EFFECT OF PROCESSING ON THE ANTIOXIDANT ACTIVITY AND OTHER QUALITY PARAMETERS OF MUSCADINE GRAPE JUICE

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ABSTRACT OF THE THESIS

Effect of processing on the antioxidant activity and other quality parameters of Muscadine grape juice

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Muscadine grapes are native to southeastern parts of the United States. They are widely processed into juices and wines. They contain high amounts of polyphenols that are generally known to have high levels of antioxidant activity, offering potential health benefits. Since the juice is always consumed after processing, the effect of processing on the antioxidant activity and other quality parameters needs to be determined.

Unpasteurized Muscadine grape juice was obtained from Paulk vineyards in Georgia. Juice was thermally pasteurized at 85 °C for 53 s. To obtain microbiologically safe conditions for High Pressure Processing (HPP) of juice, microbial equivalence between thermal processing and HPP was established using *Saccharomyces cerevisiae* strain. For HPP, the juice was vacuum packed in pouches and processed at pressures ranging from 275 - 425 MPa for 5 - 15 min. Total phenolics in juice samples were measured using Folin - Ciocalteu method. The antioxidant activity was determined using chemical antioxidant assay - Oxygen Radical Absorbance Capacity (ORAC)

and biological antioxidant assay - Cellular Antioxidant Activity (CAA) assay. ‘Difference from Control’ sensory test was performed to find the overall difference in the sensory quality of unpasteurized and processed juice samples. The effect storage time and temperature on total phenolics, ORAC value, color and ellagic acid content was determined for the processed juice samples.

Results showed no significant change in total phenolics and ORAC value, after processing. Cellular antioxidant activity increased significantly in high pressure processed sample as compared to thermally processed sample. No overall difference was perceived during sensory evaluation between any of the samples. During storage, total phenolics and ORAC value decreased with time, but the choice of process had no impact on these parameters. Browning index (BI) calculated from measured color indicators, was different for thermally processed and high pressure processed samples, but both samples showed an increase in the BI with time. Ellagic acid content was found to be higher in high pressure processed storage samples compared to unpasteurized and thermally processed samples. Thus, HPP did not offer any special benefit over traditional thermal processing for Muscadine grape juice.

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1.1 Muscadine Grapes

1.1.1 Muscadine grapes

Muscadine grapes are native to region from Virginia to East Texas and south. They grow well in fertile, sandy and alluvial soils. Areas with wet or heavy soils are not preferred. Muscadine grapes usually require 100 days on vine to mature the fruit, resulting in a long growing season. Muscadine clusters contain 6 to 24 berries. These berries do not adhere to stems when they mature (Ahmedullah et al., 1989). They are relatively resistant to insects or diseases compared to other grape species in the southern region. Their scientific classification is shown in the **Table 1.1**. below.

Table 1.1: Scientific classification of Muscadine grapes.

Kingdom	Plantae
Division	Magnoliophyta
Class	Magnoliopsida
Order	Vitales
Family	Vitaceae
Genus	Vitis
Subgenus	Muscadinia

Carlos and Noble cultivars of Muscadine grapes have been commercially planted for juice and wine production. Carlos is a bronze cultivar. It has excellent aromatic flavor. Noble is a dark cultivar. It is relatively winter hardy and makes a high quality wine. Carlos and Noble both have

perfect flowers and are self-fertile. Other cultivars that are commonly used to make commercial products are Nesbitt, Summit and Black Beauty.

Figure 1.1: Muscadine grapes – Noble (left) and Carlos (right) cultivars (Morris et al., 2004).

Grape juice from Noble cultivar was chosen for this study because grapes from this cultivar have highly pigmented, dark purple colored skin, which is a major source of antioxidants. Moreover, purple varieties, in general, have higher antioxidant activity compared to the green or bronze varieties.

1.1.2 Muscadine grape juice

Cultivars like Noble, Suppernong, Hunt, Creek, Dulcet, Yuga and Carlos have been used to produce Muscadine grape juice (Murphy et al., 1938). This juice has a unique flavor and bouquet. Changes during growth and maturation of grapes affect the quality of juice. As grapes mature, there is an increase in sugar and color levels. On the other hand, titratable acidity decreases giving the juice its flavor and aroma. Glucose and fructose are the major sugars in the grape juice. Other components contributing to the flavor are acids, volatile esters and aldehydes.

Juices can be produced by the hot press or the cold press process. A typical flow chart for Muscadine grape juice production is shown in **Fig. 1.2** (Morris et al., 2004). Muscadine grapes give a poor yield while producing juices.

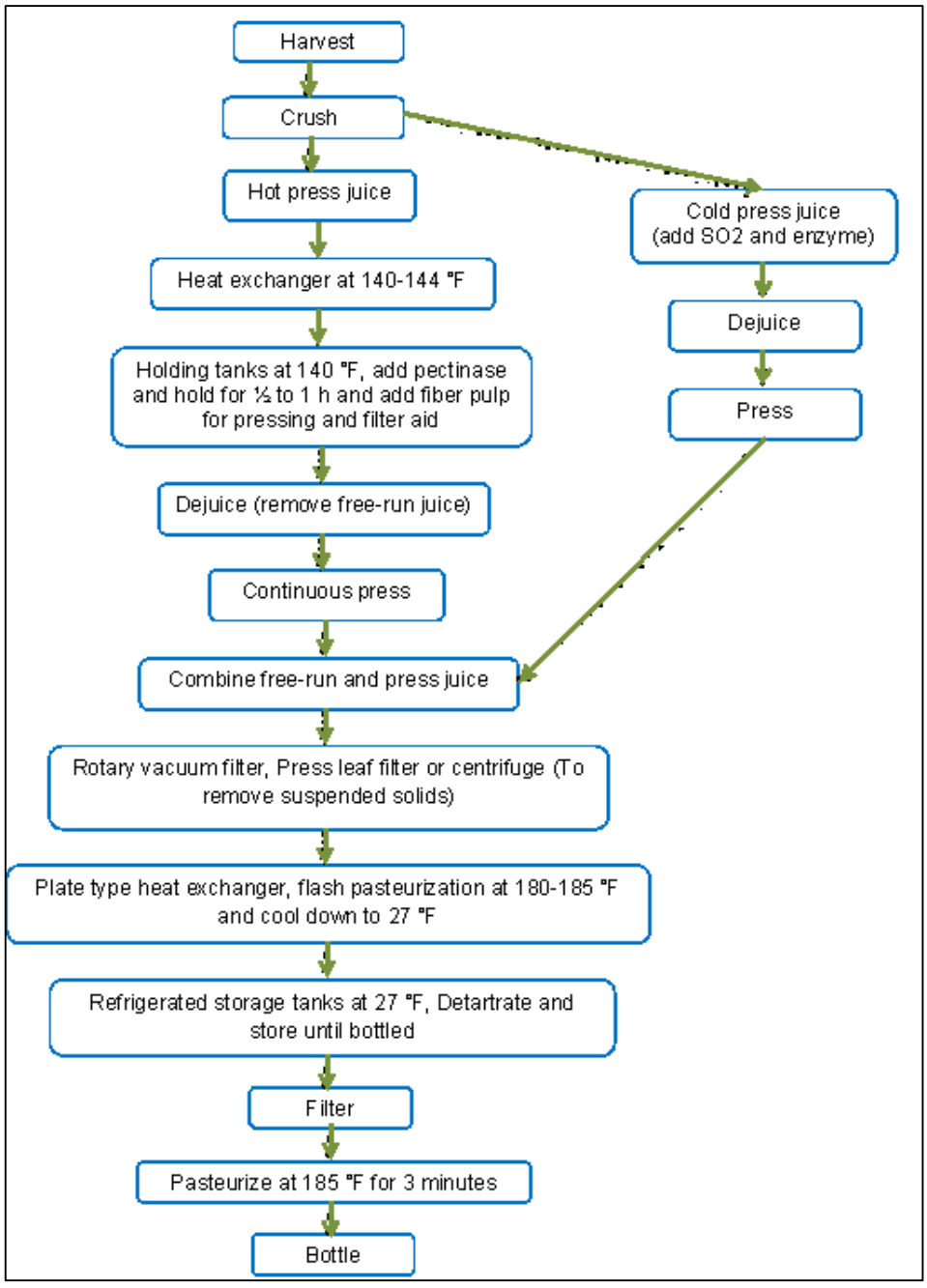

Figure 1.2: Flow chart of Muscadine grape processing (Morris et al., 2004).

For this study, Noble cultivar of Muscadine grapes, grown in Georgia and harvested in the month of January, 2010 were used to obtain the juice. The juice processing was slightly different than shown in **Fig. 1.2**. Grapes were harvested, gently crushed and deseeded. The juice was then obtained by the cold press method and treated with pectinase enzyme to increase the juice yield. No sulfites were added during this process.

1.1.3 Muscadine grapes and health

Muscadine grapes contain several phytochemicals that are associated with disease prevention in humans. Muscadine grape skins and seeds are found to contain high concentrations of polyphenols. Major phenolics found in Muscadine grape seeds were gallic acid, (+)-catechin, and epicatechin, with average values of 6.9, 558.4, and 1299.4 mg/100 g of fresh weight, respectively (Pastrana-Bonilla et al., 2003). The skins were found to contain high concentrations of ellagic acid, myricetin, quercetin, kaempferol, and trans-resveratrol, with respective average values of 16.5, 8.4, 1.8, 0.6, and 0.1 mg/100 g of fresh weight. Myricetin, ellagic acid, kaempferol, quercetin, and gallic acid were the major phenolics in muscadine leaves, with average concentrations of 157.6, 66.7, 8.9, 9.8, and 8.6, respectively (Pastrana-Bonilla et al., 2003). Average total phenolic contents were 2178.8, 374.6, 23.8, and 351.6 mg/g gallic acid equivalent in seed, skin, pulp, and leaves, respectively. The color of Muscadine grape juice is mainly due to the result of anthocyanins located in the skin of grapes, which also contribute greatly towards the antioxidant activity of the juice. Antioxidants protect the cells in body by neutralizing the damaging effects of free radicals that are produced by cell metabolism. These radicals are believed to contribute to

aging and various health problems. They can also attack normal tissues, destroy proteins and enzymes or even lead to cancer (Dani et al., 2007). Antioxidants scavenge these free radicals and hence, prevent or slow down many destructive reactions.

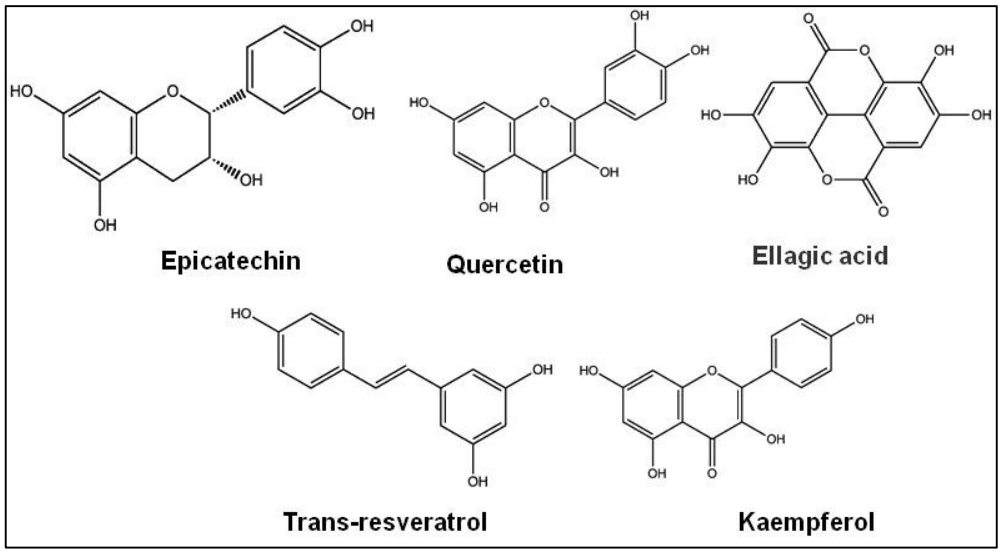

Figure 1.3: Important antioxidants present in Muscadine grape juice.

Lately, there has been interest in Trans - resveratrol as its consumption has shown to lower blood levels of low density lipoproteins. It also has cancer chemopreventative activity and reduces the risk of coronary heart diseases. It is present in both bronze and purple cultivars of Muscadine grapes. Ellagic acid is another phytochemical that has shown a number of health benefits like preventing some forms of cancer. Strawberries, raspberries and black berries are known to be great dietary sources of ellagic acid (Pastrana-Bonilla et al., 2003). However, ellagic acid content in Muscadine grapes is much more than these berries. Researchers and people in Muscadine industry have tried and are trying different ways to process these grapes and provide the products to the public, as they can contribute greatly to health.

1.1.4 Market value

According to Morris et al. (2004), there is a tremendous potential in market for Muscadine grapes as fresh fruits, as processed products and also for the production of nutraceuticals. However, it is under – developed. Marketing challenges in working with Muscadine grapes are (Morris et al., 2004)

- The flavor and aroma are completely different from that of other grapes
- Lack of familiarity with Muscadine grapes and their product
- Low demand in nontraditional marketing areas
- Lack of formal market standards to associate price and quality

To improve this, it needs substantial amount of consumer education and market development. In Arkansas, wineries are interested in Muscadine grapes in order to expand their commercial plantings. Small processors have also shown an interest to use Muscadine grapes in order to make jams, jellies and juice. But, obtaining reliable high quality Muscadine grapes has been a challenge.

Value added products can be made from Muscadine grapes if converted into new, different forms. Besides juices and wine, they can be processed into purees, vinegar, sweet spreads, and leathers. Byproducts from processing, like pomace and seeds, can be used to extract nutraceuticals and as colorants. Blending Muscadine juice with Concord or Niagara grape juice could be an option in order to have a juice with good color and refreshing taste (Morris et al., 2004). It can also improve the acceptability of strong flavored Muscadine, increasing its market potential.

1.2 Thermal Processing

1.2.1 Process

Thermal processing is heat treatment done on products mainly for food preservation. The main reasons for the heat treatment are

- Reducing the population of food pathogens below an acceptable level to ensure food safety
- Reducing the population of spoilage microorganisms in food to extend shelf life
- Destruction of toxins
- Inactivation of enzymes that degrade the quality
- Improve digestibility, color and texture in many cases

But this heat treatment adversely affects the sensory and nutritive qualities of food.

Commonly used thermal processing methods are cooking, blanching, pasteurization and sterilization. All these processes follow the equivalent time – temperature trend as shown in **Fig**. **1.4**. 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.

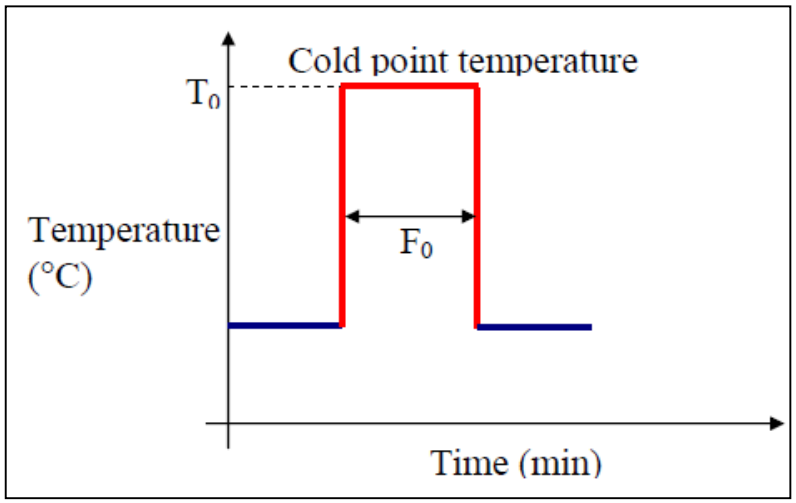

Figure 1.4: Time - Temperature trend for a typical thermal processing operation.

Selection of a heat-preservation treatment depends on:

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

1.2.2 Fruit juice processing

Preservation of fresh fruit juices is mainly done by pasteurization or sterilization. Pasteurization is done for grape juice to reduce the number of microorganisms that can grow in their environment. Microorganisms usually present in juice like grape juice having low pH (pH<4.6) are yeasts, molds and lactic acid bacteria. They are usually sensitive to heat treatment. Growth of

other bacteria commonly occurring in foods is inhibited by the acidity of juice (Meyrath, 1962). Pasteurization is a mild heat treatment in which juices are heated to target temperature range of 60-90 ℃. In the traditional batch pasteurization technique, called the Holder process, juices are held at about 60 ℃ for a relatively long period of time (~30 minutes) 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 processing. For fruit beverages, purees, and juices containing discrete particles, aseptic or ultra-high temperature (UHT) processing has become a viable option to improve product quality of heat processed, shelf-stable foods (Fellows, 2000).

1.2.3 Thermal processing and its effect on quality attributes

In fruit-based applications, one factor that largely affects antioxidant activity is the thermal processing step. Thermal treatment may lead to the deterioration of antioxidant activity due to the heat exposure. This was observed in clarified blackberry juice (Hager et al., 2008). Pasteurization at 90 ℃ for 3 minutes caused 67% decrease in the total monomeric anthocyanin as well as 55% decrease in the antioxidant activity measured using ORAC assay. However, it may also increase the antioxidant activity in some cases. This is because it releases compounds from the cell matrices.

It reduces the enzymatic browning by inactivating enzymes like polyphenol oxidases. But on the other hand, it accelerates the non-enzymatic

browning reactions in food. It affects the color pigments like chlorophyll and carotenoids by chemical reactions like degradation and isomerisation. Anthocyanins undergo polymerization and depolymerisation reactions and are degraded to brown pigments (Boyles et al., 1993). Thermal processing also has an impact on the flavor molecules. Cooked flavor was observed in guava juice when treated at 95 ℃ for 5 min.

Vitamin degradation can also occur during heat treatment and it depends on oxygen, light, pH and water solubility. Heat sensitive vitamins are the fat-soluble Vitamins A (in the presence of oxygen), D, E and water-soluble Vitamin C (ascorbic acid), Vitamins B_1(thiamine), B_2 (riboflavin) in acid environment, nicotinic acid, pantothenic acid and biotin C (Awuah et al., 2007). In general, the largest loss of Vitamin C in non-citrus foods occurs during heating. Reduction of ascorbic acid content by 2 – 6% was observed in black currant nectars (Iversen, 1999) and up to 25% loss in yellow passion fruit (Talcott et al., 2003) post pasteurization. Thus, ascorbic acid is often times used as a marker of quality changes in fruits and vegetables due to the severity of food processing. It is necessary to study the conditions for pasteurization carefully as excessive heat affects the delicate flavor and aroma components.

1.3 High Pressure Processing

1.3.1 The process

High Pressure Processing (HPP) is a cold pasteurization technique. In this technique, the products are introduced into a vessel. This vessel is then subjected to high level of isostatic pressure that is transmitted with the help of water. The pressures used are in the range of 200 MPa to 1000 MPa,

maintained for duration of few seconds to few minutes, depending on the product. **Figure 1.5** depicts the magnitude of pressure compared to many other situations.

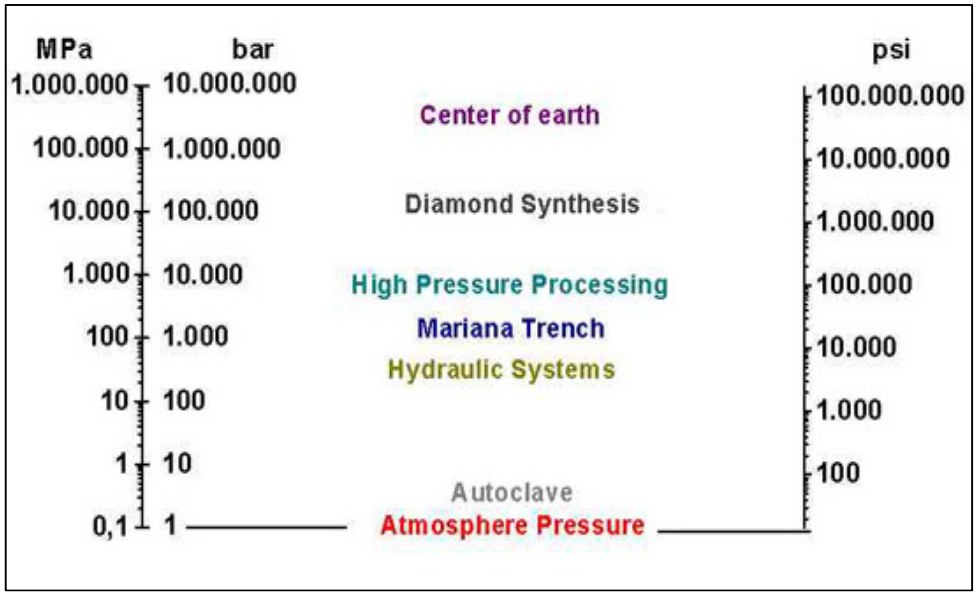

Figure 1.5: High pressure conditions used for processing.

Usage of pressures above 400 MPa at cold or ambient temperatures inactivates the vegetative flora like bacteria, virus, yeasts, moulds and parasites present in food. This helps in extending the shelf life of the products. **Figure 1.6** shows the damage done to the cell structure by high pressure processing and hence it is a potential alternative to traditional thermal and chemical treatments.

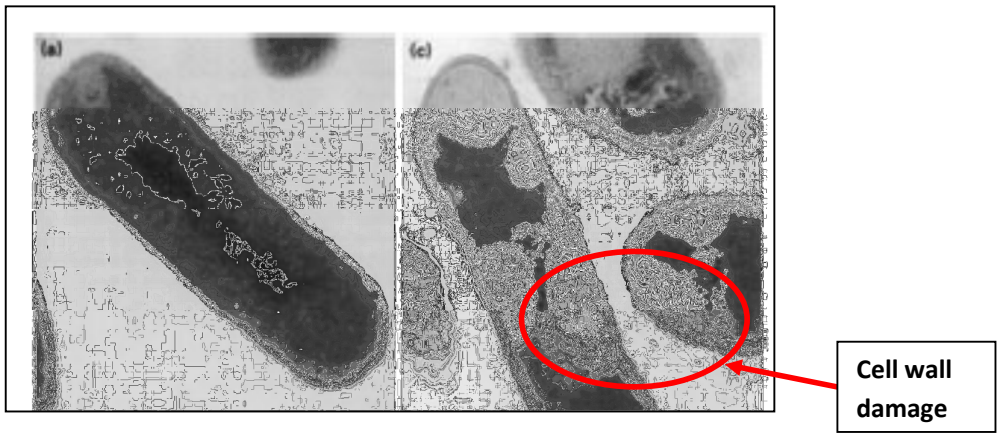

Figure 1.6: The effect of processing on Listeria monocytogenes (http://www.hiperbaric.com/Cold-Pasteurization/).

The four steps of the HPP are shown in the **Fig. 1.7.** The process starts with loading the product in high pressure vessel. Later pre-filling of the tank takes place, followed by pressurizing to a given pressure mark with the help of water. The vessel is held at that pressure for required duration of time. Once that is achieved, the vessel is depressurized and the product is unloaded from the high pressure vessel.

During high pressure processing, different pressure and temperature combinations can be used to achieve desired effects on texture, color and flavor of foods (Oey et al., 2008).

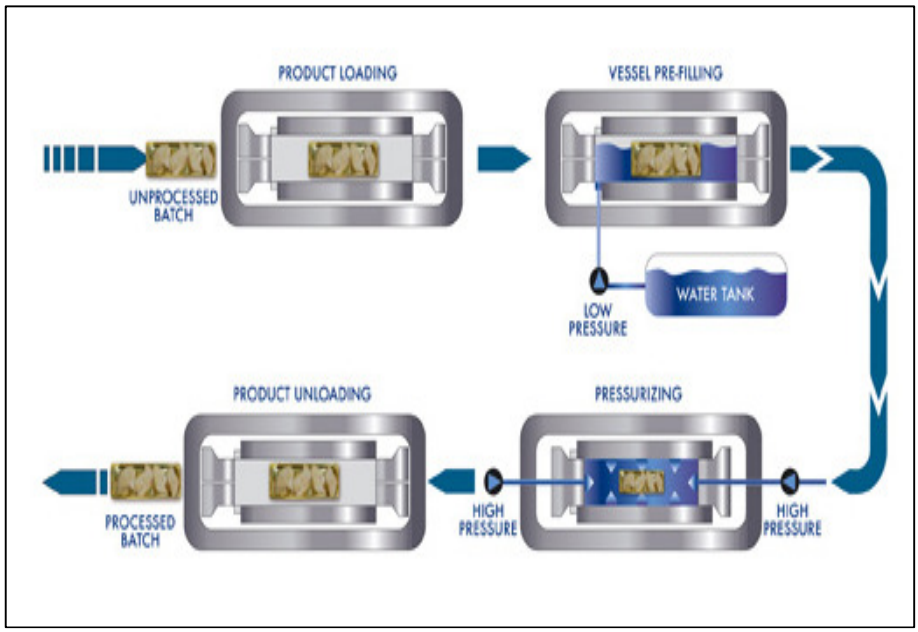

Figure 1.7: Steps during HPP of products (http://www.hiperbaric.com/Cold-Pasteurization/).

