MILD HEAT AND ULTRAVIOLET RADIATION IN SEQUENCE TO INACTIVATE *ALICYCLOBACILLUS ACIDOTERRESTRIS* SPORES IN APPLE JUICE

By

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Written under the direction of

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ABSTRACT OF THE THESIS

Mild heat and ultraviolet radiation in sequence to inactivate *Alicyclobacillus acidoterrestris* spores in apple juice

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Mild heat followed by UV treatment was investigated in this study on apple juice inoculated with the spores of *Alicyclobacillus acidoterrestris.* Commercially pasteurized apple juice was inoculated with the spores of *Alicyclobacillus acidoterrestris* and subjected to mild heat treatment to ensure at least 90 % spore germination. The juice was then subjected to UV-C light of intensity between (1.4-1.6) mW/cm^2 for various times between 10 s to 600 s. A 4.2 log reduction was obtained upon combined heat treatment at 52 °C for 38 min followed by UV-C treatment of (1.4-1.6) mW/cm^2 for 60 s. This treatment was compared with commercial pasteurization by head space GC-MS analysis for volatile fraction in apple juice and sensory evaluation of taste, color, and smell. Our results indicated that the quality attributes did not change due to combined heat and UV-C treatment of the commercially pasteurized juice.

Key words: multiple hurdle technology, *Alicyclobacillus acidoterrestris,* ultraviolet light, heat treatment, GC-MS and sensory evaluation.

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LITERATURE REVIEW

1.1. Apple:

1.1.1. History: Apple Growth and Cultivation

Apples originated in the Middle East more than 4000 years ago and have been cultivated in the UK as a crop since the Roman occupation (http://www.ifr.ac.uk/science-society/spotlight/apples/). According to a report from Institute of Food Research (http://www.ifr.ac.uk/science-society/spotlight/apples/), the special varieties of apples started to spread and flourish across Europe to France in the 13th century. For around 300 years from then, the apple produce was considered as a symbol of luxury and was sold at the London Luxury Market. Explorers in this region found new varieties from all over the world and brought them to Brogdale in Kent, which was developing its orchards and gardens. Even today, the Brogdale National Apple Festival, which is the biggest and the best apple festival, is celebrated in an extravagant way, every year. It houses around 3500 varieties of apple across 30 acres of orchards. Vista is one of the earliest flowering dessert apple which blooms in early May, followed by Idared, Discovery, Jonagold and Cox's Orange Pippin in mid-May. Gala and Worcester Pearmain flower at later times, thus the maturation dates for apples could span more than a 100 days. Apples are members of the rose family of plants as they blossom like wild-rose. Today, there are approximately 10,000 different varieties of apples grown in the world with more than 7,000 varieties grown in the United States (http://whatscookingamerica.net/Fruit/Apples).

1.1.2. Forms of Apple Juice:

We eat apples in many forms other than the raw fruit form. Agro products, Inc. (http://www.agriculturalproductsindia.com/beverages-juices/beverages-juices-apple-juice.html) mentioned that, by the late 1980s, the United States had become a major and stable producer of the fresh apple juice beverage, which was soon followed by Argentina, West Germany, Austria, Italy and many other countries in the 1990s.

Apple juice concentrate was considered a major development because this led to reduction in packaging volume and thus shipping cost. It also reduced the chances of spoilage since the products were evaporated and concentrated.

There are two types of apple juice concentrates namely, clear apple juice concentrate and cloudy apple juice concentrate.

- Cloudy haze like appearance is caused by the small pulp suspensions present evenly in the juice.
- The clear apple juice concentrate is produced by removing the pectin and starch component during the production process.

1.1.3. Conventional Processing Techniques:

According to Institute of Food Research (IFR) (http://www.ifr.ac.uk/science-society/spotlight/apples/), to maintain the distinct flavor of apple, the juices are generally flash pasteurized by heating between 71 °C and 74 °C for about 15 s - 30 s. This process kills spoilage micro-organisms making the product safer and giving it a longer shelf-life of up to two years. (http://www.ifr.ac.uk/science-society/spotlight/apples/). The main components present in apple that give it a characteristic taste and smell are sugars, oligosaccharides, amides and other nitrogenous compounds, soluble pectin, vitamin C, minerals, malic, quinic and citramalic acids,

tannins (i.e., polyphenols) and a diverse range of esters (e.g., ethyl-methyl-butyrate and iso-butyl acetate). The relative proportion of the above mentioned components in apple juice depend on several factors such as the apple cultivar, growing conditions, maturity of fruit at the time of pressing, physical, biological damage due to mold contamination and the efficiency with which the juice was pressed from the fruit.

1.1.4. World Market and Value:

US is one of the top importers of apple juice concentrate. According to the report by USDA Foreign Agricultural Service in April 2008, it is estimated to import 30% more than what was imported in the year 2006/2007. Around 1 million tons of apple juice concentrate is imported from China alone. USDA has also shown concern over the decrease in the production of apple juice in US while there is an enormous increase in the import. Figure 1 depicts the distribution of share of each country on the apple juice imported to the US. The trend in the production-import-export of apple juice over a decade is outlined in Fig. 2.

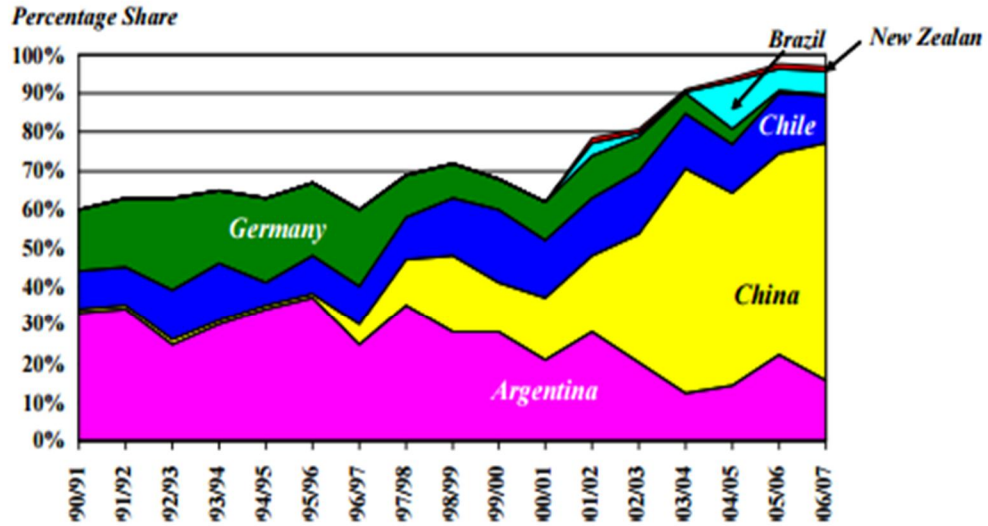


Figure 1: U.S. Apple Juice Import Market Share

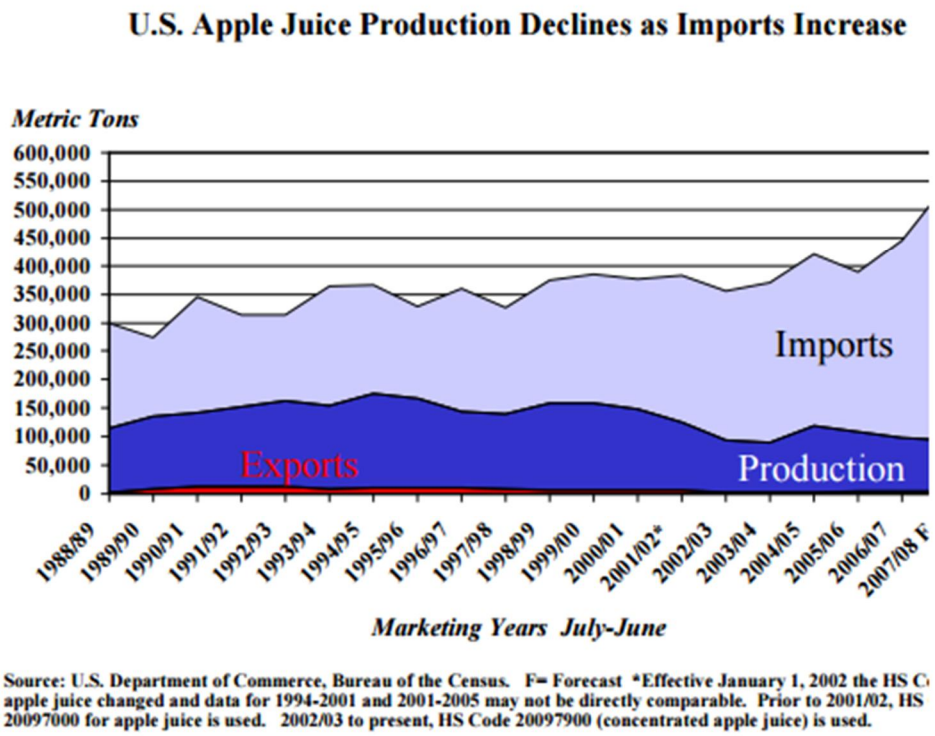


Figure 2: U.S. Apple Juice Production Report

The UK apple market is worth around £115 million (2007) as per the information on GOV.UK. In a span of 5-10 years the imports of apples to the UK have also risen by approximately 17 % from 446,400 tons to 522,100 tons. The world market for apple juice is drastically increasing year after year with increasing demand, which is depicted in Figure 3. Statistical evidence from USDA Foreign Agricultural Service, April 2008, also confirms the increase in demand for apple juice products. It has been quoted that, “In 2007, the value of juice imports boomed to $1.5 billion, from $1 billion in 2006. By value, the EU-27 and the U.S. are tied at 35 % or together make up 70 % of global imports. By volume, the EU-27 just surpasses the U.S. as the top world importer with 39 % of trade compared to the U.S. at 36 %. Other importers include Japan, Russia, Canada, Australia, and South Africa.”

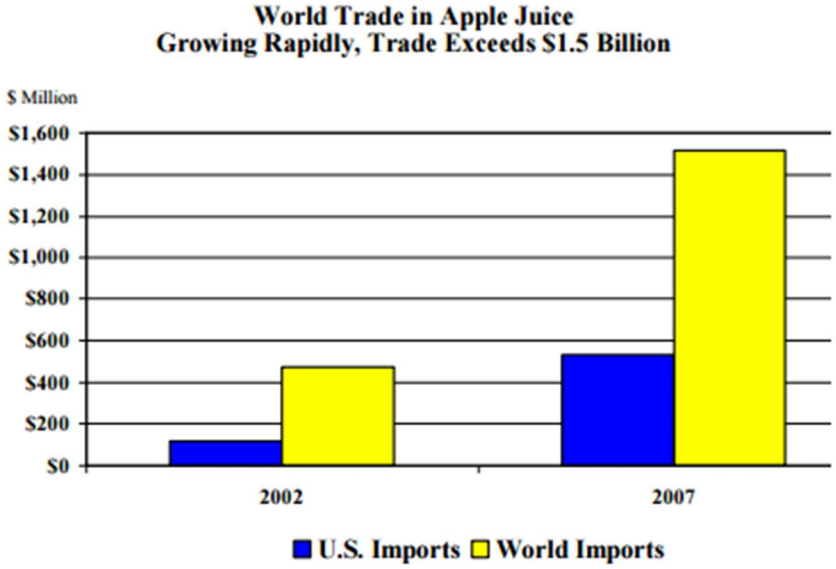


Figure 3: World Market Apple Juice

The college of ACES, University of Illinois Extension at Illinois University, Urbana-Champaign and the USDA national apple processing report, 2015 has quoted and reported the following facts and statistical data on apple. (http://www.ams.usda.gov/mnreports/fvwaplproc.pdf,http://extension.illinois.edu/apples/facts.cfm).

- A total of 992,580 apples were utilized for processing in 2014, of which 517,729 apples are utilized for processing juices. The top apple producing states are Washington, New York, Michigan, Pennsylvania, California, and Virginia.
- 63 % of the 2005 U.S. apple crop was eaten as fresh fruit and the per capita consumption of fresh market apple was around 16.9 pounds. 36 % of apples were processed into apple products; 18.6 % of this was for juice and cider, 2.0 % was dried, 2.5 % was frozen, 12.2 %

was canned and 0.7 % was fresh slices. Others were used for making of vinegar, baby food and apple butter.

- In 2005, there were 7,500 apple growers with orchards covering 379,000 acres. Total apple production in the United States was 234.9 million cartons valued at $1.9 billion. One out of every 4 apples harvested in the US is exported. The apple variety 'Red Delicious' is the most widely grown in the United States with 62 million bushels harvested in 2005.
- In 2006/2007 the People's Republic of China led the world in commercial apple production with 24,480,000 metric tons followed by the United States with 4,460,544 metric tons. The commercial world production of apples was at 44,119,244 metric tons.

1.1.5. Microbial Contamination of Apple Juice:

In October 1996 an outbreak of *E. coli* O157:H7 linked to unpasteurized apple cider occurred in 2 states in the United States, namely, Connecticut and New York (CDC. http://www.cdc.gov/mmwr/preview/mmwrhtml/00044358.htm. November 08, 1996/45(44); 975) causing illness in 66 people and 1 death. A similar outbreak, with the same organism happened in California, Colorado and Washington in October 1996, in which 45 people were affected (CDC.http://www.cdc.gov/mmwr/preview/mmwrhtml/00045558.htm.January 10, 1997/ 46(01); 4-8). Spoilage organisms also pose an economic threat to the beverage sector. The organisms that cause spoilage can produce haze and sediment in the juice and produce gas or undesirable odors (Worobo *et al.,* 2005).

