VOLATILE ANALYSES OF SINGLE-STRENGTH ORANGE JUICE INOCULATED WITH *PENICILLIUM DIGITATUM*

By

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A THESIS PRESENTED TO THE GRADUATE SCHOOL
OF THE UNIVERSITY OF FLORIDA IN PARTIAL FULFILLMENT
OF THE REQUIREMENTS FOR THE DEGREE OF
MASTER OF SCIENCE

UNIVERSITY OF FLORIDA

2011

To my fiancée, for without you this would have been an interesting paper

ACKNOWLEDGMENTS

The first person I must acknowledge is Dr. Russell Rouseff, for without him I would not be where I am today. His knowledge and expertise guided me as I developed knowledge in flavor chemistry. His trust and devotion allowed me to push myself and learn through experience, always knowing he would be there as a safety net. Dr. Rouseff also understood the value of internships, especially in the current economic situation, granting me a leave of absence to experience industry through my summer internship with Kraft Foods. This internship led to my first job offer, securing employment immediately upon graduation. The tools he bestowed upon me go beyond those necessary to understand flavor chemistry, but also include life lessons, which helped shape me as a person.

My gratitude must also be extended to each of my committee members: Dr. Jan Narciso, Dr. Renée Goodrich-Schneider, Dr. Steve Roberts, and Dr. Michelle Danyluk. Dr. Narciso offered extensive use of her lab, equipment, and expertise in fungal microbiology. This allowed me to successfully complete the microbial portion of this study while furthering my knowledge in food microbiology. My knowledge of food science grew immensely with each class I took with Dr. Goodrich. Her class on food toxicology sparked my interest in toxicology as a whole, which led me to Dr. Roberts. His classes in general toxicology and toxic substances established a strong foundation for my further interest in the field. Dr. Danyluk was also always present for support and offered the use of her lab during the government shutdown scare. Without these members' compassion and understanding, I would never have successfully completed my graduation requirements.

I owe sincere gratitude to the lab technician in Dr. Rouseff's lab, Jack Smoot, and Dr. Narciso's lab, Chris Ference, who were behind the scenes making sure the show ran smoothly. They were always there to offer a helping hand and make sure I had the supplies I needed. If an instrument ever went offline, I could call on them for the help to get my experiment back up and running. Both were even on call during my long nights and weekends if an issue ever arose in the lab.

My sniffers, Dr. Xiaofen Du, Song Mei Cau, and June Rouseff, always had a smile on their faces when they were called upon to sit and sniff at the GC – O for 30 minutes. I knew the task was not the most enjoyable and each had their own work to accomplish, so taking 30 minutes out of their day was a lot to ask. Without them, however, I would not have been able to successfully complete an objective of this study.

To my lab mates, Whitney Johnson and Kyle Edwards, it has been quite a ride, and I appreciate you lending a helping hand and providing a listening ear. We formed a bond through our experiences that no one else will understand. I hope to keep in touch as the years progress and we each go our separate ways.

Finally, none of this would have been possible without my family and friends. My mom and dad have been there through thick and thin, always supporting my endeavors without question. Zach and Jon, you are the fuel to my competitive fire and without your amazing achievements I may not have been as inspired to set the bar so high for myself. To all my friends, I thank you for being there to lend your support and advice. This especially includes Marianne Fatica, who has been there from the beginning of my journey through graduate school, lending advice and support from her own experiences through this crazy ride of ups and downs. Lastly, I must acknowledge my fiancée,

Rachel, for without you I do not know where I would be today. I do know this paper would not be as well written without your amazing editing abilities. We have had quite a journey so far and I cannot wait to travel down the road of life with you by my side.

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LIST OF ABBREVIATIONS

A_w	Water activity
DI	Deionized
DVB	Divinylbenzene
FID	Flame ionized detector
GC – MS	Gas chromatography – mass spectrometry
GC – O	Gas chromatography – olfactory
GC – S	Gas chromatography – sulfur
LRI	Linear retention index
OJ	Orange juice
PDA	Potato dextrose agar
PDMS	Polydimethylsiloxane
PFPD	Pulsed flame photometric detector
RT	Retention time
SDE	Steam distillation extraction
SPME	Solid phase microextraction
USFDA	United States Food and Drug Administration

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Requirements for the Degree of Master of Science

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August 2011

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Major: Food Science and Human Nutrition

Post-pasteurization contamination of orange juice does occasionally occur, resulting in appearance and aroma changes. Previous microbial research regarding citrus has focused on bacteria and fermentative yeasts. Molds were not considered a microbial problem because the majority of juice was 65 °Brix concentrate and its low water activity did not favor mold growth. An increased potential for spoilage from mold was a result of the shift in consumer orange juice products from concentrate to single strength, higher water activity juice. *Penicillium digitatum* is responsible for up to 90% of the citrus fruit loss experienced during storage and readily grows in juice. This study examined the changes in volatiles, and their impact on aroma, produced by *P*. *digitatum* in single strength orange juice. The primary objective was to determine qualitative and/or quantitative differentiating volatiles in inoculated and uninoculated (control) samples. A secondary objective was to correlate differentiating volatiles with aroma active volatiles. These objectives were accomplished by sampling inoculated and control juices using headspace, solid phase microextraction and analyzing the volatile profiles with gas chromatography – mass spectrometry, – sulfur, and – olfactometry. Total cloud destruction and an 11% reduction in °Brix was observed in inoculated juices

at four days. Methanol, ethyl propanoate, ethyl 2-methylbutanoate, 3-methylbutanol, (Z)-3-hexenol, and 1-octen-3-ol were qualitative differentiating volatiles found only in inoculated samples. Neryl acetate was a qualitative differentiating volatile observed only in control samples. Other volatiles, including sulfur volatiles, were found to be quantitative differentiating volatiles, either greater than or less than that in the control. Neryl acetate was a differentiating volatile that also had aroma activity. The net effect of *P*. *digitatum* growth in orange juice produced an overall decrease in total volatiles as well as total aroma active volatiles.

CHAPTER 1
INTRODUCTION

Despite all precautions, post-pasteurization contamination of orange juice (OJ) does occasionally occur and can produce changes in the juice's appearance, aroma, and taste (Huis in't Veld 1996; Larsen and Frisvad 1995a; Parish 1997). The majority of microbial research regarding citrus fruits and products has focused on the detection and prevention of spoilage caused by bacteria and fermentative yeasts such as *Lactobacillus* and *Saccharomyces* species, respectively. Of primary concern is the production of volatile metabolites that contribute to off-flavors in the juice. For example, a buttery off-note, caused by diacetyl, is associated with the presence of bacteria, including *Lactobacillus* and *Leuconostoc* species, and an alcoholic off note is associated with the presence of fermentative yeasts, such as *Saccharomyces* (Parish 1991; Parish and Higgins 1989; Wyatt and others 1995). Molds were not considered a microbial problem initially with OJ, as the majority of juice globally distributed was 65 °Brix concentrate. Fungi do not easily grow in the freezing conditions (< 0°C) or low water activity (A_w) of the concentrate. With recent technological advances in processing and storage, a shift from concentrate to single-strength, not from concentrate juice has occurred. This shift resulted in an increased potential for spoilage from mold, once not considered a problem (Parish 1991; Wyatt et al. 1995).

Molds associated with citrus include *Penicillium digitatum* and *Penicillium italicum*. Both of these molds are capable of thriving under current storage temperatures (0 – 4°C), A_w (>0.98), and increased shelf life (35 – 65+ days) of single strength, not from concentrate OJ (Filtenborg and others 1996; Narciso and Parish 1997; Parish 1991; Parish and Higgins 1989; Raccach and Mellatdoust 2007; Tournas and others 2006).

P. *digitatum* is responsible for up to 90% of the loss of citrus fruit during storage and is considered one of the most damaging and important pathogenic fungi of harvested citrus (Ariza and others 2002; Droby and others 2008; Kavanagh and Wood 1971; Pesis and Marinansky 1990). Spores of this fungus have been shown to readily grow in post-pasteurized OJ (Eckert and Ratnayake 1994).

The United States Food and Drug Administration (USFDA) requires a 5-log pathogen reduction during processing of OJ; however, post-pasteurization contamination is of great concern due to the high amounts of *P*. *digitatum* observed in the atmosphere of juice processing plants (Davé and others 1981; Narciso and Parish 1997; Parish 1991; Pelser and Eckert 1977; Raccach and Mellatdoust 2007; Tournas et al. 2006; Wyatt and Parish 1995). A survey of single strength, not from concentrate OJ in the Washington DC area found 22% of juice was contaminated with *Penicillium* species, including *P*. *digitatum* (Parish 1991; Tournas et al. 2006). The gable top juice cartons containing the juice are also a source of contamination. The multilayer cartons are not aseptic and many species of fungi have been isolated from the cardboard. Unfinished edges of the packaging can allow fungi access to the juice. The organism grows and forms a mat with spore production upon reaching the juice/air interface at the top of the carton (Narciso and Parish 1997).

P. *digitatum* produces a wide spectrum of enzymes that are stable and active at low pH and low temperature (Alaña and others 1990; Bush and Codner 1968; Bush and Codner 1970; Filtenborg et al. 1996). These enzymes are known for causing softening and destruction of the citrus peel and analogous destruction in the juice cloud (Alaña et al. 1990; Barmore and Brown 1979; Wyatt et al. 1995). Enzymes associated with *P*.

digitatum have been shown to catalyze the bioconversion of volatiles, altering the metabolic profile (Adams and others 2003; Demyttenaere and others 2001; Janssens and others 1992). For example, P-450 monooxygenases are considered the primary enzymes causing the bioconversion of limonene to α-terpineol (Demyttenaere et al. 2001; Duetz and others 2003). The organism also produces additional secondary metabolites through various metabolic pathways, which can impact aroma and taste (Ariza et al. 2002; French 1985; Fries 1973; Huis in't Veld 1996; Larsen and Frisvad 1995a).

The specific volatile metabolite profile, also known as differentiating volatiles, produced by bioconversion and production of secondary metabolites, can be used to differentiate two organisms down to the species level (Larsen and Frisvad 1994; Larsen and Frisvad 1995b). For example, in the study performed by Vikram and others (2004b), dimethyl ether and propanal were found to be specific to *Penicillium* growing on Cortland apples. In contrast, methyl acetate and styrene were associated with *Monilinia* on the same apple (Vikram and others 2004b). Studies have also shown the ability to differentiate various *Penicillia* species based on a specific or series of differentiating volatiles produced as the organism grows on media, including potato dextrose agar (PDA) (Larsen and Frisvad 1995b). Ethyl acetate was found to be the volatile produced in largest amount specific to *P*. *digitatum,* while *P*. *italicum* could be distinguished by the presence of both ethyl acetate and isopentanol on PDA (Larsen and Frisvad 1995a).

Metabolomics is the study of all the metabolites produced by a specific organism, while chemosystematics is the use of this information to identify and classify the

organism (Cevallos-Cevallos and others 2009). The use of differentiating volatiles to identify various organisms, and the disease associated with the specific organism, has been examined in potato tubers, mangos, carrots, and apples. Studies have found that organisms, and the resulting disease they cause, can often be differentiated from one another based on differentiating volatiles (Lui and others 2005; Moalemiyan and others 2007; Moalemiyan and others 2006; Vikram and others 2006; Vikram and others 2004a; Vikram et al. 2004b). Based on this and previous studies that demonstrated the ability of *Penicillia* to produce differentiating volatiles, the presence of *P*. *digitatum* should be detectable in OJ through volatile analysis (Larsen and Frisvad 1995b; Schnürer and others 1999).

Since fungi that contaminate juice initially grow while submerged, the present study focused on determining the changes in volatiles produced by *P*. *digitatum* submerged in single strength, not from concentrate OJ. The primary objective was to determine qualitative and/or quantitative differentiating volatiles in inoculated and uninoculated (control) samples. A secondary objective was to correlate differentiating volatiles with aroma active volatiles.

CHAPTER 2
LITERATURE REVIEW

Microbial Spoilage of Foods

As food products are harvested, processed, and handled, the potential of microbial contamination by bacteria, yeasts, and molds increases. This contamination could lead to microbial spoilage of the food product, rendering it unacceptable for consumption (Huis in't Veld 1996; Lacey 1989). Microbial spoilage of citrus fruits and products involves acid tolerant bacteria, yeasts, and molds. The primary bacterial contaminates include *Lactobacillus* and *Leuconostoc* species, which produce 2,3-butanedione, also known as diacetyl, as a secondary metabolite. Diacetyl imparts a buttery flavor to the contaminated product and is considered an undesirable attribute. Yeast species associated with citrus include *Saccharomyces* and *Candida* spp. These are fermentative yeasts and will ferment sugars under anaerobic conditions to produce alcohols and other metabolic end products, all of which are undesirable. Various molds have been associated with citrus products, including *Penicillium*, *Geotrichum*, *Fusarium*, *Cladosporium*, *Trichoderma*, and *Trichophyton* (Parish 1991). In rare cases, pathogenic microbes such as *Escherichia coli* are introduced post-pasteurization through contamination and lead to foodborne disease outbreaks (Parish 1997).

All microorganisms have a series of pathways that allow them to adapt readily to their environment. By-products of such pathways are known as secondary metabolites. These secondary metabolites can alter the food product’s appearance, aroma, and taste (Huis in't Veld 1996; Larsen and Frisvad 1995c). Besides safety, one of the primary concerns is the alteration of taste, which results from an increase or decrease in volatiles known as aroma impact compounds through enzymatic pathways specific to

the individual organisms (French 1985; Fries 1973; Moalemiyan et al. 2007). Bioconversion is also one of the pathways that result in the synthesis or breakdown of aroma active compounds (Janssens et al. 1992).

The majority of microbial research regarding citrus fruit and citrus products involves the detection and prevention of spoilage due to growth of lactic acid bacteria and fermentative yeasts mentioned previously (Parish and Higgins 1989; Wyatt et al. 1995). Molds were previously not an issue, as the majority of juice was concentrated to 65 °Brix and frozen, creating an inhospitable environment due to low A_w and low storage temperature (Wyatt et al. 1995). With the increase in availability of single strength, not from concentrate OJ and technology that allows for greater shelf life, molds are now becoming a problem in citrus products, specifically OJ (Parish 1991; Parish and Higgins 1989). The aforementioned molds can utilize a variety of substrates such as pectin, carbohydrates, and organic acids found in OJ and have the ability to thrive in low pH (3 – 8) and low temperatures (0 – 30°C) (Huis in't Veld 1996; Lacey 1989). The ability to tolerate these conditions associated with single strength, not from concentrate OJ affords them an advantage over other yeasts and bacteria (Lacey 1989). Prior studies that examined fungal growth of the two primary fungi associated with citrus – *P. digitatum* and *P. italicum* – on citrus fruit demonstrated the organism's ability to substantially soften the fruit and produce a range of secondary metabolites. No previous studies have examined volatiles produced by this organism in contaminated citrus juice, specifically OJ (Filtenborg et al. 1996; Schnürer et al. 1999).

Penicillium digitatum

Overview

P. *digitatum*, also known as green mold, is classified as a soft rot wound parasite that grows primarily on the surface of post-harvest oranges. Green mold is considered the major cause of orange fruit deterioration during storage and transportation and one of the most important and damaging pathogenic fungi of harvested citrus (Ariza et al. 2002; Cole and Wood 1970; Droby et al. 2008; Eckert and Ratnayake 1994; Kavanagh and Wood 1971; Pesis and Marinansky 1990). Responsible for 90% of citrus losses during storage, this mold has not been reported to cause progressive decay on any other fruits or vegetables (Ariza et al. 2002; Droby et al. 2008; Eckert and Ratnayake 1994; Pelser and Eckert 1977). Post harvest decay begins when spores enter micro-injuries in the flavedo of the orange peel and germinate. Once germination begins, a mycelia mat and yellow-green spores develop and the fruit begins to soften (Bush and Codner 1970; Caccioni and others 1998). As the organism develops it produces enzymes and secondary metabolites that lead to the biotransformation of volatiles. This causes an increase or decrease in volatile metabolites present (Achilea and others 1985; Adams et al. 2003; Bush and Codner 1970).

Growth Conditions

Growth results when specific conditions and interactions of temperature, pH, A_w, and various stimulators are present (French 1985; Fries 1973). Filamentous fungi, especially *P*. *digitatum,* have been shown to survive and grow under extreme conditions, including low temperatures, pH, A_w and oxygen, not suitable for other microorganisms (Narciso and Parish 1997). *P*. *digitatum* spores lay dormant on the surface of oranges for extended periods of time and grow rapidly upon peel injury

(Droby et al. 2008). The organism's temperature growth range is 6 - 37°C with optimum growth occurring between 20 - 25°C; however, growth can occur at lower temperatures between 0 - 6°C with extensive growth at 7.6°C (Lacey 1989; Parish and Higgins 1989; Raccach and Mellatdoust 2007; Wyatt and Parish 1995). Studies have also shown the organism's tolerance for acids associated with citrus. Germination occurred 90% of the time in OJ with a pH of 3.5 with rapid germ tube development at pH 4. The organic acids in OJ buffer the juice, aiding in the maintenance of a favorable growth pH (Filtenborg et al. 1996; Fries 1973; Pelser and Eckert 1977). These extreme temperature and pH conditions give this organism an advantage over others by decreasing competition, while components and metabolites specific to OJ facilitate and stimulate fungal infection (Ariza et al. 2002; Lacey 1989).

Specificity to Orange Juice

Sugars found in OJ, including fructose, glucose, sucrose, and xylose, were associated with over 2/3 of *P*. *digitatum* growth, with glucose supporting 50 – 70% of germination (Filtenborg et al. 1996; Kavanagh and Wood 1971; Pelser and Eckert 1977). Pectin was also found to assist in the growth of the organism, causing increased infection (Kavanagh and Wood 1971). Fungal growth increased with volatile and non-volatile extracts emitted from wounded orange peel tissue. This demonstrated host selectivity. These extracts included limonene, α-pinene, sabinene, β-myrcene, acetaldehyde, ethanol, ethylene, L-ascorbic acid, citric acid, and L-malic acid (Droby et al. 2008; Eckert and Ratnayake 1994; Filtenborg et al. 1996; French 1985; Kavanagh and Wood 1971; Pelser and Eckert 1977). Research performed by French (1985) and Fries (1973) concluded nonanol, methyl heptenone, β-ionone, and benzaldehyde were associated with the greatest stimulatory effects (French 1985; Fries 1973). One

component alone, however, did not stimulate growth. Mixtures of various volatile and non-volatile extracts at specific concentrations were needed (Droby et al. 2008; Eckert and Ratnayake 1994; Stange and others 2002). Once infection begins, the fungus possesses the ability to transform its environment making it more hospitable. It does this by inhibiting other organisms through activating or blocking enzymatic reactions, removing or introducing inhibitors, and influencing nutrient uptake. An example of this is *P*. *digitatum*'s ability to produce mycotoxins that are only harmful to bacteria (Filtenborg et al. 1996; Fries 1973).

Association with Orange Juice

Even under ideal extraction conditions, pre-pasteurized juice contains a wide range of microorganisms (Faville and Hill 1951). The USFDA requires a 5-log pathogen reduction during processing, which is stated in the USFDA Juice Hazard Analysis and Critical Control Point Regulation. This pathogen reduction normally occurs during pasteurization, in order to eliminate most pathogens and heat sensitive microbes (Narciso and Parish 1997; Raccach and Mellatdoust 2007; Tournas et al. 2006). Post-pasteurization contamination, however, remains a concern (Parish 1991).

Technological advances that allow for increased shelf life of single strength, not from concentrate OJ, such as the gable top multilayer carton, have led to a shift from 65 °Brix juice concentrates to the more convenient pour and serve single strength, not from concentrate juice. This shift made mold contamination, which was not a major concern with concentrated juice, a growing problem (Parish 1991; Wyatt et al. 1995). The storage conditions of single strength, not from concentrate juice involve non-freezing conditions (0 - 4°C). In addition, single strength, not from concentrate juice has a higher A_w (>0.98) then concentrate. These conditions allow for mold growth and spoilage

(Parish 1991; Parish and Higgins 1989; Raccach and Mellatdoust 2007; Tournas et al. 2006). *P*. *digitatum* has been isolated from pasteurized juice. In a recent sampling of pasteurized, single strength, not from concentrate OJ from grocery stores in the Washington DC area, 22% contained fungal contamination of *Penicillium* species (Parish and Higgins 1989; Tournas et al. 2006).

Contamination

Spores of *P*. *digitatum* have been isolated from and are abundant in the atmosphere of citrus groves, packinghouses, and juice processing plants, and on food contact surfaces. Some isolated samples are tolerant of presently used fungicides, including 2-amino butane, sodium orthophenylphenate, thiabendazole, and benomyl, and an increasing incidence of resistance is occurring. The ubiquitous presence of spores is making post-pasteurization contamination of OJ a primary concern, as neither cartons nor filling systems are typically aseptic (Davé et al. 1981; Narciso and Parish 1997; Pelser and Eckert 1977; Wyatt and Parish 1995; Wyatt et al. 1995). Gable top cartons themselves are an area of contamination. Fungi have been isolated from the cardboard layer used in their construction. Inappropriately sealed or unfinished areas of the cartons allow the organism access to the juice. Eventually, the germ tube will reach the juice/air interface at the top of the carton and a mycelia mat will form (Narciso and Parish 1997).

Known Effects in Citrus

Enzyme production

P. *digitatum* has the ability to produce a wide range and vast number of enzymes that are stable and active at low pH and low temperature. These enzymes drive reactions that result in the production and destruction of compounds, including volatiles,

some of which aid in the proliferation of the organism as previously discussed. Even after the removal of the organism, the enzymatic reactions will continue until they are de-activated (Alaña et al. 1990; Bush and Codner 1968; Bush and Codner 1970; Filtenborg et al. 1996). Some of the enzymes associated with this organism as it grows on infected citrus fruit include pectin lyase, pectin methylesterase, pectin esterase, pectin transeliminase, olygalacturonase, exopolygalacturonase, cellulase, α-amylase, proteinases, and cytochrome P-450 enzymes (Alaña et al. 1990; Barmore and Brown 1979; Bush and Codner 1968; Cole and Wood 1970; Duetz et al. 2003; Kavanagh and Wood 1971).

Cloud destruction, pH, and °Brix changes

Some of the enzymes produced, such as pectin lyase and pectin esterase, are involved in cloud destruction by randomly splitting α-1,4 glycosidic bonds between galacturonic acid and carbohydrates. This results in OJ cloud destruction (Alaña et al. 1990; Barmore and Brown 1979; Wyatt et al. 1995). The reaction of pectin esterase breaks down pectin into pectate and methanol (Bush and Codner 1968). The primary roles of these enzymes is to allow the mold greater access to the fruit once it has entered the micro-injuries and breakdown compounds into simpler components for use as energy sources (Barmore and Brown 1979).

These enzymes have shown acidity resistance (Barmore and Brown 1979; Patrick and Hill 1959). The process of peel softening and cloud destruction is considered a three-step process, beginning with the collapse of the cytoplasm, followed by the swelling of cell walls, and finally cell separation. When this occurs in OJ, the viscosity decreases as the juice clarifies (Alaña et al. 1990; Barmore and Brown 1979). A slight

reduction in °Brix was noted after 12 days of *P*. *digitatum* incubation at 10°C, while the pH and total titratable acidity did not change (Wyatt et al. 1995).

Volatile metabolites and biotransformation

One hundred, ninety-six volatile metabolites, consisting of monoterpenes, alcohols, esters, ketones, and alkenes, most of which are considered secondary metabolites, have been associated with *P*. *digitatum* inoculated citrus fruit. Substrate composition greatly influenced the type and amount of metabolites produced (Ariza et al. 2002; Lacey 1989; Schnürer et al. 1999). Of the metabolites mentioned previously, alcohols, including ethanol, 1-octene-3-ol, and methanol, are considered to be extraordinary indicators of this organism, as they are produced in large amounts through various pathways (Ariza et al. 2002; Larsen and Frisvad 1995a; Nilsson and others 1996; Pesis and Marinansky 1990; Schnürer et al. 1999). Following alcohols, esters are the second largest group of volatiles identified and associated with this organism in whole fruit. The production of alcohols and esters is the result of fungal metabolic pathways, seen in Figure 2-1, and enzymatically driven reactions (Ariza et al. 2002; Larsen and Frisvad 1995a; Schnürer et al. 1999). For example, an increase in ethyl acetate is considered a quantitative indicator for the presence of green mold. Ethyl acetate formation results from the metabolic pathway that bioconverts acetaldehyde to acetic acid by acetyl CoA. Acetic acid then undergoes an esterification reaction with ethanol to form ethyl acetate (Pesis and Marinansky 1990; Schnürer et al. 1999).

Microbiologists have been aware of the ability of fungi to bioconvert compounds since the late 1960's. The mechanisms by which they accomplish these conversions are not well understood but include oxidation, reduction, dehydration, degradation, and

C-C bond formation reactions (Demyttenaere et al. 2001; Janssens et al. 1992). The majority of reactions are considered enzymatically driven, as they are not noted with simple acid catalyzation at pH 3.5, which is the pH of most OJ (Adams et al. 2003).

