MICRO-OXYGEN PROCESSING AND BIOCHEMICAL DETERIORATIVE VECTORS IN BANANAS AND ORANGE JUICE

By

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(Under the Direction of Louise Wicker)

ABSTRACT

The effect of micro-oxygen (MO) and atmospheric processing on polyphenol oxidase (PPO) in bananas and orange juice quality was evaluated. PPO activity decreased non-linearly with decreasing oxygen concentration from 198 Units/ml to 45 Units/ml, at atmospheric and micro-oxygen (8ppm) concentrations. The decrease of PPO activity followed a second order polynomial regression (R^2>0.9). There were no quantifiable differences between Raman spectra taken on the PPO extracts at different times and different oxygen concentrations. A decreased in absorbance at longer times of assay was observed, and may be an indicative of non-Henri-Michaelis-Menton kinetics. Dissolved oxygen (DO) in orange juice processed under MO was low and consistent (34ppb to 100ppb) and not statistically different from juice processed under atmospheric conditions (92ppb to 200ppb) ($p<0.05$). The shelf life studies performed suggest that there are no significant differences in vitamin C retention and browning for juices processed under both conditions ($p<0.05$).

INDEX WORDS: micro-oxygen, PPO, catechol, ascorbic acid, color, dissolved oxygen

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CHAPTER 1

INTRODUCTION

Many deteriorative reactions in foods are due to oxidation. Oxygen allows growth of aerobic microorganisms and it is responsible for oxidation of lipids, flavors, and loss of some nutritional value. Oxygen in the headspace, entrained and dissolved in the food, and the one that migrates from outside to the inside through the package are the principal sources of oxygen availability in the product. Some products that are especially sensitive to oxidative reactions are bakery products, dry milk, meats, beer, juices, coffee, nuts and cereal products, and several fruits and vegetables. Even though the foods may be microbiologically sterile due to heat treatment, such as canned foods, they still undergo biochemical deteriorative reactions.

The fruit and vegetable industries suffer economic losses every year due to browning, off-flavors, and overall loss of quality (Ramirez et al. 2003). Browning in fruits and vegetables may be controlled by reducing oxygen concentration, addition of chelating agents and inactivation of polyphenol oxidase (PPO) (Marshall et al. 2000).

Polyphenol oxidase is found in the chloroplast thylakoid membranes, and the substrate, phenolic compounds, has been found in the vacuole of plants. If any cell disruption occurs, as the product suffer bruises, cuts, or mechanical damage, both the substrate and enzyme come into contact, as well as atmospheric oxygen, producing browning (Charraza et al. 2001). Polyphenol has the ability to react with different substrates (Ramirez et al, 2003), and there is evidence that PPO in bananas is specific towards *o*-diphenols (Palmer, 1963), being dopamine the specific

substrate used in the oxidation reaction (Griffiths, 1959). Banana PPO catalyzes the hydroxylation of *o*-diphenols to produce *o*-quinones that are rapidly, non-enzymatically polymerized to brown pigments (Vamos-Vigyazo, 1981).

Since oxygen is necessary to start the enzymatic reaction (Martinez and Whitaker, 1995), it has been hypothesized that reduction of O_2 will delay enzymatic browning. Several studies have shown that reduction or elimination of oxygen, satisfactorily reduces PPO activity, i.e. storage of longan fruit in 5% oxygen atmosphere (Cheng et. al. 2009), however, other studies suggest that low oxygen does not eliminate or reduce browning in carambola fruit, stored at 0.4% O_2, and apple slices stored at 0.25 kPa O_2 (Teixeira et al. 2008; Gil et al. 1998) respectively. In addition, air is present, intracellularly and intercellularly, in fruit and vegetable tissues. The entrained and dissolved oxygen in the fruit may be responsible of deteriorative reactions (Garcia-Torres et al. 2009) being enough to cause browning.

Several methods to reduce oxygen in foods have been developed, through the use of oxygen scavengers, vacuum, oxygen- barrier packaging, and flush with inert gases. In this study, the use of micro-oxygen processing is proposed to extend shelf life of foods. Micro-oxygen processing is defined as the use of oxygen levels in both the product and the processing environment that would reduce biochemical reactions to levels that are too low to be measured by conventional methods (Brody, 2005). Since there is equilibrium between the oxygen present in the headspace and the oxygen dissolved or occluded in the food, if micro-oxygen levels are achieved in the processing environment, therefore the same levels of oxygen would be present in the food. The oxygen concentration necessary to be achieved for micro-oxygen processing has been targeted as 30 ppm.

The first objective of this work is to study the effect of different oxygen concentrations on PPO activity in bananas. The second part of this work focuses on shelf life studies of orange juice processed and packaged under micro-oxygen conditions. Orange juice is one of the most common in juice production (Garcia-Torres et al., 2009). During conventional juice processing, the cells of the fruit break allowing air to come into the mix. Biochemical reactions start to take place due to the release of enzymes that were previously in compartments within the cells (Garcia-Torres et al. 2009). The enzymes, and the oxygen present in the atmosphere, are responsible for chemical and biochemical reactions that result in browning and loss of Vitamin C in orange juice (Joslyn et al. 1934) as well as oxidation of oils; therefore there is a decrease in quality and nutritional value.

Ascorbic acid degradation occurs both in presence and in absence, although insignificant, of oxygen, and it is catalyzed by metal ions, like Cu^{2+} and Fe^{3+}, accelerated by heat, and it is influence by pH (pH optimum for aerobic degradation is ~ 2.5-5.5 and pH optimum for anaerobic degradation is ~ 3 to 4 for anaerobic), oxygen and water activity (Gregory, 1996).

Color changes are a result of non-enzymatic and enzymatic browning. In orange juice processing, the pasteurization step to reduce the microbial load is 5 log, is enough to inactivate enzymes in the raw fruit that may be responsible for enzymatic browning, such as PPO (Garcia-Torres et al. 2009). However, non-enzymatic browning is of concern in the orange juice industry. Non-enzymatic browning is the product of ascorbic acid and sugar degradations (Adams and Brown, 2007). Oxygen plays an indirect role in this type of browning. By oxidizing ascorbic acid to dehydroascorbic acid with formation of furfural, a brown color is imparted to the juice (Garcia-Torres et al. 2009). Particularly, during oxidation of ascorbic acid, carbonyls are formed

which then react with amino groups and polymerize resulting in brown pigments formation (Graumlich et al. 1986).

In this study the use of micro-oxygen processing is proposed to extend shelf-life of orange juice. Quality parameters such as vitamin C retention, browning, cloud, and particle size are measured for juice processed under micro-oxygen and atmospheric conditions.

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CHAPTER 2

LITERATURE REVIEW

Many deteriorative reactions in foods are due to oxidation. Oxygen allows growth of aerobic microorganisms and it is responsible for oxidation of lipids, flavors, color and loss of nutritional value. Hence the importance of reducing oxygen in foods in order to have longer shelf life. Oxygen in the headspace, entrained in the food, and the one that migrates from outside to the inside through the package are the principal sources of oxygen availability in the product. Some products that are especially sensitive to oxidative reactions are bakery products, dry milk, meats, beer, juices, coffee, nuts and cereal products, and most fruits and vegetables.

Several methods to reduce oxygen in foods have been developed, through the use of oxygen scavengers, vacuum, oxygen- barrier packaging, flush with inert gases, among others. A new processing and packaging method is being proposed in this work, the use of micro-oxygen processing to extend shelf life of foods.

2.1. Micro-oxygen processing

Micro-oxygen processing is defined as the use of oxygen levels in both the product and the processing environment that would reduce biochemical reactions to levels that are too low to be measured by conventional methods (Brody, 2005). In personal communications with Dr. Brody and Dr. Wicker, they have hypothesized the existence of bound oxygen, as it has been proven for water (Bell, 2007), that cannot be removed and that it may be available for biochemical

reactions. Since there is equilibrium between the oxygen present in the headspace and the oxygen dissolved or occluded in the food, if micro-oxygen levels are achieved in the processing environment, therefore the same levels of oxygen would be present in the food. The oxygen concentration necessary to be achieved for micro-oxygen processing has been targeted as 30 ppm and 30 ppb for nano-oxygen (Personal communication with Dr. Aaron Brody and Dr. Louise Wicker, 2008).

An important challenge in this technology is the growth of anaerobic microorganisms, such as *Clostridium botulinum*. Due to this challenge, research has been done with high acid foods acids foods (pH<4.5) to avoid this problem. Once the technology has been studied and proved for high acid foods, research will be done on low acid foods.

2.2. Rationale behind micro-oxygen processing

Several factors affect the quality of foods such as microbial growth, enzymatic activity, moisture, and oxidative and non-oxidative reactions. Even though the foods may be microbiologically sterile due to heat treatment, such as canned foods, they still undergo biochemical deteriorative reactions. For instance, spoilage caused by aerobic bacteria, enzymes that catalyze oxidative reactions, as well as other biochemical adverse changes such as lipid oxidations, protein oxidations, flavor oxidation, color changes and loss of nutritional value. Generally, oxygen is a co-substrate of several deteriorative reactions; therefore, reducing oxygen would retard those reactions and would help extend quality and shelf life.

Products like orange juice, tomato sauce, chilled fresh meats, peanuts, peanut butter, oily seafood, beer, green vegetables, light colored fruits and salty snacks, may benefit from this

technology. The objective is to produce foods that are shelf stable for longer time than those necessary to go through the regular distribution channels, or even for voyages that go to space for prolonged periods (Brody, 2005).

2.2.1. Lipid oxidation

Environmental oxygen causes chemical deteriorative reactions of lipids. Unsaturated fatty acids react with molecular oxygen through a free radical chain mechanism forming fatty acid hydroperoxides (Nawar, 1996). Although less frequent, oxidation of saturated fatty acids is also possible. The resulting compounds are those that impart 'rancid' characteristics with strong flavors and odors caused by acids, alcohols, carbonyls, esters, aldehydes and ketone formation (Nawar, 1996). The lipid oxidation reaction, also as auto-oxidation is shown below (Richards, 2007):

- Initiation:

$$LH + X \rightarrow L\bullet + XH$$

- Propagation:

$$L\bullet + O2 \rightarrow LOO\bullet$$

$$LOO\bullet + LH \rightarrow LOOH + L\bullet$$

$$LOOH + metal(n) \rightarrow LO\bullet + metal(n+1) + OH-$$

$$LO\bullet + LH \rightarrow LOH + L\bullet$$

- Termination:

$$LOO\bullet + L\bullet \rightarrow LOOL$$

$$LO\bullet + L\bullet \rightarrow LOL$$

Where:

LH= molecule of lipid

L● = lipid free radical

LOO●= peroxyl free radical

LO●= Alcoxyl free radical

LOOH= lipid hydroperoxide

X= molecule from which hydrogen may be extracted

Oxygen reduction may retard the auto-oxidation reaction by reducing peroxyl free radical formation (Richards, 2007). The potato chip industry uses nitrogen flushing in the packaging step to displace oxygen and retard deteriorative reactions that cause off-flavors. Also, argon has been used with promising results in extension of shelf life and acceptability of the product (Spencer and Humphreys, 2003).

2.1.1. Nutrient loss

As a result of oxidative reactions, some nutrients are lost. For instance, ascorbic acid (Vitamin C) can be oxidized by oxygen, decreasing the nutritional value of citrus products. Vitamin E, also oxidizes donating hydrogen atoms in the presence of oxygen (Richards, 2007). Also, as mentioned above, lipids like polyunsaturated fatty acids, such as omega 3 and omega 6

can be easily oxidized (Decker et al. 2005) which have been proved to be highly beneficial in healthy diets (Venegas-Caleron et al. 2010).

2.1.1. Color oxidation

Browning reactions in foods maybe either non-enzymatic or enzymatic (Martinez and Whitaker, 1995). Non-enzymatic browning, known as the Maillard reaction, is the result of chemical reactions that involves an amino compound, a reducing sugar and water. The reaction is a nucleophilic attack by the nitrogen electrons of the carbonyl group of the open chain sugar. Water is lost and the ring closes forming glycosamine. Then Amadori compound rearrangements take place to eventually produce melanoidin, brown pigments, and Strecker degradation of other dicarbonyl compounds occur (Daniel et al. 2007). This kind of browning does not require oxygen to happen.

Enzymatic browning requires oxygen to take place. This kind of oxidation is seen in fruits, such as bananas, or apple slices that are left exposed to air. It is also seen in vegetables such as potatoes and lettuce (Daniel et al. 2007) and even seafood (Marshall et al. 2000). The reaction involves a phenolic substrate and oxygen along with polyphenol oxidase that catalizes the reaction.

2.3. Polyphenol oxidase and oxidative reactions

The fruit and vegetable industries suffer economic losses every year due to browning, off-flavors, and overall loss of quality (Ramirez et al. 2003). Polyphenol oxidase (PPO) is a copper-containing enzyme that catalyzes the browning reaction; therefore it is important in determining quality of the food.

Polyphenol oxidase is found in the chloroplast thylakoid membranes, and the substrate, phenolic compounds (Table 1.1), has been in the vacuole in plants. If any cell disruption occurs, as the product suffer bruises, cuts, or mechanical damage, both the substrate and enzyme come into contact, as well as atmospheric oxygen, producing browning (Charraza et al. 2001). Polyphenol oxidase also known as tyronase, phenolase, catechol oxidase, catecholase, o-diphenol oxidase, monophenol oxidase, and cresolase has the ability to react with different substrates (Ramirez et al, 2003). Polyphenol oxidase may be specific towards monophenolic and diphenolic substrates. The reaction consists of the hydroxylation of monophenols to *o*-diphenols by monophenolase (EC 1.14.18.1) (Sojo et al. 1998), or the hydroxylation of *o*-diphenols to produce *o*-quinones that are rapidly, non-enzymatically polymerized to brown pigments (Vamos-Vigyazo, 1981). PPO that catalyzes this reaction is classified as EC 1.10.3.1 (Ramirez et al. 2003) (figure 1.1). There is a third type of this enzyme, laccase (EC 1.10.3.2) that uses a different mechanism for oxidation than the other two (Ramirez et al. 2003).

Apricots, apples, peaches, strawberries, bananas, potatoes, lettuce, olives, and seafood are subjected to this kind of reaction (Martinez and Whitaker, 1995), (Ramirez et al. 2003), (Segovia-Bravo et al. 2009). Several methods have been implemented to reduce PPO. One of them is inactivation by heating (Ramirez et al. 2003), application of ascorbic acid (Gil et al. 1998) (Arias et al. 2007) (Ding et al. 2007), and reduction of oxygen (Gil et al. 1998), for instance, by inert gas flushing in the juice and wine industry (Martinez and Whitaker, 1995), use of cyclodextrin and polyvinylpyrrolidone for polyphenol removal, and biotechnology (Ramirez et al. 2003).

In some instances browning is necessary and even desirable. Such is the case of tea, coffee, cocoa, prunes, and fig processing (Ramirez et al., 2003), (Martinez and Whitaker, 1995), (Amiot et al. 1997)

Since oxygen is necessary to start the enzymatic reaction (Martinez and Whitaker, 1995), it has been hypothesized that elimination of oxygen will delay enzymatic browning. Several studies have shown that reduction or elimination of oxygen, satisfactorily reduces PPO activity, i.e. storage of longan fruit in 5% of oxygen atmosphere (Cheng et al. 2009), however, other studies suggest that low oxygen does not eliminate or reduce browning in carambola fruit (at 0.4% O_2) and apple slices (0.25 kPa O_2) (Teixeira et al. 2008; Gil et al. 1998) respectively. In addition, air is present, intracellularly, in fruit and vegetable tissues. The dissolved and entrained oxygen in the fruit may be responsible of deteriorative reactions (Garcia-Torres et al. 2009) being enough to cause browning.

It has been proposed that the browning reaction by PPO may be first order for 80% of the reaction (Duckworth and Coleman, 1969). The two substrates of the reaction are phenolic compounds and oxygen. It has been proposed that oxygen is the first substrate that binds to PPO (Amiot et al. 1995). If foods are processed under low oxygen levels, one of the substrates is limiting, therefore it may be considered as a first order reaction. However, the order of the reaction is not fully understood, since PPO can still be active at low oxygen concentrations.

2.4. Use of low oxygen processing and packaging in the beer and citrus industry

Unless avoided, oxygen contacts food during preparation or processing. Moreover dissolved or occluded oxygen may be responsible for many of the deteriorative reactions

affecting the quality of several products. In addition, presence of residual oxygen in sealed and barrier packages that diffuses the headspace, and oxygen that permeates through plastic materials, and any opening of the package will promote undesirable changes. Both the beer and citrus industries are affected by oxygen, and use low oxygen processing and packaging in order to decrease oxygen negative effects. An overview of both will be presented.

2.4.1. Oxygen and beer

Beer is one product that benefits from oxygen, but also suffers quality damage due to it, depending on the processing stage. When oxygen is limited in the fermentation process, it has been found that esters compounds increase, imparting off-flavors (Lewis and Young, 1995). Also, oxygen is necessary for the germination of the barley embrio in the malting process. Oxygen is also necessary in the oxidation and polymerization of polyphenolic compounds that clarify the final product (Kuchel et al. 2005). Other authors say that when the mashing step during brewing is done under low oxygen pressures, the enzymatic oxidation of polyunsaturated fatty acids is lower than at higher oxygen pressures and higher antioxidant capacity (like tannins and anthocyanogens released from the barley) and that provide protection against non-enzymatic browning (Arts et al. 2007).

However, when the beer is finished and stored, oxygen becomes a problem. Oxidation reactions may affect the flavor, aroma, and color, decreasing the shelf life of the product (Kuchel et al. 2005). Numerous efforts have been made to keep the total package oxygen as low as possible. BP Corporation of North America has created a bottle, U.S. Patent No. 6,558,762 (Cahill et al. 2003) that allows just 1 ppm of oxygen to ingress the package through the use of an oxygen scavenger, extending the beer's shelf life for up to 6 months (Kuchel et al. 2005).

Changes in color may be desirable or undesirable, depending on the type of beer. Both enzymatic and non-enzymatic (Maillard reaction) browning take place in beer (Bamforth, 2000). A formation of a purple pigment, hordeumin, that is an anthocyanin-tannin pigment formed during the fermentation of barley, is the result of an oxygen mediated reaction that causes it to precipitate. The use of antioxidants inhibits the formation of this pigment (Deguchi et al. 1999).

Oxygen that ingresses the package is responsible for oxidation of fatty acids, alcohols, and iso-alpha acids (Kuchel et al. 2005). In presence of oxygen, carbonyl compounds are formed, developing stale characteristics in beer (Bamforth, 2000). Carbonyls are formed by Strecker degradation of aminoacids, melanoidin mediated oxidation of higher alcohols, oxidative degradation of iso-alpha acids, aldol condensation of short chain aldehydes and enzymatic and non-enzymatic degradation of fatty acids (Kuchel et al. 2005). Also, oxygen radical formation from hydrogen peroxide is responsible for aldehyde and ketone formation affecting beer flavor (Kuchel et al. 2005). This reaction may happen through metal catalysis:

- Haber-Weiss reaction (Kuchel et al. 2005)

$$Cu^{2+} + O_2^- \rightarrow Cu^+ + O_2$$

$$Cu^+ + H_2O_2 \rightarrow Cu^{2+} + OH^{\cdot} + OH$$

$$\text{Net: } O_2^- + H_2O_2 \rightarrow O_2 + OH^{\cdot} + OH$$

- Fenton reaction (Kuchel et al. 2005)

$$Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + OH^{\cdot} + OH^-$$

$$Fe^{3+} + H_2O_2 \rightarrow Fe^{2+} + O_2^- + 2H^+$$

Net: $2H_2O_2 \rightarrow OH^{\cdot} + OH^- + 2H^+$

Today, for the beer industry is highly important to measure and control the total package oxygen that refers to the oxygen dissolved and the oxygen in the headspace (Kuchel et al. 2005). An oxygen level of 0.27 ppm oxygen delays the formation of carbonyl compounds that decrease the shelf-life of beer (Narziss, 1993). Typical shelf life of beer is 120 days; however, with the use of oxygen scavengers in the bottle, it may be extended for up to 360 days, when 1 ppb of total package oxygen is achieved. Improvements in packaging have brought the total package oxygen to less than 50 ppb (Kuchel et al. 2005), but it is thought than even levels of 0.1 ppb are enough to cause oxidative damage (Brody, 2005).

2.4.2. Oxygen and orange juice

During juice processing, the cells of the fruit break allowing air to come into the mix. Biochemical reactions start to take place due to the release of enzymes that were previously in compartments within the cells (Garcia-Torres et al., 2009). The enzymes, and the oxygen present in the atmosphere, are responsible for chemical and biochemical reactions that result in browning and loss of Vitamin C in orange juice (Joslyn et al. 1934); therefore there is a decrease in quality and nutritional value.

Oxygen concentration in liquids is described by Henry's law, where the gas in the headspace and dissolved in the liquid are in equilibrium, and the oxygen concentration increases with pressure and decreases with temperature (Ringblom, 2004). In addition, presence of salts, such as NaCl, solutes like sugars, and acids, such as citric and ascorbic acid, reduce oxygen solubility (Sadler et al. 1988). However, measurements of dissolved oxygen in liquids are a

difficult task, since oxygen is present in air, and gets incorporated in the sample (Garcia-Torres et al. 2009).

2.5. Ascorbic acid degradation

Loss of ascorbic acid due to oxidation is extremely important in orange juice, since it decreases its nutritional value. Ascorbic acid is a polar compound, which makes it soluble in aqueous solutions. Its acidic and reducing capabilities are given by the 2,3-enediol moiety. Vitamin C, or ascorbic acid, may be present in the form of L-ascorbic acid, or L-dehydroascorbic acid, product of the oxidation and dissociation of hydrogen (figure 1.2). However, L-dehydroascorbic acid has the same vitamin activity, since it is reduced in the body to L-ascorbic acid (Gregory, 1996).

Ascorbic acid degradation occurs both in presence and in absence, although insignificant, of oxygen, and it is catalyzed by metal ions, like Cu^{2+} and Fe^{3+}, accelerated by heat, and is influenced by pH (pH optimum for aerobic degradation is ~ 2.5-5.5 and pH optimum for anaerobic degradation is ~ 3 to 4), oxygen and water activity (Gregory, 1996). One electron oxidation is shown in figure 1.3.

The oxidation process of ascorbic acid to dehydroascorbic acid is reversible; however, the hydrolytic ring cleavage that produces 2,3-diketogulonic acid is irreversible (Garcia-Torres et al. 2009). 2,3-diketogulonic acid undergoes oxidation, dehydration, and polymerization (Gregory et al. 1996). Several researchers attribute the degradation of vitamin C to a first order reaction with respect to ascorbic acid (Joslyn and Miller, 1949). However, it has been also found that at low oxygen concentrations the reaction is second order (Singh et al. 1976) (Zerdin et al. 2003). Some

other authors have concluded that in a closed system it follows first order with respect to oxygen (Wilson et al. 1995). The discrepancies between research may be because most models take into account just ascorbic acid concentration, when there are other components that may be limiting substrates, such as dissolved and entrained oxygen, and also competition for oxygen (corrosion reactions in canned juice) and other reactions that occur all together in juice (Garcia-Torres et al. 2009).

2.6. Color changes

Color changes are a result of non-enzymatic and enzymatic browning. In orange juice processing, the thermal pasteurization step to reduce the microbial load is 5 log, is enough to inactivate enzymes in the raw fruit that may be responsible of enzymatic browning, such as PPO (Garcia-Torres et al. 2009). However, non-enzymatic browning is of concern in the orange juice industry.

Non-enzymatic browning is the product of ascorbic acid and sugar degradations (Adams and Brown, 2007). Oxygen plays an indirect role in this type of browning. By oxidizing ascorbic acid to dehydroascorbic acid with formation of furfural, a brown color is imparted to the juice (Garcia-Torres et al. 2009).

2.7. Aroma changes

As mentioned earlier, oxygen is responsible for ascorbic acid degradation to dehydroascorbic acid. Dicarbonyls are present in dehydroascorbic acid, and they participate in the Strecker degradation with further formation of aldehydes with a carbon chain, such as

methional and acetaldehyde, responsible for imparting aroma (Garcia-Torres et al. 2009). However, oxygen does not have a direct effect on aroma of orange juice (Ruiz Perez-Cacho and Rouseff, 2008). The stability of orange juice during five months at 22℃ and dissolved oxygen concentrations of 0.6, 1.8, 6.5 and 10.1 ppm showed that reduction of oxygen did not improve sensory characteristics of the juice (Trammell et al. 1986). Another study used oxidase-catalase to reduce the dissolved oxygen content to 1ppm, but observed no improvements in the shelf-life of pasteurized juice (Sagi et al. 1990).

2.8. Dissolved oxygen during storage

The oxygen concentration in a packaged liquid food depends on the initial concentration at the moment of packaging, the oxygen permeation through the package and the oxygen consumption by deteriorative reactions. The mass transfer of oxygen from the atmosphere to the package is described in three steps. The first is the movement of molecules from the atmosphere to the package with the subsequent dissolution; the second, the diffusion through the packaging material, and lastly desorption of oxygen and dissolution in the food (Ahrne et al.1997).

The partition coefficients outside and inside the barrier material depend on the system characteristics. For instance, the concentration of oxygen during storage of orange juice on Tetra Brik aseptic cartons changes considerably the first days of storage due to oxidative reactions (Graumlich et al., 1986), and then is kept constant because the oxygen that enters the package and the consumption by oxidation reactions balance each other (Ahrne et al.1997).