1.1.6. Apple and Health

1.1.6.1. Nutrition Information

Nutrition Facts

Serving Size 1 Medium Apple (182g / 6.4oz)

Amount Per Serving	
Calories 95	Calories from Fat 3
	% Daily Value*
Total Fat 0g	**1%**
Saturated Fat 0g	**0%**
Trans Fat 0g	
Cholesterol 0mg	**0%**
Sodium 2mg	**0%**
Total Carbohydrates 25g	**8%**
Dietary Fiber 4g	**17%**
Sugars 19g	
Protein 0g	

Vitamin A 2%	•	Vitamin C 14%	
Calcium 1%	•	Iron 1%	

*Percent Daily Values are based on a 2,000 calorie diet. Your Daily Values may be higher or lower depending on your calorie needs.

	Calories	2,000	2,500
Total Fat	Less than	65g	80g
Sat Fat	Less than	20g	25g
Cholesterol	Less than	300mg	300mg
Sodium	Less than	2,400mg	2,400mg
Total Carbohydrate		300mg	375mg
Dietary Fiber		25g	30g

Figure 4: Nutrition facts for 1 medium apple (on an average) which weighs 182 g (http://valleyendfarm.blogspot.com/2012_05_01_archive.html)

Fruit and vegetables are an important part of a balanced diet. According to health advisory organizations recommendations the risk of heart problems are to be reduced by consuming 5 portions of fruit and vegetable, and one medium-sized apple constitutes a portion. Apples are a rich source of nutrition [Institute of Food Research, IFR]. They contain dietary fiber in their peels and core. Only about 17 % of an apple is made up of carbohydrate and 18 % from a variety of vitamins and minerals. The rest of the apple (about 60 %) is water. Figure 4 shows the nutrition

data for a medium sized apple which provides about 95 calories. Excluding the peel and core of apple from the diet halves the amount of vitamin C and dietary fiber consumed but makes very little difference to the sugar intake.

1.1.6.2. Apple juice preparation and pasteurization:

The apple juice is prepared by the process of pasteurization, where the juice is heated to the appropriate temperature to essentially eliminate all harmful bacteria. The juices are prepared from concentrate where the exact amount of water that was eliminated is added back into the product making it a 100 % juice. The apple juice used for the experiments described below is a clarified apple juice, so its fiber content is essentially zero. The nutritional profile of the apple juice used for the experiments in this thesis is shown in Figure 5. A temperature-time condition of 71.7 °C (161 °F) for 15 s, which is the required pasteurization condition for milk, is also considered adequate (Penn State University 2010).

APPLE & EVE - APPLE
10% JUICE AT MEDIUM STRENGTH

Nutrition Facts

Serving Size .21 fl. oz. (6.2 mL)
(Makes 8 fl. oz.)
Servings 14

Amount Per Serving

Calories 10	Calories from Fat 0
	%Daily Value*
Total Fat 0g	**0%**
Saturated Fat 0g	**0%**
Trans Fat 0g	
Cholesterol 0mg	**0%**
Sodium 10mg	**0%**
Total Carbohydrate 3g	**1%**
Dietary Fiber 0g	**0%**
Sugars 2g	
Protein 0g	

Vitamin A 0% • Vitamin C 0%
Calcium 0% • Iron 0%

*Percent Daily Values are based on a 2,000 calorie diet.

Figure 5: Nutritional profile of clarified and commercially pasteurized apple juice from Apple & Eve ®.

1.1.7. Apples and Health

Apples are associated with a number of health benefits. It has been the most cultivated and consumed fruit for generations. Woods *et al*. (2003) reported that consumption of apples lowers the risk of asthma. It has also been shown to reduce the risk of degenerative conditions such as Alzheimer's (Dai *et al.*, 2006).

There are allergies associated with apples. Most common are the two types: allergy to birch pollen which causes mild reaction including itching and inflammation of tongue, mouth and throat and an allergic reaction to apple protein which includes severe symptoms include abdominal pain and vomiting, asthma and a rash. Birch pollens do not survive cooking or pasteurization processes

intact, thus a person with the first type of allergy can consume pasteurized or cooked apple products.

1.2. *Alicyclobacillus acidoterrestris*:

1.2.1. History of *Alicyclobacillus* in fruit juices:

Alicyclobacillus was first isolated in 1982 in association with spoilt apple juice (Cerny *et al.,* 1984). Later reports suggested that the organism can survive in soil environments too (Cerny *et al*., 1984). Deinhard *et al*. (1987) proposed is be named *Bacillus acidoterrestris,* meaning acid loving and isolated from the soil. Further investigation revealed that the organism showed the presence of unique ω-alicyclic fatty acids as the major membrane lipid component, which resulted in reclassifying into a new genus, *Alicyclobacillus* (Wisotzkey *et al.,* 1992). This unique lipid membrane provides stability and resistance (Bae *et al.,* 2009; Hippchen *et al*., 1981; Kannenberg *et al*., 1984). Yamazaki *et al*., (1996) provided molecular evidence is support of its classification as *Alicyclobacillis acidoterrestris* based on the DNA-DNA homology and the 16s ribosomal DNA sequencing.

Splittstoesser *et al*., (1994) isolated the acidic spore former from apple juice and subsequently several authors including Yamazaki *et al*., 1996; Pontius *et al*., 1998; Pettipher *et al*., 1997; and Komitopoulou *et al*., 1999; identified the organism in orange, apple and grapefruit juices stored at 30 °C. *Alicyclobacillus acidoterrestris* is considered one of the prime organisms associated with spoilage in fruit juices and often the target organism during the pasteurization of acidic fruit juices (Silva *et al*., 2001).

The first instance of a large scale spoilage associated with *Alicyclobacillus* occurred in Germany, in 1982, in pasteurized apple juice contaminated with *Alicyclobacillus acidoterrestris*

(Yokota, 2007). Spoilage was later observed in Japan in an acidic beverage and the results confirmed the role of *Alicyclobacillus* in production of a chemical called guaiacol, which causes the spoilage of acidic beverages (Suzuki, 1989; Splittstoesser *et al*., 1994; Jensen. 2000). Detection methods are not efficient and the spoilage is generally not noticeable during production as the organism does not produce any haze (Walker *et al*., 2005), so spoilage is noticed only by the end consumer due to a distinct medicinal off flavor which is associated with production of guaiacol and halophenols.

There are 4 common species of *Alicyclobacillus* depicted in Table 1. *Alicyclobacillus acidoterrestris* is far more resistant to heat and acid than the other species, and thus is responsible for most juice spoilage incidents.

Species	Source	pH	Temperature (°C)	References
A. acidocaldarius	Acid soil	2.0 – 6.0	25 – 70	Goto *et al.,* 2006
Alicyclobacillus acidoterrestris	Acid soil	2.0 – 7.5	20 - 60	Goto *et al.,* 2006
A. acidophilus	Tainted apple juice	2.5 – 5.5	20 – 55	Yokota *et al*., 2007
A. herbarius	Dried hibiscus flower	3.5 – 6.0	35 - 65	Yokota *et al*., 2007

Table 1: Species of the genus *Alicyclobacillus* as identified in the beverage sector

1.2.1.1. Characteristics of *Alicyclobacillus acidoterrestris*:

Alicyclobacillus acidoterrestris is a nonpathogenic, gram positive, thermo acidophilic, spore forming rod shaped bacterium. The organism possesses oval spores in the central, subterminal or terminal position (Baysal *et al*., 2013). The organism is classified as acidophilic-thermophilic bacteria (ATB) as it grows well in the pH range of 2.5-6.0 and temperature range of 25 °C to 60 °C (Yamazaki *et al*., 1996). ω-cyclohexanoic fatty acids present in the cellular membrane is the main factor responsible for the high resistance noticed in the organism (Krischke and Poralla, 1990; Murakami *et al*., 1998; Alpas *et al*., 2003). It requires water activity greater than 0.9 for growth and survival.

The organism is generally detected in fruit juices when the postharvest washing and cleaning procedures or the factory cleaning and processing procedures are compromised and also as a result of cross contamination by employees.(Chang and Kang *et al*., 2004).

1.2.2. Isolation and detection procedures:

Soil is the most predominant source of *A.acidoterrestris* (Goto *et al*., 2006). The strains of *Alicyclobacillus* grow well in Orange serum broth (OSB)/Agar (OSA), acidified Potato Dextrose Agar (PDA), K Agar, Yeast Starch Glucose Agar (YSG) and Tomato Juice Agar (TJAS), in the pH range of 3.0-5.0. Pettipher *et al*., (1997) showed that OSA has the best recovery of *Alicyclobacillus acidoterrestris*. K-Agar is specific for the strain *Alicyclobacillus acidoterrestris* and is said to be the defining medium for the species (Orr and Beuchat, 1999). The organism generally grows slowly, taking (3-5) days to reach log phase, when incubated at a conducive germination temperature. The cells do not show growth in media containing brain heart infusion and trypticase soy agars and broth even when the pH is dropped down to 3.5 (Splittstoesser *et al*.,

1994). Murray *et al*. (2007) noted that recovery by surface plating was greater than by pour plating at incubation temperatures of 43 ℃ - 50 ℃.

All the species of *Alicyclobacillus acidoterrestris* are capable of metabolizing sugar but do not produce gas, which can make it difficult to identify spoilage except by the customer (Sawaki, 2007). When extremely low numbers of *Alicyclobacillus acidoterrestris* are present in the product, the preferred method for identification of the species is membrane filtration technique (Chang and Kang, 2004). Lee *et al*. (2007) showed that the recovery rate with filters was 126.2 % greater than the K-Agar plating technique, which is considered to be highly specific for *Alicyclobacillus acidoterrestris.*

1.2.3. Spores:

The spores of *Alicyclobacillus acidoterrestris* are highly heat and pH resistant. The organism forms endospores under adverse conditions such as environment and nutrition (Goto et al. 2006). More than 90 % of the vegetative cells are inactivated by the conventional pasteurization 80 °C for 20 min at a pH of 4.0 (Terano *et al.*, 2005). The D-value of A. *acidoterrestris* in juices at 95 °C can be as high as 5.3 min and the Z-values range between 7.2 °C - 12.9 °C (Silva and Gibbs, 2001). The spores lose their property of heat resistance during germination (Terano *et al.*, 2005).

1.2.4. Spoilage and associated off flavors:

*Alicyclobacillus acidoterrestris*is causes spoilage in acidic fruit juices by formation of two distinct off flavors.

1.2.4.1. Guaiacol:

Guaiacol is a phenolic compound generally associated with a medicinal smell in spoiled fruit juices. It also provides a characteristic odor in Arabica coffee (Mayer *et al*., 1999) and barley

malt (Fickert and Schieberle, 1998) and is attributed for production of distinct off flavor in many beverage products (Yamazaki *et al*., 1996; Simpson *et al*., 1986).

In fruit juices, guaiacol is formed from ferulic acid via vanillin (Bahçeci *et al*., 2005). Ferulic acid is a major component in lignin and can be found abundantly in plant cell walls. It can be metabolized by bacteria and fungi (Rosazza *et al*., 1995) and converted to vanillin, vanillic acid, and protocatechuic acid. Vanillic acid is further converted to guaiacol by the microorganism, which is depicted in the Figure 6. *Alicyclobacillus* would not produce guaiacol in the absence of precursors nor could guaiacol be formed from ferulic acid or vanillin without the presence of *Alicyclobacillus* (Figure 7).

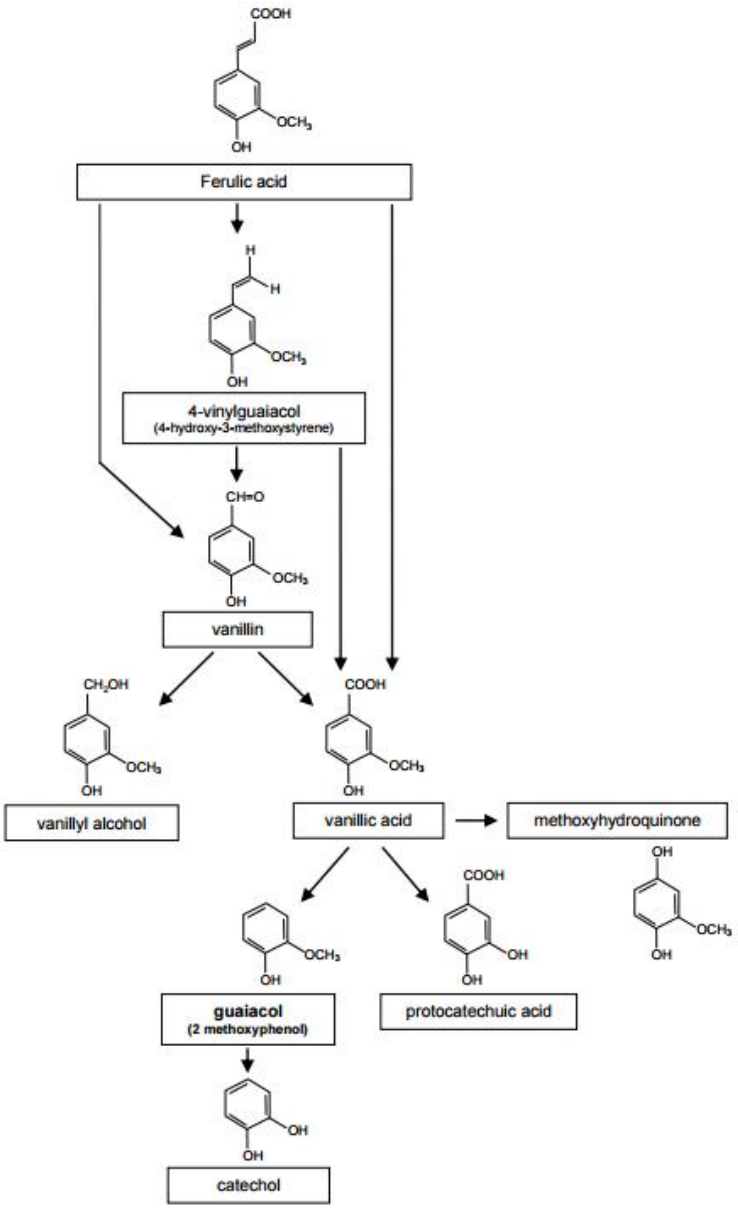


Figure 6: Metabolic Pathway of guaiacol (Ishikawa *et al*., 1963; Huang *et al*., 1993)

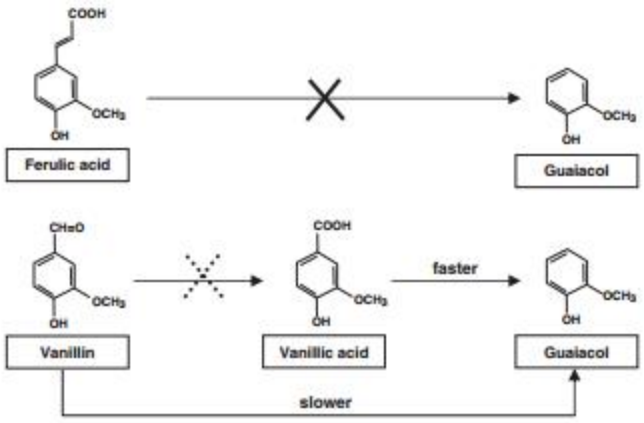


Figure 7: Production of Guaiacol by *Alicyclobacillus* (Van Der Merwe *et al*., 2011)

Crawford and Olson (1978) demonstrated that several strains of *B. megaterium* and a strain of *Streptomyces* convert vanillic acid to guaiacol and CO_2 by a non-oxidative decarboxylation mechanism. They also suggested that the ability to decarboxylate vanillic acid to guaiacol is quite common among soil *Bacilli*.