One of the most well studied bioconversions involving *P*. *digitatum* is the conversion of (*R*)-(+)-limonene to (*R*)-(+)-α-terpineol. (*R*)-(+)-limonene converts more readily then (*S*)-(-)-limonene at pH 3.5. The conversion has low specificity and leads to the formation of other volatiles including trans-carveol, cis-carveol, trans-limonene oxide, trans-*p*-menth-2-en-1-ol, carbone, γ-terpinene, terpinolene, and menth-3-en-1-ol. At a temperature of 28°C and pH 4.5, the bioconversion occurred within 8 – 12 hours, with the highest concentrations of α-terpineol noted between 1 and 2 days of incubation. Products were not recovered from a control, uninoculated flask at pH 3.5, confirming the reaction was enzymatically driven rather than simply acid-catalyzed (Adams et al. 2003; Demyttenaere et al. 2001; Duetz et al. 2003; Tan and Day 1998a; Tan and Day 1998b; Tan and others 1998). The mechanism of this bioconversion remains a great debate. An early suggested mechanism included hydrolysases and hydratases; however, a newly proposed mechanism involves P-450 monooxygenases with the initial step being an epoxidation of the 8-9 double bond followed by reductive cleavage of the epoxide (Demyttenaere et al. 2001; Duetz et al. 2003). The majority of studies examining volatile metabolites produced by *P*. *digitatum* involve the whole, contaminated orange or media containing orange pump-out, an industry term for OJ concentrates whose major volatiles have been removed during the concentration process. The reactions and metabolites observed in infected oranges were shown to be unique and associated with

individual species, furthering the idea that differentiating volatiles can be used as indicators of fungal growth (Larsen and Frisvad 1995a; Pesis and Marinansky 1990).

Metabolomics and Chemosystematics

Overview

Metabolomics is the study of all metabolites in a system, while chemosystematics, also referred to as chemotaxonomy, is the classification and identification of organisms based on all the metabolites produced in a system (Cevallos-Cevallos et al. 2009). Volatile metabolites are metabolites in a gaseous phase or those that have a high vapor pressure allowing for their liberation from a cell or substance into the headspace (Cai and others 2001; Hutchinson 1973). By focusing on the volatile metabolites produced by an organism, metabolomics and chemosystematics can be employed to develop a database of volatiles and methodology for examining volatiles for the use of identifying the microorganism based on specific differentiating volatiles (Cevallos-Cevallos et al. 2009; Larsen and Frisvad 1994; Schnürer et al. 1999). Previous metabolomic and chemosystematic studies have shown microbes can be distinguished with a clear separation of taxa down to the species level, based on their differentiating volatiles (Larsen and Frisvad 1994; Larsen and Frisvad 1995b).

Previous Applications

The previously discussed concept of metabolomics for the detection of microbes has been applied to the food industry. One study examined Russet Burbank potato tubers for the identification and discrimination of potato tuber diseases caused by *Phytophthora infestans*, *Pythium ultimum*, and *Botrytis cinerea*. Each organism produced two to three differentiating volatiles that allowed for their identification and subsequent identification of potato tuber disease (Lui et al. 2005). Other studies

examined mangos for the ability to identify and discriminate stem-end rot disease caused by *Lasiodiplodia theobromae* and anthracnose caused by *Colletotrichum gloeosporioides*. Thirty-seven volatiles were identified, with several being specific to each organism. When common to both organisms, the volatile varied in its abundance (Moalemiyan et al. 2007; Moalemiyan et al. 2006). Carrots were examined for the ability to identify *Botrytix cinerea*, *Erwinia carotovora* subsp. *Carotovora*, *Aspergillus niger*, and *Fusarium avenaceum*. As with the previous studies, each organism produced a specific series of differentiating volatiles that allowed for its identification (Vikram et al. 2006). The concept of differentiating volatiles was also applied to studies involving McIntosh, Cortland, and Empire apples for the identification and differentiation of *Botrytix cinerea*, *Penicillium expansum*, *Mucor piriformis*, and *Monilinia* spp. Each organism produced a specific set of volatiles that allowed for it to be differentiated (Vikram et al. 2004a; Vikram et al. 2004b). This concept has also been applied outside the food industry for the identification of mold in building supplies. Similar results to the previous studies were seen with the organisms tested (Wady and others 2003).

The methodology developed for testing the food products involved the use of a portable GC – MS. The intact samples were placed in mason jars and their headspace was sampled using the portable GC – MS. Volatile metabolites were concentrated on a Carbopack-X carbon trap prior to being desorbed and analyzed using the GC – MS (Lui et al. 2005; Moalemiyan et al. 2007; Moalemiyan et al. 2006; Vikram et al. 2006; Vikram et al. 2004a; Vikram et al. 2004b). The identification of mold in building supplies was performed using a SPME fiber to sample the headspace of a vial containing the specific

building material. The fiber was then desorbed and analyzed using a GC – MS (Wady et al. 2003).

Volatile Analysis

Overview

Traditional applications of volatile evaluation involved a trained sniffer who would sniff storage containers for distinctive odors associated with specific diseases. This method was highly subjective, required well-trained personnel, and by the time odors were at detectable concentrations, significant damage to the product had already occurred. Despite its faults, this method has been used for years in taxonomic classification of some microbes (Janssens et al. 1992; Moalemiyan et al. 2007). Newer methodology, which allows for improved and more standardized detection, involves the use of various methods to extract, concentrate, and analyze volatiles (Cai and others 2001). Some techniques currently employed to sample volatile metabolites include steam distillation extractions (SDE), purge and trap, direct headspace sampling with a syringe, and diffusion, which includes HS SPME (Larsen and Frisvad 1994; Larsen and Frisvad 1995c; Schnürer et al. 1999). SDE lacks sensitivity for certain volatiles, is time consuming, requires laborious manipulations, and utilizes heat. The heat can lead to the development of artifacts and/or the breakdown of volatiles (Cai et al. 2001; Larsen and Frisvad 1995c; Schnürer et al. 1999). Purge and trap is a good alternative to SDE, but requires a pump. Direct headspace sampling with a syringe or diffusion with a SPME fiber is considered optimal (Larsen and Frisvad 1994; Larsen and Frisvad 1995c).

Headspace Solid Phase Microextraction

HS SPME is a solvent-free extraction technique that uses a small fused silica fiber coated with a polymeric organic liquid. The fiber is introduced into the headspace above a sample. Volatiles in the headspace are adsorbed and concentrated in the coating of the fiber, which is then desorbed into analytical equipment such as a GC – MS for analysis (Cai et al. 2001; Nilsson et al. 1996; Zhang and Pawliszyn 1993). The type of fiber used determines the selectivity of extraction, as various fibers have differing affinities for assorted volatiles (Cai et al. 2001; Jeleń 2003). Studies examining these fibers to determine an optimal fiber for use in the HS SPME method determined that polydimethylsiloxane/divinylbenzene (PDMS/DVB) fibers have the highest sensitivity and lowest detection limits (Cai et al. 2001; Demyttenaere et al. 2001; Jeleń 2003). Temperature was also shown to play an important role in the equilibration of volatiles between a liquid medium and the headspace and the absorption of volatiles to the fiber. The optimum temperature was found to be 40 - 50°C, as this allowed for the most rapid equilibration and absorption times (Cai et al. 2001; Jeleń 2003). Agitation of the liquid medium also quickens the partitioning of volatiles between the medium and the headspace, decreasing the equilibration time (Zhang and Pawliszyn 1993).

As compared to SDE, SPME can identify and differentiate more volatiles without using a high temperature extraction (Jeleń 2003). Volatiles collected using HS SPME were comparable to those collected under similar conditions using a purge and trap methodology; however, the purge and trap technique required a pump (Nilsson et al. 1996; Schnürer et al. 1999). HS SPME has many advantages including simplicity, solvent-free nature of the extraction, short extraction time, no apparent sample hydrolysis, possibility of automation, and suitability for use in routine screening (Cai et

al. 2001; Jeleń 2003; Larsen and Frisvad 1994; Zhang and Pawliszyn 1993). Studies have shown the validity and advantage of this rapid, solvent-free extraction method and its capabilities to detect volatile metabolites produced by *P*. *digitatum* (Nilsson et al. 1996; Wady et al. 2003; Zhang and Pawliszyn 1993).

Application to Orange Juice

Filamentous fungi, specifically *P*. *digitatum*, are known to produce secondary volatile metabolites that can be detected using HS SPME (Filtenborg et al. 1996; Hutchinson 1973). By applying volatile metabolomics and chemosystematics to OJ and fungal contaminants, the detected volatiles can be associated with specific organisms and subsequently used to identify fungi down to the species level more rapidly than conventional microbiological methods (Cevallos-Cevallos et al. 2009; Hutchinson 1973; Larsen and Frisvad 1995a; Larsen and Frisvad 1995b). Studies have shown that *Penicillia* species can be distinguished from each other solely on their differentiating volatiles when grown on various media, such as PDA. These volatile metabolites can be detected after two days of fungal growth. This precedes the time required for the identification by morphological differences on the PDA plates (Larsen and Frisvad 1995b; Schnürer et al. 1999). By detecting the contamination early, appropriate steps can be taken to significantly reduce losses if adequate control measures are implemented (Lacey 1989; Vikram et al. 2006).

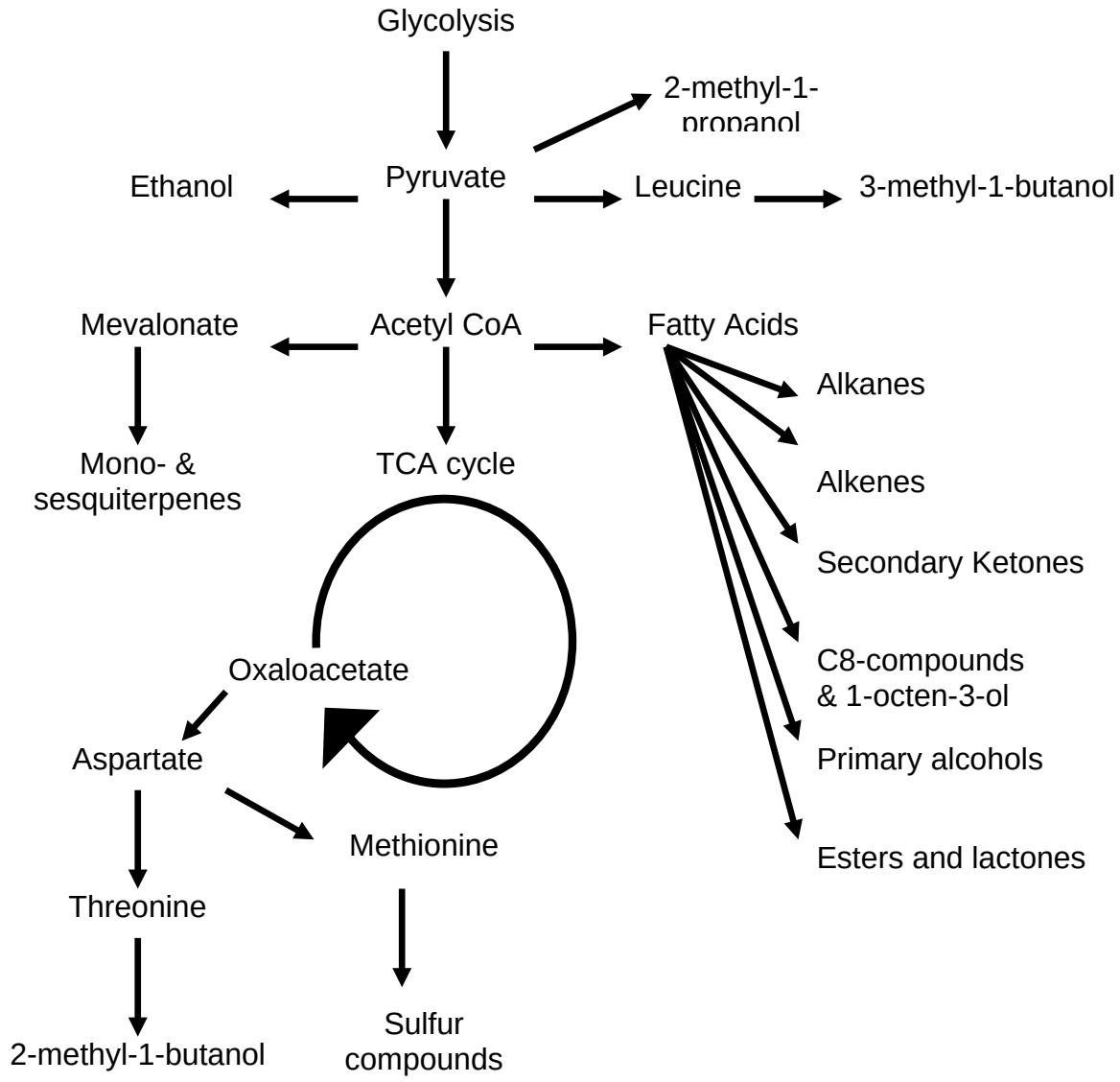


Figure 2-1. Fungal metabolic pathways and their resulting volatile metabolites from the initial substrate glucose

CHAPTER 3
MATERIALS AND METHODS

This study was undertaken to evaluate the biochemical changes that occur in single strength, not from concentrate OJ when contaminated with *P*. *digitatum*. Volatile changes in juice stored at room temperature (~22° C) occurring as a result of the submerged fungal colony within the first four days of inoculation were assessed. The experiment was repeated three times.

Penicillium digitatum **Preparation**

P. *digitatum* (Link) is an imperfect fungus commonly associated with decaying citrus. The culture for this study was obtained from the USDA/ARS, Citrus and Subtropical Products Research Laboratory, Winter Haven, FL. Spores of *P*. *digitatum* were plated on PDA plates (Difco, NJ) and incubated at room temperature until sporulation occurred, which was determined by morphological changes, such as the development of an olive-green mat. Mature colony plates were stored under refrigeration (5°C) in plastic bags to prevent dehydration.

Inoculum was prepared by placing a small amount of sterile 0.1% Tween 20 on the sporulating mat and slightly spreading the liquid to release spores. Spores were picked up from the plate surface with an inoculating loop and transferred into 9 mL of sterile 0.1% phosphate buffer and vortexed to keep the cells in suspension. Spore concentration was calculated using a haemocytometer and adjusted to 10^6/mL.

Sample Preparation

Simply Orange, pulp-free not from concentrate 100% pure pasteurized OJ (The Coca-Cola Company, FL) was purchased from a local Publix grocery store. The bottles of juice were frozen at -20°C at the Citrus Research and Education Center, Lake Alfred,

FL. When needed, a bottle was thawed in a refrigerator at 5°C and 100 mL of thawed juice was poured into sterile 250 mL Erlenmeyer flasks with screw caps. The juice was re-pasteurized by submerging the flasks 2/3 in a circulating water bath (model#2864, Thermo Scientific, NH), at 90°C for 5 minutes. The juice reached an internal temperature of 72°C. Flasks were immediately transferred to an ice water bath, removed and then left to equilibrate to ambient temperature (22°C).

When the juice was cooled, it was inoculated with *P*. *digitatum* using 0.01 mL of inoculum per pasteurized flask. A juice sample treated as the experimental with no inoculum was used as a control. Samples were placed on an Innova 2100 platform shaker plate (New Brunswick Scientific, NJ) set to 150 RPM and allowed to incubate at ambient room temperature (22°C).

Sampling

Samples from the control and inoculated juices were collected on days one, two, three, and four. An aliquot (10 mL) of juice was aseptically transferred to a 40 mL glass vial with a silicone/PTFE septa screw cap that contained a stir bar. On days one and three, 0.25 mL samples from the control and inoculated flasks were transferred to PDA plates to confirm *P*. *digitatum*.

°Brix and pH Analysis

Procedures to determine °Brix and pH were adapted from Wyatt and others (Wyatt et al. 1995). On days one, two, three, and four of incubation, prior to collecting the 10 mL samples used for volatile analysis, °Brix and pH analysis was performed. For °Brix, 0.25 mL of sample was placed on the lens of a PR-101 Palette digital refractometer (Atago, WA) that was previously calibrated using deionized (DI) water. The reading was taken. Subsequently, the sample was removed and the lens was cleaned thoroughly

with KimWipes. This process was repeated in triplicate for each sample on each day, and the average and standard error was calculated. A MiniLab, ISFET pH meter (model# IQ125, IQ Scientific, Loveland, CO) was calibrated using a standard buffer solution pH 7.00 (SB107-500) and pH 4.00 (SB101-500) (Fisher Scientific, NC). After calibrating, a 0.25 mL sample from the control flask was transferred to the probe to obtain the pH reading. The procedure was then repeated with a 0.25 mL sample from the inoculated flask, with adequate cleansing with DI water in between samples.

Volatile Analysis

Headspace Solid Phase Microextraction

The 40 mL glass vial prepared earlier was 1/3 submerged in a 40°C water bath containing a stir bar spinning at low speed and allowed to equilibrate for 15 minutes. After the equilibration period, a 1 cm 50/30 µm DVB/Carboxen™/PDMS StableFlex™ fiber (Supelco, PA) was inserted into the vial, suspended over the headspace, and exposed for 5 minutes. The exposure time was lowered to 10 seconds for the analysis of limonene only on the GC – MS. The apparatus setup can be seen in Figure 3-1. At the end of the exposure time, the fiber was fully retracted into the needle, removed from the bottle, and injected into the appropriate GC as seen below.

Gas Chromatography – Mass Spectrometry

A Claurus 500 GC – MS system (Perkin Elmer, MA) was used for volatile analysis and identification. The system contained a Stabilwax, polar (wax) column 60 m, 0.25 mm ID, 0.5 µm (Restek, PA) using helium as the carrier gas at 2 mL/min. Both the GC and MS were controlled using TurboMass software, version 5.4.2 (Perkin Elmer, MA).

GC – MS general program

The general program was developed to examine the overall volatile spectrum while not identifying limonene, as the high concentration of limonene overloaded the detector and overshadowed smaller peaks. The GC temperature program began at 40°C for 2 minutes followed by a ramp of 7°C/min up to 240°C with a sampling rate of 1.5625 pts/sec for a total run time of 30.57 minutes. The injector temperature was set at 220°C, while the detector was set at 275°C. The MS program consisted of a spectrometer scanning 25 to 300 m/z from 2.00 to 30.10 minutes with a delay at 13.25 to 13.79 minutes.

GC – MS limonene and SIR program

This program was established to examine limonene only, as the SPME exposure time had to be reduced to 10 seconds. The GC temperature program began at 40°C for 2 minutes followed by a ramp of 7°C/min up to 140°C. At 140°C, a second ramp of 20°C/min began and went up to a final oven temperature of 240°C. The sampling rate was 1.5625 pts/sec, and the program had a total run time of 21.29 minutes. The injector temperature was set at 220°C, while the detector was set at 275°C. This GC temperature program was run with two different MS programs, each used with separate injections. The first program, denoted limonene, consisted of a spectrometer scanning 25 to 300 m/z from 2.00 to 19.40 minutes, while the second program, denoted SIR, scanned from 3.01 to 20 minutes while only detecting masses at 121 and 136 m/z.

GC – MS peak identification

Chromatograms were produced and analyzed using the TurboMass software. Peak area minimums were established at 500,000 for detection. Identification of peaks was first performed by examining the spectral analysis followed by confirmation using

web database linear retention index (LRI) values, as compared to observed (±1%) which was calculated by a standard curve generated from a series of low and high alkane standards comprised of compounds with various carbon chain lengths and known retention times.

Gas Chromatography – Sulfur

Volatile sulfur metabolites were analyzed using a 7890A GC System (Agilent Technologies, CA) with a pulsed flame photometric detector (PFPD) and a Stabilwax, polar (wax) column 30 m, 0.32 mm ID, 0.5 µm (Restek, PA). The instrument data was collected by Chrom Perfect® Data software, version 6.0.4 (Justice Laboratory Software, NJ). The oven temperature program began at 40°C followed by a ramp of 7°C/min up to 240°C with a 5 minute end hold for a total run time of 34 minutes. The injector temperature was set at 200°C, and the PFPD detector was set to 250°C.

The chromatograms were analyzed using Chrom Perfect ®. LRI values were calculated based on a standard curve generated from a series of low and high alkane standards with various carbon chain lengths and known retention times. These were then compared to LRI values from standards with various carbon chain lengths and known retention times run on the GC – S with a Stabilwax, polar (wax) column in Dr. Russell Rouseff's lab (±1%).

Gas Chromatography – Olfactometry

A 6890N Network GC System (Agilent Technologies, CA) in split-mode with a flame-ionized detector (FID) was used for the detection of aroma impact compounds. The column inside the machine was a DB-Wax, polar 30 m, 0.32 mm ID, 0.25 µm column (J&W Scientific, Agilent Technologies, CA). The instrument data was collected by Chrom Perfect® Data software, version 6.0.4 (Justice Laboratory Software, NJ). The

oven temperature program began at 40°C followed by a ramp of 7°C/min up to 240°C with a 5 minute end hold for a total run time of 34 minutes. The injector temperature was set at 220°C, and the FID detector was set to 275°C.

Aroma Impact Compound Identification

Sniffing began approximately 2 minutes after the injection to allow for retained volatiles to purge from the column. The nose was positioned ½ inch from the olfactometer while breathing normally. As an odor was detected, the indicator bar of the Tigre Laboratory Interface (Justice Laboratory Software, NJ) was moved in proportion to the intensity of the odor. The bar was quickly returned back to zero after indicating the intensity, recorded as peak height. A descriptor of the odor was recorded, along with the time of elution, by the sniffer. This data was recorded as an aromagram by the Chrom Perfect® software. The set-up of this instrument can be seen in Figure 3-2. The aromagrams were analyzed using Chrom Perfect ® and the LRI values were calculated based on a standard curve generated from a series of low and high alkane standards with various carbon chain lengths and known retention times and compared to those identified on the GC – MS (±1%).

Data Treatment

The experiment was performed three times. Percent difference from control for GC – MS and GC – S volatiles was calculated based on averages. The averages, along with the standard error for the averages, were calculated using Microsoft Excel software. For GC – O, peak heights were averaged and standard errors calculated also using Microsoft Excel software. Values were analyzed and subjected to principal component analysis using The Unscrammbler® X, versions 10.0.1 (Camo Software, NJ). The principal component analysis was used as a tool to assess differences

between days based on totals from volatiles determined to be differentiating. To determine differentiating volatiles, the specific volatile in question had to be significantly different when comparing control and inoculated samples. From consultation with a statistician, equation 3-1 was used to calculate a highly conservative percent difference (80%), based on the number of control samples ($N_1 = 3$) and inoculated samples ($N_2 = 3$), required to determine significant difference. This calculated value, 80% difference, was too conservative for the study and lowered to 50% difference required to state significant difference between samples on recommendation from the statistician. With the ability to state a specific volatile was significantly different from control and inoculated samples allowed for the volatile to be classified as differentiating.

3-1. Percent difference for significant difference = $(1.96 \times \sqrt{(1/N_1 + 1/N_2)}) \times 100$

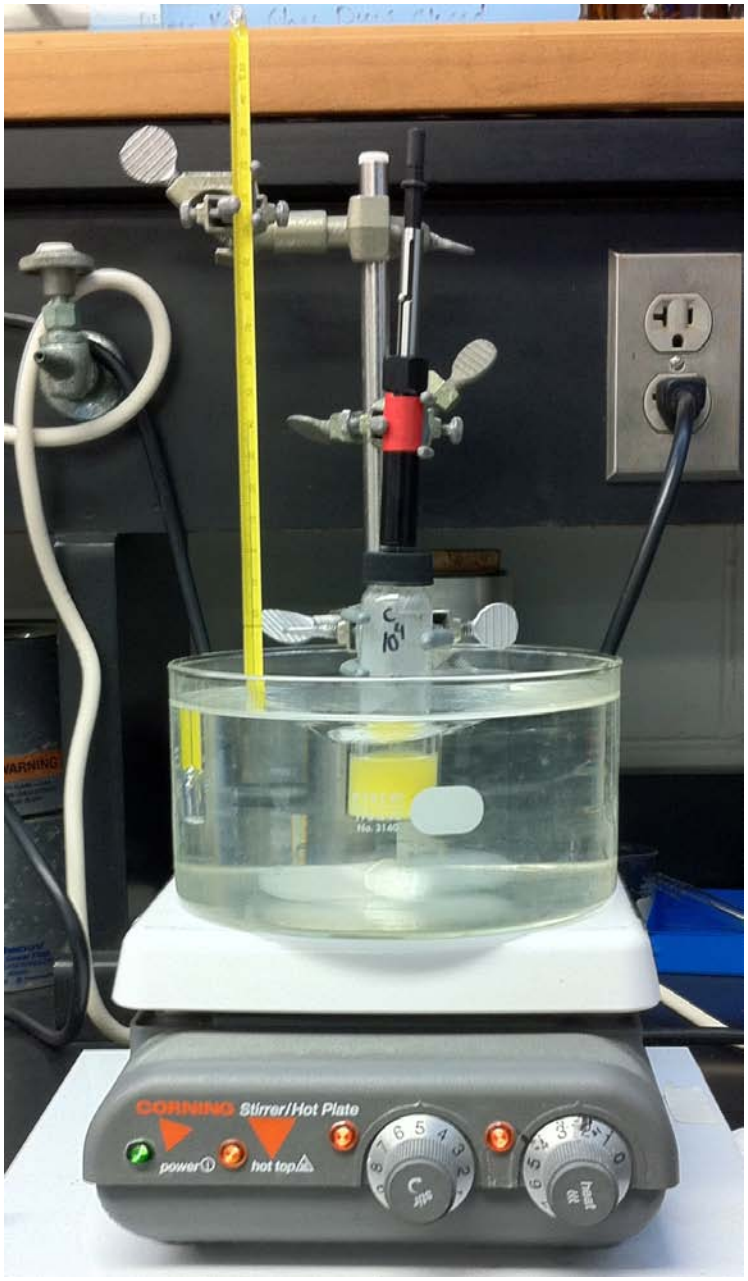


Figure 3-1. HS SPME volatile sampling apparatus setup


A

B

Figure 3-2. GC – O instrument with A) tube aromas elute from and B) Tigre Laboratory Interface aroma intensity indicator bar

CHAPTER 4
PHYSICAL AND CHEMICAL CHANGES

Cloud Destruction

Cloud destruction as a result of *P. digitatum* contamination was noticeable after two days of incubation. In the Erlenmeyer flask containing the inoculated sample, a mycelia puck, ~30 mm diameter and ~10 mm thick, began forming at incubation day two and was fully formed by day three. This mat was absent from the control sample (Figure 4-1, A). The mycelia puck was a bright orange color, similar to the color of the control juice. When the juice was extracted for sampling, the control sample was an opaque orange while the inoculated sample was clear with a yellow hue (Figure 4-1, B). By day three of incubation, the juice achieved maximum cloud destruction and there were no visible differences in the inoculated juice between further incubation days.