2.9. Oxygen removal. Processing techniques and packaging

The deaeration method using a vacuum (Figure 1.4) removes oxygen, along with flavors, when the pressure decreases in the headspace, releasing the air that is dissolved in the juice (Braddock, 1999). Another method that removes greater quantities of oxygen is the 'Gas sparging' (Figure 1.5). This method removes oxygen by bubbling another gas, such as nitrogen, argon, or helium to the juice, displacing oxygen (Garcia-Torres et al. 2009). The use of membrane deaerators (Figure 1.6) has also been put in practice. It consists of mass transfer between gas and liquid or two liquids without dispersion (Garcia-Torres et al. 2009). For instance, 96% of dissolved oxygen can be removed using a polypropylene hollow fiber separator from water (Cole and Genetelli, 1970). It has also been used to remove oxygen from beer, and other food applications (Gabelman and Hwang, 1999). Enzyme-based deaeration is a method that consists of the use of an enzyme, such as glucose oxidase, that consumes oxygen from both the headspace and the juice. Since it is an enzyme, it is dependent of factors such as pH, which limits applications. The product of the reaction is gluconic acid and peroxide that is later converted to water and oxygen by catalase (Garcia-Torres et al. 2009). The consumption of oxygen in the glucose oxidase reaction is of 0.5 mol of oxygen by one mol of D-glucose, and dissolved oxygen may be reduced in 30 minutes to 1 ppm at 20℃ (Sagi and Mannheim, 1990).

Lastly, the use of oxygen scavengers may reduce the oxygen content present in the headspace and that may ingress through the package during storage (Garcia-Torres et al. 2009). A comprehensive description of oxygen scavenger will be given later in this review.

2.10. Current methods to achieve low oxygen

The food packaging industry, in an effort to reduce oxygen levels in foods to extend the shelf life by retarding biochemical deteriorative reactions, have created new ways of packaging. Traditional packaging does not offer a prolonged shelf life of foods, reason why the active packaging concept arose, to extend shelf–life by changing the conditions of the packaging over time. Major concerns in the food industry are absorption of oxygen, ethylene, moisture, carbon dioxide, flavors and odors that affect the quality of the food (Vermeiren et al. 1999).

Reduction of oxygen levels during processing and packaging can be achieved by vacuum, modified atmosphere packaging (MAP), or by the use of oxygen scavengers. MAP of foods was developed in 1961(Personal communications with Dr. Aaron L. Brody, 2010), and consists of replacing the air with a mixture of gases usually nitrogen, carbon dioxide and reduced levels of oxygen (Damaj et al. 2009). It has been found that oxygen scavengers have the higher reduction potential of the three methods. A study that compared modified atmosphere packaging for potato chips, found that vacuum provides good results in fatty acid profiles, but deforms (crushes) the product. When iron based oxygen absorbents were used, the oxygen levels decreased to less than 1000 ppm. The potato chips packaged with oxygen scavengers presented the minor changes after three months compared with vacuum and modified atmosphere packaging (MAP), presenting the most effective solution (Sanches-Silva et al. 2004). Also, it has been found for other products, such as orange juice, that the use of oxygen scavengers reduces the oxygen dissolved and oxygen in the headspace to much lower levels than those in MAP (Zerdin et al. 2004)

Sometimes presence of oxygen is required to maintain some characteristics in products such as pork sausages, and fresh red meats. Oxygen enhances the bright-red color of meat but at

the same time it causes myoglobin oxidation, which is increased by mincing and further processing. Some authors have reported that 0.2% residual oxygen concentration in CO_2 atmospheres led to oxidation of myoglobin (Rousset and Renerre, 1991). A way to reduce oxygen below the critical level for discoloration, with a subsequent extension of shelf life, is through the use of oxygen scavengers (Martinez et al. 2006).

2.10.1. Oxygen scavenging technology

As mentioned before, food deterioration is caused by oxidation of food constituents or spoilage by molds in the presence of oxygen. MAP and vacuum offer appropriate packaging to oxygen sensitive food. However, they do not remove oxygen completely, and more important, oxygen that permeates through the packaging film cannot be removed by either of these techniques. On the other hand, oxygen scavenging technology absorbs residual oxygen after packaging. Iron powder oxidation, ascorbic acid oxidation, photosensitive dye oxidation, enzymatic oxidation, unsaturated fatty acids, and immobilized yeast on a solid material are some of the concepts used in developing oxygen scavenging materials. Most oxygen scavengers are iron powder based, and the oxidation is as follows (Vermeiren et al. 1999):

$$Fe \rightarrow Fe^{2+} + 2e^-$$

$$½O_2 + H_2O + 2e^- \rightarrow 2OH^-$$

$$Fe^{2+} + 2OH^- \rightarrow Fe(OH)_2$$

$$Fe(OH)_2 + ¼\ O_2 + ½H_2O \rightarrow Fe(OH)_3$$

Irreversible oxygen absorption happens immediately in the package, hence the importance of rapidly putting the scavengers into the pouches (Charles et al. 2006). Ageless® (Mitsubishi

Gas Chemical Co., Japan) is one of the most used iron-based oxygen scavengers. The sachets reduce oxygen levels to less than 0.01% (Vermeiren et al. 1999). Knowing the initial oxygen concentration at the moment of packaging, as well as the oxygen permeability of the packaging material, the needed absorbent capacity can be calculated. Therefore a scavenging system with a higher capacity will ensure absence of oxygen during storage life of the product (Vermeiren et al. 1999. The Freshilizer® Series (Toppan Printing Co., Japan), Vitalon® (Toagosei Chem. Industry Co., Japan), Sanso-cut (Finitec Co., Japan) and Freshpax (Multisorb Technologies Inc., USA), also reduce oxygen levels to less than 0.01% (http://www.multisorb.com/products/pdfs_new/FRESHPAX.pdf) (Vermeiren et al. 1999).

The sachets can be used in dry products such as roasted nuts, coffee, dried fish, cereals, species; in high fat products like potato chips and chocolate; in minimally processed foods like fresh and precooked pasta; in meat and dairy; frozen products, like fish and vegetables; bakery products, like pizza crust, bread, cakes, cookies, pastries; and beverages such as beer, fruit juice, and wine (Floros et al. 1997).

Other oxygen scavenging systems use an enzyme reactor surface that reacts with a substrate, such as polypropylene or polyethylene, to scavenge incoming oxygen. For instance, glucose oxidase, an oxidoreductase, is used in bottled beer and wine to eliminate oxygen. An example of these scavengers is Bioka O_2-absorber (Bioka, Findland) (Vermeiren et al. 1999) that reduces the oxygen concentration in the headspace to 0%. (http://www.bioka.fi/products/quality.htm). However, they are dependent of physical chemical factors such as pH, water activity, salts, temperature (Graff, 2004).

In addition to the sachet presentation, the scavengers may be incorporated into the packaging structure. The ingredients may be dispersed in a plastic, or the plastic may be made of a polymeric scavenger. Examples of these systems are Amosorb® and Amosorb®SolO2 (ColorMatrix, USA) that offer a shelf life of four to six months (colormatrix.com). The Oxbar™ system (Carnaud-Metal Box, UK) involves a cobalt-catalyzed oxidation of a nylon polymer blended in PET bottles for plastic packaging of wine, beer, sauces and beverages (Vermeiren et al. 1999). It has been found that sachets have better absorbent capacity than systems with the scavenger incorporated in the packaging film. Also, other scavengers may be incorporated as flat packets, cards, sheets or adhesive labels, such as Freshmax® (Multisorb technologies, USA).

ATCO® reduces oxygen levels without requiring moisture to activate the system. It comes pre-humidified and according to the manufacturer is supposed to be efficient regardless of the value of relative humidity. Other scavenger systems that do not need to be moisture activated are Bioka®S-100, and FreshPax®R-300. On the other hand, Ageless® and FreshPax®M-100 need moisture to be activated and therefore are recommended for moist food (Charles et al. 2006).

Other scavenging systems include ascorbic acid as an absorbent. Darex O_2-scavenging technology consists of the incorporation of the scavenger into the package barrier as crown caps, plastic or metal closures of ascorbate that is oxidized to dehydroascorbic acid and sulphite to sulphate. It is used to protect beer from oxidation (Brody, 2005).

Another study shows the oxygen reduction properties of different polymer/TiO_2 nanocomposite films, under continuous UV light. The study focuses on the scavenging effect of the photo-catalytic process. It was found that the deoxygenating process is a first order reaction and that the system presents strong oxygen scavenging activity (Xiao-e et al. 2004). Other novel

studies include the use of entrapped microorganisms in hydroxyehtyl cellulose and polyvinyl alcohol, as active compounds to be used as oxygen scavengers. The microorganisms proposed in the study are *Kocuria varians* and *Pichia subpelliculosa* (Altieri et al. 2004).

Oxygen scavengers can be used alone or in combination with MAP. Commercially, most of the atmospheric oxygen is removed by MAP and then an oxygen scavenger is applied. To increase the effectiveness of the scavenger, the packaging material should be an oxygen barrier of intermediate performance, with a permeation value of approximately 20 ml/m^2.d.atm. to protect the scavenger from saturation and subsequent loss of capability of oxygen absorption (Smith et al. 1990).

Even though all the oxygen scavengers described above present high levels of oxygen reduction to less than 0.01%, they are not widely used, in part because of their high cost and lack of sufficient technical information on their performance and how to apply them in an effective way (Damaj et al. 2008). Also, there is a possibility of being eaten along with the food (Sanchez et al. 2004).

2.11. Raman spectroscopy

Raman spectroscopy represents a very useful tool to be used in the food industry. It may help understanding chemical and structural changes in acids, such as caffeic acid (Sanchez-Cortes and Garcia-Ramos, 2000), and proteins, such as lactoglobulin (Ikeda and Li-Chan, 2004; Ikeda, 2003). Therefore the nutritional value of foods and changes due to processing may be monitored using this technology.

Raman spectroscopy allows the study of a material at the molecular level. It mainly deals with vibrations and rotations of molecules and the interactions of those with the incident electromagnetic radiation (Colthup et al. 1990). The parameters that define electromagnetic radiation are the wavelength λ, frequency ν that represents the vibrations per unit of time, and $\bar{\nu}$ representing the number of waves per unit of length:

$$\bar{\nu} = \frac{\nu}{(c/n)} = \frac{1}{\lambda} \qquad (1)$$

c = velocity of light in a vacuum (2.997925×10^{10} cm / s)

(c/n)= velocity of light in a medium with refractive index n.

ν= frequency in Hz

$\bar{\nu}$ = wavenumber in cm^{-1}

λ=wavelength in cm.

Raman spectroscopy analyses the radiation scattered by the sample that is irradiated with a monochromatic source. The photon being irradiated collides with the sample inelastically, resulting in a change on the vibrational and rotational energy of the molecule (Colthup et al. 1990). Generally, the scattered light have the same intensity as the incident beam, however just 1 in 10^7 photons present a change in energy with a subsequent change in frequency (Smith and Dent, 2005).

Vibrations of chemical bonds can be detected at different wavenumbers. Therefore, reactions can be studied and compounds can be quantified using Raman Spectroscopy. For instance, C-O stretching in phenols and OH deformations are detected at 1260-1180 cm^{-1}. Bands

have been designated for carbonyl groups, amines, aromatic rings, methyl compounds, alcohols, ethers, etc. (Colthup et al. 1990).

2.12. Summary

A comprehensive literature review that covered foods that are negatively influenced by oxygen, current methods to reduce oxygen, and tools to determine changes due to oxidation has been given in this chapter. One of the challenges the food industry faces today is the high levels of oxygen present in processing, decreasing the shelf life of food products such as beer and orange juice. In the present work, micro-oxygen processing will be evaluated and the effect of low levels of oxygen in decreasing enzymatic activity and extending shelf life.

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Table 2.1 - Phenolic compounds in fruits. Adapted from Amiot et al., 1997

Skeleton	Class	Plant
C_6-C_1	Hydroxybenzoic acids	Strawberries
C_6-C_3	Hydroxycinnamic acids/Coumarins	Apples, potatoes/Citrus
C_6-C_1-C_6	Xanthones	Mango
C_6-C_3-C_6	Flavonoids/Isoflavonoids	Fruits/soybeans
$(C_{15})n$	Tannins	Grape, persimmon

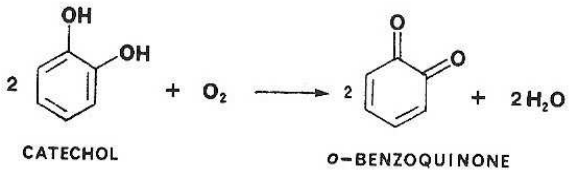

Figure 2.1 - Catechol oxidation by Polyphenol oxidase. From Ramirez et al. 2003

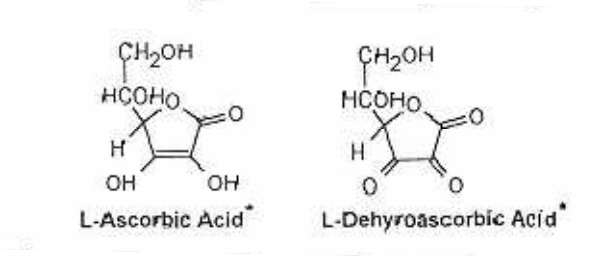

Figure 2.2 - L-ascorbic acid and L-dehydroascorbic acid. From Gregory, 1996.

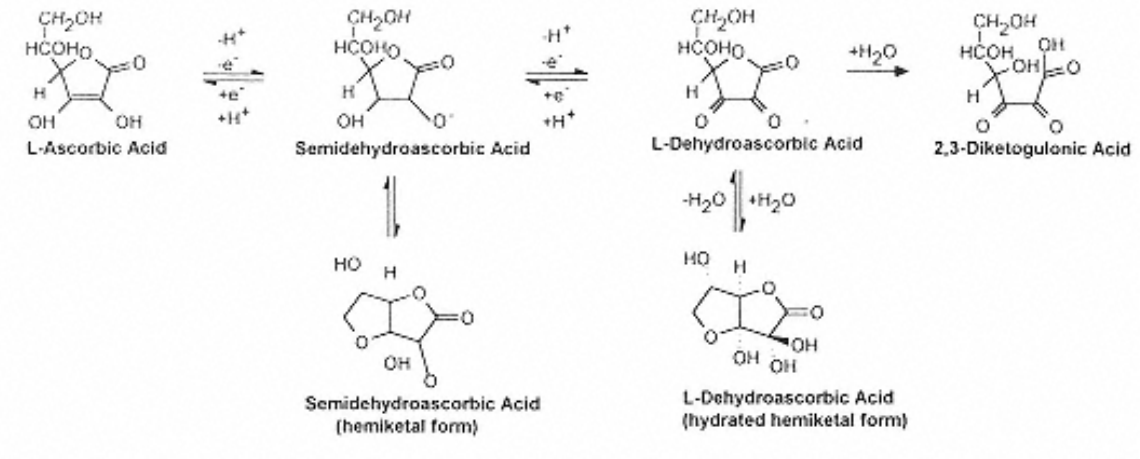

Figure 2.3 - One electron oxidation of ascorbic acid. From Gregory, 1996

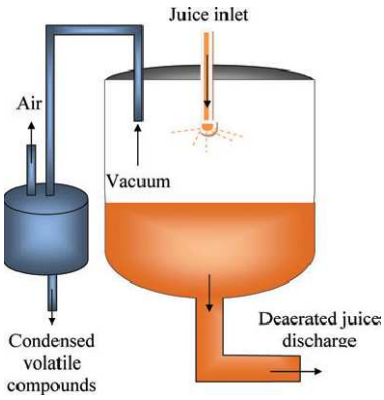

Figure 2.4 - Vacuum deaeration. From Garcia-Torres et al. 2009

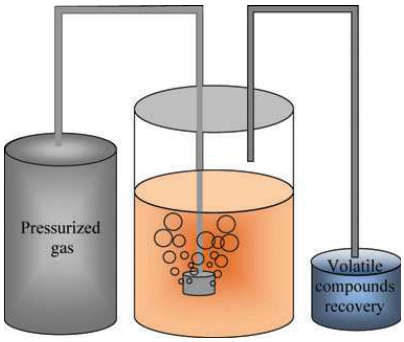

Figure 2.5 - Gas sparging. From Garcia-Torres et al. 2009

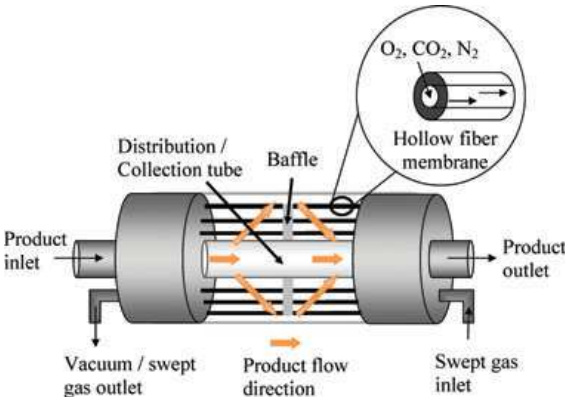

Figure 2.6 - Membrane deaerator. From Garcia-Torres et al. 2009

CHAPTER 3

MICRO-OXYGEN PROCESSING AND DETECTION OF BIOCHEMICAL DETERIORATIVE VECTORS IN BANANAS[1]

[1] Perez Almeida, S., Mulligan, J. H., Brody, A. L., Wicker, L. To be submitted to Journal of the science of food and agriculture. 2010.

ABSTRACT

The effect of micro-oxygen on polyphenoloxidase (PPO) activity was evaluated at atmospheric and ppm oxygen concentrations. PPO activity decreased non-linearly with decreasing oxygen concentration from 198 Units/mL to about 45 Units/mL at atmospheric and micro-oxygen (8 ppm), respectively. The decrease in PPO with oxygen followed a second order polynomial regression ($R^2 > 0.9$). Raman spectra taken initially and at four minutes of PPO reaction with catechol and four peaks at 1282, 1257, 1338 cm^{-1} and reference peak at 1850cm^{-1} were quantified for differences in peak ratio. There was no quantifiable difference at different times and different oxygen concentrations. The control peak ratio for catechol concentration was $7.3\pm5\times10^{-3}$. PPO activity in catechol with time increased as a hyperbolic curve under atmospheric conditions. Under micro-oxygen conditions, PPO activity in catechol decreased after longer times of assay. The decrease in absorbance at longer times of assay may be the result of non-Henri-Michaelis-Menton kinetics.

Keywords: micro-oxygen, PPO, o-diphenol, catechol

INTRODUCTION

Many deteriorative reactions in foods are due to oxidation. Oxygen allows growth of aerobic microorganisms and it is responsible for oxidation of lipids, flavors, and loss of nutritional value. Oxygen in the headspace, entrained in the food, and the one that migrates from outside to the inside through the package are the principal sources of oxygen availability in the product. Some products that are especially sensitive to oxidative reactions are bakery products, dry milk, red meats, processed meats, beer, juices, coffee, nuts and cereal products, and several fruits and vegetables. Even though the foods may be microbiologically sterile due to heat treatment, such as canned foods, they still undergo biochemical deteriorative reactions.

The fruit and vegetable industries suffer economic losses every year due to browning, off-flavors, and overall loss of quality (Ramirez et al. 2003). Browning in fruits and vegetables may be controlled by reducing oxygen concentration, addition of chelating agents, inactivation of polyphenol oxidase (PPO) (Marshall et al. 2000).

Polyphenol oxidase is found in the chloroplast thylakoid membranes, and the substrate, phenolic compounds, has been found in the vacuole of plants. If any cell disruption occurs, as the product suffer bruises, cuts, or mechanical damage, both the substrate and enzyme come into contact, as well as atmospheric oxygen, producing browning (Charraza et al. 2001). Polyphenol oxidase also known as tyronase, phenolase, catechol oxidase, catecholase, o-diphenol oxidase, monophenol oxidase, and cresolase has the ability to react with different substrates (Ramirez et al, 2003). Polyphenol oxidase may be specific towards monophenolic and diphenolic substrates. The reaction consists of the hydroxylation of monophenols to *o*-diphenols by monophenolase (EC 1.14.18.1) (Sojo et al., 1998), or the hidroxylation of *o*-diphenols to produce *o*-quinones that

are rapidly, non-enzymatically polymerized to brown pigments (Vamos-Vigyazo, 1981). PPO that catalyzes this reaction is defined as EC 1.10.3.1 (Ramirez et al. 2003). There is a third type of this enzyme, laccase (EC 1.10.3.2) that uses a different mechanism for oxidation than the other two (Ramirez et al., 2003).There is evidence that PPO in bananas is specific towards *o*-diphenols (Palmer, 1963), being dopamine the specific substrate used in the oxidation reaction (Griffiths, 1959). The enzyme is located in the pulp and peel of the banana (Palmer, 1963). One mole of molecular oxygen oxidizes two moles of catechol producing two moles of 1,2-benzoquinone along with two moles of water.

Since oxygen is necessary to start the enzymatic reaction (Martinez and Whitaker, 1995), it has been hypothesized that elimination of O_2 will delay enzymatic browning. Several studies have shown that reduction or elimination of oxygen, satisfactorily reduces PPO activity, i.e. storage of longan fruit in 5% oxygen atmosphere (Cheng et. al. 2009), however, other studies suggest that low oxygen does not eliminate or reduce browning in carambola fruit, stored at 0.4% O_2, and apple slices stored at 0.25kPa O_2 (Teixeira et al. 2008; Gil et al. 1998), respectively. In addition, air is present, intracellularly and intercellularly, in fruit and vegetable tissues. The dissolved oxygen in the fruit may be responsible of deteriorative reactions (Garcia-Torres et al. 2009) being enough to cause browning.

Enzyme kinetics studies are used to determine the factors that influence enzymatic reactions such as enzyme and substrate concentrations, pH, inhibitors, activators, and temperature (Segel, 1976). Parameters like maximum velocity (V_{max}) and Michaelis constant (K_m) may be calculated from velocity and substrate curves. The enzymatic reaction where a single substrate gives one product and where equilibrium is reached rapidly is known as the Henri-Michaelis-Menten approach. The reaction is as follows (Segel, 1976):

Where:

E= enzyme

S= substrate

ES= enzyme-substrate complex

P= product

The Lineweaver-Burk reciprocal plot is widely used to determine V_{max} and K_m and to perform a preliminary diagnosis of the order of the reaction (Segel, 1976). From the plot and rearrangeing the Henri-Michaelis-Menten equation, the paramters may be determined from the linear regression:

$$\frac{1}{v} = \frac{K_m}{V_{max}}\frac{1}{[S]} + \frac{1}{V_{max}} \qquad (2)$$

ν= velocity of reaction

Gomez et al. (2006) studied the effect of superatmospheric oxygen concentrations on PPO activity in mushrooms. They found that an inhibitor was present, and that Michaelis-Menten kinetics was not followed. Other authors also found the lacking of Michaelis-Menten kinetics in the oxidation reaction by PPO (Arias et al. 2007). When high product concentration was present, the inhibition was independent of oxygen, but at low product concentrations, oxygen had a role in the inhibition of the enzyme. A linear mixed kinetics including the product and oxygen concentration was proposed (Gomez et al. 2006).

To study the browning reaction catalyzed by PPO in fruits, Raman spectroscopy represents a helpful tool to assess changes in chemical bonds. In browning, four peaks are considered of interest: vibrations of the bonds C-O corresponding to catechol, at wavenumbers of 1282cm^{-1} and 1257cm^{-1} (Greaves and Griffith, 1991); catechol-like polymers at 1338 cm^{-1} (Sanchez-Cortes and Garcia-Ramos, 2000) and a constant peak that was common for all spectra at 1850 cm^{-1}.These peaks are selected in order to study the vibration of the C-O bond of catechol that will change during the reaction to form products. The two peaks 1282 and 1257 cm^{-1} correspond to the two sites (C-O) of the catechol molecule, which consists of a phenol ring with two hydroxyl groups in the *ortho* and *meta* position. The catechol-like polymer peak at 1338cm^{-1}, is a measure of the product being formed, since catechol polymerizes overtime.

Several methods to reduce oxygen in foods have been developed, through the use of oxygen scavengers, vacuum, oxygen- barrier packaging, flush with inert gases, among others. In this study, the use of micro-oxygen processing is proposed to extend shelf life of foods. Micro-oxygen processing is defined as the use of oxygen levels in both the product and the processing environment that would reduce biochemical reactions to levels that are too low to be measured by conventional methods (Brody, 2005). Since there is equilibrium between the oxygen present in the headspace and the oxygen dissolved or occluded in the food, if micro-oxygen levels are achieved in the processing environment, therefore the same levels of oxygen would be present in the food. The oxygen concentration necessary to be achieved for micro-oxygen processing has been targeted as 30 ppm. The objective of this work is to study the effect of different oxygen concentrations on PPO activity in bananas.

MATERIALS AND METHODS

1. Sample collection

The experiments were performed in a custom designed chamber (1.8m × 1.1m × 0.9m) that was sealed and flushed with liquid nitrogen (Airgas, Industrial grade, less than 5ppm oxygen) to displace oxygen. Oxygen concentration was reduced from atmospheric conditions (19.8%) to 8 ppm, uniformly throughout the chamber, in about 5 hours. The oxygen concentration was monitored continuously using an Orbisphere A1100 oxygen sensor, and analyzer Orbisphere 3650/55 (HachUltra™, Grants Pass, OR).

DelMonte bananas (Cavendish) were peeled inside the chamber and a puree was prepared with 10 g taken from the center portion of a ripe banana (yellow with no black spots). A volume of 40 ml of 0.1 M sodium phosphate, 0.1 M citric acid buffer, pH 6.5 with 1 g of polyvinyl pyrrolidone (PVP), was added to the banana. The puree was stirred for 3 minutes, and was filtered with Miracloth® (Calbiochem, La Jolla, CA) to remove insolubles. The filtrate was used as PPO extract for assays.

2. PPO activity

Change in absorbance at 400 nm with time was measured with a Spectronic 20+ (Genesys20 ThermoSpectronic, model 4001/4), using catechol 0.1 M, prepared in buffer without PVP, as substrate (method adapted from Soliva-Fortuny et al. 2001). The enzyme was diluted in a 1:2 ratio with phosphate buffer. The reaction rate was estimated from the slope of the linear portion of the reaction rate curve. A unit (U) of PPO activity was defined as the change in absorbance at 400 nm per minute. Activity was expressed as units per ml of enzyme. This assay procedure was repeated at different oxygen concentrations inside the chamber.

3. Particle size

Particle size of catechol and PPO extracts was measured. The cuvettes containing the enzymatic extract used for absorbance measurements inside the chamber were covered with parafilm (Pechiney Plastic Packaging, Chicago, IL) and placed in a glass jar that was closed under micro-oxygen. The samples were taken out of the chamber to atmospheric conditions to perform the particle size measurements using a 90-Plus Brookhaven particle sizer (Brookhaven Inst., NY). Also, enzymatic extract without buffer or catechol was collected in the chamber and particle size measurements were performed. The mean particle size for the control was 545±39 nm.