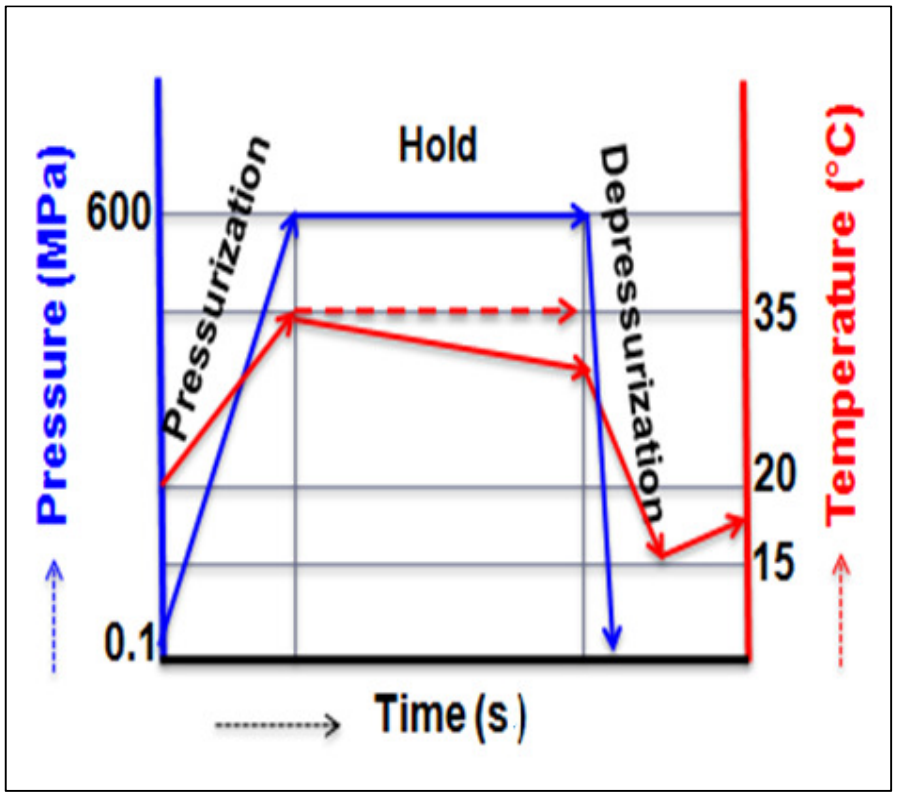

Figure 1.8: A typical time - temperature - pressure trend during HPP.

1.3.2 Advantages and disadvantages of HPP

Foods like fruits and vegetables are subjected to cooking or processing to make products like jams, jellies, etc. This, not only increases their edibility and palatability, but also is important in order to increase shelf life of the product. But this treatment might affect the original sensory and nutritional properties of food. Hence, it is important for food industries to develop and apply some novel processing techniques. HPP, being a non-thermal technology, offers several advantages over thermal processing. Main advantages of using high pressure processing are:

- Retaining sensory quality: It has limited effects on the covalent bonds of low molecular mass compounds like color and flavor compounds. Hence, helps in preserving the delicate sensory properties (Sancho et al., 1999)
- The technique provides homogeneity of treatment. This is because of the fact that the pressure is always the same at each point of the product
- HHP acts instantaneously and uniformly throughout a mass of food independent of size, shape and food composition
- Shape and integrity remains unchanged for fluids and soft solids as the pressure is equal on every part of the product
- New product development possibilities: Heat processing destroys some functional molecules. They can be preserved in high pressure processing. For example, HPP helps in retaining the anti-mutagenic components of carrot, cauliflower, kohlrabi, leek, spinach, beet, tomatoes, broccoli and many others (Sancho et al., 1999)

- It eliminates pathogenic and spoilage microorganisms and post packaging high pressure processing prevents cross-contamination.

However, in a complex matrix of food system, these sensory compounds coexist with enzymes, metal ions, etc. So high pressure processing can lead to many changes like

- Cell wall and membrane disruption
- Enzyme catalyzed reactions
- Various other chemical reactions like polymerization
- Modification of biopolymers including enzyme inactivation, protein denaturation and gel formation
- It does not have effect on spores when processed at room temperatures. Hence, it fails to give a commercially sterile low acid product.

At pressures around 600 MPa which are employed for food pasteurization, chemical reactions in the food may be accelerated or inhibited according to the Le Chatelier principle (Butz et al., 2003). An example is reduction of volume during processing due to adiabatic compression. This could lead to condensation reactions. Hence, the quality of high pressure processed fruits and vegetables may or may not change immediately after processing, but can change during storage. The other main reason being incomplete inactivation of endogenous enzymes and microorganisms that lead to reactions like oxidation. (Oey et al., 2008). Hence, HPP conditions need to be carefully optimized for a food product.

1.3.3 HPP, consumers and market

There are many companies in USA, Japan, France and Spain that use this technology for their products. The European Commission has included products obtained using HPP technology to the group of novel foods. It is also under the power of Novel Foods Legislation. Hence, every individual food product has to be tested and producer has to demonstrate that the product is safe. Later after the success and verification of this technology, Food Standard Agency of UK released the statement that the technology is not regarded as novel provided the foods are of fruit or vegetable origin having pH below 4.2 and germination of clostridia is prevented during the shelf life. A HACCP system is also required to be applied for production of high-pressure treated foods. After these fulfillments of the regulations of European Commission, Ata SpA company in Italy started processing fruit purees using high pressure processing technology. It was followed by Groupe Danone company that received the permission from European Commission to manufacture the fruit-based preparations like components for yoghurt using the high-pressure pasteurization (Houska, M. et al., 2006).

Hence, HPP is capable to secure the freshness and nutritive value. There are some products already in the market that use high pressure processing technology. They include gold oysters, orange juice, avocado sauce Guacamole, stewed packed ham, cooked rice and cooked rice mixtures, marinated chicken meat, etc.

Figure 1.9: High pressure processed products in the market.

The high-pressure processing is the emerging technology and can be used not only for non-thermal pasteurization of foods but also for pressure assisted quick freezing and thawing of heat and chilling sensitive products such as biological tissues. It can also be used for the development of food additives, pharmaceutical and cosmetic products and semi-products. But, due to the higher costs of high pressure processing in comparison with the

existing heat treatment there is a chance to treat the high value and high quality original products with some health benefit by this process only.

There is a great amount of interdependency between consumer needs and new technology research to be recognized by the food industry. Food packaging and labeling are also very important aspects to promote new technologies like high pressure processing. Besides protecting food and the shelf life of the product, packaging is the first point of contact between the consumer and the product. It plays an important role in its selection because it is the major source of information for consumers, permitting them to make better choices in the marketplace.

According to some studies done, ‘fresh’’ remains the most desirable food label claim (Deliza, 2005). Consumers also continue to look for positive health benefits and they desire to avoid artificial ingredients. Hence, high pressure processing can be best suited for all the modern consumer needs. But, effective communication between the producer and the consumer about food and nutrition is needed. This relies on delivering messages that consumers find believable and that also convinces them that making healthy food choices is achievable. For example, an extra couple of words explaining the meaning and advantages of high pressure technology on the package may lead to a higher product satisfaction, and will contribute to the market introduction of a product that offers higher nutritional and sensory qualities. Despite the recognized advantages of the pressurized products as mentioned above and in the following sections, a positive consumer attitude towards them is necessary to guarantee the success of the product in the market.

1.3.4 Effect of HPP on anthocyanins and flavor

Anthocyanins are usually stable during high pressure treatment at around room temperature. Patras et al. (2009) showed that when strawberry and blackberry purees were treated thermally, there was 28% loss in the anthocyanin content. Against this, high pressure processed products had no change in their anthocyanin content.

Treatment	Anthocyanin, mg/100 g DW	
	Strawberry[f]	Blackberry[g]
Unprocessed	202.27 ± 0.50^{a}	1004.90 ± 8.60^{a}
Thermally processed	145.82 ± 6.40^{b}	975.28 ± 7.90^{b}
HPP400MPa / 15min	173.34 ± 6.51^{ab}	1039.21 ± 4.51^{a}
HPP500MPa / 15min	202.53 ± 5.40^{a}	1014.21 ± 0.10^{a}
HPP600MPa / 15min	204.30 ± 1.60^{a}	1014.47 ± 1.00^{a}

Figure 1.10: Effect of thermal and high pressure processing on anthocyanin content. (Patras et al., 2009*).* Different alphabets indicate statistically significant differences ($p<0.05$).

However, it was seen that the anthocyanins degrade during the storage of products. There are various reasons for this degradation mechanism of anthocyanins. One reason could be incomplete enzyme inactivation. A link between inactivation of enzymes like β-glucosidase, peroxidase and polyphenoloxidase and anthocyanin stability has been found in several fruits (Garcia-Palazon et al., 2004). For example, inactivation of polyphenoloxidase

during high pressure processing of red raspberry and strawberry at 800 MPa, 18-22 ℃ for 15 min lead to the stabilization of anthocyanins, pelargonidin-3-glucoside and pelargonidin-3-rutinoside, during storage.

Another reason for the degradation of anthocyanins could be the substrate specificity of β-glucosidase (Gimenez et al., 2001). Different residual levels of anthocyanin compounds like pelargonidin-3-glucoside and pelargonidin-3-rutinoside were found in strawberry after HP treatments of 200, 400, 600 and 800 MPa at18 to 22 ℃ for 15 min. A higher loss in the amount of pelargonidin-3-glucoside was found as compared to pelargonidin-3-rutinoside at the same level of residual enzyme activity. This was probably due to the reason that β-glucosidase has higher substrate specificity to pelargonidin-3-glucoside than to pelargonidin-3-rutinoside. The degradation of anthocyanins during storage after high pressure treatment could also occur as a result of the effect of ascorbic acid. Ascorbic acid tends to accelerate the degradation of anthocyanins, apart from being an antioxidant. Talcott et al., (2003) investigated the stabilization of anthocyanins during heat or high pressure processing of ascorbic acid fortified Muscadine grape juice by the copigmentation method. Water-soluble rosemary extract was used for anthocyanin copigmentation. The results showed an increased antioxidant activity with the addition of rosemary extract. However, the presence of ascorbic acid, along with rosemary extract, resulted in the overall loses of the anthocyanin content, ascorbic acid content and antioxidant activity. The study also stated that the HPP resulted in higher loses of anthocyanins post processing due to the action of residual oxidase enzymes. Therefore, prior to assessing the copigmentation method to improve the processing stability of

anthocyanins in presence of ascorbic acid, residual enzymes have to be inactivated. Also, anthocyanin losses could also be reduced by storing high pressure treated products at low temperature (Kouniaki et al., 2004).

1.3.5 Effect of HPP on the browning reaction

Browning also plays a major role in the discoloration of high pressure treated fruits and vegetables. There might not be any significant color changes in fruit and vegetables, or their products immediately after the treatment. But, the discoloration due to browning can occur during the storage. This phenomenon was observed in mango pulps treated at 100 MPa to 400 MPa at 20 ℃ for 30 min. Ahmed et al. (2005) observed that color parameters such as a* (redness), b* (yellow to blue), Chroma and Hue values of mango pulps remained constant after high pressure treatment indicating pigment stability. But during storage at 30 ºC, discoloration of pressurized food products occurred due to enzymatic browning.

Additions of ascorbic acid and cysteine inhibited the polyphenoloxidase activity resulting in less browning. This inhibition was enhanced by HPP and can be found based on L*, a* and b* values. An increase in storage temperature resulted in higher rates of browning of fruit and vegetable products. The activation energy for color degradation of HP-treated food due to browning was higher than that of temperature pasteurized juice (Oey et al., 2008).

1.3.6 Effect of HPP on flavor

HPP does not affect the flavor directly. It leaves the small flavor compounds unaltered. But it can indirectly affect flavor. It retards and

accelerates chemical reactions that may change the content of flavor compounds. These reactions may also disturb the flavor profile. It was observed in some cases as stated by Oey et al. (2008) that the flavor of processed food was comparable to untreated ones with minor changes. Some examples of flavor changes are mentioned in **Table 1.2.**

Table 1.2: Effect of HPP on flavor (Constructed from Oey et al., 2008).

Sample	**Processing conditions**	**Observations**
Tomato juice	500-900 MPa, room temp., 3-9 min	Increase hexanal content due to lipid oxidation – rancid flavor
Strawberry juice	400 MPa, room temp., 5 min	No major change
Orange juice	500-800 MPa, room temp., 90-40 s	Slight difference from untreated, better than heat treated
Orange-Lemon-Carrot juice	500 MPa, room temp., 5 min	Carrot flavor more intense
Grape juice	600 MPa, 40 $^{\circ}$C, 10 min	No major change
Guava juice	500 MPa, 25 $^{\circ}$C, 6 min	No major change

Many factors need to be considered when optimizing high pressure processing conditions of foods. Some characteristics may be retained at a particular pressure. But same conditions might lead to loss in other characteristics. Hence, desired parameters must be prioritized in order to decide the high pressure processing conditions.

2 HYPOTHESIS, RATIONALE AND OBJECTIVES

2.1 Hypothesis

- HPP might be a promising alternative technology to traditional thermal processing in order to process Muscadine grape juice.
- HPP might retain antioxidant activity as well as give better sensory characteristics to Muscadine grape juice.

2.2 Rationale

This study will help us to determine the effect of processing methods on the antioxidant activity and other quality parameters of Muscadine grape juice. The success of this investigation will help us to find a suitable method to process Muscadine grape juice. Although Muscadine grapes contain high levels of health compounds, very little research exists on their bioavailability. This study will help us know more about the *in vivo* activity of grape juice. It will, eventually, lead to an increased value and marketability of the fruit. An increase in dietary consumption of the polyphenolics contained in these fruit products will provide health benefits as these compounds have been linked to reduce the risk of cancer and cardiovascular diseases.

2.3 Objectives

The objectives of this study were as follows:

- Study the effect of thermal processing and HPP on total phenolic levels of Muscadine grape juice
- Measure antioxidant activity using a chemical assay (ORAC) and a biological assay (CAA)

- Conduct and evaluate sensory evaluation of fresh, unpasteurized and processed Muscadine grape juice samples
- Monitor effect of storage on total phenolics, antioxidant activity, color and ellagic acid content of Muscadine grape juice samples.

3 MATERIALS AND METHODS

3.1 Approach

The overall approach of the study is shown in the **Fig. 3.1.** For all the experiments, fresh and unpasteurized Muscadine grape juice (Noble cultivar) was obtained from Paulk Vineyards in Georgia. It was stored in the blast freezer at -18 °C. Single strength, unpasteurized juice was then subjected to thermal processing and high pressure processing. Total phenolics, chemical antioxidant activity and cellular antioxidant activity of the juice before and after processing were measured and compared. All juice samples were then subjected to storage study. Total phenolics, chemical antioxidant activity and color were monitored during storage. HPLC analysis of ellagic acid was also performed on the storage study samples.

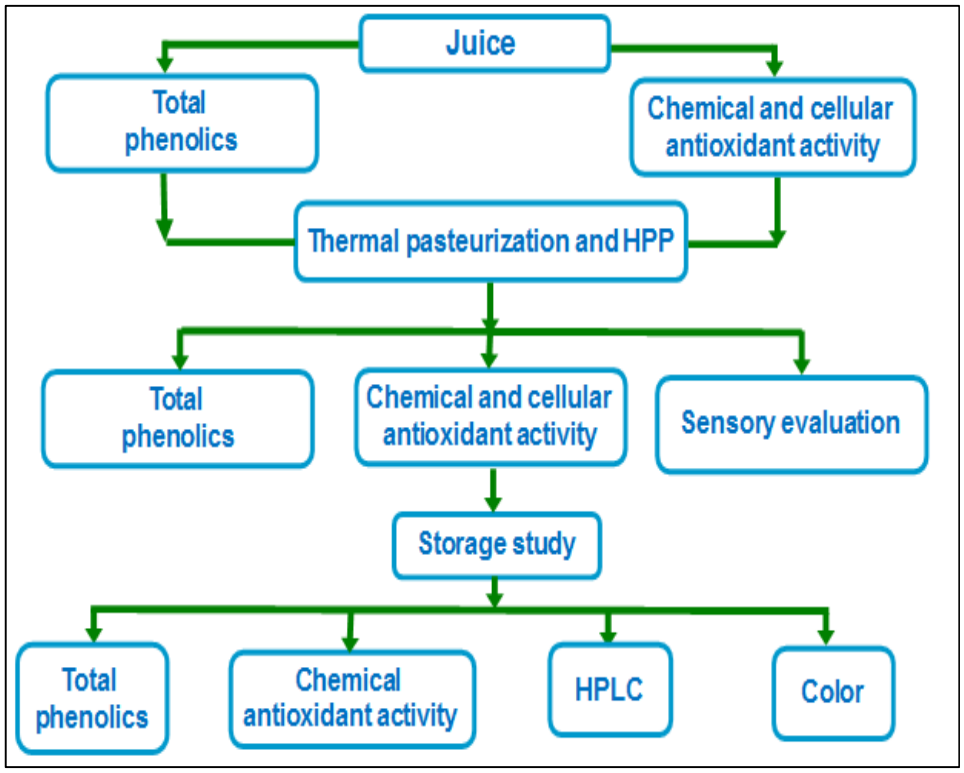

Figure 3.1: Flowchart for the experimental approach followed.

3.2 Thermal Processing

3.2.1. Materials used

- Stainless steel vessel
- Hot water baths
- Type T thermocouple
- Data acquisition system
- Meals Ready to Eat (MRE) pouches
- Steam jacketed kettle
- Metal wire basket with a stretched slinky.

3.2.2. Small batch pasteurization

Small batch pasteurization was carried out by taking single strength fresh Muscadine grape juice in a stainless steel vessel (around 7 ml) as shown in **Fig. 3.2.** The vessel had a thermocouple inserted into the lid in order to measure the temperature of the juice during pasteurization. The thermocouple was attached to a high speed USB data acquisition system (National Instruments, Austin, TX).

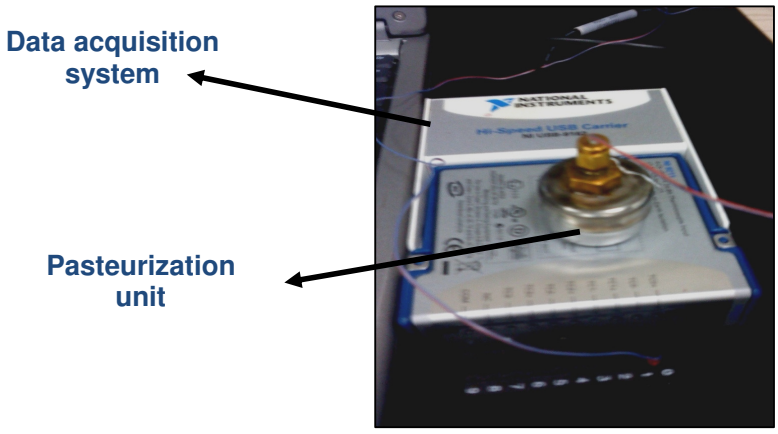

Figure 3.2: Small batch pasteurization system.

This system was connected to a computer that used LabView software to record time and temperature data. It also calculated cumulative F - value during the process using the trapezoidal method

$$F = \int 10^{\left(\frac{T-Tref}{z}\right)} dt \quad \text{.....(Eq. 1)}$$

A graph plotted using this data as shown in **Fig. 3.3.**

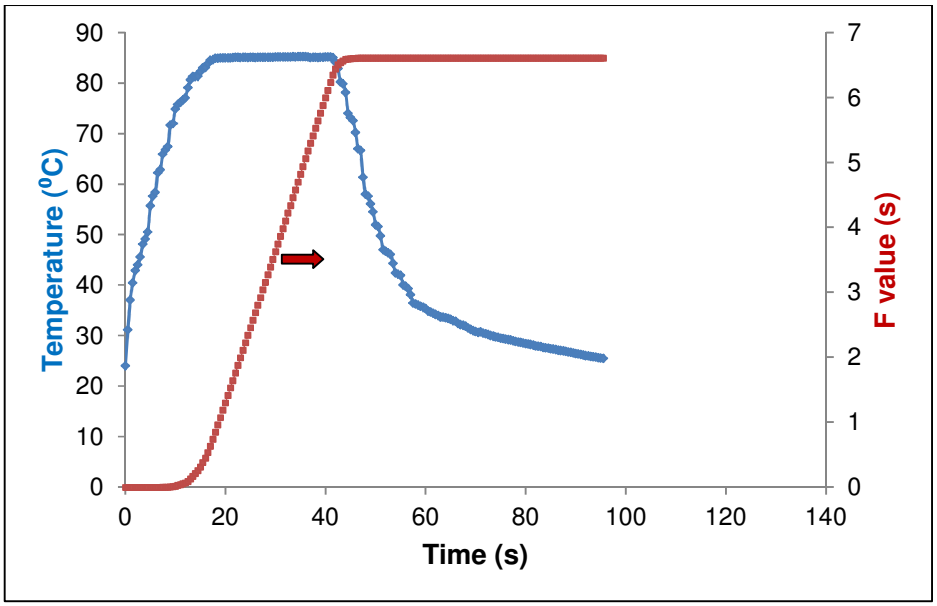

Figure 3.3: Time - Temperature plot for thermal process.

Based on the pH of Muscadine juice (pH 3.7), it is recommended to process the juice at 93.33 °C as the reference temperature for the F - value of 6 s, with z – value of 8.8 °C (http://www.gmaonline.org/resources/science-education-foundation/). Using this data, the time required at 85 °C was calculated as follows

$$\frac{\text{F at 85}}{\text{F at 93.33}} = 10^{\left(\frac{93.33-85}{8.8}\right)} \quad \text{.....(Eq. 2)}$$

Where,

F at 93.33 ℃ = 6 s.

Based on this, the juice was processed at 85 ℃ for approximately 53 s to get equivalent processing. When the juice reached the desired F – value, the vessel was immediately removed from the water bath and cooled in an ice bath till the juice reached room temperature.

3.2.3. Bulk pasteurization

Bulk quantity of juice was required for sensory tests and storage studies. Bulk pasteurization was a way to process all the required quantity of juice at once. This will help to lower the inconsistencies in results that may arise because of batch – to – batch variations. For this, approximately 20 ml of juice was vacuum packed in each MRE pouch. Sixty such pouches were placed in a stretched slinky kind of arrangement. This slinky was placed in a mesh wire basket, which in turn, was placed in a steam jacketed kettle. A thermocouple was inserted in the pouch that was placed at the geometric center of the wire basket. This thermocouple was attached to a data acquisition system. Time, temperature and F – value data were obtained using LabView software, as done in batch pasteurization. Following that, pasteurization was carried out by pouring hot water in the kettle at 85 °C for approximately 53 s, till it reaches the desired F – value. Once the processing was done, hot water was drained off and mixture of cold water and ice was poured in the kettle in order to cool the product to the room temperature.