This physical change was observed in previous studies performed by Alaña and others (1990), Barmore and Brown (1979), Bush and Codner (1968), and Wyatt and others (1995). Various pectin degrading enzymes, including pectin esterases, cause this change by splitting α-1,4 glycosidic bonds between galacturonic acid and carbohydrates (Alaña et al. 1990; Barmore and Brown 1979; Bush and Codner 1968; Wyatt et al. 1995). The rapid cloud destruction supports the idea that multiple enzymes were present in abundance and worked in conjunction (Alaña et al. 1990; Bush and Codner 1968; Doyle and Beuchat 2007; Filtenborg et al. 1996). In addition, the juice pH was initially 3.7, close to the optimum pH of 4.5 for some enzymes including polygalacturonase and exopolygalacturonase, which assisted with the rapid cloud destruction (Barmore and Brown 1979; Patrick and Hill 1959).

Change in pH

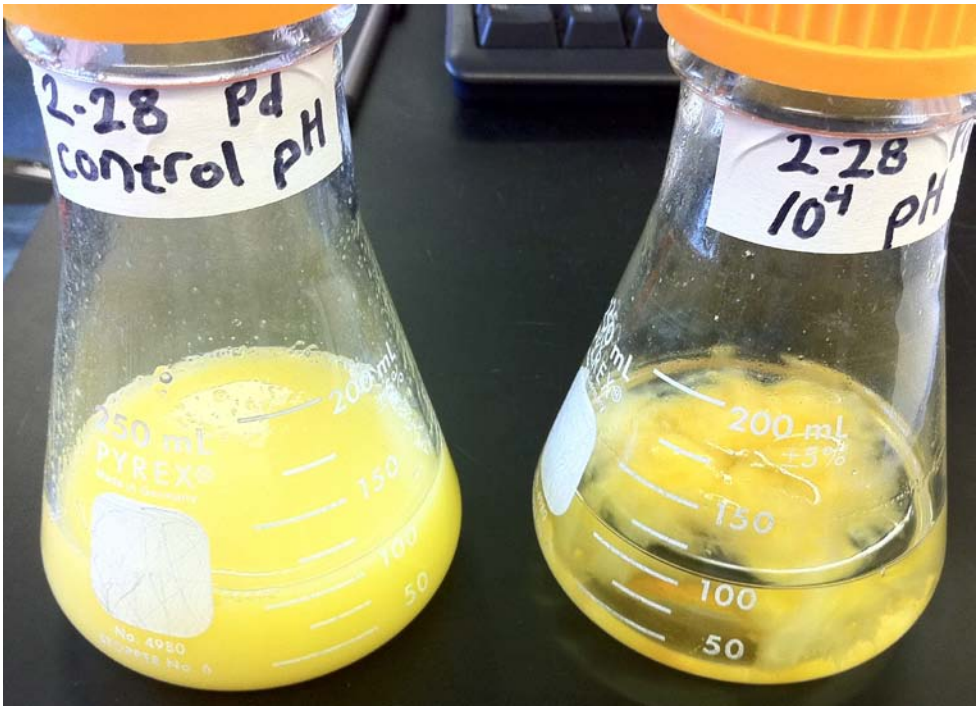
The pH of the control and inoculated juice was monitored over the four day incubation period. The pH of juice was ~3.7. It can be seen in Table 4-1 that the percent difference between samples was at most 2.6 ± 1.52% on incubation day three and decreased to a percent differences of 1.8 ± 0.90 on day 4. Other incubation days' percent differences were less than 2.6 ± 1.52%. The relative stability in the juice pH was noted in literature and is explained by the buffering capabilities of the organic acids such as citric acid (Filtenborg et al. 1996; Fries 1973; Pelser and Eckert 1977; Wyatt et al. 1995). pH stability helps to maintain an optimum environment for *P*. *digitatum* and its enzymes while inhibiting competition from others (Ariza et al. 2002; Lacey 1989).

Change in °Brix

Noted in previous studies by Wyatt and others (1995) and seen in this study was a reduction in °Brix within a few days of inoculation. Wyatt found that °Brix began decreasing after 12 days at 10°C (Wyatt et al. 1995). This study, however, found an 11.05 ± 1.52% reduction in °Brix began after day three at 22°C (Table 4-1). The higher incubation temperature (22°C as compared with 10°C) of this study can explain the earlier decrease in sugar content, as the organism was more active at the elevated incubation temperature.

One would expect the sugar content to decrease starting at day one of incubation, as sugars are one of the primary energy sources entering the metabolic pathway through glycolysis seen in Figure 2-1 (Filtenborg et al. 1996; Kavanagh and Wood 1971; Pelser and Eckert 1977). A relationship exists between sugar content and breakdown of pectin. The enzymes causing cloud destruction are dissolving the cellulose, a component of cell walls, into simpler components including sugars (Barmore and Brown

1979). This production and supply of sugar could be relatively equal to the amount being used by the organism for the first three days of incubation. Starting at the fourth day, the supply of sugar from the breakdown of cellulose becomes depleted, so the sugars used by the organism are not being replenished, explaining the observed decrease. Visual observation of cloud destruction reaches its optimum between day three and four, after which no increase in cloud destruction is noted. This timeline of cloud destruction coincides with the beginning of a decrease in °Brix, supporting the correlation between observed cloud destruction and decrease in °Brix.



A


B

Figure 4-1. Control and inoculated sample, respectively at incubation day four, A) taken while in Erlenmeyer flasks and B) after 10 mL samples were extracted.

Table 4-1. pH and °Brix percent difference from control with standard error for the four day incubation period

	Percent difference from control ± standard error	
Incubation period	pH	°Brix
Day 0	(-) 0.90 ± 0.90	(-) 0.38 ± 0.19
Day 1	0.88 ± 0.88	(-) 0.18 ± 0.25
Day 2	(-) 1.76 ± 0.88	(-) 0.37 ± 0.34
Day 3	(-) 2.63 ± 1.52	(-) 2.61 ± 1.31
Day 4	(-) 1.80 ± 0.90	(-)11.05 ± 1.52

CHAPTER 5
CHANGES IN ORANGE JUICE VOLATILES DUE TO *PENICILLIUM DIGITATUM*

Gas Chromatography – Mass Spectrometry Volatiles

The HS SPME analysis of the samples analyzed using a GC – MS with a polar (wax) column identified 102 volatiles (Table A-1). Of those 102, 44 volatiles differentiated from the control at least ~50% by incubation day four. The ~50% difference from control was established to identify the compound as a differentiating volatile (Table 5-1). The exception to this rule was 4-terpineol as its maximum percent difference was 32.9 ± 4.7% on day four. It was retained as a differentiating volatile for further analysis of the bioconversion of limonene. These differentiating volatiles are either only present in the control or inoculated sample (qualitative difference) or are present in both, but differ in quantity, either greater than or less than that in the control by at least ~50% (quantitative difference). These qualitative and quantitative differences between the inoculated sample and the control sample can be used to distinguish the sample contaminated with *P*. *digitatum*.

Chromatographic Differences

Comparing chromatograms of the control sample to those from inoculated samples for each day allowed for an initial analysis of both qualitative and quantitative differences. The chromatogram for day one, with the inoculated sample's chromatogram above the control's chromatogram, can be seen in Figure 5-1. Days two and three are a progression of increasing and decreasing peaks culminating at day 4. The chromatogram for day four can be seen in Figure 5-2. On first observation, it is easy to see that some peaks are either greater or lesser in the inoculated as compared to the control sample. The volatile that eluted at a retention time (RT) of 7.06 minutes

with an LRI of 947, identified as ethanol, increased over the four day incubation time. Limonene, at a RT of approximately 13.67 minutes, LRI 1230, decreased in percent difference from (-) 7.3 ± 4.8 at incubation day 1 to (-) 99.0 ± 0.3 at incubation day four. Two other obvious peaks include those at RT 16.16 minutes (LRI 1354), identified as hexanol, and RT 20.15 minutes (LRI 1569), identified as octanol, which both increased initially, but decreased on days three and four. The visual analysis is an extremely rudimentary method, however, and more quantitative evaluation is required.

Volatiles of Greatest Difference

By examining the percent difference of inoculated volatiles, as compared to the control, volatiles of greatest percent difference for each incubation day can be determined. Figures 5-3, 5-4, 5-5, and 5-6 display the top 10 volatiles with the greatest percent increase from control and the top 10 volatiles with the greatest percent decrease from control. For incubation day one, the top two volatiles with the greatest percent increase are hexanol and α-terpineol. For day two, however, the top two are α-terpineol and α-terpinolene. These change again for day three, hexanol and α-terpinolene, and day four, hexanol and ethanol. The top two volatiles of greatest decrease for day one are neryl acetate and hexanal. For incubation day two, it is neryl acetate and benzaldehyde, for day three, neryl acetate and citronellyl, and day four carvone and α-selinene. The percent difference changes between each incubation day for each quantitative volatile, except when the volatile decreased to undetectable limits, making it a qualitative volatile. Once below detectable limits, the volatile would only be detected in the control. Since each day is unique, it may be possible to not only identify *P*. *digitatum* from differentiating volatiles, but also determine how long the organism has been present based on the percent difference of various volatiles. For application of the

concept, further studies and validation would be required under different incubation conditions.

Volatile Changes During Incubation

Analysis of the FID chromatograms initially identified that there were at least 102 volatiles. Of those 102 volatiles, 44 were determined to be differentiating volatiles as discussed previously. The differentiating volatiles were categorized based on whether they were differentiating from the control qualitatively or quantitatively. Volatiles with quantitative differences were then classed as whether their percent difference from control was increasing, decreasing, or intermediate. Intermediate volatiles initially increased in percent difference then decreased during days three and four. Changes from the control represent the differentiating volatiles used to detect contamination and identify *P*. *digitatum*.

Figure 5-7 is a representation of the overall trend in volatiles throughout the four days of incubation. An initial increase in the amount of volatiles in the inoculated sample is noted. This increase in volatiles is possibly due to the organism's metabolic pathway (Figure 2-1) using sugar as an energy source, resulting in the production of volatiles. The decrease in volatiles seen in days three and four is representative of the bioconversion or degradation of volatiles due to enzymes into non-volatile compounds or compounds with a lower affinity to the SPME fiber used, making them less detectable.

Qualitative differences: Volatiles present in only inoculated or control samples

Seven volatiles, consisting of alcohols and esters, were found to be qualitative differentiating volatiles, either only in inoculated or control juices (Table 5-2). The majority of these volatiles, including methanol, ethyl propanoate, ethyl 2-

methylbutanoate, 3-methylbutanol, (Z)-3-hexenol, and 1-octen-3-ol, were only present in the inoculated sample. Neryl acetate, an ester, was the only qualitative volatile present in the control and not in inoculated juices.

The qualitative differences between samples represent the most promising potential target volatiles. Target volatiles are volatiles that can be used to easily identify the presence of contamination since they are either present or absent in the juice. Their presence or absence would signify contamination. For example, if the juice was sampled and either of the volatiles only seen in the inoculated sample was detected, or in the case of neryl acetate, not detected, it would be an important indicator that *P. digitatum* contamination has occurred.

Quantitative differences: Increasing volatiles

Of the 44 differentiating volatiles, a mixture of seven alcohols and esters, were found to be increasing in percent difference as compared to the control over the four day incubation period (Table 5-3). This was expected because alcohols, followed by esters, are volatiles consistently associated with *P. digitatum*, discussed previously (Ariza et al. 2002; Larsen and Frisvad 1995a; Nilsson et al. 1996; Pesis and Marinansky 1990; Schnürer et al. 1999).

Volatiles that increased as a result of inoculation can be used to detect contamination and determine the presence of *P. digitatum* based on the increase in percent difference when there is significant difference (>50% difference). Unlike qualitative differences, which are either present or absent, quantitative differences can only be determined as such when compared to an uninoculated control sample or baseline concentration of the differentiating volatiles. This comparison is required to determine the percent difference in order to classify the volatile as increasing in amount.

For example, ethyl acetate is considered a quantitative indicator for the presence of *P*. *digitatum* (Ariza et al. 2002; Larsen and Frisvad 1995a; Schnürer et al. 1999). This study found that ethyl acetate does increase by 361.4 ± 173.6% by incubation day four as the result of *P*. *digitatum* contamination. The determination that ethyl acetate did increase in amount, however, was only accomplished by comparing the inoculated sample to the control sample and calculating percent difference. Without the comparison, the percent difference and overall change in amount would not have been able to be determined.

Quantitative differences: Decreasing volatiles

The majority of differentiating volatiles, as compared to the control, decreased during the four day incubation period. This helps explain the overall decrease in volatile peak area seen in table 5-7. Table 5-4 contains the 25 volatiles, consisting of aldehydes, alcohols, esters, monoterpenes, cyclic terpenes, bicyclic terpenes, sesquiterpenes, bicyclic sesquiterpenes, tricyclic sesquiterpenes, a terpinoid, and a furanoid, that decreased in percent difference compared to the control. Some volatiles, such as ethyl hexanoate, decreased to undetectable levels four days after inoculation.

As with the volatiles which increased as a result of inoculation, those that decreased can be used to detect contamination and determine the presence of *P*. *digitatum*. A value to compare the differentiating volatiles to is required in order to determine that a percent decrease occurred. With some compounds, like ethyl hexanoate, this comparison is not needed since the compound decreases to undetectable limits by day four. This means the quantitative ethyl hexanoate is now a qualitative volatile and its absence signifies contamination. In addition to signifying contamination, since the compound is present in the inoculated sample until day four,

the absence of the compound signifies the organism has been present in the sample for at least four days.

Quantitative differences: Intermediate volatiles

Consisting of esters, alcohols, a cyclic terpene, and a bicyclic terpene, 12 intermediate volatiles were identified (Table 5-5). These volatiles, like those found to be increasing or decreasing, can only be determined as an intermediate when compared to a control or standard baseline in order to calculate percent difference. As seen in Table 5-5, each volatile has a specific day it reaches its maximum percent difference. Based on this information, these volatiles and their maximum percent difference could be used as indicators of how long the organism has been present. For example, if a sample was tested and hexyl acetate was determined to be at its maximum percent difference, it could be assumed that the organism has been present for two days, based on this study. Application of this concept would require not only a baseline concentration for comparison, as is required to determine an increasing or decreasing volatile, but it would also require specific studies performed under the storage and quality conditions of the applicable OJ. Alterations in storage conditions, such as fluctuations in temperature or pH, would alter the day a maximum percent difference is observed since substrate composition and environmental factors influence the production of metabolites (Larsen and Frisvad 1995a).

Gas Chromatography – Sulfur Volatiles

The purpose of running samples on the GC – S with a PFPD detector was to examine sulfur volatiles specifically, as they normally do not appear with analysis using a GC – MS with FID detector (Acree and Arn 2004; Reineccius 2006). Since the GC – S was not attached to a MS and multiple columns per sample could not be used,

tentative identification was based on the comparison of observed LRI values with standards run on the GC – S with a Stabilwax, polar (wax) column in the lab.

The analysis using the GC – S observed 14 volatiles (Table A-2), with 12 of those being differentiating volatiles, four of which could be tentatively identified (Table 5-6). The differentiating volatiles were determined based on the same requirements as the GC – MS volatiles, a percent difference from control of at least ~50%. Some of these differences in sulfur volatiles were noticeable upon initial analysis of the control and inoculated chromatogram. Figure 5-8 is the chromatogram from incubation day four with the inoculated sample chromatogram above the control chromatogram. It is clear that the compound at RT of 6.82 minutes (LRI 1093), tentatively identified as dimethyl disulfide, has decreased in the inoculated sample from control.

As with the GC – MS, the overall trend for differentiating sulfur volatiles in the inoculated sample was a decrease in amount, seen in Figure 5-9, as compared to the control. This decrease did not begin until incubation day four. Until day four, the amount of sulfur volatiles in the inoculated sample was similar to that of the control. When examining the differentiating sulfur volatiles in more detail, they were observed to differentiate from the control sample qualitatively and quantitatively. For example, Sulfur volatile LRI 1484 was a qualitative volatile being only present in the control sample. Sulfur volatile LRI 738, tentatively identified as dimethyl sulfide, is observed to decrease in the inoculated sample as compared to the control, classifying it as a quantitative difference.

These differentiating volatiles, both qualitative and quantitative, were further examined. Volatiles of greatest difference for each incubation day can be seen in

figures 5-10, 5-11, 5-12, and 5-13. Sulfur volatile LRI 1789 was the volatile of greatest increase at day one; however, this compound begins to decrease on day two and by day three and four, this sulfur volatile was of greatest decrease. Dimethyl disulfide was of greatest increase during incubation days two and three, identifying it as an intermediate volatile. The applications of these findings are similar to the GC – MS. Each day, the inoculated sample contained a series of differentiating volatiles, both qualitative and quantitative, which can be used to identify *P. digitatum* contamination along with how long the organism has been present.

These differentiating volatiles utilize the same application as those identified with the GC – MS. The differentiating sulfur quantitative volatiles require a baseline for comparison in order to determine any change. Further studies are needed to accurately identify these volatiles. The addition of this knowledge further supports the potential of the concept that the differentiating volatiles can be used as indicators of *P. digitatum* contamination.

Table 5-1. Differentiating volatiles

Compound	Observed LRI	Literature LRI*	Day 1 percent difference from control (% ± standard error)	Day 4 percent difference from control (% ± standard error)
Ethyl acetate	900	890	109.1 ± 73.1	361.4 ± 173.6
Methanol	909	899	0.0 ± 0.0	100.0 ± 0.0
Ethanol	947	947	19.6 ± 11.2	965.5 ± 29.2
Ethyl propanoate	974	951	0.0 ± 0.0	100.0 ± 0.0
Methyl butanoate	1002	990	(-) 34.4 ± 0.0	585.5 ± 441.7
Ethyl butanoate	1050	1048	13.4 ± 13.3	(-) 47.1 ± 29.7
Ethyl 2-methylbutanoate	1065	1062	0.0 ± 0.0	100.0 ± 0.0
Hexanal	1098	1099	(-) 61.8 ± 11.4	(-) 95.8 ± 2.1
Δ-3-carene	1161	1148	(-) 8.3 ± 4.0	(-) 62.6 ± 5.7
Myrcene	1171	1172	69.9 ± 62.5	(-) 97.2 ± 1.7
Methylbutanol, 3-	1212	1212	0.0 ± 0.0	100.0 ± 0.0
Limonene	1230	1205	(-) 7.3 ± 4.8	(-) 99.0 ± 0.3
B-phellandrene	1238	1241	3.4 ± 2.0	(-) 91.0 ± 3.5
Ethyl hexanoate	1245	1246	2.8 ± 1.0	(-) 100.0 ± 0.0
Γ-terpinene	1267	1260	(-) 5.4 ± 1.9	(-) 100.0 ± 0.0
Hexyl acetate	1286	1267	(-) 4.2 ± 3.6	(-) 53.3 ± 26.3
A-terpinolene	1300	1298	(-) 6.6 ± 3.5	353.6 ± 101.2
Octanal	1310	1309	(-) 38.8 ± 9.0	(-) 95.9 ± 1.2
Hexanol	1359	1364	357.2 ± 125.0	3078.2 ± 829.8
(E)-2-hexenol	1383	1388	(-) 44.1 ± 28.0	(-) 100.0 ± 0.0
(Z)-3-hexenol	1398	1399	0.0 ± 0.0	100.0 ± 0.0
Nonanal	1417	1416	(-) 14.9 ± 3.9	(-) 90.1 ± 1.7

*Database LRIs (Rouseff 2006)

Table 5-1. Continued

Volatile	Observed LRI	Database LRI*	Day 1 percent difference from control (% ± standard error)	Day 4 percent difference from control (% ± standard error)
Perillene	1442	1409	15.7 ± 1.9	(-) 100.0 ± 0.0
Ethyl octanoate	1451	1450	11.4 ± 9.2	(-) 86.7 ± 1.4
Octen-3-ol, 1-	1465	1452	0.0 ± 0.0	100.0 ± 0.0
Octyl acetate	1492	1480	10.2 ± 1.7	(-) 92.1 ± 2.3
Decanal	1526	1523	(-) 6.6 ± 3.9	(-) 85.1 ± 3.7
A-copaene	1531	1536	13.6 ± 2.3	(-) 100.0 ± 0.0
Linalool	1558	1557	8.7 ± 6.9	(-) 46.9 ± 10.3
Octanol	1569	1565	177.8 ± 50.7	57.1 ± 51.6
Benzaldehyde	1575	1555	(-) 10.4 ± 7.1	(-) 100.0 ± 0.0
B-elemene	1626	1595	13.5 ± 1.4	(-) 100.0 ± 0.0
Terpineol, 4-	1636	1616	(-) 4.6 ± 3.3	20.0 ± 2.0
B-caryophyllene	1646	1641	15.6 ± 1.3	(-) 85.1 ± 4.8
Citronellyl acetate	1680	1672	28.7 ± 5.6	(-) 100.0 ± 0.0
B-selinene	1690	1711	11.8 ± 5.5	(-) 89.0 ± 3.0
A-terpineol	1730	1724	264.6 ± 112.5	178.8 ± 108.1
Neryl acetate	1746	1742	(-) 100.0 ± 0.0	(-) 100.0 ± 0.0
Valencene	1770	1763	11.8 ± 5.3	(-) 98.4 ± 0.5
A-selinene	1779	1724	7.2 ± 1.5	(-) 100.0 ± 0.0
Carvone	1788	1779	(-) 5.4 ± 4.4	(-) 100.0 ± 0.0
Δ-cadinene	1798	1794	11.8 ± 0.1	(-) 74.4 ± 7.2
A-panasinsen	1819	1840	10.1 ± 0.3	(-) 80.5 ± 5.9
Cis-carveol	1864	1855	10.6 ± 0.5	(-) 61.4 ± 5.5

*Database LRIs (Rouseff 2006)

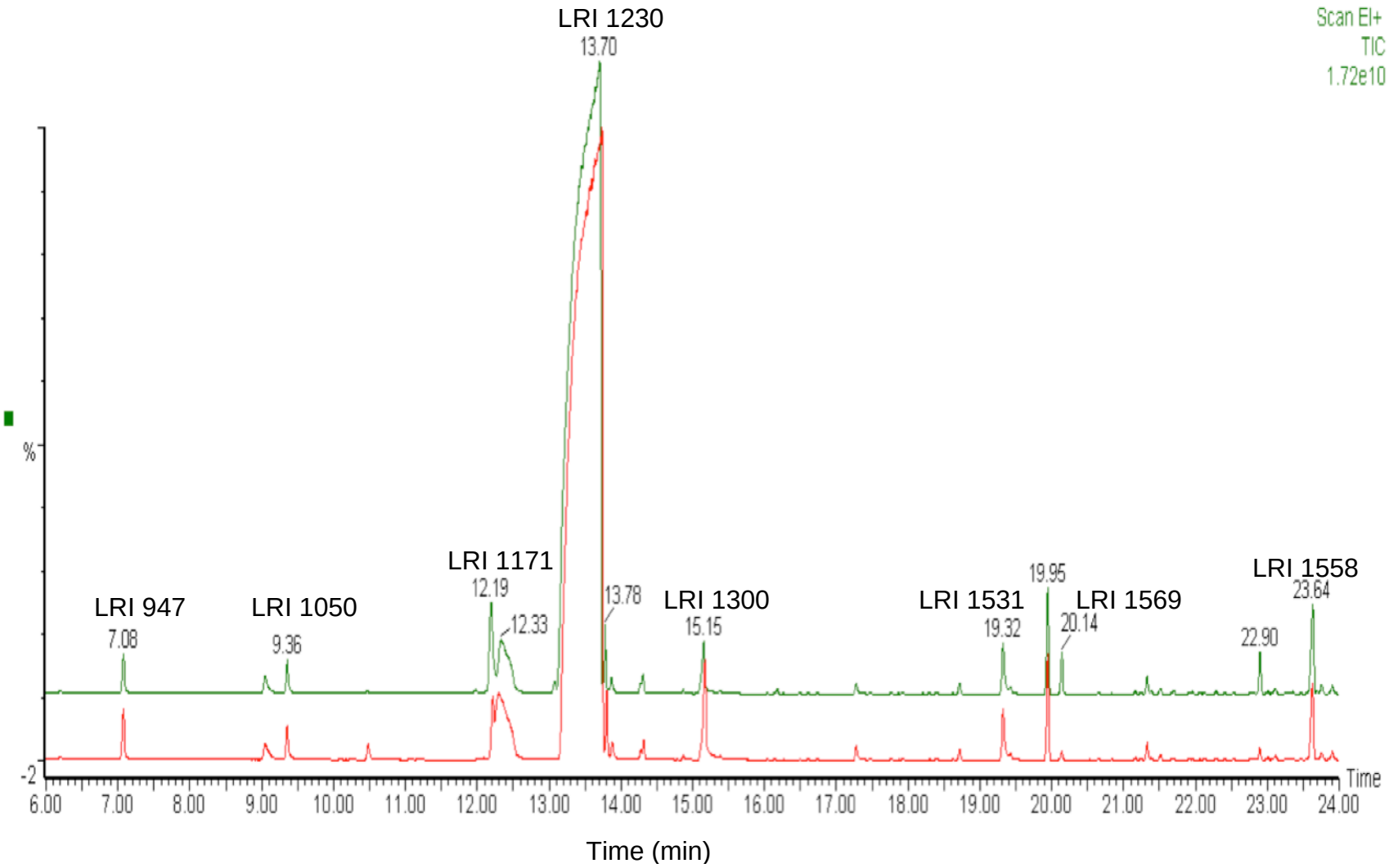


Figure 5-1. MS chromatograms of control (lower) and inoculated (upper) OJ at incubation day one