4. Transmission and backscattering

Transmission and backscattering were measured using a Turbiscan Classic MA2000 (Sci-Tec Inc., Sandy Hook, CT) of PPO in catechol sealed under micro-oxygen. A volume of 7.2ml composed by 120µl of the PPO extract prepared at micro-oxygen conditions (8 and 10 ppm), 1080 µl of phosphate-citrate buffer, and 6ml of catechol, was placed in a test tube that was introduced in the Turbiscan. Scans were taken hourly for a period of 48h.

5. Raman spectroscopy

Raman spectroscopy using a HRC-10HT Bruker Optics Sentinel Raman spectrometer (Bruker Optics, Billerica, MA) was performed on the PPO extract in catechol to collect evidence of browning. The laser (750nm, 500mW) was placed inside the chamber and was connected through fiber optic cables to the spectrometer placed outside the micro-oxygen processing chamber. A volume of 5ml of catechol was added to 100 µl of the PPO extract and 900 µl of phosphate-citrate buffer. Raman measurements were taken every 40 seconds to the solution.

Spectra were collected at time intervals of 1, 2, 3, 4, and 5 min. Raman spectra were collected at multiple oxygen concentrations. The catechol concentration was defined as:

$$[catechol]_t = \frac{I_{1282} + I_{1257}}{I_{1282} + I_{1257} + I_{1338}} \quad (3)$$

The peak ratio (P.R.) was defined as:

$$Peak\ ratio = \frac{[catechol]_t}{I_{1850}} \quad (4)$$

The peak at 1850cm^{-1}, was observed consistently in all spectra, and therefore it was considered a common peak to calculate peak ratios. Control P.R. was determined with catechol prepared with sodium phosphate buffer at atmospheric conditions. PPO extract was not added to the control. Peak ratios for the spectra were estimated at initial time and at 4 min, time after completion of enzyme assay at discrete oxygen concentrations. These times were chosen in order to compare the results with the enzymatic activity assay determined by the change in absorbance with the Spectronic+20 and to study changes between the begging and the end of the colorimetric reaction.

6. Statistical analysis

The extraction of PPO from bananas under atmospheric and micro-oxygen conditions was repeated in experimental triplicates. Data presented are extractions and assays at specific time intervals. Due to the dynamic change in oxygen concentrations between triplicate experiments, extractions at specific oxygen concentrations could not be made. Data presented are values from a single run. Similar trends were observed in replicates. Microsoft Excel was used to determine the polynomial regression for enzymatic activity with respect to oxygen concentration.

RESULTS AND DISCUSSION

1. Enzymatic activity

The increase in absorbance at 400 nm of PPO in catechol with time at atmospheric conditions is presented in Figure 3.1a. The initial slope of the line was used to estimate PPO activity. The estimated time to add PPO extract to substrate, mix and place the cuvette in the sample holder was about 10 sec. In replicate assays, the initial absorbance values were less than 0.05. The absorbance of PPO in catechol under reduced oxygen levels is presented in Figs. 3.1b and 3.1c at the indicated oxygen levels in the chamber. The PPO activity is much lower under reduced oxygen than atmospheric oxygen (note different absorbance scale). Also note that the absorbance decreases near 100 sec at all low oxygen levels. The difference between the plateau and the last point recorded at 4 min of the enzyme activity assay was between 0.078 and 0.007 absorbance units.

The data in Figure 3.2 is a compilation of PPO activities for the three replications with atmospheric, intermediate, and low (8 ppm) oxygen. As observed in figure 2.2, activity decreases with oxygen concentration, but does not achieve zero activity. Further, the activity decreases non-linearly with oxygen concentration.

Enzymatic activity was plotted against oxygen and a model was fit to each replication. A second order polynomial regression presented the highest R^2 values (table 3.1). Therefore, the oxidation reaction involving polyphenol oxidase in bananas at decreasing oxygen concentrations follows a second order model. This may be due to the nature of the catechol molecule, that has two hydroxyl groups (*ortho* and *meta* position) and that are oxidized to produce brown pigments.

The Lineweaver-Burk double reciprocal plot was graphed for oxygen concentrations from atmospheric to micro-oxygen (8ppm) conditions. A linear fit was not applicable for all the

oxygen concentration range (figure 3.3). However, a linear regression: y =0.0843x + 0.0077; R^2=0.4664, was determined for oxygen concentrations from 52 to 8ppm (figure3.4). The K_m value determined from the Henri-Michaelis-Mentis equation was 10.94 ppm. Since the substrate concentration is neither very low nor very high compared to K*m*, the reaction is neither first nor zero order (Segel, 1976) for micro-oxygen concentrations. Similar results were found in other studies, where at high substrate concentrations and low oxygen levels, Henri-Michaelis-Menten equation is not followed and inhibition is observed (Duckworth and Coleman, 1970).

2. Particle size

The PPO catalyzed polymerization of phenolics to produce brown pigments, involves the conversion of benzoquinones to other products that do not absorb at the benzoquinones wavelength (Whitaker, 1995), this may be an explanation to the decrease in absorbance in the enzymatic activity assay. In addition, the reaction may precede the formation of condensation and polymerization products (Cheynier et al. 1989; Fortea et al. 2009). The decrease in absorbance after achieving maximum absorbance under micro-oxygen conditions (figure 3.1b, 3.1c) is not typical in colorimetric enzymatic assays, but the decrease in absorbance is not related to polymerization of phenolics and increase in particle size. The particle size of the extracts prepared at different oxygen concentrations, at the end of the colorimetric assay is reported in figure 3.5. The particle size of the PPO extracts was about 1064±80 nm and did not change from atmospheric to micro-oxygen concentrations. The percent transmission and back scatter of extracts prepared at atmospheric and 8 ppm oxygen concentrations were in average 80%, for transmission, and 50% for back scatter (data not shown). Same values were observed for both extracts taken at atmospheric and micro oxygen conditions. There is no evidence of sedimentation, serum, or particle size growth.

3. Raman spectroscopy

The peak ratios of catechol C-O vibrations and catechol polymers are presented in figures 3.6 a, b and c, representing O_2 concentration of about 22%, <2% O_2, and <15 ppm O_2, respectively. Raman spectra taken at initial time and at 4 min at atmosphere conditions, oxygen levels between 1.61% and 1.84% and micro-oxygen, 15ppm and 9 ppm, were used to study the effect of time and oxygen on the enzymatic reaction. These peak ratios are estimated from spectra that are representative of three replications.

Peak ratios for the spectra were estimated at initial time and at 4 min, time after completion of enzyme assay at discrete oxygen concentrations (figures 3.6a, b, and c). Negligible differences were observed between the initial time and 4 minutes at any O_2 concentration. Also, differences in peak ratios between atmospheric and low oxygen conditions were negligible (figures 3.6 a, b, and c). The control peak ratio for catechol concentration was 0.0073 ±0.005. The Raman measurements for the control were taken at atmospheric conditions without PPO enzyme. However, it may be assumed, from the rest of the results that catechol concentration is likely to be the same at all oxygen concentration.

It was observed that the surface of the banana indeed turned brown under micro oxygen conditions. This result suggests the presence of entrained O_2 and that micro-oxygen conditions may be still enough to trigger oxidative reactions.

Enzymatic activity results showed that there is difference between PPO extracts that were prepared at atmospheric and micro-oxygen conditions. Raman spectral analysis corroborated the results. The peak ratio of catechol concentration at time zero and 4 minutes after the reaction started was the same, indicating that any change in substrate occurred between the 10 s needed to

prepare the sample for analysis. Similar results were obtained for the different oxygen concentrations. In addition, the catechol concentration for samples prepared at atmospheric and micro-oxygen conditions were basically the same (P.R. varied between 0.0003 and 0.0004). The control was noticeably higher than the rest of the samples.

The results suggest that processing at oxygen levels of 8 ppm may be still enough to produce browning. Also, even though the oxygen concentration in the chamber where the experiments were performed was 8 ppm, dissolved oxygen in the buffers and substrate, as well as dissolved oxygen in the banana tissue may have been higher than 8 ppm, however equilibrium between the headspace and dissolved oxygen was assumed.

It is possible that in the case of activity of banana PPO, a high concentration of product is present; therefore the effect of oxygen concentration is negligible as other researchers have found (Gomez et al, 2006). Moreover, it has been found that at high substrate concentration and low oxygen levels (3.78%) there is substrate inhibition (Duckworth and Coleman, 1970). All these evidence show that PPO mechanisms of reaction are complicated and the simple reduction of the co-substrate, oxygen, may not have an impact on decreasing PPO activity.

It may be also suggested that very small quantities of PPO are needed for the reaction. The molecular weight of the enzyme is about 30,000 Daltons (Galeazzi and Sgarbieri, 1981), which indicates a small molar concentration. Oxygen concentrations of 8 ppm may be enough to saturate the enzyme; therefore PPO activity is not eliminated.

CONCLUSIONS

This study shows that micro-oxygen levels, as low as 8ppm, decrease PPO activity but does not eliminate it. It has been suggested that dissolved oxygen on the banana tissue as well as mechanisms of reaction of the enzyme may play a role in PPO activity. Browning reaction by PPO is neither first nor zero-order.

Results show that Raman may represent an effective way to quantify enzymatic activity, since it allows the identification and quantification of compounds that are being formed throughout the reaction. In addition, the sample preparation time necessary for the absorbance assay, may be reduced to less than 10s, eliminating the possibility of catechol changes before the activity is measured.

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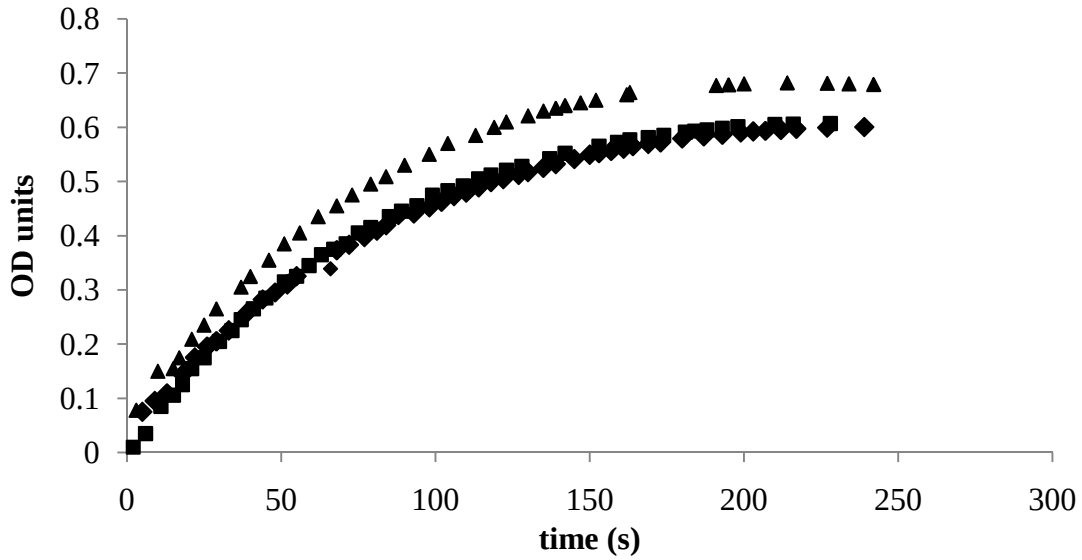

Figure 3.1a - Absorbance vs. time at atmospheric conditions. Each line corresponds to a different replication. ♦ 22.6%, ■ 22.5%, ▲ 23%.

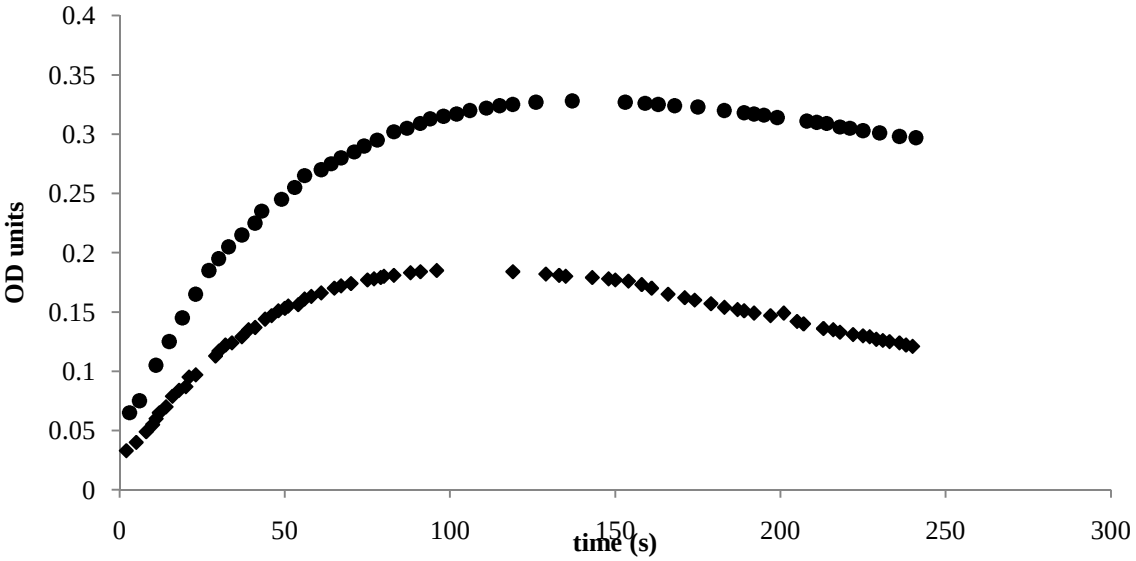

Figure 3.1b - Absorbance vs. time at 1.8% and 1.6% oxygen. Each line corresponds to a different replication. ● 1.8%, ♦ 1.6%.

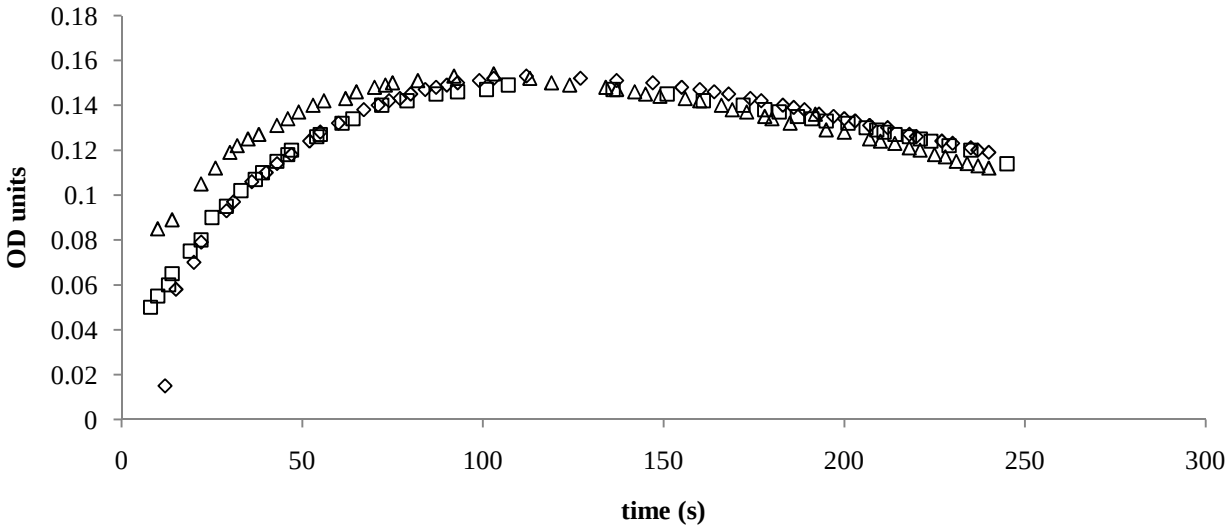

Figure 3.1c. - Absorbance vs. time at 15 and 8 ppm oxygen. Each line corresponds to a different replication. ◇ 15 ppm, □ 15 ppm, △ 8 ppm.

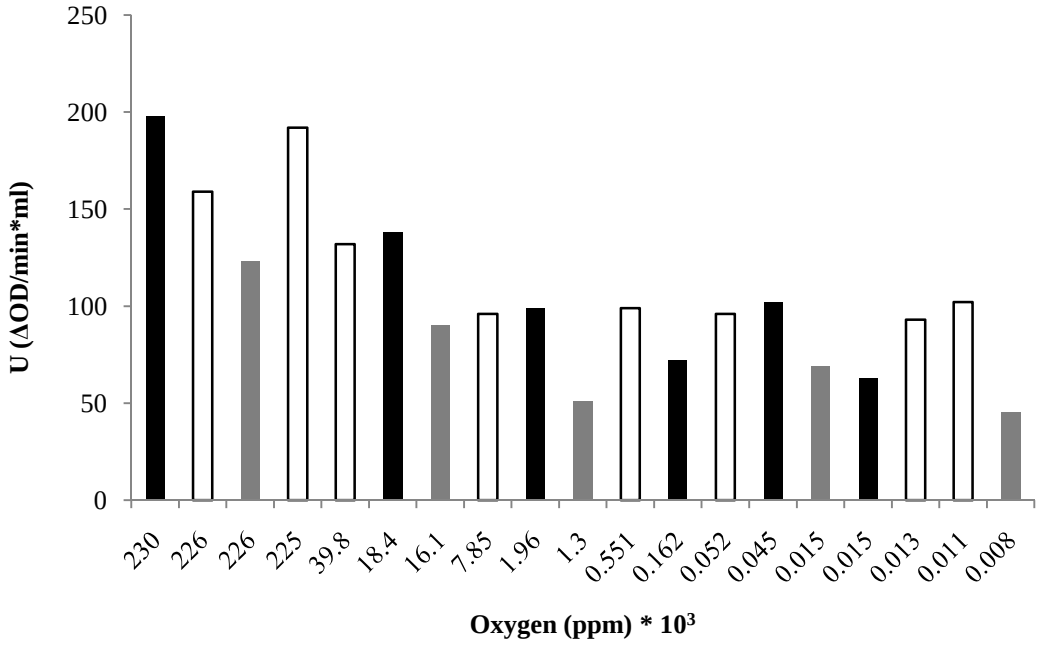

Figure 3.2. - PPO activity with respect to oxygen concentration. Solid black, solid white and grey bars represent replications 1, 2, and 3, respectively.

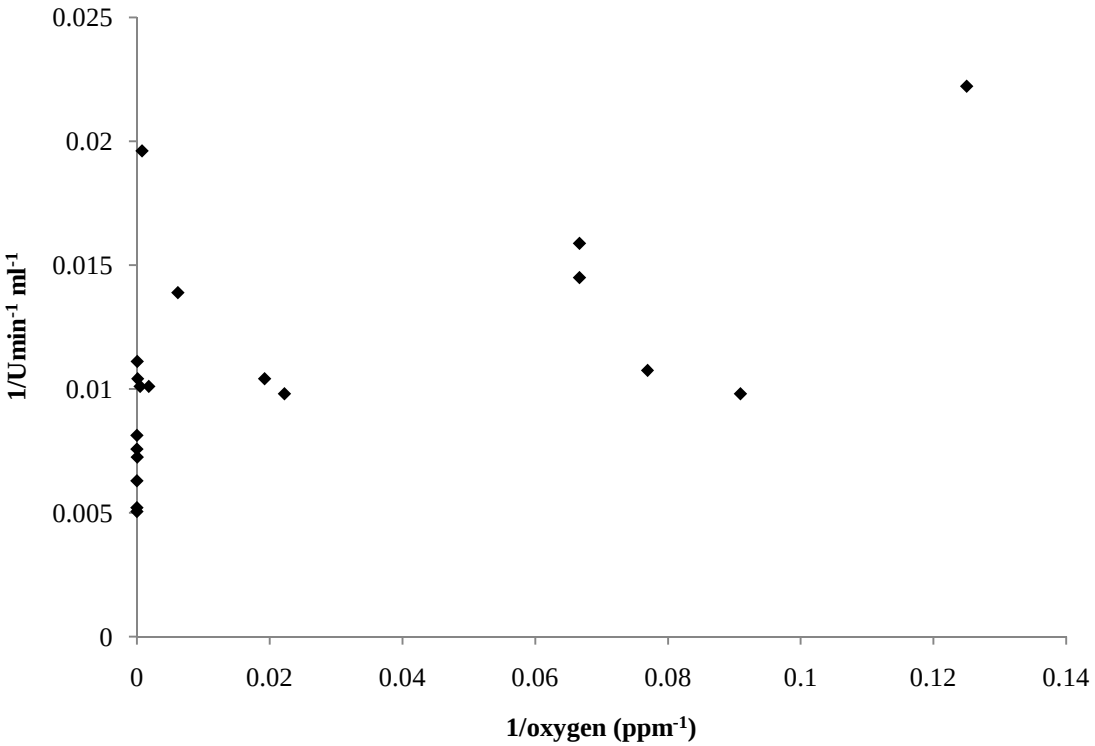

Figure 3.3. - Lineweaver-Burk double reciprocal plot including oxygen concentrations from atmospheric conditions to micro-oxygen (8ppm) concentrations.

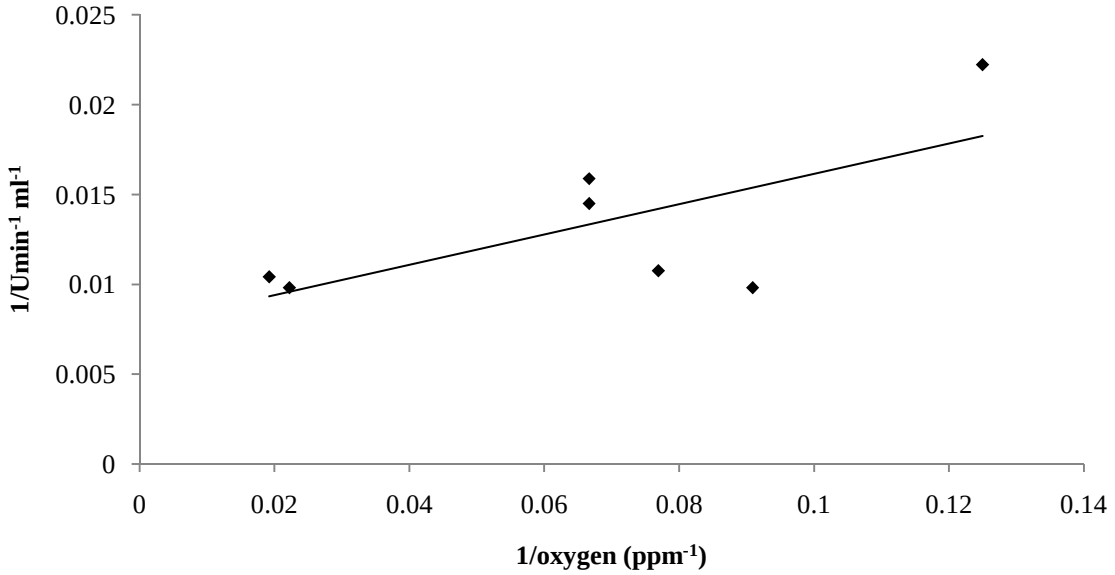

Figure 3.4. - Lineweaver-Burk double reciprocal plot including micro-oxygen concentrations from 52 to 8 ppm. The linear regression is y=0.0843x + 0.0077; R^2=0.4664.

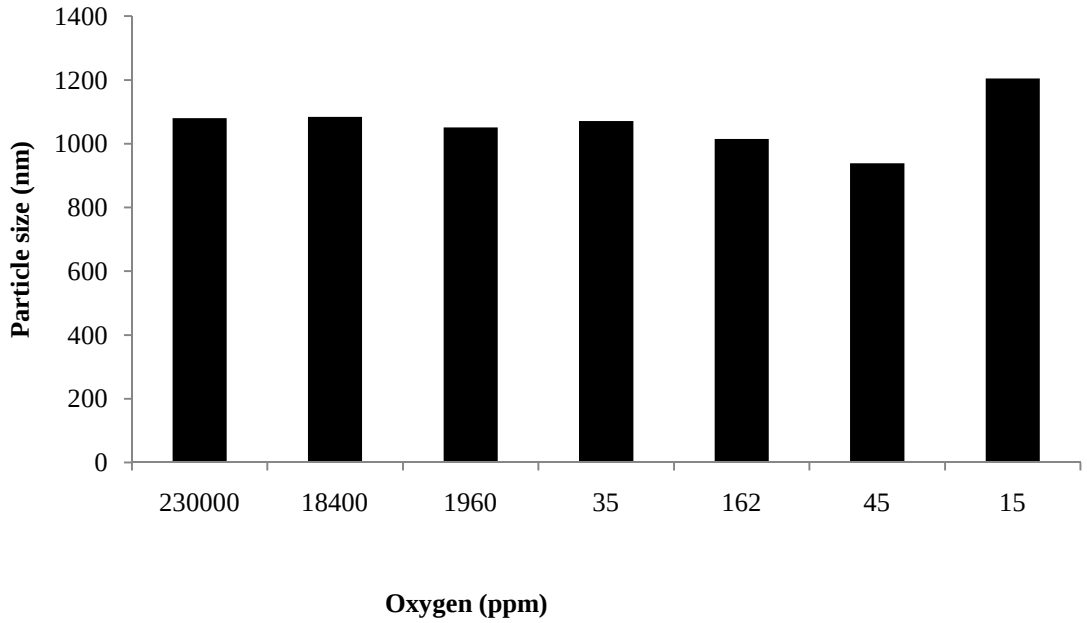

Figure 3.5. - Particle size with respect to different oxygen concentrations. The linear regression was: y= 1.54x+1057.4, R^2=0.0017. The data shown corresponds to one replication.