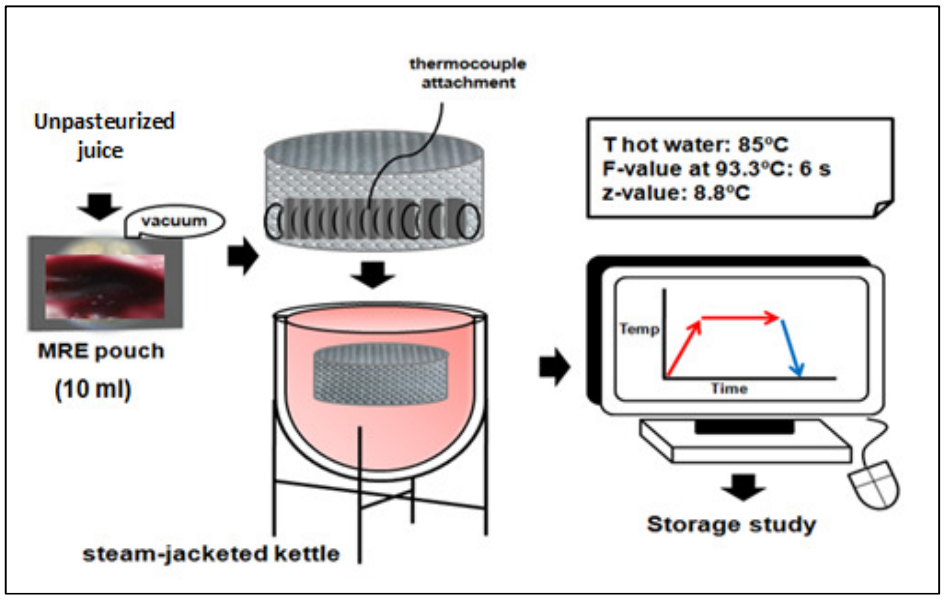

Figure 3.4: Bulk pasteurization assembly.

3.3 Microbial Equivalence Studies

Yeasts are important group of spoilage microorganisms and represent a substantial economic threat to the food industry. Microbial fermentation and spoilage of fruit juices and other fruit products are most frequently associated with the *Saccharomyces cerevisiae* yeast strain. Their growth results in ethanolic spoilage, carbonation, and production of hydrogen sulfide as well as other off-odors (Parish, 1991). Because of these problems, FDA developed more stringent measures for microbiological quality of juices. All juice processors must follow the FDA regulation that states 'The HACCP regulation requires you to use treatments capable of consistently achieving at least a 5-log reduction (using ten as the base number) in the level of the pertinent microorganism in your juice' (FDA, 2004). This can be done by pasteurization or many other non-thermal technologies. Zook et al. (1999) reported that ultra high pressure is an alternative to traditional thermal treatments for eliminating yeasts while retaining acceptable quality standards. Hence, to obtain safe

conditions for high pressure processing of juice, microbial equivalence between thermal and HPP was established using *Saccharomyces cerevisiae* strain. This would help to find conditions of high pressure processing that would give at least 5 log reduction in Muscadine grape juice.

3.3.1. Materials used

- *Saccharomyces cerevisiae* strain BY4741
- Yeast extract – peptone – dextrose (YPD) agar Fisher Scientific, Pittsburgh, PA
- Yeast extract – peptone – dextrose (YPD) broth Fisher Scientific, Pittsburgh, PA
- Peptone water
- Petri dishes from Fisher Scientific, Pittsburgh, PA
- 30 °C incubator.

3.3.2. Method

A BY4741 strain of *Saccharomyces cerevisiae* was obtained from Dr. Carman's lab (Food Science Department, Rutgers University, NJ). A loopful of culture was inoculated in 40 ml of YPD broth in a 50 ml centrifuge tube. The tube was incubated overnight at 30 °C. Following that, optical density of the tube was measured at 600 nm. Concentration of yeast cells in broth was then calculated using the following conversion:

1 O.D. unit at 600 nm = 3×10^7 cfu / ml……(Eq. 3)

Juice to be tested for log reduction, was sterilized at 121 °C / 15 psi steam pressure for 15 min. This will reduce the organisms that may be present in juice before the test. It helps in eliminating the interference in calculating log reduction of inoculated yeast achieved by processing. After

sterilization, the juice was inoculated with yeast, such that the concentration in juice was at least 10^7 cfu / ml. It was vortexed to ensure uniform dispersion of inoculum in the juice. Then the inoculated juice was subjected to thermal processing and high pressure processing. For HPP, preliminary experiments were performed at the conditions as stated in **Table 3.1.**

Table 3.1: Preliminary pressure - time conditions for microbial studies.

Pressure (MPa)	200	200	200	300	300	300
Time (min)	5	10	20	5	10	20

Unpasteurized and processed, inoculated juice samples were then diluted with peptone water in sterilized culture tubes in the ratio 1:9. The sample was further diluted 3 times to give a dilution factor of 10^4 before plating. 1 ml of diluted juice was spread plated on YPD agar plates. The dilutions and plating method followed gave the detection limit of 100 cfu / ml. The plates were then incubated at 30 °C for 3 days. The plates were then enumerated for the number of colonies. The difference between number of colonies in unpasteurized and processed juice samples helps to calculate log reduction caused by the process. The entire plating process was done in a sterilized environment. **Figure 3.5** shows the process on a flow chart. These preliminary results are plotted as shown in **Fig. 3.6.**

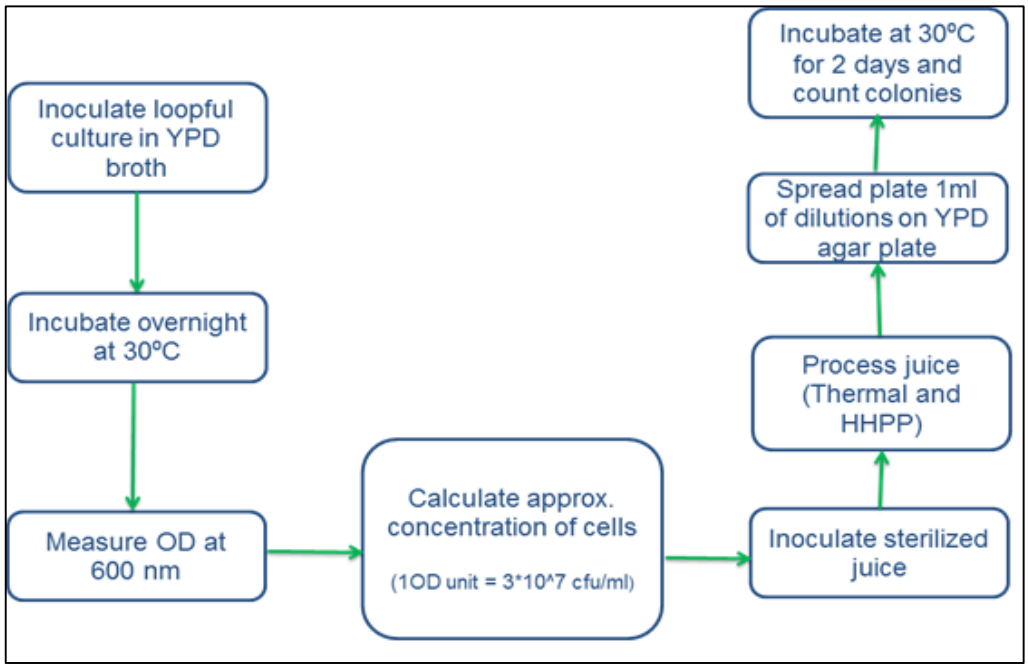

Figure 3.5: Process followed to find log reduction.

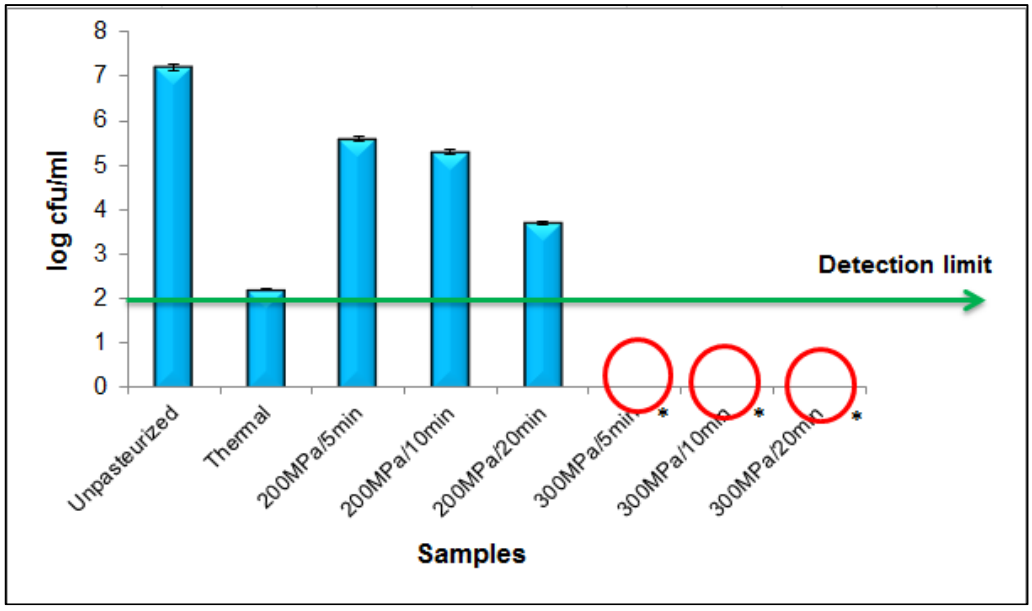

Figure 3.6: Results on microbial equivalence studies (* indicates that the results were below the detection limit of 2 cfu/ml).

Figure 3.6 shows that 5 log reduction was obtained in the thermal pasteurization process. However, for HPP, necessary reduction was not

achieved for the conditions tried at 200 MPa. But as the pressure was increased to 300 MPa, yeast inactivation was below the detection limit. This experiment gave us the boundary conditions for HPP of juice, in order to obtain juice with acceptable microbiological juice quality.

3.4 High Pressure Processing (HPP)

3.4.1. Materials used

- Plastic pouches
- Vacuum sealer
- A 10 L High Hydrostatic pressure unit from Elmhurst Research Inc., Albany , NY
- K type Thermocouples.

3.4.2. Method

Based on the microbial equivalence studies, the design of experiments for HPP was obtained using SAS 9.2 (Statistical Analysis Software). Two variables, five levels, and rotatable Central Composite Circumscribed (CCC) design was chosen for all the HPP runs (Eriksson et al., 2008). **Table 3.2** gives the codes of the factors and **Table 3.3** gives the sequence of runs.

Table 3.2: Uncoded and coded factors for the design of experiments.

Code	0	0.146	0.5	0.853	1
Pressure (MPa)	275	297	350	403	425
Time (min)	5	6	10	14	15

Table 3.3: Sequence of HPP runs after randomizing.

Run No.	Pressure (MPa)	Time (min)
1	350	10
2	350	15
3	403	14
4	350	10
5	275	10
6	350	10
7	425	10
8	350	10
9	297	14
10	297	6
11	350	10
12	403	6
13	350	5

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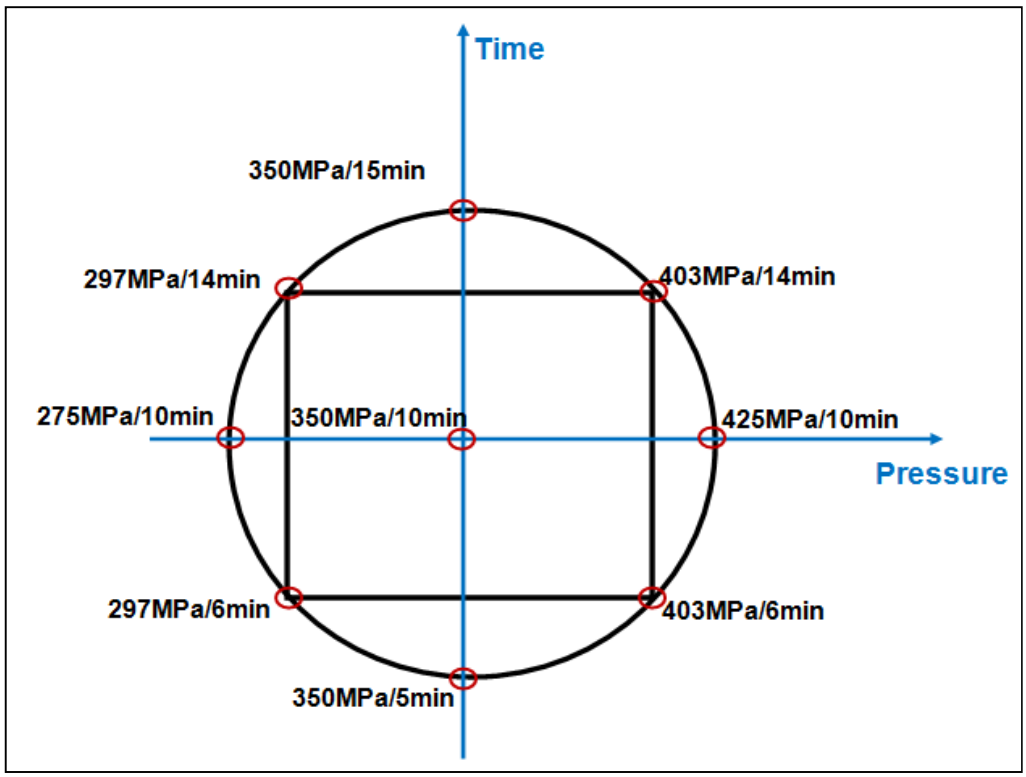

Figure 3.7: Grid for design of experiments.

For HPP, 20 ml of juice samples was vacuum-packed in opaque plastic pouches and processed in a 10 L High Hydrostatic Pressure unit (**Fig. 3.8**) (Elmhurst Research Inc., Albany, NY), with water as the pressure transmitting medium.

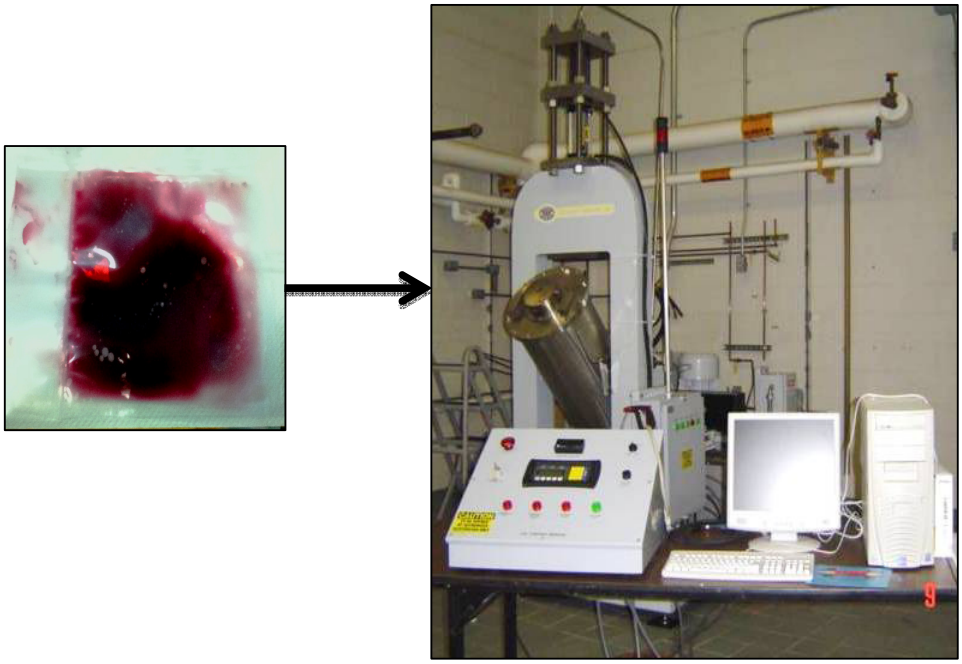

Figure 3.8: Vacuum packed pouch containing Muscadine grape juice and High Pressure Processing unit at the Rutgers University.

The vessel of the high pressure unit has an internal diameter of 127 mm, its length is 800 mm and the wall thickness is 145 mm. The maximum working pressure of the high pressure unit is 690 MPa or 100,000 psi, which can be reached in less than 3 minutes, using a 20 HP pump. Starting pressure of the process is at atmospheric pressure (0.1 MPa = 14.7 psi = 1 atm). The maximum processing temperature is 80 ℃. The operation of the high pressure process is monitored using a tabletop PC, where pressure, temperature and time data are logged using LabView 7® software (National

Instruments, Texas). Temperature inside the vessel was measured using a K type thermocouple.

In order to process samples using HPP, the vessel was brought to horizontal position and the top closure was removed pneumatically. The closure was closed after loading the juice sample pouches. The vessel is then returned to the vertical position and filled with water using a water hose. Following that, the desired operating parameters for the run, like pressure and time were entered in the control module. Once the run is started, the vessel is pressurized to the desired pressure. It is then held at that pressure for the preset time and then the vessel is depressurized. A typical plot of Pressure and Temperature vs. Time during a processing operation is shown in **Fig. 3.9**. Once this process is over, the vessel is emptied and the top closure removed to unload the samples.

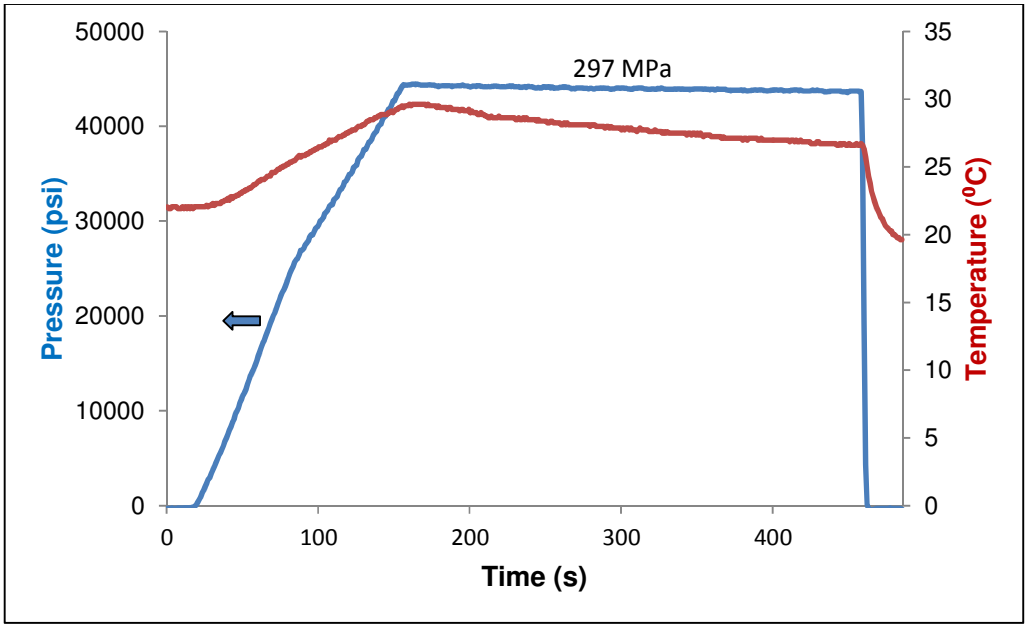

Figure 3.9: Actual Pressure and Temperature vs Time plot for a typical HPP run.

3.5 Total Phenolics: Folin-Ciocalteu Assay

3.5.1. Materials used

- Folin-Ciocalteu reagent from Sigma-Aldrich, St. Louis, MO
- Gallic acid from Sigma-Aldrich, St. Louis, MO
- Sodium carbonate from Sigma-Aldrich, St. Louis, MO
- UV – Vis Spectrophotometer from Varian, Palo Alto, CA.

3.5.2. Method

Total phenolics in juice were measured using Folin – Ciocalteu method as described by Singleton et al. (1999). This method uses Folin – Ciocalteu (FC) reagent which is a mixture of phosphomolybdate and phosphotungstate. This reagent reacts with reducing agents like phenols. It forms blue colored products on reduction, the absorbance of which can be measures at 765 nm. The intensity of light absorption is directly proportional to concentration of total phenols. The reaction is sensitive to high temperature and color is degraded. The standard polyphenol used in the assay is gallic acid. Hence, the total phenolic content is measured in terms of Gallic Acid Equivalents (GAE).

3.5.3. Reagent and sample preparation

- Standard gallic acid solutions: For developing the standard curve, different concentrations of gallic acid (w/v) were made in distilled water. They ranged from 50 – 500 µg / ml
- Folin – Ciocalteu (FC) reagent: Folin – Ciocalteu reagent was diluted with distilled water in the ratio 1:1 for experimental purposes. It was freshly prepared for every experiment and stored in dark before it was used

- Sodium carbonate solution: For the total phenolics assay, 20% (w/v) solution of sodium carbonate for prepared
- Grape juice samples: In order to get the absorbance readings of juice samples within the range of standard curve, they were appropriately diluted. 1 ml of juice sample was diluted with 11 ml of distilled water and used for the experiment.

3.5.4. Experimental protocol

For gallic acid standards, 250 µl of the sample was taken in the test tube. To work with juice samples, 125 µl of diluted juice was mixed with 125 µl of distilled water. To these samples, 250 µl of FC reagent was added, followed by 4 ml of distilled water. 500 µl of 20% sodium carbonate solution was then added to this mixture. The test tubes were vortexed for 1 min and then incubated in dark for around 30 min at room temperature. After incubation time, 300 µl of each sample was pipetted in a 96 well plate. The absorbance of the samples in the plate was measured at 765 nm in a 96 well plate BIO – TEK spectrophotometer (**Fig. 3.10).** For each sample, absorbance reading was corrected by subtracting the blank reading measured using distilled water.

Figure 3.10: 96 well plate spectrophotometer.

The standard curve of absorbance (O.D.) vs. concentration of gallic acid is plotted using the absorbance values of gallic acid standards at 765 nm. The corrected absorbance readings of samples obtained were then interpolated back to the standard curves in order to find total phenolics value. It was expressed as mg Gallic Acid Equivalents (GAE) per 100 ml of juice.

Polyvinylpolypyrrolidone (PVPP) binding was tried to avoid overestimating the phenolic value due to interfering compounds such as ascorbic acid, sugars, and protein. Ascorbic acid and sugars are additive interfering compounds since they reduce Folin-Ciocalteu reagent and thus, their presence increases total phenolics value. (Singleton et al., 1999). This PVPP binding method had been adopted in determining corrected total phenolics content of oak acorns as influenced by thermal processing (Rakić et al., 2007). For the Muscadine grape juice, the ascorbic acid level was found to be very low. Moreover, some preliminary studies showed that the phenolic values before and after PVPP binding were not significantly different. So, PVPP binding method was not used further for the study.

3.6 Chemical Antioxidant Activity: Oxygen Radical Absorbance Capacity (ORAC) Assay

3.6.1. Materials used

- 2,2'-azobis-2-amidinopropane dihydrochloride (AAPH or ABAP) from Sigma-Aldrich, St. Louis, MO
- Fluorescein from Sigma-Aldrich, St. Louis, MO
- Sodium phosphate dibasic from Sigma-Aldrich, St. Louis, MO
- Sodium phosphate monobasic from Sigma-Aldrich, St. Louis, MO

- 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox) from Sigma-Aldrich, St. Louis, MO
- Cary Eclipse Fluorescence spectrophotometer (Varian, Palo Alto, CA).

3.6.2. Method

Chemical antioxidant activity of juice samples was measured using ORAC assay. Cao et al., (1993) first developed this assay to measure antioxidant activity. It basically measures the effectiveness of an antioxidant to scavenge peroxyl radicals generated 2,2'-azobis(2-amidinopropane) dihydrochloride (AAPH) at 37 ℃. The assay used B-phycoerythrin (B - PE), a protein isolated from *Porphyridium cruentum*, as the fluorescent probe. This probe loses fluorescence when oxidized and thus indicates the extent of damage done by peroxyl radicals. The presence of antioxidants show a protective effect for probe as it scavenges the radicals and reduces the rate of decrease in fluorescence. This effect of an antioxidant is measured by assessing the area under the fluorescence decay curve (AUC) of the sample as compared to that of the blank in which no antioxidant is present. The schematic illustration of this process is shown in **Fig. 3.11** (Huang et al., 2002). 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox) is used as the standard antioxidant. All the values are expressed in terms of Trolox Equivalents.