The human sensory threshold for guaiacol is low, so it is easily detected. Wasserman (1966) reported that the threshold concentration of guaiacol in water is 0.021 ppm for odor and 0.013 ppm for taste. The threshold for smelling guaiacol in 12 % aqueous ethanol is reported to be 0.03 ppm (Chang and Kang, 2004). Pettipher *et al*. (1997) used a GC-MS method and found that the odor threshold for guaiacol in orange, apple juice, and a non-carbonated fruit juice drink was about 2 ppb. Orr *et al*. (2000) also showed similar results by another study using sensory panel and the forced choice ascending concentration. They reported the best estimate threshold of guaiacol in apple juice is 2.23 ppb.

1.2.4.2. Halophenols:

Halophenols are another compound that can be present in various food products and is characterized by a distinct "medicinal" or "disinfectant odor" odor (Whitfield, 1998). Halophenols are formed in the presence of haloperoxidase which catalyzes the reaction of phenolic precursor with hydrogen peroxide. The taste threshold in water of 2, 6-Dichlorophenol (DCP) is 6.2 ppt (Young *et al*. 1996) and 0.5 ppt for 2, 6-Dibromo phenol (DBP) (Whitfield *et al*., 1988). The taste threshold is reported to be 0.5 ppt for 2, 6-DBP and 30 ppt for 2, 6-DCP in juices (Jensen, 1999). According to Jensen and Whitfield (1993), 2, 6-DBP and 2, 6-DCP are produced by some enzymes like haloperoxidase in some strains of *A. acidoterrestris* that are capable of halogenation (Lamikanra *et al*., 2005).

Past recalls associated with spoilage by *Alicyclobacillus acidoterrestris* are summarized in Table 2. But considering the economic stress faced by the beverage industries it is important to take the measures to ensure the quality of products.

Spoilage by *Alicyclobacillus acidoterrestris*	**Region/Year**	
Aseptically packaged Apple juice (Cerny *et al*., 1984)	Germany	1982
Fruit Juice (Suzuki, 1989; Splittstoesser *et al*.,1994; Jensen, 2000)	Japan Europe USA	1990's
Isotonic water and Lemonade (Yamaziki *et al*.,		1996

1996)		
Carbonated Fruit Juice (Pettipher *et al*., 2000)		2000
Fruit pulps (Gouws *et al*., 2005)		2005
Shelf stable ice tea with berry juice (Duong and Jensen, 2000)		2000
Canned diced Tomato (Walls and Chuyate, 1998)		1998

Table 2: Recalls of different juices associated with *Alicyclobacillus acidoterrestris* spoilage

1.3. Thermal Processing/Pasteurization

The heat treatment is done on food products for the purpose of preservation. It also plays an important role in food safety to reduce the population of food pathogens below an acceptable level, in shelf life to extend the shelf life by reducing the population of spoilage microorganisms in food and in quality to inactivate the enzymes that degrade the quality and to improve digestibility, color and texture in many cases.

In many cases the sensory and the nutritive properties of the food gets affected during thermal processing. Cooking, blanching, pasteurization and sterilization are the common types of thermal processing. All these processes follow time- temperature trends as shown in Figure 8. During the process, the product is heated to a specific temperature (T_0), held at the temperature for a certain time till it achieves the desired lethality (F_0) and then cooled to the initial temperature. F_0

is the time required to cause a stated reduction in population of a specific species of microorganisms at a specified reference temperature. F_0 value depends on what kind of thermal processing operation is being carried out, pH of food, a_w, etc.

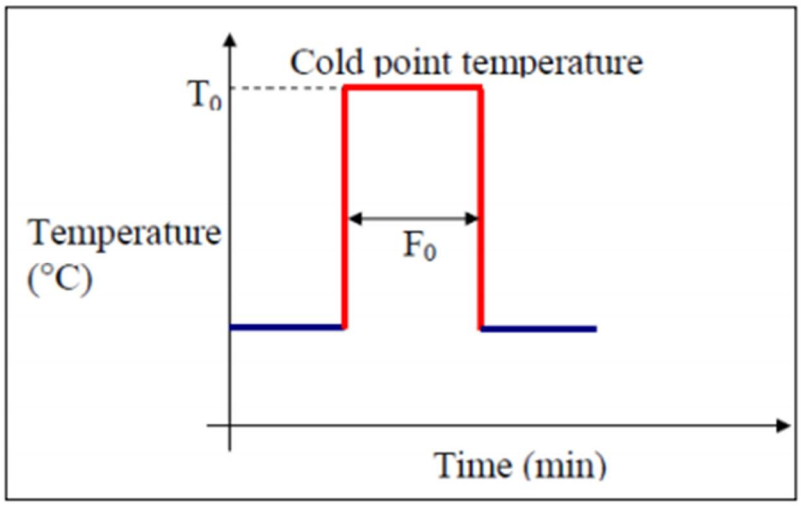


Figure 8: Time - Temperature plot (ideal) for a typical thermal processing operation.

Selection of a heat-preservation treatment depends on the time-temperature combination required to inactivate the most heat resistant pathogens and spoilage organisms in a particular food, the heat-penetration characteristics of a particular food, including the can or container of choice, the physical properties of the food (solid vs. liquid) and the chemical properties of the food (pH, fat content, other food components that will interfere on the thermal resistance of microorganisms)

1.3.1. Fruit juice processing

Sterilization/Pasteurization is the main method by which the fresh fruit juices are commonly preserved. The pH of apple juice is ~3.5, at this pH very few microorganisms survive. Pasteurization is a mild heat treatment in which juices are heated to target temperature range of 60 °C to 90 °C (Lewis, 2006). In the traditional batch pasteurization technique, (Lewis, 2006), juices are held at about 60 °C for a relatively long period of time (~30 min) in an open pan or vat and

then hot-filled into containers, sealed and inverted, thus sterilizing the upper part of the containers and lids (Lewis, 2006). This type of hot-fill process is simple and suitable for fruit-based products with pH below 4. It also has the additional advantages of creating a partial vacuum in the sealed container as vapor condenses upon cooling. High Temperature Short Time (HTST) process can also be implemented for continuous pasteurization. If fruit juices contain discrete particles, aseptic processing helps to improve product quality of heat processed shelf-stable foods (Fellows, 2000). Aseptic processing is the process by which a sterile (aseptic) product (typically food or pharmaceutical) is packaged in a sterile container in a way that maintains sterility. Table 3 explains the quality attributes affected upon thermal processing on different substrates.

Quality attributes affected	**Supported references**
Deterioration of antioxidant activity in clarified blackberry juice. Reduces enzymatic browning by inactivating oxidative enzymes. Increased non enzymatic browning.	(Hager *et al.*, 2008)
Color pigments like chlorophyll and carotenoids by chemical reactions like degradation and isomerization. Cooked flavor was observed in guava juice when treated at 95 °C for 5 min.	(Boyles *et al.*, 1993)

Vitamin degradation can also occur during heat treatment and it depends on oxygen, light, pH and water solubility Reduction of ascorbic acid content by 2 % – 6 % was observed in black currant nectars and up to 25 % loss in yellow passion fruit post pasteurization	(Iversen, 1999) (Talcott *et al*., 2003)

Table 3: Thermal processing and its effect on quality

1.4. Ultraviolet radiation

1.4.1. Introduction:

For centuries people have been using the conventional processing technology to process and preserve food products. Some of the well-known conventional processing techniques are heating, salting, blanching, freezing etc. New technologies includes the non-thermal processing techniques such as high hydrostatic pressure (HPP), pulsed electric field and ultraviolet light (UV) (Aplas *et al*., 2003; Lee *et al*., 2002; Vercammen *et al*., 2012; Gould, 2001; Koutchma, 2009). Multiple hurdle technology uses two or more processes rather than a single step treatment. Among the non-thermal processing techniques UV light has found a niche for itself in the food production as it has been approved by US FDA for application in the industry (US FDA 2000). The UV-C light is thought to inactivate microorganism by damaging DNA (Franz *et al*., 2009). UV light is a non-ionizing radiation and can bring about reduction in spores within a short during of time, using minimal energy, compared to other non-thermal treatments (Geveke *et al*., 2005; Beltran *et al*., 2004). This treatment also ensures that the quality of the product is preserved (Allende *et al*., 2003; Keyser *et al*., 2008). Previous researches have shown that UV light alone 1.31 mW/cm^2 has the potential to bring about a 2 log reduction of *Alicyclobacillus acidoterrestris* spores in apple juice (Baysal *et al.,* 2013). A greater reduction in the concentration of spoilage microorganism could mean reduced chance of spoilage and a longer shelf life.

Historically ultraviolet technology has been used as a disinfecting agent in water purifying plants. Ultraviolet-C (UV-C) light of 254 nm is used to inhibit or inactivate foodborne microorganisms in liquid food products (Guerrero-Beltrán and Barbosa-Cánovas 2004). UV-C technology is considered a “cold pasteurization step” to control the microbial load in the food products. UV-C light is easy to use and lethal to most types of microorganisms (Bintsis *et al*.,

2000). Generated by UV mercury lamps, UV-C light (between 220 nm and 300 nm) can have a germicidal effect on microorganisms such as bacteria, viruses, protozoa, molds and yeasts (Morgan, 1989; Sizer and Balasubramaniam, 1999; Bintsis *et al*., 2000).

1.4.3. UV light and Spores:

Ultraviolet (UV) radiation covers a small part of the electromagnetic spectrum. Figure 10 shows the electromagnetic spectrum and where the UV radiation is located in the spectrum. The UV radiation between the visible range and the X- rays, with a wavelength of between 100 nm-400 nm. There are 3 types of UV radiations, UV-A, UV-B and UV-C (Table 4). Figure 9 depicts the role of each range UV radiation.

Ultraviolet Radiation type	**Wavelength (nm)**	**Property**
UV-A	400 -315	Blacklight UV
UV-B	315 -280	Dangerous UV
UV-C	280 – 200	Germicidal ultraviolet
UV-V	200 – 100	Vacuum UV

Table 4: Types of UV-C radiation: (*http://www.americanairandwater.com/uv-facts/uv-types.htm*)

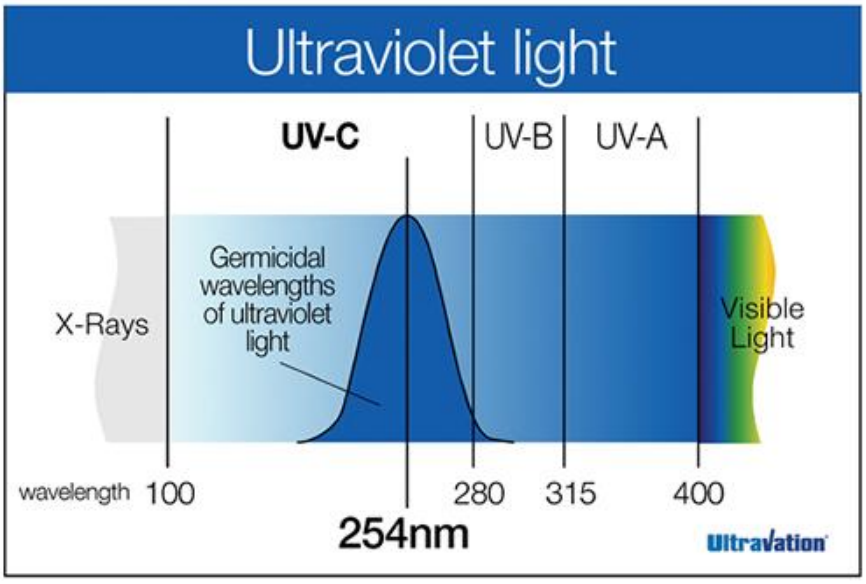


Figure 9: Types of UV radiation (https://cwgoodguys.com/uvphotomax/)

UV-A rays are of longer rays of the wavelength between 400 nm - 315 nm and cause premature aging of skin. They are associated with skin damage including wrinkles and skin cancer. These are the rays associated with tanning. UV-B rays are the “harmful rays” that also cause cancer, but which are primarily absorbed in the ozone layer. UV-C rays are short wave length UV rays which possess germicidal properties and have been in use for disinfection of air, water and surfaces.

