ABSTRACT

LAFOUNTAIN, LISA JANE. Quality and Safety of Refrigerated Cucumber Pickles. (Under the direction of Dr. Suzanne Johanningsmeier and Dr. Fred Breidt, Jr.).

Refrigerated cucumber pickles are produced by direct acidification of pickling cucumbers followed by refrigerated storage for the entirety of their shelf-life. They do not undergo a thermal process, such as pasteurization, which allows them to maintain fresh-like qualities, such as crisp texture, white flesh and characteristic cucumber flavor. There is growing concern related to the survival of acid resistant *Escherichia coli* O157:H7 strains in low pH products that have not been subjected to a thermal treatment. Prior research showed that this pathogen is capable of surviving in a refrigerated cucumber pickle environment for over one month. One potential method of reducing the likelihood of foodborne illness relating to cucumber pickles is to apply a brief blanch step to cucumbers prior to processing refrigerated pickles in order to reduce microbiota. It was hypothesized that a brief blanch step could reduce microbiota without negatively impacting quality characteristics of the final product. Blanch treatments (15, 90, or 180 seconds) in 80 °C water were conducted in duplicate for two independent lots of whole cucumbers, along with an un-blanch control. Enumeration of total aerobes (PCA), lactic acid bacteria (LAB), and *Enterobacteriaceae spp.* (VRBG) was conducted immediately following blanching. Quality measurements, including firmness, exocarp color and flesh cure appearance were taken of cucumbers and refrigerated pickles during a one-year shelf life. Microbiological samples were taken in order to determine microbial reduction following blanching. A tetrad discrimination test (n=110) was conducted after 62 days of refrigerated storage. Additionally, volatile compound analysis was conducted to determine if any changes in flavor-active lipid oxidation products were evident between un-blanch cucumber pickles and blanched (90 seconds) cucumber pickles. It was found that a 90 second blanch in 80 °C water applied to whole cucumbers is
capable of consistently reducing background microbiota without significantly deteriorating quality of refrigerated cucumber pickles. Consumers were unable to detect differences between cucumber pickles produced with either un-blanched or blanched cucumbers. Pickles made with cucumbers that received a 90 second blanch maintained an average exocarp chroma value (color saturation) of 24.7 across the one year shelf life, while pickles made with unblanched cucumbers underwent a significant reduction in average chroma (25.9 to 14.0) over the same time period. Additionally, pickles made with cucumbers that received a 90 second blanch exhibited less cure appearance development (CAD) than those made with unblanched cucumbers, suggesting that this treatment may extend the shelf life of refrigerated cucumber pickles. No differences in the abundance of volatile, secondary lipid oxidation products were found between blanched and unblanched cucumber pickles (P > 0.05). A second study was conducted utilizing *Escherichia coli* O157:H7 in cucumber homogenate in order to determine the kinetics of thermal inactivation of this pathogen. The z-value was found to be 9.49 °C. This z-value was used along with a COMSOL thermal model in order to determine predicted pathogen reduction within the cucumber during blanching. Based on this model, a 90 second blanch of whole cucumbers in 80 °C water was predicted to achieve a 5-log reduction of *Escherichia coli* O157:H7 up to 1.1mm into the cucumber fruit. Both microbiological and quality results suggest that a 90 second blanch could be used by refrigerated cucumber pickle processors in order to maintain a safe product as it will not negatively affect the product in terms of quality or consumer perception.
Quality and Safety of Refrigerated Cucumber Pickles.

by
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DEDICATION

To my husband, Ryan, whose love and support has allowed me to follow this path.

I know that it’s not been an easy ride, but I’m glad we got to endure it together.

Thank you for believing in me, especially when I didn’t believe in myself.
BIOGRAPHY

Lisa Jane LaFountain (née Davies) was born on September 22nd, 1986 to John and Marian Davies. She was raised in Shropshire, United Kingdom with her brother, Robert, and sister, Annie. In 2007, she moved to Sydney, Australia. While on a work project in the USA, she met her husband, Ryan. They were married in October 2011. Lisa earned her Bachelor’s degree in food science technology at North Carolina State University in May 2017. Upon completion of her undergraduate studies, Lisa remained at NC State in order to pursue her Master’s degree in food science under the advisement of Dr. Suzanne Johanningsmeier and Dr. Fred Breidt, Jr. in the USDA-ARS Food Science Research Unit. During her time in the Department of Food, Bioprocessing and Nutrition Sciences, Lisa was an active member of the Food Science Club, serving as the 2016-2017 College Bowl Committee Chair, the 2017-2018 Product Development Committee Chair, the 2017-2018 Club Secretary, and the 2016-2019 FBNS Awards Banquet Committee Chair. Lisa will continue to be a member of the Wolfpack following her graduation, working in the College of Agriculture and Life Sciences.
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Chapter 1: Factors influencing safety and quality of refrigerated-type cucumber pickles

1.1 Introduction to cucumbers and cucumber processing

Cucumbers, *Cucumis sativus*, can be categorized into three classes, namely field grown slicers, greenhouse grown slicers and processing cucumbers (USDA ERS, 2007). Approximately 3.2 pounds of pickling (processing) type cucumbers were consumed per capita in the US in 2016 alone (USDA ERS, 2017), and they are the most commonly pickled vegetable in the US (USDA ERS, 2007). Cucumber pickles are produced by fermentation, direct acidification or a combination of both, resulting in a final pH of 4.6 or below, that must be maintained throughout the shelf life (USDA AMS, 1991). Fermented, or ‘cured’, cucumber pickles are brined with a salt solution and fermented in natural or controlled conditions (Franco et al., 2016) and may contain added acids, aromatics or preservatives (USDA AMS, 1991). Non-fermented cucumber pickles are directly acidified, using acetic acid liquor containing salt, calcium and, typically, some form of preservative compound (Pérez-Díaz et al., 2013). Once directly acidified, ‘fresh pack’ pickles are thermally processed using pasteurization, while ‘refrigerated pickles’ are refrigerated immediately upon acidification and remain under refrigeration for the entirety of their shelf life (USDA AMS, 1991).

Acidified, refrigerated pickles are characterized by a fresh, white flesh and crisp texture (USDA AMS, 1991) which is achieved by foregoing a thermal process that could potentially bring about negative changes in texture (Rodrigo and Alvarruiz, 1988) or flesh appearance (Fellers and Pflug, 1968). Similarly, since most refrigerated pickles are not fermented, they are less likely to exhibit the translucent appearance or ‘cure’ that is characteristic of fermented cucumber pickles. In refrigerated cucumber pickles, end of shelf life is typically dictated by a
change in color or texture perceived by consumers rather than microbial spoilage (Personal Communication with Processors) due to the addition of preservatives to the cover brine.

### 1.2 Safety concerns of directly acidified, non-thermally processed pickles

In a study conducted from 1998-2008, Painter at al. (2013) found that 46% of foodborne illnesses reported in the US were attributed to fresh produce. Correspondingly, produce was indicated as the vehicle in 38% of hospitalizations and 23% of deaths attributed to foodborne illnesses (Painter et al., 2013). More recently the CDC identified seeded vegetables, such as cucumbers and tomatoes, as being the single food category with the most linked foodborne illnesses in the US in 2015 (CDC, 2017). The prevalence of produce-linked foodborne illness outbreaks could be attributed to increased demand driving suppliers to expand their sourcing of these items, which in turn could lead to changes in growing, harvesting and post-harvest processing of the raw cucumbers (Beuchat and Ryu, 1997). Cucumbers are classified as fresh produce prior to any further preservation and, as such, there is a risk of pathogenic microbial contamination of the raw product. Pathogenic contamination has the potential to occur at every stage of the production and supply chain of fresh produce including contamination from soil, manure, irrigation water, human hands, shipping to the processing facility and at the processing facility prior to preservation. The Food Safety Modernization Act’s Produce Safety Rule focuses on water quality, manure application, animal contact and worker hygiene on produce farms (FDA, 2015) to improve the quality and safety of produce, which should benefit pickle producers.

Following a number of foodborne outbreaks attributed to pathogenic *Escherichia coli* O157:H7 in apple cider, the FDA mandated the use of a Hazard Analysis and Critical Control
Point (HACCP) food safety management system for fruit and vegetable juice processors in 2001 (HACCP Systems, 2001). Since then, research focused on the safety of acidified food products has used a 5-log reduction in pathogens as a gold standard, likely due to the similarities in pH between apple cider and acidified foods. In cucumber pickle products, a 5-log reduction of *Escherichia coli* O157:H7, *Salmonella enterica* and *Listeria monocytogenes* was achieved by holding products acidified with acetic acid, with an equilibrated pH of 3.3 or below, for 2 days at 25 °C or 6 days at or above 10 °C (Breidt et al., 2007) without the need for a heat treatment. Storage temperature and duration for assuring safety of non-thermally processed acidified products with a pH of up to 3.8 has previously been determined and validated by Breidt et al. (2013) but not for products with the acid and pH conditions typical of refrigerated cucumber pickles. Although *L. monocytogenes* is a common pathogen of concern in refrigerated foods, *E. coli* O157:H7 is more acid resistant than other pathogens under conditions typical of refrigerated pickles (Breidt et al., 2007), making it the primary pathogen of concern in this particular product category.

Refrigerated type pickles are exempt from federally mandated acidified food regulations (21 CFR 114), so processors have not had to comply with the safety requirements associated with this regulation including process filing. However, under FSMA's new legislation, most food producers will be required to perform a hazard assessment and determine preventive controls, such as process or sanitation controls, to ensure product safety. Since directly acidified, refrigerated pickles achieve a final, equilibrated pH of approximately 3.8-4.0, the time temperature combinations of pathogenic destruction mentioned previously do not apply and, in fact, could require a much longer storage duration to ensure safety (Lu et al., 2013). Moreover, since lengthy heat treatments could negatively affect the texture and color attributes of the
finished product, another method to reduce potential pathogenic microorganisms must be sought to ensure a safe and high quality finished product. Ultimately, a combined heat and acid approach will be needed to ensure safety.

1.2.1 Brine Formulation

Refrigerated type pickles contain brine composed of acetic acid (added as vinegar), salt, calcium chloride and, typically, some form of preservative compound such as sodium benzoate (Pérez-Díaz et al., 2013). Processors currently rely on a combination of acidification and refrigerated storage to produce a stable refrigerated pickle product. The acetic acid in directly acidified, refrigerated cucumber pickles acts to decrease the survival time of potential pathogens (Breidt et al., 2004). However, it has been found that some strains of *Escherichia coli* O157:H7 exhibit acid-resistance, which could pose problems for processors (Lu et al., 2013). In fact, Lu et al. (2013), found that some strains of *Escherichia coli* O157:H7 were able to survive for up to 1 month in commercially available refrigerated cucumber pickle brines containing both acetic and benzoic acids. Lu et al. (2013) were able to achieve a 5-log reduction in pathogenic *Escherichia coli* within a 2-day holding period at 10 °C by reformulating the cucumber pickle cover liquor to reduce the acetic acid component and include fumaric acid, without negatively impacting the overall, flavor, and texture liking scores in a consumer acceptance test of cucumber pickles when compared to a commercially available control. Despite this promising finding, fumaric acid has not yet become a common ingredient in cucumber pickle processing. Moreover, considering the emerging popularity of ‘clean label’ foods by consumers (Asioli et al., 2017); a process adopted to reduce microorganisms rather than the addition of an extra ingredient during formulation could result in a more favorable customer perception.
Another method of reducing the pathogenic microbial population prior to acidification is the application of chemical sanitizers to the fresh produce. However, previous studies have shown that the utilization of chemical sanitizers on the surface of cucumber fruit are not entirely effective in decreasing the microbial load of fresh produce (Goodburn and Wallace, 2013; Adams et al., 1988). This ineffectiveness may be due to pits and crevices that are found on the surface of produce, or may be due to the hydrophobicity of the waxy cuticle, which does not allow for sufficient surface wetting and thus contact between the sanitizer and produce (Adams et al., 1988). Additionally, bacteria are known to transfer to the interior of the fruit through stomata which decreases effectiveness of surface sanitizers (Reina et al., 2002). The application of chemical treatments on fruits and vegetables in order to preserve quality and render them safe for consumption is increasingly questioned by consumers (Basha et al., 2015). Additionally, treatments such as chlorine soaking could affect the sensorial qualities such as flavor and texture of the produce (Goodburn and Wallace, 2013), so the development of non-chemical heat treatments to enhance postharvest quality and safety is important to food processors.

1.2.2 Thermal Treatments to Ensure Safety

Thermal processing has historically been used to preserve foodstuffs by reducing or eliminating spoilage microorganisms and eliminating pathogenic microorganisms (Ling et al., 2015). During the production of pasteurized cucumber pickles, jars filled with cucumbers and brine undergo a thermal treatment designed to penetrate to the middle of the jar for a certain time/temperature combination, depending on the pH of the finished product and jar geometry (Etchells and Jones, 1944; Monroe et al., 1969). The thermal process conditions for achieving a 5-log reduction in pathogens in acidified cucumber has been established for products with a pH up to 4.1 (Breidt et al., 2005) and with pH values from 4.1 to 4.6 (Breidt et al., 2014). In
refrigerated type pickles, no thermal process occurs, and processors instead rely on the composition of the brine formulation and refrigerated storage to yield a stable and safe product (Breidt et al., 2013; Breidt et al., 2007). While pasteurization is designed to deliver a microbial kill, it can also negatively affect color and texture (Fellers and Pflug, 1968; Rodrigo and Alvarruiz, 1988), thus a less harsh treatment could begin to reduce microbiota while preserving the characteristic quality of refrigerated pickles.

Blanching is a common thermal treatment used in vegetable processing, especially prior to freezing in order to inactivate degradative enzymes. Breidt et al. (2000) achieved a 2-3 log reduction (cfu/ml) in microbial cell counts in cucumbers blanched for 15 seconds at 80 °C, but their study did not include a quality component to determine differences between the treatment and a control. Blanching also has the ability to reduce pathogenic bacteria in order to render produce safe to consume. In a study conducted by Ceylan et al. (2017), hot water blanch treatments at 85 °C resulted in a greater than 5-log reduction of *Listeria monocytogenes* and *Salmonella spp* that were inoculated onto the surface of several different vegetables including potatoes, carrots and broccoli within 30 seconds.

Mattos et al. (2005) conducted a study to estimate localization and type of microbiota in cucumbers, and the susceptibility of those microbiota to blanch treatments. Their findings can be applied to determine microbial reduction of microorganisms due to heat treatments, both on the surface and subsurface of the cucumber fruit. Upon utilization of a heat transfer and microbial kinetic model, aerobic microorganisms were found to be located between the surface and 0.65mm from the surface deeming this the critical depth for heat transfer, the minimum point to which any thermal treatment must be able to penetrate to in order be effective in the reduction of microbial load (Mattos et al., 2005). Furthermore, it was found that blanching the cucumbers at
80°C for 12.5 seconds was able achieve a 2-log reduction in total aerobic microorganism counts for the entire cucumber (Mattos et al., 2005). Since this treatment may be implemented as a method to reduce microbiota on cucumbers, the quality of pickle products processed using heat treated cucumbers is of interest.

1.3 Quality of refrigerated pickles

1.3.1 Texture of pickles

Since refrigerated pickles are characterized by a ‘crisp’ texture (USDA AMS, 1991), texture quality degradation is a determining factor for end of shelf life. It is therefore of utmost importance to understand how processing factors affect pickle texture over the shelf life of the product.

The texture of plant-part foodstuffs can be attributed to a number of factors, which vary from plant to plant (Sajnin et al., 2003), and even between cultivars (Kapitsimadi et al., 1990). The primary attributes that affect texture of fruits and vegetables are turgor and cell wall composition, which are undeniably interconnected. During ripening of fruit tissue, polysaccharide components including pectins, hemicellulose and cellulose of the plant cell wall structure are modified (Brummell, 2006). The texture of a cucumber is dependent on the structure comprising the cell wall, cellular membrane and middle lamella of the cells of the cucumber. These structural components have the ability to resist to deformation during the consumer’s bite or chew (Sajnin et al, 2003) and this resistance results in crispness associated with a refrigerated pickle.