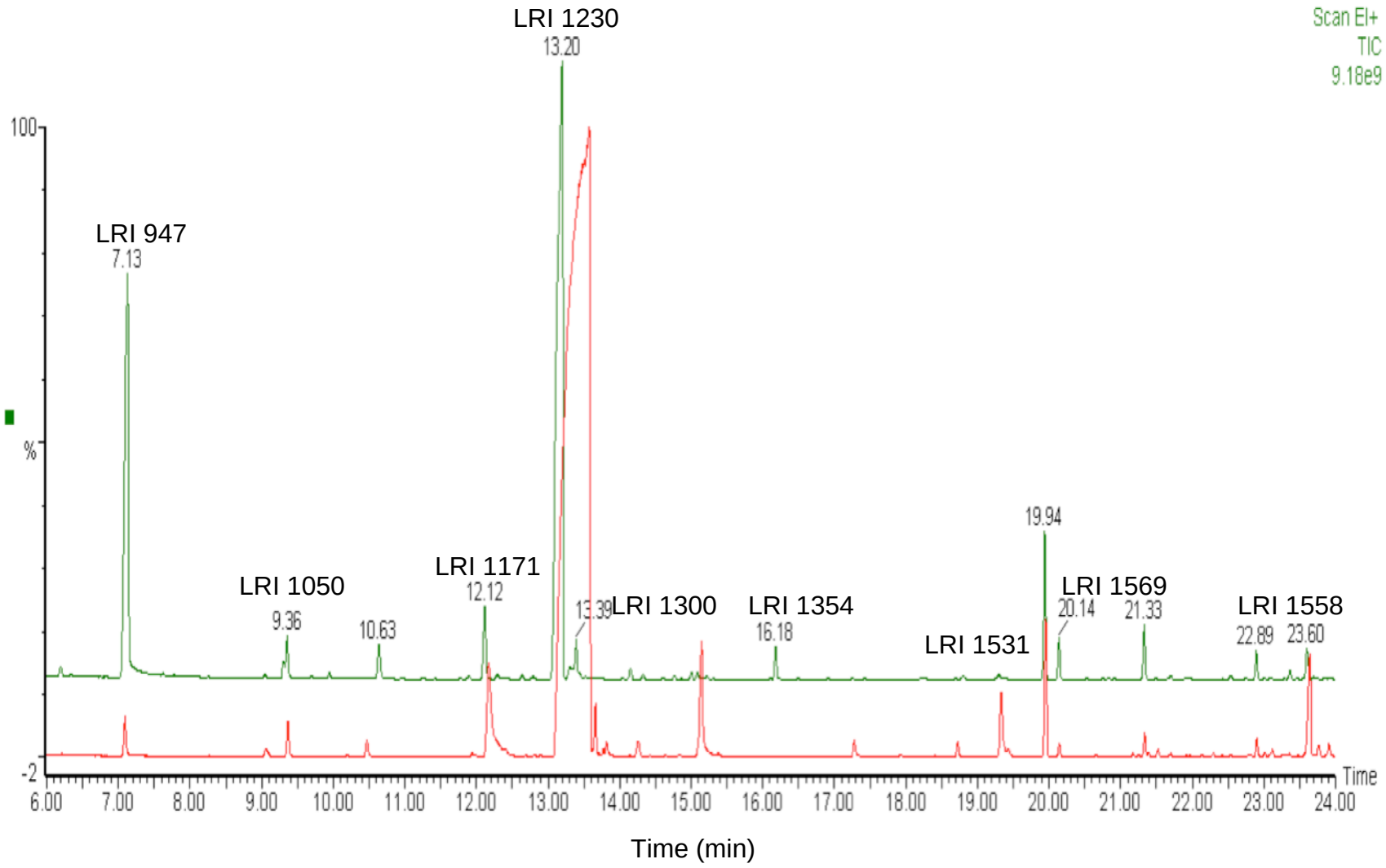


Figure 5-2. MS chromatograms of control (lower) and inoculated (upper) OJ at incubation day four

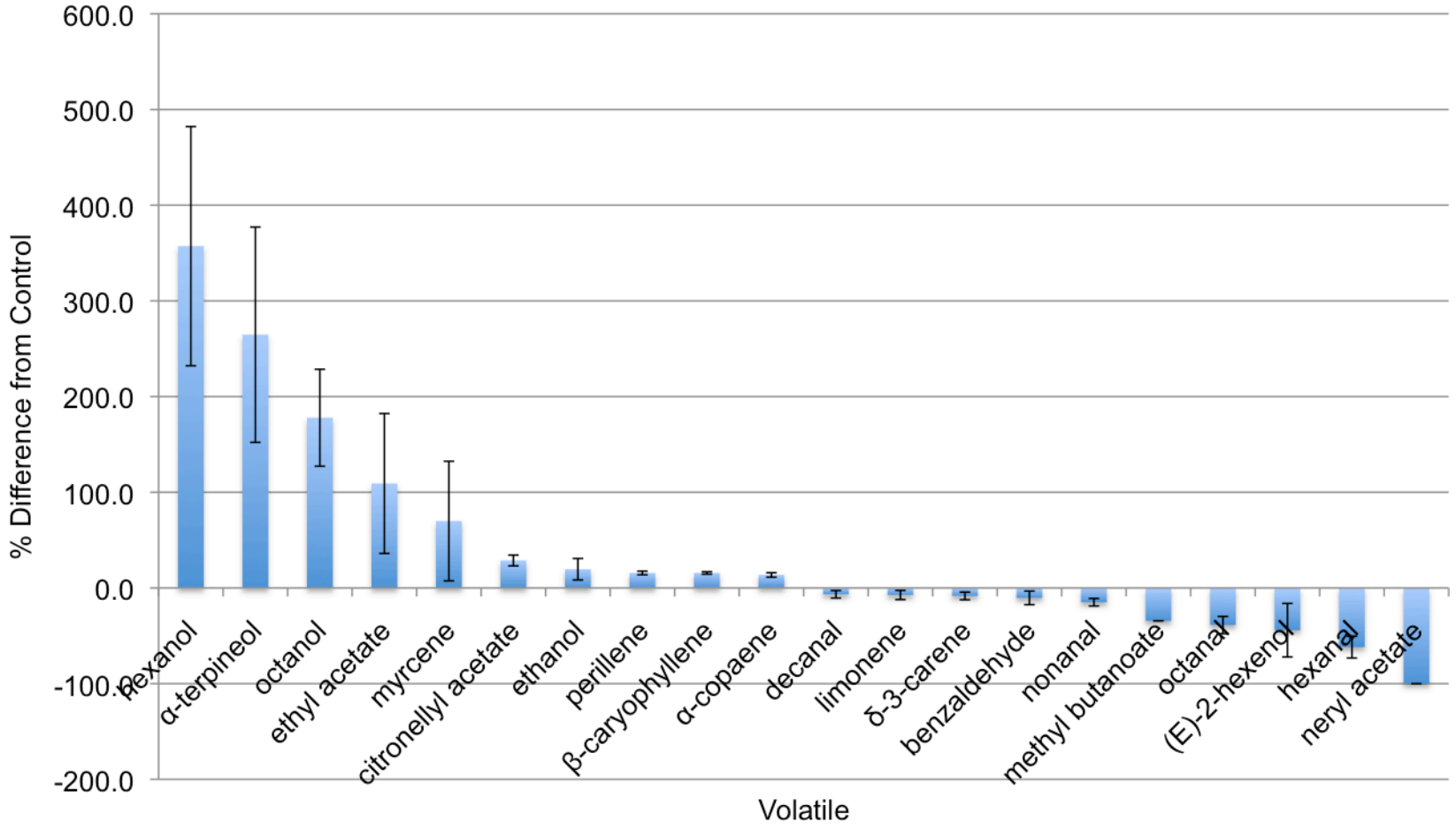


Figure 5-3. Top 20 differentiating volatiles of greatest difference from control at incubation day one

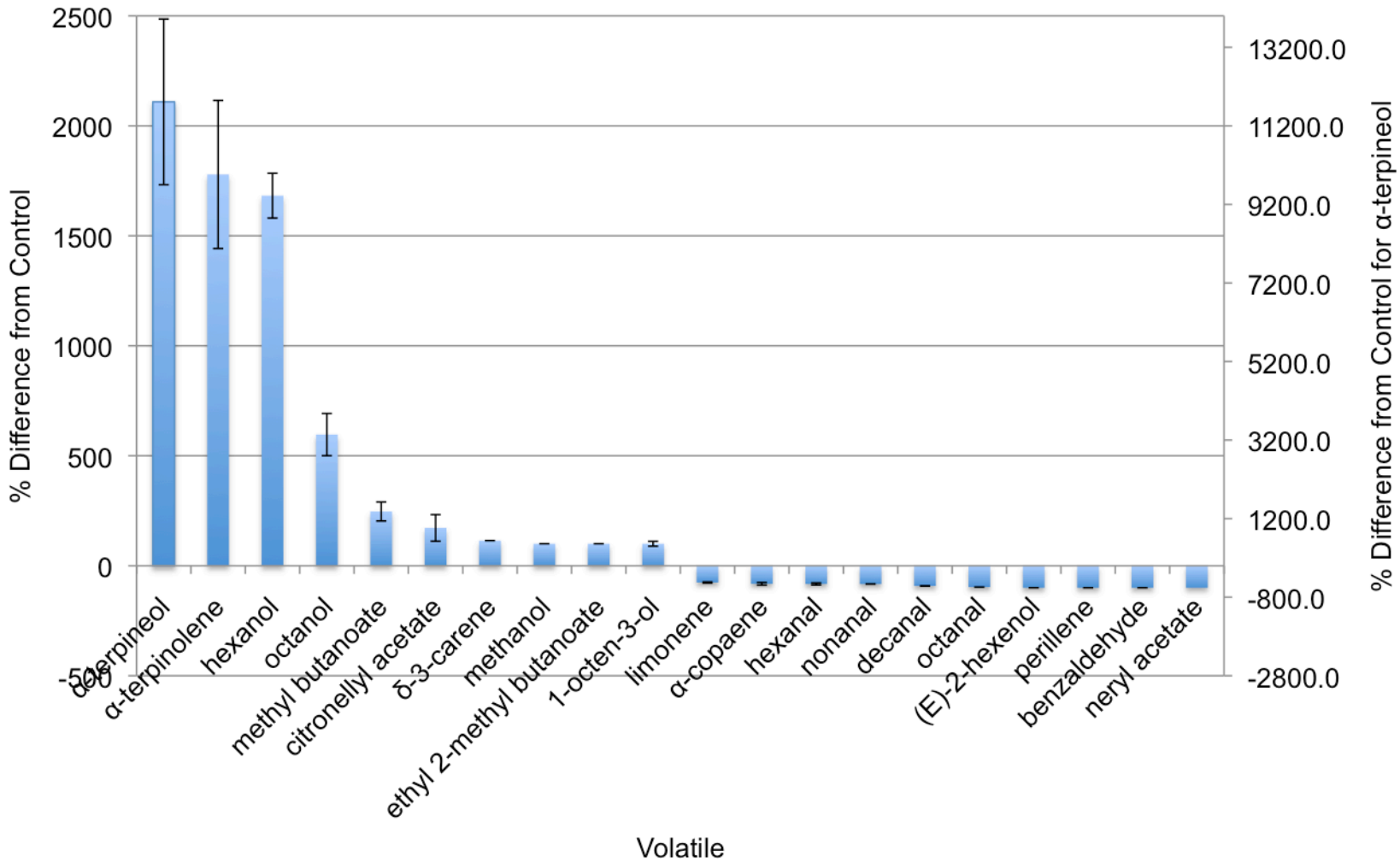


Figure 5-4. Top 20 differentiating volatiles of greatest difference from control at incubation day two

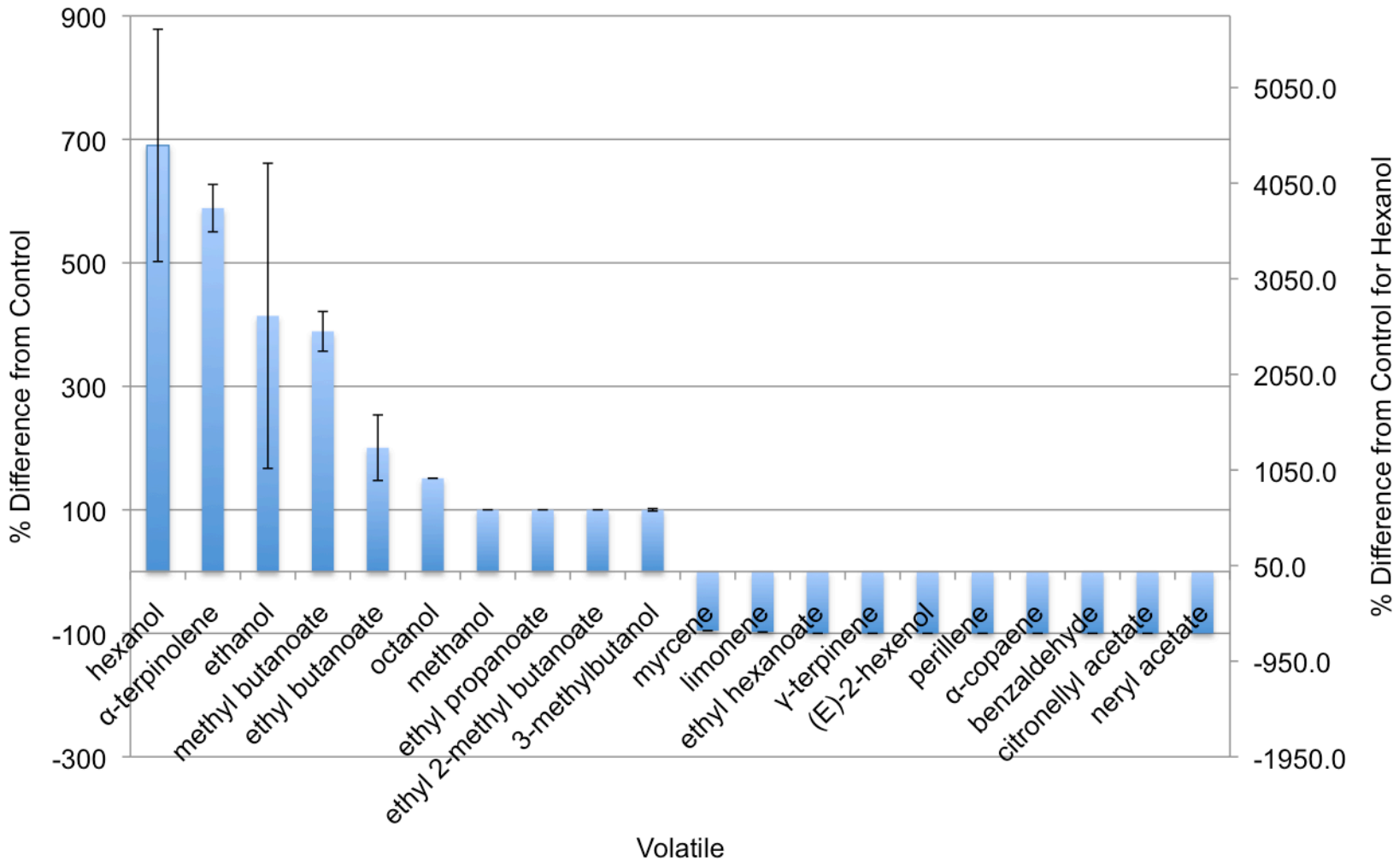


Figure 5-5. Top 20 differentiating volatiles of greatest difference from control at incubation day three

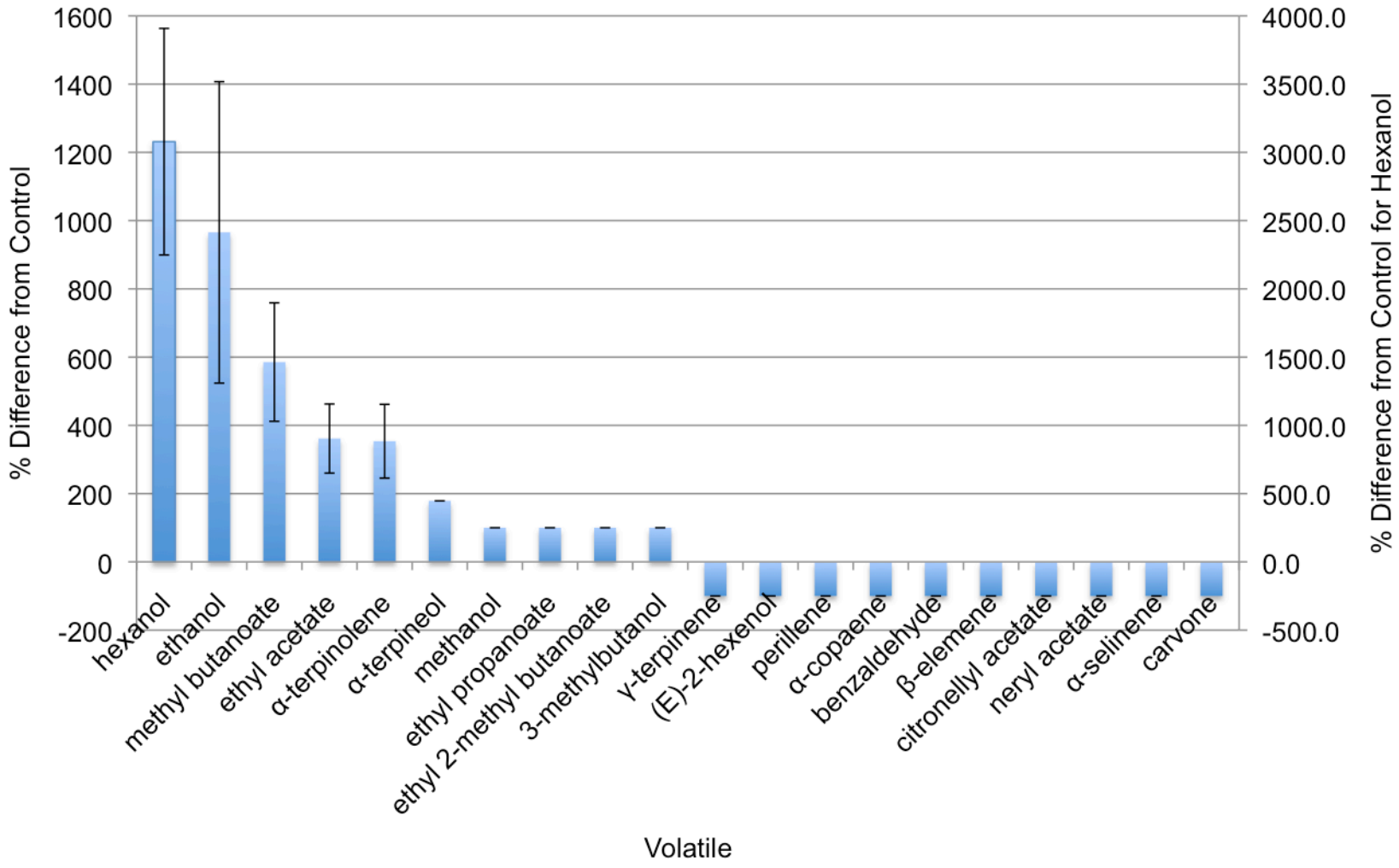


Figure 5-6. Top 20 differentiating volatiles of greatest difference from control at incubation day four

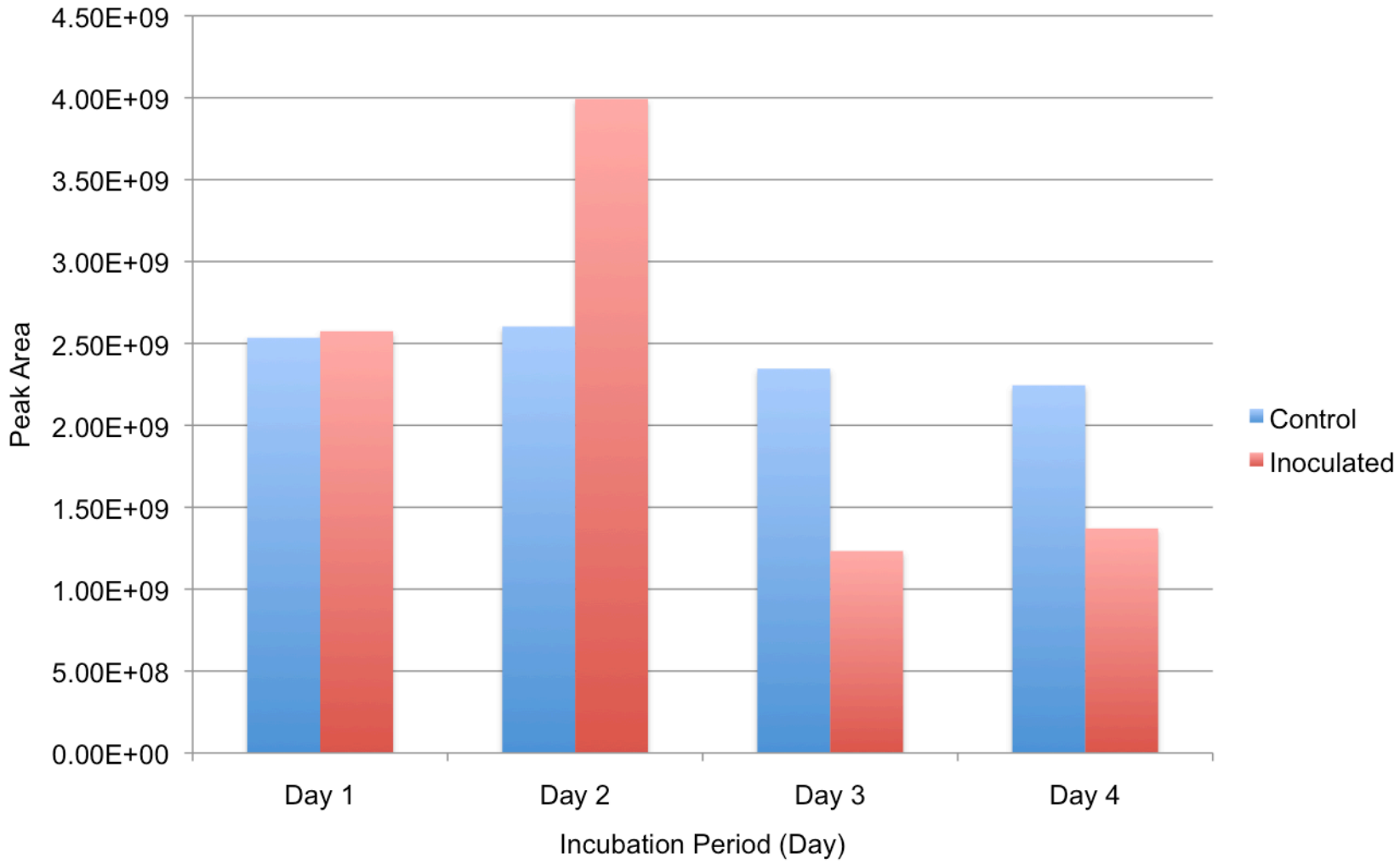


Figure 5-7. Overall trend in volatiles

Table 5-2. Qualitative differentiating volatiles

Volatile	Observed LRI	Database LRI*	Day one percent difference from control (% ± standard error)	Day four percent difference from control (% ± standard error)
Methanol	909	899	0.0 ± 0.0	100.0 ± 0.0
Ethyl propanoate	974	951	0.0 ± 0.0	100.0 ± 0.0
Ethyl 2-methylbutanoate	1065	1062	0.0 ± 0.0	100.0 ± 0.0
Methylbutanol, 3-	1212	1212	0.0 ± 0.0	100.0 ± 0.0
(Z)-3-hexenol	1398	1399	0.0 ± 0.0	100.0 ± 0.0
Octen-3-ol, 1-	1465	1452	0.0 ± 0.0	100.0 ± 0.0
Neryl acetate	1746	1742	(-) 100.0 ± 0.0	(-) 100.0 ± 0.0