Microorganisms suspended in air are the most sensitive, followed by those suspended in water and then by those in juices. This is because the effectiveness of UV radiation is based on the transmittance. Since UV light has a low penetrating power due to its low inherent energy of photons it is classified as a nonionizing radiation.

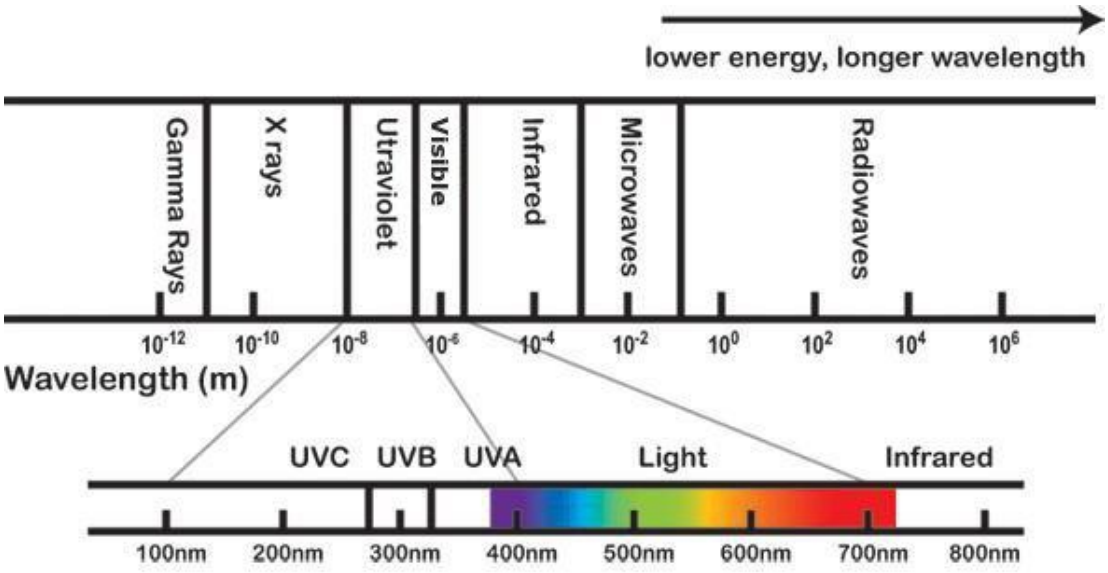


Figure 10: Electromagnetic spectrum (http://healthycanadians.gc.ca/healthy-living-vie-saine/environment-environnement/sun-soleil/radiation-rayonnement-eng.php)

Factors that affect the efficacy of UV-C radiation are colored compounds, organic solutes and suspended matter. Koutchma (2009) showed that low transmission lowers the performance efficiency of UV pasteurization process which is supported by the Figure 11, where an experiment on water and different juices show that the absorbance of fresh juices is higher than water.

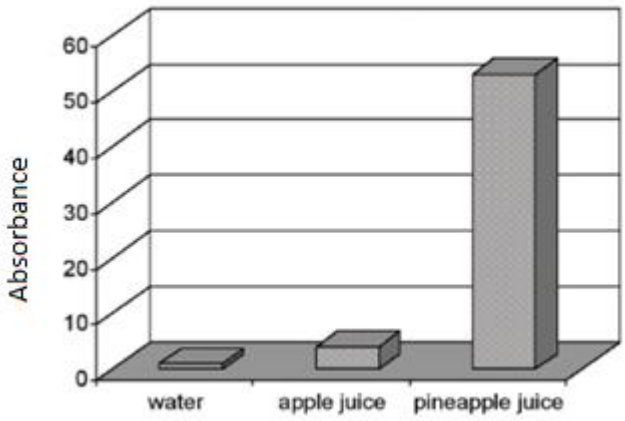


Figure 11: Comparison of viscosity of water and fresh juices (Koutchma *et al*., 2009b)

US FDA has concluded that usage of UV radiation is safe and approved it as an alternative to thermal processing of fresh juice products (U.S. Food and Drug Administration. 2000.21 CFR Part 179). Low pressure mercury lamps (LPM) are most widely approved for food processing application.

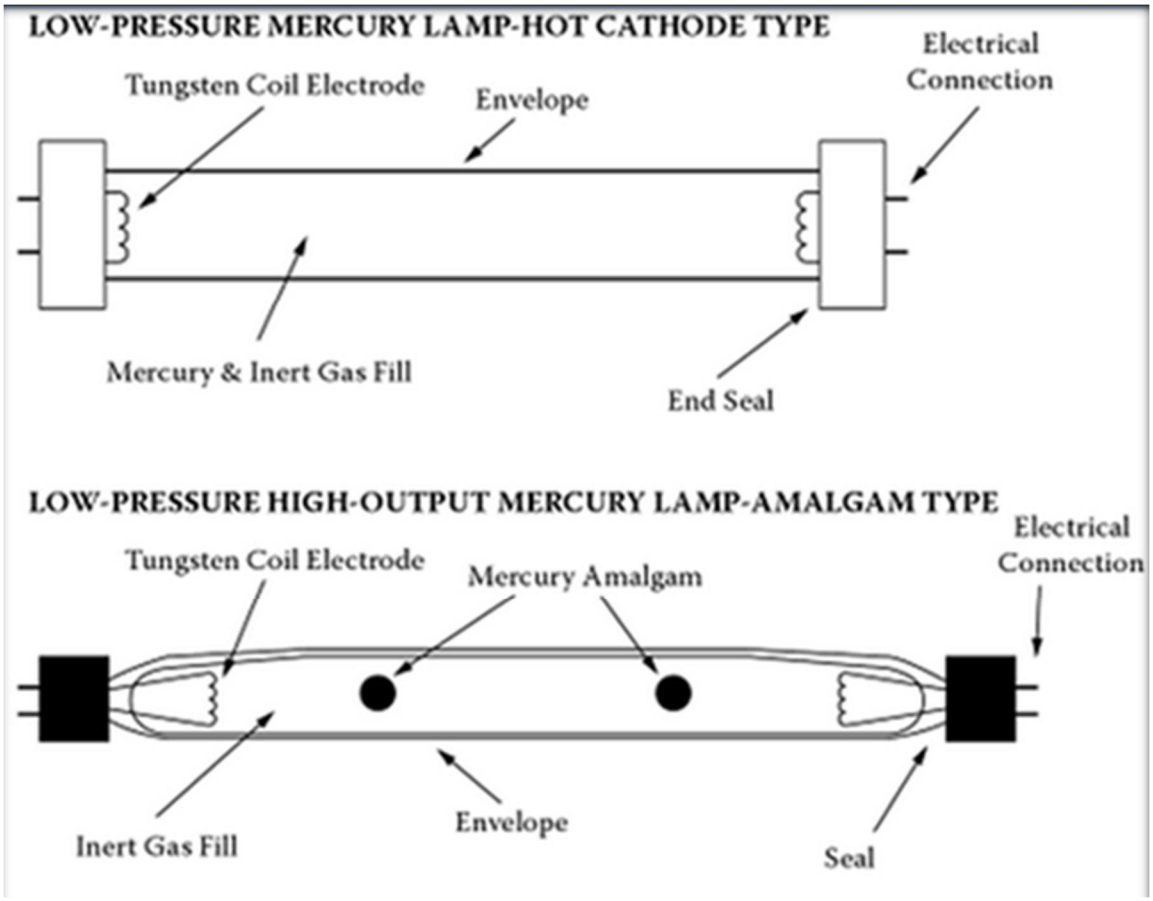


Figure 12: UV-C Radiation source (Koutchma *et al*., 2009b)

Lethality in UV processing depends on wavelength (level of UV light photon energy), intensity (number of photons), exposure time, temperature, pH, chemical composition and physical structure of the product, density and the type of microorganism, and UV-C absorptivity of liquid,

suspended and soluble solids in liquid (Shama, 1999; Bintsis *et al*., 2000) as well as color of the liquid.

The greater the concentration of soluble and suspended solids there are in the food product, the lower the intensity of penetration of the UV-C light in the liquid. The UV light emitted interacts with materials through absorption, reflection, refraction and scattering, thus the positioning of UV source and the distance plays a very critical role in maximizing the efficacy (Figure 13). Any obstruction in the path of UV light results in reduction in the efficacy, including dust particles or clumping of bacteria. UV light is more effective on smooth surfaces as compared to rough surfaces (Rahman, 1999).

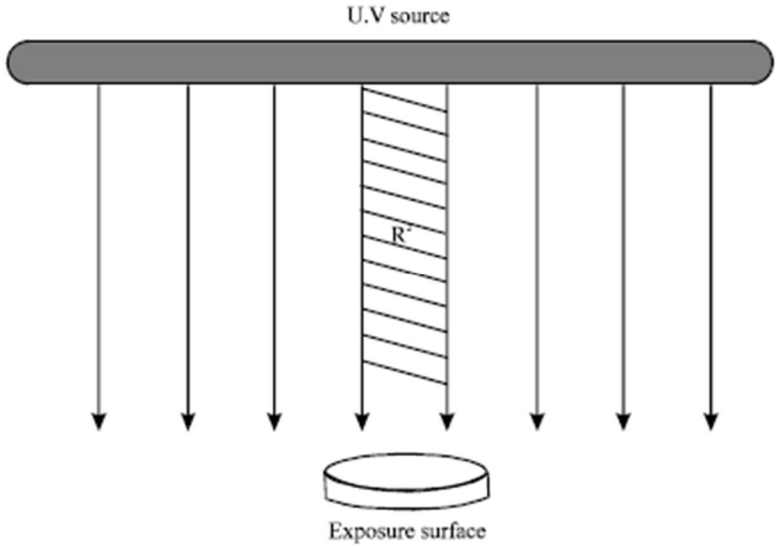


Figure 13: Factors affecting the effectiveness of UV-C radiation are distance and position

1.4.2. Mechanism of inactivation by UV light:

The UV light affects the microorganisms at DNA level disrupting both replication and transcription of the nucleic acid. Photoproducts (chemicals produced after applying UV light, such as pyrimidine dimers and pyrimidine hydrates) can encourage cross-linking within proteins (Shama, 1999). Figure 14 depicts how UV-C inactivates microorganisms. The movement of RNA/DNA polymerase is stalled due to the dimers that are formed. Therefore the DNA injury within microorganisms is advantageous in UV-C treatment of liquid food products because the process works as a "cold pasteurization" to obtain minimally heat-treated products. Table 5 indicates that the use of UV-C light is a promising approach to obtaining liquid food products with fresh-like sensory characteristics.

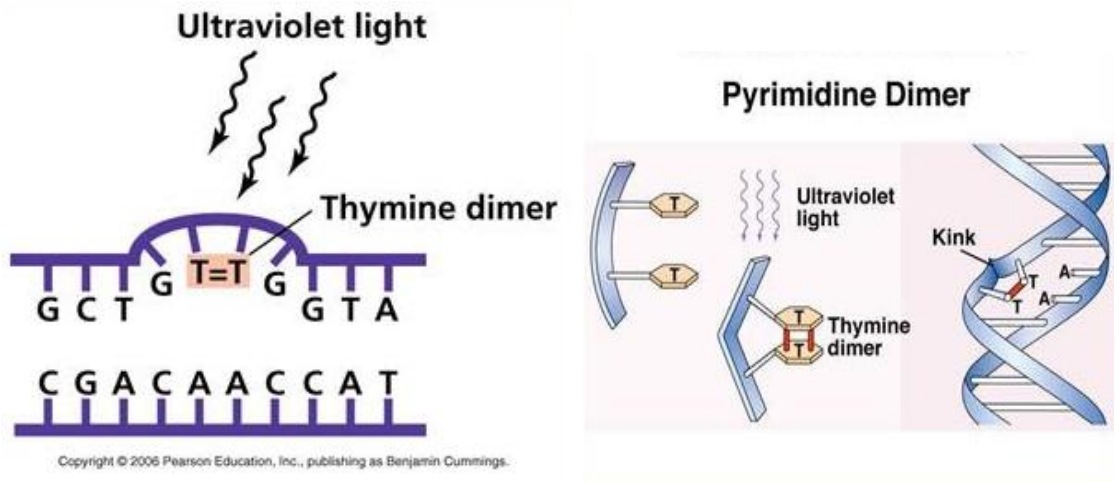


Figure 14: Mechanism of microbial inactivation by UV-C radiation (http://mol-biol4masters.masters.grkraj.org/html/Gene_Expression_I4Regulation_of_Gene_Expression.htm)

PRODUCT	REFERENCE
Apple cider	Harrington and Hills (1968); Worobo (1999); Wrigh et al.(2000)
Orange juice	Farid et al. (2001)
Red beet juice	López-Malo *et al.* (2001a)
Carrot juice	López-Malo *et al.* (2001b)
Apple juice	Guerrero-Beltrán and Barbosa-Cánovas (2005)
Mango nectar	Guerrero-Beltrán and Barbosa-Cánovas (2006)
Water	Snowball and Hornsey (1988); Anon (2005); Hoyer (1998)

Table 5: Past research with UV-C radiation on beverages

CHAPTER 2

HYPOTHESES AND RATIONALE

HYPOTHESIS

- Multiple hurdle technology of sequential heat and UV treatment might be a promising alternative to traditional thermal processing of clarified apple juice to inactive *Alicyclobacillus acidoterrestris.*
- Sequential application of mild heat followed by UV technology might retain the color, flavor characteristics of clarified apple juice.