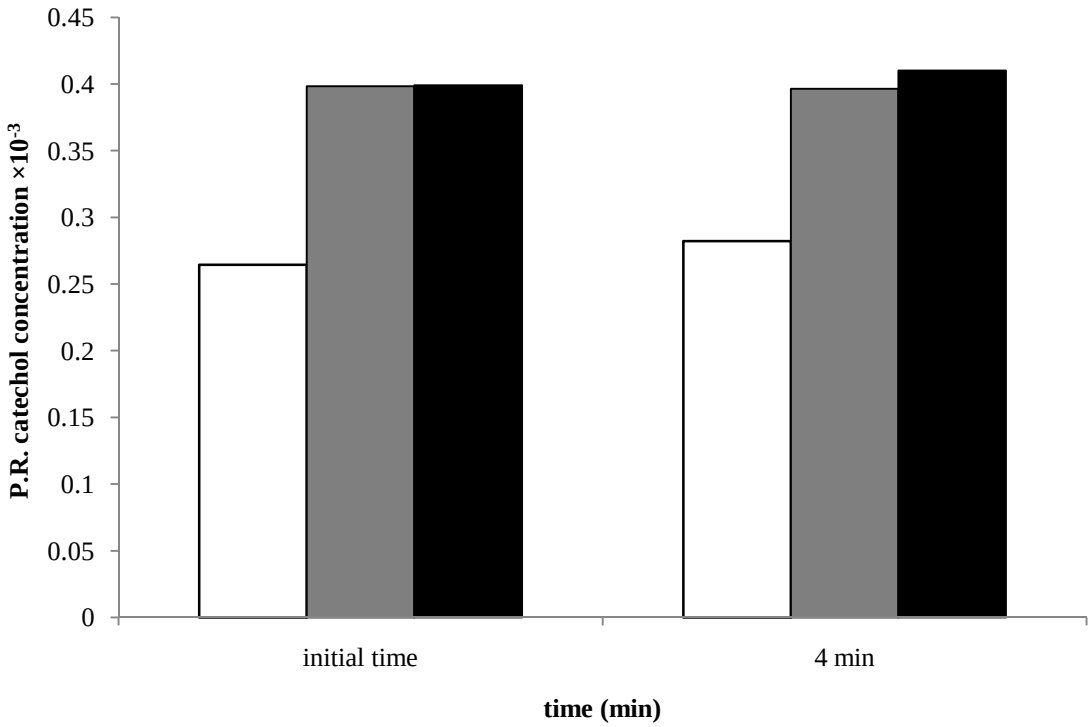

Figure 3.6a. - Peak ratio of catechol concentration with added PPO extract obtained at atmospheric conditions. Each solid white, black and gray bars represent a replication. Control P.R. was 7.3 ±5×10^{-3}. □ 23.0% O_2, ■ 22.6% O_2, ■ 22.5% O_2.

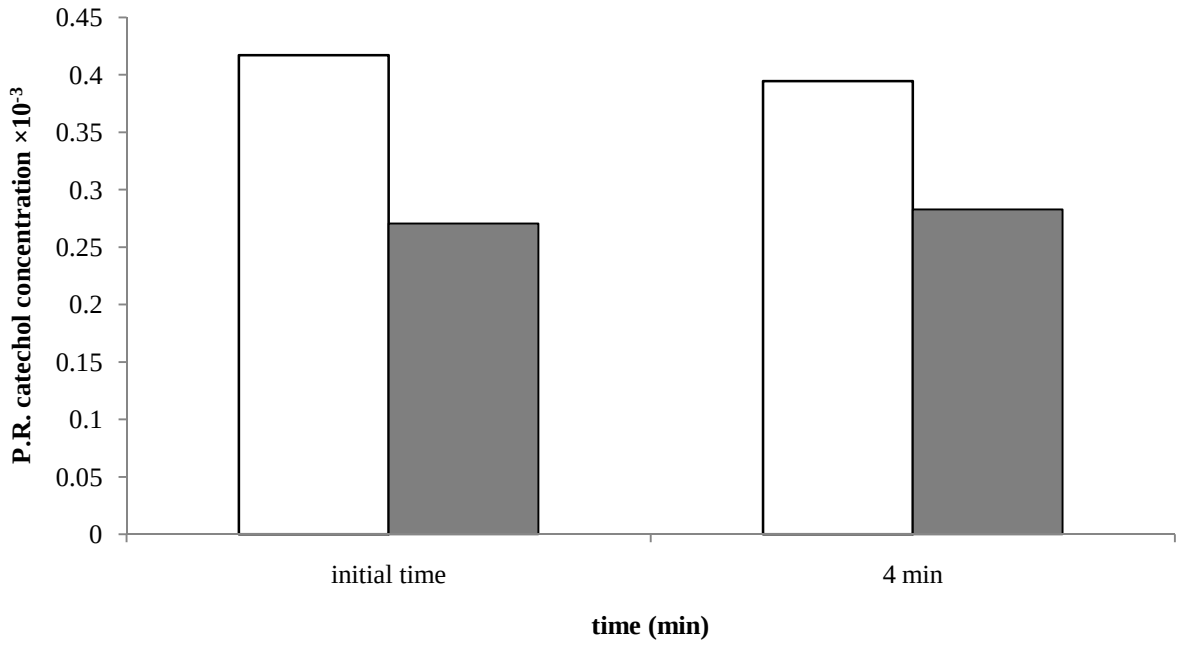

Figure 3.6b.- Peak ratio of catechol concentration with added PPO extract obtained at oxygen levels of 1.61% and 1.84%. The solid white and gray bar represents a different replication. Control P.R. was 7.3 ±5 $\times 10^{-3}$. □ 1.6% O_2, ■ 1.8% O_2.

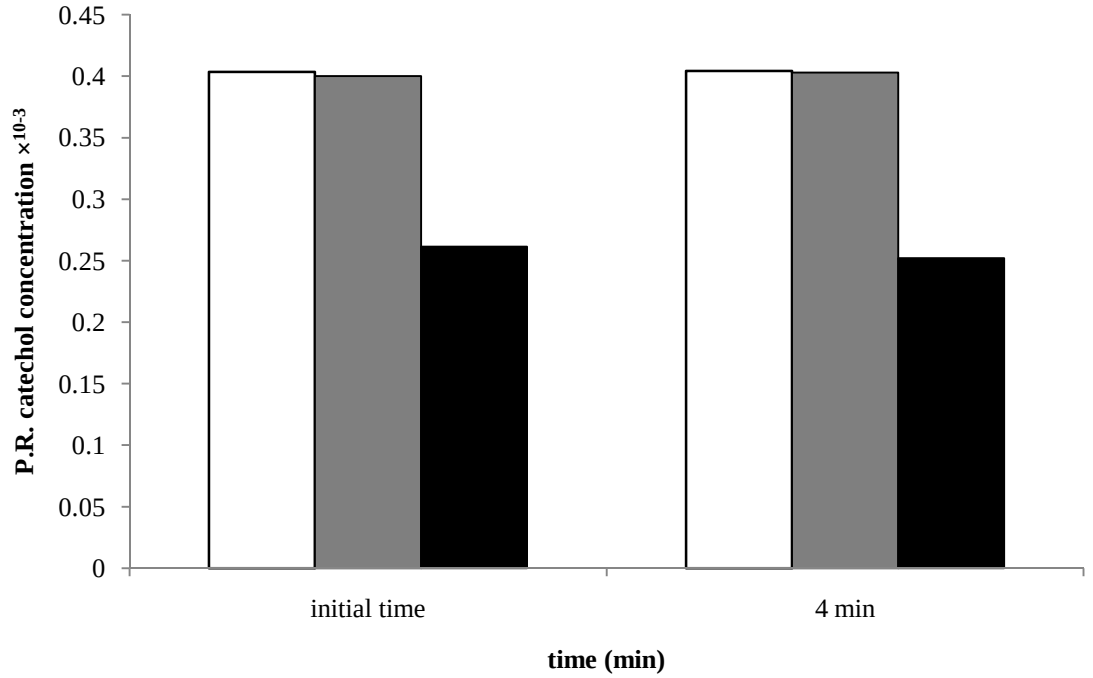

Figure 3.6c. - Peak ratio of catechol concentration with added PPO extract obtained at micro-oxygen conditions. Solid white, gray and black bars represent different. Control P.R. was 7.3 ±5 $\times 10^{-3}$. □ 15 ppm O_2, ■ 9 ppm O_2. ■ 15 ppm O_2.

Table 3.1.- Polynomial regression for enzymatic activity with respect to oxygen concentration

Regression	R^2
$y = -6E\text{-}11x^2 + 2E\text{-}05x + 1.9162$	$R^2 = 0.93$
$y = -2E\text{-}10x^2 + 7E\text{-}05x + 1.6381$	$R^2 = 0.92$
$y = -2E\text{-}10x^2 + 5E\text{-}05x + 1.0829$	$R^2 = 0.92$

CHAPTER 4

MICRO-OXYGEN PROCESSING AND DETECTION OF BIOCHEMICAL DETERIORATIVE VECTORS IN ORANGE JUICE[2]

[2] Perez Almeida, S., Mulligan, J. H., Brody, A. L., Wicker, L. To be submitted to Journal of the science of food and agriculture. 2010.

ABSTRACT

The effect of processing at micro-oxygen (MO) and atmosphere (ATM) was evaluated in orange juice. Dissolved oxygen was measured for the different processing conditions, achieving oxygen levels as low as 34ppb to 100ppb in juices processed at MO conditions that were considered constant throughout the shelf-life studies and levels from as low as 200ppb to 1041 ppb for juices processed at ATM conditions. Dissolved oxygen levels for juices processed at ATM and MO conditions are not statistically different throughout the study, except day 0, which explains the observation of no differences in ascorbic acid retention, color, and particle size for juices subjected to the different processing conditions. Juice did not clarify throughout the shelf-life study (%T<36). Ascorbic acid degradation for juices processed at ATM is zero order for all storage temperatures, with exception of juice stored at 50℃ (first order). For juice processed at MO conditions, ascorbic acid degradation is first order, with exception of juice stored at 50℃, where neither first nor zero order was followed.

Keywords: micro-oxygen processing, ascorbic acid, color, cloud, particle size, dissolved oxygen

INTRODUCTION

Orange juice is one of the most common in juice production (Garcia-Torres et al. 2009). During juice extraction from the fruit, the cells of the fruit break allowing biochemical reactions to take place due to the release of enzymes that were previously in compartments within the cells (Garcia-Torres et al. 2009). The enzymes and the oxygen present in the atmosphere are responsible for chemical and biochemical reactions that result in browning and loss of Vitamin C in orange juice (Joslyn et al. 1934); therefore there is a decrease in quality and nutritional value.

Loss of ascorbic acid due to oxidation is extremely important in orange juice, since it decreases its nutritional value. Ascorbic acid is a polar compound, which makes it soluble in aqueous solutions. Its acidic and reducing capabilities are given by the 2,3-enediol moiety. Vitamin C, or ascorbic acid, may be present in the form of L-ascorbic acid, or L-dehydroascorbic acid, product of the oxidation and dissociation of hydrogen (figure 3.1). However, L-dehydroascorbic acid has the same vitamin activity, since it is reduced in the body to L-ascorbic acid (Gregory, 1996).

Ascorbic acid degradation occurs both in presence and in absence, although insignificant, of oxygen, and it is catalyzed by metal ions, accelerated by heat, and it is influenced by pH (pH ~ 2.5-5.5 for aerobic degradation and pH ~ 3 to 4 for anaerobic degradation), oxygen and water activity (Gregory, 1996).

The oxidation process of ascorbic acid to dehydroascorbic acid is reversible; however, the hydrolytic ring cleavage that produces 2,3-diketogulonic acid is irreversible (Garcia Garcia-Torres et al. 2009). 2,3-diketogulonic acid undergoes oxidation, dehydration, and polymerization (Gregory et al. 1996).

There have been several studies to determine the reaction order of ascorbic acid degradation (Garcia-Torres et al. 2009). Some authors have suggested the ascorbic acid degradation reaction to be first order with respect to dissolved oxygen (Trammell et al. 1986), others, zero order (Kennedy et al. 1992) and second order reaction, when low oxygen concentrations are present (Singh et al. 1976). According to some authors, other substrates may limit the reaction, such as dissolved oxygen, that is present in orange 10 times less than ascorbic acid (Garcia-Torres et al. 2009).

Color changes are a result of non-enzymatic and enzymatic browning. In orange juice processing, the pasteurization step to reduce the microbial load in 5 log, is enough to inactivate enzymes in the raw fruit that may be responsible for enzymatic browning, such as PPO (Garcia-Torres et al. 2009). However, non-enzymatic browning is of concern in the orange juice industry. Non-enzymatic browning is the product of ascorbic acid and sugar degradations (Adams and Brown, 2007). Oxygen plays an indirect role in this type of browning. By oxidizing ascorbic acid to dehydroascorbic acid with formation of furfural, a brown color is imparted to the juice (Garcia-Torres et al. 2009). Particularly, during oxidation of ascorbic acid, carbonyls are formed which then react with amino groups and polymerize resulting in brown pigments formation (Graumlich et al. 1986).

The oxygen concentration in the orange juice package depends on the initial concentration at the moment of packaging, the oxygen permeation through the package and the oxygen consumption by deteriorative reactions. The mass transfer of oxygen from the atmosphere to the package is described in three steps. The first is the movement of molecules from the atmosphere to the package with the subsequent dissolution; the second, the diffusion through the packaging material, and lastly desorption of oxygen and dissolution in the food (Ahrne et al.1997).

The partition coefficients outside and inside the barrier material depend on the system characteristics. For instance, the concentration of oxygen during storage of orange juice on Tetra Brik Aseptic cartons changes considerably the first days of storage due to oxidative reactions (Graumlich et al. 1986), and then is kept constant because the oxygen that enters the package and the consumption by oxidation reactions balance each other (Ahrne et al.1997).

Nowadays several methods are used in industry to eliminate dissolved oxygen in the juice. The deaeration method, using a vacuum, removes oxygen, and also flavor, when the pressure decreases in the headspace, releasing the air that is dissolved in the juice (Braddock, 1999). Another method that removes greater quantities of oxygen is the 'Gas sparging'. This method removes oxygen by bubbling another inert gas, such as nitrogen, argon or helium to the juice, displacing oxygen (Garcia-Torres et al. 2009). The use of membrane deaerators has also been put in practice. It consists of mass transfer between gas and liquid or two liquids without dispersion (Garcia-Torres et al. 2009). For instance, 96% of dissolved oxygen can be removed using a polypropylene hollow fiber separator from water (Cole and Genetelli, 1970). It has also been used to remove oxygen from beer, and other food applications (Gabelman and Hwang, 1999). Enzyme-based deaeration, is a method that consists of the use of an enzyme, such as Glucose oxidase that consumes both dissolved oxygen and oxygen from the headspace, producing gluconic acid and peroxide. Since it is an enzyme, it is dependent of factors such as pH, what limits its applications (Garcia-Torres et al. 2009). Lastly, the use of oxygen scavengers may reduce the oxygen content present in the headspace and that may ingress through the package during storage (Garcia-Torres et al. 2009). In this study we are proposing the use of micro-oxygen processing to extend shelf-life of orange juice. Micro-oxygen processing is defined as the use of oxygen levels in both the product and the processing environment that would reduce

biochemical reactions to levels that are too low to be measured by conventional methods (Brody, 2005), and has been targeted in 30ppb. The aim of this work is to study quality parameters in orange juice that has been processed under micro-oxygen conditions. The parameters to be measured are ascorbic acid, color, cloud stability, pH, particle size and dissolved oxygen.

MATERIALS AND METHODS

1. Orange juice

The experiments were performed at atmosphere conditions and in a custom designed chamber (1.8m × 1.1m × 0.9m) that was sealed and flushed with liquid nitrogen to displace oxygen. Oxygen concentration was reduced from atmospheric conditions (19.8%) to about 80ppm, uniformly throughout the chamber, in about 5 hours. The oxygen concentration was monitored continuously using an Orbisphere A1100 oxygen sensor, and analyzer Orbisphere 3650/55 (HachUltra™, Grants Pass, OR).

The orange juice was processed at the lowest oxygen concentration (80 ppm). Sunkist Valencia oranges, from California, were washed and packaged in vacuum the day before the experiments were performed. Once the 80 ppm was achieved in the chamber, the oranges were cut in half and squeezed with a juice extractor model No. Mil-E2053 (Strite-Anderson Mfg. Co. , Minneapolis, Minnesota). The juice was immediately filtered using Miracloth® (Calbiochem, La Jolla, CA) to remove left over pulp. Then, 200 ml of juice were placed in retortable pouches provided by Printpack (Atlanta, GA) with an oxygen transmission rate of 0.01 cc/m^2. The pouches were sealed with a heat sealer from the Heating Sealer Company Equipment Mfg. Co. Model 10P (Cleveland, OH). All the pouches were put in an ice bath until the end of the processing day (4 L of orange juice).

The chamber was opened, and the pouches were resealed to obtained a better seal with a Packworld Sealer, model A-17-2993 (Packworld, USA). The orange juice was pasteurized in boiling water, to achieve 99% inactivation of pectinesterase (1 min at 90 ℃) (Versteeg, 1980). The pouches were taken out of the boiling water and immediately submerged in an ice bath to

stop heating of the juice. Later, samples were identified and stored at 0 ℃, 4 ℃, 25 ℃ and 50 ℃. Samples were taken out from storage when time was pertinent to perform the shelf-life stability tests.

2. Shelf-life stability

Accelerated shelf-stability test were performed in orange juice that was stored at different temperatures: 0℃ (control), 4℃ (refrigeration), 25℃ (ambient temperature) and 50℃. Tests for ascorbic acid retention, color, particle size, cloud stability and pH were performed in each sample. Orange juice stored at 50℃ was tested every other day for two weeks, at 25 ℃ once a week, 4 ℃ every two weeks and 0 ℃ every week. Assays were carried out for 5 weeks and changes over time were recorded.

3. Ascorbic acid determination

Ascorbic acid content was determined using the AOAC Method 967.21 of 2,6-dichloroindophenol titration. Kinetics studies were performed to determine the order of reaction for degradation of ascorbic acid. Plots of ascorbic acid content versus time, and ln (ascorbic acid) versus time were graphed. If a linear regression fits the content of ascorbic acid vs. time, the reaction is zero order. If a linear regression fits ln (ascorbic acid) vs. time, then the reaction is first order. In order to determine activation energy (E_a), the log (ascorbic acid) against time was plotted for first order reactions. From the slope, the rate of reaction is calculated as:

$$slope = -\frac{k}{2.3}$$

Then with k values, the effect of temperature was studied plotting the log (k) against the reciprocal of absolute temperature (1/T). From the slope of the curve, activation energy (E_a) was determined (Arrhenius equation):

$$slope = -\frac{Ea}{2.3R}$$

Where R is the constant of gases.

4. Color

A colorimeter (Minolta CR-310, Japan) was used to determine L, a, and b values. A volume of 30 ml of orange juice was put on a petri dish and the lid was put on. The colorimeter probe was placed under the petri dish that was previously wiped with Kimwipes.

Also, orange juice USDA color standards were used (Agtron®, Nevada, USA). Six color references were given from the the darkest one and very high orange color (Orange juice 1) to the lightest and less orange color (Orange juice 6). Color standards are used in the orange juice industry to evaluate color of fresh, canned, chilled, and concentrated orange juice.

5. Cloud

Cloud was determined measuring the percent transmittance at 650nm with a Spectronic 20+ (Milton Roy, Thermoscientific, Waltham, MA). A volume of 15ml was added to a centrifuge tube. The juice was centrifuged for 10 min at 500xg (2060rpm) at 25℃ (Fisher Scientific Marathon 3200). The supernatant was poured into a beaker, and then poured to a cuvette to be introduced in the Spectronic20+. It is considered that at 36%T clarification has occurred (Redd et al. 1986).

6. Particle size

A Malvern Mastersizer, Model: MSS (Malvern Instruments Limited, UK) was used to measure particle size distribution. The real relative particle refractive index used was 1.3, the imaginary relative refractive index 0.1, and the dispersant index 1.33 (taken from the Malvern Manual).

7. pH

The pH was measured using a 718 Stat Titrino and 728 Stirrer Metrohm (Brinkmann, Westbury,NY). Also the titrator was used to determine that pectinesterase was inactivated.

8. °Brix

The °Brix was measured on the same day the juice was extracted using a refractometer (Milton Roy, Ivyland, PA)

9. Titratable acidity

It was determined using the titration method with 0.1 N NaOH described on the Technical Manual of Reconstituted Florida Orange Juice, Method No. 1.05 (Florida department of Citrus, 1985).

10. Dissolved oxygen

In order to measure the dissolved oxygen in the juice, the retortable pouch was pierced with a needle No. 16. The juice passed through flexible nylon tubing (McMaster-Carr, Atlanta, GA) that was connected to the oxygen sensor, Orbisphere A1100, and analyzer Orbisphere 3650/55 (HachUltra™, Grants Pass, OR). At the same time, the sensor and analyzer equipment

was also connected to a nitrogen source to generate a positive pressure and let the juice pass though the electrode.

11. Statistical analysis

The extraction of orange juice at atmospheric and micro-oxygen conditions was repeated in experimental triplicates identified as rep 1, 2 and 3, atmosphere referring to replication 1 at ATM conditions, and rep 1, 2 and 3 MO, referring to rep1, 2 and 3 at micro-oxygen conditions, respectively. Due to the dynamic change in oxygen concentrations in the chamber, orange juice extractions at specific oxygen concentrations could not be made. Data presented are averages of the three runs. Statistica®, version 7.0 (Statsoft, Tulsa, OK) was used to performed statistical analysis. ANOVA, was carried out using Tukey's test *post hoc* multiple comparison tests. Probabilities of occurrence greater than 95% ($P<0.05$), were considered statistically different.

RESULTS AND DISCUSSION

1. °Brix and Titratable Acidity

At day zero of the experiments, when the orange juice was extracted, °Brix and titratable acidity were determined. °Brix for juice processed at atmosphere conditions was 11.5 ± 0.6 and for juice processed at micro-oxygen conditions 12±1. For titratable acidity in atmosphere (ATM) juice 0.76±0.04 % citric acid w/w and 0.72±0.02 % citric acid w/w for juice at micro-oxygen (MO) conditions.

2. Dissolved oxygen

Figures 4.2a, b, c and d, show the dissolved oxygen values. At 50 ℃ for juice processed at ATM conditions there is a decrease and then an increase going down from 600 ppb, passing through 95 ppb and then up to 526 ppb. At 25 ℃, dissolved oxygen in ATM juice decreased over time from 600 ppb to 86 ppb, at 4℃, oxygen decreased from 600 ppb to 1041 ppb at week 3 to go down to 92 ppb at week 5. For the juices stored at the control temperature, there was a decrease in oxygen concentration for ATM samples from 600 ppb to about 200 ppb. It is important to mention that for all MO juice, the oxygen content varied between 34 and 100 ppb, and it may be considered constant throughout the study, since no important variations were observed with time and storage temperature. Dissolved oxygen concentrations for ATM and MO were not statistically different throughout the study ($p<0.05$). However, dissolved oxygen at day 0 for MO, 48±17ppb, was statistically different from ATM, 660±218 ppb.

3. Ascorbic acid

Juice samples processed at atmosphere and micro-oxygen conditions were sampled for retention of vitamin C (ascorbic acid). Figures 4.3 a, b, c and d, show the results. In addition, kinetics studies to determine the order of reaction of degradation of ascorbic acid were performed. The plots that include the linear regression for ATM and MO juices are shown in figures 4.4a, b to 4.11a, b. The results are summarized in tables 4.1 and 4.2.

The E_a was determined for ATM juices, and two linear regressions fit the data, from 50℃ to 25℃, E_a= 58.22KJ/mol, and another for 25℃ to 0℃, E_a= 3.61 KJ/mol, corroborating the existence of mixed order reaction (figure 4.11c). However, it was not possible to determine the activation energy for MO juices. Positive slopes were obtained when log (ascorbic acid) against time was plotted; therefore log(k) was not possible to calculate and E_a was not determined. Degradation of ascorbic acid for juices processed under MO does not follow the Arrhenius equation.

4. Color

Color was determined by ‘L’, ‘a’ and ‘b’ values. In addition Hue value was calculated. ‘L’ values close to 100 represents white, being 0 black; decrease in L values is an indication of browning (Kanner et al. 1982); ‘a’ values that are positive represent red, and the ‘a’ negative values green. Lastly, ‘b’ positive values represent the yellow color and ‘b’ negative values blue. In figures 4.12a, b, c and d, L values are graphed against time for samples processed at ATM and MO.

For juice stored at 50 ℃ (figure 4.12a), a decrease in L values is observed for both ATM and MO juice. L values decreased from about 50 to 47. ATM and MO are not statistically different throughout the shelf-life study at 50 ℃. At 25 ℃ (figure 4.12b), there is also a decrease for juices processed at ATM, and MO. ATM and MO juice at week 2 are statistically different ($p<0.05$). However, they are statistically the same for the rest of the shelf-life study at 25 ℃, 4 ℃ and 0 ℃, from day 0 to week 5. With respect to changes in temperature, ATM juices stored at 25 ℃ and 0 ℃ are statistically different in week 2. Also, in week 3, ATM juice at 50 ℃ is different from 25 ℃, 4 ℃ and 0 ℃, presenting lower L values, and in week 5 ATM juice at 25 ℃ is statistically different from juice stored at 0 ℃. For MO juice, in week 4 samples stored at 25 ℃ are statistically different from the ones stored at 0℃.

Hue angle was also calculated (data not shown). MO and ATM juices are only statistically different in week 2 and week 3, for juice stored at 25 ℃ (MO more negative). For juice stored at 4 ℃, in weeks 3 and 5, ATM and MO are statistically different. Lastly, for juice stored at 0 ℃, in week 3 and 5, ATM and MO were different. Regarding changes with temperature, ATM juices were statistically different in week 4 for juices stored at 25℃ and 0℃, and in week 5 for those stored at 0 ℃, 4 ° and 25℃. For MO, in week 3, those stored at 4 ℃, 25℃ and 50 ℃ were statistically different.

In addition, USDA color standards were used to evaluate color. The results are shown in figures 4.6a, b, c, and d. Orange juice stored at 50 ℃ was darker (figure 4.15a), being the one processed at ATM darker than juice at MO. Also, juice stored at this temperature was darker than the juice stored at the rest of the temperatures. For juice stored at 25 ℃ (figure 4.15b) the juice gets darker over time, with exception of week 5, where juice at MO conditions was specially

light. For weeks 3 and 4, MO juice was darker than ATM juice. For juice stored at 4℃ (figure 4.15c) orange juice processed at ATM gets lighter whereas the one at MO gets darker over time. The juice at the control temperature (figure 4.15d) was constant over time, being the juice processed at MO conditions darker that OJ processed at ATM.