*Database LRIs (Rouseff 2006)

Table 5-3. Quantitative differentiating volatiles that increased in percent difference from control

Compound	Observed LRI	Database LRI*	Day one percent difference from control (% ± standard error)	Day four percent difference from control (% ± standard error)
Ethyl acetate	900	890	109.1 ± 73.1	361.4 ± 173.6
Methanol	909	899	0.0 ± 0.0	100.0 ± 0.0
Ethanol	947	947	19.6 ± 11.2	965.5 ± 29.2
Ethyl propanoate	974	951	0.0 ± 0.0	100.0 ± 0.0
Methyl butanoate	1002	990	(-) 34.4 ± 0.0	585.5 ± 441.7
Methylbutanol, 3-	1212	1212	0.0 ± 0.0	100.0 ± 0.0

*Database LRIs (Rouseff 2006)

Table 5-4. Quantitative differentiating volatiles that decreased in percent difference from control

Volatile	Observed LRI	Database LRI*	Day one percent difference from control (% ± standard error)	Day four percent difference from control (% ± standard error)
Hexanal	1098	1099	(-) 61.8 ± 11.4	(-) 95.8 ± 2.1
Myrcene	1171	1172	69.9 ± 62.5	(-) 97.2 ± 1.7
Limonene	1230	1205	(-) 7.3 ± 4.8	(-) 99.0 ± 0.3
B-phellandrene	1238	1241	3.4 ± 2.0	(-) 91.0 ± 3.5
Ethyl hexanoate	1245	1246	2.8 ± 1.0	(-) 100.0 ± 0.0
Γ-terpinene	1267	1260	(-) 5.4 ± 1.9	(-) 100.0 ± 0.0
Octanal	1310	1309	(-) 38.8 ± 9.0	(-) 95.9 ± 1.2
(E)-2-hexenol	1383	1388	(-) 44.1 ± 28.0	(-) 100.0 ± 0.0
Nonanal	1417	1416	(-) 14.9 ± 3.9	(-) 90.1 ± 1.7
Perillene	1442	1409	15.7 ± 1.9	(-) 100.0 ± 0.0
Ethyl octanoate	1451	1450	11.4 ± 9.2	(-) 86.7 ± 1.4
Octyl acetate	1492	1480	10.2 ± 1.7	(-) 92.1 ± 2.3
Decanal	1526	1523	(-) 6.6 ± 3.9	(-) 85.1 ± 3.7
A-copaene	1531	1536	13.6 ± 2.3	(-) 100.0 ± 0.0
Linalool	1558	1557	8.7 ± 6.9	(-) 46.9 ± 10.3
Benzaldehyde	1575	1555	(-) 10.4 ± 7.1	(-) 100.0 ± 0.0
B-elemene	1626	1595	13.5 ± 1.4	(-) 100.0 ± 0.0
B-caryophyllene	1646	1641	15.6 ± 1.3	(-) 85.1 ± 4.8
B-selinene	1690	1711	11.8 ± 5.5	(-) 89.0 ± 3.0
Valencene	1770	1763	11.8 ± 5.3	(-) 98.4 ± 0.5
A-selinene	1779	1724	7.2 ± 1.5	(-) 100.0 ± 0.0
Carvone	1788	1779	(-) 5.4 ± 4.4	(-) 100.0 ± 0.0
Δ-cadinene	1798	1794	11.8 ± 0.1	(-) 74.4 ± 7.2
A-panasinsen	1819	1840	10.1 ± 0.3	(-) 80.5 ± 5.9
Cis-carveol	1864	1855	10.6 ± 0.5	(-) 61.4 ± 5.5

*Database LRIs (Rouseff 2006)

Table 5-5. Quantitative intermediate volatiles

Volatile	Observed LRI	Database LRI*	Day of maximum percent difference	Day one percent difference from control (% ± standard error)	Day four percent difference from control (% ± standard error)
Ethyl butanoate	1050	1048	3	13.4 ± 13.3	200.6 ± 32.3
Ethyl 2-methylbutanoate	1065	1062	3	0.0 ± 0.0	100.0 ± 0.0
Δ-3-carene	1161	1148	2	(-) 8.3 ± 4.0	114.3 ± 60.4
Hexyl acetate	1286	1267	2	(-) 4.2 ± 3.6	70.2 ± 8.4
A-terpinolene	1300	1298	2	(-) 6.6 ± 3.5	1779.0 ± 94.4
Hexanol	1359	1364	3	357.2 ± 125.0	4445.1 ± 1214.8
(Z)-3-hexenol	1398	1399	3	0.0 ± 0.0	100.0 ± 0.0
Octen-3-ol, 1-	1465	1452	2	0.0 ± 0.0	100.0 ± 0.0
Octanol	1569	1565	2	177.8 ± 50.7	596.4 ± 101.8
Terpineol, 4-	1636	1616	3	(-) 4.6 ± 3.3	32.9 ± 4.7
Citronellyl acetate	1680	1672	2	28.7 ± 5.6	172.0 ± 43.3
A-terpineol	1730	1724	2	264.6 ± 112.5	11811.1 ± 2109.6

*Database LRIs (Rouseff 2006)

Table 5-6. Differentiating sulfur volatiles

Sulfur volatile	Observed LRI	Database LRI*	Difference from control	Day one percent difference from control (% ± standard error)	Day four percent difference from control (% ± standard error)
Dimethyl sulfide	738	736	decrease	(-) 6.4 ± 13.7	(-) 67.1 ± 3.0
Unidentified	942	-	only in inoculated	0.0 ± 0.0	100.0 ± 0.0
Dimethyl disulfide	1093	1064	intermediate	8.8 ± 27.7	54.0 ± 61.1
Unidentified	1413	-	decrease	12.6 ± 0.6	(-) 100.0 ± 0.0
Unidentified	1484	-	only in control	(-) 100.0 ± 0.0	(-) 100.0 ± 0.0
Unidentified	1557	-	only in inoculated, increase	0.0 ± 0.0	(-) 100.0 ± 0.0
Unidentified	1563	-	only in inoculated, intermediate	0.0 ± 0.0	(-) 100.0 ± 0.0
Dithiane, 1,4-	1597	1589	decrease	(-) 23.0 ± 13.4	(-) 100.0 ± 0.0
Unidentified	1670	-	decrease	5.5 ± 8.0	(-) 100.0 ± 0.0
Unidentified	1743	-	only in inoculated, increase	0.0 ± 0.0	(-) 100.0 ± 0.0
Phenethyl mercaptan	1779	1753	decrease	4.3 ± 1.7	(-) 100.0 ± 0.0
Unidentified	1789	-	decrease	15.2 ± 9.3	(-) 100.0 ± 0.0

*LRI values from standards run on the GC – S with a Stabilwax column in Dr. Russell Rouseff's lab

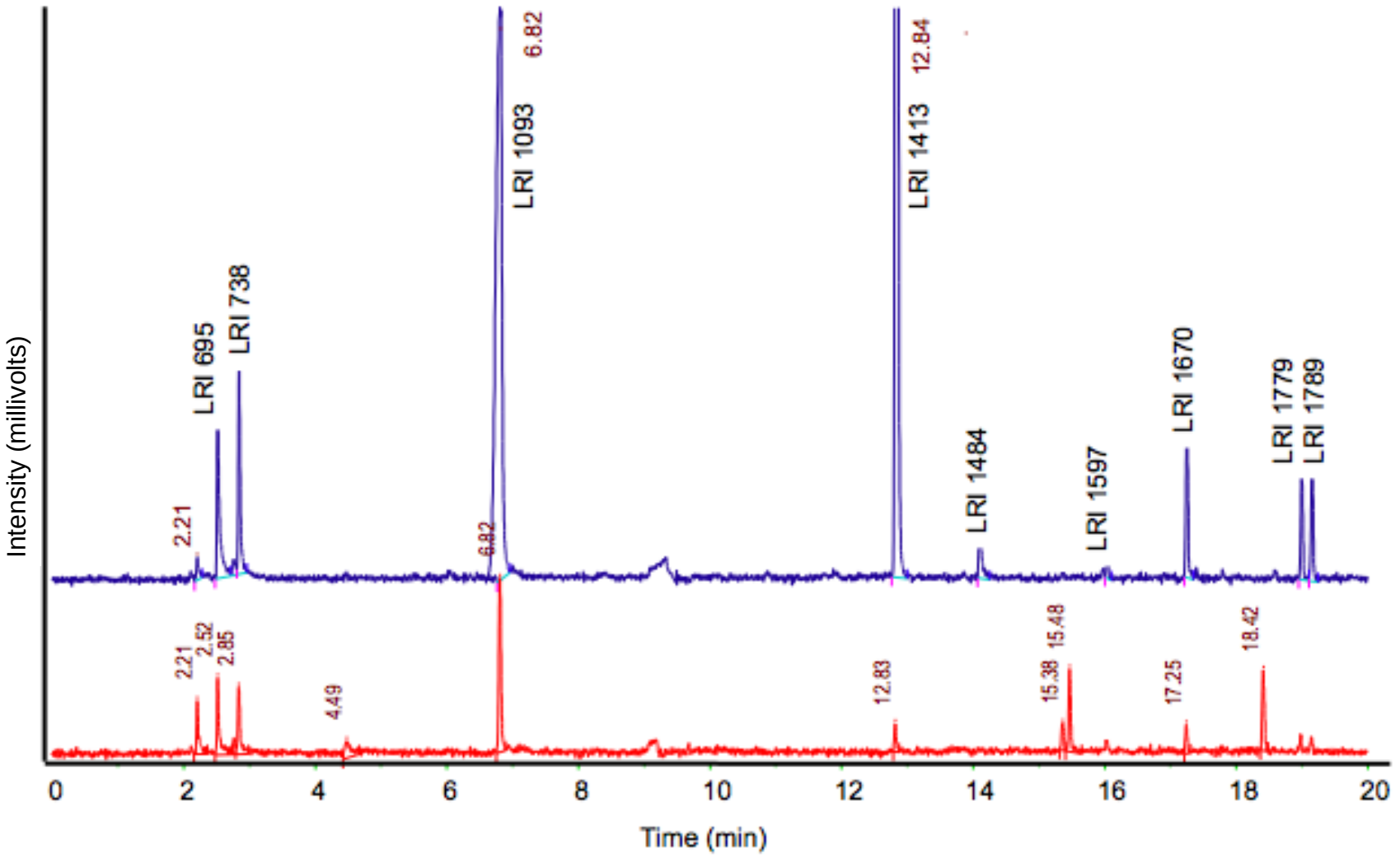


Figure 5-8. Sulfur chromatograms of control (upper) and inoculated (lower) OJ at incubation day four

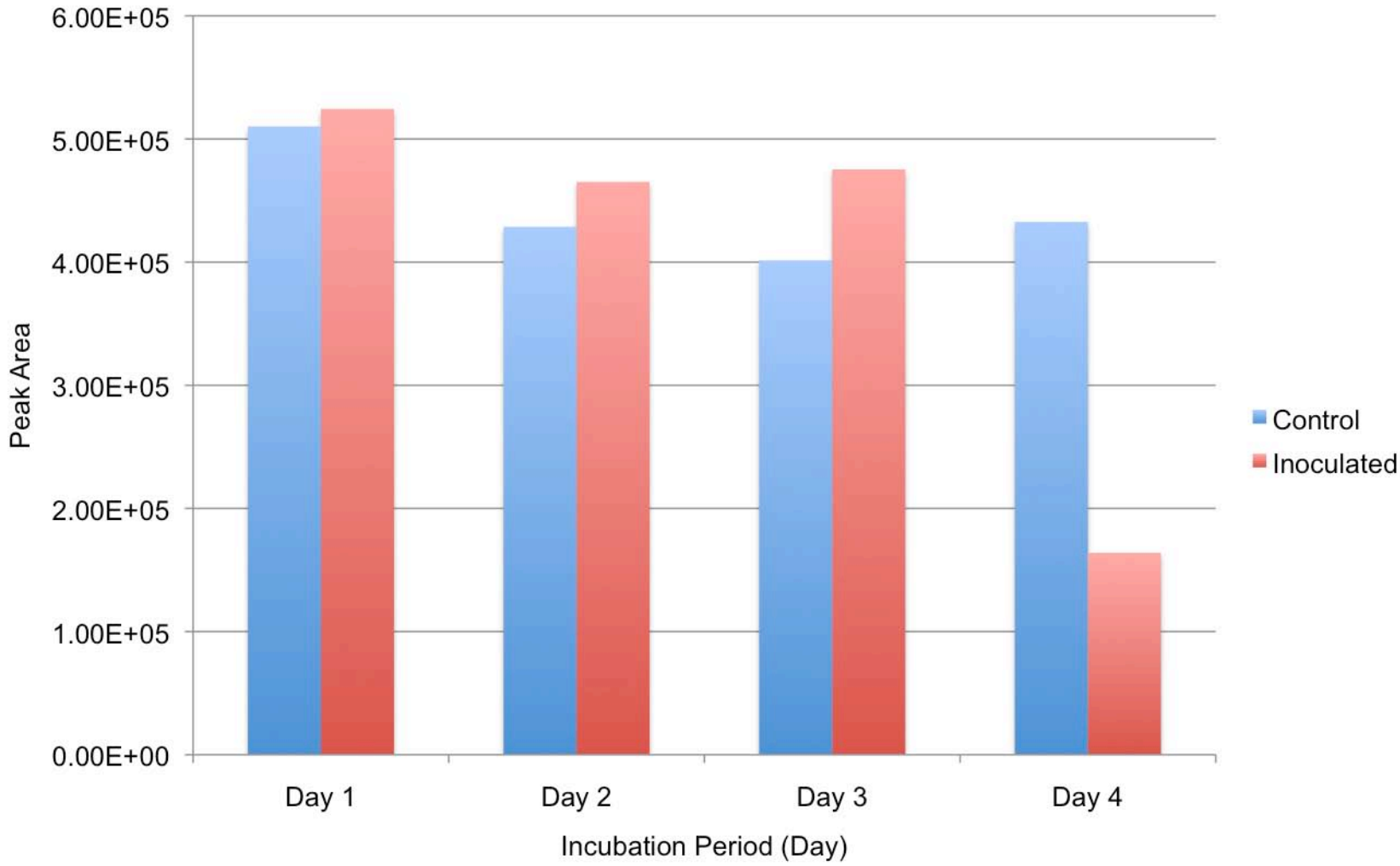


Figure 5-9. Overall trend in sulfur volatiles

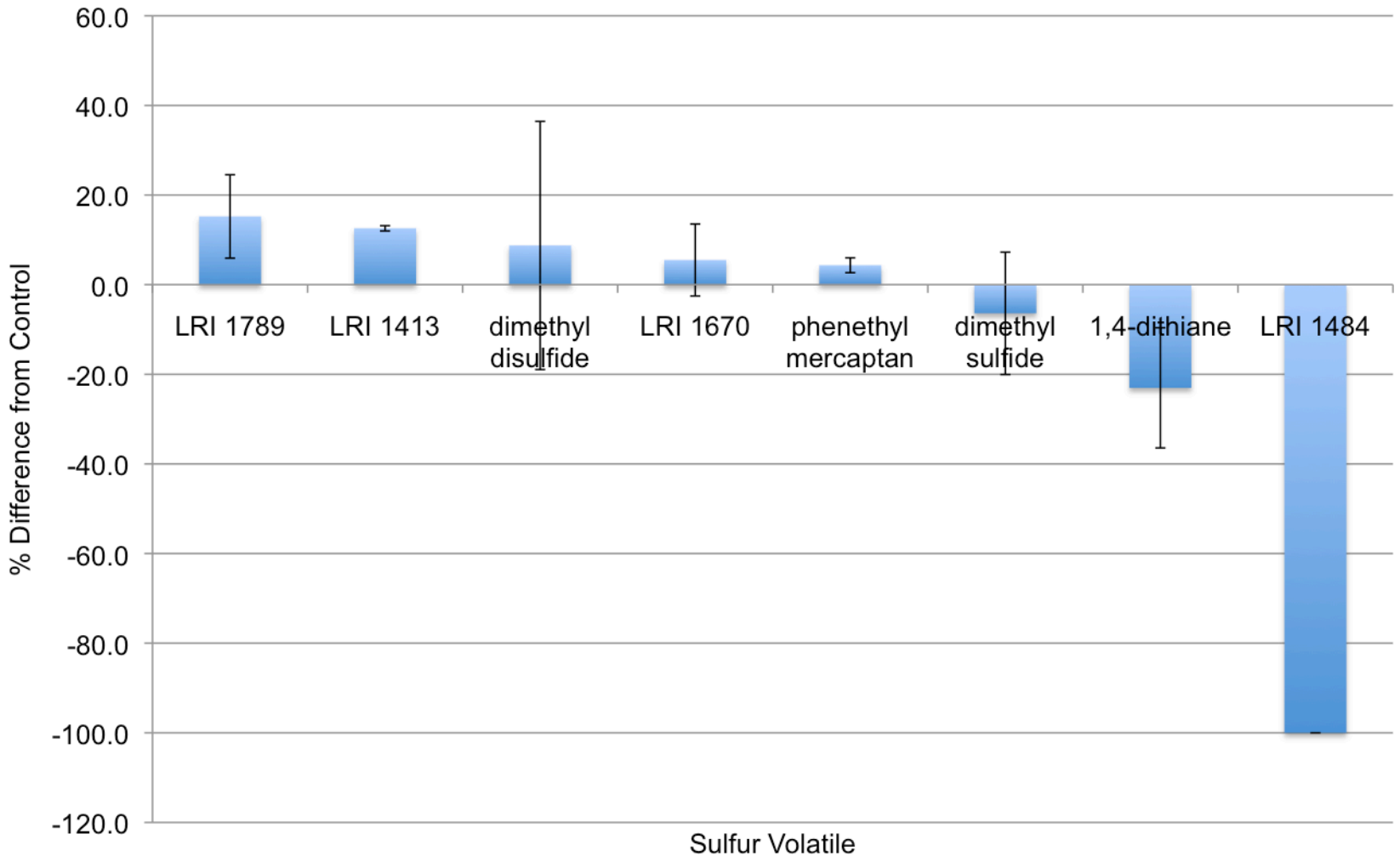


Figure 5-10. Major differentiating sulfur volatile differences from control at incubation day one

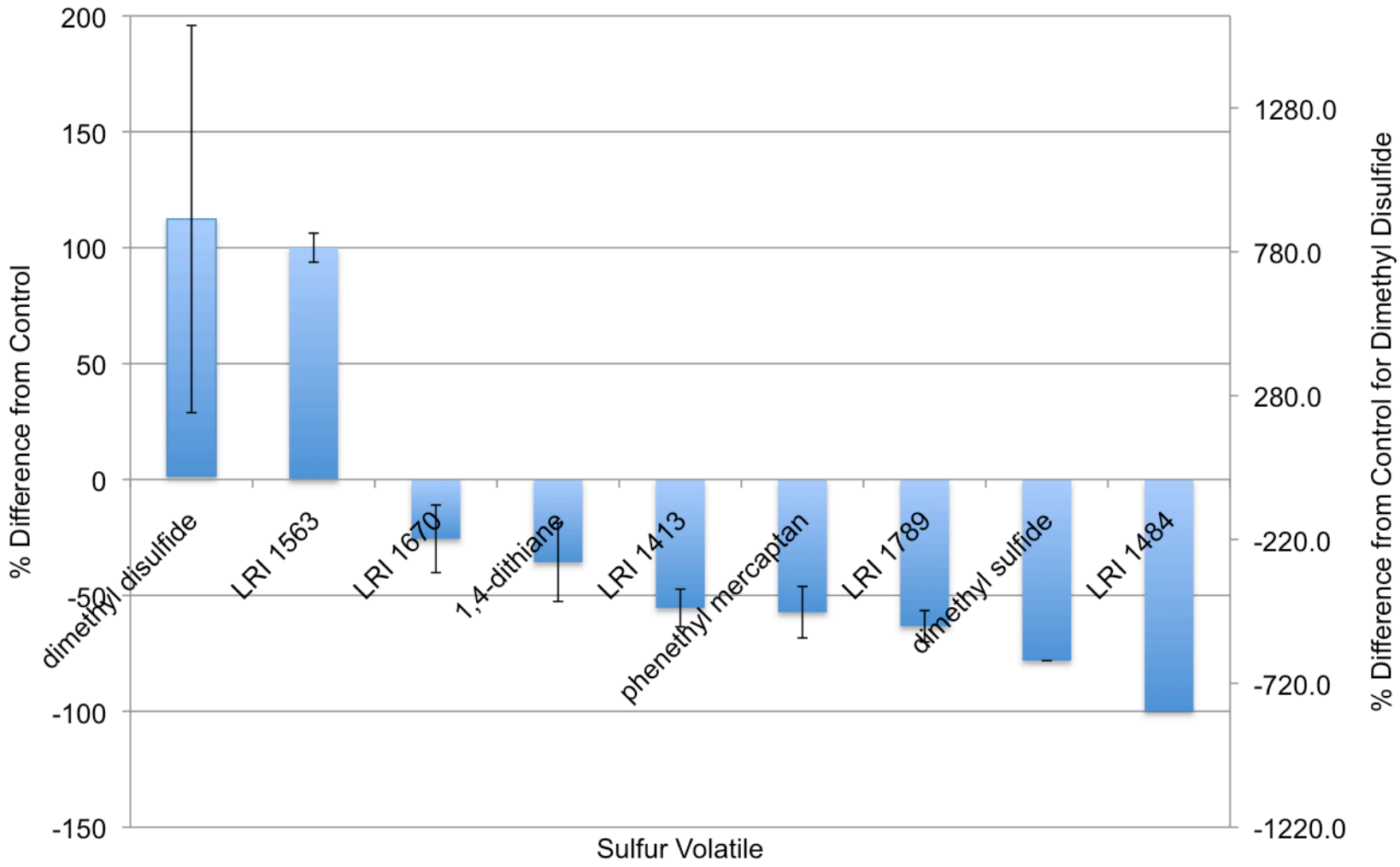


Figure 5-11. Major differentiating sulfur volatile differences from control at incubation day two

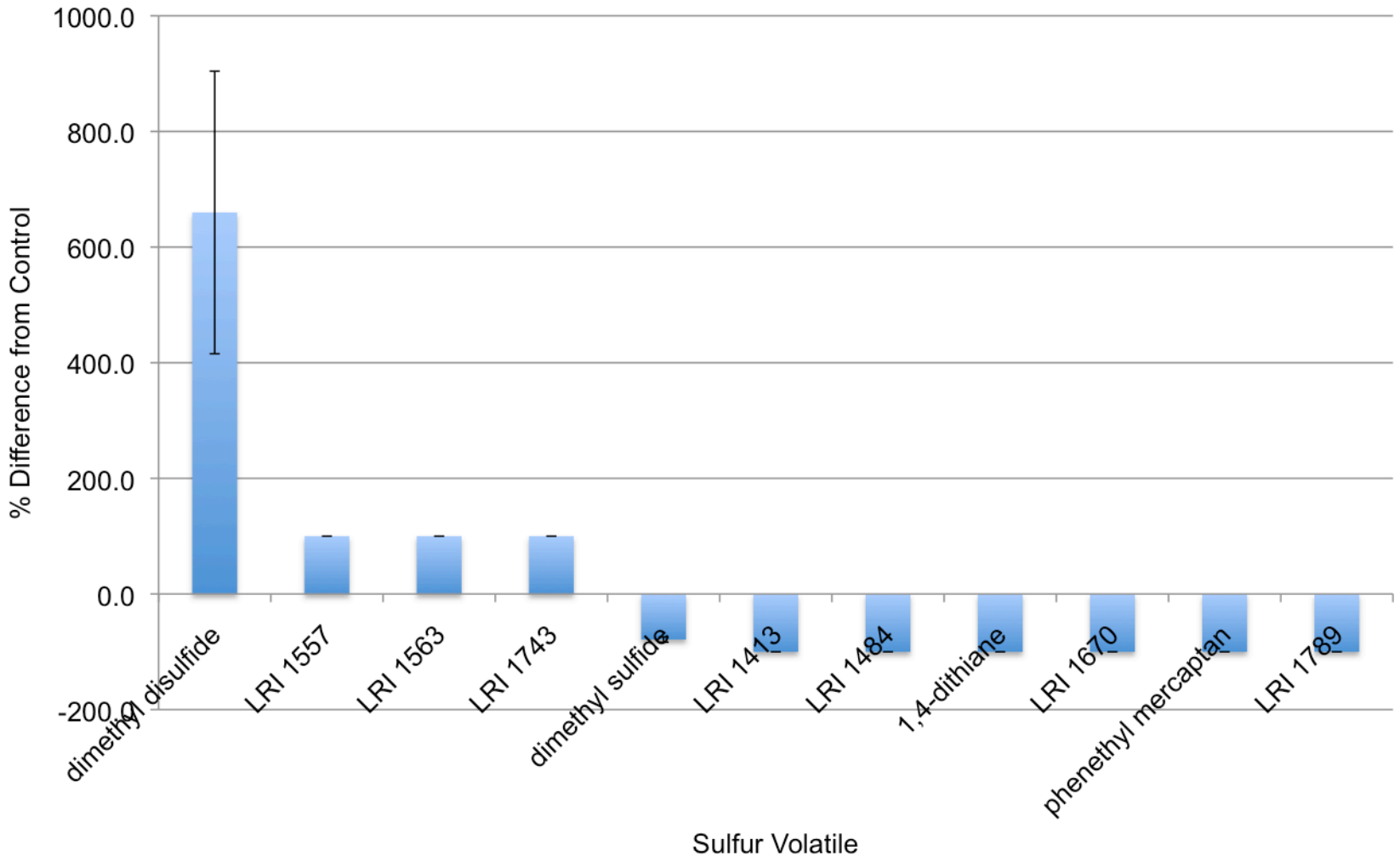


Figure 5-12. Major differentiating sulfur volatile differences from control at incubation day three