RATIONALE:

Beverage industries around the world need to manage the presence of spore forming, nonpathogenic spoilage organism *Alicyclobacillus acidoterrestris* (Sapers *et al*., 2005). This organism produces a distinct medicinal odor in fruit juices, particularly in apple juice (Goto *et al*., 2003), which is associated with the production of guaiacol and halophenols. This organism is thermophilic and acidophilic, and can survive the normal pasteurization process of juices (Pena *et al*., 2009). An alternative method of treatment of apple juice that would preserve the quality of the product and also bring about 4-5 log reduction of *Alicyclobacillus acidoterrestris* spores would be beneficial. This research work was targeted to find a suitable combination of thermal and non-thermal technologies, especially mild- heat and ultraviolet radiation that can be used to process clear apple juice to reduce *Alicyclobacillus acidoterrestris* spores to a desired level while maintaining the sensory quality of apple juice.

CHAPTER 3

OBJECTIVES

The specific objectives of this study were as follows:

- To study the effect of mild thermal processing and subsequent UV radiation on the reduction of *Alicyclobacillus acidoterrestris* spores in clarified apple juice.
- To optimize the temperature, UV intensity and time required for the effective inactivation of spores in the juice using multiple hurdle technology.
- To conduct and evaluate the flavor and sensory properties of pasteurized and processed clarified apple juice samples.
- To analyze and compare headspace volatiles of the treated and commercially pasteurized clarified apple juice in order to elucidate flavor profiles.

CHAPTER 4

MATERIALS AND METHODS

4.1. Culture and culture conditions

Alicyclobacillus acidoterrestris ATCC 49025 was used in this study (American Type Culture Collection, Rockville, MD). The culture was stored at -80 °C in 80 % sterile glycerol. A loop of glycerol stock was streaked onto potato dextrose agar acidified with 10 % sterile tartaric acid and incubated at 43 °C for 48 h. A single colony was transferred to 10 ml of potato dextrose broth (Thermo Scientific Oxoid, Dallas, Texas) containing 10 % sterile tartaric acid and incubated at 43 °C for 48 h. One ml of the bacterial suspension was taken from the above potato dextrose broth and transferred to fresh broth with the same conditions in a shaker incubator (New Brunswick Scientific, New Brunswick, NJ), to obtain a uniform culture. The cells were harvested by centrifugation at 5000 rpm, for 15 min at 4 °C in 1 mL micro centrifuge tubes (Fisher Scientific accuSpin Micro 17, Pittsburgh, PA). The cells were re-suspended in sterile distilled water and centrifugation repeated three more times. The cells were re-suspended into 1 ml of sterile distilled water, and this suspension was used for all the bacterial inoculation procedures.

4.2. Preparation of spores:

Sporulation was induced by transferring a loop of cells from the PDA slant spread plating onto acidified Potato Dextrose Agar (approximately 50 numbers of petri plates) incubated at 43 °C for 7 days. The 7 day incubation ensures spore formation. Spore staining using Fulton and Schaeffer stain and phase contrast microscopy of magnification 300X confirmed sporulation.

The spores were harvested by pipetting 5 mL of sterile distilled water onto the plates and swabbing using a sterile swab. The resulting suspension was collected in a 50 mL centrifuge tube and centrifuged (5000 rpm, 15 min at 4 °C). The procedure was repeated three times and the resulting pellet was re-suspended in sterile distilled water. This suspension was vortexed then heated at 80 °C for 3 min to inactivate vegetative cells. The resulting suspension was viewed under phase contrast microscopy of magnification 300X and spore staining to confirm > 95 % spores. The spore suspension was stored at 4 °C.

4.3. Apple Juice Sample

Commercially pasteurized apple juice (Apple & Eve, 100% Apple juice) was purchased from a local market. The pH values of the clarified apple juice were measured using a pH meter (Metrohm, Switzerland) while soluble solid content (°Brix) was determined by a refractometer (Mettler, Toledo). The absorbance of the apple juice was also measured in a 1 cm path quartz cuvette using a UV-VIS spectrophotometer (Cary 50 Spectrophotometer from Agilent Technologies, Santa Clara, CA, USA) at 254 nm. The transparency of juices to UV-light was determined by measuring the absorbance. Absorbance coefficient (A_e) was calculated by measuring the absorbance of dilutions of the juices and determining the slope of absorbance against concentration (Caminiti *et al*., 2012).

4.5. Heat treatment

Once the cells are sporulated, heat treatment is performed to ensure maximum germination of the cells with apple juice as the medium. Spores were treated over a range of temperature-time conditions from 45 °C for 10 min to 70 °C for 120 min. This range was used in the study to determine the most optimal temperature-time condition for maximum germination, since the most optimal temperature for the growth of *Alicyclobacillus acidoterrestris* 45 °C and 72 °C is the

optimal temperature for pasteurization. The cells were observed in phase contrast microscope and checked with Schaeffer Fulton stain to ensure sufficient germination. The samples were plated on PDA to get an accurate count of germination. Calculation of percentage germination is done as below: The sample after the germination step is further treated at 80 °C for 3 minutes, this step will inactive the germinated (vegetative cells) and then plated. Thus the cells that grow on the plate are the spores. The percentage germination is calculated by comparing the reduction in the number of cells compared to the control.

4.5.1. The process of mild heat treatment

Small batch heat treatment was carried out by taking 20 mL of commercially pasteurized Apple & Eve apple juice in a small glass beaker with lid. A sterile magnetic stirrer was introduced into the beaker. The vessel had a thermocouple with data acquisition system inserted into the lid in order to measure the temperature of the juice during the various heat treatments. The heat up and cool down time was recorded for the most optimal time temperature condition and a graph was plotted.

4.5.2. Bulk pasteurization

Bulk quantity of juice was required for sensory tests and storage studies. Approximately 200 ml of juice was introduced into each glass beaker with lid. Ten such beakers were placed in a water bath with thermocouple. The beakers were constantly agitated to ensure even mixing of the samples. This thermocouple was attached to a data acquisition system. Once the appropriate time-temperature condition was reached the heating was discontinued and samples were allowed to reach room temperature prior to UV treatment.

4.6. UV treatment

UV-C irradiation of samples was conducted using Spectrolinker XL-1500 UV Crosslinker (254 nm wavelength, six 15 watt tubes, shorter wavelength, 120 V, 60 Hz - 2 A). The UV lamp with a single tube on was switched on for (10 -15) min prior to experiment to minimize fluctuations. The samples were placed in lidless 6 cm diameter glass petri dishes directly below the collimated UV beam. The intensity of the UV lamp was measured by a UV–VIS radiometer supplied with UVX–25 sensor (UVX, UVP Inc., CA, USA) placed at the same distance from the UV lamp as the plates. The inner and the outer chamber of UV Spectrolinker are depicted in Figure 15. The 6 UV tubes were placed equidistant from one another, at a distance of 15.9 cm from the base of the Spectrolinker.

Figure 15: Images of spectrophotometer (a) external and (b) internal shows the presence of UV tubes

4.7. Inactivation treatments

Spores (1×10^8 CFU/mL) were inoculated into the commercially pasteurized and sterilized clarified apple juice, germinated by mild heat treatment and then exposed to UV-C radiation of a known intensity level (1.6 mW/cm^2) for times from 10 s to 600 s. The intensity calculations are explained below in results section.

4.8. Microbiological analysis

Following UV-C irradiation, the plates were incubated at 43 °C for 5 days. Spore counts (since the vegetative cells are inactive by mild heat and UV treatment) were determined by spread plating the diluted samples onto PDA agar (pH 3.5). Microbial count determinations were performed in two replications and expressed as CFU/mL.

4.9. Statistical analysis

Data presented are averages ± standard deviations of six independent UV inactivation experiments for three independent spore batches. The mean and standard deviation of the treatments were calculated using Microsoft Excel. A one-way analysis of variance (ANOVA), F-test and T-test were performed using Minitab software (2015b, New Brunswick) for the treatment of inactivation of *Alicyclobacillus acidoterrestris* spores in apple juices.

4.10. Sensory Evaluation

A triangle, "FIZZ" test (http://www.biosystemes.com/fizz.php) was performed in order to determine if any sensory difference existed between mid-heat-UV processed and commercially pasteurized apple juice samples. This method typically requires 20-50 panel members to provide meaningful results (BASF306B Sensory Science of Food, 2012).The sensory evaluations were conducted in the Rutgers University sensory evaluation laboratory with 55 untrained panel members consisting primarily of Food Science faculty, staff and students. The age of the panel members ranged from 20 years to 55 years. Each subject was presented with 3 samples marked with random 3 digit code (Figure 16). The total number of possible combinations is 6, which are shown below in Table 6. The panel members were asked to determine which of the three samples was different from other two based on taste and/or smell (Figure 17).

Combinations	1st sample (A)	2nd sample (B)	3rd sample (C)
1	Treated	Treated	Control
2	Treated	Control	Control
3	Treated	Control	Treated
4	Control	Treated	Treated
5	Control	Treated	Control
6	Control	Control	Treated

Table 6: Sensory Evaluation: Triangle test

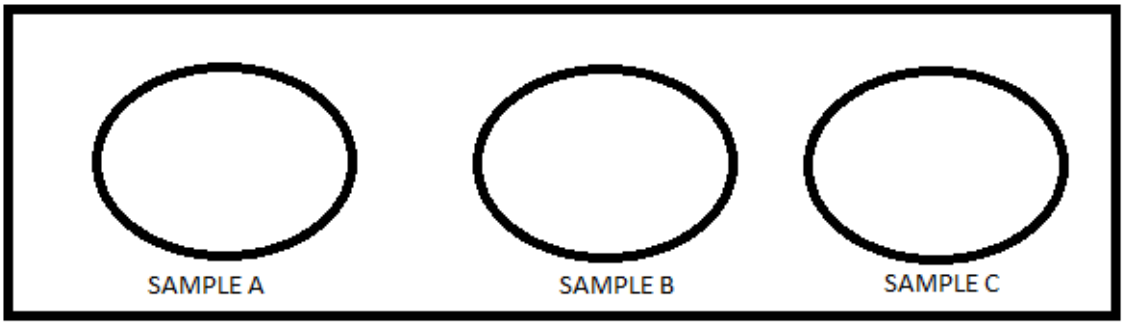
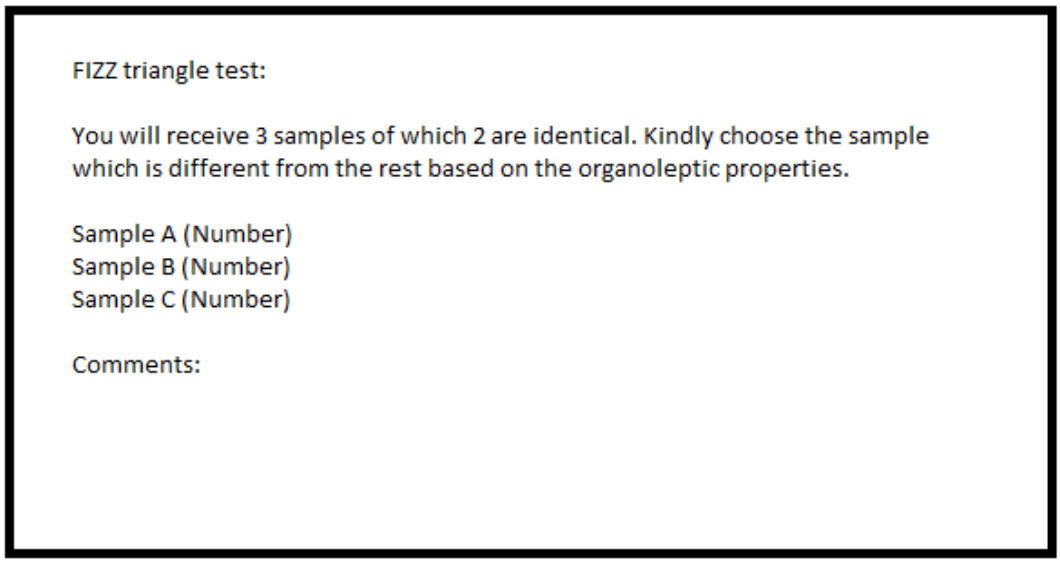


Figure 16: Sensory Evaluation- Samples presented to participants


FIZZ triangle test:

You will receive 3 samples of which 2 are identical. Kindly choose the sample which is different from the rest based on the organoleptic properties.

Sample A (Number)
Sample B (Number)
Sample C (Number)

Comments:

Figure 17: Sample questionnaire for the samples presented to the panel members.

4.11. Headspace Volatiles analysis:

Static headspace GC-MS followed by purge and trap GC-MS was performed to determine the volatiles in apple juice. Each peak on the chromatogram represents a specific compound and the area under the peak corresponds to the relative amount of that particular compound in the sample mixture.

4.11.1. Static headspace analysis:

One mL of the apple juice sample was placed in a closed glass vial with 5 mL of NaCl sealed and incubated at 100 °C for 30 min. The volatile components, approximately 10 mL were extracted from the headspace with a headspace syringe and injected into a gas chromatograph for separation of all the volatiles. The temperature ramp program used for the separation was -20 °C to 280 °C at the rate of 10 °C / min. The sample was held at -20 °C for 3 min. It took about 35 min to obtain the profile of the apple juice samples.