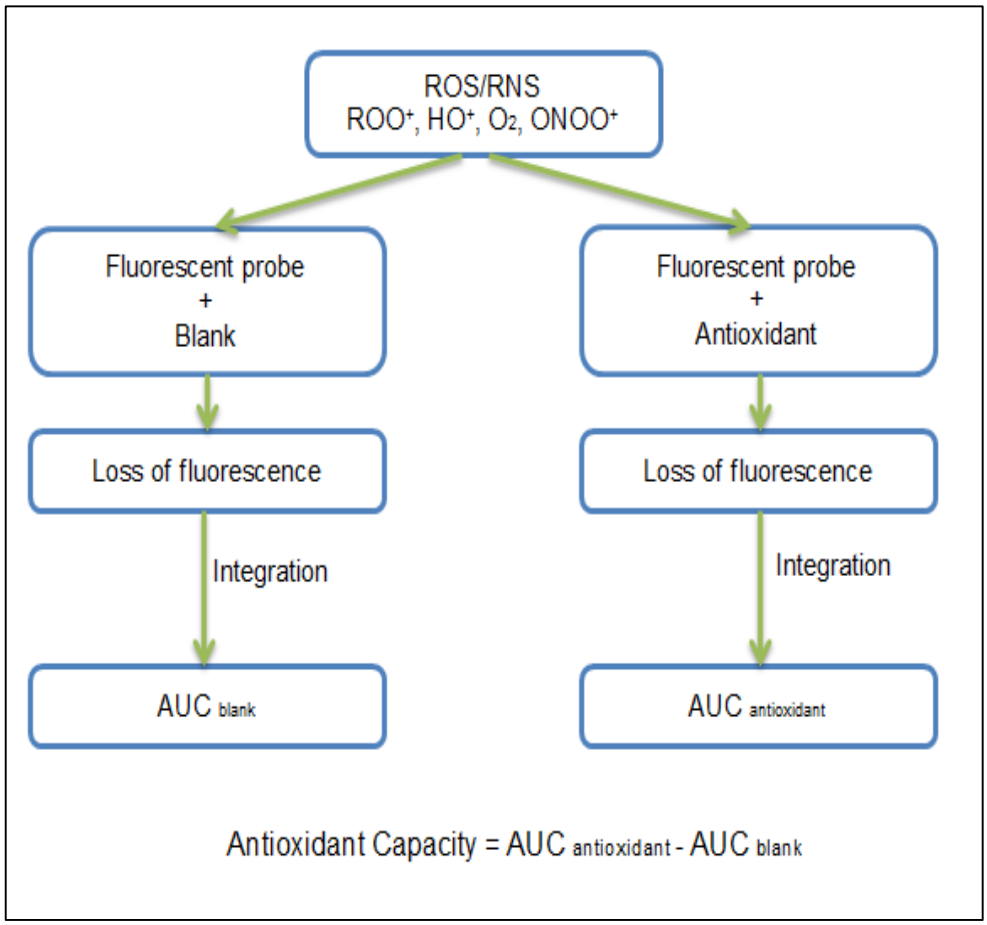

Figure 3.11: Schematic illustration of the ORAC process.

However, the major limitation of this assay was the usage of B - PE as the probe (Ou et al., 2001). Firstly, B - PE produced inconsistency from batch to batch, which resulted in variable reactivity to peroxyl radical. Secondly, it was not photostable. This led to photobleaching of B – PE on exposure to excitation light. Thirdly, B - PE also interacted with polyphenols due to the nonspecific protein binding. Hence, there was a need of a stable fluorescent probe to replace B – PE in ORAC assay. Ou et al., (2001) utilized and validated a stable fluorescent probe called fluorescein (FL) (3',6'-dihydroxyspiro[isobenzofuran-1[3*H*],9'[9*H*]-xanthen]-3-one) for this assay.

3.6.3. Reagent and sample preparation

- Phosphate buffer: A 75 mM phosphate buffer (pH = 7.4) was made by dissolving 1.58 g of NaH_2PO_4 and 8.78 g of Na_2HPO_4 in distilled water and made to 1 liter
- Trolox: Trolox was used as a standard antioxidant. A 100 µM stock solution of trolox was made in phosphate buffer and stored at - 51 ℃. Trolox solution stored at such conditions is stable for several months. Fresh dilutions (6.25, 12.5, 25, 50 and 100 µM) were made from stock solution for developing the standard curve
- Fluorescein: Fluorescein stock solution 1 (1 mM) was prepared in 75 mM phosphate buffer (pH = 7.4). Stock solution 2 (4.19 × 10-3 mM) was made by further diluting solution 1 with buffer and stored in dark at 4 ℃. Fluorescein stored at such conditions lasts for several months. The 8.16 ×10-5 mM fresh working solution of fluorescein was made as required by further diluting the second stock solution
- AAPH: AAPH solution (radical generator) was made by dissolving 0.414 g of AAPH in 10 ml buffer to obtain the final concentration of 153 mM and stored in dark in ice bath. AAPH solution was made fresh every time and discarded after experiments
- Grape juice sample*:* For the assay, the juice sample was diluted with buffer in the ratio of 1:1000. This was done in order to get the readings in the range of the standard curve.

3.6.4. Experimental protocol and calculations

2.25 ml of fluorescein solution was taken in fluoremetric cuvette. To this, 375 µl of sample (trolox or grape juice) was added. Buffer solution was

used as sample for blank measurements. The mixture was stirred continuously with magnetic stirrer and was allowed to incubate at 37 ℃ for 10 min. This is important step since ORAC assay is sensitive to temperature. After incubation, 375 µl of AAPH was added quickly in the cuvette and fluorescence was measured after every 1 min for approximately 70 min till it dropped to 5% of the starting value using a Varian Cary Eclipse fluorescence spectrometer in Dr. Ludescher's laboratory (Food Science Department, Rutgers University, NJ). The instrument is shown in **Fig. 3.12.** During the measurement, the chemicals were stirred continuously by magnetic stirrer.

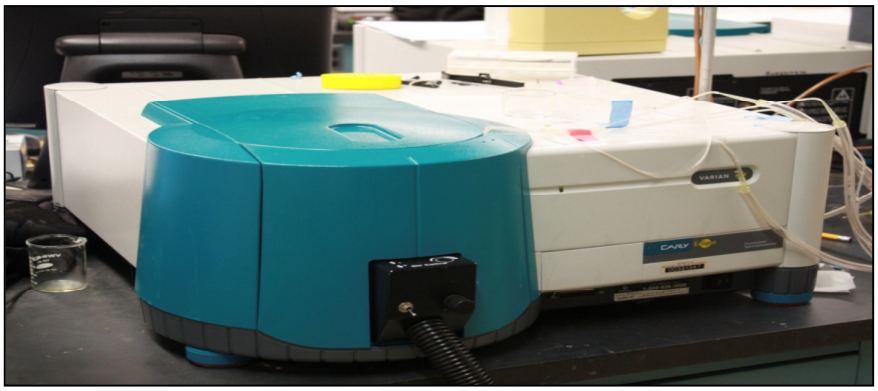

Figure 3.12: Fluorescence meter used for ORAC measurements.

The operating parameters for the measurement were

- Excitation wavelength 485 nm
- Emission wavelength 530 nm
- Excitation slit width 20 mm
- Emission slit width 20 mm
- Temperature of the block 37 ℃

Once the observation readings of fluorescence were collected, the relative fluorescence was calculated as fluorescence at given

time/fluorescence at t = 0. AUC was then calculated from the curve of relative fluorescence vs. time by following formula which is based on trapezoidal rule.

AUC = [0.5 + *f*1/*f*0 + ... *fi* /*f*0 + ... + *f*n-1/*f*0 + 0.5(*f*n/*f*0)] Δt….(Eq. 4)

Where *f*0 is the initial fluorescence at 0 min and *fi is* fluorescence at time i (Ou et al., 2001).

Net AUC = $AUC_{juice/trolox} - AUC_{blank}$……(Eq. 5)

AUC vs. concentration of trolox was plotted to obtain the standard curve. AUC of grape juice samples is then used to obtain antioxidant activity when interpolated with the help of standard curve. This gives antioxidant activity (ORAC value) in terms of Trolox Equivalents and is back calculated to express in terms of Trolox Equivalents (μM TE) per 100 ml juice.

3.7 Biological Antioxidant Activity: Cellular Antioxidant Assay

3.7.1. Materials used

- 2',7'-Dichlorofluorescin diacetate (DCFH-DA) from Sigma-Aldrich, St. Louis, MO
- 2, 2'-azobis (2-amidinopropane) (ABAP) from Sigma-Aldrich, St. Louis, MO
- Dimethyl sulfoxide (DMSO) from Sigma-Aldrich, St. Louis, MO
- L-glutamine from Sigma-Aldrich, St. Louis, MO
- Hepes from Sigma-Aldrich, St. Louis, MO
- Hydrocortisone from Sigma-Aldrich, St. Louis, MO
- Insulin from Sigma-Aldrich, St. Louis, MO
- Phosphate buffer saline (PBS) from Sigma-Aldrich, St. Louis, MO
- Quercetin from Sigma-Aldrich, St. Louis, MO
- 0.25% Trypsin-EDTA from Fisher Scientific, Pittsburgh, PA

- Fetal Bovine Serum (FBS) from Invitrogen, Carlsbad, CA
- Hanks' Balanced Salt Solution (HBSS) from Invitrogen, Carlsbad, CA
- Penicillin-streptomycin from Invitrogen, Carlsbad, CA
- Williams' Medium E (WME) from Invitrogen, Carlsbad, CA
- CellTiter 96® AQueous One Solution Cell Proliferation Assay from Promega, Madison, WI
- HepG2 cells from American Type Culture Collection (ATCC), Rockville, MD
- Synergy HT Multi- Detection Microplate Reader from BioTek Instruments, Inc., Winooski, VT.

3.7.2. Method

Cellular Antioxidant Assay (CAA) is a biological method to measure the antioxidant activity. There are some chemical methods that are in use to measure this parameter like ORAC, total radical-trapping antioxidant parameter (TRAP), Trolox equivalent antioxidant capacity (TEAC), total oxyradical scavenging capacity (TOSC), the peroxyl radical scavenging capacity (PSC), ferric reducing/antioxidant power (FRAP) assay and the DPPH free radical method. But, the validity of these chemical assays in in vivo environment still remains questionable (Wolfe et al., 2008). This is because these assays are performed at non physiological pH and temperature. Additionally, none of them take into account the bioavailability, uptake and metabolism. The protocols often do not include the appropriate biological substrates to be protected, relevant types of oxidants encountered, or the partitioning of compounds between the water and lipid phases and the influence of interfacial behavior (Wolfe et al., 2007). There was a need for

such a method which could be biologically more relevant. The best way to do it is from animal models and human studies. But, these studies are more expensive and time consuming. So, we use cell culture models to determine cellular antioxidant activity as this approach is cost-effective, relatively fast, and address some issues of uptake, distribution, and metabolism.

Figure 3.13 depicts the proposed principle of this assay (Wolfe et al., 2007). HepG2 cells are used since they are a better model to simulate and address metabolism issues. Using HepG2 model, studies have been done to evaluate bioactivities of fruits including cranberry, apple, red grape, strawberry, peach, lemon, pear, banana, orange, grapefruit and pineapple (Yi et al., 2006). DCFH - DA is used in this assay due to its rapid uptake and stable final concentrations. Furthermore, a wide array of ROS (Reactive Oxygen Species) are able to oxidize DCFH to its fluorescent DCF. So, it offers a versatile technique to measure general oxidative stress in cells. (Wang et al., 1999). ABAP is a radical initiator used as an oxidant source and acts in dose dependent manner.

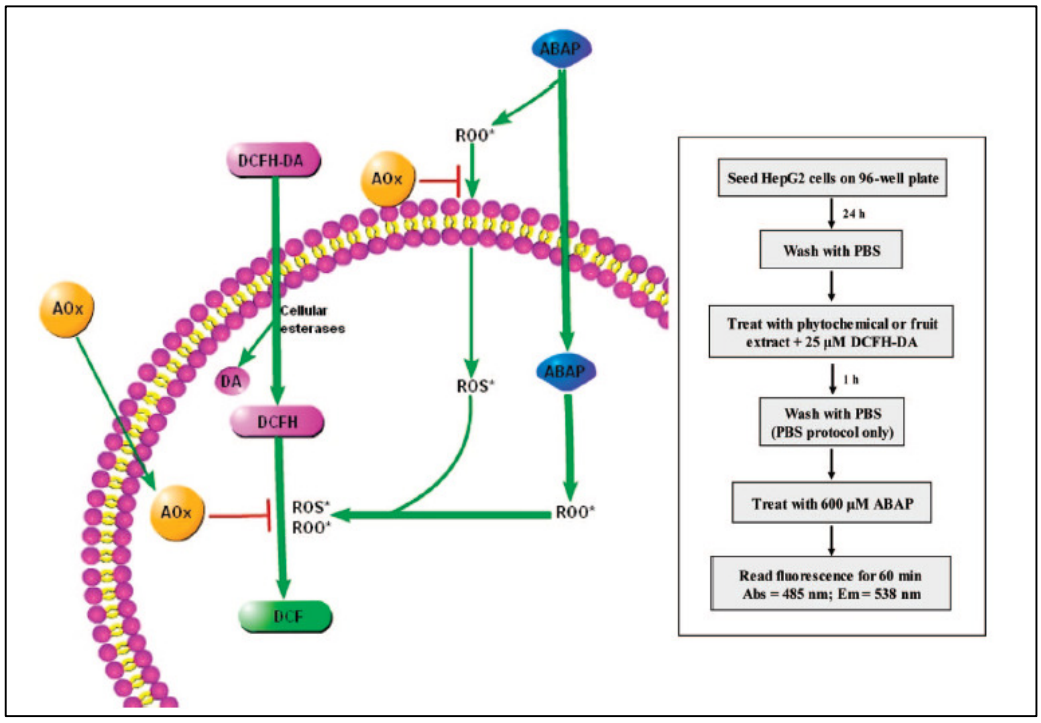

Figure 3.13: Proposed principle of CAA.

When applied to intact cells, the nonionic, nonpolar DCFH - DA crosses cell membranes and is hydrolyzed enzymatically by intracellular esterases to nonfluorescent DCFH. It is trapped within the cell. Cells are then treated with ABAP, which diffuses into cells. ABAP spontaneously decomposes to form peroxyl radicals. These peroxyl radicals attack the cell membrane to produce more radicals and oxidize the intracellular DCFH to the fluorescent DCF. Antioxidants, when present in the system, prevent oxidation of DCFH and membrane lipids and reduce the formation of DCF. Hence, more the antioxidant activity of the sample, less is the fluorescence of DCFH.

Quercetin is recommended to be used as a standard because it has high CAA activity in comparison to other phytochemicals. Another reason is that its pure form can be economically obtained and it is ubiquitously present in fruits, vegetables, and other plants. It is also chemically stable.

3.7.3. Reagent and sample preparation

- DCFH – DA: Stock solution of 20 mM DCFH-DA was prepared in methanol. It was aliquoted and stored at -20 ℃
- ABAP: A 200 mM ABAP stock solution was prepared, and aliquots were stored at -40 ℃
- Quercetin: A 10 mM stock solution of quercetin dihydrate, as a standard of CAA, was freshly made in DMSO prior to use. Different dilutions (1 µM – 10 µM) of quercetin in treatment medium were made for CAA assay
- Growth Medium (GM): WME supplemented with 5% FBS, 10 mM Hepes, 2 mM L-glutamine, 5 *µ*g / mL insulin, 0.05 *µ*g / mL hydrocortisone, 20 *µ*L / mL penicillin – streptomycin mixture. Cells in growth medium were maintained at 37 ℃ and 5% CO2
- Treatment Medium (TM): WME supplemented with 2 mM L-glutamine and 10 mM Hepes
- Juice samples: For CAA, various concentrations (1% – 30%) of single strength, filtered juice were made in treatment medium

3.7.4. Experimental protocol and calculations

3.7.4.1. Cytotoxicity

Cytotoxicity test is a necessary step before performing CAA with juice samples. This test helps to detect concentrations of sample that are safe for cells and can be used for CAA test. This is performed using CellTiter 96® AQueous One Solution Cell Proliferation Assay. This test uses a mixture of [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt; MTS] and phenazine ethosulfate (PES), which is an

electron coupling reagent. When viable cells react with this mixture, they reduce the tetrazolium compound. The formazan product obtained from the reduction reaction has absorbance at 490 nm. So, the more the concentration of viable cells, the higher is the absorbance at 490 nm. Those concentrations of samples, when applied to cells, drops the absorbance to <10% of the control samples (only treatment medium), are considered cytotoxic concentrations. All the samples prepared for CAA test should have concentration below the cytotoxic concentration.

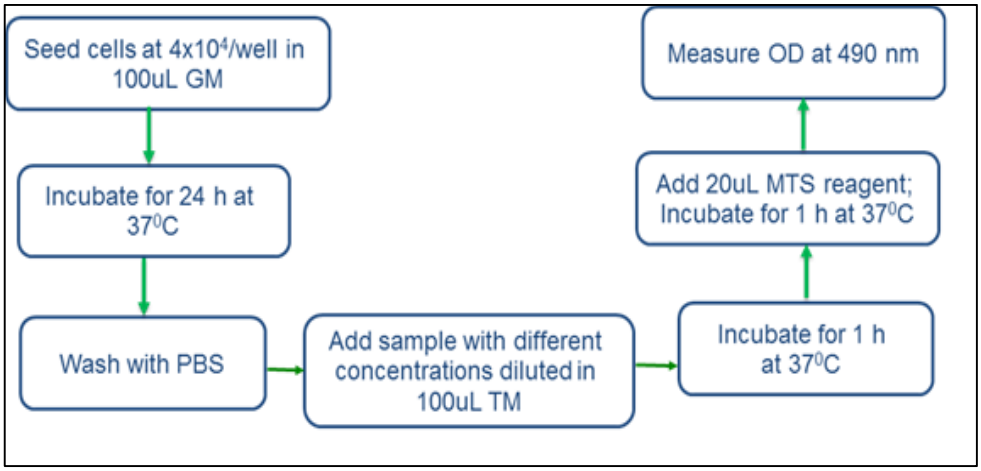

Figure 3.14: Cytotoxicity measurement assay.

HepG2 cells were seeded at 4x104/well on a 96 well-plates in 100 µl of growth medium and incubated for 24 h at 37 ℃. The growth medium was removed and the cells were washed with PBS. 100 µl of treatment medium containing different concentrations of juice and quercetin were applied to the cells followed by incubation at 37 ℃ and 5% CO_2 for 1 h. After incubation, 20 µl of CellTiter 96® AQueous One Solution Reagent (MTS reagent) was added into each well of the 96-well assay plate without removing the treatment medium. The plate was incubated for around 60 – 90 min till sufficient blue – purple color is developed in the plate wells. Absorbance was then measured

at 490 nm. **Figure 3.14** describes the process followed. For quercetin, the highest concentration chosen for this test was 10 µM. The results showed that this concentration was not toxic to cells. All juice concentrations taken (5% to 40%) were non-toxic. Hence, for CAA, all juice concentrations tested were 40% or lower.

3.7.4.2. Cellular Antioxidant Activity assay

The method followed for CAA is as per mentioned in Wolfe et al., (2007) and is shown in **Fig. 3.15.** For CAA, HepG2 cells were seeded into 96-well plate at a density of 6 x 104/ well in 100 µL of growth medium. The outer wells were filled with PBS to create a thermal mass and reduce fluctuations. The plates are incubated for 24 h at 37 °C. After that, the growth medium was removed and the cells were washed with PBS. Triplicate wells were treated for 1 h with 100 µl of different concentrations of juice samples made in treatment medium containing 25 µM DCFH-DA. The cells were then washed with 100 µl PBS. Following that, 100 µl of HBSS containing 600 µM ABAP was applied to each well. The plate was immediately placed in a pre-heated (37 ℃) Synergy HT Multi- Detection Microplate Reader (BioTek Instruments, Inc., Winooski, VT) for kinetic fluorescence measurement. The emission filter of 485 nm and excitation filter of 528 nm was used, with a sensitivity setting of 50. The fluorescence reading was taken every 5 min for 1 h. In each plate, triplicate wells of control and blank were also included. Control wells contained treatment medium with DCFH-DA and HBSS with oxidant. Blank wells contained treatment medium with DCFH-DA and HBSS without oxidant.

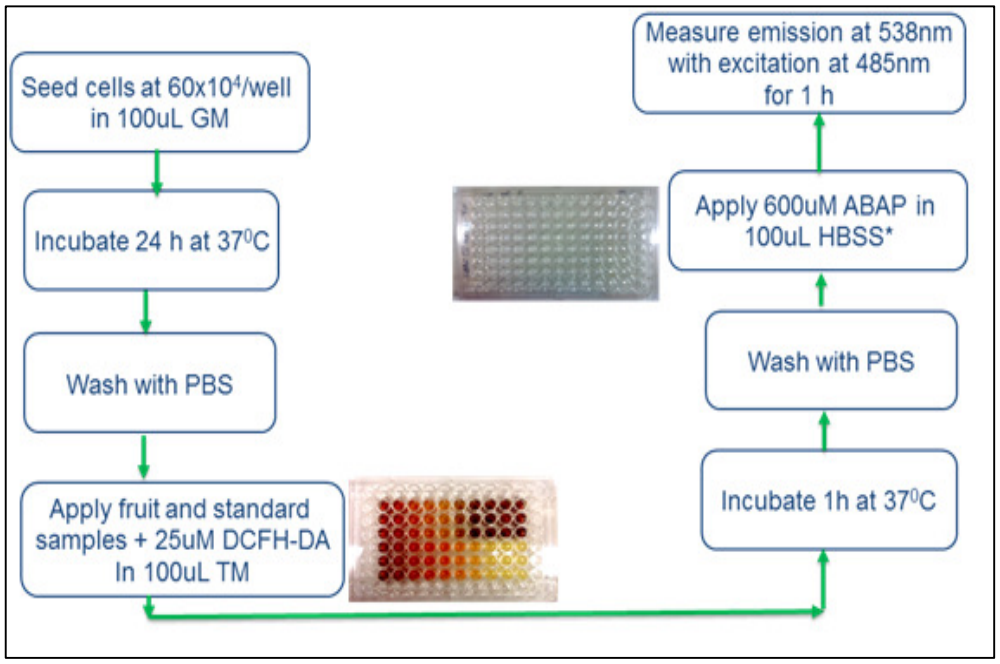

Figure 3.15: Method followed for CAA assay.

The blank is subtracted from all the fluorescence readings. Following that, the net area under curve was calculated from the fluorescence versus time curve of each concentration of quercetin and juice sample.

Then, the CAA unit was calculated using the formula as follow:

CAA unit = 100-(∫SA⁄∫CA) X 100……(Eq. 6)

Where ∫SA is the integrated area under the sample fluorescence vs. time curve and ∫CA is the integrated area from the control curve.

The dose response curve was then generated by plotting CAA units vs. concentration of quercetin or juice sample. The dose response-curve was then converted to median effect plot of log (fa/fu) versus log (concentration or dose) to determine the median effective dose (EC_{50}). fa is the fraction affected, which is equal to CAA unit, and fu is the fraction unaffected, calculated as: 100 - CAA unit. EC_{50} from the curve is the concentration needed to induce halfway reduction of AUC in a given exposure time.

In other words, EC_{50} value is the concentration at which fa/fu = 1 (log fa/fu = 0) or CAA = 50. It is calculated from the linear regression of the median effective plot. EC_{50} values were converted to CAA values by dividing EC_{50} of quercetin by EC_{50} for sample and were expressed in terms of unit of µmoles of Quercetin Equivalents (QE) / 100 ml of juice.

3.8 Sensory Evaluation

3.8.1. Materials used

- Unpasteurized and processed juice samples
- Labels
- Panel members
- Ballot sheet

3.8.2. Method

Difference from control test was performed in order to determine if any sensory difference exists between processed and unpasteurized sample. This test also determines the size of difference between samples (Kemp et al., 2009). This test is classified as an overall difference test. It requires 20-50 panel members to provide meaningful results. The sensory evaluations for were conducted in Dr. Beverly Tepper's lab (Sensory evaluation lab, Rutgers University, NJ) with untrained panel members consisting primarily of faculty, staff and students. The age group of the panel members ranged from 20 to 55 years. For the sensory tests, unpasteurized juice was the 'control' sample. Whereas, 'test' samples were thermally processed, high pressure processed and unpasteurized (blind control) samples. The test was performed with 33 panel members. Each subject is presented with a labeled control sample and one test sample marked with a random 3 digit code (**Fig. 3.16).** Within the test

samples, the control is also presented with a 3 digit code for blind-control test. The blind control helps to establish a base line for the rest of the test samples, as most blind controls will get a non-zero score due to individual variability (Lawless et al., 2010).