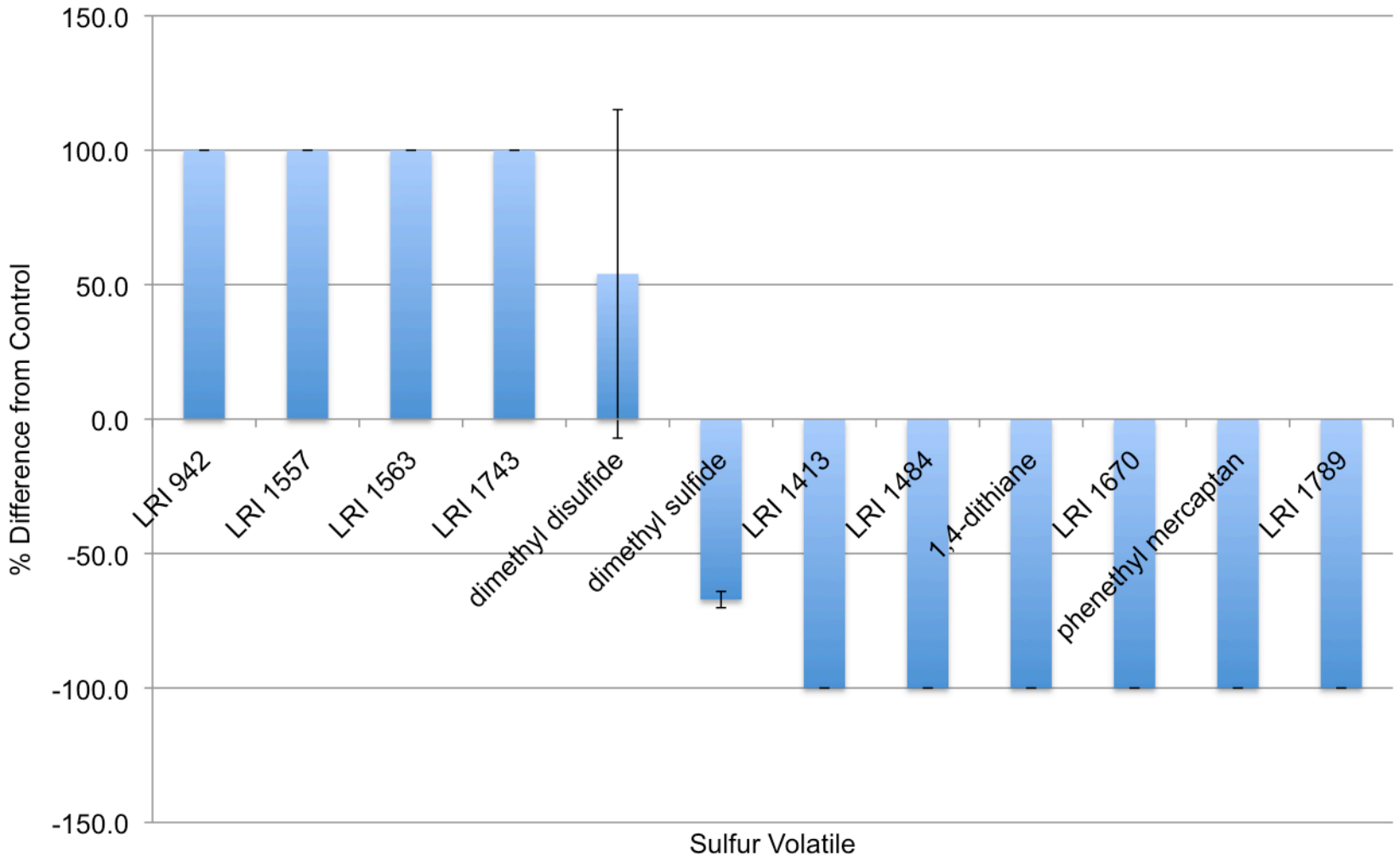


Figure 5-13. Major differentiating sulfur volatile differences from control at incubation day four

CHAPTER 6
MECHANISMS OF VOLATILE FORMATION

The production and breakdown of volatiles mentioned above results from the biosynthesis of metabolites via fungal metabolic pathways and enzymatically driven reactions. OJ is a very complex system and it cannot be assumed that the reactions taking place are independent of one another. The reactions are most likely working in conjunction, as the resulting product of one can be the substrate or reactant in another. In addition, multiple reactions produce or use similar volatiles. These reactions are specific to OJ contaminated with *P*. *digitatum*. The mechanisms are not well understood, but involve oxidation, reduction, hydrolytic, dehydration, degradation and C-C bond forming reactions (Demyttenaere et al. 2001; Janssens et al. 1992).

Volatile Formation as a Result of the Metabolic Pathway

Figure 2-1, an overview of fungal metabolic pathways and the resulting volatile metabolites, has been adapted from Schnüner et al (1999). This figure helps explain the formation of esters and alcohols seen in this study. The increase in ethyl acetate in the inoculated sample is the result of the metabolic pathway involving the conversion of glucose to pyruvate to acetic acid via acetyl CoA. Acetic acid then reacts with ethanol through the process of esterification to form ethyl acetate (Figure 6-1) (Pesis and Marinansky 1990; Schnürer et al. 1999). One of the earliest pathways seen in Figure 2-1 is the breakdown of glucose by glycolysis to produce pyruvate. Once formed, pyruvate loses carbon dioxide to form acetaldehyde, which is then reduced by NADH to form ethanol (Figure 6-2). The association between the decrease in sugar content, °Brix, and increase in ethanol due to fungal contamination can be seen in Figure 6-3. In the control samples, the °Brix and ethanol remain consistent. In the inoculated sample,

however, the ethanol begins to increase as the °Brix begins to decrease. Fungal metabolic pathways are not the sole means of producing alcohols in this system.

Volatile Formation as a Result of Enzymes

Methanol, a qualitative differentiating volatile only found in the inoculated samples, forms through a fungal metabolic pathway, but in this system also forms as a result of an enzymatic reaction. *P*. *digitatum* produces a wide range of enzymes that are active at the low pH associated with OJ (Alaña et al. 1990; Bush and Codner 1968; Bush and Codner 1970; Filtenborg et al. 1996). One of these enzymes, pectin esterase, is associated with softening of orange peel and cloud destruction. Through a de-esterification reaction, this enzyme breaks down pectin into pectate and methanol (Bush and Codner 1968). This enzyme is produced by *P*. *digitatum*, thus is only present in contaminated OJ, explaining why methanol is a qualitative volatile. Other esterases contribute to the decrease associated with some ester volatiles present and an increase in alcohols. Ethyl hexanoate, found consistently in the control sample, yet decreasing in the inoculated (Figure 6-4), is an example of such. Esterase enzymes produced by *P*. *digitatum* catalyze the de-esterification of ethyl hexanoate into ethanol and hexanoic acid (Figure 6-5). This reaction is another source of ethanol, secondary to that produced by the metabolic pathway mentioned previously. The breakdown of compounds from enzymatic reactions can help explain the overall decrease in differentiating volatiles, and sulfur volatiles, seen in Figures 5-7 and 5-9.

Volatile Formation as a Result of Bioconversions

Enzymes produced by *P*. *digitatum* also catalyze the bioconversion of volatiles present. The most well studied bioconversion is that of limonene to α-terpineol, and as expected, this bioconversion was observed in this study. Due to the reaction's low

specificity, other volatiles, such as α-terpinolene and 4-terpineol, were also produced (Figure 6-6) (Adams et al. 2003; Demyttenaere et al. 2001; Duetz et al. 2003; Tan and Day 1998a; Tan and Day 1998b; Tan and others 1998). Figure 6-7 graphically represents the decrease in limonene with the production of α-terpinolene, 4-terpineol, and α-terpineol as compared to the control samples. As observed by Tan and others, the highest amount of α-terpineol occurred on day 2, along with the highest amount of α-terpinolene (Tan and Day 1998a; Tan and Day 1998b; Tan et al. 1998). The highest amount of 4-terpineol was not observed until day 3 of incubation, suggesting the bioconversion of α-terpineol or α-terpinolene to 4-terpineol. As discussed in Chapter 2, the mechanism of this bioconversion is debated, with the current proposed mechanism involving P-450 monooxygenases causing epoxidation of the 8-9 double bond of limonene followed by reductive cleavage of the epoxide (Demyttenaere et al. 2001; Duetz et al. 2003). All of the bioconversions and metabolic pathways are under debate, and the interactions between the different pathways and bioconversion only increase the possibilities.

Figure 6-1. Esterification of acetic acid and ethanol to form ethyl acetate

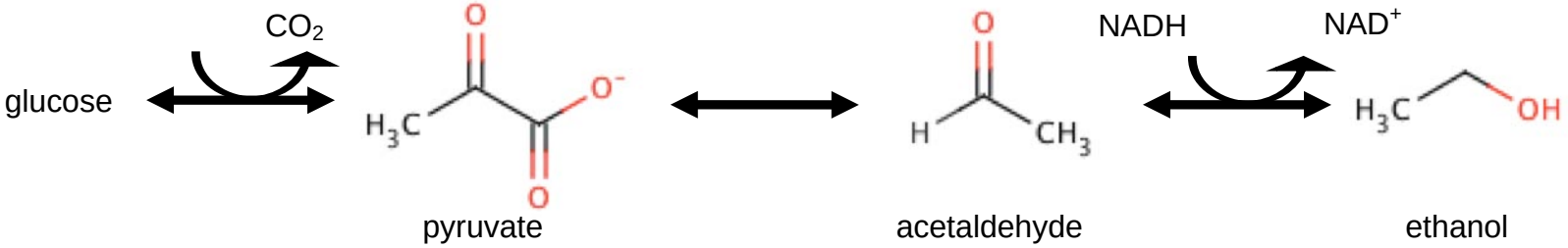


Figure 6-2. Formation of ethanol from glucose

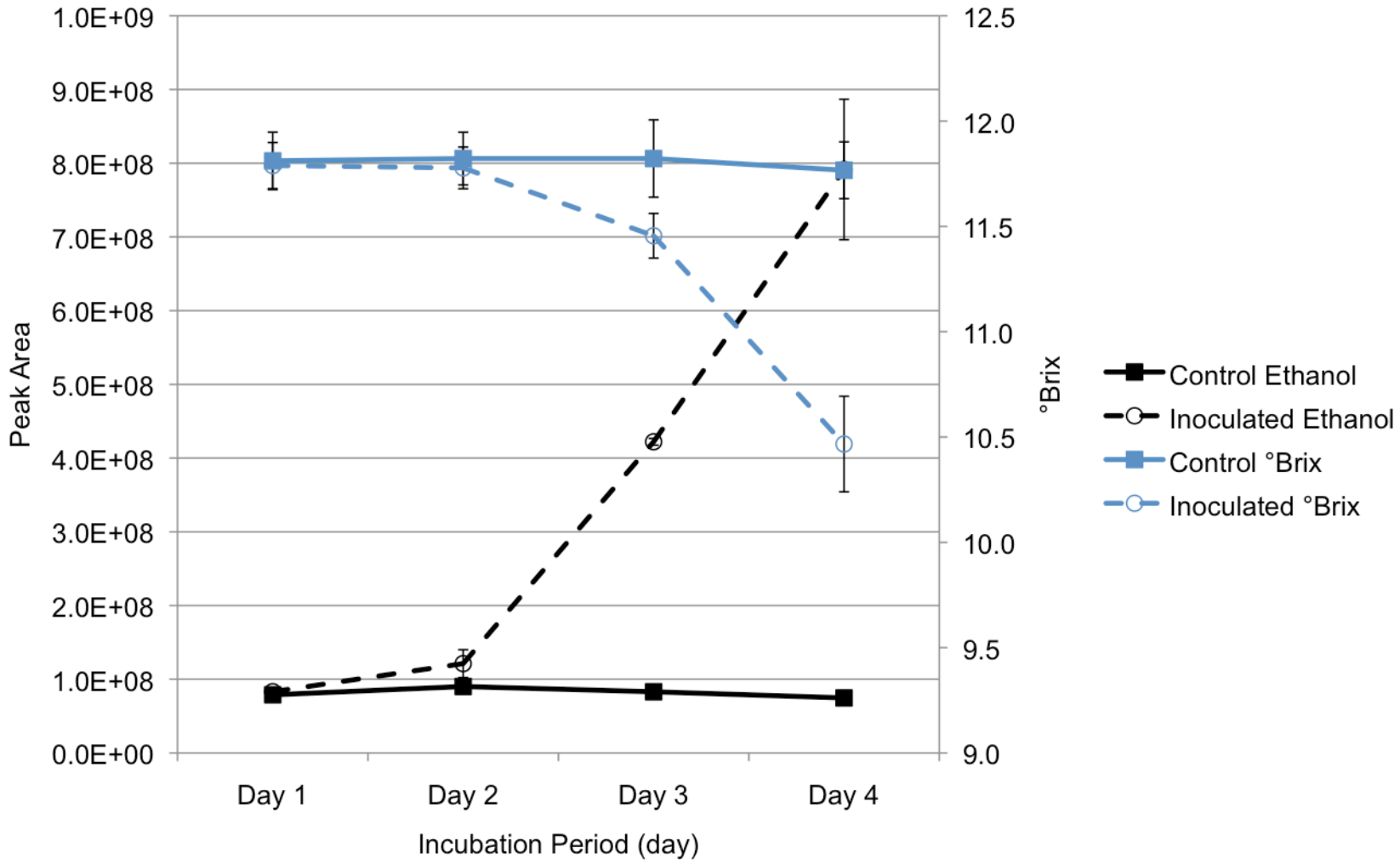


Figure 6-3. Relationship between the decrease in °Brix and the increase in ethanol

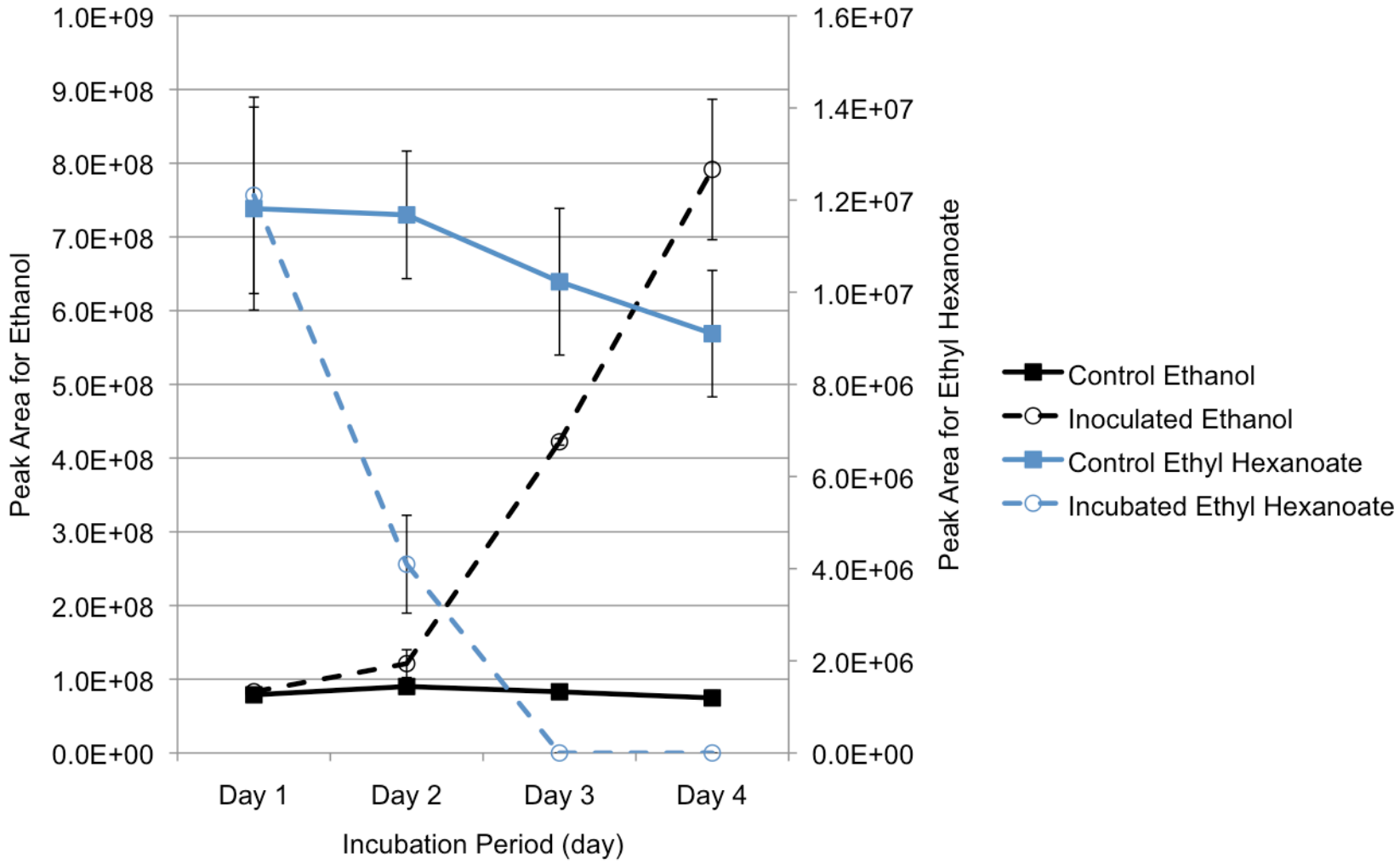


Figure 6-4. Relationship between the decrease in ethyl hexanoate and the increase in ethanol

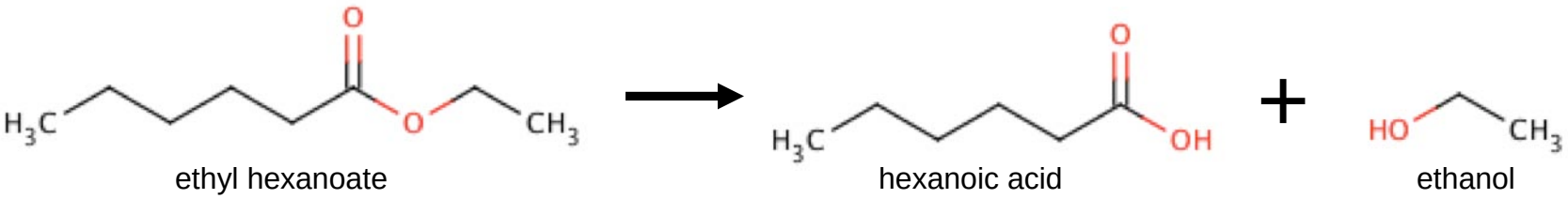


Figure 6-5. De-esterification of ethyl hexanoate to form hexanoic acid and ethanol

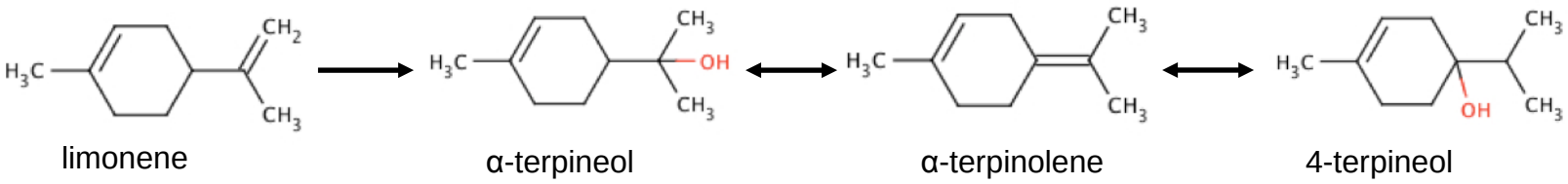


Figure 6-6. Bioconversion of limonene to α-terpineol, terpinolene, and 4-terpineol

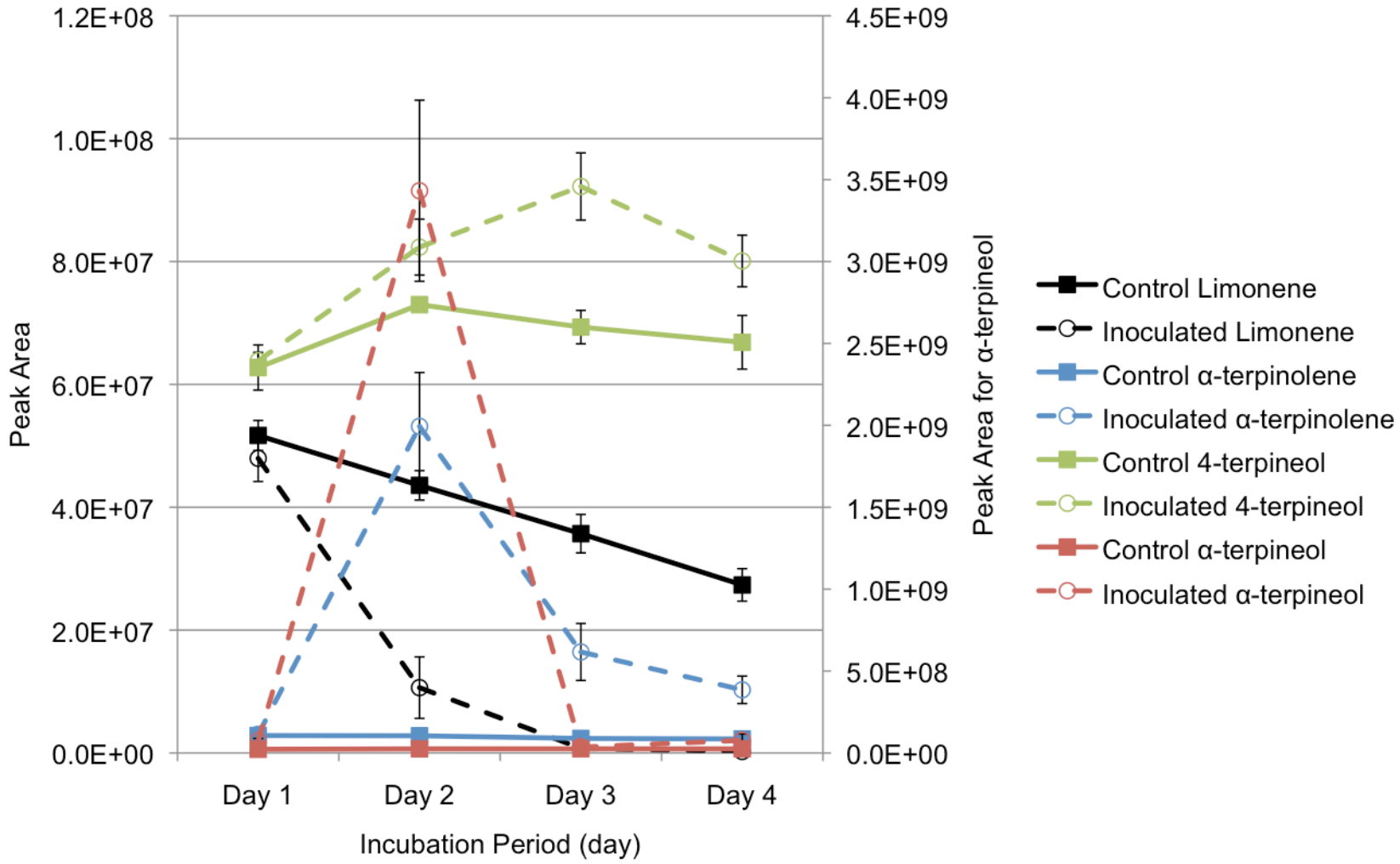


Figure 6-7. Relationship between the bioconversion of limonene to α-terpineol, terpinolene, and 4-terpineol

CHAPTER 7
CHANGES IN ORANGE JUICE AROMA IMPACT VOLATILES DUE TO *PENICILLIUM DIGITATUM*

Determination of aroma impact compounds was accomplished by the methodology stated in CHAPTER 3, performed by four different sniffers. In order to be considered an aroma impact compound, three out of the four sniffers had to identify it as such. Based on this requirement, 20 volatiles were found to impact aroma (Table B-1). These 20 volatiles were then compared to the GC – MS data (Table 7-1) to determine only nine of the 44 differentiating volatiles were aroma impact compounds.