5. Cloud

Cloud is determined as percent transmittance (%T) in figures 4.16a, b, c, and d. For all temperatures and processes (ATM and MO) the juice did not clarify (less than 30% T). Processing under low oxygen does not affect cloud in orange juice.

6. pH

In figures 4.17a, b, c and d the pH for juice is reported. pH values give indication of sterility of the product and was taken as a quality parameter to ensure that yeast and mold were not present. It may be observed that for all cases, the pH was lower than 4.5, which assures the juice was a high acid food; therefore, anaerobic microorganisms were not of concern. In addition, in general, with exception of two cases, day 4 at 50℃ (figure 4.17a), week 2 at 25℃ (figure 4.17b) and week 4 of the control temperature (figure 4.17d), pH was higher for juice processed at MO. The pH values varied from 4.2 to 4.5, however important differences were not observed.

7. Particle size

Figures 4.118 a, b and c present results of volume mean diameter, D[4,3] and figures 4.19a, b and c surface mean diameter D[3,2]. The only noticeable difference in particle size, D[4,3] values is observed at 50 ℃. The volume mean diameter is much lower at MO conditions

compared to ATM samples. When the temperature decreases, there is also a decrease in particle size, D[4,3] values ; however the values do not correlate with oxygen concentration, since there is no trend observed for both ATM and MO samples.

For surface mean diameter values, there is a more defined behavior between the ATM and MO samples. For juice stored at 50 ℃, D[3,2] values are lower for MO juice compared to ATM juice. However, for lower temperatures, the values are very similar between ATM and MO, moreover, the values at different storage temperatures (25 ℃ and 4 ℃) are also close to each other, suggesting that MO processing does not affect D[3,2] values.

Also, particle size distribution is presented in figures 4.20a, b, c and d for MO and ATM samples stored at 50℃, 4.21a, b, c and d for MO and ATM samples stored at 25℃, 4.22a, b, c and d for MO and ATM samples stored at 4 ℃ and 4.23a, b, c and d for samples stored at 0 ℃. In figures 4.20a, b and c, it may be observed that all MO samples present the same shape, with exception of Rep 2 MO that is specially higher for sizes close to 1000 μm. This increase is observed for all Rep 2 MO samples stored at each storage temperature. For samples processed at ATM (figures 4.20d and e) the shape of the curve is flatter than those for the MO samples, and for both ATM replications there is an increase in particle size after 100 μm that was not noticed for the MO samples (with exception of Rep 2 MO). For samples stored at 25 ℃, shape of the curve for MO and ATM samples are similar. It may be noticed that at week 5 for MO samples, the top of the curve moves to the left being maximum around 10μm. Same trend is observed for ATM samples (figures 4.21a, b, c and d). At 4℃, there are no differences in trends for ATM and MO samples (figures 4.22a, b, c, and d). At 0 ℃, the curves present similar shapes for ATM and MO. No difference in trends is observed (Figures 4.23a, b, c and d).

Processing under micro-oxygen conditions is an effective way to decrease the dissolved oxygen content in orange juice (figures 4.2a, b, c and d). However, not significant differences were observed between ATM and MO juices in ascorbic acid retention, browning, and particle size. During sample preparation for the indophenol titration method, agitation at atmosphere conditions was necessary. This may have incorporated oxygen to the MO samples, therefore no differences were observed between ATM and MO.

As mentioned in the introduction, oxygen is not directly responsible for juice color; however it influences ascorbic acid degradation that may result in browning due to the formation of furfurals (Garcia-Torres et al. 2009). Even though some authors have found that in the presence of oxygen scavengers there is less browning (Zerdin et al. 2003), or that browning is correlated with dissolved oxygen (Trammell et al. 1986), in the present study was found that micro-oxygen processing does not affect color, since no correlation was found between color and oxygen concentration, and in the majority of the cases, ATM was not significantly different from MO juice. Moreover, the oxygen levels achieved in the present study are much lower than other values reported in the literature. For instance, a study found that browning and ascorbic acid degradation were linearly related to oxygen levels of 0.6, 1.8 6.5 and 10.1 ppm (Trammell et al. 1986), in this study, levels of 34 to 100 ppb of oxygen did not reflect the same results. However, the same study found that after 22 weeks of storage, browning was occurring, suggesting that browning may occur independently of oxygen or there is also the possibility of oxygen ingress through the package that may be available for oxidation reactions (Trammell et al. 1986). Other authors have found that oxygen removal through glucose oxidase-catalase, achieving dissolved oxygen concentrations between 1 and 7ppm, did not improve the quality and shelf stability of

pasteurized orange juice, although it did improve it in fresh orange juice (Sagi and Mannheim, 1990).

Researchers have found that just enough oxygen is necessary to initiate changes and that additional oxygen is not necessary (Joslyn and Marsh, 1935), suggesting the idea that there is a critical level of oxygen necessary to trigger oxidative reactions and that oxygen levels achieved in this study are enough to produce such reactions. In addition, in presence of pulp, oxygen may be present as bubbles adsorbed in the surface of these cells (Ringblom, 2004), therefore giving a lower measurement of oxygen content than what is in reality present in the product. Even though the juice was filtered through Miracholoth®, in some occasions pulp was found in the packaged juice.

It has also been found that juice that has been processed under atmosphere conditions and that has surrounding air during storage, but kept at -17°C, showed important retention of ascorbic acid after months of storage (Joslyn et al. 1934). These results suggest that temperature plays an important role in ascorbic acid retention, even when oxygen is present.

The pH values are considered constant throughout the study. These results are in accordance with another study where no changes in pH in grapefruit juice were observed for samples stored at different temperatures and sampled every three weeks during a 12-week period (Smoot and Nagy, 1980).

Cloud, the turbid appearance typical of orange juice and that is composed of suspended solids (Perez-Cacho and Rouseff, 2008) was not influenced by MO processing. Both ATM and MO samples were cloudy throughout the study (no clarification was observed % T< 36), which was expected since pectinesterase was inactivated by heating. Particle size was also not affected

by MO processing. With exception of juice stored at 50 ℃, there was no difference between juices processed at ATM and MO.

CONCLUSIONS

It may be concluded from this study that even though MO processing decreases dissolved oxygen levels in orange juice, there is enough entrained oxygen that quality parameters were no statistically different between ATM and MO juice. Dissolved oxygen throughout the study was not significantly different for ATM and MO juices, which may be the reason for having similar results for both juices. In addition, dissolved oxygen concentrations of 34 ppb may be enough to initiate oxidative reactions. Also, sample preparation for shelf stability studies, required exposure of the samples to atmosphere, which may have been an important factor that affected the results.

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Table 4.1. - Reaction order for ascorbic acid degradation for juice processed at ATM and MO conditions and stored at different temperatures

	Reaction order			
Temperature (℃)	ATM	R_{ATM}^2	MO	R_{MO}^2
50	first	0.9217	neither	<0.2
25	zero	0.472	first	0.7528
4	zero	0.7237	first	0.9993
0	zero	0.5059	first	0.6198

Table 4.2. Reaction rates for ascorbic acid degradation of orange juice processed at ATM and MO conditions and stored at different temperatures

ATM			MO		
Temperature (℃)	k	R^2	Temperature (℃)	k	R^2
50	0.0483	0.92	50	0.002982	0.21
25	0.00782	0.46	25	-0.00989	0.75
4	0.0069	0.72	4	-0.00299	0.99
0	0.0069	0.51	0	-0.00345	0.62

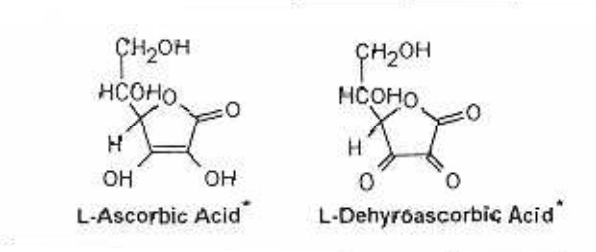

Figure 4.1. - L-ascorbic acid and L-dehydroascorbic acid. From Gregory, 1996.

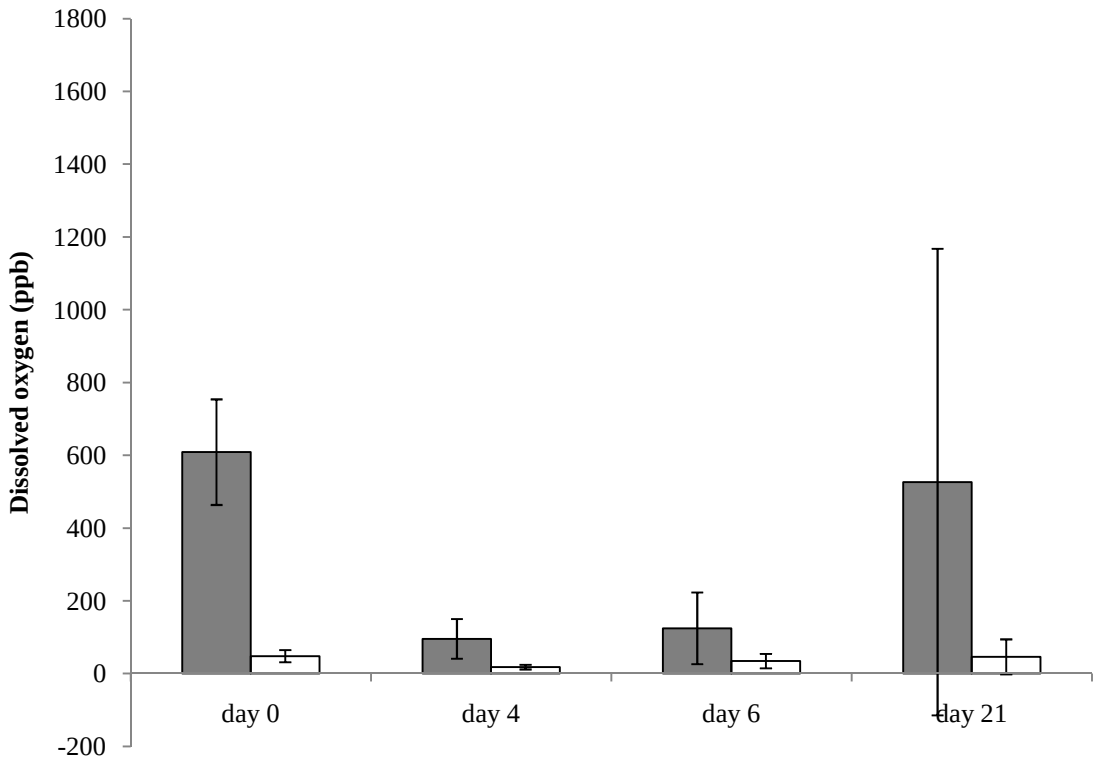

Figure 4.2a. - Dissolved oxygen for juice processed at ATM and MO conditions and stored at 50°C. ■ - ATM, □ - MO.

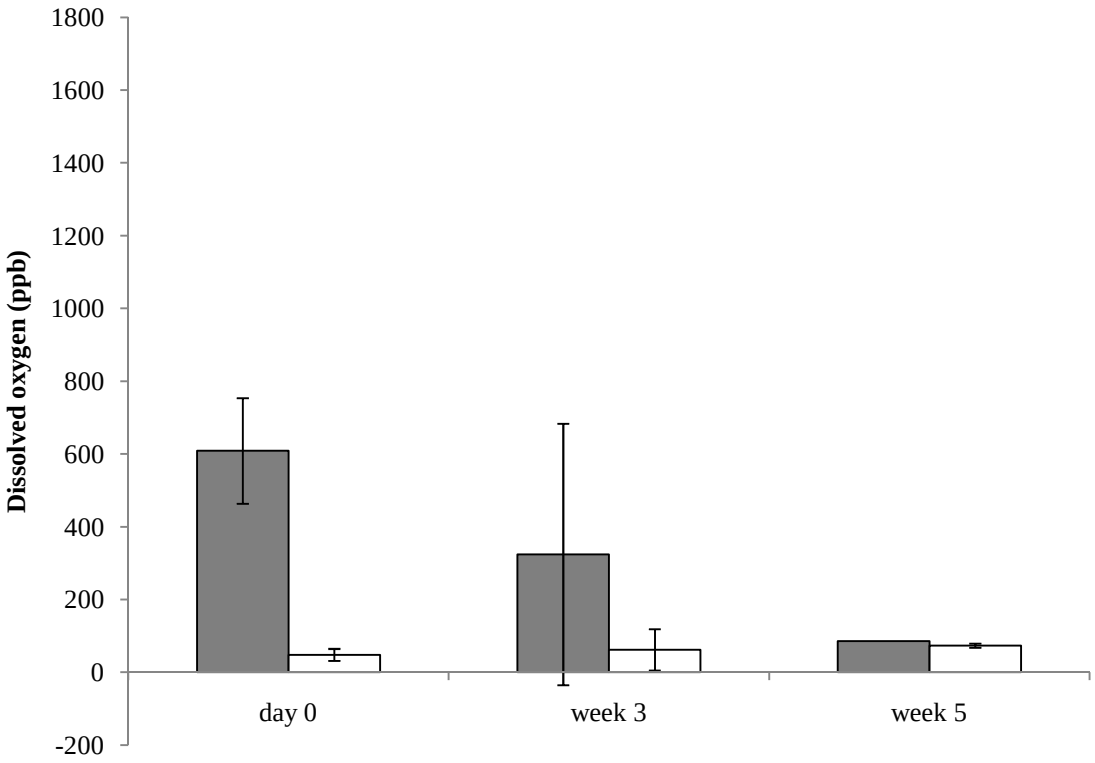

Figure 4.2b. - Dissolved oxygen for juice processed at ATM and MO conditions and stored at 25°C. ■ - ATM, □ - MO.

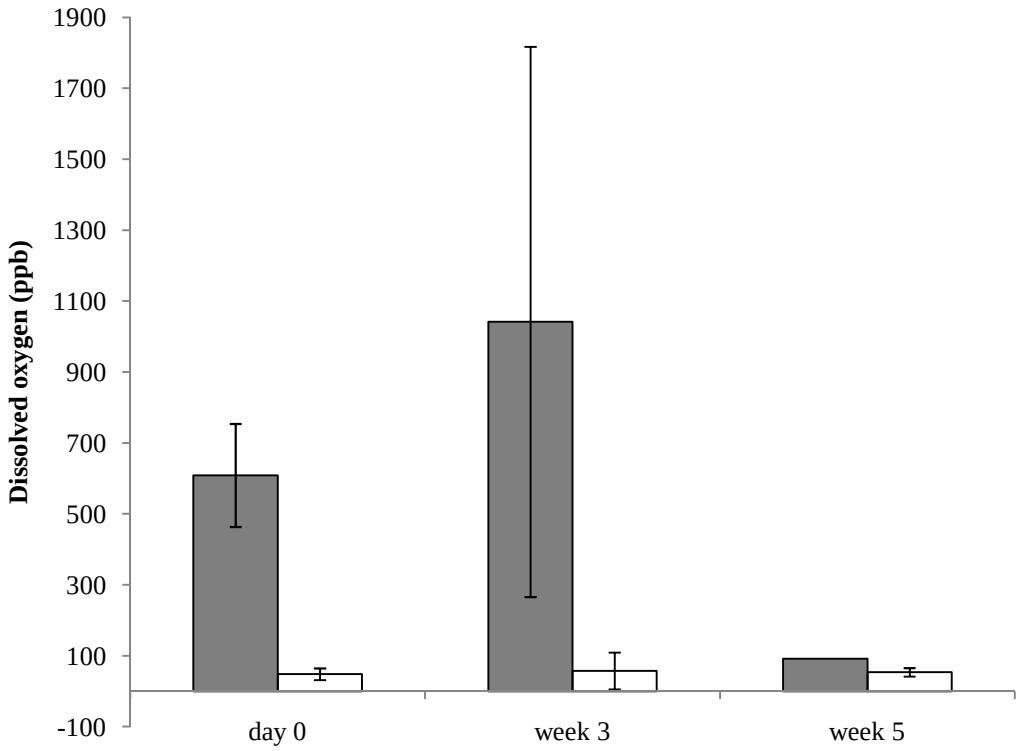

Figure 4.2c - Dissolved oxygen for juice processed at ATM and MO conditions and stored at 4°C. ■ - ATM, □ - MO.

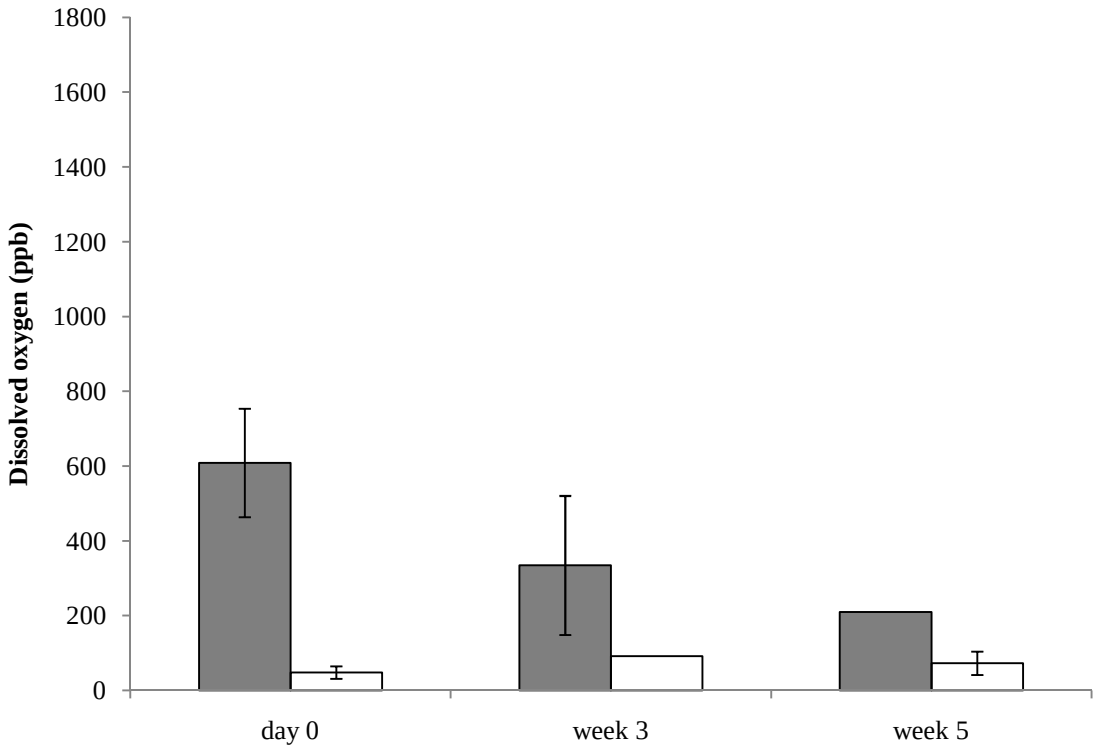

Figure 4.2d. - Dissolved oxygen for juice processed at ATM and MO conditions and stored at 0°C. ■ - ATM, □ - MO.

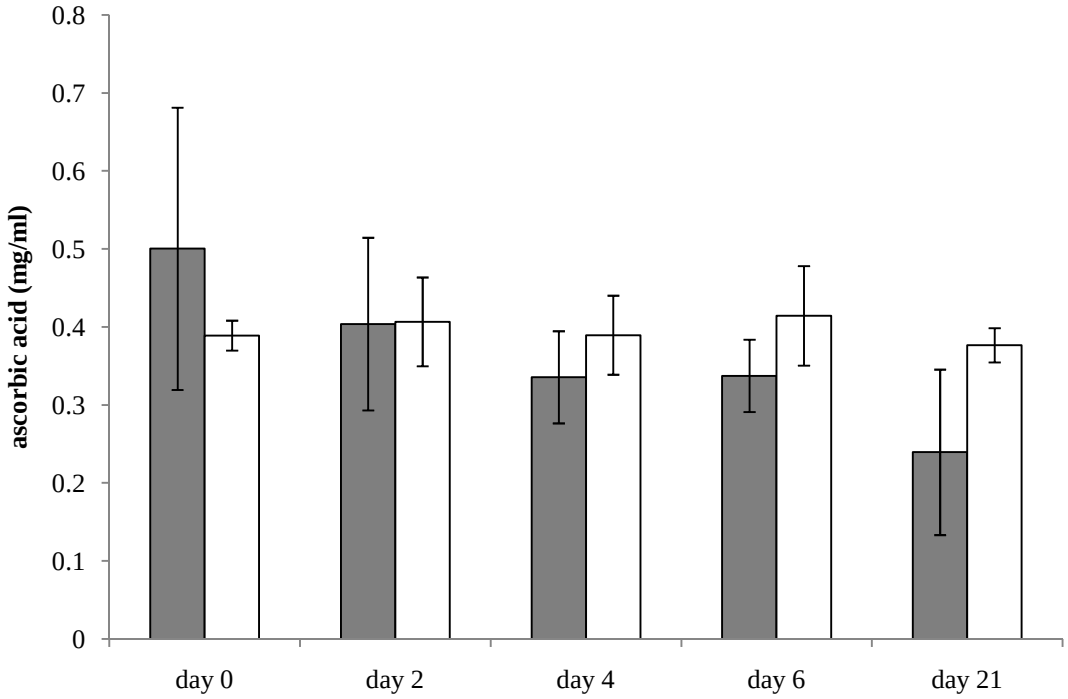

Figure 4.3a. - Ascorbic acid retention for juice stored at 50 °C. ■ - ATM, □ - MO.

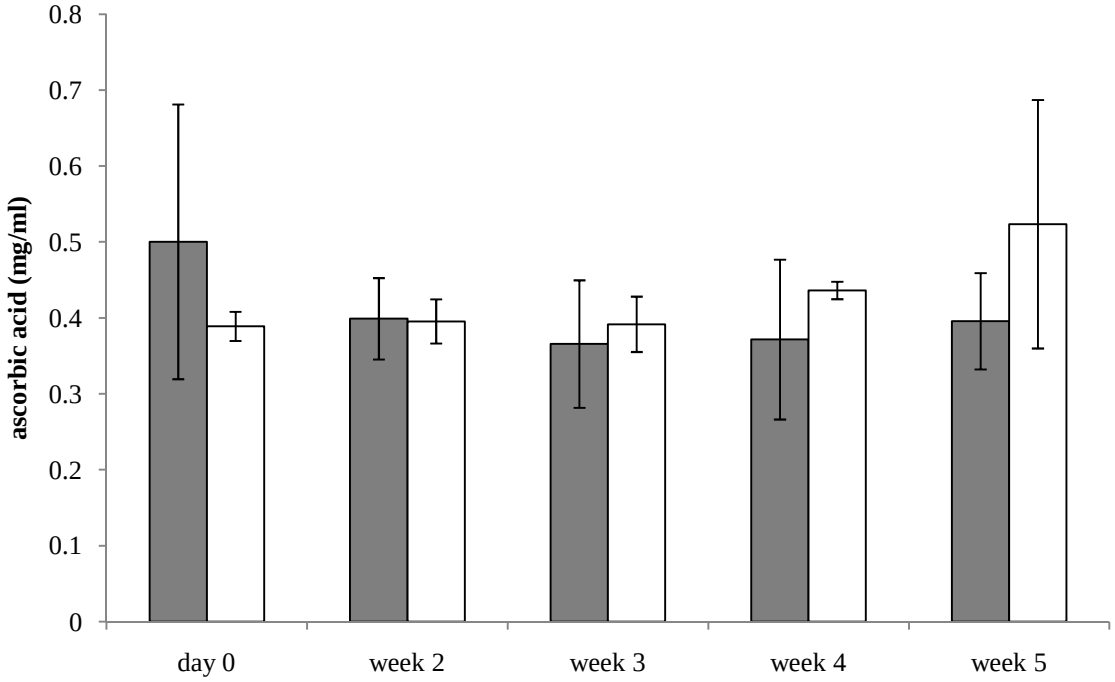

Figure 4.3b. - Ascorbic acid retention for juice stored at 25 °C. ■ - ATM, □ - MO.

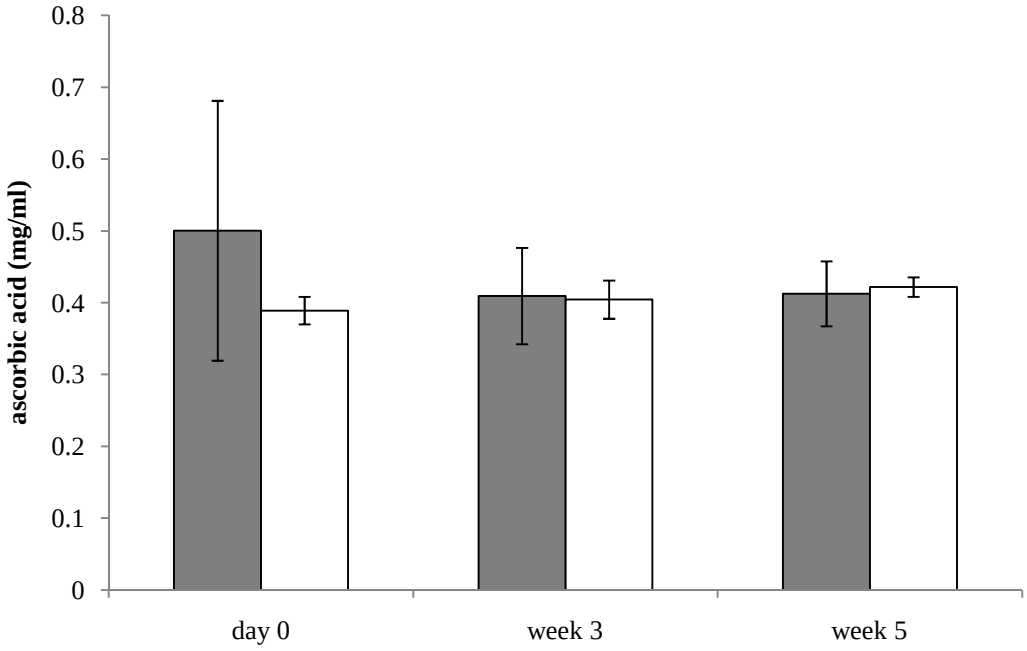

Figure 4.3c. - Ascorbic acid retention for juice stored at 4 °C. ■ - ATM, □ - MO.