Cell walls provide structural support and are constructed of polysaccharides, which fall into three categories: cellulose, hemicelluloses, and pectins. Cellulose provides mechanical strength and rigidity, while hemicelluloses and pectins assist with flexibility of the cell wall (Van
Since turgor is lost following harvest of produce, the cell wall becomes the principle structure responsible for texture of the produce. The cell wall is comprised of three layers, the middle lamella, the primary cell wall and the secondary cell wall, and is a hydrophilic gel whose primary structure is made up of cellulose (Scholey, 1971). The texture of the gel making up the cell wall can be influenced by external stressors, including heat. While cellulose is typically fairly resistant to stressors and is insoluble in water, heating in water at very high temperatures can break hydrogen bonds and therefore weaken the structure (Scholey, 1971). However, Deguchi et al. (2006) found that the crystalline arrangement of cellulose does not transform to an amorphous state until a temperature of 320 °C and pressure of 25 MPa. With that in mind, major changes to cellulose structure is not expected during brief blanches, or even pasteurization of cucumber pickles.

Pectic substances make up a large portion of fruit cell walls, and are relatively more prone to solubilization and chemical reactions than cellulose or hemicellulose, making them a key player in textural changes of produce (Van Buren, 1979). Pectin is composed of galacturonic acid chains, connected via α 1-4 glycosidic bonds, along with various side-chains (Van Buren, 1979). The α 1-4 glycosidic bonds have found to be susceptible to breakage when heated at neutral pH via β elimination and can also be broken by hydrolytic splitting in acid conditions (Van Buren, 1979). However, refrigerated pickles traditionally contain less acid than pasteurized pickle products and so the conditions may not favor acid hydrolysis, nor would the low temperature of refrigeration (Cho and Buescher, 2012). Since refrigerated pickles do not undergo a thermal treatment, enzymes are unlikely to be denatured and this might be a reason for the relatively shorter shelf life of refrigerated pickles (4-6 months) compared with their pasteurized counterparts (12 months or more) (Cho and Buescher, 2012).
1.3.1.1 Enzymatically induced textural changes

The building blocks of the cell wall and cell membrane are susceptible to enzyme degradation, which leads to a reduction of texture quality in cucumbers and cucumber products, such as pickles. Both pectin and cellulose are subject to degradative enzymes, pectinases and cellulases respectively, which can lead to severe softening of fruit tissue (Miller et al., 1988).

1.3.1.1.1 Polygalacturonase

Degradation of firm texture in refrigerated pickles could be attributed to the activity of polygalacturonase (PG) due to its native presence in fresh cucumbers (McFeeters et al., 1980). PG has been detected in fresh cucumbers and has been found to increase under certain stressors to the fruit and as the fruit matures and undergoes processing (Sajnin et al., 2003). PG activity has been indicated in commercially produced, refrigerated cucumber pickles and may be responsible for degradation in textural quality of refrigerated pickles (Cho and Buesher, 2012).

Polygalacturonase is an enzyme innately present in cucumber flowers and in locular tissue of mature cucumbers (McFeeters et al., 1980). Polygalacturonase catalyzes the hydrolytic cleavage of galacturonide linkages (Brummell and Harpster, 2001), and is the primary enzyme responsible for the breakdown of pectin, and thus the promoter of tissue softening in some fruits and vegetables (Sozzi, 2004). Excess levels of polygalacturonase can cause textural degradation; however, it is an important enzyme in produce ripening. Saltveit and McFeeters (1980) found that the small ethylene burst at cucumber maturity can increase the level of native polygalacturonase. While this increase is not desirable for pickling cucumbers, as it can lead to unwanted softening of pickle products, it could be advantageous for other produce to assist in ripening, and can be brought about, if required, by exogenous ethylene (Saltveit and McFeeters, 1980).
The amount of PG in pickles can be influenced by the stress on the cucumber during harvest and post-harvest handling. It is thought that increased PG activity is due to the expression of the PG gene in response to water stress caused by post-harvest water loss (Kubo et al., 2000). Polygalacturonase activity has been shown to increase in response to other stresses. Miller et al. (1987) determined that there was an observable increase in PG activity in cucumbers held at cool temperatures (0°C and 10°C) following mechanical injury. An increase of polygalacturonase could potentially be avoided by harvesting cucumbers before they reach peak maturity, which is a common practice (Guillou and Floros, 1992). Even if no softening of the raw cucumber tissues is obvious prior to processing, any polygalacturonase that might be present on or inside of the cucumber could have negative implications during pickle storage. Native cucumber polygalacturonase has been identified as causing softening in non-pasteurized pickles, since there is no heat step to assist denaturing the enzyme (Cho and Buescher, 2012). While polygalacturonase is often connected with deterioration of texture in cucumbers, it is highly unlikely that it works independently. It has been shown that in order for polygalacturonase to modify pectin structure by depolymerization and solubilization, the pectin must first be acted upon by pectin methyl esterase (Brummell and Harpster, 2001).

1.3.1.1.2 Pectinesterases

Pectinesterases are found in most tissue of the cucumber plant, seeds, fruit and flowers and levels are similar across all stages of development (Bell et al., 1950). They are classified as endogenous or exogenous and catalyze the deesterification of pectin, leaving alpha-galacturonide linkages susceptible to cleavage by degradative enzymes. Pectinesterases, such as pectin methylesterases (PMEs), have been implicated in part for the degradative softening of cucumber fruit (Bell et al., 1950). However, it has more recently been suggested that PME may assist in the
preservation of firmness in cucumber pickles produced with CaCl$_2$ in the brine or cover liquor by demethylating the pectins and promoting Ca$^{2+}$ ion binding to free carboxyl groups (Hudson and Buescher, 1986). Once Ca$^{2+}$ ions are bound to the carboxyl groups, this assists in protecting the pectic substances against solubilization and deesterification (Hudson and Buescher, 1986). Solubilization of pectin, leading to depolymerization can lead to greater wall porosity, which can directly affect fruit texture by allowing more access for degradative enzymes into the cell (Negi and Handa, 2008).

When pectin methyl esterase de-esterifies pectin, it creates zones of charge on the pectin backbone, which leads to calcium sensitive pectins that can be reinforced by calcium bridges (Kim et al., 2009). It has been shown that blanching temperatures of 50-70°C can activate PME and thus increase the level of demethylation, leading to greater chance of interaction between carboxyl groups and Ca$^{2+}$ ions (Bartolome and Hoff, 1972, Hudson and Buescher, 1986). However, since the optimal pH for cucumber PME is around 6.0-7.0 (Guiavarc’h, 2003), it may be retarded or inactivated by a typical refrigerated pickle pH of 3.8-4.0. McFeeters et al. (1985), found that a 3-minute blanch of cucumber slices at 81°C reduced pectinesterase activity to 0% immediately following blanching; however, the activity increased with storage time, indicating at least a partial reactivation of the enzyme. Despite this, the percent pectin methylation in acid-brined cucumbers blanched at 81°C was nearly identical to that of unblanched cucumbers, with much higher initial pectinesterase activity. In fact, there was no evidence found of a relationship between pectin methylation and firmness changes (McFeeters et al., 1985).

Although polygalacturonases and pectinesterases are not the only enzymes implicated in textural changes of fruit tissue, they are the most commonly associated with premature softening of cucumber tissue, especially in pickles. Considering the potential role that enzymes play in
softening of produce, and ultimately the deterioration of quality, it is not surprising that there is current interest in down-regulation of cell-wall enzymes (Sozzi, 2004). Brummell and Harpster (2001) suggest that the ability to suppress genes involved in fruit softening could greatly reduce deterioration and ultimate spoilage of produce. The benefit of transgenic control rather than stopping the entire ripening process is that desirable aspects of ripening, such as pigmentation changes and sugar formation, can be encouraged while softening, an undesirable aspect of ripening, can be prevented. However, a simple suppression of one gene is unlikely to have the desired effect since ripening can be attributed to multiple enzymes, which may each be controlled by multiple genes (Brummell and Harpster, 2001).

1.3.1.1.3 Cellulases

Cellulases are broadly categorized into five types: endocellulases, exocellulases, cellobiases, oxidative cellulases, and cellulose phosphorylases. While polygalacturonase is the leading cause of enzymatic softening in cucumber tissue, cellulase has also been implicated to a lesser extent (Miller et al., 1988). Buescher and Hudson (1984) conducted a study on the effect of Cx-cellulase (an endocellulase) in a polygalacturonase free environment. Their study found that Cx-cellulase had little effect on softening of cucumber pickles during the first 4 months of shelf life, but a loss of firmness was observed thereafter. Additionally, they found that inclusion of calcium chloride mitigated the effect of Cx-cellulase, preventing softening of pickles during 12 months of storage.

1.3.1.2 Other factors influencing softening

Depolymerization of pectin can occur via an enzymatic or a non-enzymatic reaction (Krall and McFeeters, 1998). While enzymatic softening is typically the main focus of research relating to softening in cucumber pickles, softening can also be protein-mediated. Expansins are
small proteins, located in plant cell walls that are suspected to cause disruption of hydrogen bonds between cellulose microfibrils and other components within the cell wall matrix (Brummell and Harpster, 2001). The disruption of hydrogen bonds can contribute to cell wall relaxation and a reduction in cell turgor (Brummell and Harpster, 2001), thereby reducing the crispness, and potentially reducing texture quality of the cucumber fruit. The amount of expansin proteins could differ between cultivars, and so lot-to-lot variation in fresh produce could result in differences in texture in cucumber pickles due to cell wall loosening.

Mechanical damage is a leading cause of produce quality loss at the wholesale and retail level and can be caused by friction, impact and compression and leads to a loss of quality in the produce such as cuts and bruising (Lamikanra, 2005), which could ultimately affect the texture of cucumber fruits. When wounded, plant cells are able to make use of both short and long distance wound signaling, which can cause the release of oligosaccharides from the damaged cell wall as well as increased hormonal activity (Lamikanra, 2005). Signaling triggers various biochemical and developmental pathways, which are responsible for releasing compounds such as phenolics, tannins, and lignins that can assist in repairing and reinforcing the cell wall (Lamikanra, 2005). In wounded cucumber fruits, Walter at al. (1990) found that significant amounts of lignin were deposited at the wound cite during the healing process. Lignification of a wound site causes cell wall thickening, which is likely to result in textural changes in the finished product. Such textural changes could be perceived as toughness or chewiness or cucumber skin, which could be deemed as a quality defect in pickle products.

Low temperature storage can have the ability to slow post-harvest respiration, but if the temperature is too low or the duration too long, it can have the negative effect of leading to chilling injury (Etchells et al., 1973). Slowing post-harvest respiration has been shown to reduce
the rate of senescence, however if respiration is reduced too dramatically, then this will lead to a rapid onset of senescence and, ultimately, cell death (Aked, 2002). Chilling injury is a major postharvest factor in reducing texture quality of cucumber fruits, and its symptoms include high water loss, surface pitting and increased mesocarp solute leakage (Cabrera and Saltveit, 1992). Chilling injury is dependent on both temperature of the storage environment and duration (Aked, 2002), as well traits of the cucumber cultivar (Cabrera and Saltveit, 1992). The optimal post-harvest storage temperature for pickling cucumbers is approximately 10 °C (Etchells 1973) with high relative humidity (Etchells 1973 and Walter et al., 1990).

1.3.1.3 Thermally induced textural changes

When a fruit is subject to high-temperature treatment, an effect on the tissue metabolism of the fruit can be expected (Lurie, 2006) including conformational changes of cell components, enzyme activity and hormone production. Textural changes to the cucumber caused by the heat treatment may be immediately evident, or may be exhibited during the life of the treated product. While thermal processing is typically connected with a decrease in produce firmness (Iborra-Bernad et al., 2015), moderate heat treatment may have a positive effect, such as assisting with resistance to chill injury (Lurie, 2006) or delaying softening (Buescher et al., 2013). Buescher et al. (2013) concluded that heat-conditioning of whole cucumbers for 60 minutes at 50 °C resulted in cucumber pickles retaining firmness for 12 months, which is double the typical shelf-life for a refrigerated pickle. Furthermore, sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) results from the same study revealed that mesocarp tissue from heat conditioned cucumbers contained low molecular mass proteins, which could be indicative of formation of protective heat-shock proteins through heat conditioning. It is unlikely, however, that a brief
blanch treatment on whole cucumbers would produce heat shock proteins since the majority of the mesocarp tissue would not reach temperatures close to 50 °C.

1.3.1.4 Measuring Texture Quality of Pickles

Instrumental measurements have the benefit of being unbiased, however this data is fairly meaningless unless it can be empirically connected with the sensory qualities of the pickle. A puncture test is the most common instrumental method of determining textural properties of cucumber pickles (Jeon et al., 1975). Fruit Pressure Testers (FPT) were originally used to quantify the texture of pears and the first documented use of this type of instrument in the texture analysis of pickle products, using a 5/16-inch tip, was in 1941 (Jones et al., 1941). A UC Fruit Firmness Tester with an Ametek LKG-14 gauge and a 5/16-inch tip has been used to measure both the mesocarp firmness, and the skin firmness by piercing perpendicularly through the exocarp (Hudson and Buescher, 1986).

An Instron UTM fitted with a 0.315cm diameter cylindrical, flat-ended punch was employed by Thompson et al. (1982) to measure the penetration force of a 0.48cm thick cucumber slice. The same study found no significant difference (P > 0.05) in force required to penetrate the mesocarp region tissues of cucumber slices among slices between 0.48 cm and 0.95 cm thick (Thompson et al., 1982). A sensory panel was conducted, using a trained panel who utilized a 10-point scale in order to judge firmness and it was determined that there was high correlation (r = 0.88) between the instrumental and sensory methods (Thompson et al., 1982). This puncture method is now accepted as an empirical test and is used by researchers and industry as a simple and expedient measure of texture quality in cucumber pickles. More recently, Yoshioka et al. (2009) developed a method using a TA.XT Texture Analyzer (Stable Micro Systems Ltd., Surrey, UK) with a 3 mm diameter blunt-tipped probe puncturing mesocarp
tissue at a speed of 150 mm per minute, and found that instrumental measurements showed a strong linear relationship with sensory crispness. Pérez-Diaz et al. (2015) reported that an approximate 1N difference in mesocarp firmness resulted in statistically significant differences in hardness, fracturability, crispness, and crunchiness as assessed by a trained descriptive analysis panel using a modified Spectrum™ method. Furthermore, Wilson et al. (2015), determined a relationship between reduced firmness and consumer liking, with consumers ranking softer pickles lower on a hedonic scale. Therefore, mesocarp puncture tests are a suitable instrumental test that can be employed to indicate consumer perception of cucumber pickle texture quality.

1.3.2 Appearance of pickles

1.3.2.1 Color

Color is especially important in the consideration of consumer perception and shelf life of refrigerated fresh-pack pickle spears and slices, since both the exocarp and mesocarp tissues are visible to consumers (Mok and Buescher, 2011). Chlorophyll a and b are green pigments found primarily in the peel of cucumbers. Chlorophyll a is the primary pigment involved in plant photosynthesis and imparts a vivid green color to the plant. Chlorophyll b, an accessory pigment, absorbs light in a different wavelength range than chlorophyll a, increasing the photosynthetic capability of the plant. Both of these pigments have a similar structure and only differ in the side chain connected to carbon 3; chlorophyll a has a methyl group while chlorophyll b has a formyl group attached (Schwartz et al., 2008).