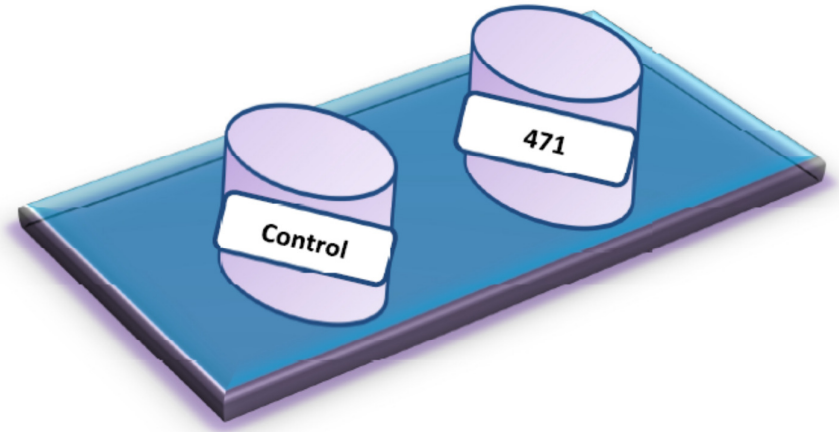

Figure 3.16: Presentation of samples to the panel members.

The panel members then rated the size of the difference between each test sample and the control on the ballot sheet as shown in **Fig. 3.17.** To interpret the results, two factor ANOVA test was performed to find out difference between the subject and samples. The entire sensory evaluation was completed on the same day.

DIFFERENCE FROM CONTROL TEST

Date: 05 – 26 – 2011

Code of the test sample: _____

Type of sample: Grape juice

Instructions:

1. You have received two samples, a control sample and a test sample labeled with a 3 - digit number
2. Taste the sample marked control first
3. Taste the sample marked with the 3 – digit code
4. Drink some water before starting the test and in between samples
5. Assess the overall difference between the two samples using the scale below
6. Indicate the size of overall difference of the sample, relative to the control, on the scale below

_____ 1 = No difference

_____ 2= Very slight difference

_____ 3= Slight/Moderate difference

_____ 4= Moderate difference

_____ 5= Moderate/Large difference

_____ 6= Large difference

_____ 7= Very large difference

REMEMBER THAT A DUPLICATE CONTROL IS THE SAMPLE SOME OF THE TIME

COMMENTS: _____

_____

Figure 3.17: Ballot sheet to mark the results for the study.

3.9 Storage Study

3.9.1. Materials used

- MRE pouches
- Different juice samples
- Controlled temperature chambers

3.9.2. Method

A 8 week long storage study was performed on unprocessed, thermally processed and high pressure processed samples (297 MPa / 6 min). The sample processed at 297 MPa / 6 min was chosen for the storage study as it gave juice with acceptable microbiological quality. Moreover, juice processed at higher conditions than 297 MPa for 6 min did not show any significant difference in antioxidant activity. Also, higher the processing parameters, more is the cost of processing. Hence, this sample was chosen. Juice samples were then vacuum packed in MRE pouches and used for this study. Three storage temperatures, 4 ℃, 25 ℃, and 37 ℃, were selected for the study. The impact of processing and storage on total phenolics content, ORAC value, color, and ellagic acid content was determined. Temperature controlled chambers were used in this storage study to store pouches of juice. The above mentioned parameters were measured bi – weekly.

3.10 Color Measurement

3.10.1. Materials

- Konica Minolta chroma meter (CR – 410) from Konica – Minolta, Tokyo, Japan
- A circular clear plastic dish

3.10.2. Method

Color of the juice samples subjected to storage study was monitored using a CR – 410 Konica Minolta chroma meter as shown in **Fig. 3.18** (left). The chroma meter was first calibrated using a white D_{65} standard disc (Y = 94.7, x = 0.3156 and y = 0.3319). The method to measure color of juice samples was followed as mentioned in Lieu et al., (2010). Juice samples were placed in a circular clear plastic dish that perfectly fits the top of light port of the chroma meter. The dish is then covered with a cover. L*, a* and b* values are then measured for the juice samples. The CIELAB color space is shown in the **Fig. 3.18** (right)**.**

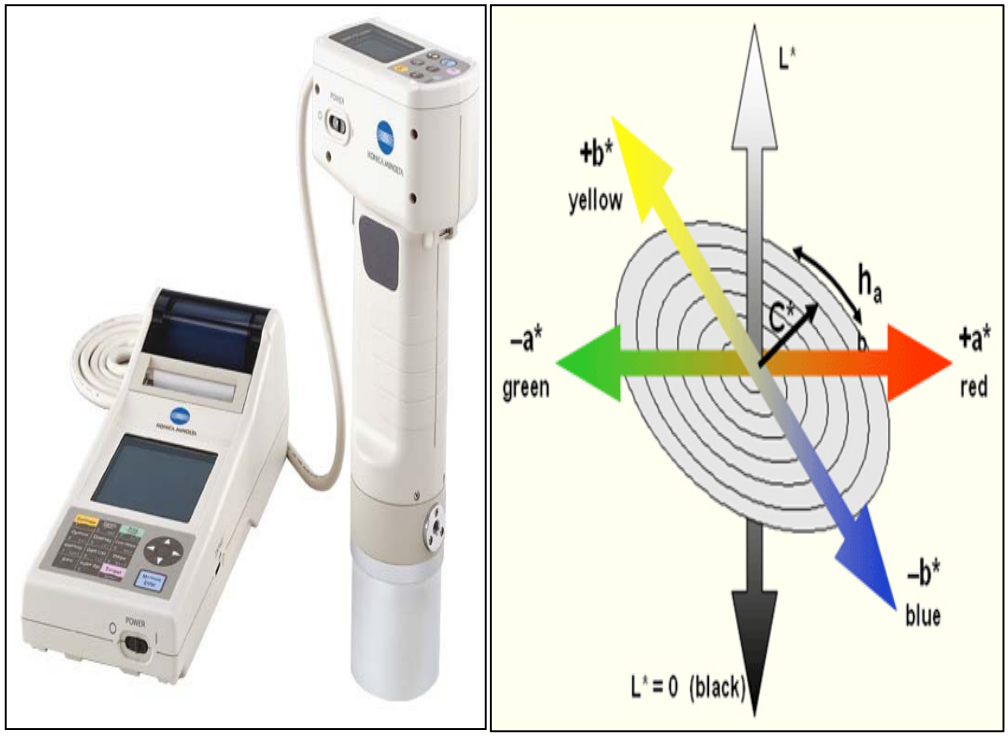

Figure 3.18: Konica Minolta chroma meter (left) and CIELAB color space (right).

Using these values from the chroma meter, the Browning Index (BI) of samples was calculated using the formula 7 (Palou et al., 1999).

$$B.I. = \frac{100\left(\frac{a^*+1.75L^*}{5.646L^*+a^*-3.012b^*} - 0.31\right)}{0.172}$$ .....(Eq. 7)

In order to find the change in color of samples from the initial unprocessed, fresh juice, ΔE* was also calculated. This was done using the Hunter – Scotfield equation

$$[(L^* - L_0^*)^2 + (a^* - a_0^*)^2 + (b^* - b_0^*)^2]^{½}$$ .....(Eq. 8)

3.11 High Pressure Liquid Chromatography (HPLC)

3.11.1. Materials

- Freeze dried juice samples
- 100% acidified methanol
- 95% HPLC grade ellagic acid standard from Sigma – Aldrich, St. Louis, MO
- 0.45 µm PTFE filter 25 mm from Fisher Scientific, Pittsburgh, PA
- HPLC grade acetronitrile from Fisher Scientific, Pittsburgh, PA
- HPLC grade water from Fisher Scientific, Pittsburgh, PA
- Orthophosphoric acid from Fisher Scientific, Pittsburgh, PA
- Supelco Ascentis™ RP – Amide column
- UV – Visible detector

3.11.2. Method

High Performance Liquid Chromatography (HPLC) was performed in order to find the change in ellagic acid content in stored juice samples.

Pastrana-Bonilla et al., (2003), stated that the major phenolic compound in Muscadine grapes in ellagic acid and not resveratrol. A thesis submitted by Joonhee Lee to the University of Florida (2004) also stated that the unique phytochemical composition in *Vitis rotundifolia* species is due to presence of ellagic acid and its derivatives. This differentiates them from *Vitis vinifera* species. It was also mentioned that thermal processing increased the free ellagic acid via ellagitannins hydrolysis. This influenced the kinetic changes of ellagic acid derivatives during storage. Hence, HPLC study was done to monitor the effect of processing and storage on ellagic acid in Muscadine grape juice as it is an important phenolic compound in Muscadine grapes due to its beneficial health properties. This also adds value and marketability to the crop (Stoner et al., 1997).

3.11.3. Solvents and reagent preparation

- Extraction solvent: Acidified methanol was used as the extraction solvent for phenolics from Muscadine grape juice samples. 80% methanol was first prepared using HPLC grade water. It was then acidified to about pH 3 using 6N HCl
- HPLC mobile phases: 100% acetonitrile was used along with 0.085% orthophosphoric acid made in HPLC water
- Ellagic acid standard: Various concentrations of ellagic acid were prepared, ranging from 20 – 200 µg / ml in 80% acidified methanol to obtain the standard curve
- Juice samples: Week 0 and Week 8 processed juice samples stored at 4 ℃ and 22 ℃ from storage study were subjected to HPLC analysis.

25 ml of juice was spread on a glass petri dish. These dishes were placed in freeze dryer for 24 h to obtain freeze dried juice samples.

3.11.4. Experimental protocol

500 mg of freeze dried juice sample was taken in a centrifuge tube. Two ml of 80% acidified methanol was then added to the tube. The tube was vortexed for 1 min and then placed in shaker for 30 min at room temperature. Following that, the sample was centrifuged and the extracted phenolics were collected in form of the supernatent liquid. These extracted samples were filtered through a 0.45 µm PTFE filter and were stored in freezer before the test. A 5 mm, 4.6 x 250 mm Ascentis C18, RP Amide column was used. It was attached to a Waters HPLC equipped with 486 UV-Vis Tunable detector and Peak Simple Chromatography 2.83 data system. The mobile phase consisted of a gradient of 0.085% phosphoric acid (A) and acetonitrile (B). The above solvent composition was selected based on an application note by Supelco that stated the conditions to analyze certain polyphenols using the Ascentis RP-amide column (http://www.sigmaaldrich.com/etc/medialib/docs/Supelco/Application_Notes/t005271.Par.0001.File.tmp/t005271.pdf). The flow rate was set at 1.0 ml/min and the sample injection volume was 10 ul. The gradient used was as follows: 15% B at 0 min, 35% B at 30 min and 85% B at 35 min. A 10 min postrun was performed with the initial gradient concentration so the column was primed and ready for the next sample. The eluents were scanned at 360 nm by UV – Vis detector and area under the curve for peaks was calculated using Peak Simple system. At periodic intervals, the column was flushed with 100% acetronitrile to clean the residues that may be present. The method

developed was based on Pastrana-Bonilla et al., 2003. Ellagic acid standards are subjected first to obtain the retention time for this phenolic compound. Peak areas for different ellagic acid standards were calculated PeakSimple 2.83 program. Peak areas were plotted against the concentration of ellagic acid to obtain the standard curve. Calculated peak area of ellagic acid peaks for juice samples were calculated and interpolated to find concentration of ellagic acid using the external standard method. The concentration of ellagic acid in juice was expressed as mg of ellagic acid per 100 ml juice.

3.12 Statistical Analyses

All the statistical analyses were done using MATLAB R2011b (The MathWorks Inc., Natick, MA). Analysis Of Variance (ANOVA) test was used to find differences between the means. In case where multiple means were to be compared, multiple comparison command was used on MATLAB. Results were considered significant if p value was <0.05. All the results are expressed as mean value ± SE (SE = Standard Error).

4 RESULTS AND DISCUSSION

The results from the experiments performed on unprocessed, thermally processed and high pressure processed Muscadine grape juice are presented and discussed in this chapter. Differences in the total phenolics content, ORAC values and CAA values were evaluated before and after processing of the juice. Changes in total phenolics, ORAC values, color and ellagic acid content were monitored during a storage study of 8 weeks. Results of the sensory evaluation test to find out the overall difference between unprocessed, thermally processed and high pressure processed juice are also presented in the following sections of this chapter.

4.1 Total phenolics

Total phenolics in the juice samples were measured using Folin – Ciocalteu method. Gallic acid was used as standard for this method. A standard curve (**Fig. 4.1**) was used to interpolate total phenolics in juice and was expressed in terms of mg Gallic Acid Equivalents (GAE) per 100 ml juice.

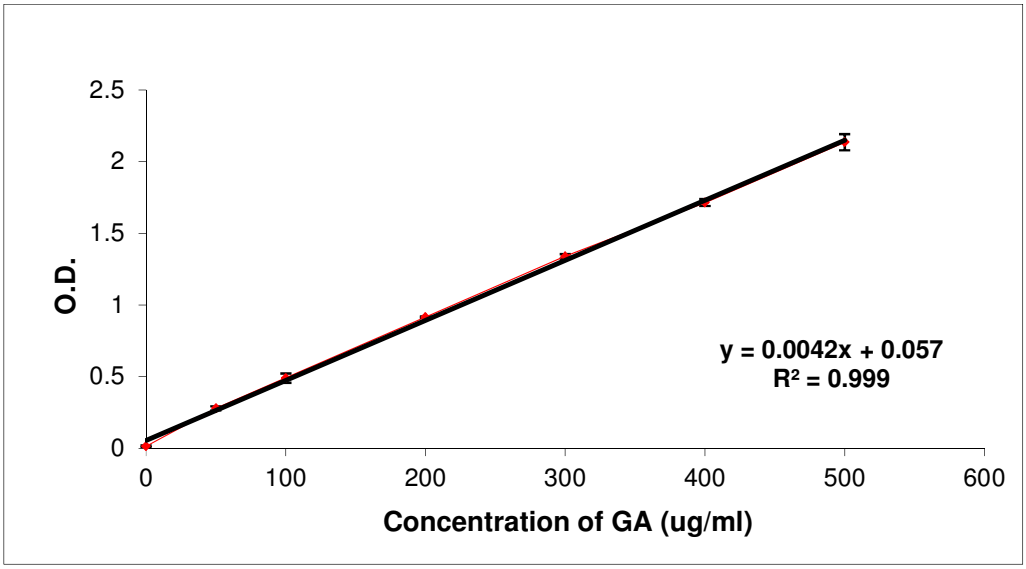

Figure 4.1: Standard curve for total phenolics. All values are mean±S.E. (n=9).

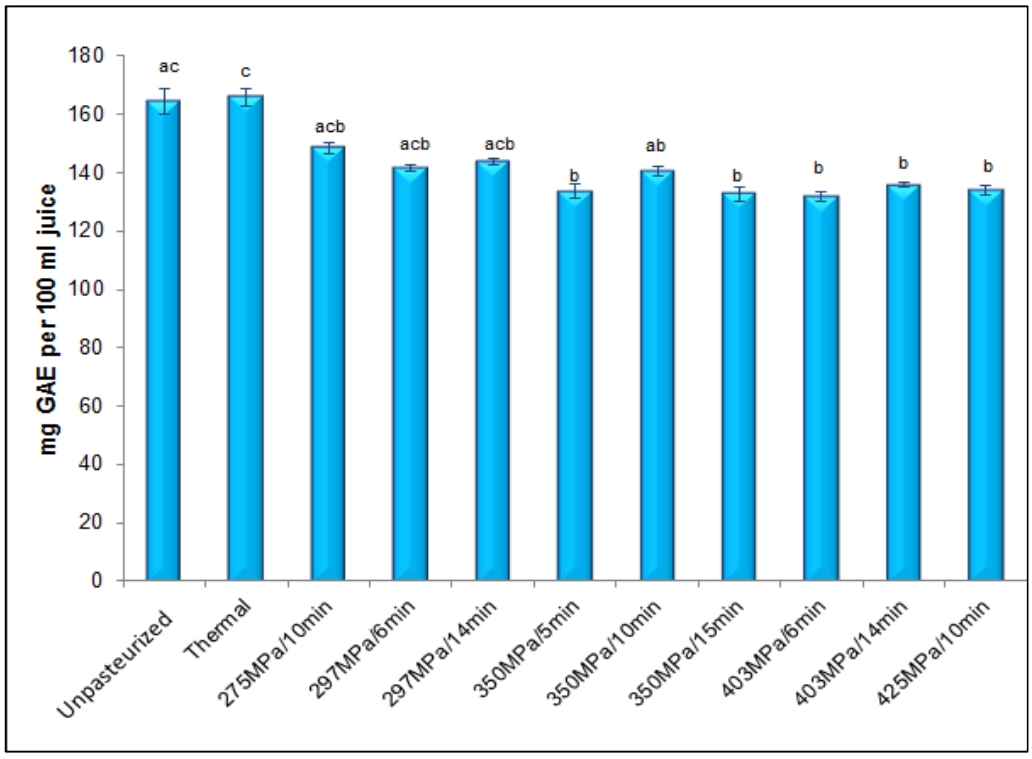

Figure 4.2: Total phenolic values of samples. All values are mean±S.E. (n=9). Different alphabets indicate statistically significant differences ($p<0.05$).

Fresh, unpasteurized juice samples were thermally processed using batch pasteurization method. Some unpasteurized samples were vacuum packed in pouches. They were then subjected to HPP at conditions stated in **Table 3.3.** The corrected absorbance values of the samples at 765 nm were then compared to the standard curve. Based on interpolation from the standard curve, total phenolic content of juice samples were calculated and are plotted in **Fig. 4.2**.

From the above **Figure 4.2**, we can observe that no statistically significant difference was found between the thermally processed juice sample and the unpasteurized juice, maintaining a value of around 165 mg GAE per 100 ml juice. It was also seen that total phenolics value did not change significantly when unpasteurized juice and thermally processed juice

were compared to high pressure processed juice samples processed at 275 MPa for 10 min, 297 MPa for 6 min and 297 MPa for 14 min. At pressure - time conditions higher than 297 MPa / 14 min, a decrease in total phenolics was seen. Comparing the average values of total phenolics of unpasteurized and thermally processed juice against that of juice samples processed at higher pressure – time combinations, around 18% decrease in total phenolics was seen. This is probably due to condensation reactions at those higher pressures. They involve covalent association of anthocyanins with other flavanols present in fruit juices leading to the formation of a new pyran ring by cycloaddition (Tiwari et al., 2009). This form may not be detected by Folin – Ciocalteu method, giving lower value for total phenolics. The value of unpasteurized and thermally processed juice is very similar to the total phenolics value for Concord grape juice (176 mg GAE per 100 ml juice) commercially available in markets. So, thermal processing did not change the total phenolics value of Muscadine grape juice. For samples that were high pressure processed, the total phenolics value decreased at pressure – time conditions beyond 297 MPa for 14 min.

4.2 Chemical Antioxidant Activity

To measure ORAC value of juice samples, trolox was used as the standard. In order to plot the standard curve, different concentrations of trolox were subjected to the ORAC assay. The fluorescence vs. time graph was plotted (**Fig. 4.4)** to find out the AUC. Once the AUC was obtained, the standard curve for trolox, as shown in the **Fig. 4.3** was plotted. The samples were then analyzed and the AUC was calculated from the fluorescence decay curves. Using the standard curve and AUC for the sample, ORAC value was

calculated in terms of µMTE per 100 ml juice. The ORAC values for all the samples tested are shown in the **Fig. 4.5** below.

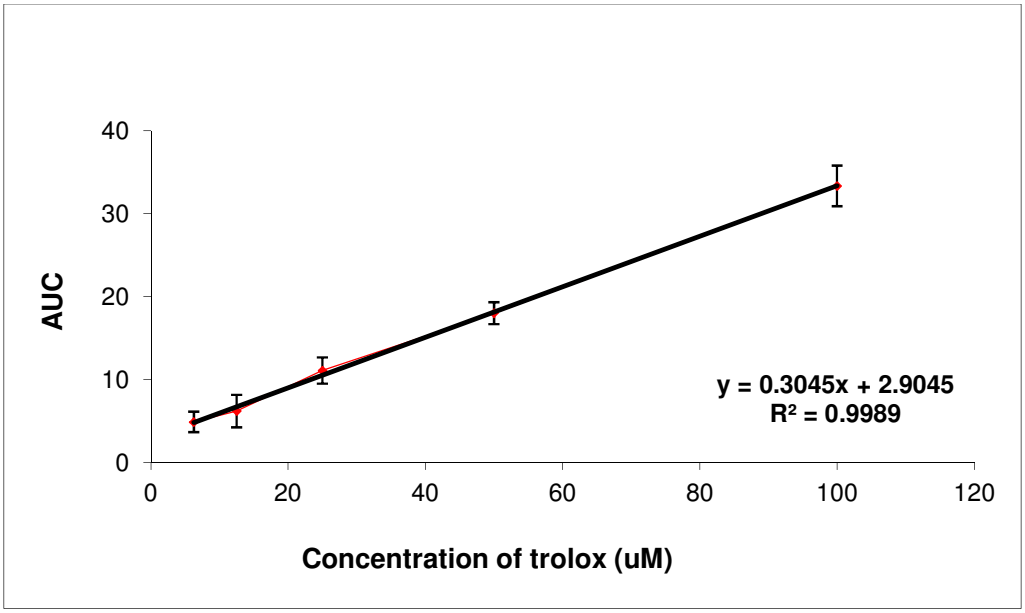

Figure 4.3: Standard curve for ORAC. All values are mean±S.E. (n=4).

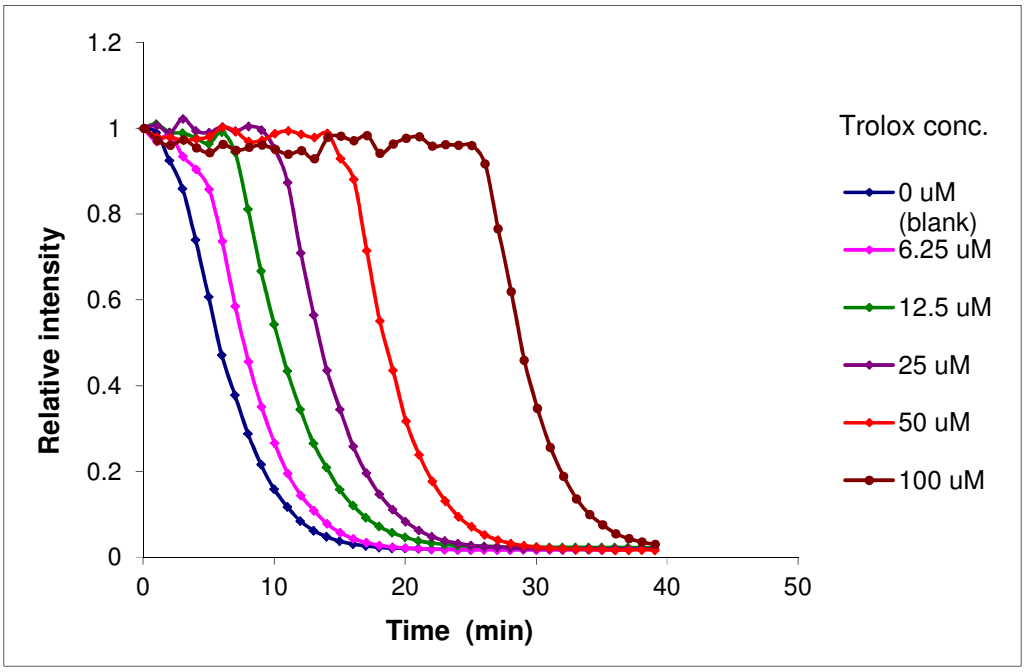

Figure 4.4: Decay curves obtained for trolox.

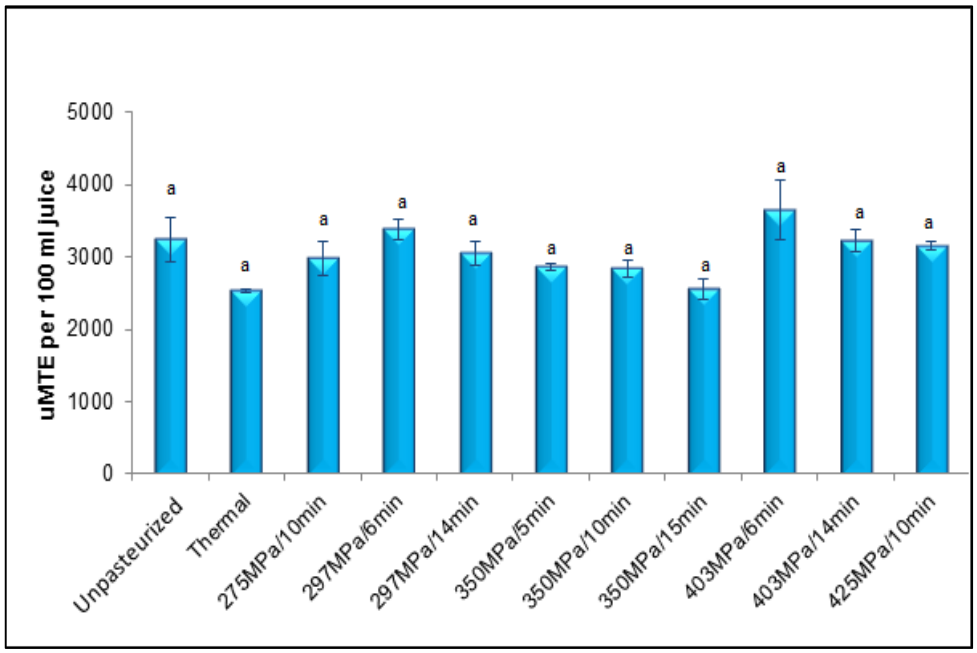

Figure 4.5: ORAC values for unpasteurized and processed samples. All values are mean±S.E. (n=9). Different alphabets indicate statistically significant differences ($p<0.05$).