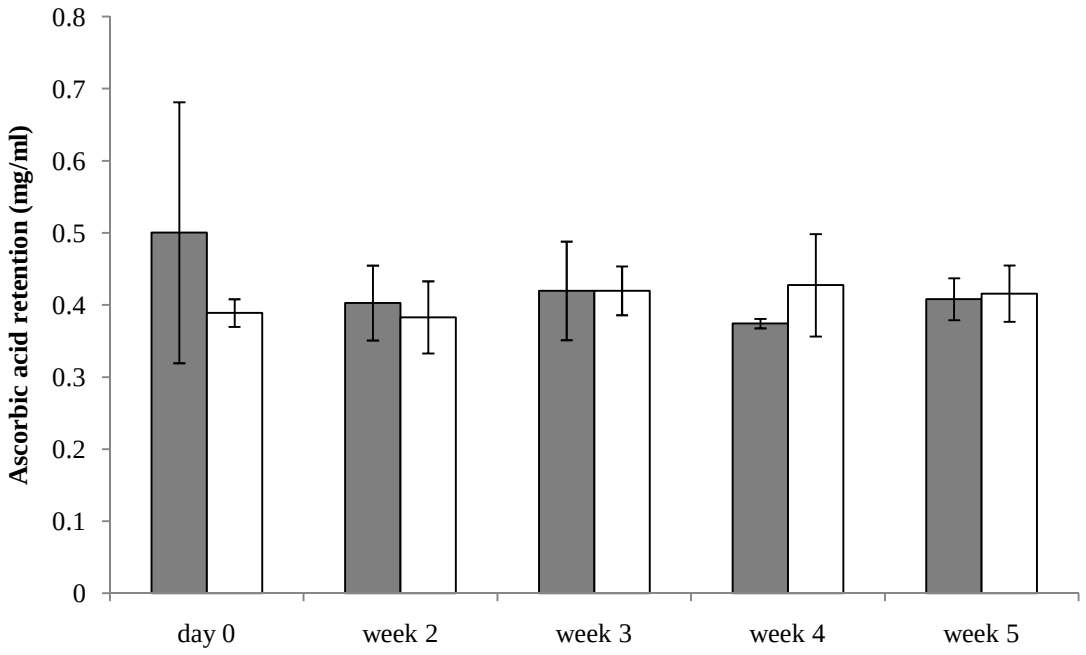

Figure 4.3d. - Ascorbic acid retention at 0 °C. . ■ - ATM, □ - MO.

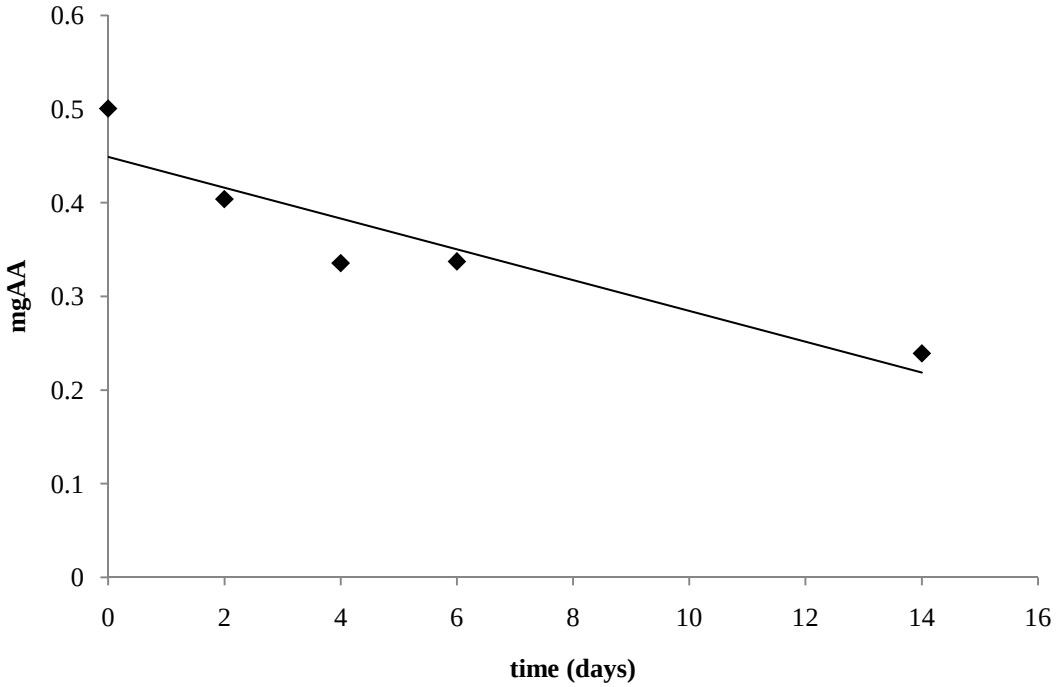

Figure 4.4 a. - Plot of ascorbic acid content versus time for juice at ATM and stored at 50°C. Linear regression is y= -0.0164x+0.4487; R^2= 0.8482

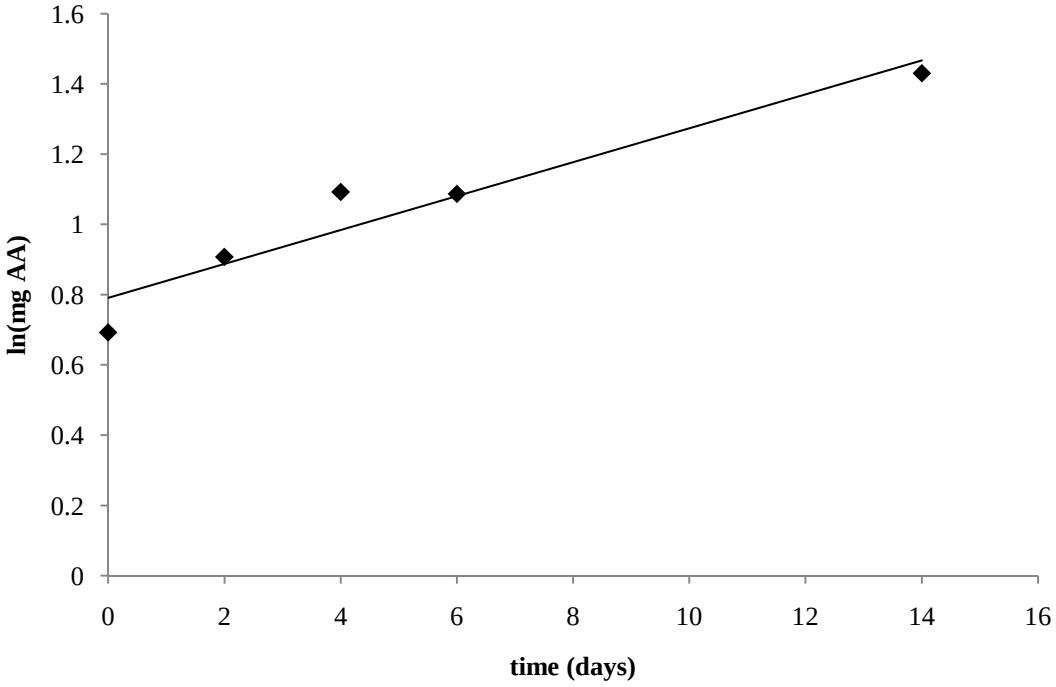

Figure 4.4b. - Plot of ln of ascorbic acid versus time for juice at ATM and stored at 50°C. Linear regression is y= 0.0483x+0.7906; R^2= 0.9217

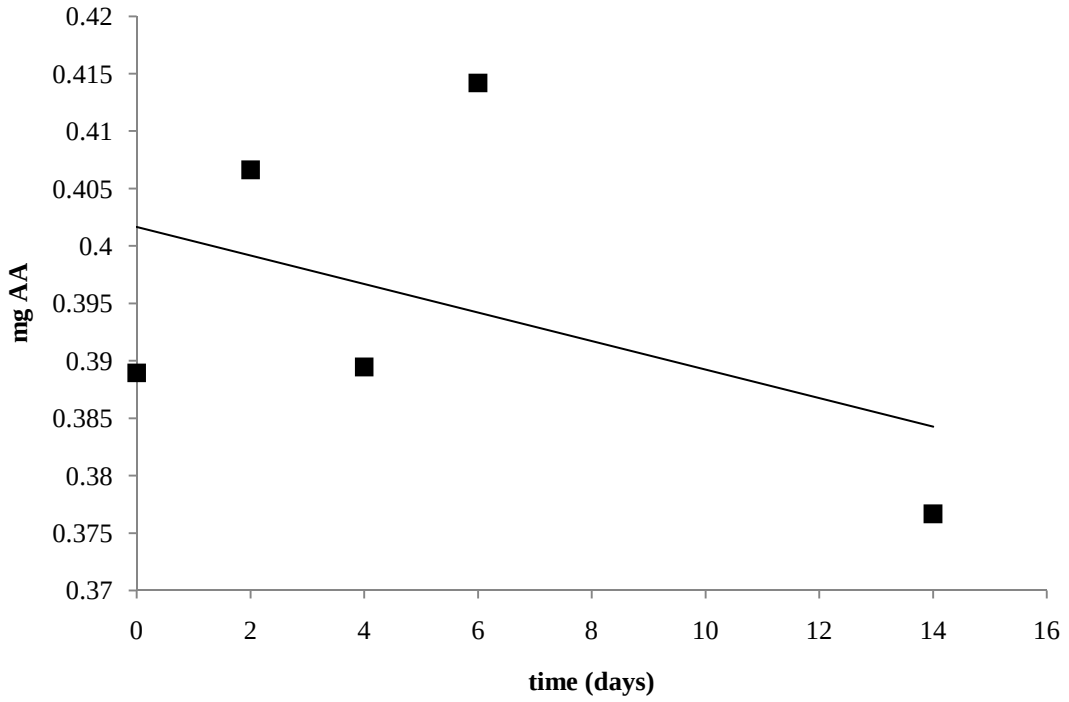

Figure 4.5a. - Plot of ascorbic acid content versus time for juice at MO and stored at 50°C. Linear regression is y= -0.0012x+0.4016; R^2= 0.1987

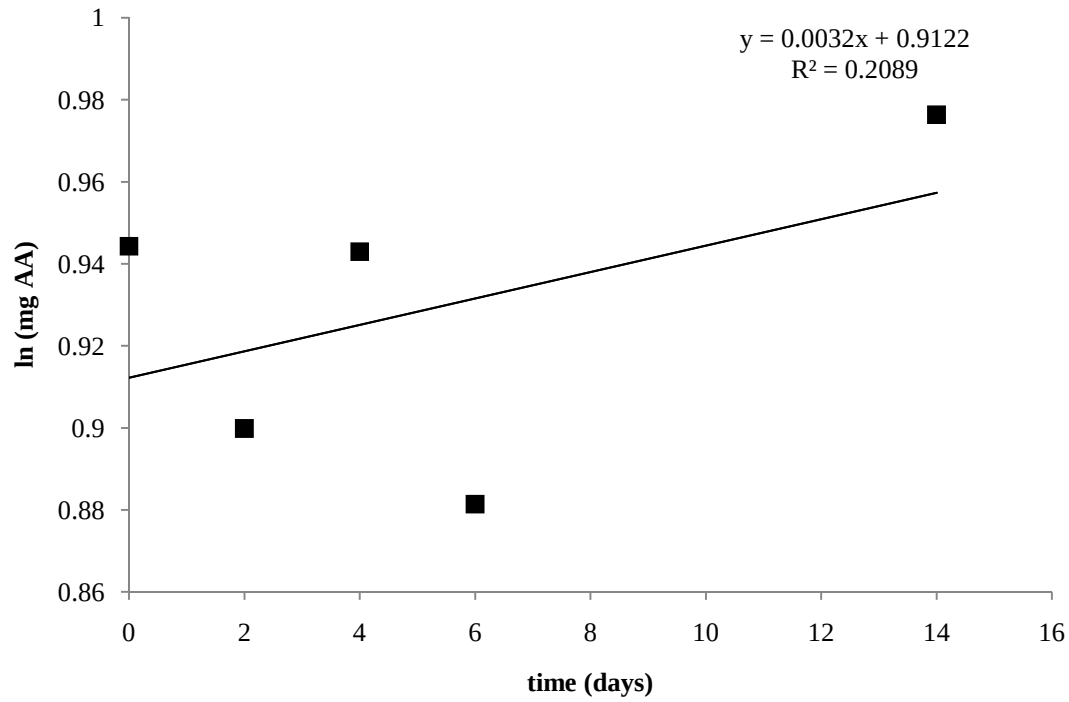

Figure 4.5b. - Plot of ln of ascorbic acid content versus time for juice at MO and stored at 50°C. Linear regression is y= 0.0032x+0.9122; R^2= 0.2089

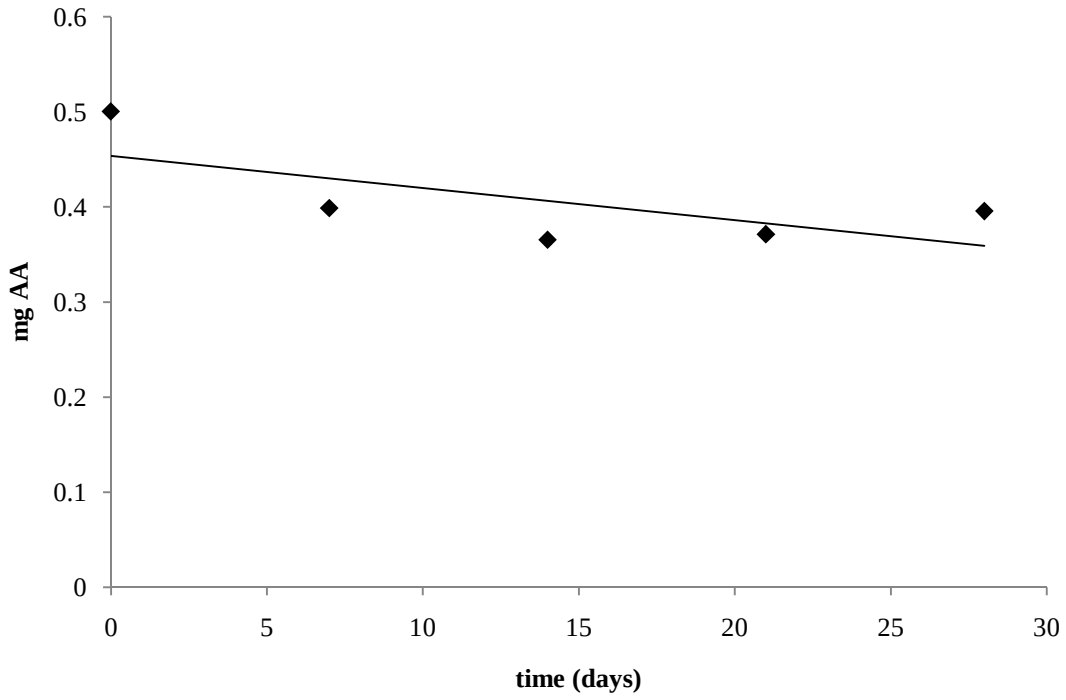

Figure 4.6 a. - Plot of ascorbic acid versus time for juice at ATM and stored at 25℃. Linear regression is y= -0.0034x+0.4538; R^2= 0.472

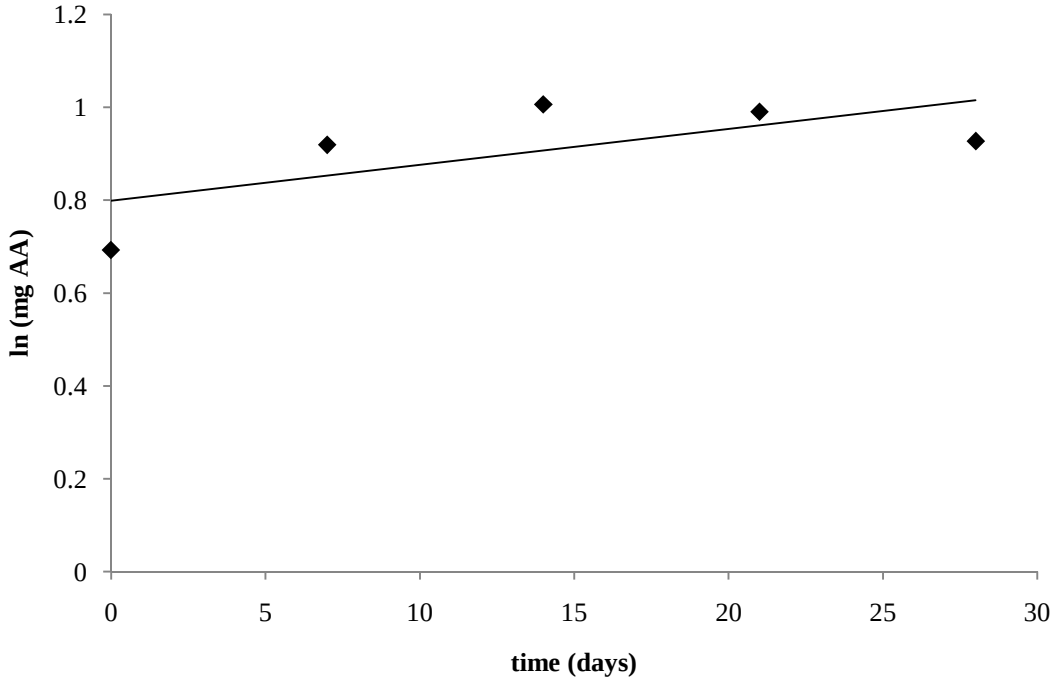

Figure 4.6b. - Plot of ln of ascorbic acid versus time for juice at ATM and stored at 25℃. Linear regression is y= 0.0077x+0.7989; R^2= 0.4616

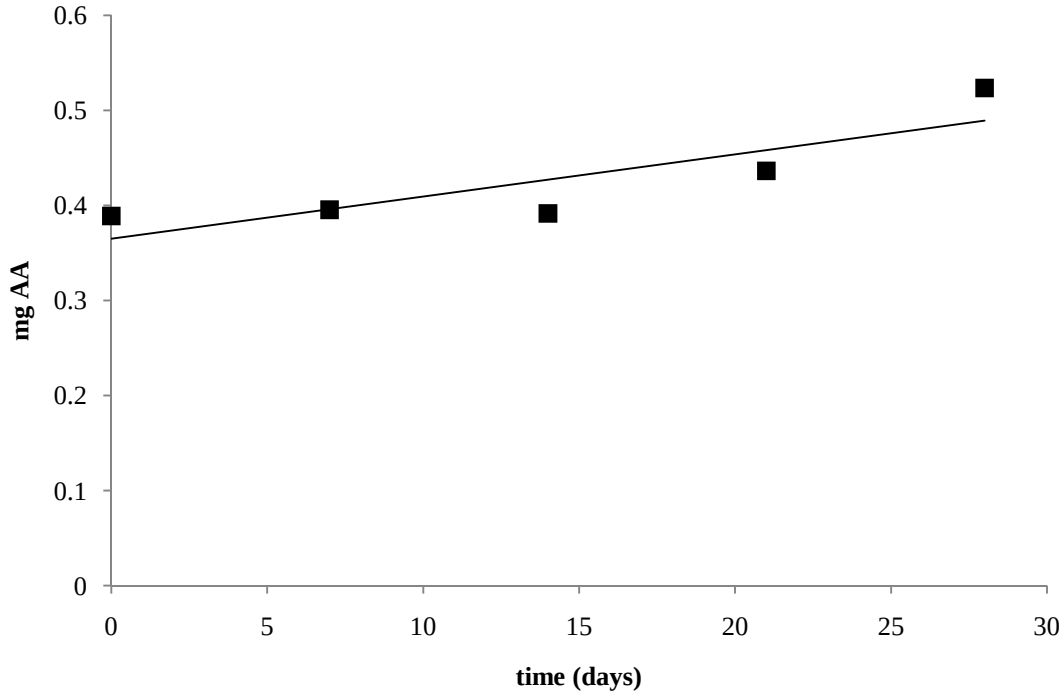

Figure 4.7a. - Plot of ascorbic acid versus time for juice at MO and stored at 25℃. Linear regression is y= 0.0044x+0.3652; R^2= 0.7338

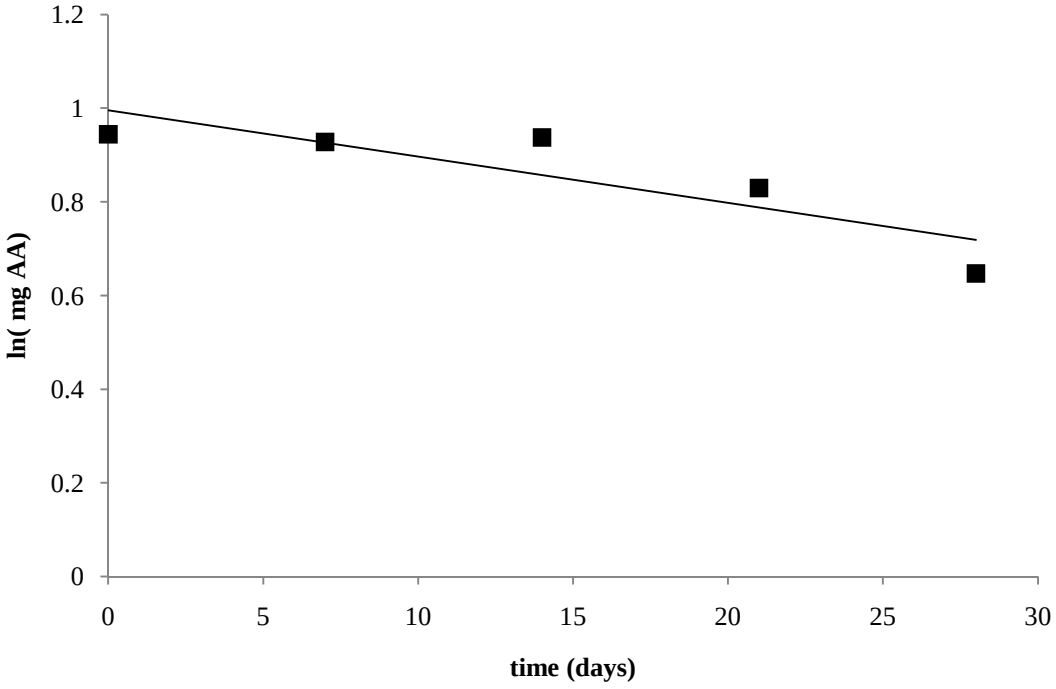

Figure 4.7b. - Plot of ln of ascorbic acid versus time for juice at MO and stored at 25℃. Linear regression is y= -0.0099x+0.9957; R^2= 0.7508

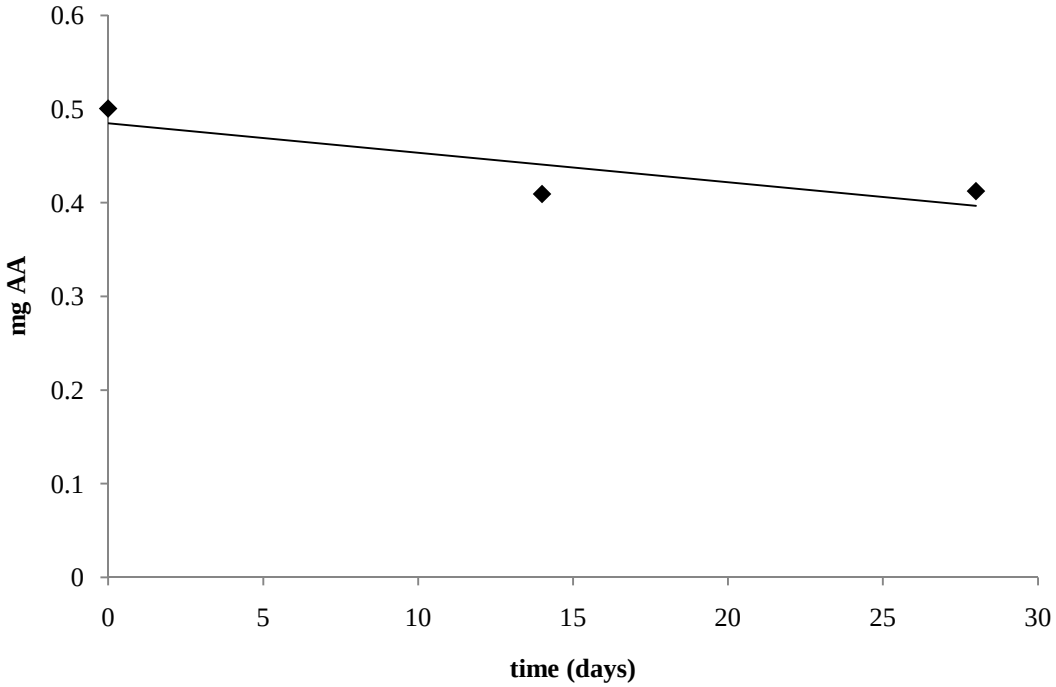

Figure 4.8a. - Plot of ascorbic acid versus time for juice at ATM and stored at 4°C. Linear regression is y= -0.0031x+0.4846; R^2= 0.7237

