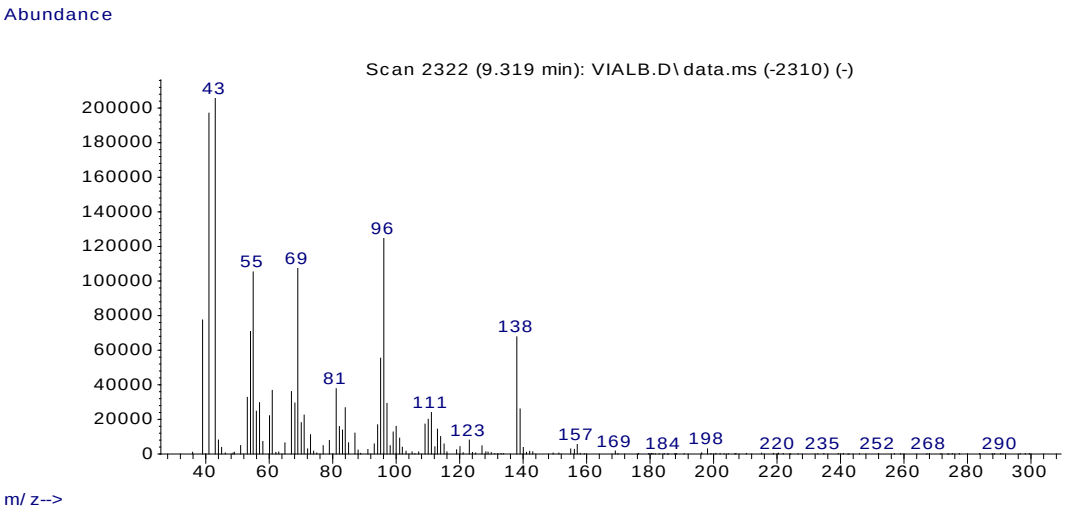
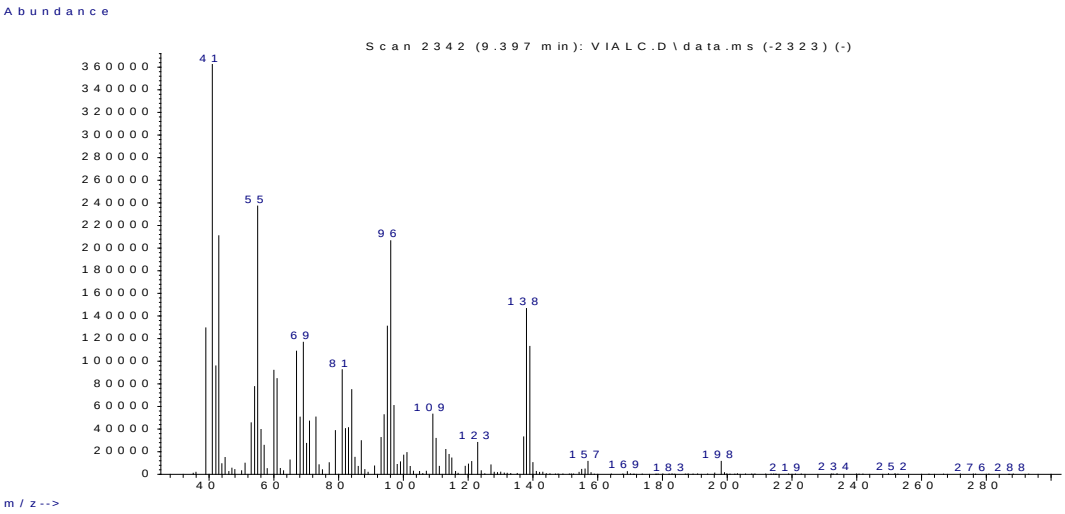