From the results, we can observe that no statistically significant difference was found between samples. The values indicate 22% loss in ORAC value of thermally processed sample when compared to unpasteurized juice. However, it was not statistically significant. It was also seen that ORAC value did not change significantly when unpasteurized juice was high pressure processed at various conditions, showing high pressure had no effect on the chemical antioxidant activity. The values ranged from around 2550 µMTE to 3650 µMTE for high pressure processed samples. There was also no difference in ORAC values of samples when they were processed thermally and high pressure processed. These results showed that the process followed had no statistically significant effect on the ORAC value of Muscadine grape juice. No particular trend was seen. Although, values have

wide range, they still show a higher ORAC value than that of commercially available Concord grape juice (2416 µMTE per 100 ml juice) (http://www.thenutritiouslife.com/pdf/orac_points_portable_guide.pdf)

4.3 Cellular Antioxidant Activity

This CAA assay was performed on HepG2 cells. Quercetin was used as the standard. As seen from the earlier experiments, the total phenolics value and the ORAC values did not change significantly between the different high pressure treatments. Hence, for this assay we chose to analyze only one high pressure processing condition and compare it to unpasteurized and thermally processed juice. This is because CAA assay is very time consuming and since, no difference was seen in the results between different high pressure conditions, one of the conditions could be approximate representation to show the effect of HPP on CAA. The condition chosen to be tested was 297 MPa / 6 min.

The fluorescence decay curves for quercetin and all the samples are shown in **Fig. 4.6.** Dose response curves and median effect plots are shown in **Fig. 4.7** and **Fig. 4.8**, respectively. The EC_{50} value for quercetin and the juice samples are plotted in **Fig. 4.9.** EC_{50} value is basically the concentration of sample required to inhibit the fluorescence by 50% as compared to blank. This means that lower the value of EC_{50} of a sample, higher is its antioxidant activity. The EC_{50} values were further converted to CAA values using the [EC_{50} Quercetin / EC_{50} Juice]*100. This was expressed in terms of µmol QE per 100 ml juice, taking the value of 17.46 for EC_{50} Quercetin. The calculated CAA values are shown in **Fig. 4.9.** For CAA, higher the value, higher is the antioxidant activity.

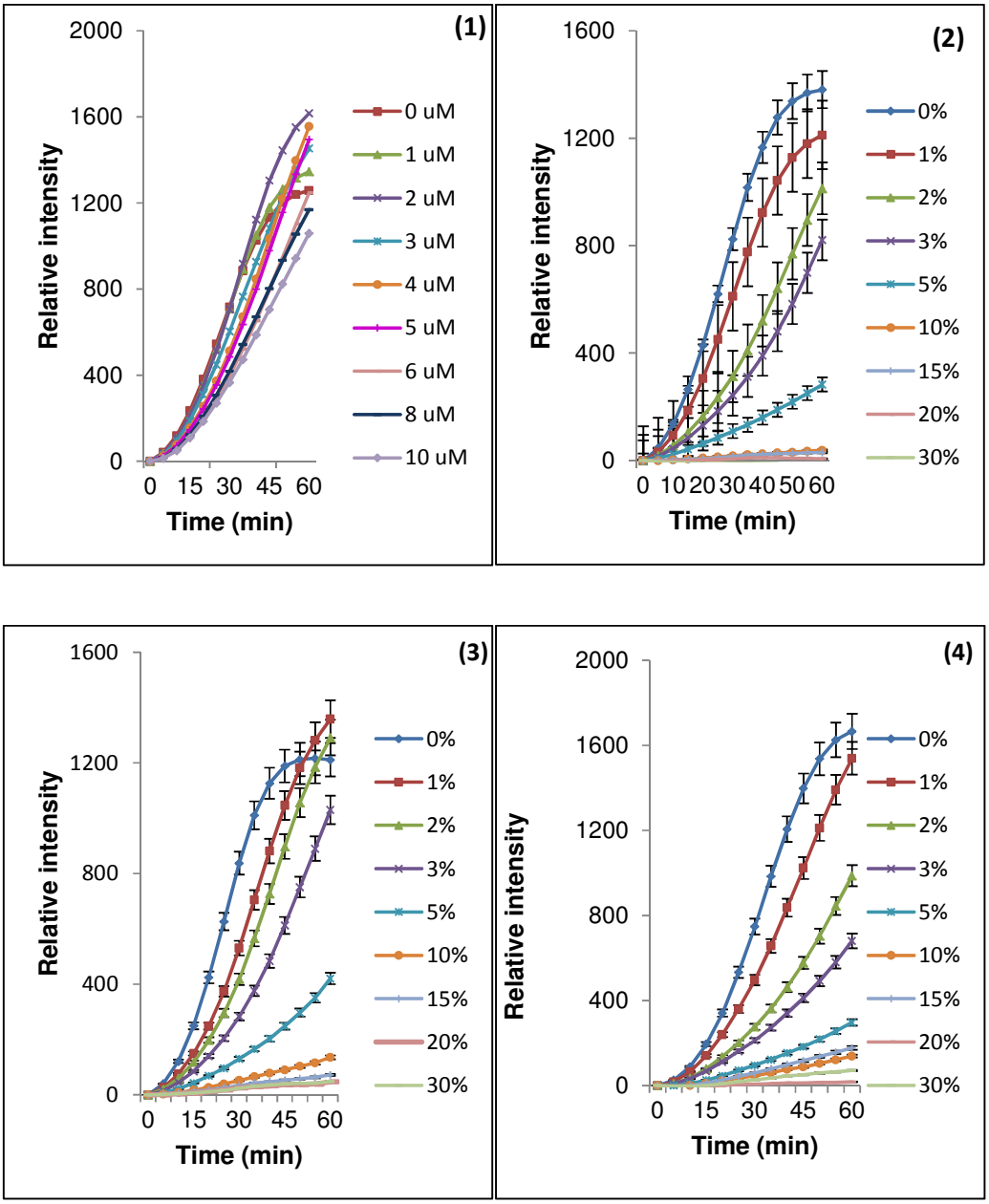

Figure 4.6: Fluorescence decay cures for (1) Quercetin; (2) Unpasteurized juice sample; (3) Thermally processed sample and (4) High pressure processed sample. All values are mean±S.E. (n=3).

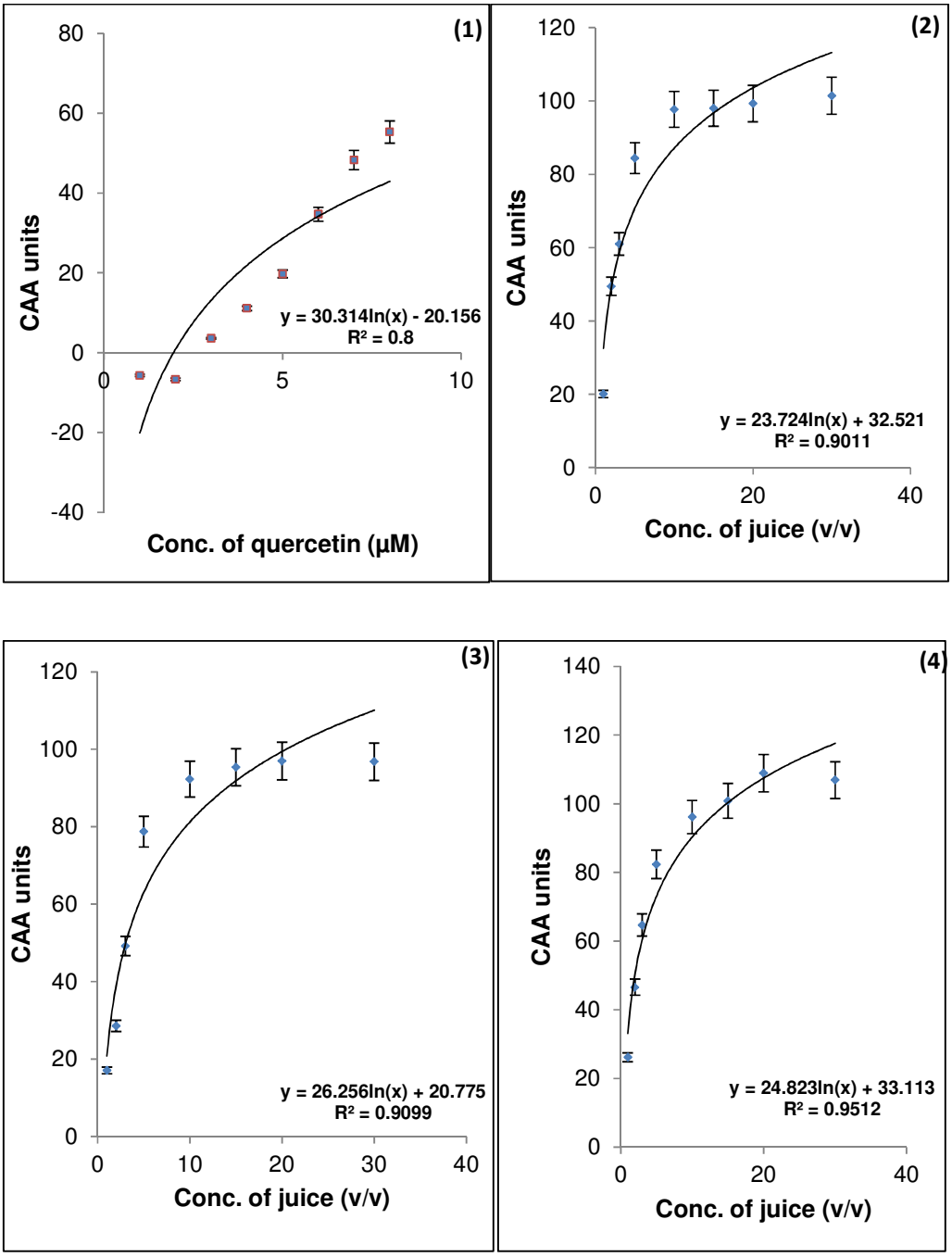

Figure 4.7: Dose response curves for (1) Quercetin; (2) Unpasteruized juice sample; (3) Thermally processed sample and (4) High pressure processed sample. All values are mean±S.E. (n=3).

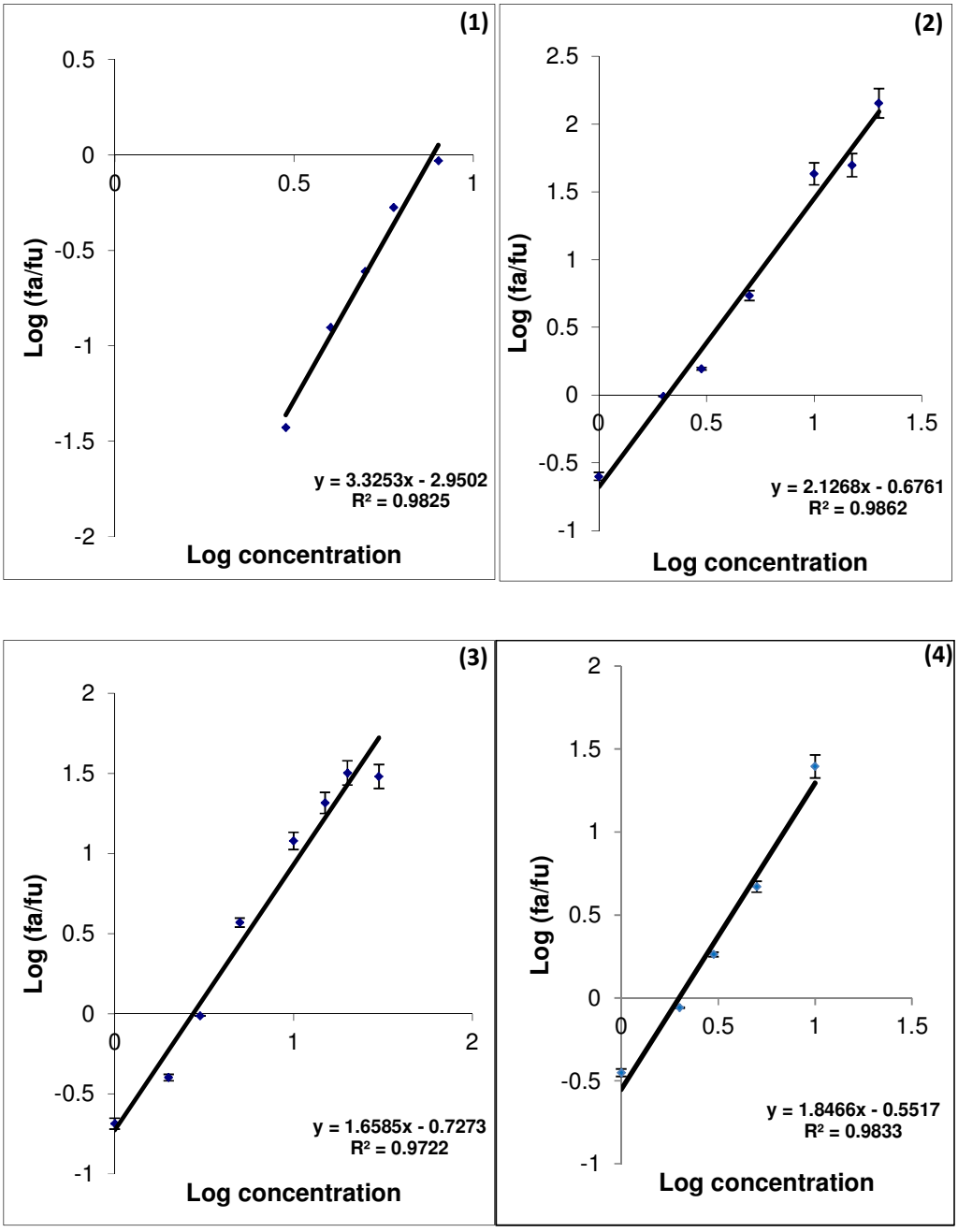

Figure 4.8: Median effect plots for (1) Quercetin; (2) Unpasteurized juice sample; (3) Thermally processed sample and (4) High pressure processed sample. All values are mean±S.E. (n=3).

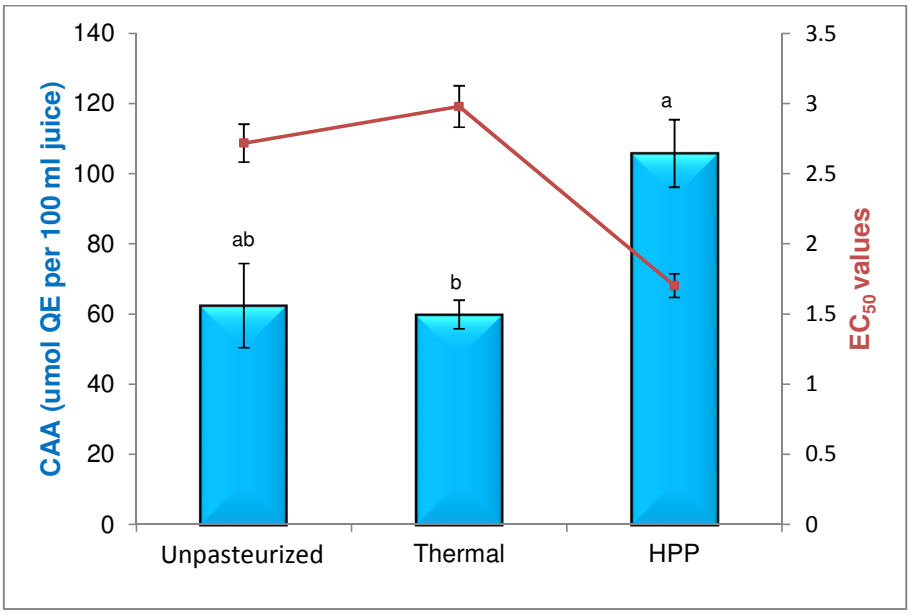

Figure 4.9: CAA and EC50 values for unpasteurized and processed juice. All values are mean±S.E. (n=3). Different alphabets indicate statistically significant differences ($p<0.05$).

From the results, the CAA value for thermally processed juice was not significantly different from that of unpasteurized juice. When the sample was high pressure processed, CAA showed an increase by 69% as compared to the unpasteurized sample. But, statistics showed no significant difference between the two samples. The CAA value for thermally processed sample was significantly different when compared to that of high pressure processed sample. It showed an increase by 77%. Raiké et al., (2007) mentioned that thermal processes can lead to formation of smaller degradation products from polyphenol compound. This might affect its bioavailability and hence gives a lower cellular antioxidant activity. In case of HPP, there might be chemical reactions like deglycosylation taking place along with structural changes in the compounds. Aglycones diffuse more efficiently in the cells as they possess

increased solubility and bioavailability (Williamson, 2004). Some compounds that are in fewer amounts like ascorbic acid might show an enhanced effect in antioxidant activity in this assay as compared to chemical assays. Main reasons that influence CAA values are degree of glycosylation, basic compound structure and molecular size, conjugation with other compounds, degree of solubility and degree of polymerization (Wolfe et al., 2007). These changes occurring during processing show a substantial effect on its cellular antioxidant activity.

4.4 Sensory Evaluation

'Difference form control' test was performed in order to find out the overall difference between the juice samples that were unpasteurized against the processed ones, consulting Dr. Beverly Tepper. In this test, the samples were presented in pairs to the panel members and they marked the magnitude of the difference based on the scale shown in **Fig**. **4.10** below.

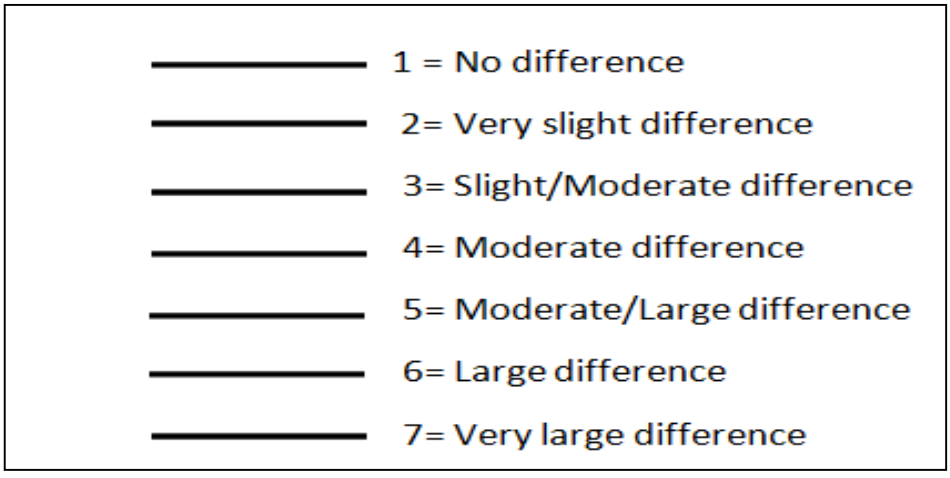

Figure 4.10: Scale for 'Difference from Control' test.

The results from the evaluation showing the average rating of difference are shown in the **Fig. 4.11.** The results were analyzed using a two factor ANOVA test and are presented in **Table 4.1.**

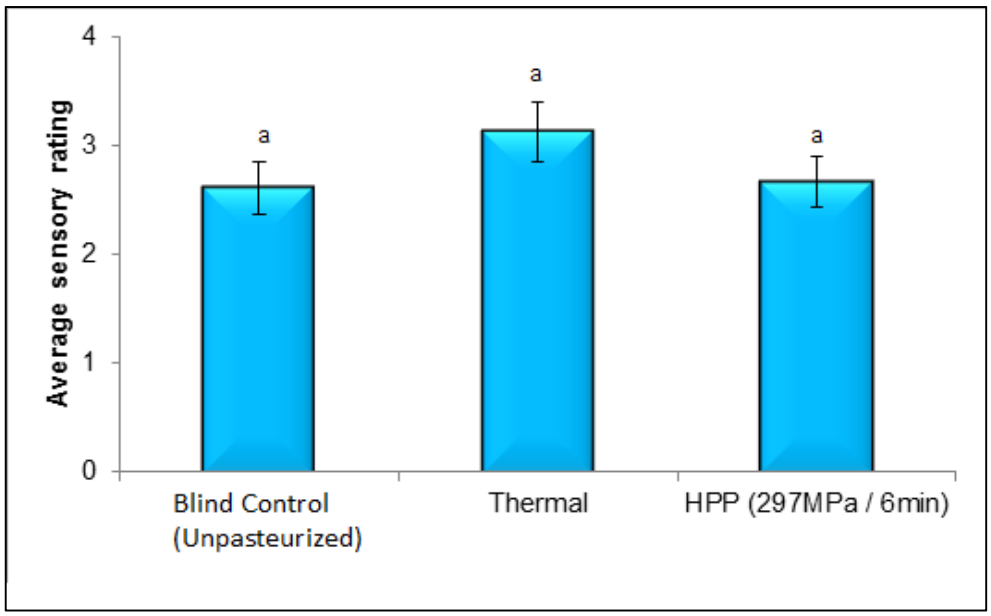

Figure 4.11: Results of sensory evaluation. All values are mean±S.E. (n=33). Different alphabets indicate statistically significant differences ($p<0.05$).

Table 4.1: 2-way ANOVA table for sensory evaluation studies.

ANOVA						
Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Panel members	101.2929293	32	3.165404	2.037383	**0.007842**	1.62386
Treatments	5.232323232	2	2.616162	1.683868	**0.193774**	3.14043
Error	99.43434343	64	1.553662			
Total	205.959596	98				

From the results, we can see that, on an average, no statistically significant differences were seen between unpasteurized and processed samples as the p value is >0.05. The ANOVA table shows that the p value is <0.05 when the source of variation was panel members. This means that every person shows a measurable difference in evaluating the same sample.

4.5 Storage studies

Storage studies for samples were done at three temperatures, 4 ℃, 22 ℃ and 37 ℃. Total phenolics, ORAC value, color measurements and HPLC analyses were done on samples in order to monitor the impact of treatment and time on the above parameters.

4.5.1 Total phenolics

Figure 4.12 represents the results for total phenolics before the samples were kept for storage studies. It represents the initial change in sample immediately after processing at time zero. This shows that there was no significant change in total phenolics after processing. Storage study results are shown in **Fig. 4.13.** It is seen from the charts that at all three temperatures, no significant difference was found in total phenolics of juice processed by the two treatments. In most of the cases, keeping temperature and treatment constant, no significant changes occurred in total phenolics with time. The amount decreased numerically, but there was no statistically significant difference. 3 - way ANOVA analysis can be found in the Appendix A.

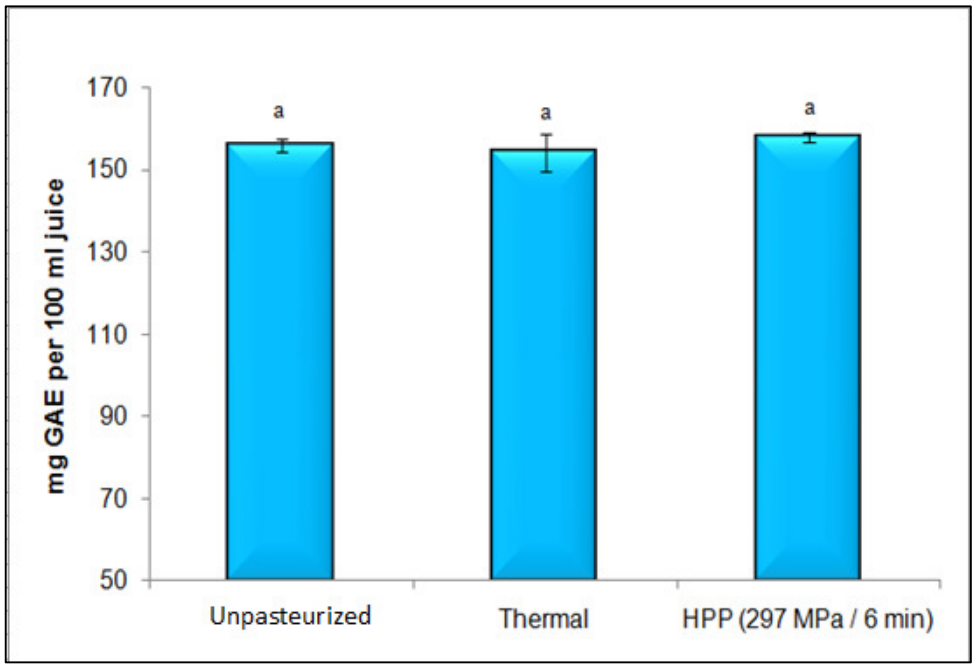

Figure 4.12: Time zero total phenolics of unpasteurized and processed juice samples. All values are mean±S.E. (n=3). Different alphabets indicate statistically significant differences ($p<0.05$).