Chlorophylls are susceptible to degradation, especially from heat, acid conditions and enzymes. During heat treatments of produce, chlorophyll undergoes a structural change whereby the molecule undergoes isomerization (Schwartz et al., 2008). The magnesium atom core of
Chlorophyll can be replaced with two hydrogen atoms, resulting in the formation of pheophytin, which is an olive brown pigment that is seen as undesirable to consumers of refrigerated type pickles. Heat treatments can severely affect the cell structure of produce, which in turn affects membrane permeability, leading to increased transfer of hydrogen ions across the cell membrane, and more chance of the magnesium core being replaced (Schwartz et al., 2008). Chlorophyll b has been found to possess greater heat stability than chlorophyll a (Koca et al., 2007; Loey et al., 1998; Steet and Tong, 1996), likely due to the formyl side chain. Koca et al. (2007) determined that chlorophyll becomes more susceptible to heat degradation in lower pH environments.

Chlorophyllase is the enzyme responsible for enzymatic degradation of chlorophyll. Once heat activated, with an optimum temperature range between 60 and 82.2 °C (Schwartz et al., 2008), the enzyme cleaves phytol from chlorophyll, resulting in chlorophyllides, which have an intense green color. Enzymatic conversion of chlorophyll to chlorophyllide may help to retain green color, when green vegetables are blanched between 54-76 °C for up to 45 mins (Schwartz et al., 2008). However, since these temperatures are lower than the typical blanches applied to fresh produce in order to inhibit degradative enzymes, it is unlikely that processors would adopt such blanch conditions.

Etchells and Thompson (1974) found that the “natural green cucumber color” of refrigerated pickle peel lasted for approximately 3-4 weeks at storage temperatures of 4 – 6 °C, after which time the peel color changed to that of “yellowish straw”. Buescher and Hamilton (2000) found that both fermented and non-fermented pickles stored in the dark for 6 months showed minimal changed in hue angle (°) or L-value during 6 months of storage. Fermented and non-fermented pickles stored in light (6 lux) exhibited a significant increase in L-value.
(becoming lighter) and decrease in hue angle (becoming more yellow), suggesting that photo-oxidation is the primary cause of color change during pickle storage.

1.3.2.2 Color measurement

While sensory perception is the primary motivation for measuring color in food, it can be troublesome when used for evaluative purposes, since perception can be influenced heavily by the environment, such as lighting, and the vision of the evaluator. Rather than rely solely on an individual to determine the color of an item, less subjective evaluation is preferred. The USDA regularly uses the Munsell color system, which relies on matching the color of a certain product to a reference color ‘chip’ that is subject to the same lighting as the product being measured (Wrolstad and Smith, 2017). These reference color chips can be a more affordable and less complex method for color evaluation than instrumental measurements, can be readily used in a number of environments, and are often used to determine color of egg yolks, farmed salmon, and milk.

Instrumental evaluation is a more objective method to determine color and is most often achieved with the use of colorimeters. Colorimeters contain three photocell receptors, a light source and a microprocessor to convert the measurement into recognizable and meaningful numbers from a certain color space (Veeramuthu, 2014). Several different color spaces exist, but all seek to give a digital representation of color which can then be used to either reproduce the color or help understand differences in color. The tristimulus color space is denoted by XYZ, whereby X indicates the amount of red, Y the amount of green, and Z the amount of blue making up the color of a certain product. Other color spaces include the CIE \( L^*a^*b^* \) and Hunter \( L, a, b \) scales, which are both based upon the tristimulus color space (Veeramuthu, 2014). Both of these
color spaces use the L(*) scale to denote lightness or darkness, a(*) to denote red (positive numbers) or green (negative), and b(*) to denote blue (positive) or yellow (negative). Another commonly used color space is CIELCh, where L denotes lightness or darkness, C denotes Chroma, or color intensity, and h denotes hue which describes the color such as red, green, blue or yellow (Pathare et al., 2013). Hue is communicated as a numerical value based on a 360-degree scale, with red falling at 0/360 degrees, yellow at ~90, green at ~180 and blue at ~270.

Previously mentioned color spaces can be used to indicate an absolute color value of an object and can indicate whether objects are different from each other in their color composition but do not allow us to determine whether the difference in color is significant, or even perceivable to the human eye. Color difference can be measured against a control sample or a reference color plate. For instance, Sistrunk and Kozup (1982) used a Gardner color difference meter (CDM) and an olive green standard plaque of L=52.0, -a=3.3 and +b=30.9 to determine the color difference between fermented cucumber samples at 1 month storage and 6 months storage. Total color difference (TCD) is denoted by delta E, and is calculated using L* a* b* values of the sample and the reference. A threshold of 1 is typically, but arbitrarily, used when determining whether there is a perceivable difference, with any TCD < 1 being unperceivable and any TCD > 1 being able to be perceived by the human eye. Furthermore, Adekunte et al. (2010) determined that a very distinct color difference would be shown by a TCD > 3, a distinct color difference would be shown by a TCD between 1.5 and 3, while a small difference would be shown by a TCD < 1.5.

1.3.2.3 Cure appearance development

Prior to acidification and storage, cucumber spears or slices have an opaque, white flesh which is preferred by consumers, who connect a loss of whiteness of the pickle to a loss of
texture or freshness (Fellers and Pflug, 1968). This loss of whiteness, or ‘cure’ occurs when gas trapped in intercellular spaces in the flesh of the cucumber is replaced by liquid (Fellers and Pflug, 1968) and is typical in fermented cucumber pickles, but not in directly acidified refrigerated pickles (Howard and Buescher, 1993). Cure appearance development is thought to be caused by alterations to the protein structure in the pickles and has been found to be retarded by the use of oxidants, and accelerated by the use of reductants (Howard and Buescher, 1993).

A 2012 study conducted by Buescher et al. found that cucumbers that were initially heat conditioned by submersion in 50°C water for 60 minutes prior to acidification and storage prevented the onset of cure development for 12 months, which is approximately double the typical shelf life of refrigerated pickles. Furthermore, the texture of the pickle was not negatively impacted by this heat treatment, and was still found to be satisfactory at 12 months (Buescher et al., 2012). The rate of cure appearance has been found to rely on storage temperature (Fellers and Pflug, 1968) and pressure changes; however, much of the currently available data focuses on cured appearance of pasteurized pickles and not cucumbers which are directly acidified and refrigerated. Ultimately, a reduction in cure appearance development could improve consumer perception of refrigerated cucumber pickles as the end of shelf life approaches.

1.3.3 Flavor of refrigerated pickles

Development of distinctive cucumber flavor does not occur until E-Z-(2,6)-nonadienal is produced during enzymatic lipid oxidation of linolenic acid, via a hydroperoxide intermediate, following disruption of cells, such as when cut or masticated (Grosch and Schwarz, 1971; Fleming et al., 1968). While enzymatic degradation of lipids can produce favorable products, it can also produce oxidative off-flavors, which can be a quality problem during shelf life of cucumber pickles. The primary fatty acids of cucumber lipids are linolenic, palmitic, and linoleic
acids. Of these, linolenic and linoleic acids are unsaturated and, thus, are prone to oxidation. Cucumbers contain roughly twice the amount of linolenic acid than linoleic acid (Peng and Geisman, 1976). Forss et al. (1962) found that (Z,Z)-2-6-nonadienal, an isomer of the aldehyde responsible for the main flavor in cucumbers, can exhibit ‘oily’ and ‘tallowy’ off-flavors at certain concentrations. Influential factors of lipid oxidation in food include: quantity of substrate, lipid constituents (degree of saturation), processing method (such as temperature), storage conditions (such as temperature, light, and oxygen availability), and levels of endogenous or exogenous inhibitors and/or catalysts. Hydroperoxides formed during enzymatic, autooxidative, and photooxidative processes are non-volatile, but are unstable and can result in the formation of volatile secondary decomposition products (Gordon, 2001; Gardner, 1975). Methods of controlling lipid oxidation include: limiting interaction of lipids and oxygen; inactivating lipoxygenase; using antioxidants; limiting pro-oxidants; and inactivating free radicals (Wąsowicz, 2004).

Enzymatic oxidation of free fatty acids begins when lipoxygenase, having access to oxygen, attacks free fatty acids containing a cis,cis-1,4-pentadiene structure. Enzymatic degradation of linoleic acid by lipoxygenase results in two potential hydroperoxide isomers, 9-hydroperoxy-trans-10,cis-12-octadecadienoic acid (9-LOOH) and 13-hydroperoxy-cis-9,trans-11-octadecadienoic acid (13-LOOH) (Gardner, 1975). Linolenic acid has two similar isomers, 9-hydroperoxy-trans-10,cis-12,cis-15-octadecatrienoic acid (9-LnOOH) and 13-hydroperoxy-cis-9,trans-11,cis-15-octadecatrienoic acid (13-LnOOH) (Gardner, 1975). Gaillard et al. (1976) concluded that at least two different enzymes work to fragment linoleic acid into volatile and non-volatile fragments. Fatty acid hydroperoxides are further broken down into volatile and non-volatile products by a secondary enzyme, hydroperoxide lyase, and the products are dependent
on the substrate, created by the first enzymatic reaction of fatty acid and lipoxygenase. Gaillard et al. (1976) found that the major volatile products of linoleic acid oxidation via 9-hydroperoxide were hexenal, cis-3-nonenal, and trans-2-nonenal and that 13-hydroperoxide cleavage resulted in hexenal with trace amounts (<1%) of cis-3-nonenal, and trans-2-nonenal. Additionally, they determined that oxidation of hydroperoxides also resulted in non-volatile carbonyls; the ratio of non-volatile to volatile products for 9- and 13-hydroperoxides of linoleic acid was 38:62 and 72:28, respectively. Oxidation of 9-hydroperoxides produced 9-oxonanoic acid and 13-hydroperoxides produced 12-oxodecanoic acid. Furthermore, Phillips and Galliard (1978) confirmed that cis-3,cis-6-nonadienal and cis-3-hexenal were produced from 9- and 13-hydroperoxides of linolenic acid. It is thought that E-Z-(2,6)-nonadienal, the compound primarily responsible for fresh cucumber flavor is formed when the double bond between carbon 3 and 4 in cis-3,cis-6-nonadienal isomerizes to a trans conformation between carbon 2 and 4 (Grosch and Schwarz, 1971).

Cucumber lipoxygenase has an optimum pH of 5.5, with moderate activity as low as pH 3.5, and an optimum temperature of 40 °C (Wardale and Lambert, 1980). Wardale et al. (1978) determined that the majority of hydroperoxide cleavage activity in cucumber flesh occurs in the plasma membrane, Golgi apparatus, and endoplasmic reticulum. Additionally, their study demonstrated that the cucumber exocarp had higher cleavage activity than the flesh, and that the majority of enzymatic activity occurred in the chloroplasts. Wardale et al. (1978) also observed that purified pellet of cucumber homogenate lost 50% hydroperoxide cleavage activity after heating at 50 °C for 10 minutes and 100% after heating at 70 °C for 2 minutes and Wardale and Lambert (1980) determined that cucumber lipoxygenase lost 50% of its activity after being heated to 50 °C for 5 minutes and 70 °C for 2 minutes. Furthermore, it was found that heating
cucumbers to an internal temperature of 77 °C resulted in > 75% reduction in carbonyl
compounds when compared to fresh, unheated cucumbers (Fleming et al., 1968).

Zhou et al. (2000), found that (E)-2-pentenal, (E)-2-hexenal, (E)-2-heptenal, and (E)-2-
ocotenal were a likely cause of oxidized off-odors in fermented cucumbers, but their study was
able to demonstrate that these aldehydes were unlikely to have been caused by enzymatic
oxidation since they found similar levels of these aldehydes after heating fermented pickles to
inactivate lipoxygenase. These findings could be due to the relatively low pH of fermented
pickles (pH < 3.5), which is much lower than the optimum pH of lipoxygenase (pH 5.5) and so
less enzymatic flavor compounds are formed in fermented pickles. These aldehydes, instead,
could be the result of autooxidation or photooxidation.

Autooxidation is the reaction of molecular oxygen with lipids and includes a three-step
free radical chain mechanism of initiation, propagation, and termination. Hydrogen abstraction
occurs during the initiation step, and depends on the bond strength of the hydrogen to the fatty
acid chain. In fatty acids with more than one double bond, the weakest bound hydrogens are
those located in the methylene group between double bonds. The relative reactivity of linoleic
acid is much higher than oleic acid, and the reactivity of linolenic acid is approximate double the
reactivity of linoleic acid (Min et al., 1989). This is due to the number and bond strengths of
allylic methylenes present (Frankel, 1989). Additionally, hydroperoxides formed from linolenic
acid are much more unstable than those formed from linoleic acid. Autooxidation of linoleic acid
produces 9- and 13-hydroperoxides, which are produced in equal amounts. Linolenic acid
produces 9-, 12-, 13-, and 16-hydroperoxides, but the isomeric distribution is not equal: 9- and
16- isomers are formed much more readily, while 12- and 13-hydroperoxides of linolenic acid
have been found to cyclize into hydroperoxy cyclic peroxides. Typical products of autooxidation
of linoleic acid include 2,4-decadienal, methyl octanoate, 3-nonenal and 9-oxononanoate from 9-hydroperoxides, and hexanal, pentane, 1-pentanol and pentanal from 13-hydroperoxides (Frankel, 1989).

Unlike free radical autooxidation, singlet oxygen photooxidation is capable of producing both conjugated and non-conjugated hydroperoxides (Min et al., 1989). Indicators of photooxidation include the presence of methyl 10-oxo-8-decenoate, 1-octene-3-ol, and 2-butenal, which are formed from the non-conjugated hydroperoxides of linoleic and linolenic acid (Min et al., 1989). Despite autooxidation and photooxidation sharing many of the same volatile decomposition products, due to the hydroperoxide intermediates, the relative quantities of those volatile compounds differ between pathways (Frankel, 2005). Ultimately, a brief blanch may result in a reduction of flavor-active volatile compounds by partially denaturing lipoxygenase or other enzymes responsible for lipid oxidation of fatty acids and their hydroperoxides, such as hydroperoxide lyase.

1.4 Overview of Proposed Research

1.4.1 Hypothesis

The application of a brief blanch step (80°C for 15 seconds) to whole cucumbers prior to production of refrigerated pickles will reduce total aerobic microorganism count by 2-log CFU/g in refrigerated cucumber pickles while improving texture quality and reducing susceptibility to cure appearance development and lipid oxidation due to partial denaturation of degradative enzymes. Researchers do not expect the blanch treatment to have a significant effect on exocarp color of refrigerated pickles, since any changes brought about by a brief blanch would likely be masked by drastic changes brought about by extended storage in an acidic environment.
1.4.2 Objectives

1. Determine the reduction of epiphytic microorganisms (aerobes, yeasts/molds, lactic acid bacteria, Enterobacteriaceae, spores) following blanch treatments of cucumbers (submersion in 80 °C water for 15, 90 or 180 seconds).

2. Determine z-value of Escherichia coli O157:H7 in cucumber homogenate and use a thermal model to predict the theoretical pathogen reduction in whole cucumber subjected to blanching.

3. Determine the changes in texture, peel color, and cure appearance development in refrigerated pickles made with blanched cucumbers (submersion in 80 °C water for 15, 90 or 180 seconds) compared to un-blanched cucumber refrigerated pickles over the shelf life of the product.

4. Optimize sample preparation of fresh cucumber and refrigerated cucumber pickles for volatile compound analysis by SPME GCxGC-TOFMS.

5. Analyze volatile compound profiles, focused on lipid oxidation products, of refrigerated cucumber pickles produced with blanched and un-blanched cucumbers.

1.4.3 Potential Impact

Since color, texture, and flavor are undeniably important quality characteristics in refrigerated pickles, it is paramount that research be conducted to determine changes in these parameters following any processing steps taken to ensure safety of the product. While a rapid pre-pack blanch treatment may reduce native microbiota, and therefore assist with an overall 5-log reduction in pathogenic bacteria, proposing such methods would be futile if the quality characteristics of the finished product were negatively altered during this process. If a brief blanch is successful in reducing microbiota and retaining pickle quality, it could be a valuable
addition to refrigerated pickle production that may also assist in extending shelf-life of a relatively perishable product.
1.5 References


Food Additives. 21 CFR § 170.3 (o) 2. 2017.