4.11.2. Purge and trap head space analysis:

N_2 gas at 100 ℃ was blown over the sample for 30 min to collect volatiles then into a small C 32 column where volatiles are adsorbed onto a solid matrix and concentrated for analysis. The volatiles were collected in the thermal desorption traps which are made up of 100 % Tenax purge and trap tubes. Volatiles in the trap were then desorbed into a gas chromatograph for analysis using the short path thermal desorption step, which was attached to the GC unit. The process of desorption takes place for 5 min at 250 ℃. In purge and trap GC-MS analysis, it is noticed that the temperature directly increases to 280 ℃ at 10 ℃ / min without a wait/hold time.

CHAPTER 5

RESULTS AND DISCUSSIONS

5.1. Apple and Eve® apple juice: The substrate

5.1.1. pH of apple juice

The pH of apple juice was 3.62±0.1.

5.1.2. ºBrix of apple juice

ºBrix of the commercially pasteurized apple juice was 11.8 as shown in Table 7.

Apple juice (mL)→	10	9	8	7	6	5	4	3	2	1
Distilled water (mL)→	0	1	2	3	4	5	6	7	8	9
Trial#										
1	11.8	10.6	9.5	8.3	7.0	5.9	4.7	3.7	2.5	1.3
2	11.8	10.6	9.5	8.3	7.0	5.9	4.7	3.7	2.1	1.2
3	11.8	10.6	9.5	8.3	7.0	5.9	4.7	3.7	2.3	1.3
4	11.1	10.5	9.6	8.3	7.0	5.9	4.7	3.7	2.3	1.3
5	11.9	10.6	9.4	8.3	7.1	5.9	4.8	3.8	2.5	1.3

Table 7: Brix values for apple juice at various concentrations

5.1.3. UV absorbance:

Figure 18 is a plot of the concentration of apple juice (vs) absorbance at 254 nm. The transparency of apple juice to UV light was determined by the absorption coefficient A_e calculated using a UV spectrometer to be 24.276 cm^{-1}. The slope of the absorption and concentration will give the value of absorption coefficient.

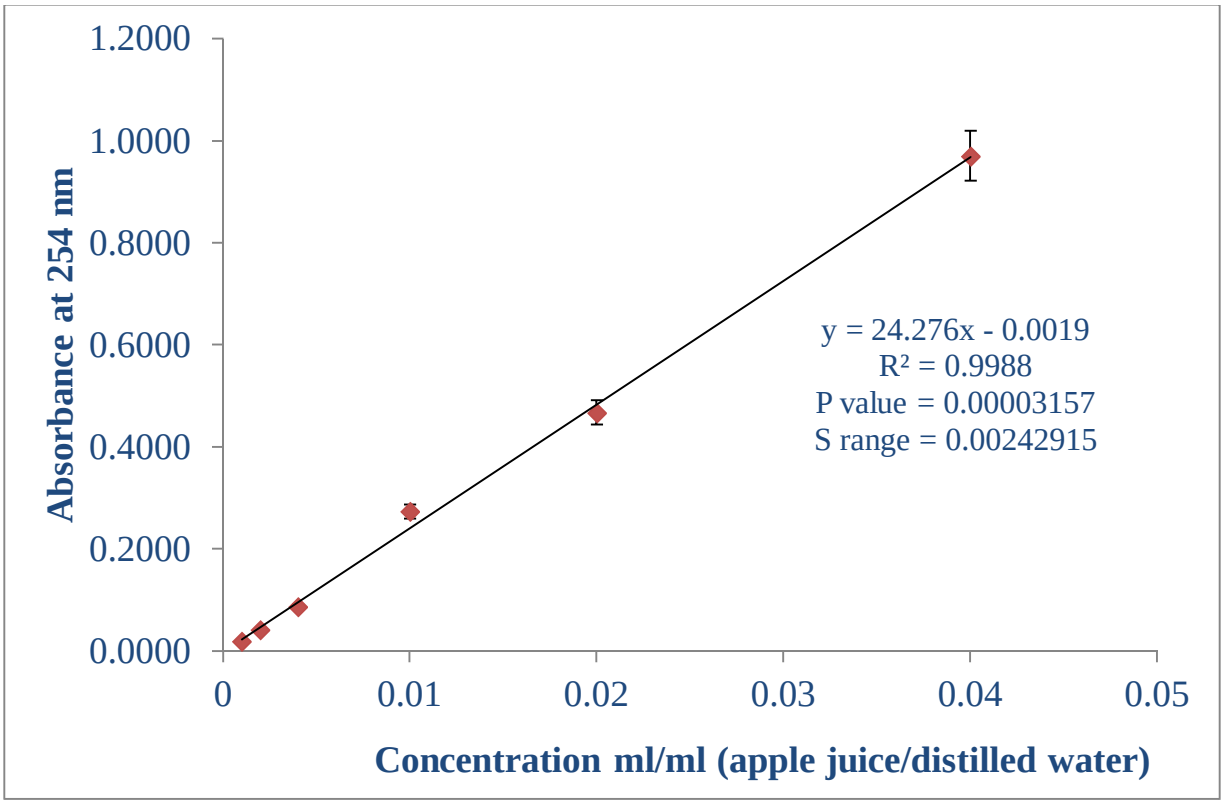


Figure 18: Absorbance curve for measuring the absorbance coefficient of apple juice using UV-spectrophotometer

5.1.4. Intensity Measurement

Power of each tube is 15 W/cm^2 and the area of base rectangle (b×h) is 1470 cm^2, where b represents the base of the Spectrolinker and h is the height. The schematic representation of the position of the UV tubes in the UV Spectrolinker is shown in the Figure 19, along with the image of the actual arrangement of tubes in the UV chamber. Intensity of the 6 tubes at the bottom of

the plate is 5500 µW/cm^2 (Based on the information on Spectrolinker). Therefore, 1 tube corresponds to 916 µW/cm^2 or 0.916 mW/cm^2.

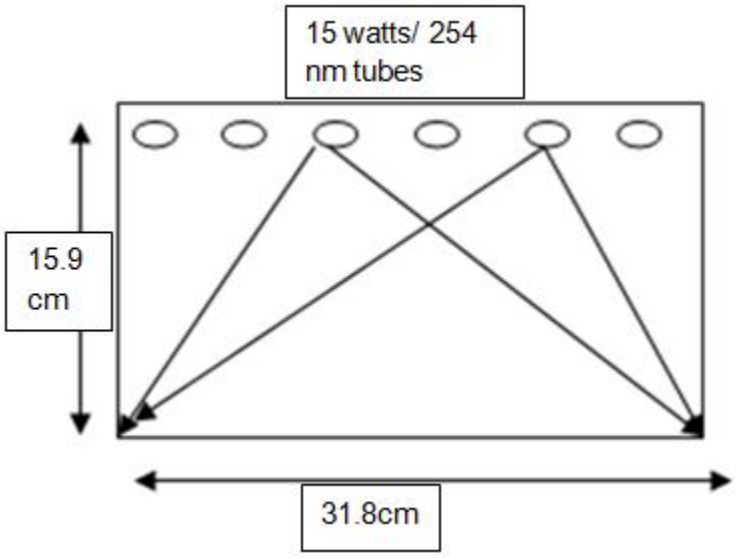


Figure 19: Schematic diagram of UV spectrolinker

Calculating the intensity:

Based on the formula, *Power = Intensity × Area.* Therefore Intensity is inversely proportional to Area. We consider the light source as a cylinder, thus the area is 2π R L. The sample is placed at the base, thus the area at the base is L × B, where L is the length and B is the breadth of the spectrolinker.

$$\frac{I}{I'} = \frac{A'}{A},$$

$$where\ I, A = Intensity, Area\ near\ the\ tube,$$

$$I', A' = Intensity, Area\ at\ the\ base\ of\ spectrolinker$$

$$\text{Intensity at the base} = \frac{[(1.333)(2\pi \times 4 \times 46.4)]}{46.4 \times 31.8}$$

$$= 1.05\ \text{mW/cm}^2$$

Where, L=length of the base of the Spectrolinker, R= radius of the UV tube, B= breadth of the UV Spectrolinker.

By UV-Vis Radiometer:

The UV-Vis Radiometer readings were taken at measured at different time of exposure with 1 centrally placed tube ON is shown in Table 8.

	Intensity (mW/cm^2)				
Time (s)	#1	#2	#3	#4	#5
10	1.07	1.06	1.03	1.06	1.04
30	1.17	1.38	1.38	1.40	1.39
60	1.3	1.44	1.48	1.49	1.49
120	1.38	1.47	1.49	1.50	1.48
300	1.43	1.50	1.50	1.50	1.50
600	1.48	1.50	1.51	1.49	1.50

Table 8: Intensity measurement based on UV-Vis Radiometer

Once the system was warmed up, the intensity was measured at the base of the chamber as 1.6 mW/ cm^2. This value was different from the value obtained by calculations. To ensure the value obtained by calculation is same as the value obtained from measurement, the following experiment was performed. The intensity of UV light right below the tube and the intensity when all the sides were closed with black cardboards was measured. The intensity of the UV light right below the tube was measured to be 4.10 mW/ cm^2. When the intensity was measured with the sides

closed gave a value of 1.07 mW/cm^2 which matches with the value obtained by calculation. Thus the increase in intensity is due to internal refection in the system. The intensity of UV light exposed during each treatment would vary between (1.4 - 1.6) mW/cm^2. The intensity measured the same (1.4 - 1.6) mW/cm^2 at every point on the base of the Spectrolinker.

5.1.5. Heat Treatment: Heat up and cool down time

The effective time for heat treatment is the sum of heat up (come up) time, actual mild heat treatment time and cool down time. The total to bring the temperature to 52 °C was approximately 15 min and from the 52nd min the process was stopped and the set up was left at room temperature to cool down which took 5 min ± 0.5 min. Figure 20 depicts the temperature time profile for the total heat treatment process.

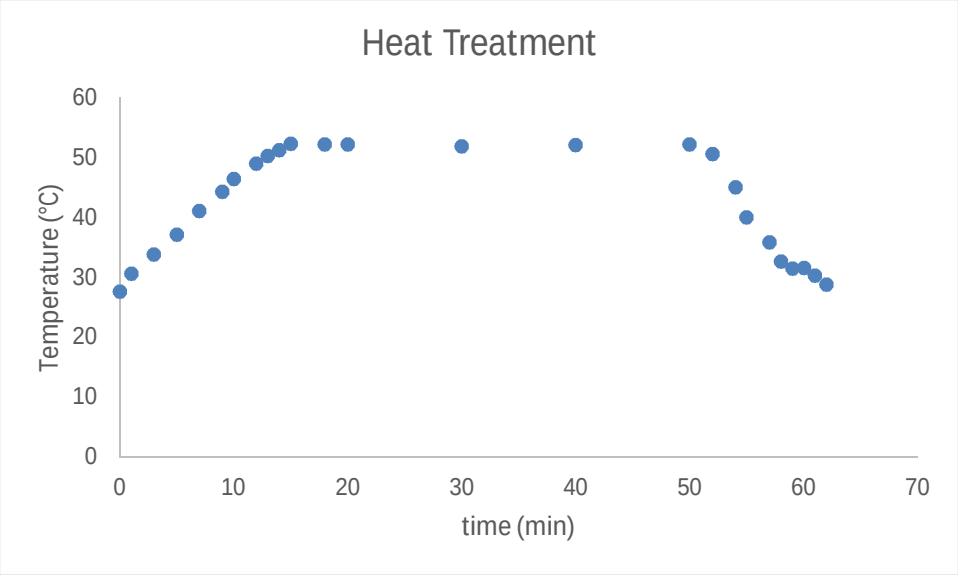


Figure 20: The total time required for heat treatment of 20 mL of pasteurized, clarified apple juice. The graph depicts the heat up, treatment and cool down time.

***5.2. Alicyclobacillus acidoterrestris* and heat treatment**

Figures 21, 22, and 23 indicate the transformation in the physical appearance of *Alicyclobacillus acidoterrestris* upon application of mild heat (52 ℃ for 38 min). Initially the batch has a mixture of vegetative cells and spores, which are sporulated by the process of sporulation. These spores are germinated by mild heat treatment. Figure 21, 22, and 23 indicates the 3 phases of the organism respectively. Figure 21, depicts the culture as how it looked on day 1. A mixture of vegetative cells and spores were observed, where the spores are in phase bright or resistant stage. From Figure 22, we can interpret that the area is predominantly populated by spores. This phase contrast image was taken after 7 days of incubation. Figure 23, appears to be very similar to the Figure 23, which helps us to visually verify that heat treatment induces spore germination. Apart from germinating the spores, it also helps in transformation of phase bright spores to phase dark spores. This step is significant as the phase dark spores or the germinated spores are less resistant compared to the phase bright spores.