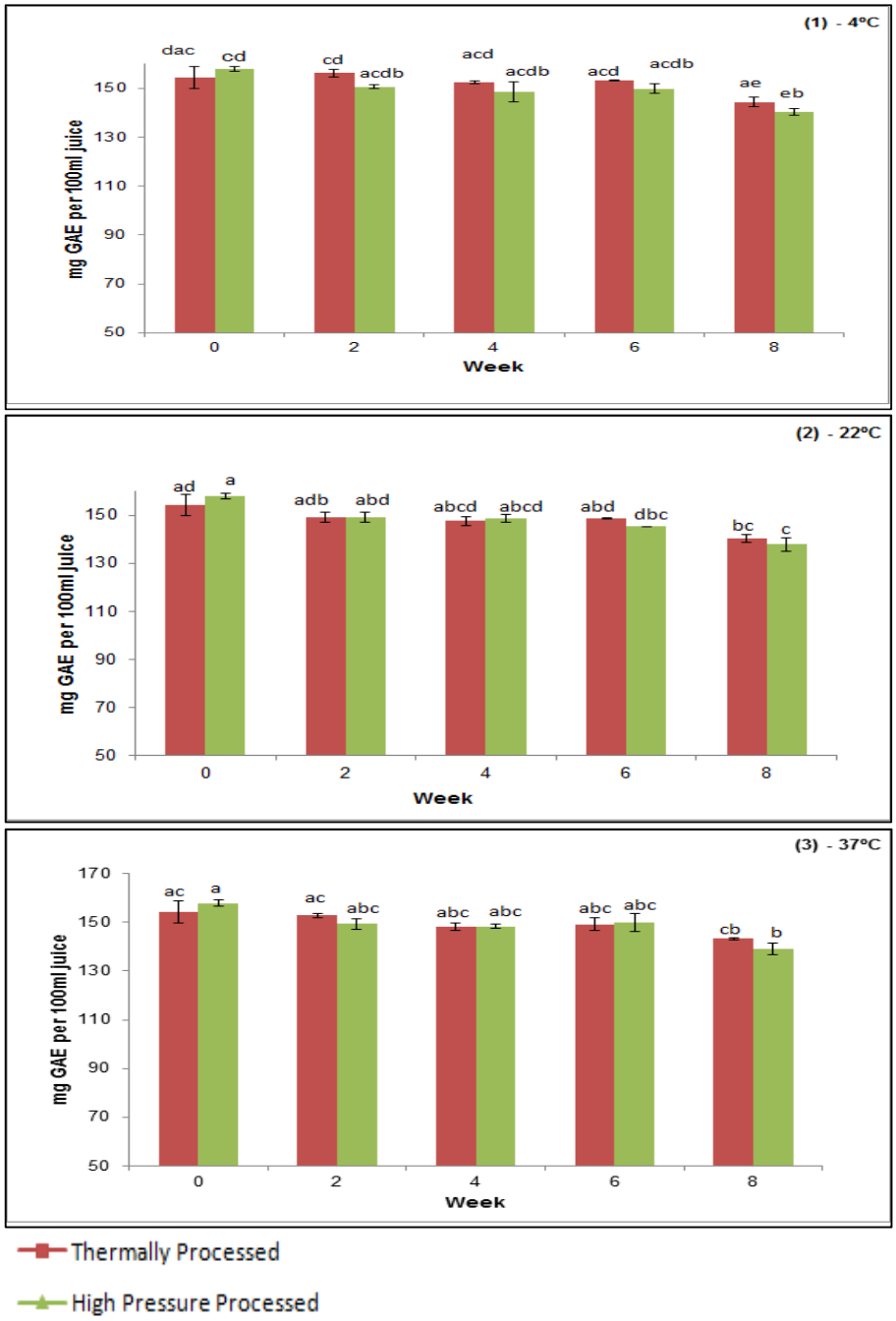

Figure 4.13: Total phenolics of thermally and high pressure processed samples during storage study at (1) 4 °C; (2) 22 °C and (3) 37 °C . All values are mean±S.E. (n=3). Different alphabets indicate statistically significant differences ($p<0.05$).

4.5.2 Chemical antioxidant activity (ORAC value)

The results for ORAC were similar to that of total phenolics. Immediately after processing, no significant change was observed in the ORAC value of unpasteurized juice, thermally processed juice and high pressure processed juice (**Fig. 4.14).** During the storage study, there was no difference in the ORAC value of thermally treated juice as against high pressure processed juice at all the temperatures for storage study. For thermal processed juice at 22 °C, a decrease in the ORAC value was seen with time. High pressure processed sample did not particularly show any such trend with time in most of the cases. It gave a lower ORAC value when stored for 8 weeks at 4 ℃.

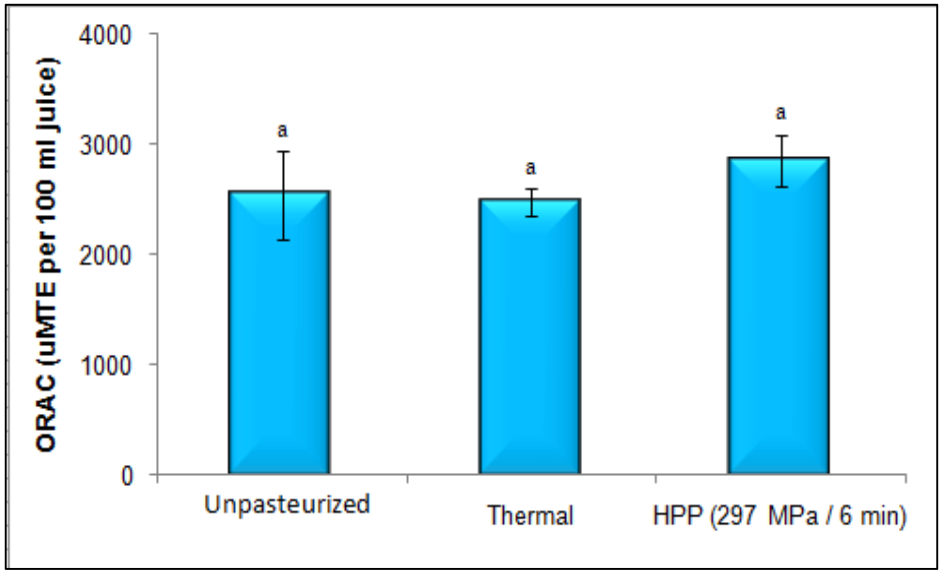

Figure 4.14: Time zero ORAC values of unpasteurized and processed juice samples. All values are mean±S.E. (n=3). Different alphabets indicate statistically significant differences ($p<0.05$).

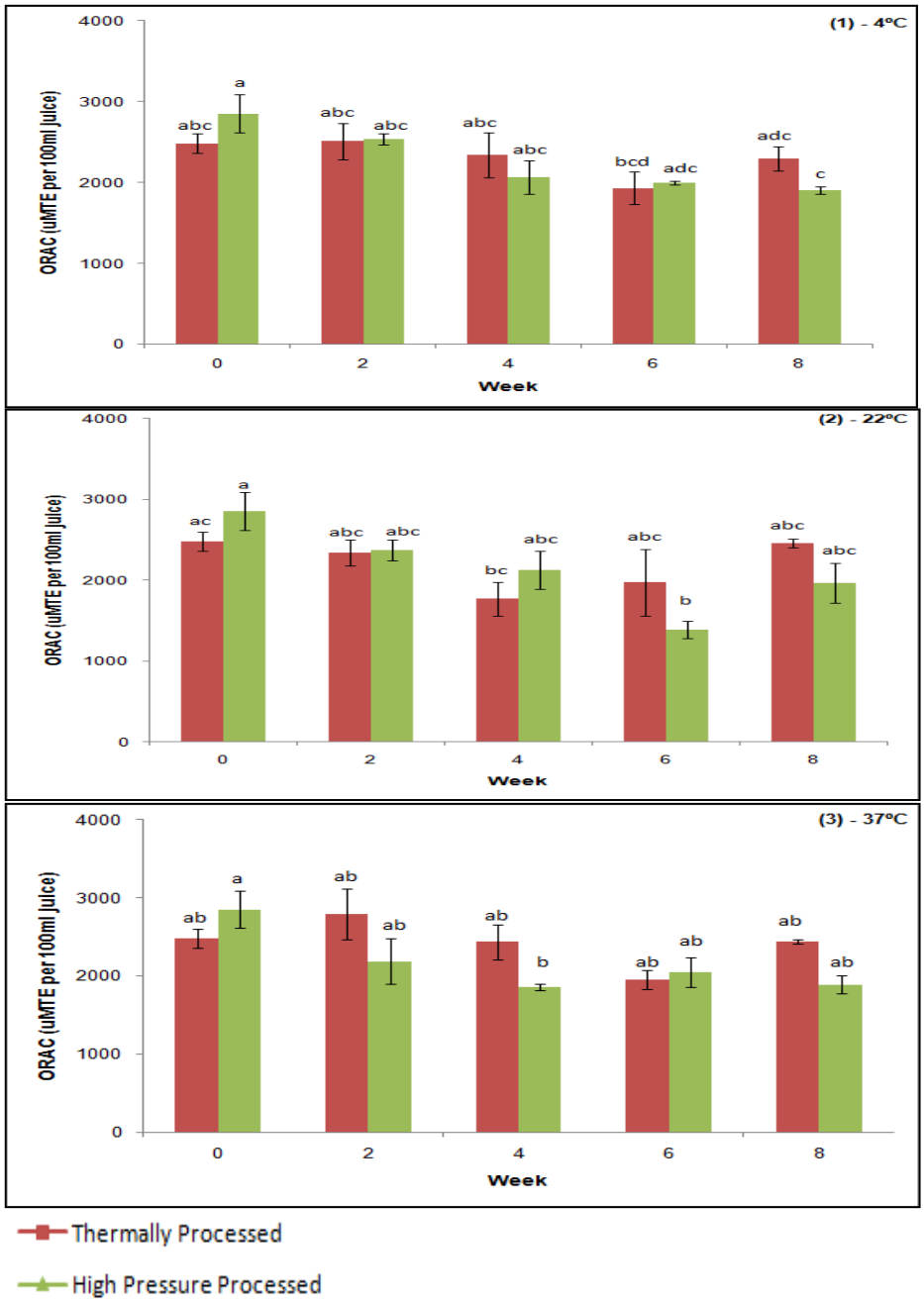

Figure 4.15: ORAC values of thermally and high pressure processed samples during storage study at (1) 4 ℃, (2) 22 °C and (3) 37 °C. All values are mean±S.E. (n=3). Different alphabets indicate statistically significant differences ($p<0.05$).

4.5.3 Color

Two parameters were studied with respect to color; browning index and ΔE*. They were calculated using equations 7 and 8, respectively. In case of browning index, there was numerical increase in the browning index with time for thermally processed juice. Similar increase with time was seen for high pressure processed juice at all three temperatures. But this increase was not significant. The rate of increase in browning index was significantly higher for thermal processed sample as compared to high pressure processed sample. The rate also increased proportionally with temperature. Higher variations in BI values exist in this case. Sensitivity analysis showed that smaller changes in b value bring about big changes in the B.I value.

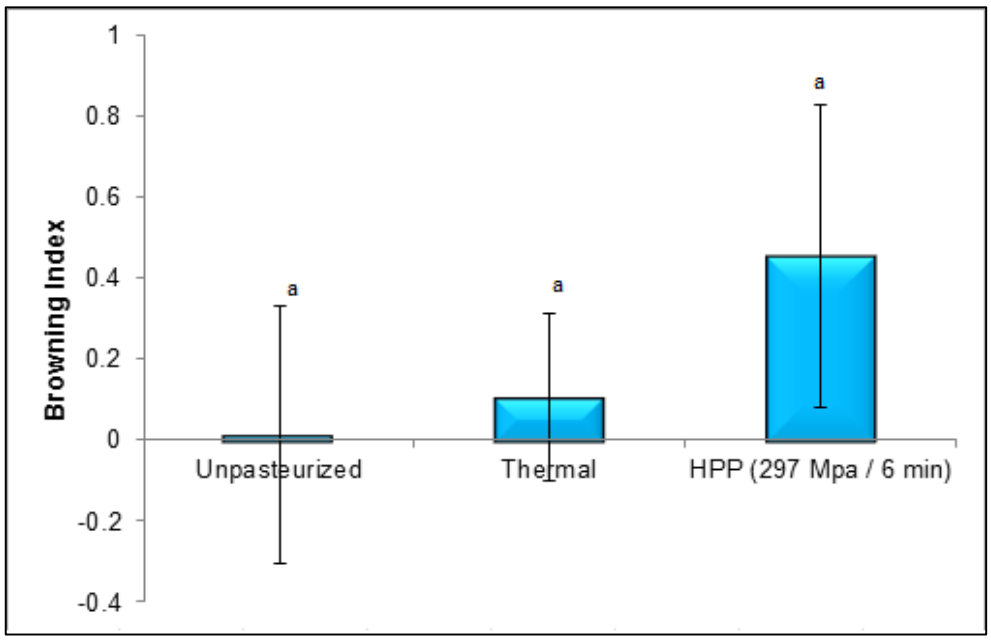

Figure 4.16: Time zero browning index of unpasteurized and processed juice samples. All values are mean±S.E. (n=3). Different alphabets indicate statistically significant differences ($p<0.05$).

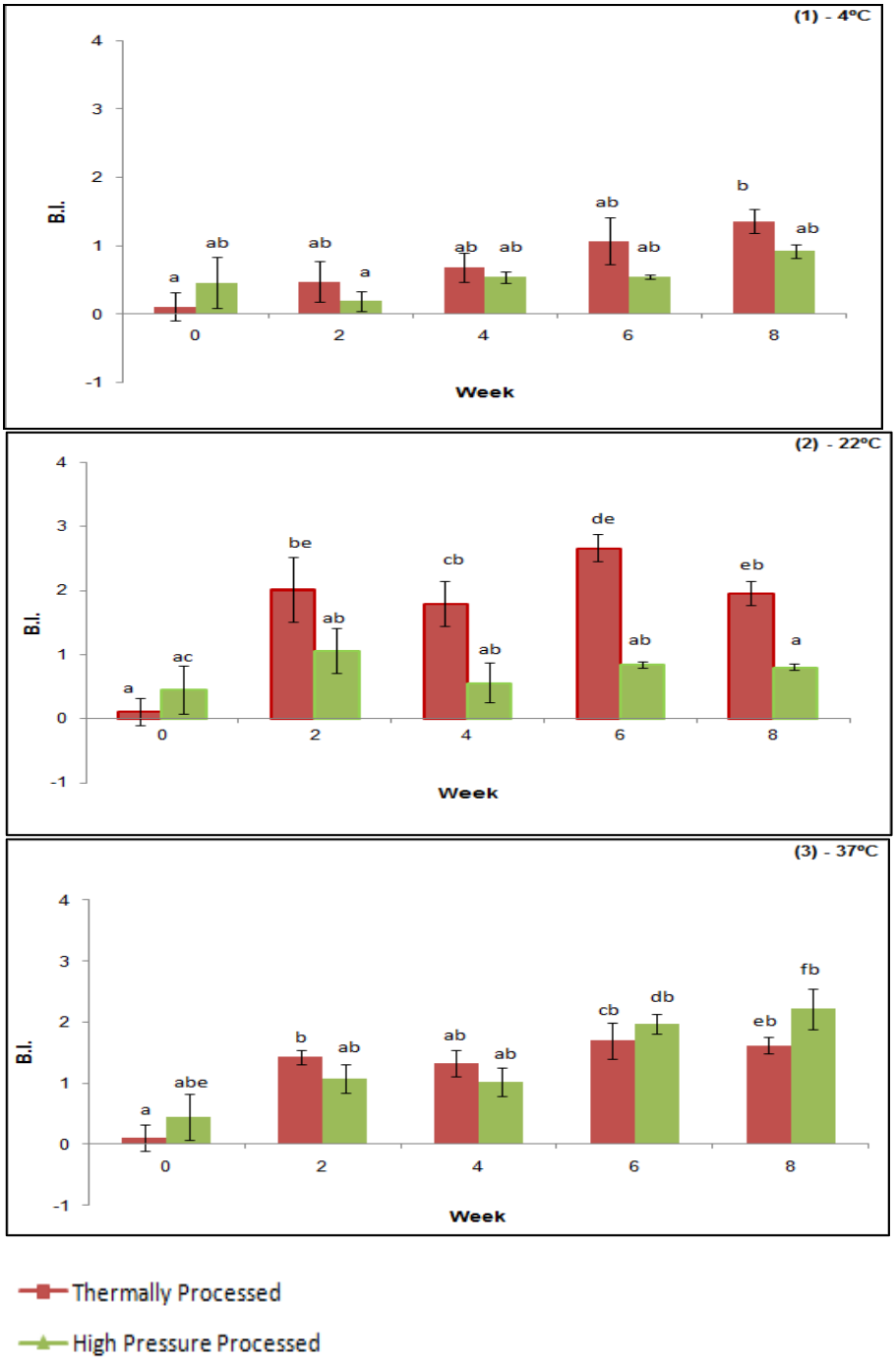

Figure 4.17: Browning index of thermally and high pressure processed samples during storage study at (1) 4 °C; (2) 22 °C and (3) 37 °C. All values are mean±S.E. (n=3). Different alphabets indicate statistically significant differences ($p<0.05$).

As mentioned earlier, ∆E* measures the difference of processed samples from the initial state. The initial state for the purpose of this study was unprocessed, fresh, single strength juice. Similar trend to browning index was observed in case of ∆E*. It was seen that this value keeps increasing with time for thermally as well as high pressure treated juice samples. Statistical analysis showed no significant difference with time, although numerical differences exist. The rate of change of color from the initial color increased at higher temperatures. Immediately after processing, it was seen that there was a rise in ∆E* values. Thus, it demonstrates that processing alters the overall color of the juice. Morris et al., (2004) stated that in a 12 month storage study, noble grape juice becomes lighter due to pigment loss.

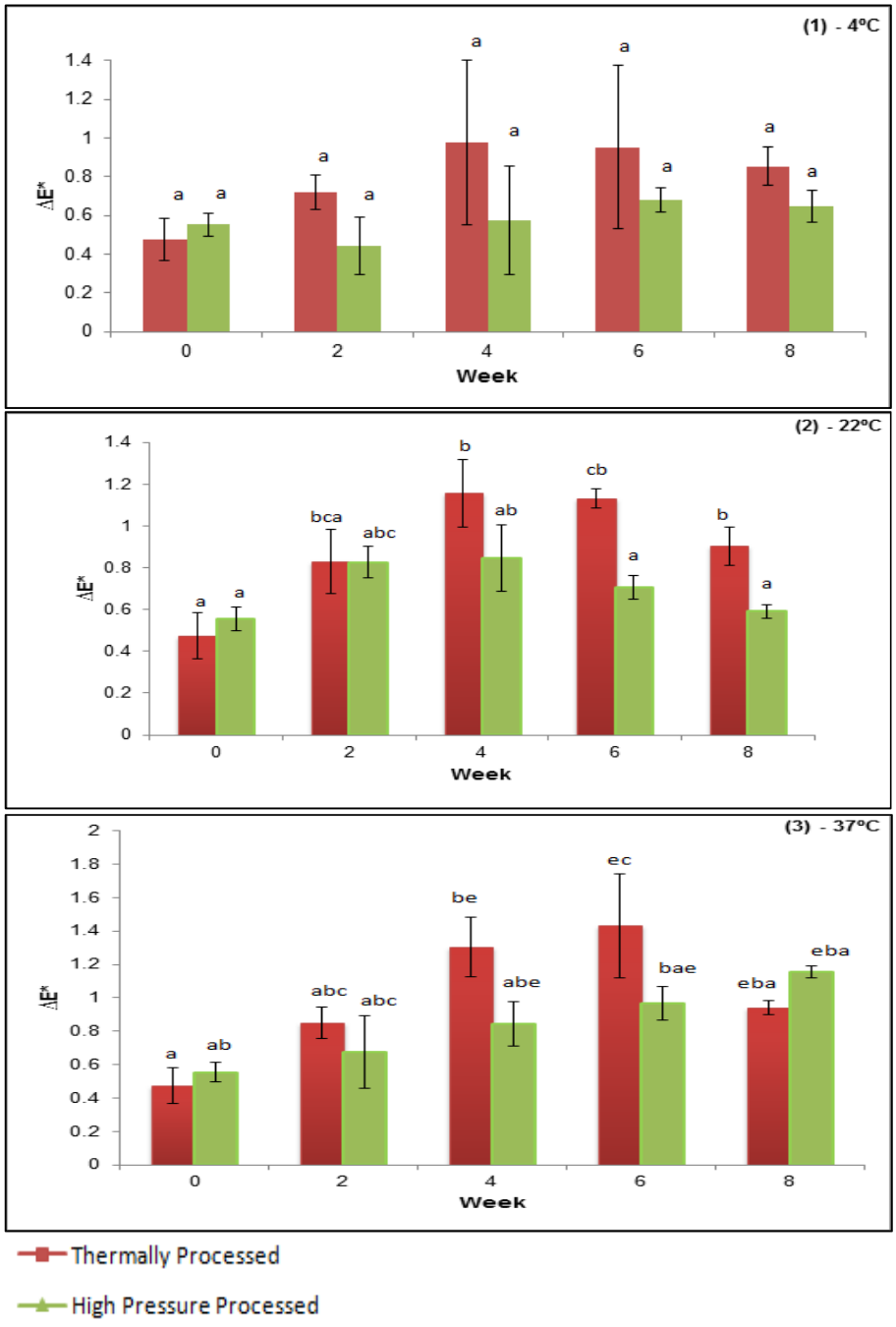

Figure 4.18: ΔE* of thermally and high pressure processed samples during storage study at (1) 4 °C; (2) 22 °C and (3) 37 °C. All values are mean±S.E. (n=3). Different alphabets indicate statistically significant differences ($p<0.05$).

4.5.4 HPLC

HPLC was performed to identify ellagic acid (EA) in juice phenols and quantify it in different samples. In order to do this, HPLC system was used with UV-Vis detector at 360 nm. External standard curve method was used for this study. External standard curve (**Fig. 4.19)** was generated by subjecting different known concentrations of ellagic acid to HPLC. Peak area was calculated and plotted against the concentration.