Chapter 2: Effects of a Brief Blanching Process on Quality of Refrigerated Cucumber Pickles

2.1 Abstract

Refrigerated pickles are characterized by a crisp, crunchy, opaque flesh. These products are not thermally processed; instead their safety relies on preventive controls during production, composition of cover brine, and hold time prior to consumption. It was hypothesized that a brief blanch of whole cucumbers prior to processing could reduce background microbiota, without negatively impacting product quality. Blanch treatments (15, 90, or 180 seconds) were conducted in duplicate for two independent lots of whole cucumbers in 80 °C water prior to cutting into spears, acidifying, and storing under refrigeration, along with an un-blanched control. Enumeration of total aerobes, lactic acid bacteria, and Enterobacteriaceae spp. was conducted on cucumber samples immediately following blanching along with samples of fresh, un-blanched cucumbers. Instrumental texture and color analyses were conducted for fresh and blanched cucumbers and pickle products during refrigerated storage, and a consumer discrimination test was conducted for pickle products stored for 62 days. The 90 second blanch treatment consistently achieved a minimum 2-log reduction in cucumber microbiota and a predicted 5-log reduction of Escherichia coli O157:H7 up to 1.1mm into the cucumber fruit. Blanch treatments had no impact on tissue firmness during refrigerated storage for one year (P > 0.098). Differences in hue were observed on blanched cucumbers (P < 0.05) but not after equilibration with brines or during refrigerated storage. Exocarp chroma was significantly lower in control samples than blanched samples during extended pickle storage. Significant development of mesocarp translucency (cure) was observed in control pickles during extended shelf-life, with the time to onset of cure depending on cucumber lot. Consumers (n=110) were unable to differentiate between control and 90 second blanched pickles after a typical shelf-life
of 62 days. Findings demonstrate that a 90 second, whole cucumber blanch at 80 °C could significantly reduce background microbiota without causing degradation of quality attributes associated with refrigerated cucumber pickles.
2.2 Practical Application

Refrigerated pickles do not undergo thermal processing, which can leave them vulnerable to microbial contamination. This study showed that addition of a brief blanch step in refrigerated pickle processing can reduce background microbiota without negatively impacting quality attributes such as appearance or texture. This process could assist processors in guaranteeing a safer product for consumers, while maintaining a high-quality product.

2.3 Introduction

Directly acidified, refrigerated pickles have a crisp texture and opaque flesh and do not undergo a thermal process in order to retain these characteristics. They are held under refrigeration for the length of the shelf life, and so do not need to comply with the thermal processing requirements of 21 CFR 114.80. However, with the introduction of the Food Safety Modernization Act (FSMA), and its focus on preventive controls, producers of refrigerated pickles must demonstrate that their products pose no risk to human health. Lu et al. (2013) found that pathogenic *Escherichia coli* 0157:H7 strains were able to survive in refrigerated pickle brines of pH 3.7 to 4.0, stored at 4°C, for more than one month. Furthermore, a 5-log reduction in *L. monocytogenes* strains in home-prepared, fermented, refrigerated dill pickles with an approximate equilibrated pH of 4.0 required about 50 d regardless of salt concentration (1.3 to 7.6 %), and, therefore, did not accomplish the pathogen kill required by the Code of Federal Regulations in a timeframe consistent with typical consumption patterns (Kim et al. 2005). Moreover, the same study found that *L. monocytogenes* was still detected at day 91 of refrigerated storage. Given that the typical shelf life of refrigerated pickles is around 4-6 months, storing product for extended periods prior to shipping to ensure a 5-log reduction of potential
pathogens is not financially feasible, so alternative methods to assist in the destruction of these pathogens must be sought.

Hurdle technology, historically used in food preservation, employs multiple treatments in order to achieve a safe and quality product. Blanching cucumbers for 15 seconds at 80°C has been found to achieve a 2-log reduction in vegetative aerobic microorganisms (Mattos et al. 2008). Since acidification alone requires an extended hold time to achieve a 5-log reduction of pathogenic microbiota, employing a rapid heat treatment to the cucumbers prior to acidification may help by reducing microbial counts along with potentially increasing microbial susceptibility to acid or salt conditions through heat-induced injury (Busta, 1975; Ray, 1979). Safety of a food product is paramount in food production; however, the quality of the food product is also of great importance. If treatments to render the product safe negatively impact the consumer’s experience of sensory properties such as color and texture, then the product will not be successful in the marketplace.

Heat conditioning of cucumbers has been shown to preserve the characteristic crisp texture of refrigerated pickles over their shelf life when compared with un-blanched cucumbers (Buescher et al. 2013). Similarly, hot water dip treatments have been shown to preserve the texture of raw produce such as strawberries, broccoli and potatoes (Lurie, 2008). However, these treatments are typically conducted at a lower temperature (50°C) and for longer periods (45-60 minutes) and rely on the penetration of the heat treatment through the entire mesocarp. Mattos et al. (2008) found that the center of size 3B cucumbers blanched at 80°C did not reach 50°C until approximately 15 minutes into the treatment. It is therefore unlikely that the brief heat treatments utilized in this study will significantly penetrate the mesocarp of the cucumber and therefore an increased retention of crisp texture is not expected. Papageorge et al. (2003) observed an
improvement of texture retention of acidified red peppers during storage when bell pepper strips were exposed to a 1 minute hot water treatment at 75°C. This treatment is closer in terms of heat and duration to the current study, but the raw commodity differs in both structure and size. Additionally, it is believed that this treatment may have caused inactivation of degradative enzymes, since the raw commodity equilibrated rapidly to the temperature of the water.

Enzymes such as polygalacturonase (PG) and pectin methylesterase (PME) play a considerable role in textural changes of fresh produce. PG is often implicated in softening of cucumber fruit (McFeeters et al., 1980 and Cho and Buescher, 2012), while activation of PME has been found to assist with firmness of fruits and vegetables, with optimal heating (McFeeters et al., 1985). Cho and Buescher (2012) found that complete inactivation of PG was achieved by blanching at 90 °C for 15 minutes, while another study found that the optimal temperature range for activation of PME in potatoes is between 50°C and 70°C (Bartolome and Hoff, 1972). Yemenicioğlu and Cemeroğlu (1999) found the optimum temperature range for PME activity was between 50 and 60 °C and inactivation increased as temperatures exceeded 65 °C. Previous research by McFeeters et al. (1985) determined that a three-minute blanch at 81 °C improved firmness retention of cucumber slices which was correlated with partial activation of PME during blanching. It is possible that a brief blanch step in processing may allow certain parts of the cucumber to reach optimum PME activation temperatures such that a firming effect is observed in cucumber tissue.

An additional quality concern in refrigerated cucumber pickles is development of translucency of the mesocarp. This translucency in cucumber pickles is termed ‘cured’ or ‘cure appearance development’, which occurs when intercellular gas is exchanged for liquid (Fellers and Pflug, 1967) due to proteolytic changes in cell walls of cucumber fruits (Mok and Buescher,
Consumers associate a loss of opaqueness of the pickle to a loss of texture or freshness, which is one of the determining factors of shelf life in refrigerated pickles (Fellers and Pflug, 1967).

Consumer perception is an incredibly important component of product success in the marketplace. When manipulating a product formulation or process, it is of utmost importance to determine whether a difference can be determined between the resulting products. Discrimination testing is used in the food industry in order to determine either differences or similarities between products. Tetrad tests are a relatively new approach to discrimination testing and have the same guessing probability as the commonly used triangle test (33%), but with significantly more power due to its greater sensitivity. For example, a tetrad test held at an alpha and beta level of 0.05 and a d’ level of 1 requires 110 participants, while a triangle test with the same power and d’ level would require 369 participants (Ennis and Jesionka, 2011).

This study aims to determine whether applying brief blanches to whole cucumbers for 15, 90, or 180 seconds in 80°C water prior to direct acidification is able to achieve a reduction of background microbiota without negatively affecting quality attributes of refrigerated cucumber pickles.

2.4 Materials and Methods

2.4.1 Cucumber procurement and preparation

Two lots of size 3B (50 ± 5 mm diameter) pickling cucumbers (n ~ 400) were procured from a local commercial pickle processor and transported to a pilot scale processing facility in Raleigh, NC. Upon receipt, diameter was measured and recorded for each cucumber using digital calipers. Each lot was then separated into 8 subgroups of 50 cucumbers. The subgroups were
randomly assigned to the following treatments, in duplicate: no blanch (control), blanch at 80°C for 15 seconds, blanch at 80°C for 90 seconds, and blanch at 80°C for 180 seconds. Cucumbers were stored at 3.9 +/- 1.5 °C overnight. Refrigerated cucumbers were rinsed under cool, potable water with mild manual abrasion in order to remove debris and then allowed to equilibrate to room temperature. Cover brine was formulated based upon typical commercial refrigerated pickle composition (Buescher and others, 2013 and Lu et al. 2013) such that cucumber pickles equilibrated to approximately 73.6 mM acetic acid, 2.2% sodium chloride, 6.9 mM sodium benzoate, and 11.0 mM calcium chloride.

2.4.2 Blanching and packing

Blanch treatments were conducted using a steam-jacketed kettle filled with 132.5 Liters of distilled water maintained at 80 °C. This temperature was measured by a calibrated thermocouple and adjusted, as required, by manually opening or closing the steam input line. Cucumbers were added to an empty five-gallon blanching vessel, and secured with a perforated lid in order to ensure that the cucumbers remained in the blanching vessel, while allowing water to flow through and make contact with the fruits. The entire vessel was submerged and a stopwatch used to measure submersion time. Once the blanch duration was reached, the blanching vessel was removed and cucumbers were immediately poured onto a table. Cucumbers (n=3) were randomly selected and sealed in a sterile stomacher bag for microbiological analysis. The remaining cucumbers were immediately cut into (6) spears using a 6-Section Vegetable Wedger (Choice, WebstaurantStore, Lititz, PA). The spears were manually mixed, 516 grams of cucumber spears were packed into each of 12 x 32 ounce jars, and 450 grams of cold brine was added to each jar (to achieve a 55:45 cucumber-to-brine pack out ratio). The jars were capped with a pre-heated metal cap and randomly assigned a jar code, signifying a shelf-life analysis
time point. Jars were then held at 3.9 +/- 1.5 °C until texture, color, and appearance analysis. An additional jar was packed with 516 grams of cucumber spears without the addition of brine and set aside for immediate quality analysis.

2.4.3 Microbiological Analysis

Whole cucumbers set aside for microbiological sampling were aseptically removed from the sterile stomacher bag, cut into small pieces, and added to a sterile blender jar. The cucumber pieces were then blended at maximum speed until they formed a slurry. The cucumber slurry was decanted into a filtration stomacher bag, and placed in a Stomacher® unit for 120 seconds on high. Filtered cucumber homogenate was diluted 1:10 using a sterile 0.85% saline solution. Undiluted and 1:10 dilution cucumber homogenate was plated on Plate Count (PCA), Violet Red Bile Glucose (VRBG), and *Lactobacilli* (MRS) agars (Difco, BD, Sparks, MD) using a spiral plater. PCA and VRBG plates were incubated at 37 °C for 24 hours. MRS plates were incubated at 30 °C for 48 hours. Points below the limit of detection (LOD), approximately 100 CFU/ml, were imputed. Nonlinear reduction curves were modeled using a Weibull function (Breidt et al., 2005).

2.4.4 pH Measurement

A Fisherbrand™ AR25 pH meter (Fisher Scientific, Hampton, NH), fitted with a Fisherbrand™ accumet™ gel-filled pH combination electrode (Fisher Scientific, Hampton, NH) was used for measurement and was calibrated using standard buffers of pH 2.00, 4.00, and 7.00. At storage day 3, pickle samples were removed from refrigerated storage, drained and blended to a slurry. The pH of both brine and slurry mixture were individually measured to determine whether the pickles had fully equilibrated.
2.4.5 Quality Analysis

Texture, color and cure appearance development analyses were conducted on fresh cucumbers (control), blanched cucumber samples post-blanching, and on refrigerated pickles at 8 time points during the shelf life of the pickle products (approximately 3, 30, 60, 120, 180, 240, 300, and 365 days). At each time point, 8 jars of pickles were removed from refrigerated storage (3 x blanch treatments and 1 un-blanch control, in duplicate) and allowed to come to room temperature. Order of analysis was randomized.

2.4.5.1 Cure Appearance Development (CAD) Analysis

Pickle spears (n=15) were removed from each jar and placed on black corrugated plastic. Photographs were taken using an iPhone 6s, using an overhead mount to ensure distance consistency between camera and spears. Images were retained for qualitative cure appearance development analysis. Each photograph was blind-coded using a three-digit numerical code in order to reduce potential bias. An evaluation panel consisting of three assessors was trained using a reference scale (Supplementary Figure 1, Appendix A) and several randomly selected photographs. During training, assessors were able to compare and discuss their scores in order to ensure correct usage of the scale and limit variability between assessors. Once training was complete, the order of presentation of each of the photographs (n=128) was randomized, by way of a random number generator, into 5 sets of 25 or 26 photographs. Assessors were instructed to score 7 photographs, take a minimum 5-minute break, and repeat this process for 3 more times per day in order to minimize fatigue. This process was repeated for 4 subsequent days, with each assessor scoring at the same time each day, in the same location, using the same computer screen, to reduce variability. Assessors had access to the same reference scale used in training throughout the entire process.
An average CAD score (%) was calculated for each individual spear based upon scores given by the three assessors, and the spear averages (n=15) for each jar were averaged to give a jar average, which was used for statistical analysis.

2.4.5.2 Exocarp Color Analysis

Exocarp color measurements were taken using a calibrated CM-700d Spectrophotometer, fitted with a 3mm aperture (Konica Minolta Sensing Americas, Ramsey, NJ, USA). The handheld spectrophotometer was placed on a lab bench with the aperture facing up. L*, a*, b*, C*, and h* measurements were taken by placing the center of each spear exocarp onto the aperture, ensuring that the center of the spear exocarp covered the entire aperture before reading the color.

2.4.5.3 Texture Analysis

A 6 mm slice from the center of each of 15 spears per sample was taken using a twin blade sample preparation tool (Stable Micro Systems, Godalming, GU7 1YL, United Kingdom). Each 6 mm slice underwent a firmness test, measuring the peak force (N) of penetration through the mesocarp region. A TA.XT Plus Texture Analyzer (Stable Micro Systems, Godalming, GU7 1YL, United Kingdom) fitted with a 3 mm blunt probe was used to puncture the mesocarp and allowed to travel through the entire sample (Thompson et al. 1982; Yoshioka et al. 2009). The peak force was recorded using Texture Exponent software (Stable Micro Systems, Godalming, GU7 1YL, United Kingdom).

2.4.6 Sensory Analysis – Consumer Testing

Subjects were recruited under approval from the NCSU Institutional Review Board (IRB #14038), and informed consent was obtained from all participants. A Tetrad discrimination test was conducted with n=110 consumers to compare un-blanch cucumber pickles (control) with
blanched cucumber pickles (90 sec) after 62 days of refrigerated storage, which is a typical consumption period. The majority of participants (78.2 %) consumed pickles at least once every 2 weeks, and 41.8% of panelists were consumers of refrigerated pickles in particular. Participants received 2 blind-coded samples of cucumber pickles produced with no blanch (control) and 2 blind-coded samples of cucumber pickles produced with a 90 second blanch. Samples were coded with 3-digit random numbers and presented in one of the 6 possible sample orders. Sample presentation order was randomized among panelists. Participants were directed to consume a portion of each sample, and group the four blind-coded samples into 2 groups of similar samples. Consumers were also asked to divulge information related to perceived differences between the two groups of samples, using a check all that apply (CATA) question. The CATA question asked for participants to select any differences from a list that differentiated the two samples. The list included ‘flesh appearance’, ‘seeds appearance’, ‘peel color appearance’, ‘peel texture’, ‘flesh texture’, ‘seeds texture’, ‘bitter taste’, ‘sour taste’, ‘sweet taste’, ‘salty taste’, ‘fresh cucumber flavor’ and an ‘other’ category to allow for a write-in description of perceived differences in the two groups. The number of correct responses (participants who had correctly grouped the samples by their corresponding treatment) was counted and compared to the chance value of guessing the correct grouping combinations (ASTM, 2015).