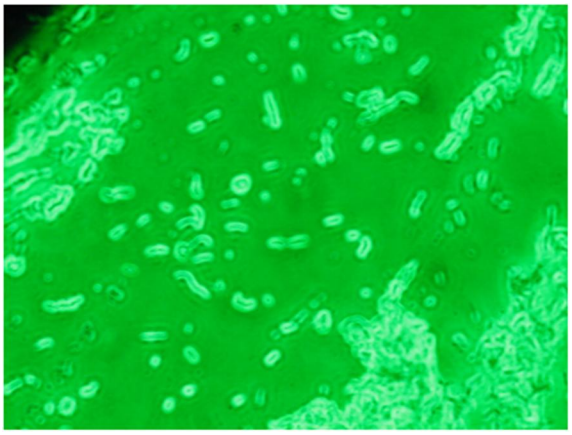


Figure 21: Phase Contrast Image of *Alicyclobacillus acidoterrestris*, mixture of spores and vegetative cells

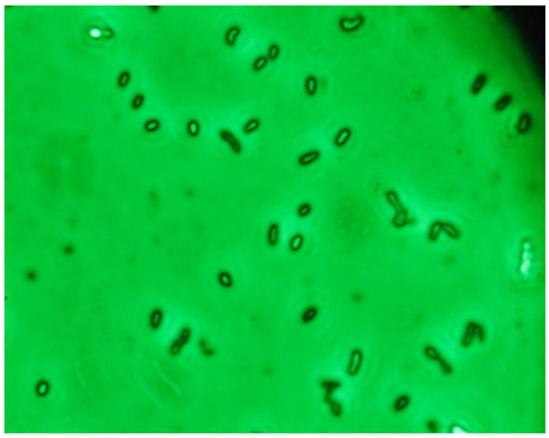


Figure 22: Phase contrast Image of *Alicyclobacillus acidoterrestris*: Predominantly spores

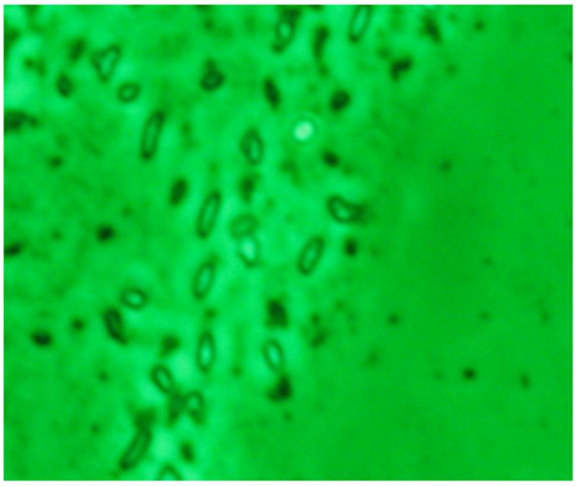


Figure 23: Phase Contrast Microscopy: Germinated *Alicyclobacillus acidoterrestris*, after initial heat treatment

5.3. Spore staining

Figure 24 indicates that on day 1 most of the cells are all in vegetative form, where the rods are clearly visible. This image indicates that some spores may be present at the sub terminal end of the vegetative cells. Figure 25 shows the presence of spores which are represented by the green circles. Figure 26 captures how the cells look like after heat treatment and we can observe that most of the spores have germinated by the predominance of purple colored colonies.

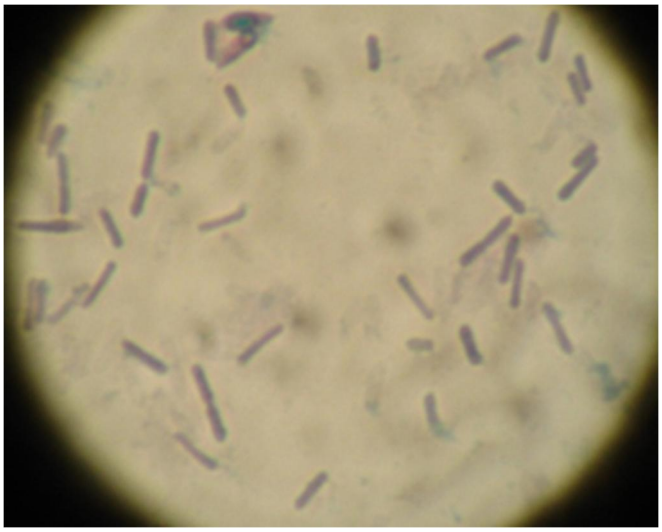


Figure 24: Day 1: Predominantly Vegetative cells

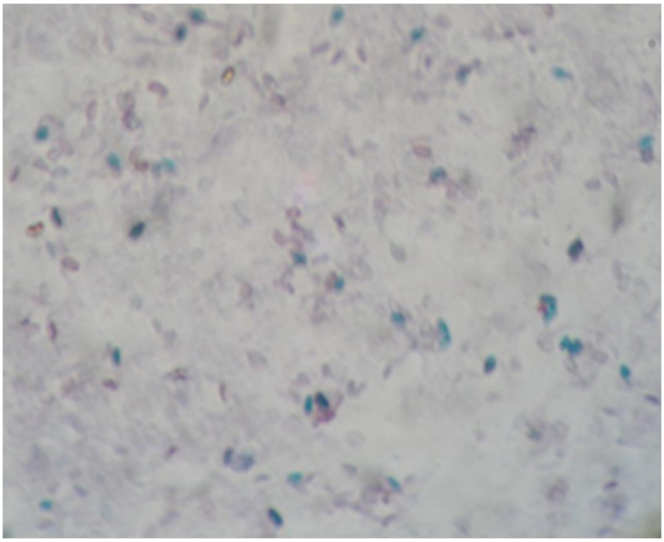


Figure 25: Day 7: Presence of spores

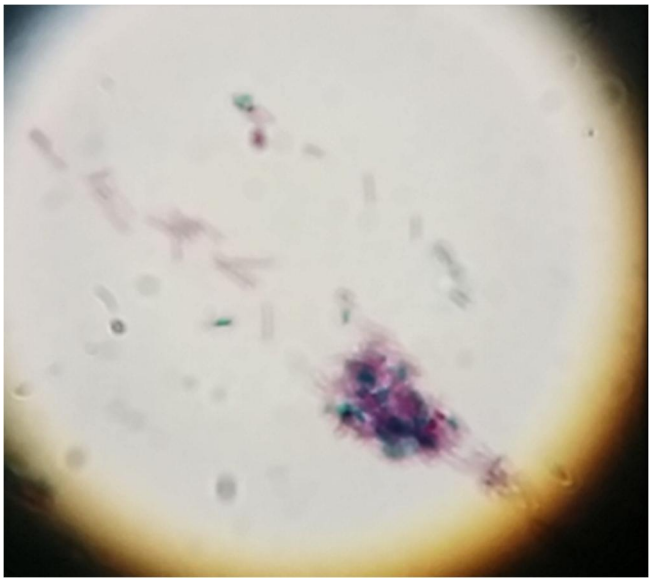
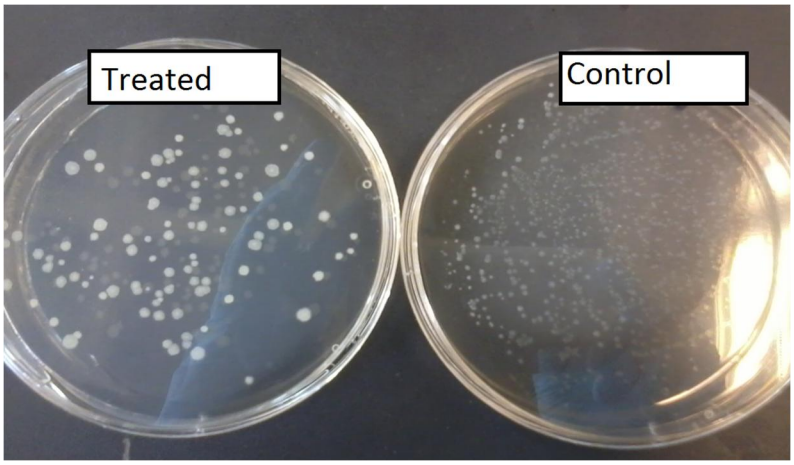


Figure 26: Fulton Schaffer Spore staining test: The purple colonies represent the vegetative cells and the Green color represents the spores. Here, in the above image 90% of the cells are vegetative after the preliminary heat treatment.

5.4. Reduction in the colony after treatment: Effect of UV followed by heat treatment



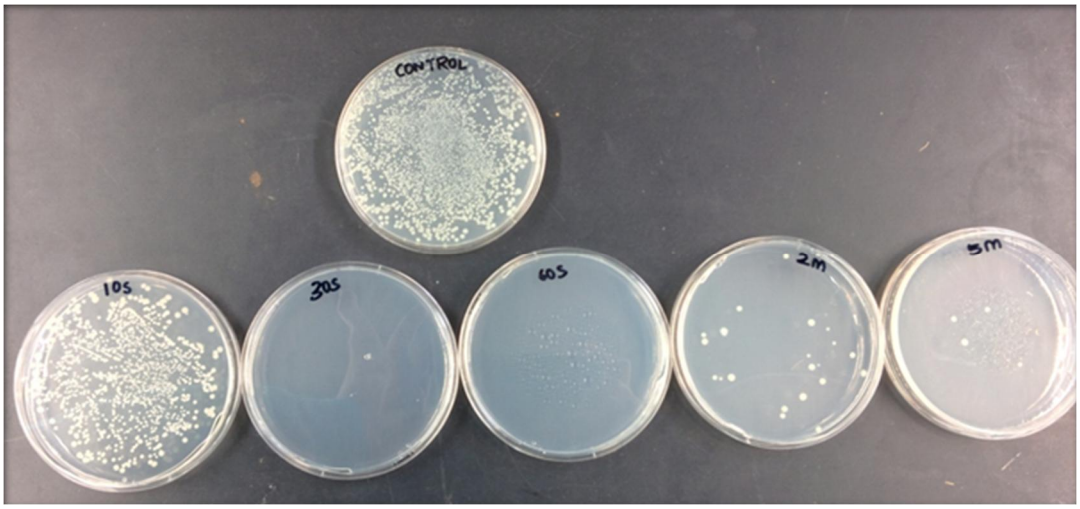


Figure 27: (a) Reduction in the number of colonies after heat treatment (top), (b) Reduction in the colonies after UV treatment (bottom)

A clear reduction in the number of colonies is seen between the control and the heat treated sample in Figure 27 (a). UV treatment for a period of 60 s at an intensity of 1 mW/cm^2 results in about 4 log reduction of the *Alicyclobacillus acidoterrestris* spores. Figure 27 (b) depicts how the exposure time plays an important role in activating *Alicyclobacillus acidoterrestris*.

The process of heat treatment causes the spores to germinate and the percentage germination for the most optimal condition is given in Table 9. Figure 28 provides statistical evidence that mid heat plus UV light is able to bring about a 4.2 log reduction of the spores of *Alicyclobacillus acidoterrestris* in commercially pasteurized, clarified apple juice. The immediate decrease in the germinated cells upon UV-C treatment provides evidence and supports the results of Baysal *et al.* (2013), who showed a 2 log reduction of the spores of *Alicyclobacillus acidoterrestris* in clarified apple juice. The small increase in the number of cells upon extended UV-C treatment is observed. This can be explained by the fact that some spores are more resistant, this is based on the temperature at which it is grown (Jay *et al*., 1970). The extended period of UV-C treatment might aid in the germination of the spores that are more resistant to heat treatment. In

the above experiment, based on statistical analysis, it is evident that the slight increase in the number of cells is not statistically significant.

Temperature (°C)	Time (min)	Germination (%)
52	38	96 ± 2

Table 9: Step 1-Heat treatment of apple juice

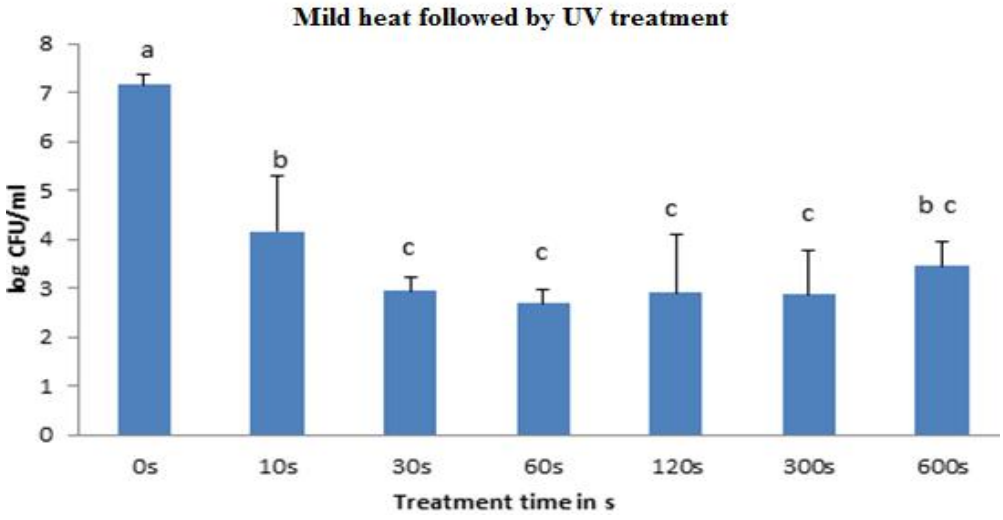


Figure 28: Graph for UV inactivation (Data that do not share the same superscript letter are significantly different (Tukey test, $p<0.05$))

5.5. Sensory Evaluation

Figure 29 and table 10 depicts the results obtained from the triangle test. The subjects evaluated a set of 3 samples based on the taste, smell and color. The critical value limit for a triangle test with n = 55 at an alpha level of 0.05 is that at least 25 correct responses are required to show significant difference in the sensory property of the treated and the untreated apple juice. Of the 55 participants, 19 were able to distinguish the treated and the control samples. Since this number is lesser than the critical limit for triangle test with n = 55. We concluded that there is no

significant difference between the sensory properties of the treated and the untreated samples. This evaluation gave us the evidence based human perception one would not be able to differentiate between the commercially pasteurized clarified apple juice and the commercially pasteurized- mild heat-UV treated clarified apple juice.