The association between the GC – MS and GC – O data was aided by identifying the aroma impact compounds based on their calculated LRI values and observed aroma descriptors as compared to literature. These tentative identifications were then compared to the identification made using the GC – MS. If both the GC – MS and GC – O had volatiles with similar LRIs (±1%) and the same identification, it was concluded that the volatile observed to impact aroma on the GC – O was the same differentiating volatile observed on the GC – MS. Based on the observed aroma descriptors seen in Table 7-1, coelution probably occurred. For example at LRI 1246, ethyl hexanoate, a fruity aroma, was observed, but this volatile is not associated with the green, grassy odor also observed. These other odors are the result of another unidentified volatile coeluting.

Trends in aroma impact compounds followed that of the GC – MS volatiles when comparing the aroma intensity (peak height) difference from control (Table 7-2). The overall trend was a decrease in perceived aroma intensity not only when comparing the inoculated to the control, but also within the inoculated sample over the four day incubation period (Figure 7-1). This decreasing trend in perceived intensity can also be

observed when comparing the aromagram from day one (Figure 7-2) to the aromagram from day four (Figure 7-3). The aromas observed during day one in the control closely mirror the aromas observed in the inoculated sample. For day four, however, only two aromas were observed in the inoculated sample while seven were observed in the control. When looking at the difference between control and inoculated peak height over the four day incubation period (Figure 7-4, 7-5, 7-6, and 7-7), the decrease is also quite evident. The only volatile observed to increase after day three is α-terpinolene. All other volatiles decrease in aroma intensity.

When the sniffers were asked to smell each sample and describe the overall odor profile, the control maintained its orange aroma throughout the 4 days. The inoculated, however, was described as decreasing in orange aroma while increasing in alcoholic aroma. The only alcohol identified as an aroma impact compound was linalool, but the aroma associated with this volatile is floral, green, and citrus (Rouseff 2006). The sniffers on the GC – O did not identify alcoholic aromas, and subsequently alcohol volatiles, possibly because the concentrations of these volatiles were below the aroma thresholds. For example, methanol, a volatile that would contribute an alcoholic odor, has a high aroma threshold at 100 ppm (Acree and Arn 2004). The lack of adequate sniffer training for the specific samples also contributed to their inability to detect aroma impact compounds eluting from the GC – O (Reineccius 2006).

Despite only 9 volatiles being identified as aroma impact compounds using this methodology, other volatiles are probably contributing to the change in aroma of the inoculated juice. Based on the relationship of aroma and flavor, it can be assumed that the inoculated juice would have a different flavor profile then the control (Reineccius

2006). These off-flavors are of primary concern when dealing with microbial spoilage because they render the juice unacceptable to the consumer and a loss to the manufacturer. The sooner the organism can be identified, the quicker control measures can be taken to limit the spoilage of the juice.

Table 7-1. Differentiating volatiles which impact aroma with aroma descriptors

Volatile	GCO LRI	GCMS LRI	Database LRI*	Observed aroma descriptor	Literature aroma descriptor*
Hexanal	1074	1098	1099	Green, grassy, fresh	Fatty, green, grassy, powerful
Myrcene	1151	1171	1172	Musty, moldy, metallic, alcoholic	Musty, wet soil
Unidentified	1191	-	-	Leafy, pungent, spicy, floral	-
Ethyl hexanoate	1246	1245	1246	Fruity, fresh, green, grassy	Fruity
A-terpinolene	1296	1300	1298	Green, citrus, fruity	Citrus, pine
Octen-3-one, 1-	1310	-	1315	Mushroom	Mushroom-like, metallic, musty
Octyl acetate	1497	1492	1480	Minty, fruity, pine-like, bamboo	Fruity, slightly fatty
Linalool	1545	1558	1557	Floral, flowery, sweet	Floral, green, citrus
Neryl acetate	1728	1746	1742	Woody, rosy	Fruity, floral

*Database LRIs and aroma descriptors (Rouseff 2006)

Table 7-2. Differentiating volatiles which impact aroma with percent difference

Volatile	GCO LRI	Aroma difference from control	Day one percent difference from control (% ± standard error)	Day four percent difference from control (% ± standard error)
Hexanal	1074	Only in control	(-) 100.0 ± 0.0	(-) 100.0 ± 0.0
Myrcene	1151	Decrease	32.3 ± 22.6	(-) 100.0 ± 0.0
Unidentified	1191	Only in control	(-) 100.0 ± 0.0	(-) 100.0 ± 0.0
Ethyl hexanoate	1246	Decrease	12.3 ± 5.9	0.0 ± 0.0
A-terpinolene	1296	Only in inoculated	0.0 ± 0.0	100.0 ± 0.0
Octen-3-one, 1-	1310	Only in control	(-) 100.0 ± 0.0	(-) 100.0 ± 0.0
Octyl acetate	1497	Decrease	8.3 ± 15.2	(-) 100.0 ± 0.0
Linalool	1545	Decrease	(-) 17.5 ± 8.8	(-) 68.4 ± 23.6
Neryl acetate	1728	Only in control	(-) 100.0 ± 0.0	(-) 100.0 ± 0.0

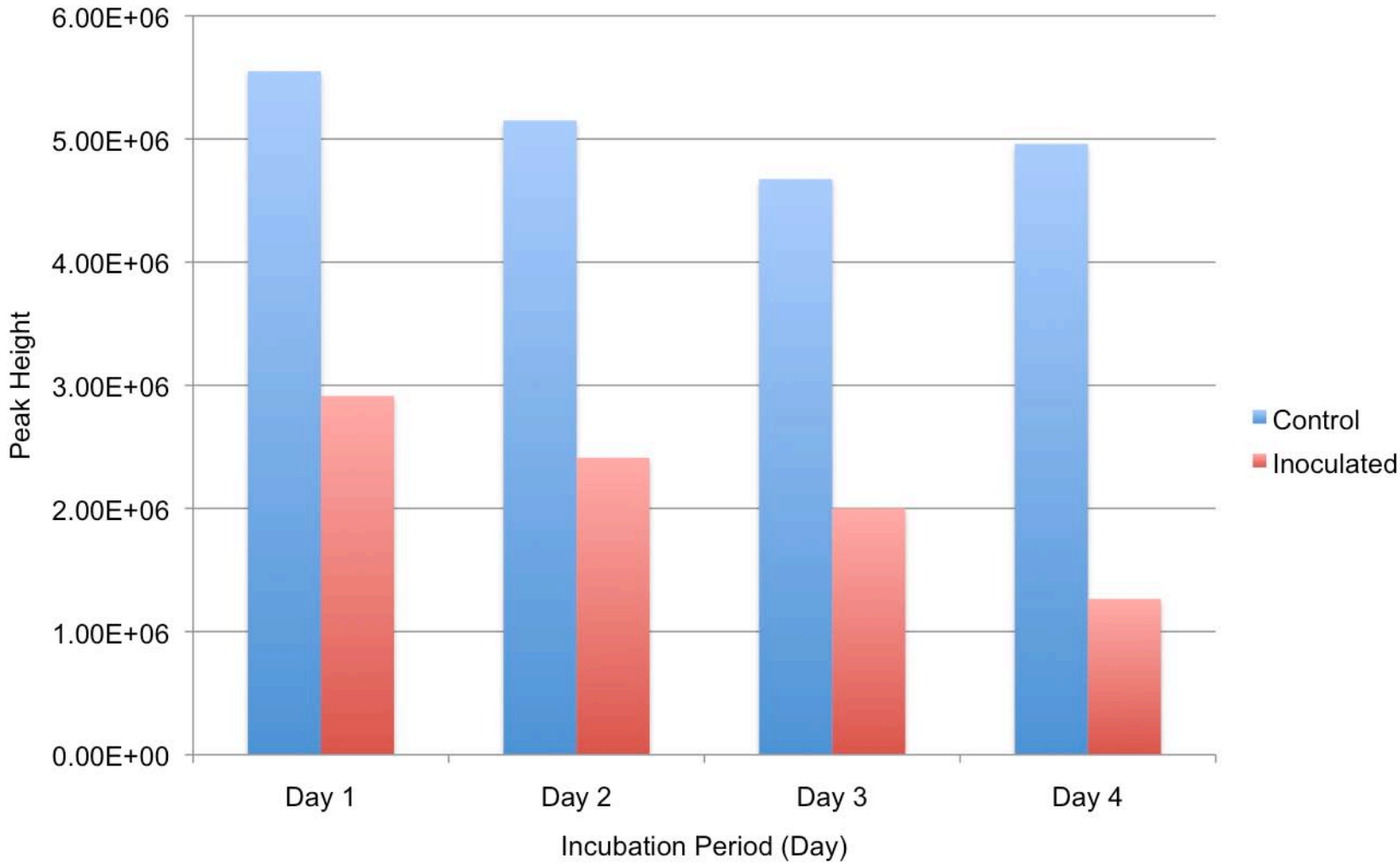


Figure 7-1. Trend in aroma impact compounds' perceived intensity

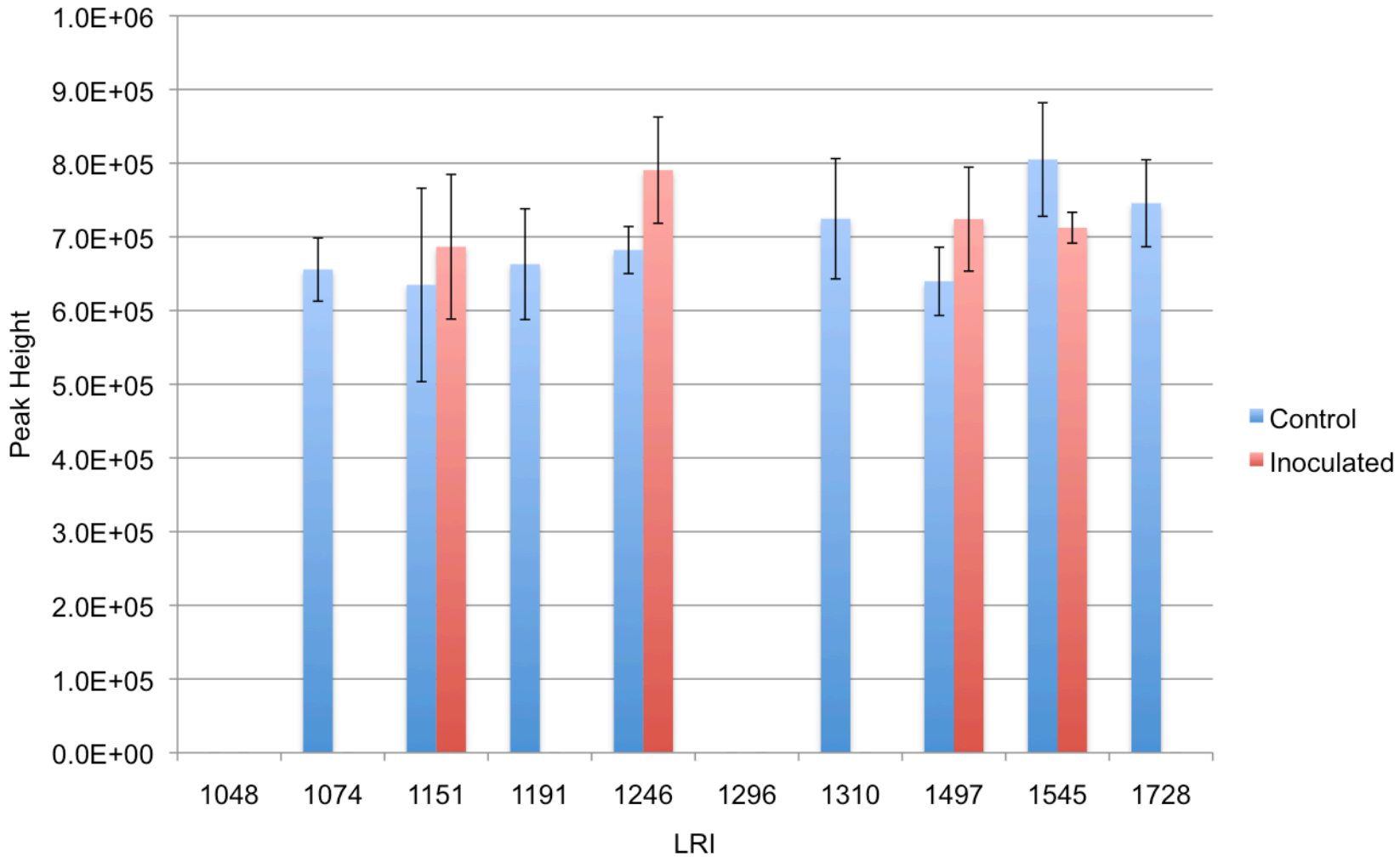


Figure 7-2. Control and inoculated aromagram at incubation day one

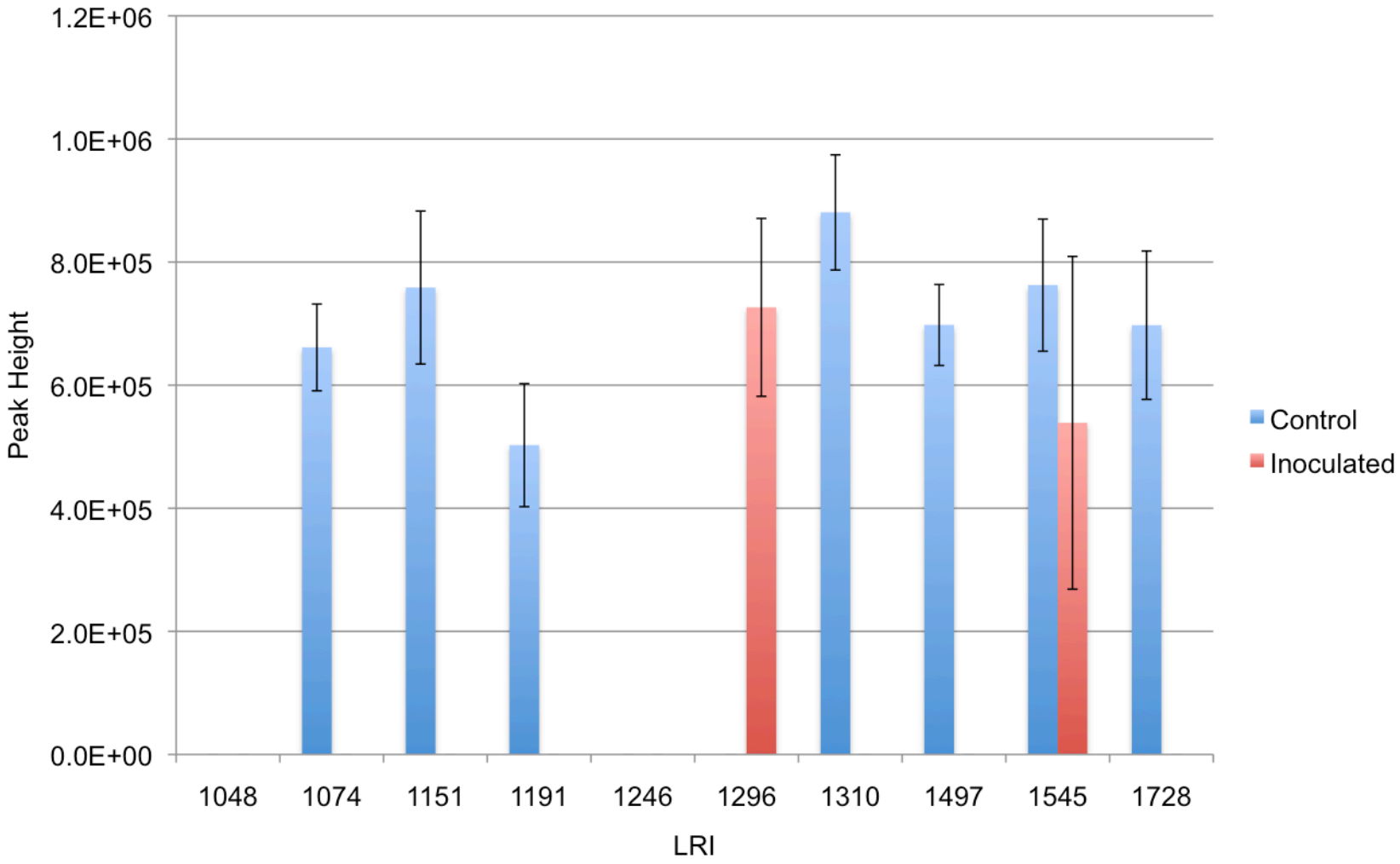


Figure 7-3. Control and inoculated aromagram at incubation day four

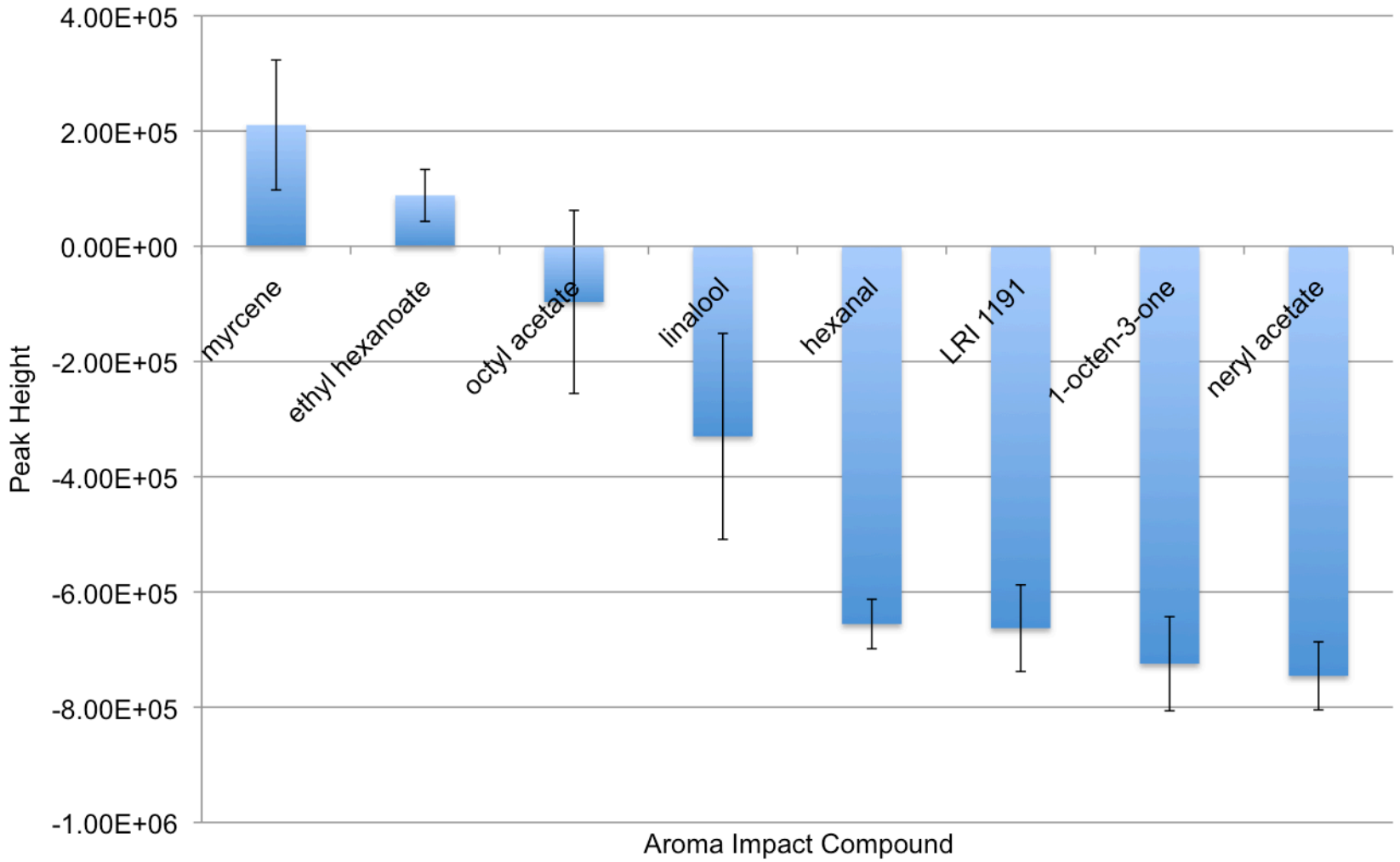


Figure 7-4. Difference in inoculated sample aroma intensity from control at incubation day one

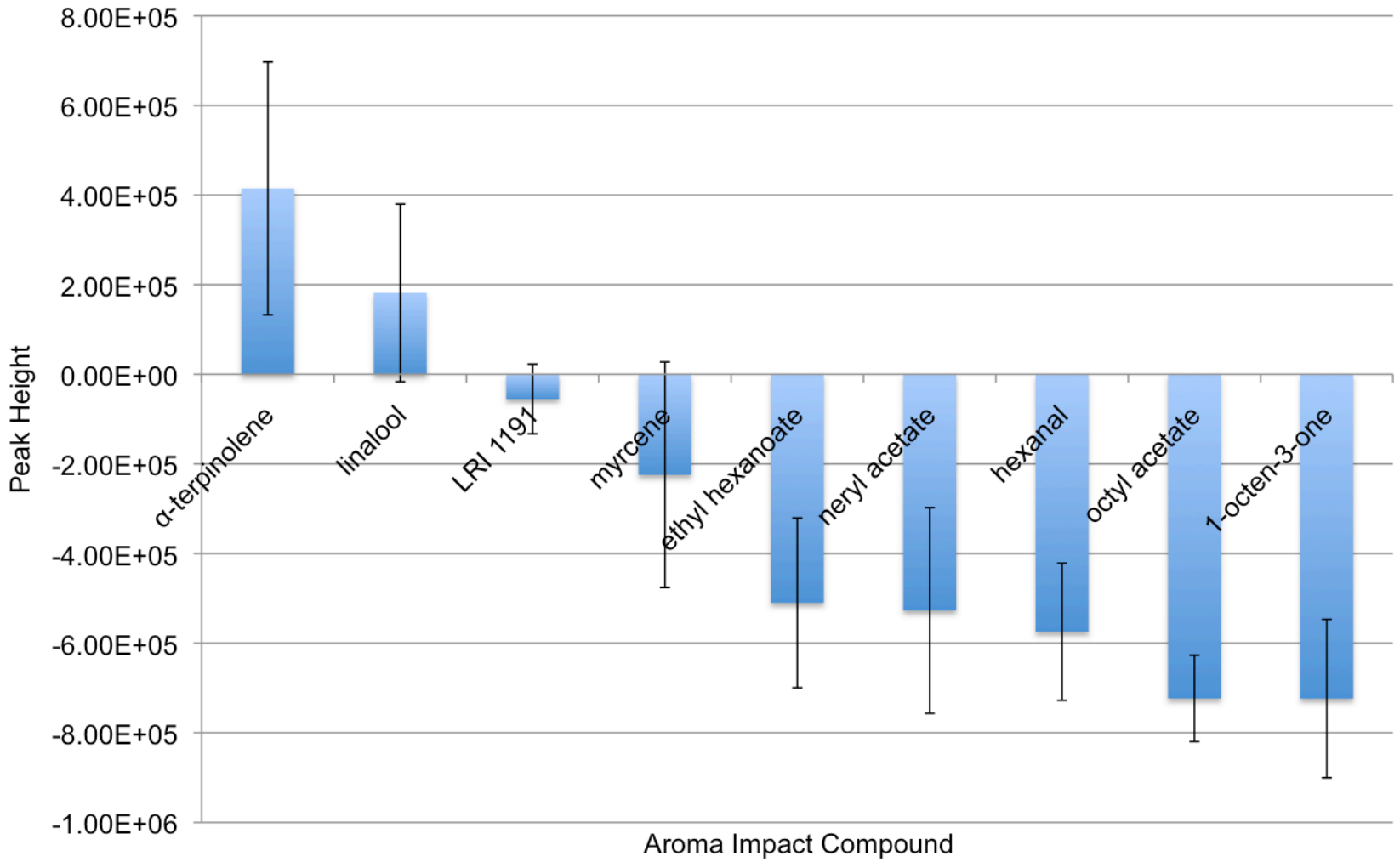


Figure 7-5. Difference in inoculated sample aroma intensity from control at incubation day two