2.4.7 Thermal Inactivation Study

A thermal inactivation study was conducted to determine the D & z values of a pathogenic Escherichia coli cocktail in fresh cucumber. Each of 5 strains of E. coli O157:H7 (B200, B201, B202, B203, B204) were inoculated in triplicate, into separate 15 ml conical centrifuge tubes containing 5 ml Luria-Bertani (LB) broth, and allowed to grow statically overnight at 37 °C. After incubation, the 5 strains of E. coli were combined in a 50 ml conical
centrifuge tube, in triplicate, to produce 3 independent bacterial cocktails. The 50 ml tubes were centrifuged at 2988 g for 15 minutes. Supernatant was poured off and the pellet re-suspended by adding 5 ml sterile filtered cucumber homogenate (pH 6.4, adjusted using 3M sodium hydroxide), and vortexing for 30 seconds.

Three sterilized, water jacketed flasks were connected in series to a hot water bath using tubing and zip-ties to ensure a secure connection. The water bath was set to 50 °C and allowed to run to ensure no leaks in the tubing or connections. Each of the three flasks were filled with 99 ml of 100% cucumber homogenate, adjusted to pH 6.4, and the magnetic stir plates were turned on. A sterilized thermocouple was inserted into each of the flasks and connected to a recording device to allow monitoring of cucumber homogenate temperature. Once the cucumber homogenate reached 50 °C, it was held at temperature for 30 minutes prior to continuing. Each independent *E. coli* cocktail (1 ml) was added to a unique flask, resulting in approximately $10^6$ CFU/ml initial cell counts. A 1 ml sample of the cucumber homogenate was removed from each flask immediately following inoculation and pipetted into a micro centrifuge tube held on ice. Subsequent 1ml samples of the inoculated cucumber homogenate were removed at 60, 120, 180, and 240 minutes post-inoculation.

Each 1 ml sample was immediately diluted 10-fold into 0.85% saline. Serial 10-fold dilutions were prepared and plated using a spiral plater (Autoplate 5000, Advanced Instruments, Norwood, MA) for enumeration on LB agar. Plates were incubated for 24 hours at 37 °C. Colonies were counted using a plate counter (SCAN 300, Interscience, Woburn MA). This study was repeated using a water bath at 52 °C, 54 °C, 56 °C, and 58 °C (Table 1). Plating times were adjusted as needed for each temperature to allow 4-5 sampling times before counts were below
limit of detection (Table 2.1). D and z-values were determined by linear regression (Breidt et al., 2010).

2.4.8 Thermal Modeling

Predictive thermal modeling of temperatures within a 3B cucumber during 80 °C blanch treatments was performed with COMSOL 5.2a software (Comsol Inc., Burlington, MA) using heat transfer equations and thermo-physical properties of cucumbers set forth by Fasina and Fleming (2001). Temperatures were predicted from the surface of the cucumber to the geometric center at 0.1 mm intervals, and were used along with D and z values determined during the thermal inactivation study (section 2.4.7) to calculate a predicted log reduction (cfu/ml) of pathogenic *Escherichia coli* during blanch treatments.

2.4.9 Statistical Analysis

Texture and appearance (color and cure appearance) data were analyzed using JMP Software (SAS, Cary, NC). Prior to statistical analysis, data for individual spears (subsamples) were averaged to provide a sample mean for each independently replicated blanch treatment and storage time samples. A general linear model two-way analysis of variance (ANOVA) was used. Statistical significance was indicated at P < 0.05. Sensory data significance was determined using ASTM Sensory Analysis—Tetrad Test (ASTM, 2015).

2.5 Results and Discussion

2.5.1 Cucumber microbiota

Microorganism survivors of total aerobes (PCA), Lactobacilli (MRS), and *Enterobactericeae* (VRBG), were plotted for all blanch treatments (Figure 2.1). The estimated 80 °C blanch treatment duration required for a 2-log reduction of total aerobes, lactic acid bacteria (LAB), and enteric bacteria was 5 ± 6.5 seconds, 28 ± 10.2 seconds, and 34 ± 5.6 seconds,
respectively. These results demonstrate that a longer blanch duration was necessary in order to obtain a 2-log reduction in microbiota than was expected based on Mattos et al. (2013). This could be due to a larger cucumber to water ratio during blanching, differences in the type of water bath used (static versus circulating), or a difference in the type or overall health of microorganisms enumerated as total aerobes. The 90 second blanch treatment delivered a minimum 2-log reduction in total aerobes, LAB, and Enterobacteriaceae counts consistently across cucumber lots and treatment replicates.

2.5.2 Cure Appearance Development (CAD)

As expected, cure appearance development was one of the major quality changes during refrigerated pickle storage. Interestingly, the onset and severity of CAD differed greatly between lots of cucumbers (Figure 2.2). While information relating to difference in CAD between cucumber lots is unavailable, Yoshioka et al. (2009) observed differences in texture among 12 cucumber cultivars. Since CAD and texture changes are often observed in parallel (Buescher et al., 2013), despite different mechanisms, it is somewhat unsurprising that these differences were noted. Despite these differences, significant interactions between treatment and time point were found for both lots (p = 0.0015 and p < 0.0001, respectively). Cucumbers in lot A remained relatively uncured (less than 20% cure) for the majority of the storage period. However, significant differences in cure were apparent between treatments after a year of refrigerated storage (p = 0.0190). Control pickles exhibited a higher average CAD (~42%) than pickles prepared with cucumbers that were first blanched for 90 seconds at 80 °C (~22% CAD). Cucumbers in lot B underwent a much more rapid cure, and differences in cured appearance between treatments were more prominent, especially between the control and 90 second treatment. Despite both the control and 90 second treatment exhibiting an increasing level of
cure throughout the shelf life of the refrigerated pickles, the rate of cure onset for control samples was much higher than 90 second treatment samples during extended storage (240 – 383 days). Interestingly, for cucumbers in lot B, the 180 second treatment appeared to maintain a similar level of cure throughout the shelf life. Buescher et al. (2013) found that heat conditioning whole cucumbers for 45 minutes aided in maintaining a minimally cured appearance (<20 % CAD over a 12-month shelf life). During the heat conditioning in Buescher’s study, mesocarp and endocarp tissue reached ~48-49 °C. The 180 second blanch during this study achieved similar temperatures up to 5.7mm deep into the cucumber, which is approximately 1/3 of the mesocarp depth and so this suggests that some heat conditioning could be taking place around the periphery of the cucumber. Based on an approximate mesocarp depth of 16mm, the predicted temperature range of the mesocarp tissue is between 21 and 71 °C after a 180 second blanch treatment. A study of consumer perception with regard to CAD would be required to determine whether a 22% average cured appearance would still be considered acceptable. It appears that a 90 second or longer blanch of whole cucumbers may extend the shelf-life of refrigerated pickles due to a delay of cure appearance development when compared with refrigerated pickles produced with un-blanchered cucumbers.

2.5.3 Exocarp Color

Exocarp color of refrigerated cucumber pickles is immediately noticeable to consumers due to the transparent glass packaging. Differences in product appearance, including color can have a significant impact on consumer perception of a product (Wilson et al., 2015). The hue of the exocarp is especially important, as it is this that determines what we refer to as ‘color’ such as green or yellow. Hue angle of cucumber exocarp blanched at 90 and 180 seconds was found to be significantly different than the un-blanchered control or 15 second blanchered cucumbers prior to
direct acidification \( (p = 0.0021) \). Significant differences were also noticed between the un-blanched control and pickles made with 90 second blanched cucumbers \( (p = 0.0069) \) and 180 second blanched cucumbers \( (p = 0.0001) \) at day 3 of refrigerated storage \( (4 \, ^{\circ}C) \). However, these differences were no longer observed when measurements were taken at 30 days, nor at any time point thereafter. Figure 2.3 shows that the rate of hue angle change was not significantly different between treatments and control cucumber pickles across the entire shelf life of \( \sim 365 \) days. The reduction in hue angle is consistent with previous research, suggesting chlorophyll is converted to pheophorbides and pheophytins in acid conditions \( (\text{White et al. 1963}) \). No significant difference was observed between the control and three blanch treatments \( (15, 90, \text{ and } 180 \, \text{seconds}) \) in terms of exocarp chroma, up to and including the 120-day time point during shelf life. However, a significant difference was observed between the control and three blanch treatments at all sampling test points thereafter. Exocarp chroma values of the control cucumber pickles measured between 180 days and \( \sim 365 \) days post-pack were significantly lower than their blanched counterparts \( (\text{Figure 2.4}) \), indicating that the blanch treatment may have a preservative effect in terms of saturation of exocarp color.

2.5.4 Mesocarp Texture

As predicted, mesocarp puncture testing showed that blanching cucumbers prior to acidification had minimal impact on texture quality of cucumber pickles, when compared to an unblanched control over the course of a one year shelf life \( (\text{Figure 5}, p \, (\text{lot A}) = 0.2388 \, p \, (\text{lot B}) = 0.1635) \). There was, however, a significant difference in terms of rate of softening between the two lots of cucumbers tested, which can be seen when comparing Figure 2.5 \( (\text{A}) \) and \( (\text{B}) \), despite the two lots having a very similar initial average peak puncture force \( (\text{firmness}) \). Differences in cucumber texture due to cucumber cultivar and growing environment has previously been
reported by Yoshioka et al. (2009). Since the two lots of cucumbers used in the current study were grown in different locations at different times of the year, it is unsurprising that rates of softening were variable.

Interestingly, despite lot B pickles softening more rapidly and developing cure appearance at a higher rate than lot A, there was no direct correlation between softening and cure appearance development (CAD) in the 180 second treatment, which maintained a low cure level throughout shelf life. This may be explained by texture degradation and CAD being caused by different components; softening has been connected with changes in pectic substances whereas CAD is linked with changes to proteins affecting membrane permeability (Mok and Buescher, 2012). This suggests that the 180 second blanch may have affected proteins but not pectic substances in refrigerated pickles from lot B. The significant influence of lot to lot variability on rate of softening in this study demonstrates that further research should be conducted to investigate potential methods to minimize the magnitude of this variability.

2.5.5 Sensory Consumer Testing

Discrimination testing has the ability to unearth significant differences in products perceivable by consumers, which may not be evident from instrumental testing alone. The ASTM Standard Test Method for Sensory Analysis – Tetrad Test uses a critical value, a minimum number of correct responses, in order to determine whether a significant difference exists between two samples. The minimum number of correct responses is the nearest whole number greater than $\frac{n}{3} + z \sqrt{\frac{2n}{9}}$, where $n$ = the number of participants and $z = 1.64$ for an alpha value of 0.05 (ASTM, 2015). For this study, the minimum number of correct responses is 45. Should the number of participants correctly grouping samples have been larger than 45, it
could be concluded that a difference can be detected between samples. Only thirty-nine of the 110 participants were able to correctly group the samples, and so it can be concluded that no difference could be detected between samples. These results are promising in terms of implementation of the proposed process in a commercial setting, since manufacturers would be unlikely to change a process that results in a perceived difference in products by consumers.

2.5.6 Thermal Modeling of Pathogen Reduction

Thermal modeling of pathogen reduction has the ability to assist researchers by predicting the effect of a thermal treatment on a product. The z value of *Escherichia coli* O157:H7 in a model cucumber homogenate system was calculated based on three independent D-values at 5 different temperatures (50, 52, 54, 56, and 58 °C) and found to be 9.49 °C (Figure 2.6). This value is similar to the 9.66 °C z-value of *Escherichia coli* O157:H7 in cucumber juice with a pH of 4.6 (Breidt et al. 2014). The reduction of *Escherichia coli* O157:H7 for a given depth up to 6 mm below the surface of the fruit was modeled using COMSOL software and this z value, for each of the three whole cucumber blanch treatments (Figure 2.7). Mattos and others (2013) determined that the critical depth, where the majority of microorganisms are found within a cucumber, was between the surface and 0.65 mm deep into the fruit. Based upon the thermal model for whole cucumber and the heat sensitivity of *E. coli* O157:H7, both the 90 second and 180 second blanch treatment should be able to achieve at least a 6-log reduction for *E. coli* O157:H7 within this depth range. This thermal model indicates that a 90 second blanch could achieve a 5-log reduction in these microorganisms up to 1.1 mm deep into the cucumber tissue. Moreover, a 5-log reduction could be achieved at the critical depth of 0.65 mm with a blanch duration of approximately 59 seconds. These results demonstrate that a blanch treatment could assist processors in reducing the potential for pathogenic survival in refrigerated pickles. Should
processors implement a blanch treatment prior to processing refrigerated pickles, blanching would likely be conducted in a continuous process rather than a batch process, as was used in this study. Considering that a continuous blanch treatment may be more effective in terms of thermal transfer, further thermal modeling would be necessary in order to determine whether the blanch duration required adjustment.

2.6 Conclusion
A 90 second blanch at 80 °C applied to whole cucumbers is capable of consistently reducing background microbiota without significantly deteriorating quality of refrigerated cucumber pickles. This treatment is predicted to achieve a 5-log reduction in pathogenic *E. coli* within the region of the cucumber fruits containing the majority of the epiphytic microorganisms. Furthermore, findings related to exocarp chroma and cure appearance development indicate that this blanch treatment could aid in the preservation of refrigerated cucumber pickle appearance and therefore assist in extending the shelf life of these products.

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Papageorge LM, McFeeters RF, Fleming HP. 2003. Factors influencing texture retention of salt-


Table 2.1 Thermal inactivation study sampling times (minutes post inoculation)

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<th>5</th>
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Figure 2.1 Reduction in natural microbiota of cucumbers following blanch treatments at 80 °C.