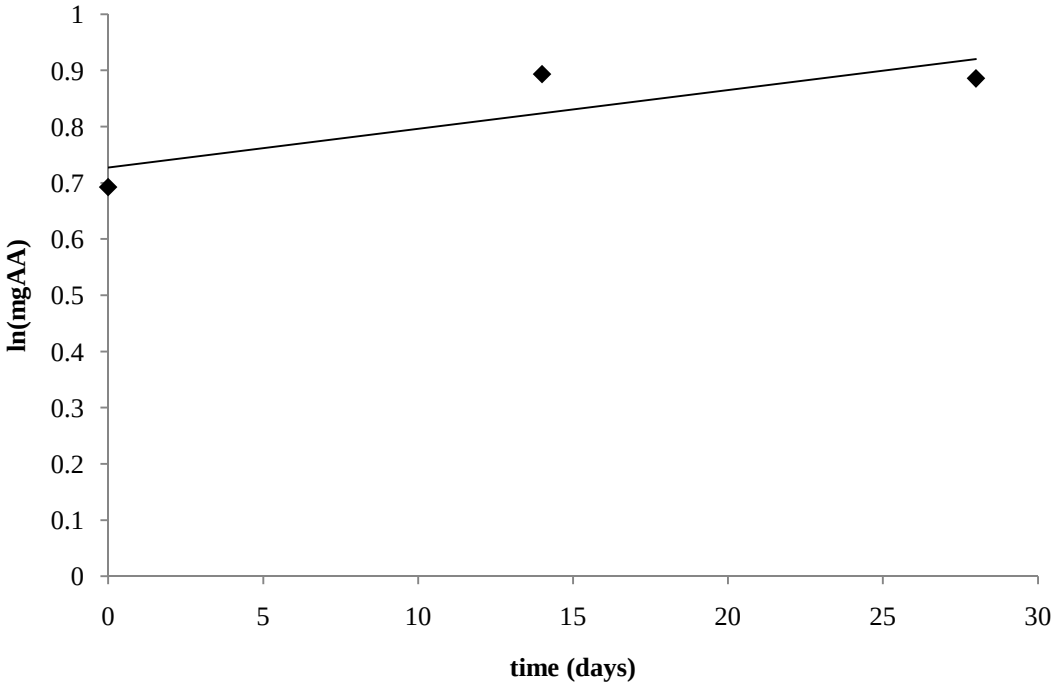

Figure 4.8b. - Plot of ln of ascorbic acid versus time for juice at ATM and stored at 4°C. Linear regression is y= 0.0069x+0.7272; R^2= 0.7209

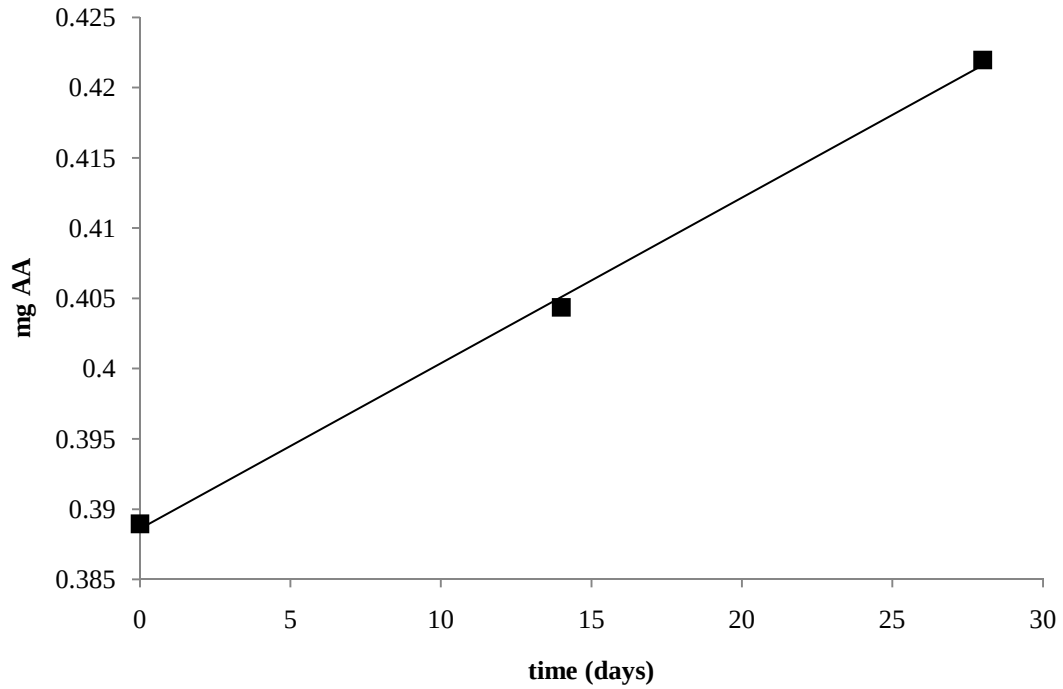

Figure 4.9a. - Plot ascorbic acid versus time for juice at MO and stored at 4℃. Linear regression is y= 0.0012x+0.3886; R^2= 0.9985

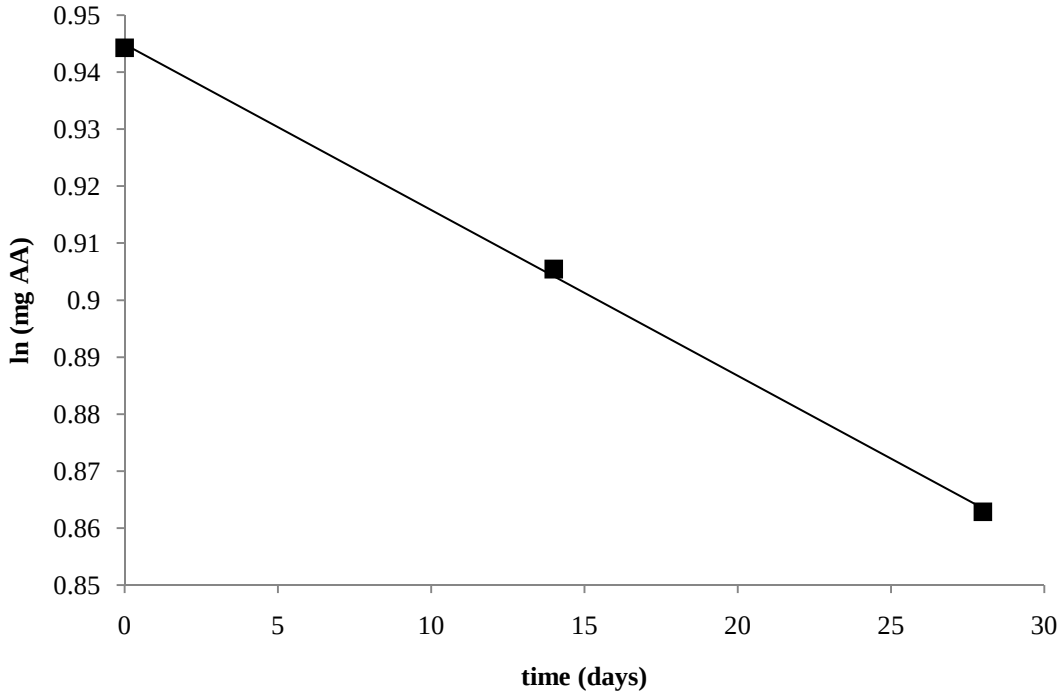

Figure 4.9b. - Plot ln of ascorbic acid versus time for juice at MO and stored at 4℃. Linear regression is y= -0.0029x+0.9449; R^2= 0.9993

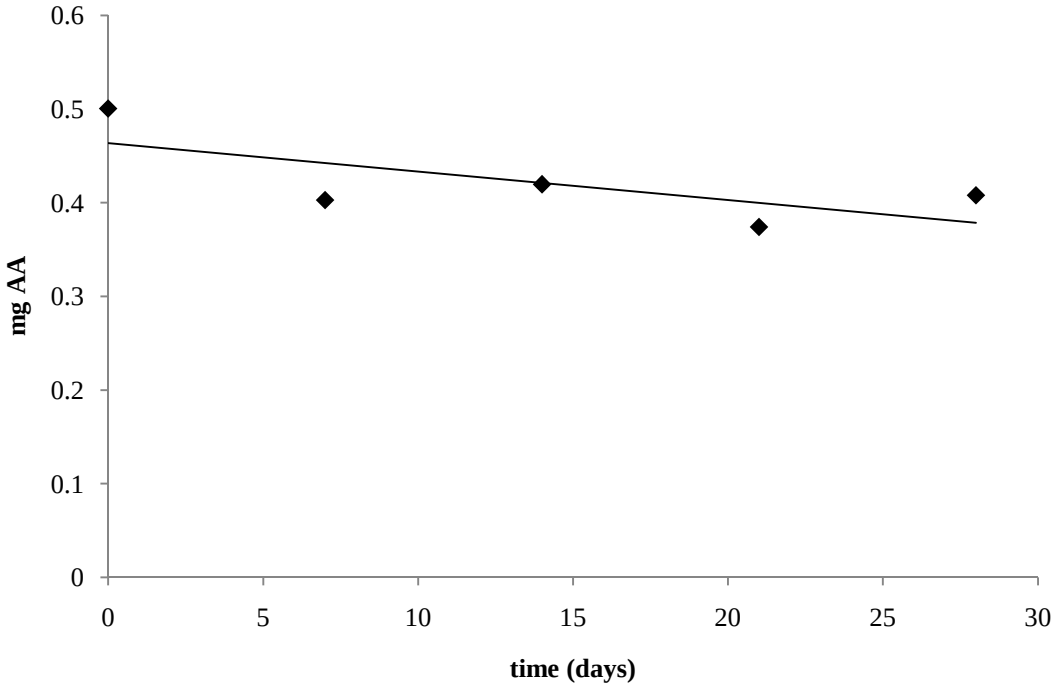

Figure 4.10a. - Plot of ascorbic acid versus time for juice at ATM and stored at 0°C. Linear regression is y= -0.003x+0.4636; R^2= 0.5059

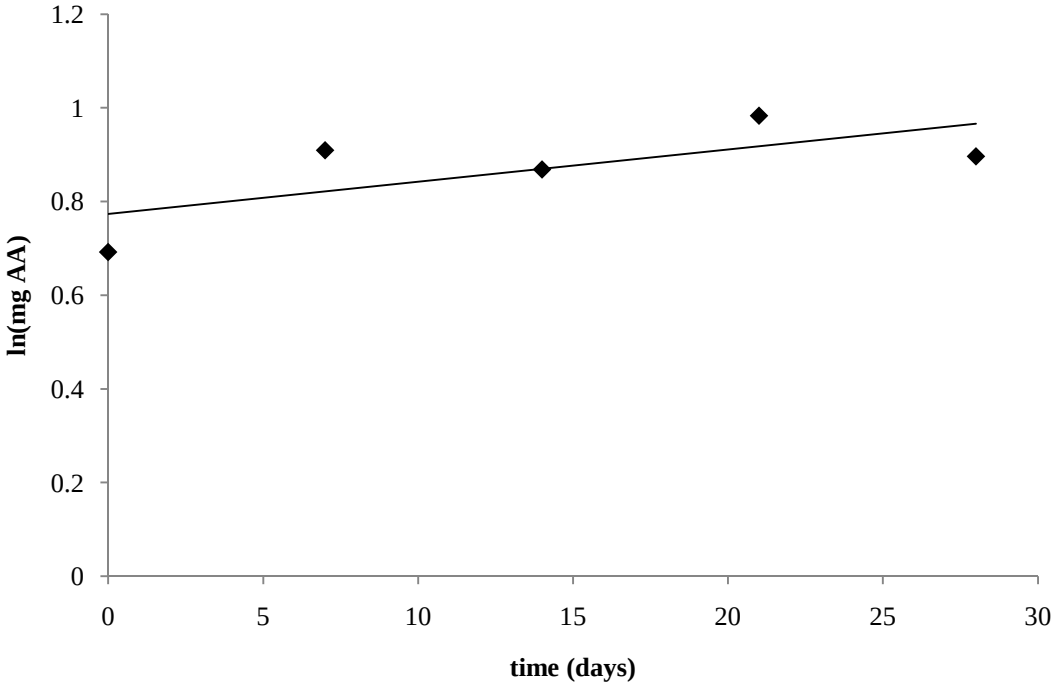

Figure 4.10b. - Plot of ln ascorbic acid versus time for juice at ATM and stored at 0°C. Linear regression is y= 0.0069x+0.4985; R^2= 0.4985

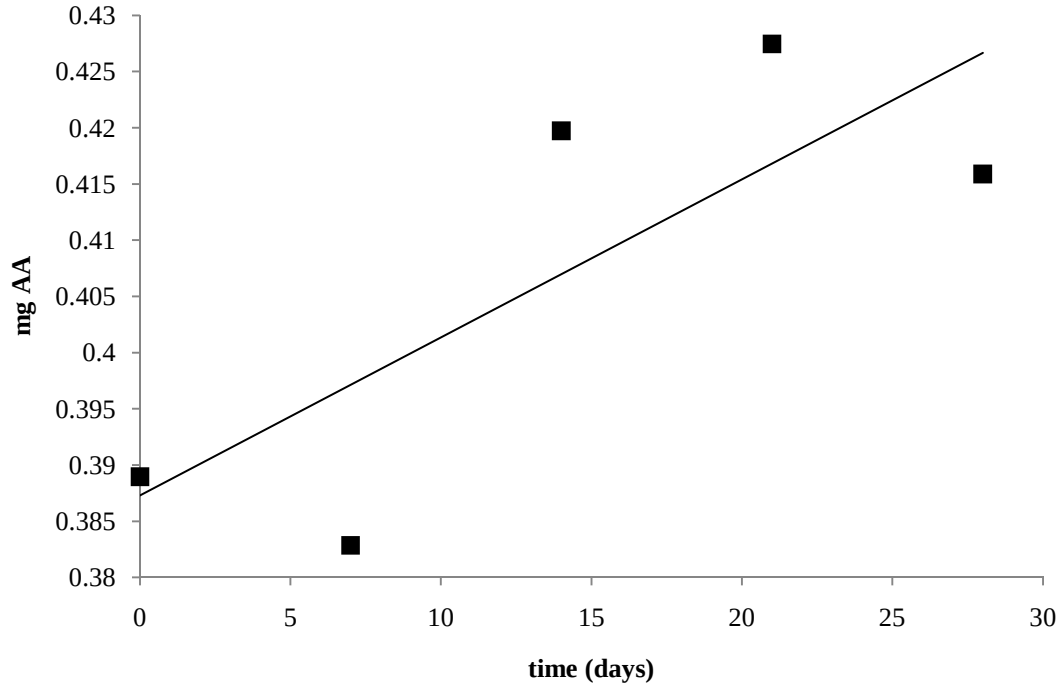

Figure 4.11a. - Plot of ascorbic acid versus time for juice at MO and stored at 0°C. Linear regression is y= 0.0014x+0.3873; R^2= 0.6182

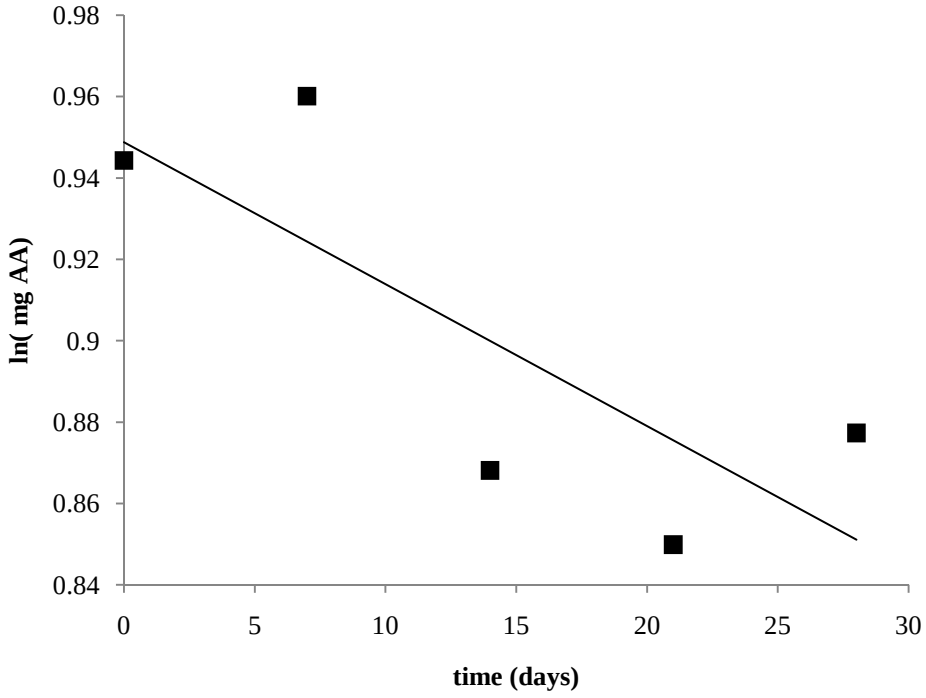

Figure 4.11b. - Plot of ln ascorbic acid versus time for juice at MO and stored at 0°C. Linear regression is y= -0.0035x+0.9488; R^2= 0.6198

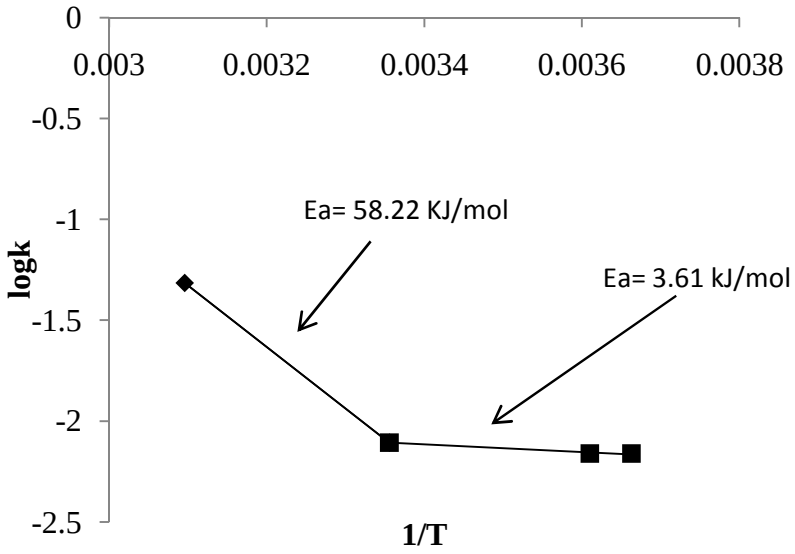

Figure 4.11c. – Plot of log k vs. the reciprocal of absolute temperature. Two linear regression fit the data, from 50℃ to 25℃: y = - 3044.5x + 8.1096; R^2= 1, and from 25℃ to 0℃: y = -188.53x -1.4751; R^2= 0.9741

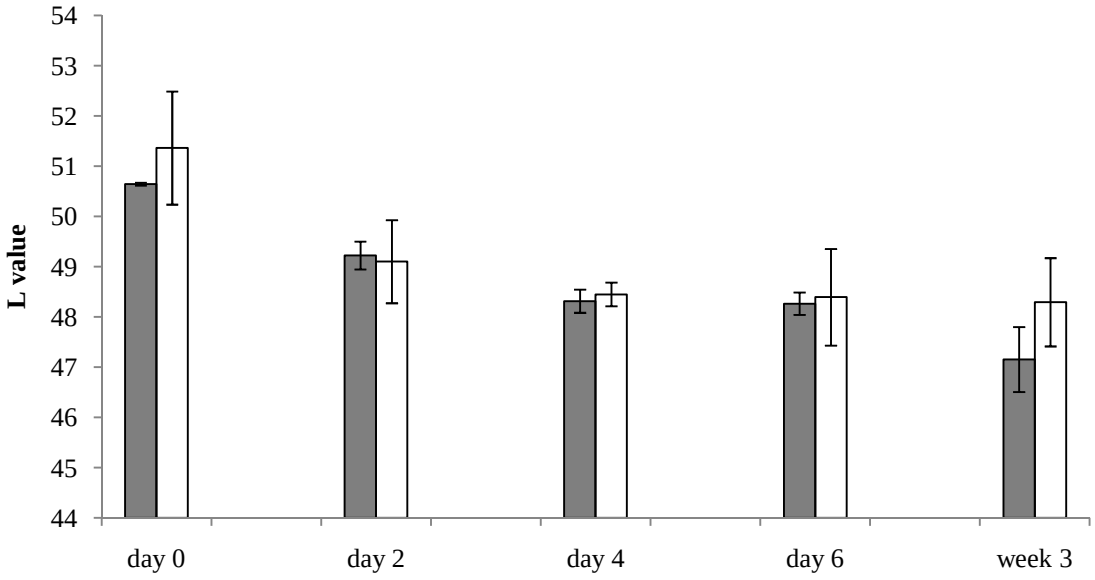

Figure 4.12a. - L values for juice processed at ATM and MO conditions and stored at 50 ºC. ■ - ATM, □ - MO.

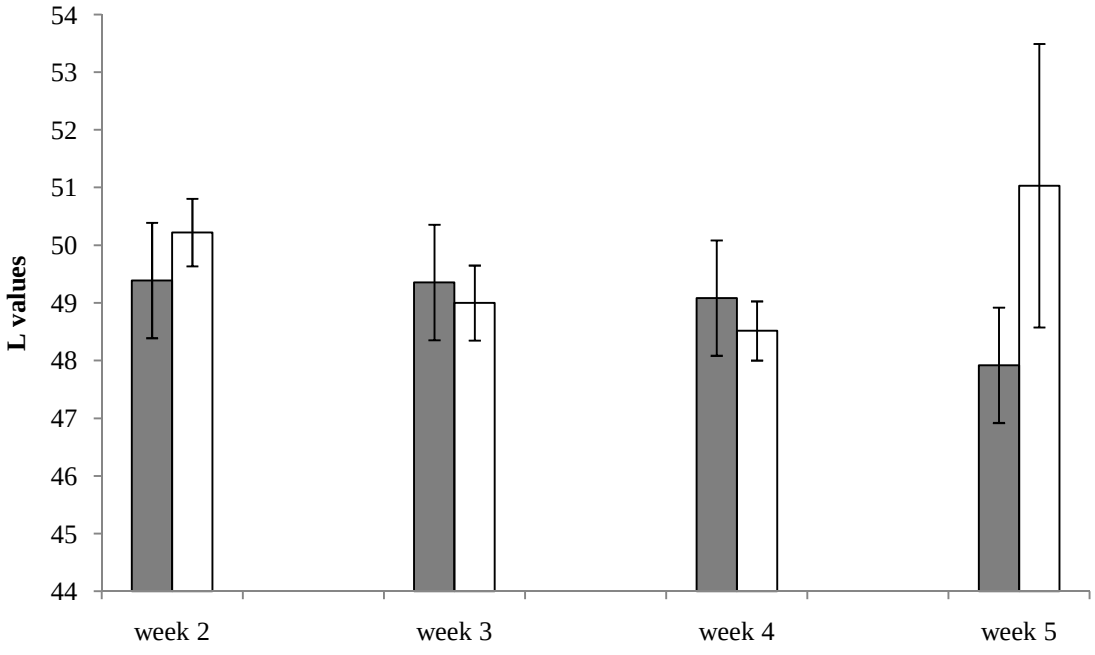

Figure 4.12b. - L values for juice processed at ATM and MO conditions and stored at 25 ºC. ■ - ATM, ⊔ - MO.

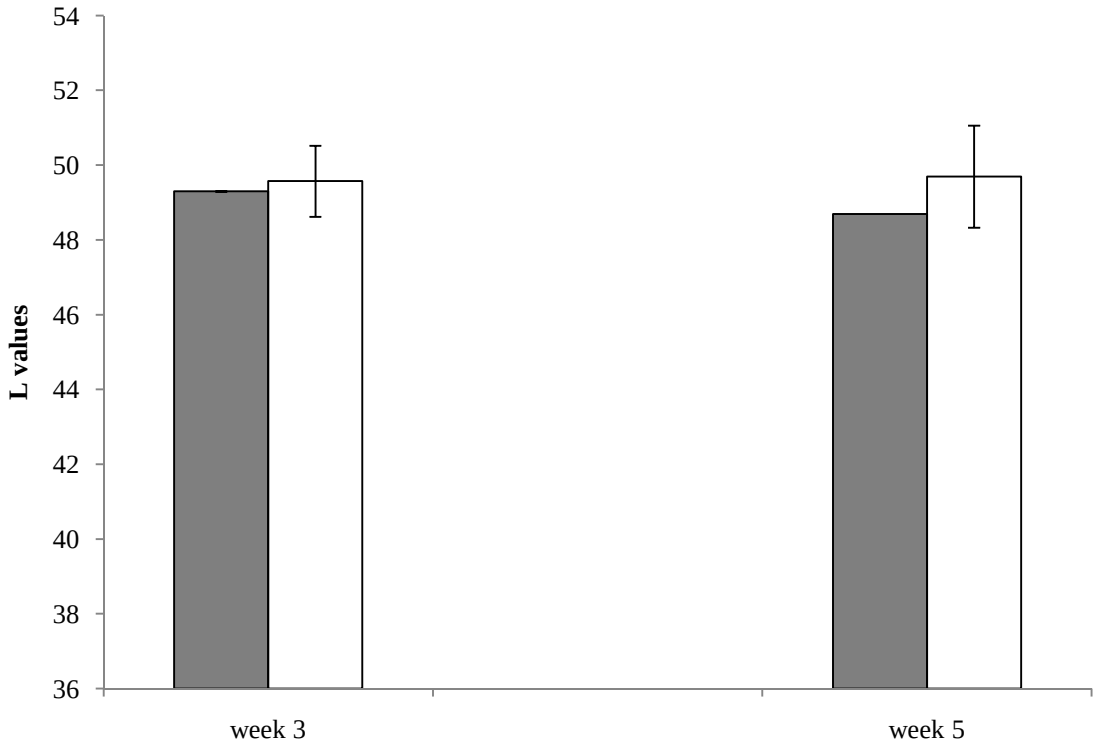

Figure 4.12c. - L values for juice processed at ATM and MO conditions and stored at 4 ºC. ▩ - ATM, ⊔ - MO.