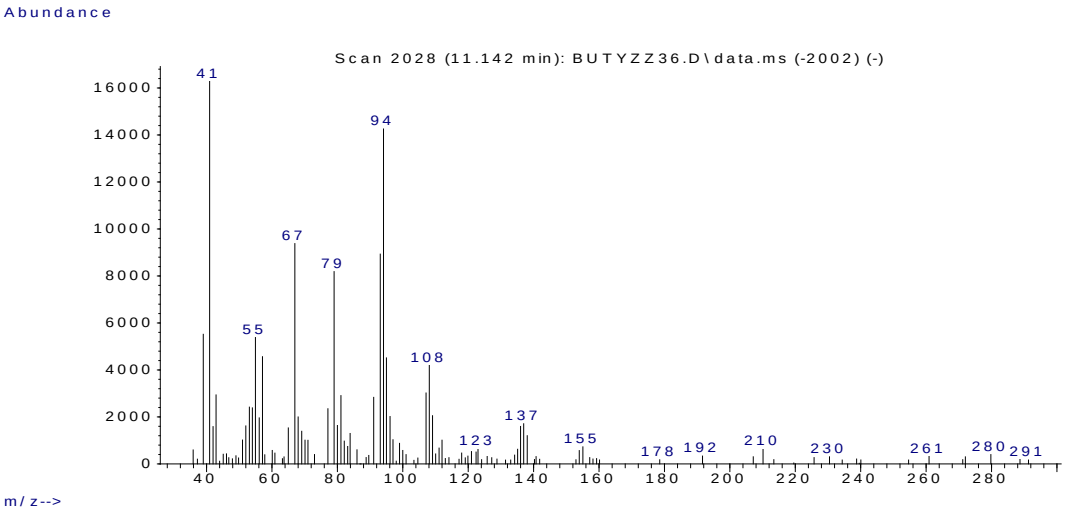
Propyl *cis,cis*-3,6-Nonadienoate



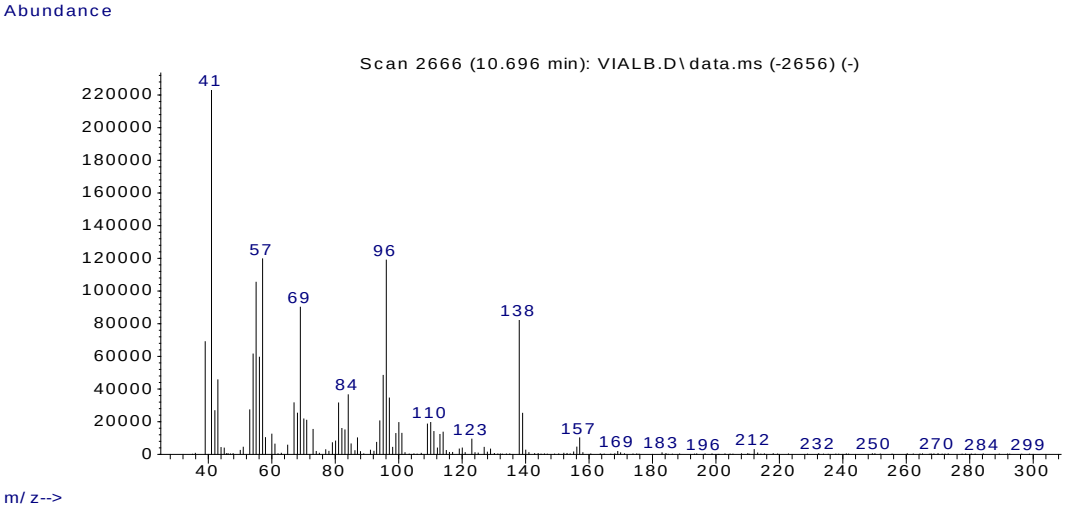
Propyl *cis*-3-Nonenoate



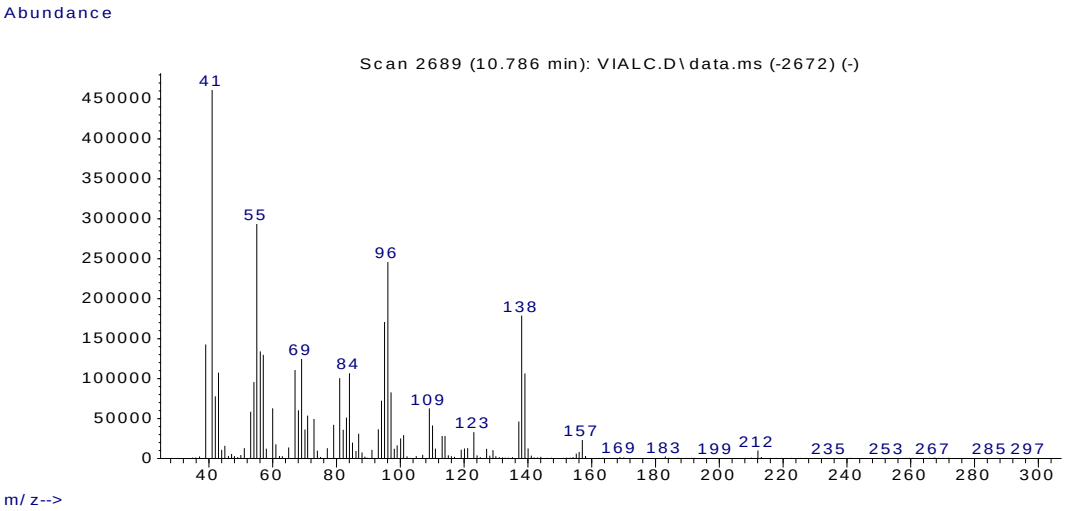
Propyl *cis*-6-Nonenoate



Butyl *cis,cis*-3,6-Nonadienoate



Butyl *cis*-3-Nonenoate



Butyl *cis*-6-Nonenoate

SENSORY MATERIALS

3-AFC Test

Instruction

1. You will perform 6 sets of 3-AFC test. Start from the first set.
2. In each set, you have received 3 samples that labeled with 3-digit number.
3. Sniff the sample from left to right as shown by **serving order** below.
4. Select the **STRONGEST ODOR** sample, and make an ✕ next to the code of that sample.
5. If samples appear the same, please make a "best guess"

STATION 1

Set	Serving Order			Description / Comments
1st	☐ ___387___	☐ ___721___	☐ ___290___	
2nd	☐ ___762___	☐ ___825___	☐ ___278___	
3rd	☐ ___410___	☐ ___781___	☐ ___181___	
4th	☐ ___520___	☐ ___389___	☐ ___762___	
5th	☐ ___263___	☐ ___289___	☐ ___739___	
6th	☐ ___978___	☐ ___614___	☐ ___681___	

Age _____ Gender_____

RANK TEST: FRESH-CUT WATERMELON AROMA

By now, you should have a concept in your mind of what a fresh-cut watermelon smells like. In this test you will sniff 5 different aromas and rank them in order of which is most like the fresh-cut watermelon smell to which one is least like fresh-cut watermelon.

Smell each aroma (in a Teflon bottle labeled with a three digit code) by gently squeezing the bottle and taking short "bunny" sniffs. Place them in the grid appropriately ranking them from "1" being the most similar to fresh-cut watermelon to "5" being least similar to fresh-cut watermelon. Next, fill out this sheet with your answers from the grid.

	RANK	3-digit code
Most similar -	1	_____
	2	_____
	3	_____
	4	_____
Least similar -	5	_____

Age _____ Gender_____

RANK TEST: FRESH-CUT WATERMELON AROMA

First smell the bottle labeled "R" for a reference by gently squeezing the bottle and taking short "bunny sniffs."

Smell each other bottle (in a Teflon bottle labeled with a three digit code) by gently squeezing the bottle and taking short "bunny" sniffs. Place them in the grid appropriately ranking them from "1" being the most similar to the reference (R) bottle to "4" being least similar to the reference (R). Next, fill out this sheet with your answers from the grid.