Curve fitted with a Weibull model. Values below the limit of detection (LOD) were imputed.
Figure 2.2 Cure appearance development (CAD) in refrigerated cucumber pickles during storage at 4 °C for two lots of size 3B cucumbers (A & B) subjected to various whole cucumber blanching times in 80 °C water. *Time points with an asterisk denote significant differences between treatments at an alpha value of 0.05.*
Figure 2.3 Hue angle (°) in refrigerated cucumber pickle exocarp during storage at 4 °C for two lots of size 3B cucumbers subjected to various whole cucumber blanching times in 80 °C water.
Figure 2.4 Chroma value of refrigerated cucumber pickle exocarp during storage at 4 °C for two lots of size 3B cucumbers subjected to various whole cucumber blanching times in 80 °C water.
Figure 2.5 Peak force (N) required to puncture 6mm cross section of cucumber pickle mesocarp during shelf life of refrigerated pickles for two lots of size 3B cucumbers (A & B) subjected to various whole cucumber blanching times in 80 °C water.
Figure 2.6 Log D values of *Escherichia coli* O157:H7 using three independent samples at five temperatures (50, 52, 54, 56, and 58 °C). Calculated z-value = 9.49 °C.
Figure 2.7 Computed log reduction of *Escherichia coli* O157:H7 on the surface and up to 6mm deep into a size 3B pickling cucumber.
Figure 2.8 Thermal model diagram of 3B pickling cucumbers subjected to various whole cucumber blanching times (15 seconds (A), 90 seconds (B) and 180 seconds (C)) at 80 °C.
Chapter 3: Sample preparation optimization for volatile compound analysis of secondary lipid oxidation products in refrigerated cucumber pickles

3.1 Abstract

Fresh cucumber flavor is evident upon disruption of cell walls, allowing enzymatic lipid oxidation to produce the compounds E-Z-(2,6)-nonadienal and, to a lesser extent, 3-nonenal. While presence of some fresh cucumber flavor is associated with high quality refrigerated cucumber pickles, the same lipid oxidation pathway can produce volatile secondary oxidation products that are associated with off-flavors. Applying a brief blanch step to whole cucumbers prior to processing cucumber pickles may reduce lipoxygenase and other enzyme activity. Refrigerated pickles were processed with un-blanced and blanched pickles, and underwent sample preparation via aerobic slicing or anaerobic blending followed by volatile compound profiling using solid-phase microextraction two-dimensional gas chromatography time of flight mass spectrometry (SPME-GCxGC-TOFMS). Preliminary data suggested that anaerobic blending led to more consistent volatile compound profiles and less secondary oxidation product sampling artifacts, thought to be generated by autooxidation. Further studies were conducted in order to optimize anaerobic blending duration. A 60 second blend at 22,000 rpms was determined to yield the least variability between technical replicates of all durations tested, suggesting that this sample preparation method is the most suitable for further volatile compound studies.
3.2 Introduction

Fresh cucumber flavor is evident upon disruption of cell walls, allowing enzymatic lipid oxidation to produce the compounds E-Z-(2,6)-nonadienal and, to a lesser extent, 3-nonenal. Disruption of cell walls may occur during processing of cucumbers and cucumber products, or when a consumer bites or chews cucumber tissue. While presence of fresh cucumber flavor is associated with high quality refrigerated cucumber pickles, the same lipid oxidation pathway can produce volatile secondary oxidation products that are associated with off-flavors. Applying a brief blanch step to whole cucumbers prior to processing cucumber pickles may reduce lipoxygenase and other enzyme activity (Wardale and Lambert, 1980) and thus reduce the potential for both fresh cucumber flavor and off-flavor production. However, quantifying lipid oxidation products in plant tissues is inherently difficult, since sample preparation can promote lipid oxidation. Sample preparation is likely to disrupt or damage cell walls or cell wall constituents, allowing greater interactions between lipid substrate and enzymes. Additionally, if surface area is increased during sample preparation, such as is the case in blending or homogenizing, then plant tissue has a greater chance of being autooxidized due to increased lipid interaction with oxygen if processed in an aerobic environment. In order to determine the effect of blanching on volatile compound composition of refrigerated pickles, samples must be prepared effectively prior to extraction and subsequent volatile analysis.

Zhou et al. (2000) determined that differences in identified volatile compounds between fermented cucumber slurries prepared in either aerobic or anaerobic environments were minimal, when using purge and trap GC-MS. Only 2 of 37 identified key compounds resulted in a significant difference in one dimensional GC chromatogram peak areas. However, Fleming et al. (1968) found a rapid production of (E,Z)-2,6-nonadienal, a volatile compound created
enzymatically, in blended fresh cucumber tissue. Since refrigerated cucumber pickles retain some of their fresh cucumber quality, we predict that they are more likely to behave like fresh cucumber tissue rather than fermented cucumber tissue when blended aerobically. Furthermore, new methods for volatile compound profiling (Palma-Harris et al., 2002; Johanningsmeier and McFeeters, 2011) are more sensitive to changes in volatile compound profiles than those used in earlier studies.

If researchers wish to determine differences in secondary lipid oxidation products of cucumber pickles produced with blanched versus un-blanced cucumbers, measures should be taken to reduce formation of lipid oxidation products while processing samples for volatile compound analysis. Additionally, care should be taken to use a suitably sensitive volatile compound profiling method, such as SPME-GCxGC-ToF-MS.

3.3 Hypothesis and Research Objectives

We hypothesize that volatile compound profiles of refrigerated cucumber pickles, determined by SPME-GCxGC-ToF-MS, will contain more lipid oxidation products in aerobic sample preparation, when compared to anaerobic sample preparation, due to lipid oxidation brought about by oxygen availability. In order to investigate this hypothesis, research objectives include: determining whether a difference exists in volatile profiles of refrigerated pickles between anaerobic blending and aerobic slicing sample preparation methods and, if a significant difference exists between sample preparations in the first research objective, selecting the most suitable method and optimizing that method for sample preparation.
3.4 Materials and Methods

3.4.1 Refrigerated pickle processing

Refrigerated pickles were processed with un-blanched and blanched pickles, as described previously in sections 2.4.1 and 2.4.2. Pickles were then held at 3.9 +/- 1.5 °C until volatile analysis.

3.4.2 Headspace vial, blank, and alkane standard preparation

Headspace vials (10 ml clear glass, screwtop) used for volatile compound analysis of either cucumber pickle homogenate or sliced cucumbers were prepared by adding 0.4g NaCl, 886 μl deionized H₂O, 4 μl 3N H₂SO₄. Headspace vials for blank samples were prepared by adding 0.4g NaCl, 986 μl deionized H₂O, 4 μl 3N H₂SO₄, and 10 μl d-11 hexanoic acid (internal standard) and capped immediately. Headspace vials for anaerobically prepared samples and blanks were added to an anaerobic chamber 24 hours prior to preparation in order to purge them of oxygen. Headspace vials for aerobically prepared samples were prepared in an aerobic environment. C₈ – C₂₀ alkane standards were prepared, to allow for the creation of a retention index, by adding 1 μl of a prepared alkane mix (CAS 110-54-3, Sigma Aldrich) to an empty headspace vial immediately prior to analysis.

3.4.3 Anaerobic blending versus aerobic slicing

A single jar of refrigerated pickles, stored for 252 days at 3.9 +/- 1.5 °C, for each of two treatments (un-blanched control, 90 second blanch) was used for this experiment. 15 spears were randomly selected from each jar. Each spear was dissected and two, 1-inch samples were removed from either side of the central point. One of the samples from each spear was put into a vacuum sealer bag, along with the other 14 samples of the same treatment, and vacuum sealed before storage at -80 °C until volatile sample preparation. This process was repeated for the
second sample from each spear. This resulted in 4 vacuum sealed bags (2 x un-blanch control samples and 2 x 90 second blanch treatment samples), with one of the bags from each treatment reserved for anaerobic blending and the other for aerobic slicing. Vacuum sealed bags were then placed into a -80 °C freezer until further sample preparation.

3.4.3.1 Anaerobic blending sample preparation

Both vacuum sealed samples bags (un-blanch and 90 second blanch) were removed from the -80 °C freezer and placed into a temperature monitored, insulated ice water bath maintained between 0 °C and 1 °C for the duration of the experiment. Samples were allowed to thaw and the entire water bath, containing the samples, was added to an anaerobic chamber. The entire contents of each bag were added to a 250ml stainless steel blender jar and blended for 20 seconds at 22,000 rpm using a 1-speed, 700G Waring blender base (Model WF2212112, Conair Corporation, Stamford CT). Aliquots (100 mg) of the cucumber homogenate were taken, in triplicate, and added to three separate pre-prepared sample headspace vials (section 3.4.2), which were then immediately capped and placed on ice. This process was replicated for the second sample bag, using a clean blender jar. Once all blended samples were prepared, 10 μl d-11 hexanoic acid (internal standard) was added to each headspace vial and the vial was immediately capped and placed on ice. The ice bath containing the samples along with five blanks was removed from the anaerobic chamber and each of the headspace vials was then vortexed for 20 seconds and placed in the refrigerated sample tray (~4 °C) of a CombiPal autosampler (Model CTC Analytics (Switzerland), LEAP Technologies, Carrboro, N.C., U.S.A.).

3.4.3.2 Aerobic slicing sample preparation

Both vacuum sealed samples bags (un-blanch and 90 second blanch) were removed from the -80 °C freezer and were placed into a temperature monitored, insulated ice water bath
maintained between 0 °C and 1 °C for the duration of the experiment. Samples were allowed to thaw and the bags were then opened, a single piece of refrigerated pickle was selected, and a 100 mg sample was sliced from the piece using a scalpel. This was repeated for an additional 4 pieces, each of which were added to a separate pre-prepared (section 3.4.2) headspace vial. The entire process was repeated for the second treatment. Headspace vials were immediately capped and placed in ice. Once all samples were prepared, 10 μl d-11 hexanoic acid was added to each headspace vial and the vial was immediately capped and returned to the ice. Each of the headspace vials was then vortexed for 20 seconds and placed the refrigerated sample tray (~4 °C) of a CombiPal autosampler (Model CTC Analytics (Switzerland), LEAP Technologies, Carrboro, N.C., U.S.A.), along with blended samples and blanks (section 3.4.3.1), and two alkane standards (section 3.4.2).

3.4.3.3 Volatile analysis

Volatile compound profiles were generated for blended and cut samples, blanks, and alkane standards, using Solid Phase Microextraction (SPME) and two-dimensional gas chromatography time of flight mass spectrometry (GCxGC-TOFMS), as described in Johanningsmeier and McFeeters (2011) with modified sample preparation and dilutions (as described in section 3.4.2 and 3.4.3, above) and a 3 second 2nd-dimension separation time with 0.6 second hot pulse.

3.4.4 Anaerobic blending optimization

A single jar of refrigerated pickles, prepared from cucumbers blanched for 90 seconds and stored for 274 days at 3.9 +/- 1.5 °C, was used for this experiment. Fifteen spears were randomly selected from the jar. Each spear was dissected and three, 1-inch samples were removed from close to the central point of the spear. One of the samples from each spear was put
into a vacuum sealed bag, along with the other 14 samples of the same treatment, and vacuum sealed before storage at -80 °C until volatile sample preparation. This process was repeated for the second and third sample from each spear. This resulted in 3 vacuum sealed bags, with each of the bags reserved for a unique blend duration (20, 40, and 60 seconds). A 10 ml aliquot of brine was taken from the jar and pipetted into a screw cap conical centrifuge tube in order to compare brine volatile composition to cucumber pickle homogenate. Vacuum sealed bags and the centrifuge tube were then placed into a -80 °C freezer until further sample preparation.

3.4.4.1 Anaerobic blending duration, filtration, and brine sampling

Sample preparation followed the procedure described in section 3.4.3.1, with some differences. Samples underwent either 20, 40, or 60 second blend durations. Temperature of the blended homogenate was monitored to ensure that the sample remained below 4 °C, typical refrigeration temperatures, to minimize formation of additional volatile compounds by heating. Some of the samples blended for 60 second underwent further preparation (homogenate was passed through an LDPE filter bag and a 100 μl aliquot of the filtrant was added to a pre-prepared headspace vials (section 3.4.2) in triplicate). Additionally, brine samples from each of the jars were collected and added to pre-prepared headspace vials (section 3.4.2) in triplicate. This resulted in 15 samples (20 second blend, 40 second blend, 60 second blend, 60 second blend & filter, and brine, in triplicate). Volatile analysis was conducted in the same manner as that described in section 3.4.3.4.

3.4.5 Data processing and statistical analysis

Data was processed using ChromaTOF® software (Leco Corporation, St. Joseph, MI) as described Johanningsmeier and McFeeters (2011), and exported to Excel 2010. Peak area data underwent log transformation, as described Johanningsmeier and McFeeters (2011). ANOVA
followed by hierarchical clustering of significantly different means was conducted on log transformed peak areas, using an FDR adjusted p-value of 0.05, in JMP Genomics 9.4 (SAS Institute, Cary NC).

### 3.5 Results and Discussion

Volatile compound profiles of refrigerated pickle samples prepared by anaerobic blending differed significantly to profiles of those prepared by aerobic slicing. In order to reduce the potential for lipid oxidation, aerobic sample preparation was done by slicing the pickles using a sharp blade to minimize lipid-oxygen interaction caused by increase of surface area and time taken for sample preparation prior to capturing the sample in a sealed headspace vial. Additionally, researchers could then determine whether slicing a cross-section of cucumber pickles resulted in a relatively homogeneous volatile compound profile between technical replicates. A dendrogram of hierarchical clustering can be seen in Figure 3.1, which shows that sample processing method had a greater impact on volatile profiles than did the difference in cucumber blanch treatment. A greater number of volatile compounds were detected in blended samples than in sliced samples (Figure 3.3 section A), which is likely caused by the ability for compounds to volatilize from a blended homogenate more readily than from a slice of tissue. Sliced tissue exhibited several lipid oxidation products, for example, 3-penten-2-one, a ketone and secondary oxidation product that has been associated with ‘sharp’ and ‘fishy’ off flavors (Kochhar, 1993), was observed at high levels in samples prepared aerobically, but not those prepared anaerobically. Many of the compounds identified in the sliced samples and not the blended samples were known lipid oxidation products including (E,E)-3,5-Octadien-2-one, 2-Pentanone, 3-Hexenal, (E,E)-2,4-hexadienal (Figure 3.1, section 1). That being said, there were...
still differences in volatile compound profiles between blanch treatments, such as much higher levels of 2-nonen-1-ol (a product of lipid oxidation that has been known to cause ‘green’ and ‘fatty’ off-flavors) in un-blanched samples than was detected in blanched samples. Additional compounds that appeared to be influenced by the blanch treatment more so than the sample preparation include (Z)-2-nonenal, benzoic acid methyl ester, 2-nonenolic acid, and (E,Z)-3,6-Nonandien-1-ol (Figure 3.1, section 2).

Not only did the samples prepared aerobically show higher levels of several oxidation products, but there was also much more variability between technical replicates, likely due to individual cucumber to cucumber variability and the reduced surface to volume ratio of 100 mg slices compared to 100 mg cucumber pickle homogenate. This variability is illustrated by % RSD histograms (Figure 3.2). The average % RSD for volatile compounds in sliced technical replicates was 39.3 for un-blanched cucumber pickles and 44.9 for blanched cucumber pickles, which is much higher than the typical threshold of 30% accepted in SPME assisted volatile analysis. The average % RSD for volatile compounds in blended cucumber pickles technical replicates was 24.5 for un-blanched and 33.7 for blanched cucumber pickles.

In order to reduce variability and artifacts in samples for volatile compound analysis, anaerobic blending was chosen as the sample preparation method. Visual inspection of the cucumber homogenate after 20 seconds of blending revealed some pieces of cucumber tissue that were not fully homogenized, so researchers focused on optimizing blend duration in order to obtain the most representative sample for volatile compound analysis. Peak area averages of three well-known oxidation products in cucumbers can be found in Table 1.1. There appears to be little difference in peak area of each of the three compounds when comparing between blend duration times (20, 40, and 60 seconds). TIC chromatograms of the 60 second blend and filtered
counterpart shows a similar overall volatile profile (Figure 3.3 section B). Figure 3.4 (A, B), however, shows that some of the compounds were less abundant in the filtered sample, while Figure 3.4 (C) shows a peak that was lost in its entirety in the filtered sample. These differences could be attributed to the polarity of the compounds and the use of a low-density polyethylene (LDPE) filtration bag.

Technical replicates (n=3) for each sample preparation method allowed for calculation of % RSD, which can be seen for key compounds in Table 3.1. Brine samples had a very low % RSD (< 5%), which is unsurprising considering the homogeneity of liquid sample when compared to cucumber pickle homogenate. Interestingly, the (Z)-2-Heptenal peak area average was much smaller than the area found in any of the 20, 40, and 60 second blended samples (182230 compared to 718238, 777797, and 726492, respectively), which may have been partitioning of less polar compounds to the cucumber flesh and not water. This result would suggest that conducting volatile analysis of lipid oxidation products in refrigerated pickles using brine samples is likely to be less sensitive than using cucumber homogenate. Samples blended for 60 seconds maintained a relatively low % RSD (< 15 %), and consistently large peak area averages, suggesting that the cucumber homogenate was somewhat homogenous, without causing any creation or degradation of key volatile compounds.