No of Participants	No of right answers	Significance (Risk)
55	19	0.4746

Table 10: Sensory Evaluation results-Generated by Fizz software

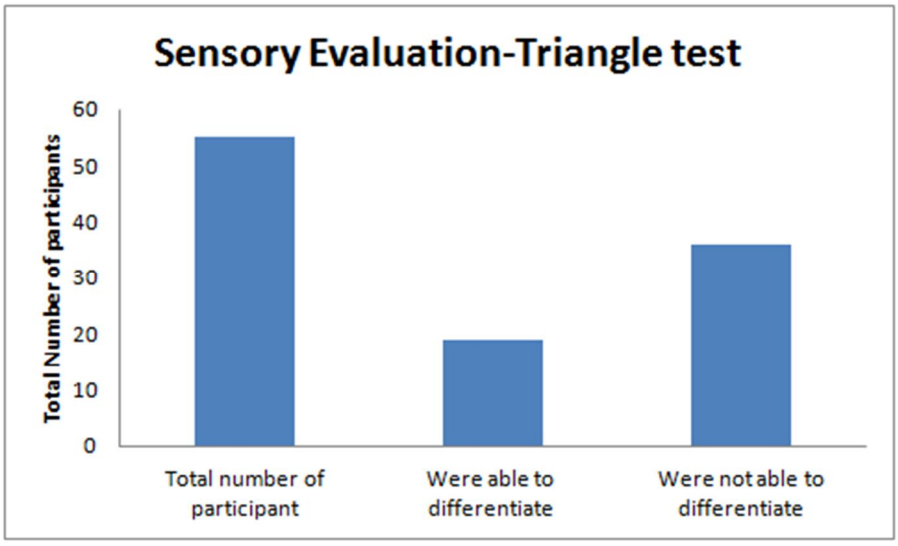


Figure 29: Sensory evaluation- the triangle test outcome reveals that there is not a significant difference between the control and treated sample

5.6. Headspace Analysis

Head space analysis through GC-MS supports the results of sensory evaluation. Over 20 distinct flavor compounds were separated and detected in the headspace volatile matter of apple

juice using GC-MS. Each compound was present in varied amount in the samples, but the compounds were in comparable amounts in both the control and the test samples. Thus the instrumental technique provides conclusive evidence that there is no significant difference in the volatile profile of commercially pasteurized apple juice and commercially pasteurized- mild heat-UV treated apple juice. Figure 30(a) compares the results obtained from static GC MS and Figure 30(b), purge and trap GC MS analysis. Based on the graph of the headspace analysis through the static GC MS and through purge and trap method we can observe that the peaks in both the samples are similar and separates out at the same time The static head space analysis gives evidence that no volatiles have been lost in the process of mild heat-UV treatment. This claim is further strengthened by purge and trap GC-MS, being 100 times more efficient than the former also shows the volatile peaks are consistent with that of the control. No new compounds are generated by the treatment of mild heat and UV. Though there is an argument that the intensity of few compounds has decreased due to the treatment, it is not pronounced. Thus it can be concluded that the flavor is similar to the control, although the percentage of volatile fraction has decreased when compared to that of the control.

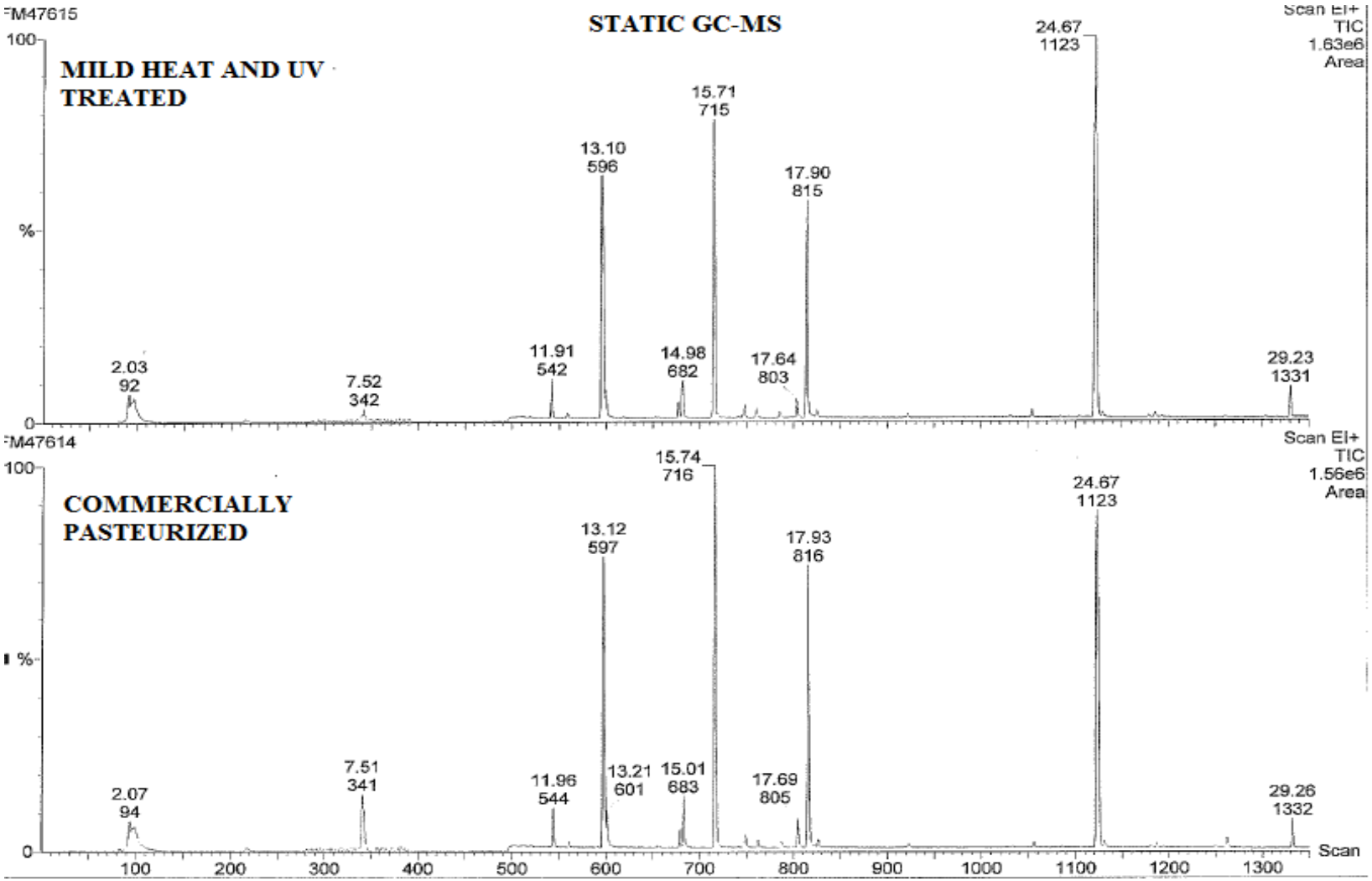


Figure 30 (a): Gas chromatogram of the head space analysis of untreated and treated by Static GC MS

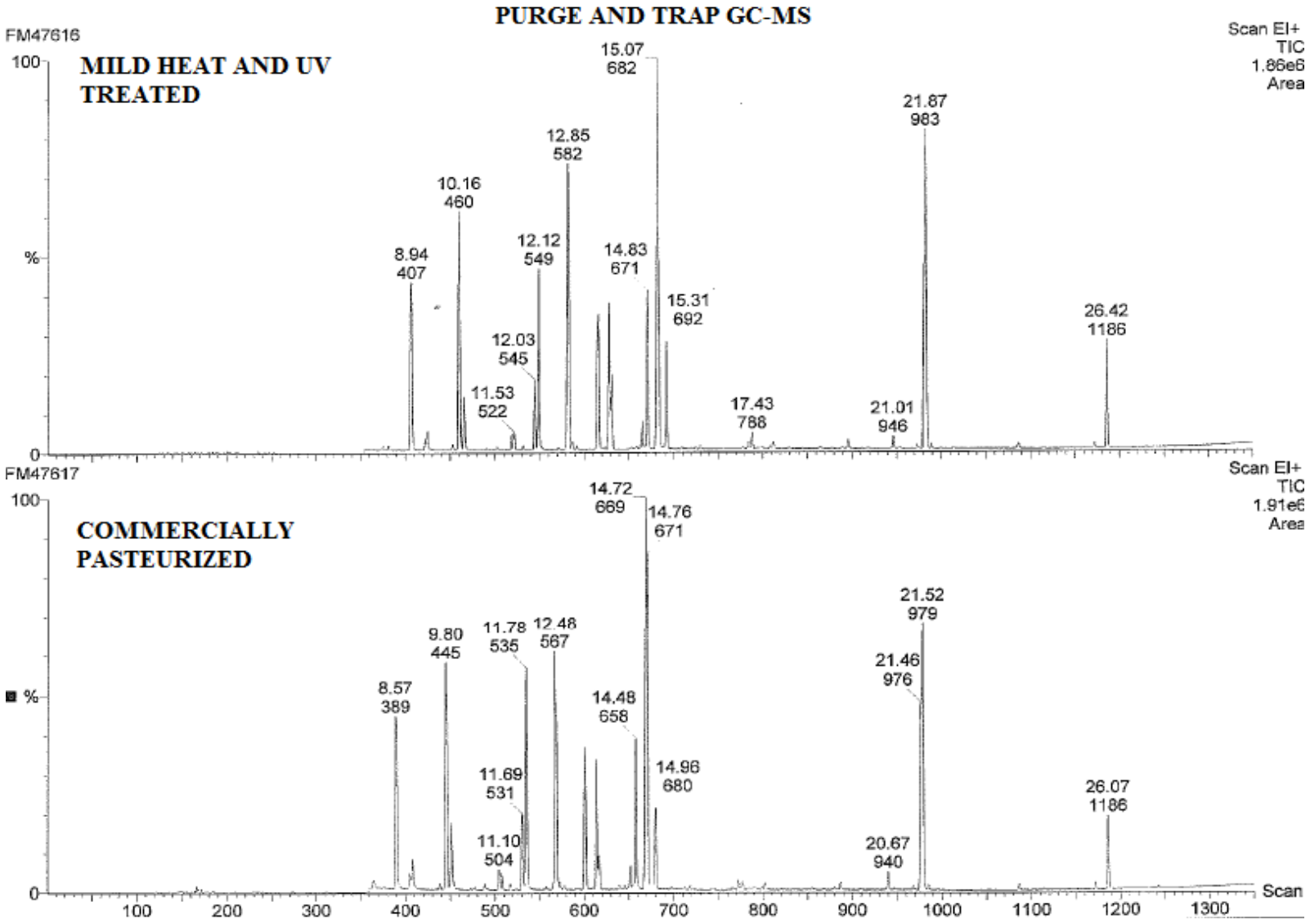


Figure 30 (b): Gas chromatogram of the head space analysis of untreated and treated by Purge and trap GC MS

CHAPTER 6

CONCLUSIONS

This research has contributed in understanding the effect of multiple hurdle technology of sequential treatment of mild heat followed by UV treatment on the inactivation of *Alicyclobacillus acidoterrestris* in apple juice. Sequential application of mild heat followed by UV technology retains the sensory and volatile characteristics of clarified apple juice.

Various combinations of time-temperature conditions were exploited to bring about germination of *Alicyclobacillus acidoterrestris* in apple juice. Both time and temperature are critical parameters that dictate the germination of the spores. A temperature time condition of 52 ºC for 38 min was ideal in bringing >90% germination of the spores of *Alicyclobacillus acidoterrestris.* A reduction of about 3.8 - 4.2 log was obtained when >90% germinated *Alicyclobacillus acidoterrestris* cells were subjected to an intensity of (1.4 - 1.6) mW/cm^2 for 60 s.

Mild heat and UV treatment played a synergistic role to inactive *Alicyclobacillus acidoterrestris* in apple juice. The sensory evaluation using Fizz triangle test conducted on commercially pasteurized apple juice and compared with the mild heat and UV treated apple juice revealed that there is no significant difference between both the treatments. Thus proving that multiple hurdle technology of mild heat followed by UV could potentially replace the conventional pasteurization process. Headspace analysis was performed using static headspace GC-MS analysis followed by headspace GC-MS purge and trap analysis on commercially pasteurized apple juice and mild-heat-UV treated apple juice. The results indicated that multiple hurdle technology used in this thesis did not result in the loss of any volatile component.

In summary, the spores of *Alicyclobacillus acidoterrestris* can be inactivated by sequential treatment of mild heat followed by UV treatment in apple juice. This process is sufficient in reaching a 4 log reduction of *Alicyclobacillus acidoterrestris.* Mild heat alone or UV processing alone does not manage to control the spoilage causing spores of *Alicyclobacillus acidoterrestris.* The current treatment can be fine-tuned to fit the requirements of industrial application (US FDA 2000).

CHAPTER 7

FUTURE WORK

In the past, studies with UV technology have been conducted on several substrates such as apple cider (Harrington and Hills, 1968; Worobo 1999), orange juice (Farid *et al*., 2001), pomegranate juice (Pala and Toklucu, 2011), apple juice (Guerrero-Beltrán and Barbosa-Cánovas, 2005). However, these studies have mostly been focused on vegetative cells and pathogenic strains. Very few studies have been conducted on effect of UV light on *Alicyclobacillus acidoterrestris* spores in fruit juice as a substrate. Recently several authors such as Baysal *et al*. (2013) and Bevilacque *et al*. (2013) have studied the effect of inactivating *Alicyclobacillus acidoterrestris* spores in apple juice by UV light and mild heat treatment respectively. But these studies were able to bring about only a 1-3 log reduction in the number of spores. These research studies along with the work of Koutchma (2009) on multiple hurdle technology paved the way for us to use a combination of mild heat followed by UV light treatment to inactive spores.

In future, work can be done to design a set up for continuous heat and UV treatment rather than a sequential treatment. Future research can be done on studying the germination kinetics of *Alicyclobacillus acidoterrestris* and compare how two non-thermal processing techniques such as HPP and UV could help in replacing the heating step. This would potentially lead the way to make a transition from thermal to completely a non- thermal processing.

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