SET 1 - RANK	3-digit code
Most similar - 1	_____
2	_____
3	_____
Least similar - 4	_____

SET 2 – RANK	3-digit code
Most similar – 1	_____
2	_____
3	_____
Least similar - 4	_____

SET 3- RANK	3-digit code
Most similar – 1	_____
2	_____
3	_____
Least similar - 4	_____

UNIVERSITY OF ILLINOIS
AT URBANA-CHAMPAIGN

Office of the Vice Chancellor for Research
Institutional Review Board
528 East Green Street
Suite 203
Champaign, IL 61820

May 7, 2010

Keith Cadwallader
Food Science & Human Nutrition
102 ABL
MC 640

RE: *Determining Sensory Attributes of watermelon-like aroma compounds*
IRB Protocol Number: 10508

Dear Keith:

Thank you for submitting the completed IRB application form for your project entitled *Determining Sensory Attributes of watermelon-like aroma compounds*. Your project was assigned Institutional Review Board (IRB) Protocol Number 10508 and reviewed. It has been determined that the research activities described in this application meet the criteria for exemption at 45CFR46.101(b). Category 6 applies because participants will sniff fruit-like aroma compounds to determine the detection threshold for individual compounds in water. Using sensory descriptive analysis, panelists will determine definitions and commercially available reference products for the sample compounds.

This determination of exemption only applies to the research study as submitted. **Exempt protocols are approved for a maximum of three years.** Please note that additional modifications to your project need to be submitted to the IRB for review and exemption determination or approval before the modifications are initiated. To submit modifications to your protocol, please complete the IRB Research Amendment Form (see http://irb.illinois.edu/?q=forms-and-instructions/research-amendments.html).

We appreciate your conscientious adherence to the requirements of human subject research. If you have any questions about the IRB process, or if you need assistance at any time, please feel free to contact me or the IRB Office, or visit our website at http://www.irb.illinois.edu.

Sincerely,

Sue Koehn, Director, Institutional Review Board

c: Elizabeth Gardner

[illegible] • fax [illegible] • email IRB@illinois.edu