3.6 Conclusion

Of the sample preparations surveyed in this study, anaerobic blending produced more consistent volatile compound profiles than slicing cucumbers in an aerobic environment. Moreover, a 60 second blend duration (22,000 rpm using a 250ml stainless steel blender jar) appeared to be the optimum duration of those tested, suggesting that a 60 second anaerobic blend could be reliably used for analyzing volatile compounds relating to lipid oxidation in refrigerated
cucumber pickles. Filtering the cucumber homogenate is not recommended due to partial or complete loss of some compounds, altering the overall volatile compound profiles observed.
3.7 References


Table 3.1 Peak areas of key lipid oxidation products in refrigerated pickle samples produced with various blending durations, filtered cucumber homogenate, and brine. Refrigerated pickle samples were approximately 240 days into storage.

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Figure 3.1 Dendrogram of hierarchical clustering of volatile compounds found in anaerobically blended un-blanced (A), anaerobically blended blanched (B), sliced un-blanced (C), and sliced blanched (D) cucumber pickles.
Figure 3.2 Histograms of % RSD peak area of volatile compounds in sliced un-blanch (A), sliced blanched (B), anaerobically blended un-blanch (C) and anaerobically blended blanch (D) cucumber pickles. Mean values are A = 39.3, B = 44.9, C = 24.5, and D = 33.7.
Figure 3.3 Overlayed total ion current (TIC) chromatograms of blended refrigerated cucumber samples. Samples are pickle homogenate (orange) and sliced cucumber pickles (green) (A) and blended refrigerated cucumber pickle homogenate (orange) and blended then filtered cucumber pickle homogenate (green) (B). The x-axis represents elution time (0-2000 seconds) and the y-axis represents relative abundance of a compound as total ion current intensity (TIC).
Figure 3.4 Chromatograms of volatile constituents of blended refrigerated cucumber pickle homogenate (orange) and blended then filtered cucumber pickle homogenate (green).

Compounds are A = (E,Z)-2,6-nonadienal, B = Hexenal, and C = Unknown. The x-axis represents elution time (0-2000 seconds) and the y-axis represents relative abundance of a compound at m/z of 27 (A), m/z of 41 (B), and total ion current intensity (C).
Chapter 4: Volatile lipid oxidation products of refrigerated cucumber pickles
during storage

4.1 Abstract

The primary fatty acids found in cucumber tissue are palmitic, linoleic, and linolenic acids. Of these acids, linoleic and linolenic are polyunsaturated and are therefore prone to lipid oxidation. E-Z-(2,6)-nonadienal is the compound responsible for distinctive fresh cucumber flavor and is a product of enzymatic lipid oxidation. The decomposition of fatty acids can also result in the production of aldehydes, ketones, and alcohols that have flavors associated with a loss of quality or development of an ‘off-flavor’. It was hypothesized that blanching whole cucumbers in 80 °C water prior to production of refrigerated pickles may limit production of volatile lipid oxidation products by partially denaturing lipoxygenase (LOX). Volatile compound profiles of blended cucumber and refrigerated pickle homogenate were determined using SPME-GCxGC-TOFMS followed by data processing and ANOVA analysis. Blanching cucumbers prior to processing into refrigerated pickled resulted in no significant differences in major lipid oxidation products, such as (E,Z)-2,6-nonadienal and (E)-2-nonenal, when compared to an un-blanched control. Storage time of refrigerated pickles had a significant effect on volatile compound profiles across a one year shelf-life. One interesting difference between the blanched and un-blanched pickles was the significant difference in levels of methyl benzoate, which is not a product of lipid oxidation, but is an odor active compound. While no significant differences in volatile lipid oxidation products were detected between treatments, this study has revealed several changes in aroma active compounds that could affect consumer perception of refrigerated pickles during shelf life.
4.2 Introduction

Refrigerated pickles are sought after by consumers because of key quality characteristics including crisp texture, white flesh, and fresh cucumber flavor (Etchells et al., 1976). Since cucumber flavor is an important characteristic in refrigerated cucumbers, understanding how flavor changes over the shelf life is of utmost importance. Food flavor can be heavily influenced by lipid oxidation. Cucumbers contain approximately 103 mg of total lipid per 100 g of tissue; of these lipids, the primary fatty acids are palmitic, linoleic, and linolenic acids (Kinsella, 1971). Peng and Geisman (1976) determined that fresh cucumbers contain 27.5, 22.7, and 45.8 mg/100 g fresh tissue of palmitic, linoleic and linolenic acids, respectively. Polyunsaturated fatty acids, such as linoleic and linolenic acids, are susceptible to lipid oxidation. The decomposition of fatty acids can result in the production of hydroperoxides which then further decompose into small volatile molecules such as aldehydes, ketones, and alcohols (Tressl et al., 1981), which may be aroma active or inactive. One such aldehyde is E-Z-(2,6)-nonadienal, the compound responsible for distinctive fresh cucumber flavor, which is produced during enzymatic lipid oxidation of linolenic acid. This compound is not produced until lipoxygenase (the enzyme) gets access to the linoleic acid (the substrate), which happens during injury of cucumber cell walls (Tressl et al., 1981). Despite the benefits of lipid oxidation, such as production of compounds leading to characteristic food flavors such as cucumber and strawberry, these reactions can also cause degradation of quality in food products by producing less-than-desirable flavor compounds, broadly categorized as ‘off-flavors’ (Wasowicz, 2004).

While cucumbers contain a relatively small amount of total lipids, products of lipid oxidation have the ability to overwhelm the consumer’s senses, given the low detection threshold of some classes of compounds such as ketones and aldehydes (Kochhar, 1993). Such products
can be produced via three main lipid oxidation pathways: autooxidation, photooxidation, and enzymatic oxidation. Similar volatile secondary oxidation products are produced, regardless of the lipid oxidation pathway, due to lipid oxidation pathways having the same hydroperoxide intermediates. This is not the case, however, for products formed from non-conjugated hydroperoxides, as is the case in photooxidation of both linoleic and linolenic acid. Additionally, relative quantities of oxidation products differ between pathways (Frankel, 2005). Indicators of photooxidation include the presence of methyl 10-oxo-8-decenoate, 1-octene-3-ol, and 2-butenal, which are formed from the non-conjugated hydroperoxides of linoleic and linolenic acid (Min et al., 1989). Methyl linoleate monohydroperoxides can also undergo 1,4-cycloaddition, forming six-membered hydroperoxy epidioxides which can further break down into similar volatiles as monohydroperoxides, as well as 2-heptanal and 9-oxononanoate.

Monohydroperoxides undergo beta-scission following thermal degradation to alkoxyl radicals, to produce aldehydes, hydrocarbons (via an alkyl radical intermediate), alcohols (via an alkyl radical intermediate), and olefins (via an olefinic radical) (Frankel, 2005). However, some compounds resulting from decomposition of hydroperoxides do not appear to result from beta scission cleavage mechanisms. These compounds include, but are not limited to, acetaldehyde, 2-pentylfuran, 2,4-nonadienal, and 2-octenal (linoleic acid) and butanal, 2-butyl furan, methyl 8-oxooctanoate, and methyl 10-oxo-8-decenoate (linolenic acid). Some of these compounds may have formed from further oxidation of secondary oxidation products such as unsaturated aldehydes, or could be a result of competing secondary reactions or reactions dependent on the environment, such as acid conditions.

Heterolytic cleavage occurs when hydroperoxides are decomposed in acid conditions and can produce 9-oxononanoate, 3-nonenal, and methyl 12-oxo-9-dodecenoate (from linoleic acid
hydroperoxides) and methyl-9-oxononanoate, 3,6-nonadienal, methyl 12-oxo-9-dodecenoate, 3-hexenal, propanal and methyl 15-oxo-9,12-pentadecadienoate (from linolenic acid hydroperoxides) (Frankel, 2005).

Two broad classes of methods are used in order to identify and/or quantify volatile metabolites from food matrices: analytical methods using instruments or the use of sensory testing, using human subjects. One method of instrumental analysis is gas chromatography. Gas chromatography, at its most fundamental level, is separating mixtures into their constituent molecules based upon their affinity for a column or columns. Solid phase microextraction, two-dimensional gas chromatography, time of flight mass spectrometry (SPME-GC×GC-TOFMS) has previously been used to both detect and identify volatile compounds found in cucumber pickles (Johanningsmeier and McFeeters, 2011). This system uses a coated fiber to adsorb volatile compounds from the headspace above a sample, which are then thermally desorbed. The volatile compound constituents elute from the first column, small portions of the eluent are cryo-focused and then enter the second column in sections in order to further separate compounds, improving the ability to identify the compound (Lubes and Goodarzi, 2017). The detector in this system is a mass spectrometer (MS), which detects metabolites based upon mass-to-charge ratio (m/z), analyzed using a time of flight (TOF) analyzer. Compounds are identified based upon matches of the mass spectra and identifiers, such as quantitative mass and retention times, with mass spectral libraries and databases. Instrumental quantification of lipid oxidation products found in food can help manufacturers to predict shelf-life stability of the product (Wasowicz, 2004). However, sensory analysis is still required if researchers wish to determine human perception of odor active compounds and consumer acceptability.
In order to understand changes in volatile compounds brought about by processing cucumber pickles and storing pickles post-processing, detecting and identifying compounds is an important first step. Changes in volatile composition brought about by processing may significantly impact the flavor quality of pickles, by either producing or limiting production of aroma active secondary oxidation products. Should processing limit production of these compounds, which often cause off-flavors, it is plausible that a higher quality product could be achieved. Research exists about changes in fresh cucumber flavor of refrigerated pickles during storage (Palma-Harris et al., 2005; Buescher and Buescher, 2001) however, studies regarding changes in compounds responsible for off-flavors in refrigerated pickles have yet to be conducted.

Blanching of whole cucumber as a pre-treatment for refrigerated pickles was proposed for improving safety and quality of refrigerated pickles (Chapter 2). It was found that a 90 second blanch treatment resulted in refrigerated pickles that had a slower rate of softening and cure appearance development (CAD). Considering heat sensitivity of lipoxygenase, it was predicted that a 90 second blanch treatment at 80°C on pickling cucumbers prior to production of refrigerated cucumber pickles would result in less lipid oxidation products than an unblanched control due to thermal destruction of lipoxygenase during blanching. If lipoxygenase is unable to decompose linoleic and linolenic acids to hydroperoxide intermediates, the pathway to producing secondary, volatile, lipid oxidation products will be blocked. The objective of this study was to determine the differences in volatile secondary lipid oxidation products in refrigerated cucumber pickles made with blanched cucumbers (90 seconds at 80°C) compared with refrigerated cucumber pickles made with un-blanched cucumbers, over twelve months of refrigerated storage.
4.3 Materials and Methods

4.3.1 Cucumber and Refrigerated Pickle Samples

Cucumber and refrigerated pickle samples (~1 inch sections of 15 individual spears, per jar) were taken during a previous shelf life study (Chapter 2), vacuum sealed, and frozen at -80 °C until sample preparation. In total, 72 samples were frozen. These samples represented 2 independent treatment replicates for 8 time points during storage for two lots of un-blanched and blanched (90 seconds) cucumbers processed into pickles (64 samples) plus 2 independent replicates for 1 time point during storage (120 days) for two lots of cucumbers blanched for either 15 or 180 seconds (8 samples). A summary of these samples can be found in Appendix B (Table B.1), along with their randomized run order and batch information. This study used a randomized complete block design for preparing samples and a randomized incomplete block design for sample analysis.

4.3.2 Volatile Sample Preparation

Headspace vials (10 ml) were placed in an anaerobic chamber, with their caps removed, 24 hours prior to sample preparation in order to purge them of oxygen. Prior to sample preparation, blank headspace vials were prepared as follows: 0.4g NaCl, 986 μl deionized H₂O, 4 μl 3N H₂SO₄, and 10 μl d-11 hexanoic acid (internal standard) were added to each of the blank vials, in that order, followed by immediate capping to mitigate loss of the volatile internal standard. Sample headspace vials were prepared with the addition of 886 μl deionized H₂O and 4 μl 3N H₂SO₄. Samples (n=12) in vacuum sealed bags (Appendix B) were removed from -80 °C storage and placed into a temperature monitored, insulated ice water bath maintained between 0 °C and 1 °C for the duration of the experiment. Samples were allowed to thaw and the entire water bath, containing the samples, was placed in an anaerobic chamber. The contents of each
bag was added to a 250 ml stainless steel blender jar and blended for 60 seconds using a 1-speed, 700G Waring blender base (Model WF2212112, Conair Corporation, Stamford CT). An aliquot of the blended slurry (100 μg) was added to a sample vial, which was immediately capped and placed on ice. This process was replicated for each sample, using a clean blender jar. Once all samples were prepared, 10 μl d-11 hexanoic acid was added to each headspace vial and the vial was immediately capped and placed on ice. The ice bath containing the samples along with blanks was removed from the anaerobic chamber and each of the headspace vials was then vortexed for 20 seconds and placed in the refrigerated sample tray (~4 °C) of a CombiPal autosampler (Model CTC Analytics (Switzerland), LEAP Technologies, Carrboro, N.C., U.S.A.). This process was repeated for each of 6 sample batches analyzed on 6 different days.

4.3.3 Volatile compound analysis

Volatile compound analysis was conducted for blended samples, blanks, and alkane standards, using Solid Phase Microextraction (SPME)GCxGC-TOFMS, as described in Johanningsmeier and McFeeters (2011) with the modified sample preparation and dilutions described above, and a 3 second 2nd-dimension separation time with 0.6 second hot pulse.

4.3.4 Data processing and statistical analysis

Data was processed using ChromaTOF® software (Leco Corporation, St. Joseph, MI) as described by Johanningsmeier and McFeeters (2011), and exported to Excel 2010. Peak area data underwent log transformation, as described Johanningsmeier and McFeeters (2011). Several analyses of variance (ANOVAs) were conducted of log transformed peak areas, using an FDR adjusted p-value of 0.05, in JMP Genomics 9.4 (SAS Institute, Cary NC). ANOVA models included: blanched versus un-blanched cucumbers prior to pickle processing; blanched and un-blanched cucumbers versus refrigerated pickles at 3 days of storage; blanched versus un-
blanched cucumber pickles throughout refrigerated storage (3-375 days); and blanched versus un-blanched cucumber pickles at each individual time point throughout storage.

4.4 Results and Discussion

Nine hundred and forty-six compounds peaks were detected during volatile compound analysis. Of those compounds, 215 were consistently detected in cucumber pickle homogenate during SPME-GCxGC-TOFMS, tentatively identified, and subjected to statistical analysis. Of these compounds, the primary compound classifications were found, as predicted, to be aldehydes and ketones. It was of interest to determine changes in volatile compounds between cucumbers and refrigerated pickles, as well as changes throughout the shelf life of refrigerated pickles.

4.4.1 Volatile compounds in short term storage of pickles

Differences in volatile compounds between un-blanched and blanched cucumbers, as well as pickles made with un-blanched or blanched cucumbers at day 3 of storage were determined by an ANOVA. Key compounds were selected from the 83 compounds identified (Appendix B) as having significantly different peak areas among the four treatments. The 10 compounds used in Figure 4.3 had the largest peak area of the 83 compounds, and all have a p value < 0.001. Interestingly, (E,Z)-2,6-nonadienal did not differ among these treatments, indicating that fresh cucumber flavor is likely to be present in refrigerated pickles very early in their shelf-life, with or without a 90 second blanch of whole cucumber in 80 °C water prior to pickling. All of the compounds found in Table 1 can be produced by lipid oxidation of linoleic or linolenic acids. These compounds are classified as secondary lipid oxidation products, as they are produced from lipid hydroperoxides as an intermediate. Cleary and McFeeters (2006)
determined that two of these compounds, pentenal and (E)-2-hexenal, were present in fresh-pack dill pickle samples that had been prepared both anaerobically and aerobically, and so it is expected that they are found in refrigerated cucumber pickles. All 10 compounds are known to be odor active, and their characteristic odors can be found in Table 4.1. No significant differences in these compounds were found between blanch treatments for either cucumbers or cucumber pickles. These findings are consistent with Zhou et al. (2000) whom determined that (E)-2-hexenal, (E)-2-heptenal and (E)-2-pentenal are capable of being formed non-enzymatically and would therefore be unaffected by thermal denaturation of lipoxygenase.