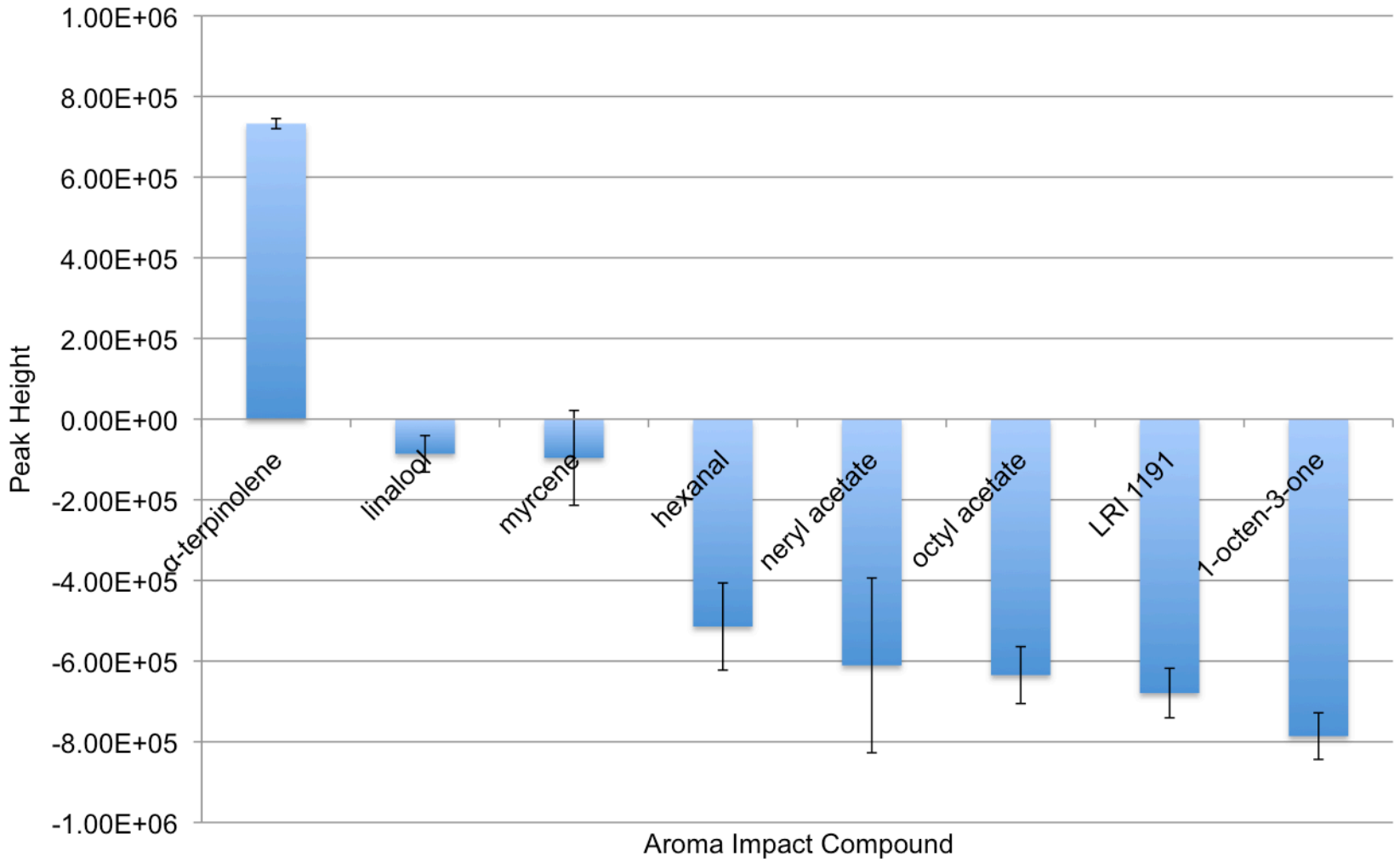


Figure 7-6. Difference in inoculated sample aroma intensity from control at incubation day three

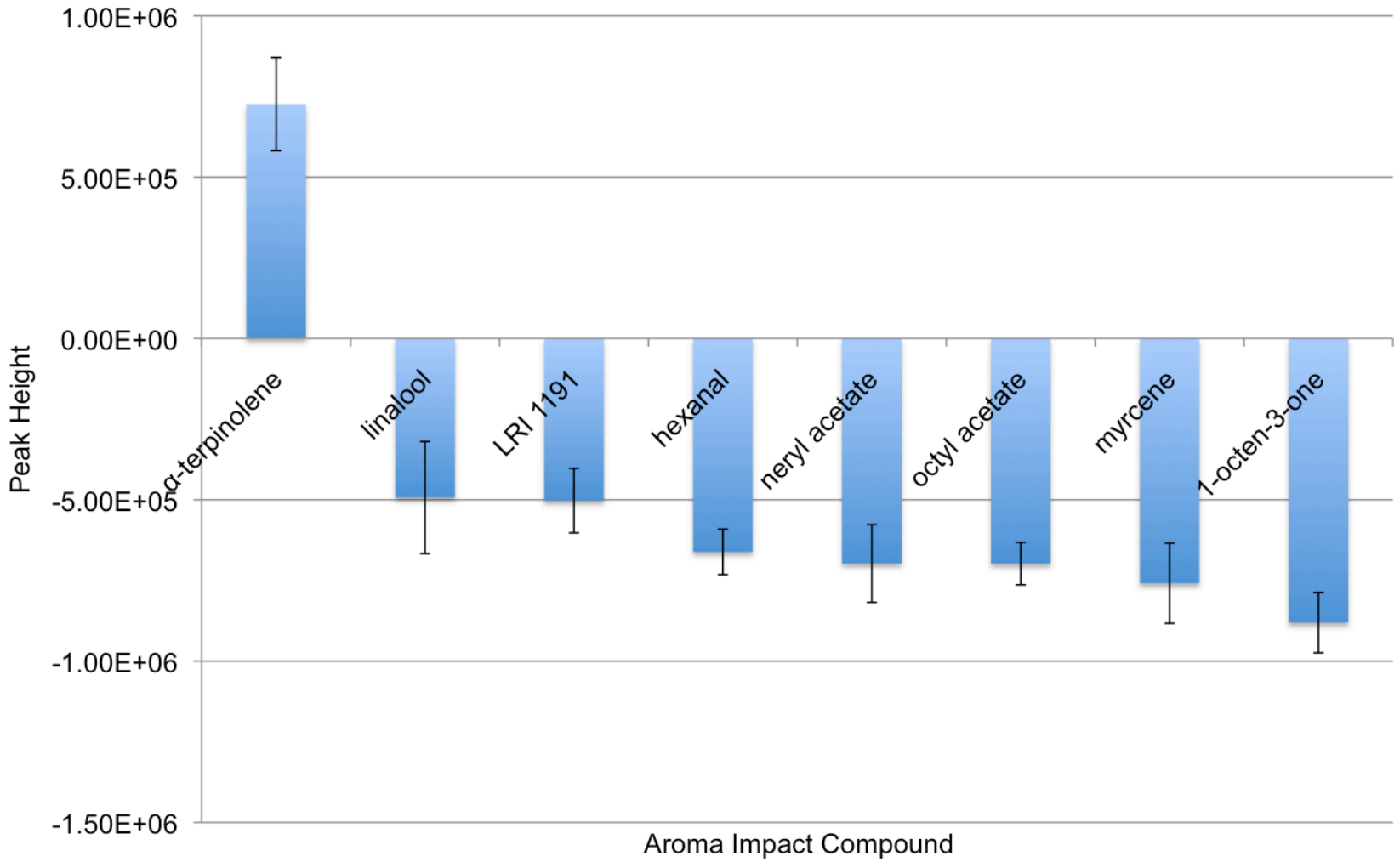


Figure 7-7. Difference in inoculated sample aroma intensity from control at incubation day four

CHAPTER 8
CONCLUSIONS AND FURTHER WORK

Overall, there was a decrease in volatile quantity and aroma intensity in the inoculated sample when compared to the control. In addition, various differentiating volatiles were observed. Of most importance are the qualitative differences including methanol, ethyl propanoate, ethyl 2-methylbutanoate, 3-methylbutanol, (Z)-3-hexenol, and 1-octen-3-ol, which are only identified in the inoculated sample. Neryl acetate, another qualitative difference, is also of importance, as this was the only volatile identified in the control, but not the inoculated. These volatiles, along with others, make a series of 44 differentiating volatiles that could be used to identify *P*. *digitatum* contamination in single strength OJ under carefully control conditions. The differences between control and inoculated percent difference for some volatiles, such as ethyl butanoate, demonstrate the ability to determine length of contamination. The day of maximum percent difference depends on specific storage conditions, such as temperature, and would fluctuate with alteration in conditions. Further study and validation is required to apply this concept. Sulfur compounds observed from the GC – S analysis could also be classified as qualitative or quantitative differentiating volatiles. Their application in determining the presence of contamination is the same as the differentiating volatiles from the GC – MS analysis.

The formation and breakdown of volatiles results from the fungal metabolic pathway and enzymatically driven reactions, including bioconversions. These reactions help explain relationships between volatiles, such as the increase in ethanol and the 11% reduction in °Brix. The increase or decrease in a certain volatile is usually the

result of multiple reactions. Enzymes also aid in the bioconversion of volatiles, such as limonene to α-terpineol.

Some of the volatiles that change are aroma impact compounds. These volatiles, including neryl acetate and octyl acetate, decreased overall resulting in a decrease in perceived aroma intensity in the inoculated sample. A-terpinolene was the only aroma impact compound found to increase over the incubation period.

The qualitative and quantitative differences in volatiles, including sulfur volatiles and aroma impact compounds, between control and inoculated samples demonstrate that contamination from *P*. *digitatum* results in unique reactions. These reactions have profound effects on the volatiles present, causing the development of differentiating volatiles. The differentiating volatiles consist of both qualitative and quantitative differences that are distinctive and can be used as indicators of *P*. *digitatum* growth. Studies of this nature, however, are only examinations of potential (Hutchinson 1973). The results here do show some potential for industry application as a means to identify *P*. *digitatum* in OJ.

This study, however, was performed under controlled laboratory conditions and validation under commercial conditions would be needed prior to application of this methodology for quality control. Further studies examining more organisms would be required to determine volatiles specific only to *P*. *digitatum*. In addition, running more replicates and exploring the results in different OJ would allow for greater determination of the specificity of the volatiles found here to *P*. *digitatum* and to *P*. *digitatum* grown in Simply Orange OJ.

APPENDIX A
VOLATILE TABLES

Table A-1. All peaks detected using GC – MS

LRI	Volatile	LRI	Volatile	LRI	Volatile
714	Acetaldehyde	1262	Unidentified	1591	Unidentified
825	Unidentified	1267	Γ-terpinene	1599	Unidentified
829	Unidentified	1270	Unidentified	1608	Unidentified
900	Ethyl acetate	1276	Unidentified	1626	B-elemene
909	Methanol	1286	Hexyl acetate	1631	Unidentified
947	Ethanol	1292	Unidentified	1636	Terpineol, 4-
974	Ethyl propanoate	1296	P-cymene	1646	B-caryophyllene
991	Unidentified	1300	A-terpinolene	1657	Unidentified
997	Unidentified	1310	Octanal	1666	Unidentified
1002	Methyl butyrate	1321	Unidentified	1671	Unidentified
1022	Unidentified	1354	Unidentified	1680	Citronellyl acetate
1037	A-pinene	1359	Hexanol	1690	B-selinene
1050	Ethyl butanoate	1365	Unidentified	1697	Unidentified
1065	Ethyl2-methylbutanoate	1378	Unidentified	1704	Unidentified
1077	Unidentified	1383	(E)-2-hexenol	1720	Unidentified
1082	Unidentified	1388	Unidentified	1730	A-terpineol
1090	Unidentified	1398	(Z)-3-hexenol	1233	Unidentified
1098	Hexanal	1417	Nonanal	1738	Unidentified
1105	Unidentified	1427	Unidentified	1746	Neryl acetate
1120	B-pinene	1442	Perillene	1750	Unidentified
1132	Sabinene	1451	Ethyl octanoate	1753	Unidentified
1161	Δ-3-carene	1465	Octen-3-ol, 1-	1760	Unidentified
1171	Myrcene	1468	Unidentified	1767	Unidentified
1175	Unidentified	1471	A-p-dimethylstyrene	1770	Valencene
1180	Unidentified	1476	Unidentified	1779	A-selinene
1186	Unidentified	1488	Unidentified	1784	Unidentified
1194	A-terpinene	1492	Octyl acetate	1788	Carvone
1203	Unidentified	1500	Pentadecane	1798	Δ-cadinene
1212	Methylbutanol, 3-	1526	Decanal	1812	Unidentified
1228	Limonene	1531	A-copaene	1819	A-panasinsen
1240	B-phellandrene	1558	Linalool	1830	Unidentified
1245	Ethyl hexanoate	1569	Octanol	1842	Perillaldehyde
1249	E-2-hexenal	1575	Benzaldehyde	1856	Unidentified
1256	Unidentified	1580	Unidentified	1864	Cis-carveol

Table A-2. All peaks detected using GC – S

LRI	Sulfur Volatile
695	Unidentified
738	Dimethyl sulfide
942	Unidentified
1093	Dimethyl disulfide
1413	Unidentified
1484	Unidentified
1557	Unidentified
1563	Unidentified
1597	Dithiane, 1,4-
1670	Unidentified
1743	Unidentified
1779	Phenethyl mercaptan
1789	Unidentified
2125	Unidentified

APPENDIX B
AROMA IMPACT COMPOUNDS

Table B-1. All aroma impact compounds detected using GC – O

LRI	Observed Aroma Descriptor	Volatile
836	Sulfuric, sour, fruity	Unidentified
1031	Fruity, apple, sweet, cotton candy	Unidentified
1048	Fruity, citrus, bubble gum, pineapple	Unidentified
1074	Green, grassy, fresh	Hexanal
1151	Musty, moldy, metallic, alcoholic	Myrcene
1191	Leafy, pungent, spicy, floral	Unidentified
1209	Rubber, soil	Unidentified
1230	Sweet, plants, floral, spicy	Unidentified
1246	Fruity, fresh, green, grassy	Ethyl hexanoate
1284	Minty, sweet, citrus	Unidentified
1296	Green, candy, fruity	A-terpinolene
1310	Mushroom	Octen-3-one, 1-
1370	Musty, moldy, earthy, green bean	Unidentified
1391	Celery, herbal	Unidentified
1445	Cooked potato	Unidentified
1497	Minty, fruity, pine-like, bamboo, leaf	Octyl acetate
1530	Dusty, earthy, damp, herbal	Unidentified
1545	Floral, flowery, sweet	Linalool
1660	Medicinal	Unidentified
1728	Woody, rosy	Neryl acetate

LIST OF REFERENCES

Achilea O, Fuchs Y, Chalutz E & Rot I. 1985. The contribution of host and pathogen to ethylene biosynthesis in *Penicillium digitatum*-infected citrus fruit. Physiologial Plant Pathology 27(1):55-63.

Acree TE & Arn H. 2004. Flavornet and human odor space. Gas chromatography - olfactometry (GCO) of natural products*.* Geneva, NY.

Adams A, Demyttenaere JCR & De Kimpe N. 2003. Biotransformation of (R)-(+)- and (S)-(-)-limonene to α-terpineol by *Penicillium digitatum*-- investigation of the culture conditions. Food Chemistry 80(4):525-534.

Alaña A, Alkorta I, Domínguez JB, Llama MJ & Serra JL. 1990. Pectin lyase activity in a *Penicillium italicum* strain. Applied Environmental Microbiology 56(12):3755-3759.

Ariza MR, Larsen TO, Petersen BO, Duus JØ & Barrero AF. 2002. *Penicillium digitatum* metabolites on synthetic media and citrus fruits. Journal of Agricultural and Food Chemistry 50(22):6361-6365.

Barmore CR & Brown GE. 1979. Role of pectolytic enzymes and galacturonic acid in citrus fruit decay caused by *Penicillium digitatum*. Phytopathology 69(9):675-678.

Bush DA & Codner RC. 1968. The nature of macerating factor of *Penicillium digitatum* saccardo. Phytochemistry 7(5):863-869.

Bush DA & Codner RC. 1970. Comparison of the properties of the pectin transeliminases of *Penicillium digitatum* and *Penicillium italicum*. Phytochemistry 9(1):87-97.

Caccioni DRL, Guizzardi M, Biondi DM, Agatino R & Ruberto G. 1998. Relationship between volatile components of citrus fruit essential oils and antimicrobial action on *Penicillium digitatum* and *Penicillium italicum*. International Journal of Food Microbiology 43(1-2):73-79.

Cai J, Liu B & Su Q. 2001. Comparison of simultaneous distillation extraction and solid-phase microextraction for the determination of volatile flavor components. Journal of Chromatography A 930(1-2):1-7.

Cevallos-Cevallos JM, Reyes-De-Corcuera JI, Etxeberria E, Danyluk MD & Rodrick GE. 2009. Metabolomic analysis in food science: A review. Trends in Food Science & Technology 20(11-12):557-566.

Cole ALJ & Wood RKS. 1970. Pectic enzymes associated with *Penicillium digitatum* decay of citrus fruit. Annals of Botany 34(1):211-216.

Davé BA, Kaplan HJ & Petrie JF. 1981. The isolation of *Penicillium digitatum* Sacc. strains tolerant to 2-AB, SOPP, TBZ and benomyl. Proceedings of the Florida State Horticultural Societ 93:344-347.

Demyttenaere JCR, Van Belleghem K & De Kimpe N. 2001. Biotransformation of (*R*)-(+)- and (*S*)-(-)-limonene by fungi and the use of solid phase microextraction for screening. Phytochemistry 57(2):199-208.

Doyle MP & Beuchat LR. 2007. Food microbiology: Fundamentals and frontiers. 3rd ed. Washington, DC: American Society for Microbiology Press. p. 1038.

Droby S, Eick A, Macarisin D, Cohen L, Rafael G, Stange R, McColum G, Dudai N, Nasser A, Wisniewski M & Shapira R. 2008. Role of citrus volatiles in host recognition, germination and growth of *Penicillium digitatum* and *Penicillium italicum*. Postharvest Biology and Technology 49(3):386-396.

Duetz WA, Bouwmeester H, van Beilen JB & Witholt B. 2003. Biotransformation of limonene by bacteria, fungi, yeasts, and plants. Applied Microbiology and Biotechnology 61(4):269-277.

Eckert JW & Ratnayake M. 1994. Role of volatile compounds from wounded oranges in induction of germination of *Penicillium digitatum* conidia. Phytopathology 84(7):746-750.

Faville LW & Hill EC. 1951. Incidence and significance of microorganisms in citrus juices. Food Technology 5(10):423-425.

Filtenborg O, Frisvad JC & Thrane U. 1996. Moulds in food spoilage. International Journal of Food Microbiology 33(1):85-102.

French RC. 1985. The bioregulatory action of flavor compounds on fungal spores and other propagules. Annual Review of Phytopathology 23(1):173-199.

Fries N. 1973. Effects of volatile organic compounds on the growth and development of fungi. Transactions of the British Mycological Society 60(1):1-21.

Huis in't Veld JHJ. 1996. Microbial and biochemical spoilage of foods: An overview. International Journal of Food Microbiology 33(1):1-18.

Hutchinson SA. 1973. Biological activities of volatile fungal metabolites. Annual Review of Phytopathology 11(1):223-246.

Janssens L, De Pooter HL, Schamp NM & Vandamme EJ. 1992. Production of flavours by microorganisms. Process Biochemistry 27(4):195-215.

Jeleń HH. 2003. Use of solid phase microextraction (SPME) for profiling fungal volatile metabolites. Letters in Applied Microbiology 36(5):263-267.

Kavanagh JA & Wood RKS. 1971. Green mould of oranges caused by *Penicillium digitatum* Sacc.; effect of additives on spore germination and infection. Annals of Applied Biology 67(1):35-44.

Lacey J. 1989. Pre- and post-harvest ecology of fungi causing spoilage of foods and other stored products. Journal of Applied Microbiology 67(s18):11s-25s.

Larsen TO & Frisvad JC. 1994. A simple method for collection of volatile metabolites from fungi based on diffusive sampling from Petri dishes. Journal of Microbiological Methods 19(4):297-305.

Larsen TO & Frisvad JC. 1995a. Characterization of volatile metabolites from 47 *Penicillium* taxa. Mycological Research 99(10):1153-1166.

Larsen TO & Frisvad JC. 1995b. Chemosystematics of *Penicillium* based on profiles of volatile metabolites. Mycological Research 99(10):1167-1174.

Larsen TO & Frisvad JC. 1995c. Comparison of different methods for collection of volatile chemical markers from fungi. Journal of Microbiological Methods 24(2):135-144.

Lui L, Vikram A, Hamzehzarghani H & Kushalappa A. 2005. Discrimination of three fungal diseases of potato tubers based on volatile metabolic profiles developed using GC/MS. Potato Research 48(1):85-96.

Moalemiyan M, Vikram A & Kushalappa AC. 2007. Detection and discrimination of two fungal diseases of mango (cv. Keitt) fruits based on volatile metabolite profiles using GC/MS. Postharvest Biology and Technology 45(1):117-125.

Moalemiyan M, Vikram A, Kushalappa AC & Yaylayan V. 2006. Volatile metabolite profiling to detect and discriminate stem-end rot and anthracnose diseases of mango fruits. Plant Pathology 55(6):792-802.

Narciso JA & Parish ME. 1997. Endogenous mycoflora of gable-top carton paperboard used for packaging fruit juice. Journal of Food Science 62(6):1223-1239.

Nilsson T, Larsen TO, Montanarella L & Madsen JØ. 1996. Application of head-space solid-phase microextraction for the analysis of volatile metabolites emitted by *Penicillium* species. Journal of Microbiological Methods 25(3):245-255.

Parish ME. 1991. Microbiological concerns in citrus juice processing. Food Technology 45(4):128-136.

Parish ME. 1997. Public health and nonpasteurized fruit juices. Critical Reviews in Microbiology 23(2):109-119.

Parish ME & Higgins DP. 1989. Yeasts and molds isolated from spoiling citrus products and by-products. Journal of Food Protection 52(4):261-263.

Patrick R & Hill EC. 1959. Microorganisms in juice from freeze-damaged oranges of various acidities and their resistance to heat. Applied Microbiology 7(4):193-195.

Pelser PdT & Eckert JW. 1977. Constituents of orange juice that stimulate the germination of conidia of *Penicillium digitatum*. Phytopathology 67(6):747-757.

Pesis E & Marinansky R. 1990. Volatile production induced by *Penicillium digitatum* in orange fruit and in culture. Journal of Phytopathology 128(4):306-314.

Raccach M & Mellatdoust M. 2007. The effect of temperature on microbial growth in orange juice. Journal of Food Processing and Preservation 31(2):129-142.

Reineccius G. 2006. Flavor chemistry and technology,2nd ed. Boca Raton, FL: CRC Press.

Rouseff R. 2006. Citrus flavor and color. In: Smoot, J., editor). Flavor database: Retention index wax. Lake Alfred, FL: University of Florida - Citrus Research and Education Center.

Schnürer J, Olsson J & Börjesson T. 1999. Fungal volatiles as indicators of food and feeds spoilage. Fungal Genetics and Biology 27(2-3):209-217.

Stange RR, Midland SL, Sims JJ & McCollum GT. 2002. Differential effects of citrus peel extracts on growth of *Penicillium digitatum*, *P. italicum*, and *P. expansum*. Physiological and Molecular Plant Pathology 61(5):303-311.

Tan Q & Day DF. 1998a. Bioconversion of limonene to α-terpineol by immobilized *Penicillium digitatum*. Applied Microbiology and Biotechnology 49(1):96-101.

Tan Q & Day DF. 1998b. Organic co-solvent effects on the bioconversion of (R)-(+)-limonene to (R)-(+)-α-terpineol. Process Biochemistry 33(7):755-761.

Tan Q, Day DF & Cadwallader KR. 1998. Bioconversion of (R)-(+)-limonene by *P. digitatum* (NRRL 1202). Process Biochemistry 33(1):29-37.

Tournas VH, Heeres J & Burgess L. 2006. Moulds and yeasts in fruit salads and fruit juices. Food Microbiology 23(7):684-688.

Vikram A, Lui LH, Hossain A & Kushalappa AC. 2006. Metabolic fingerprinting to discriminate diseases of stored carrots. Annals of Applied Biology 148(1):17-26.

Vikram A, Prithiviraj B, Hamzehzarghani H & Kushalappa AC. 2004a. Volatile metabolite profiling to discriminate diseases of McIntosh apple inoculated with fungal pathogens. Journal of the Science of Food and Agriculture 84(11):1333-1340.

Vikram A, Prithiviraj B & Kushalappa AC. 2004b. Use of volatile metabolite profiles to discriminate fungal disease of Cortland and Empire apples. Journal of Plant Pathology 86(3):215-225.

Wady L, Bunte A, Pehrson C & Larsson L. 2003. Use of gas chromatography-mass spectrometry/solid phase microextraction for the identification of MVOCs from moldy building materials. Journal of Microbiological Methods 52(3):325-332.

Wyatt MK & Parish ME. 1995. Spore germination of citrus juice-related fungi at low temperatures. Food Microbiology 12:237-243.

Wyatt MK, Parish ME, Widmer WW & Kimbrough J. 1995. Characterization of mould growth in orange juice. Food Microbiology 12:347-355.

Zhang Z & Pawliszyn J. 1993. Headspace solid-phase microextraction. Analytical Chemistry 65(14):1843-1852.

BIOGRAPHICAL SKETCH

Gabriel Louis Shook graduated summa cum laude from the University of Florida with a Bachelor of Science in food science and human nutrition in May 2009. Throughout his undergraduate years, he has authored a food microbiology undergraduate thesis entitled “Permeability of *Staphylococcus aureus* through Latex Finger Cots,” been accepted into The National Society of Collegiate Scholars, Delta Epsilon Iota, and Golden Key International Honor Society, and was the recipient of the food science department’s Outstanding Senior award. In order to further his knowledge in food chemistry, he chose to study flavor chemistry under Dr. Russell Rouseff at the University of Florida and entered into the Master of Science program in the food science and human nutrition department. Gabriel’s project was a mixture of food microbiology and food chemistry. While working towards his master’s degree, Gabriel interned with Kraft Foods in East Hanover, New Jersey. Upon graduation, he returned to East Hanover, NJ, to work as a food product developer for Kraft Foods, Nabisco.