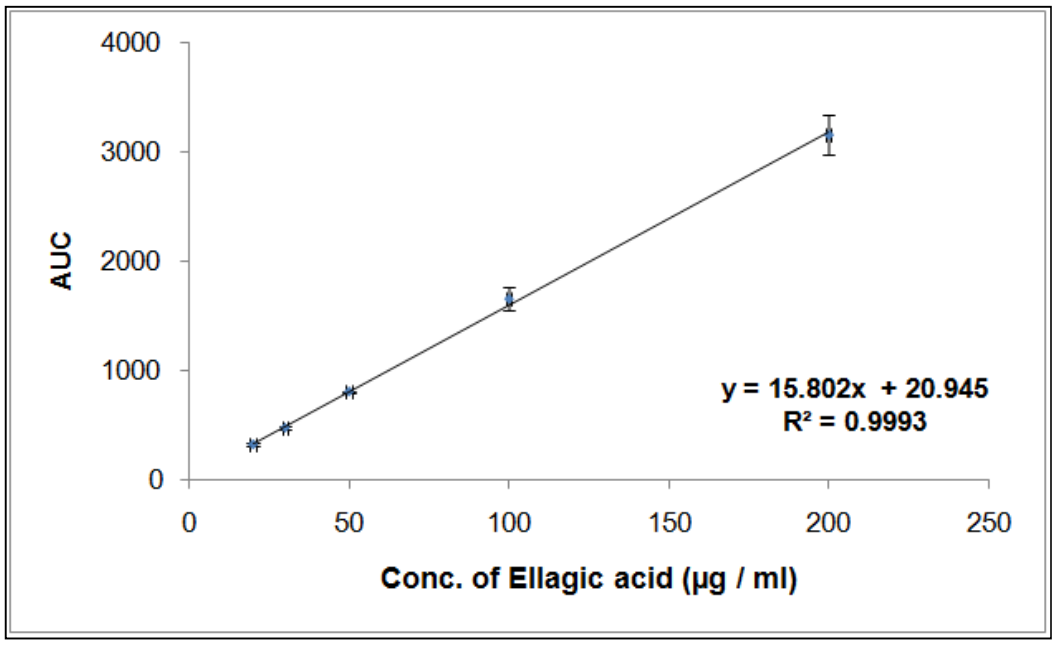

Figure 4.19: Standard curve for ellagic acid determination. All values are mean±S.E. (n=3).

Following the standard curve, different juice extracts were subjected to HPLC and the results are shown in the **Fig. 4.20** and **Fig. 4.21.** The first one shows the immediate difference in ellagic acid content before it goes for storage. It was found that no significant changes were found in grape juice samples that are processed differently. During storage, no significant change was observed in the ellagic acid content of thermally processed samples with

respect to temperatures. On the other hand, in the case of high pressure processed juice, there was a 54% increase in ellagic acid content when stored at 22 ℃. When the two treatments were compared, they showed a significant difference, high pressure being higher in EA content. Slight increase in the ellagic acid content immediately after thermal treatment could be due to hydrolysis of ellagitannins giving free ellagic acid. HPP might lead to similar changes in several precursors of ellagic acid during storage, thus giving higher result for free ellagic acid after 8 weeks. It still needs to be determined if this is desirable as free ellagic acid is partially responsible for the formation of insoluble sediments in juice in addition to other juice constituents such as metal ions or insoluble pectins (Lee, 2004).

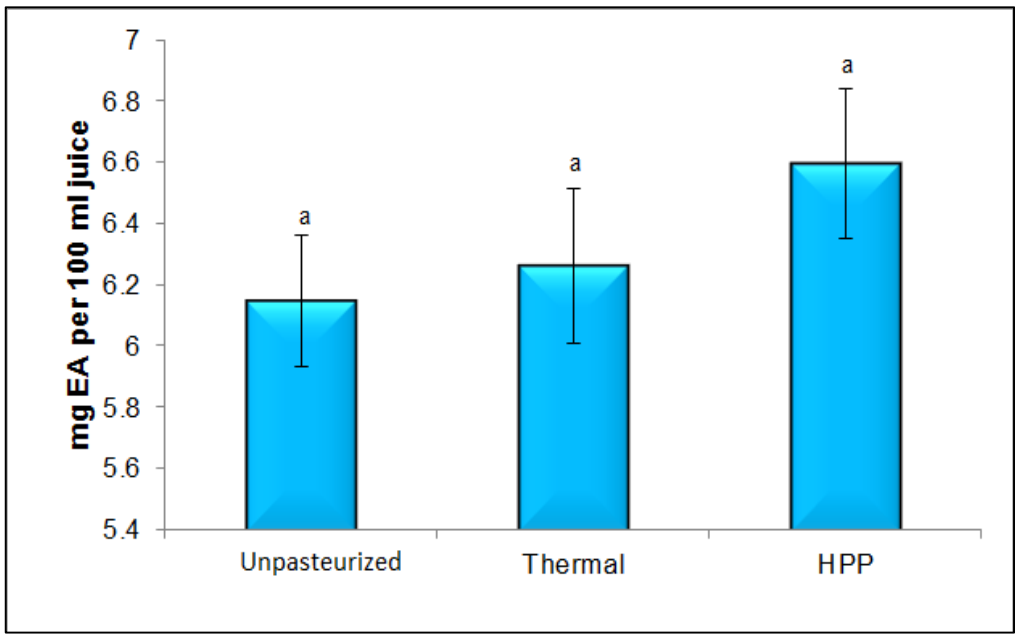

Figure 4.20: Time zero ellagic acid content of unpasteurized and processed juice samples. All values are mean±S.E. (n=3). Different alphabets indicate statistically significant differences ($p<0.05$).

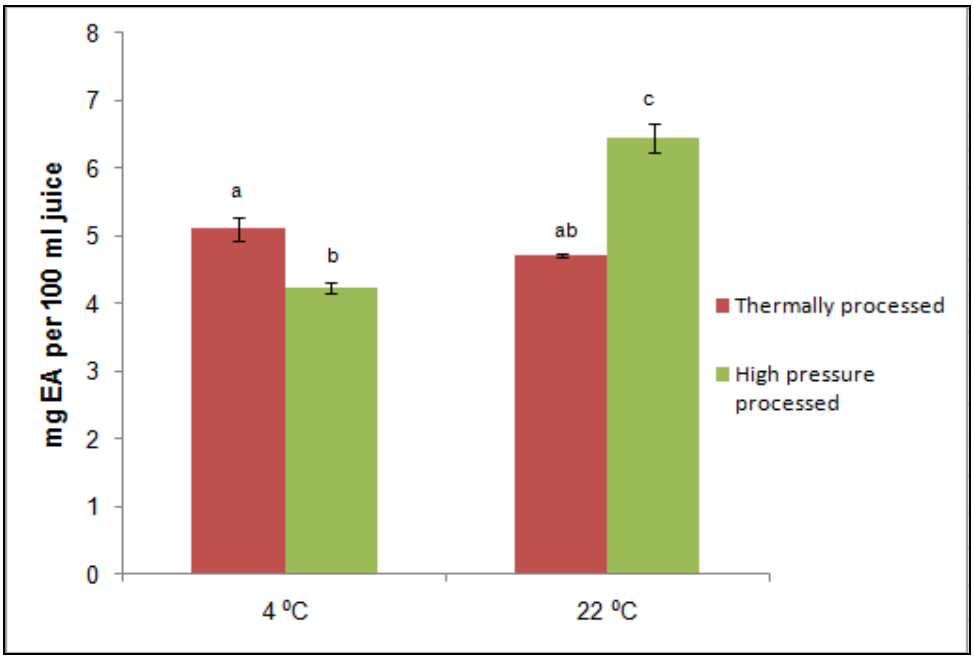

Figure 4.21: Ellagic acid content in thermally and high pressure processed samples after 8 weeks of storage. All values are mean±S.E. (n=3). Different alphabets indicate statistically significant differences ($p<0.05$).

5 CONCLUSIONS

Based on the study to evaluate the effect of processing on the quality parameters, the overall conclusions drawn were as follows:

- Total Phenolics: It was done using Folin – Ciocalteu method. No significant difference was found between unprocessed, thermally processed and some HPP processed juices (275MPa / 10min, 297MPa / 6min and 297MPa / 10 min). They all gave values around 165 mg GAE per 100 ml juice. These values were comparable to that of commercially available Concord grape juice (176 mg GAE per 100 ml juice). As pressure was increased, there was on an average 8% drop in total phenolic value. This may be due to condensation reactions of polyphenols. So, HPP did show an impact on total phenolic value
- Chemical Antioxidant Activity: This was found using the ORAC assay. It was found that processing did not have any significant impact on the ORAC value of juices. Before and after processing, the ORAC value still remained higher than that of Concord grape juice (2416 µMTE per 100 ml juice)
- Cellular Antioxidant Activity: No significant difference was found between the CAA values of unpasteurized and thermally processed sample. Similar result was obtained when comparing unpasteurized and high pressure processed sample. But there was 77% increase in CAA value when thermally processed sample was compared to high pressure processed product, showing that the type of processing did

affect CAA. This could be due to bioavailability and metabolism of antioxidants in physiological environment

- A 8 week storage study of samples at 4 °C, 22 °C and 37 °C showed the effect of storage and processing on grape juice quality parameters. During storage studies, it was seen that time and treatment had no effect on the total phenolics level of grape juice samples. ORAC value showed a decrease with time at 22 °C. However, there was no significant difference with respect to the treatments given to juice
- L*, a* and b* values were found using Konica Minolta chroma meter. These values were used to calculate browning index and ∆E* for the samples. In case of browning index, it was seen that the rate of increase in browning was higher for thermally processed samples than high pressure processed samples. However, both the samples showed increase in browning with time. ∆E* had similar results as browning index. It increased with time for all the samples. The increase was higher at higher temperature. The main reason for color change could be pigment loss and enzymatic activity
- HPLC results showed a higher ellagic acid content in juice that is treated by high pressure processing than thermally treated juice. This could mainly result because of structural changes in precursors during storage leading to formation of free ellagic acid.

6 FUTURE WORK

- Based on the results obtained for cellular antioxidant activity of juice samples, further investigation needs to be done to find out detailed mechanism of this assay. This will help to understand obtained results in a better manner
- When studying color changes during the storage study, it was observed that the rate of increase in BI of high pressure processed samples was less than that of thermally processed samples. But in both cases, BI increased with time when compared to the fresh, unpasteurized juice. Enzyme activity is one of the important speculated reasons for this. So studies on enzyme activity and enzymatic reactions can be done to support these results
- HPLC analysis for ellagic acid can be done for all the storage study samples to get the entire trend of the changes in ellagic acid content occurring during storage.

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8 APPENDIX

Appendix A

1. 3-way ANOVA table for total phenolics storage study

Analysis of Variance

Source	Sum Sq.	d.f.	Mean Sq.	F	Prob>F
Temperature	124.5	2	62.248	4.01	0.0232
Time	2181.89	4	545.473	35.14	0
Treatment	30.71	1	30.708	1.98	0.1647
Temperature*Time	43.96	8	5.495	0.35	0.9403
Temperature*Treatment	23.3	2	11.65	0.75	0.4765
Time*Treatment	148.23	4	37.058	2.39	0.0609
Temperature*Time*Treatment	39.5	8	4.938	0.32	0.9561
Error	931.27	60	15.521		
Total	3523.35	89			

2. 3-way ANOVA table for ORAC storage study

Analysis of Variance

Source	Sum Sq.	d.f.	Mean Sq.	F	Prob>F
Temperature	284404.2	2	142202.1	1.25	0.2949
Time	6886830.7	4	1721707.7	15.09	0
Treatment	321733.2	1	321733.2	2.82	0.0983
Temperature*Time	422013	8	52751.6	0.46	0.8776
Temperature*Treatment	200350.9	2	100175.5	0.88	0.4209
Time*Treatment	1700968.2	4	425242	3.73	0.0089
Temperature*Time*Treatment	1327492.5	8	165936.6	1.45	0.1932
Error	6845163.3	60	114086.1		
Total	17988956	89			

3. 3-way ANOVA table for color (BI) storage study

Analysis of Variance

Source	Sum Sq.	d.f.	Mean Sq.	F	Prob>F
Temperature	7.9233	2	3.96165	20.03	0
Time	17.1602	4	4.29005	21.69	0
Treatment	2.8064	1	2.80639	14.19	0.0004
Temperature*Time	3.9938	8	0.49923	2.52	0.0195
Temperature*Treatment	4.5977	2	2.29884	11.62	0.0001
Time*Treatment	3.0522	4	0.76305	3.86	0.0074
Temperature*Time*Treatment	2.5691	8	0.32113	1.62	0.1371
Error	11.8666	60	0.19778		
Total	53.9693	89			

4. 3-way ANOVA table for color (ΔE*) storage study

Analysis of Variance

Source	Sum Sq.	d.f.	Mean Sq.	F	Prob>F
Temperature	0.8067	2	0.40333	4.79	0.0118
Time	2.583	4	0.64575	7.66	0
Treatment	0.8202	1	0.82017	9.73	0.0028
Temperature*Time	0.5061	8	0.06327	0.75	0.6468
Temperature*Treatment	0.0114	2	0.00568	0.07	0.9349
Time*Treatment	0.7302	4	0.18254	2.17	0.0837
Temperature*Time*Treatment	0.3245	8	0.04057	0.48	0.8647
Error	5.057	60	0.08428		
Total	10.8391	89			

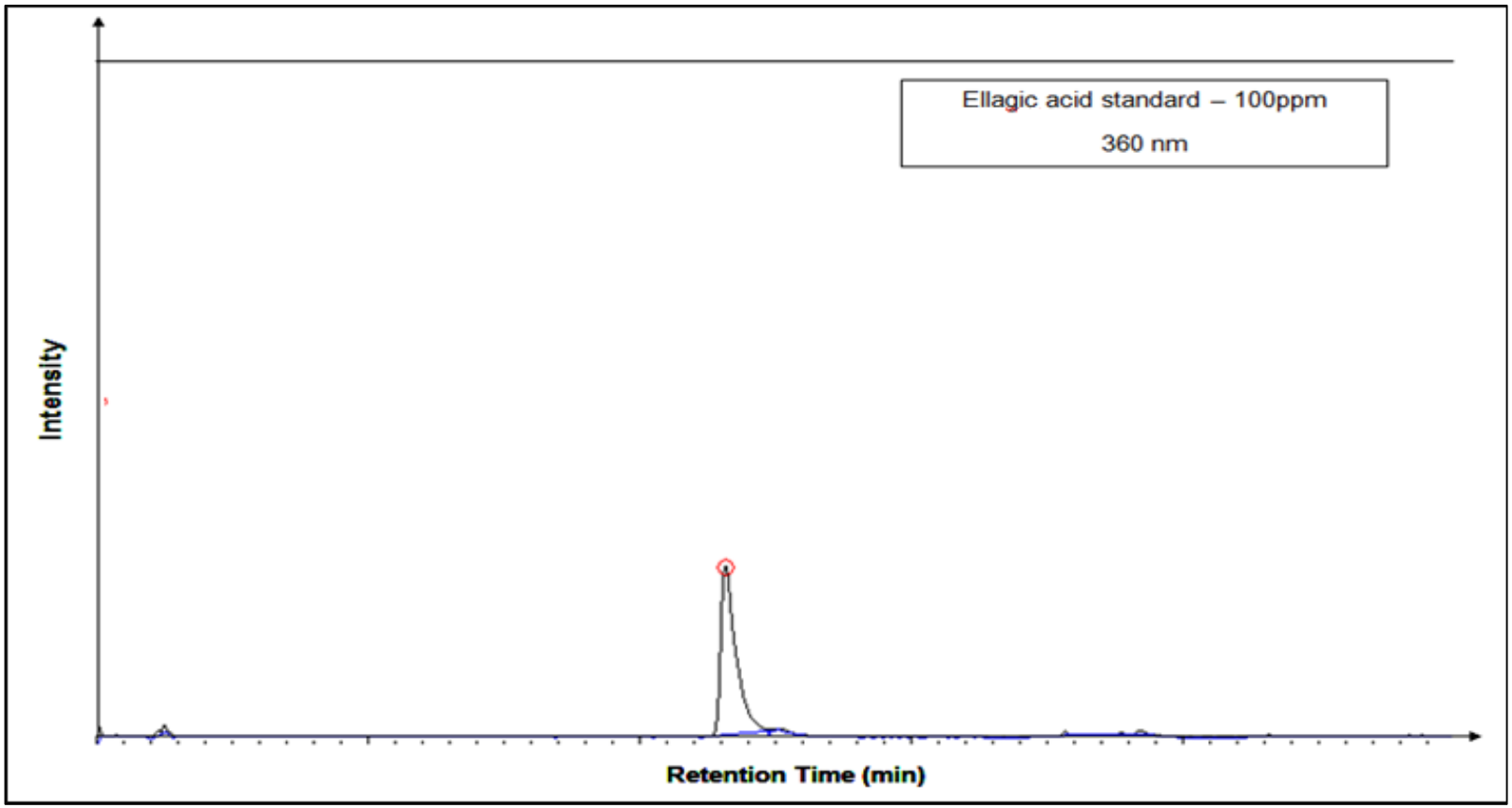
5. 3-way ANOVA table for ellagic acid content storage study

Analysis of Variance

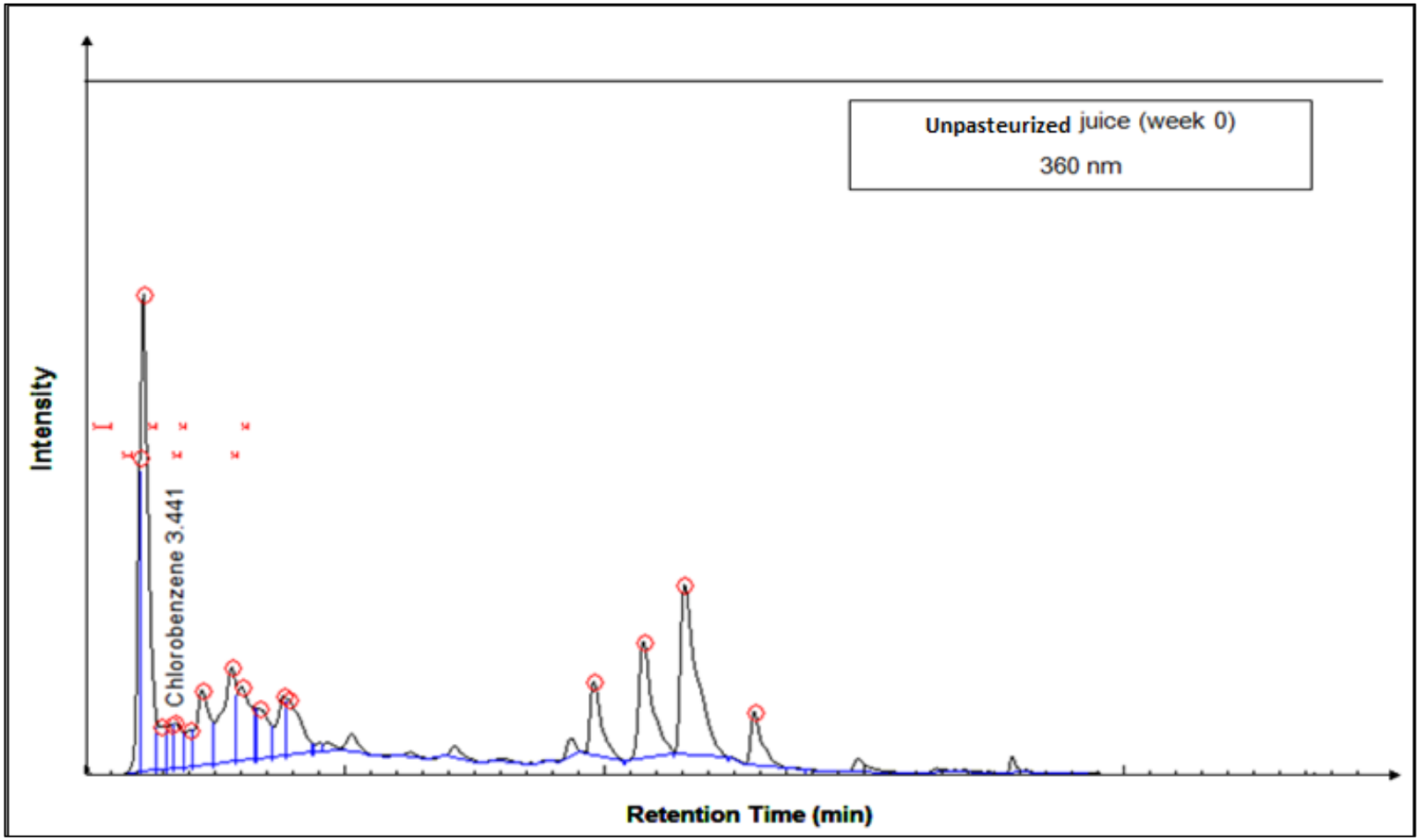
Source	Sum Sq.	d.f.	Mean Sq.	F	Prob>F
Temperature	1.2573	1	1.2573	9.97	0.0061
Time	10.21	1	10.21	80.94	0
Treatment	0.872	1	0.872	6.91	0.0182
Temperature*Time	1.2573	1	1.2573	9.97	0.0061
Temperature*Treatment	2.5903	1	2.5903	20.53	0.0003
Time*Treatment	0.0142	1	0.0142	0.11	0.7416
Temperature*Time*Treatment	2.5903	1	2.5903	20.53	0.0003
Error	2.0183	16	0.1261		
Total	20.8097	23			

Appendix B

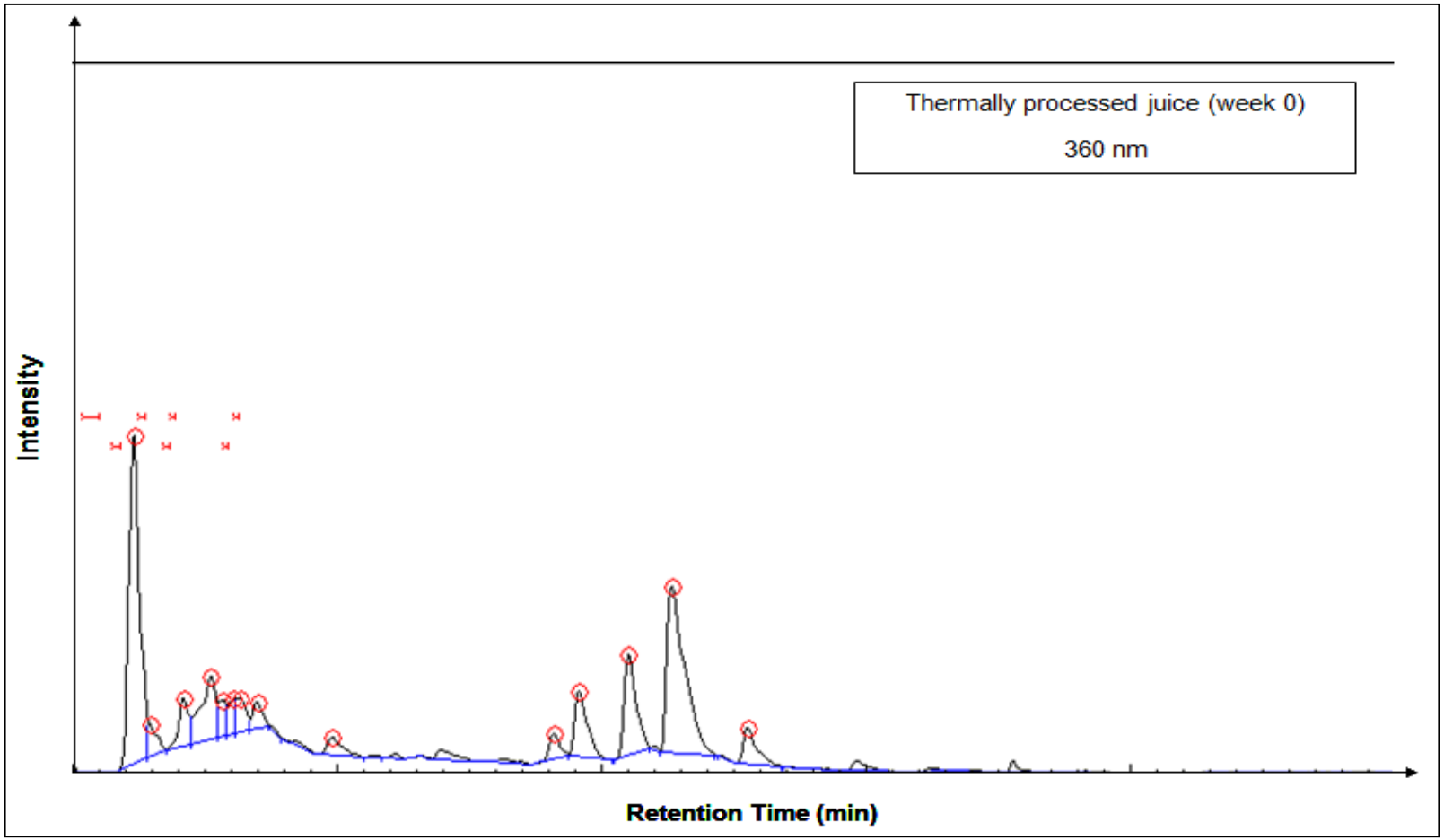
1. HPLC chromatogram: Ellagic acid standard



2. HPLC chromatogram: Unpasteurized juice sample – Week 0



3. HPLC chromatogram: Thermally processed juice sample – Week 0



4. HPLC chromatogram: High pressure processed juice sample – Week 0