4.4.2 Volatile compounds in long term storage of pickles

An ANOVA was conducted to determine differences between pickles produced using either un-blanched or blanched cucumbers at several time points during 375 days of storage. Table 4.2 shows the 59 compounds that were identified as significantly different. Of these 59 compounds, 19 were classified as aldehydes, 12 as ketones, while the remainder were alcohols, alkanes, alkenes, carboxylic acids, esters, ethers, furans, and sulfur containing compounds.

4.4.2.1 (E,Z)-2-6-nonadienal

Data presented in Figure 4.1 is consistent with previous findings (Palma-Harris et al., 2002), and demonstrates a decline in (E,Z)-2,6-nonadienal and (E)-2-nonenal production during shelf-life, despite having an initial pH much lower than pickles in the previous study. Average peak area of (E,Z)-2,6-nonadienal was approximately 2,500,000 in un-blanched cucumbers prior to pickling compared with 3,600,000 for cucumbers blanched for 90 seconds. This finding is not consistent with the original hypothesis that blanching could inactive lipoxygenase and therefore reduce lipid oxidation products.
Buescher and Buescher (2001) determined that refrigerated pickles produced with cucumbers that had previously been frozen at -20 °C were unable to produce (E-Z)-2,6-nonadienal, and that refrigerated pickles produced with fresh cucumbers lost the ability to produce (E-Z)-2,6-nonadienal within 6 days. Furthermore, their study found that production of (E-Z)-2,6-nonadienal was dramatically reduced at pH levels below 5. It is interesting, therefore, that this study, featuring freezing and acidification to a pH of ~ 3.8, demonstrates that (E-Z)-2,6-nonadienal was present up to and including 375 days post-pack. This could be explained by (E-Z)-2,6-nonadienal being present in refrigerated pickle tissue before sub-zero storage, and not produced during storage. This finding indicates some stability of the compound during extended refrigerated storage, albeit around 20% of the initial amount. Thanks to the low detection threshold of this compound (0.0001 mg/kg, Forss (1972)), it is likely that some of the fresh cucumber flavor would still be evident at 365 days, as long as there were no high levels of off-flavors, capable of flavor masking. Levels of (E-Z)-2,6-nonadienal were not significantly different between treatments at any of the time points used in this study. Several other lipid oxidation products exhibited similar trends to (E-Z)-2,6-nonadienal, starting at high levels at day 3, decreasing significantly by day 60 of storage, and remaining relatively consistent thereafter (Figure 4.2). This trend could indicate that such compounds are produced by the same method of enzymatic oxidation. Once again, there was no significant difference between blanch treatments in these compounds and so it appears as though blanching cucumbers prior to processing refrigerated pickles did not change levels of these lipid oxidation products. One plausible explanation of these findings is that the blanch duration and/or temperature was ineffective at denaturing lipoxygenase, likely due to the heat treatment not being able to fully penetrate the cucumber mesocarp.
4.4.2.2 Increasing compounds during shelf life

Peak area of 18 volatile compounds increased over the course of 375 days of storage. Diphenyl ether, a compound with a characteristic “geranium, leafy, green, phenolic, metallic, medicinal” odor (www.goodscentscompany.com), was not detected in samples up to and including 60 days during storage, but was detected at time points thereafter (Figure 4.4 A). Additionally, (E) 3,7-dimethyl-2,6-octadienoic acid, a compound associated with “waxy, green, fruity” notes, was detected in low levels in samples at 3 days, and increased in a linear fashion thereafter (Figure 4.4 B). 3-Hexen-1-ol, an alcohol with linked with odors such as green, foliage, vegetable and herbal, was similarly found in low levels at 3 days and increased by approximately 300 % (Figure 4.4 C). None of these compounds showed significantly different levels between blanch treatments.

4.4.3 Volatile compounds found to be significantly different between treatments

An ANOVA was conducted for each individual storage time point to determine whether significant differences in volatile compounds were evident between pickles produced with un-blanched or blanched cucumbers. One compound, methyl benzoate, was found to be substantially different between treatments (Figure 4.5). The level of methyl benzoate was significantly higher in pickles made with un-blanchester cucumbers. Interestingly, methyl benzoate is not a product of lipid oxidation, and is produced from methanol and benzoic acid during a condensation reaction. Methyl benzoate is odor active and has a phenolic, wintergreen, and camphorous aroma at certain concentrations. Given that typical shelf-life of refrigerated pickles is between 4-6 months, and there are significant differences in methyl benzoate between treatments at certain time points within that range, further investigation should be conducted. In order to further determine potential changes in consumer perception of flavor, sensory testing
would need to be conducted. This could be accomplished by training and running a descriptive analysis (DA) panel. However since it is only one compound and DA is labor intensive, it would be advisable to conduct a consumer difference test to determine whether a difference is perceivable by the general public.

4.5 Conclusion

Applying a 90 second blanch treatment to cucumbers prior to processing had no clear impact on levels of secondary lipid oxidation products during storage and extremely minimal impact on the overall volatile compound profile of refrigerated pickles. This suggests that, if a blanch treatment is required for another purpose such as preservation of texture or reduction in microbial numbers, it could be conducted without significant risk of changing the flavor characteristics of refrigerated pickles.
4.6 References


Table 4.1 Characteristic odors of compounds found to be more abundant in refrigerated cucumber pickles after 3 days of refrigerated storage than fresh cucumbers.

<table>
<thead>
<tr>
<th>Compound Name</th>
<th>Characteristic Odors¹</th>
</tr>
</thead>
<tbody>
<tr>
<td>pentanal</td>
<td>fermented, bready, fruity, nutty, berry</td>
</tr>
<tr>
<td>1-penten-3-one</td>
<td>pungent, peppery, mustard, garlic, onion</td>
</tr>
<tr>
<td>2-pentyl-furan</td>
<td>Fruity, green, earthy, beany, vegetable, metallic</td>
</tr>
<tr>
<td>1-pentanol</td>
<td>Fusel, oily, sweet, balsamic</td>
</tr>
<tr>
<td>(E)-2-hexenal</td>
<td>Green, leafy</td>
</tr>
<tr>
<td>1-octen-3-ol</td>
<td>Mushroom, earthy, fungal, green, oily, vegetable, savory, brothy</td>
</tr>
<tr>
<td>(E,E)-2,4-heptadienal</td>
<td>Fatty, green, oily, aldehydic, vegetable, cinnamon</td>
</tr>
<tr>
<td>(Z)-2-heptenal</td>
<td>Green, fatty</td>
</tr>
<tr>
<td>2-ethyl-furan</td>
<td>Dirty, musty, brown, earthy, beany, malty</td>
</tr>
<tr>
<td>(E)-2-pentenal</td>
<td>Pungent, green, fruity, apple, waxy</td>
</tr>
</tbody>
</table>

Table 4.2 Changes in volatile compounds during shelf-life of refrigerated cucumber pickles produced with un-blanched and blanched cucumbers. Fifty-nine compounds were identified as significantly different with a false discovery rate (FDR)-adjusted p-value of 0.05.

UB= Un-blanced. B = Blanched.

<table>
<thead>
<tr>
<th>Compound Class</th>
<th>Peak Name</th>
<th>Average RI</th>
<th>Quant Mass</th>
<th>Day 3</th>
<th>Day 60</th>
<th>Day 120</th>
<th>Day 180</th>
<th>Day 240</th>
<th>Day 300</th>
<th>Day 375</th>
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<tr>
<td>Alcohol 2-Penten-1-ol, acetate, (Z)-</td>
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<td>43</td>
<td>286</td>
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<td>7,092</td>
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<td>41</td>
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<td>130</td>
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<td>Alcohol Heptanal*</td>
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<td>123,453</td>
<td>61,339</td>
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<td>98,925</td>
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<td>134,447</td>
<td>240,274</td>
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<td>1266</td>
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<td>84,136</td>
<td>98,763</td>
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<td>164,719</td>
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<th>Day 60</th>
<th>Day 120</th>
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<th>Day 240</th>
<th>Day 300</th>
<th>Day 375</th>
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<td>Carboxylic Acid</td>
<td>Benzoic acid, methyl ester*</td>
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\(^1\) Tentative compound identification based on mass spectral match to the NIST library with >750 similarity. Compounds designated with an * have been formally identified based on mass spectral and retention index match to an authentic standard.
Figure 4.1 Changes in fresh cucumber flavor compounds, (E,Z)-2,6-nonadienal and (E)-2-nonenal, during shelf-life of refrigerated pickles produced with un-blanced and blanced cucumbers.
Figure 4.2 Decrease in lipid oxidation products 2-hexenal and 2-pentylfuran during shelf-life of refrigerated pickles. Peak areas of blanched and un-blanched cucumbers were averaged following an ANOVA determination of no significant difference between treatments for these compounds ($p > 0.05$).
Figure 4.3 Differences in the most abundant volatile compounds in cucumbers and pickles prepared with or without whole cucumber blanching for 90 seconds in 80°C water. Pickles were sampled on day 3 of refrigerated storage following equilibration with the brine.
Figure 4.4 Increases in Diphenyl ether (A), (E,E)-3,7-dimethyl-2,6-octadienoic acid (B), and 3-hexen-1-ol (C) in refrigerated cucumber pickles during 375 days of refrigerated storage.
Figure 4.5 Differences in methyl benzoate between refrigerated pickles produced with un-blanch and blanched cucumbers during 375 days of refrigerated storage.
APPENDICES
Appendix A Supplementary Material to Chapter 2

Figure A.1 Cure Appearance Development Scale for Refrigerated Cucumber Pickle Spears
Figure A.2 Images indicating level of cure appearance development (CAD) in refrigerated cucumber pickles. Top left: refrigerated pickles made from un-blanched cucumbers at 120 days of storage. Top right: refrigerated pickles made from 90 second blanched cucumbers at 120 days of storage. Bottom left: refrigerated pickles made from un-blanched cucumbers at 383 days of storage. Bottom right: refrigerated pickles made from 90 second blanched cucumbers at 383 days of storage.
Appendix B Supplementary Material to Chapter 4

Table B.1 Summary of fresh cucumber and refrigerated cucumber pickle samples for volatile compound analysis.

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<tr>
<td>71</td>
<td>6</td>
<td>C</td>
<td>90</td>
<td>301</td>
<td>A</td>
</tr>
</tbody>
</table>
Table B.1 (continued).

<p>| | | | | | |</p>
<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>14</td>
<td>2</td>
<td>C</td>
<td>90</td>
<td>375</td>
<td>B</td>
</tr>
<tr>
<td>35</td>
<td>3</td>
<td>C</td>
<td>90</td>
<td>375</td>
<td>A</td>
</tr>
<tr>
<td>1</td>
<td>1</td>
<td>C</td>
<td>180</td>
<td>121</td>
<td>B</td>
</tr>
<tr>
<td>28</td>
<td>3</td>
<td>C</td>
<td>180</td>
<td>121</td>
<td>A</td>
</tr>
</tbody>
</table>
List of 83 compounds determined to be different between blanched and unblanched cucumber pickles at day 3 of storage

<table>
<thead>
<tr>
<th>Compound</th>
</tr>
</thead>
<tbody>
<tr>
<td>Furan, 3-methyl-</td>
</tr>
<tr>
<td>Pentane, 1-chloro-</td>
</tr>
<tr>
<td>Furan, 2-ethyl-</td>
</tr>
<tr>
<td>1,4-Pentadiene</td>
</tr>
<tr>
<td>2,3-Butanedione</td>
</tr>
<tr>
<td>Pentanal</td>
</tr>
<tr>
<td>3-Pentanone, 2-methyl-</td>
</tr>
<tr>
<td>1-Penten-3-one</td>
</tr>
<tr>
<td>Furan, 2-propyl-</td>
</tr>
<tr>
<td>Hexane, 1-chloro-</td>
</tr>
<tr>
<td>2,3-Pentanediene</td>
</tr>
<tr>
<td>Butanal, 2-ethyl-3-methyl-</td>
</tr>
<tr>
<td>2-Penten-1-ol, acetate, (Z)-</td>
</tr>
<tr>
<td>3-Penten-2-one, (E)-</td>
</tr>
<tr>
<td>1-Butanol, 2-methyl-, acetate</td>
</tr>
<tr>
<td>2-Pentenal, (E)-</td>
</tr>
<tr>
<td>2-n-Butyl furan</td>
</tr>
<tr>
<td>2-Heptanone</td>
</tr>
<tr>
<td>Heptanal</td>
</tr>
<tr>
<td>2-Hexenal</td>
</tr>
<tr>
<td>Furan, 2-(2-propenyl)-</td>
</tr>
<tr>
<td>Furan, 3-methyl-</td>
</tr>
<tr>
<td>Eucalyptol</td>
</tr>
<tr>
<td>2-Hexenal</td>
</tr>
<tr>
<td>Phenol, 4-ethyl-</td>
</tr>
<tr>
<td>Furan, 2-pentyl-</td>
</tr>
<tr>
<td>4-Heptenal, (Z)-</td>
</tr>
<tr>
<td>Oxepine, 2,7-dimethyl-</td>
</tr>
<tr>
<td>1-Pentanol</td>
</tr>
<tr>
<td>3-Heptanone, 5-methyl-</td>
</tr>
<tr>
<td>Octanal</td>
</tr>
<tr>
<td>2-Heptenal, (Z)-</td>
</tr>
<tr>
<td>1-Octen-3-one</td>
</tr>
<tr>
<td>trans-2-(2-Pentenyl)furan</td>
</tr>
<tr>
<td>2-Methoxythiophene</td>
</tr>
<tr>
<td>2-Penten-1-ol, (E)-</td>
</tr>
<tr>
<td>2-Penten-1-ol, (Z)-</td>
</tr>
<tr>
<td>2-Heptenal, (Z)-</td>
</tr>
<tr>
<td>1-Hepten-6-one, 2-methyl-</td>
</tr>
<tr>
<td>1-Hexanol</td>
</tr>
<tr>
<td>Cyclopropane, (3-chloropropyl) methylene-</td>
</tr>
<tr>
<td>CH3C(O)OCH(CH3)C(O)CH3</td>
</tr>
</tbody>
</table>
2,4-Hexadienal, (E,E)-
Furan, 2-(1-pentenyl)-, (Z)-
Nonanal
2,4-Hexadienal, (E,E)-
trans-2-Hexenol
1-Chloro-3-pentanone
1-Octen-3-ol
2,4-Heptadienal, (E,E)-
(R,S)-5-Ethyl-6-methyl-3E-hepten-2-one
2,4-Heptadienal, (E,E)-
Decanal
3,5-Octadien-2-one, (E,E)-
Benzaldehyde
3,5-Octadien-2-one, (E,E)-
5-Methylene-4,5,6,6a-tetrahydro-3ah-pentalen-1-one
2-Octen-1-ol, (Z)-
Benzoic acid, methyl ester
Acetophenone
2-Decenal, (Z)-
Butanoic acid, 3-methyl-
2,4-Nonadienal
Benzoic acid, ethyl ester
2,7-Octadien-1-ol
2,4-Nonadienal
Pentanoic acid
Carvone
2-Undecenal
2,4-Decadienal
Formamide, N,N-dibutyl-
2,4-Decadienal
Pentanoic acid
2-Sec-Butylcyclohexanone
2-Dodecenal, (E)-
Benzyl alcohol
(1-Oxa-2-aza-spiro[2.5]oct-2-yl)-phenylmethanone
Cyclohexane, 1-butenyldiene-
trans-á-Ionone
4-(2,6,6-Trimethylcyclohexa-1,3-dienyl)but-3-en-2-one
Undecanal
3-Nonenoic acid
2-Nonenoic acid
Benzyl Benzoate