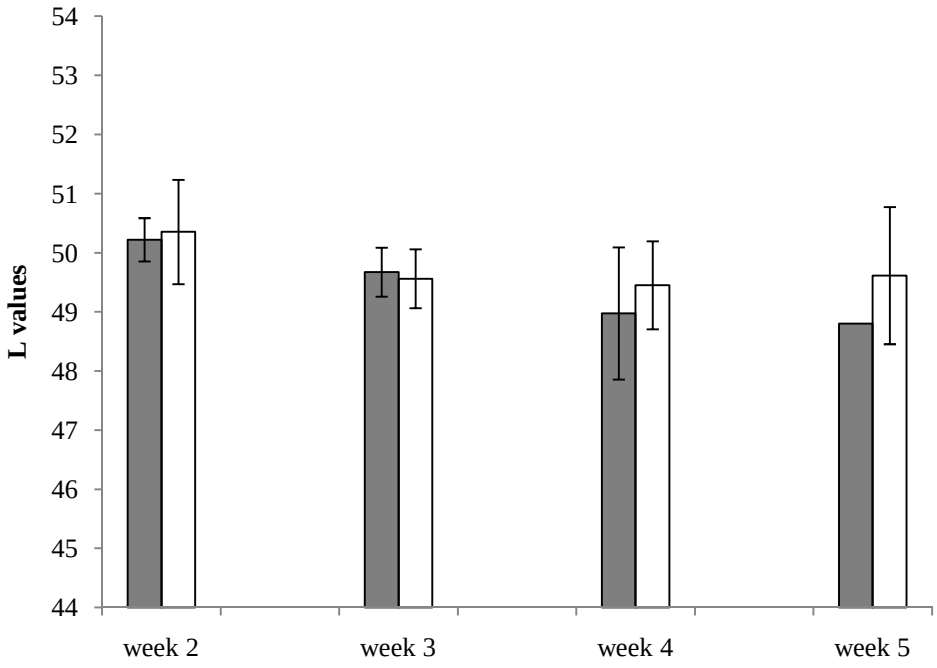

Figure 4.12d. - L values for juice processed at ATM and MO conditions and stored at 0°C. ■ - ATM, □ - MO.

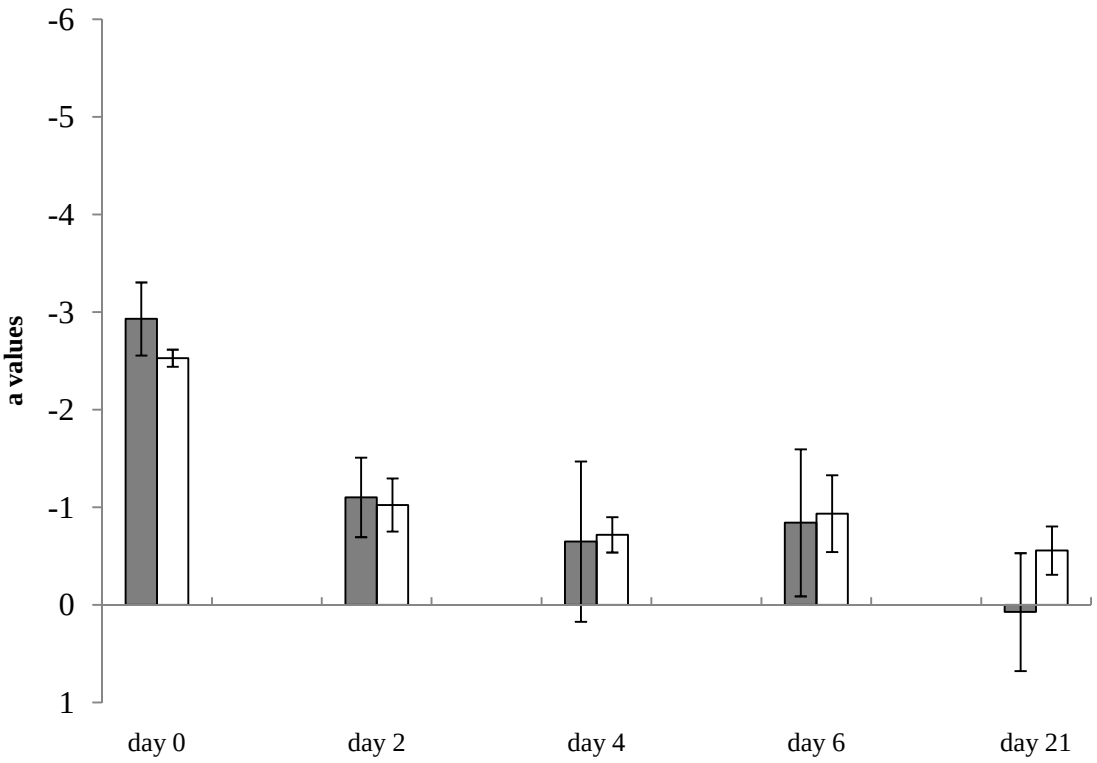

Figure 4.13a. - 'a' values for juice processed at ATM and MO and stored at 50 °C. ■ - ATM, □ - MO.

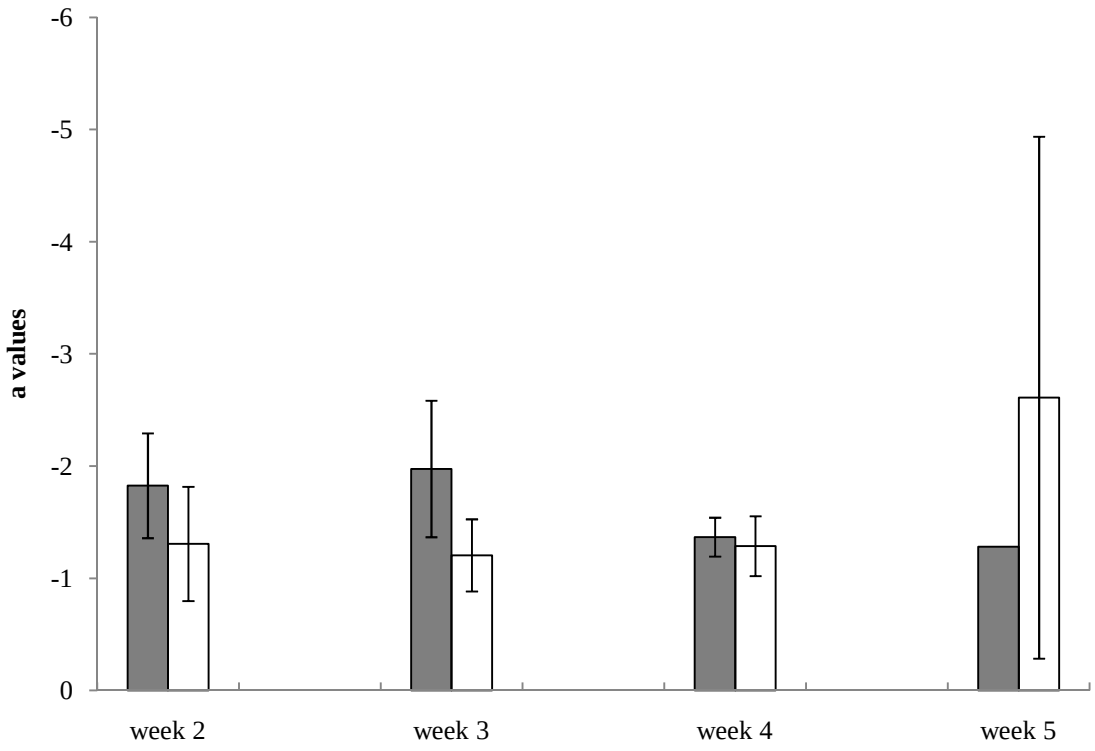

Figure 4.13b. - ‘a’ values for juice processed at ATM and MO and stored at 25 ℃. ■ - ATM, □ - MO.

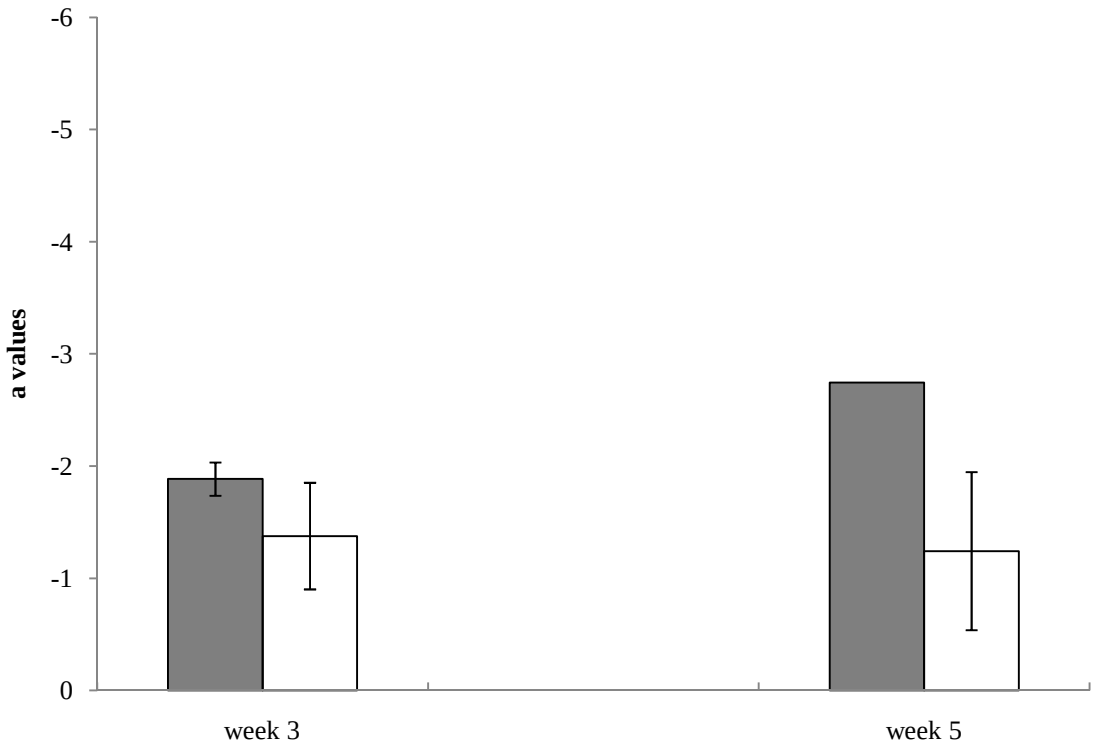

Figure 4.13c. - 'a' values for juice processed at ATM and MO and stored at 4 ℃. ■ - ATM, □ - MO.

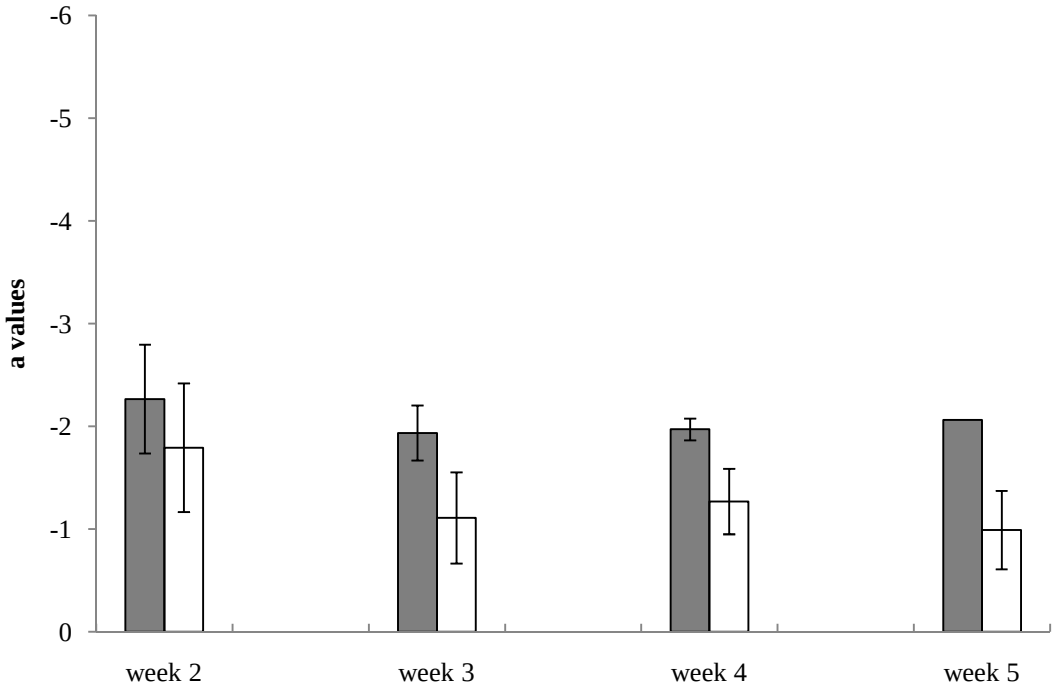

Figure 4.13d. - ‘a’ values for juice processed at ATM and MO and stored at 0 ℃. ■ - ATM, □ - MO.

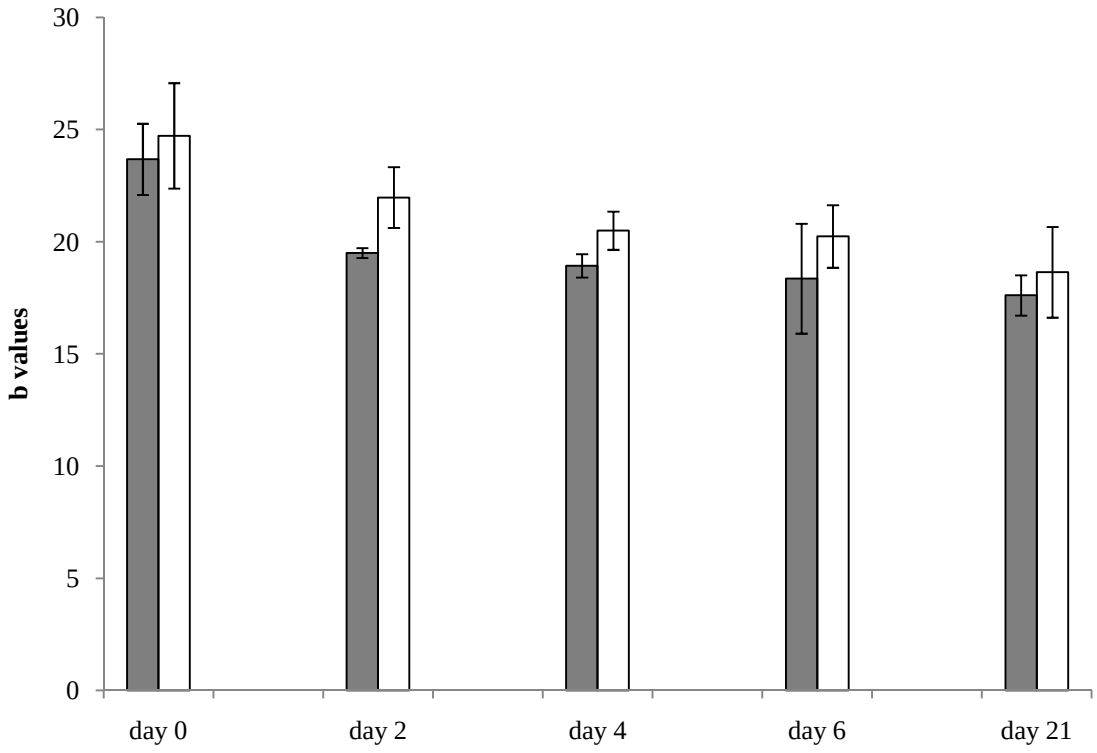

Figure 4.14a. - 'b' values for juice processed at ATM and MO condition and stored at 50°C. ■ - ATM, □ - MO.

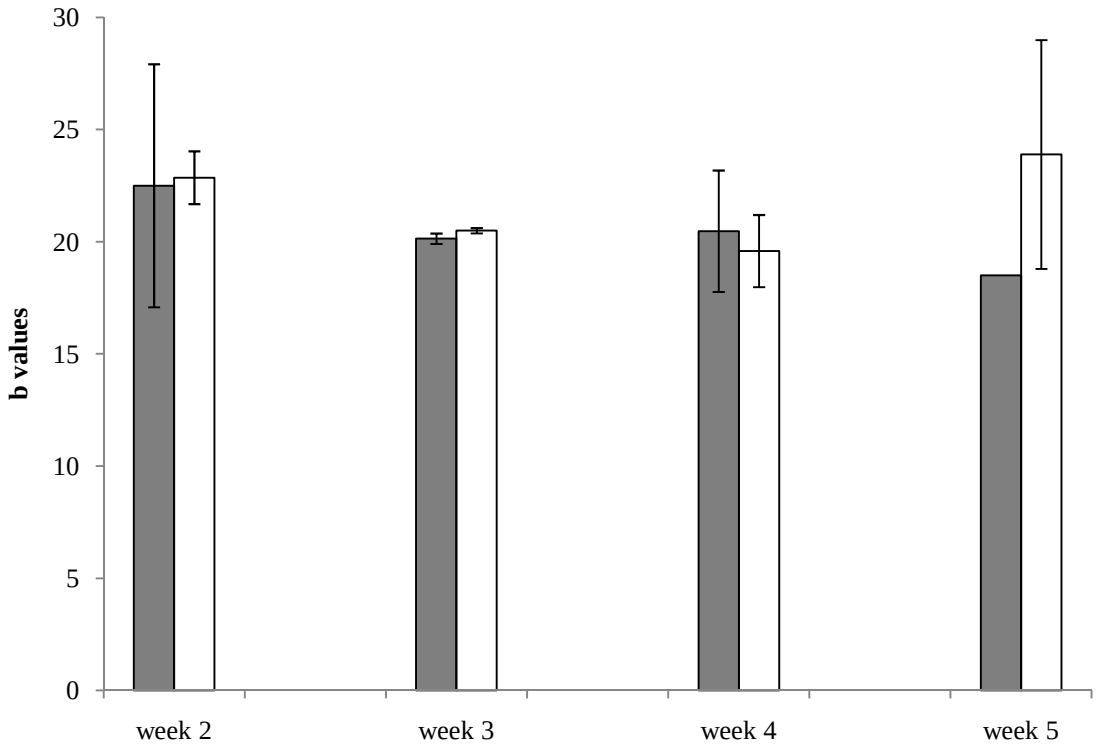

Figure 4.14b. - 'b' values for juice processed at ATM and MO condition and stored at 25 ℃. ■ - ATM, □ - MO.

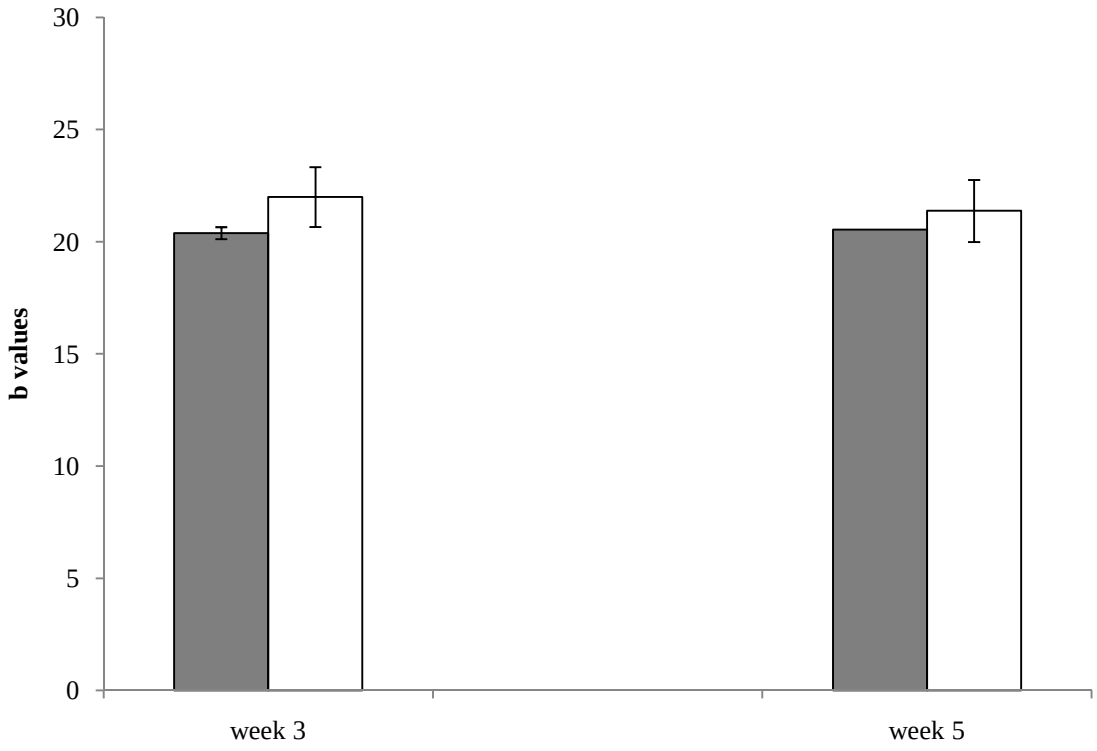

Figure 4.14c. - 'b' values for juice processed at ATM and MO condition and stored at 4 ℃. ■ - ATM, □ - MO.

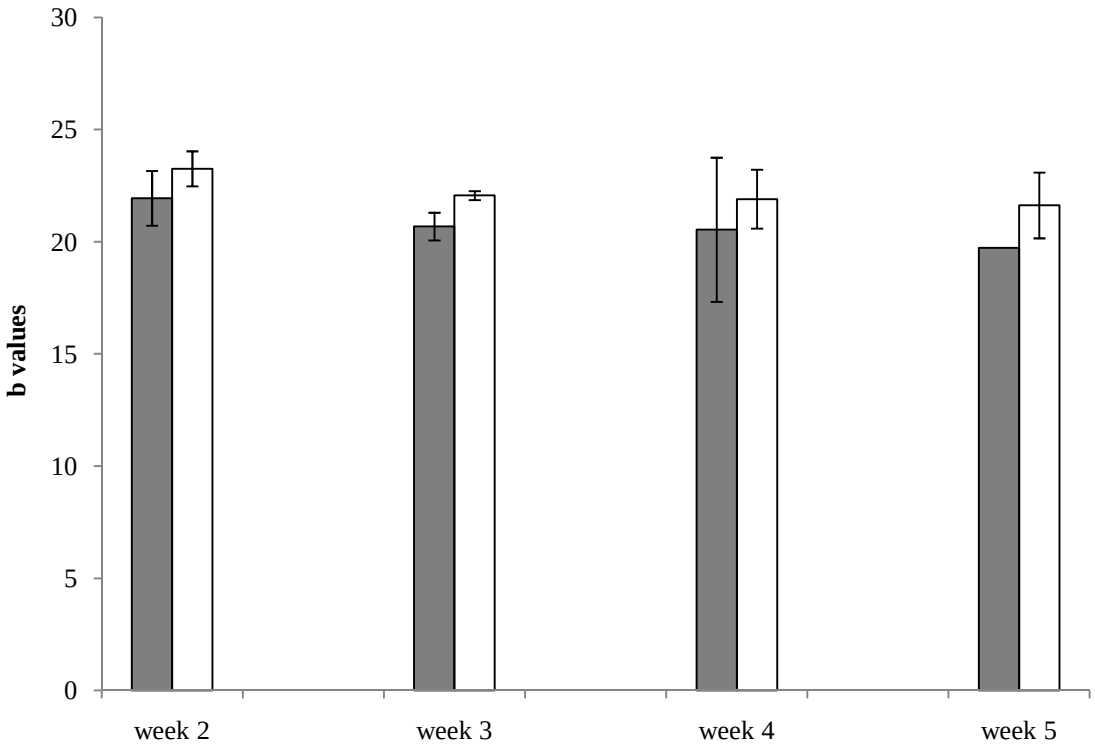

Figure 4.14d. - 'b' values for juice processed at ATM and MO condition and stored at 0 ℃. ■ - ATM, □ - MO.

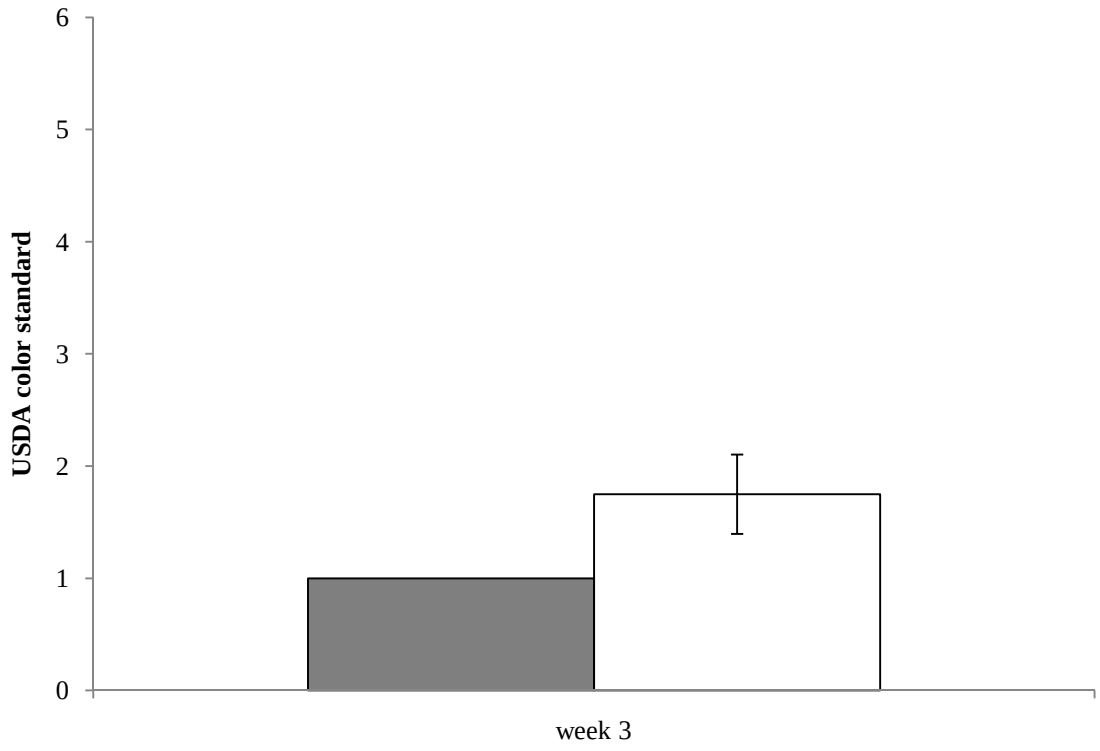

Figure 4.15a. - USDA color standard for juice processed at ATM and MO stored at 50 ℃. ■ - ATM, □ - MO.

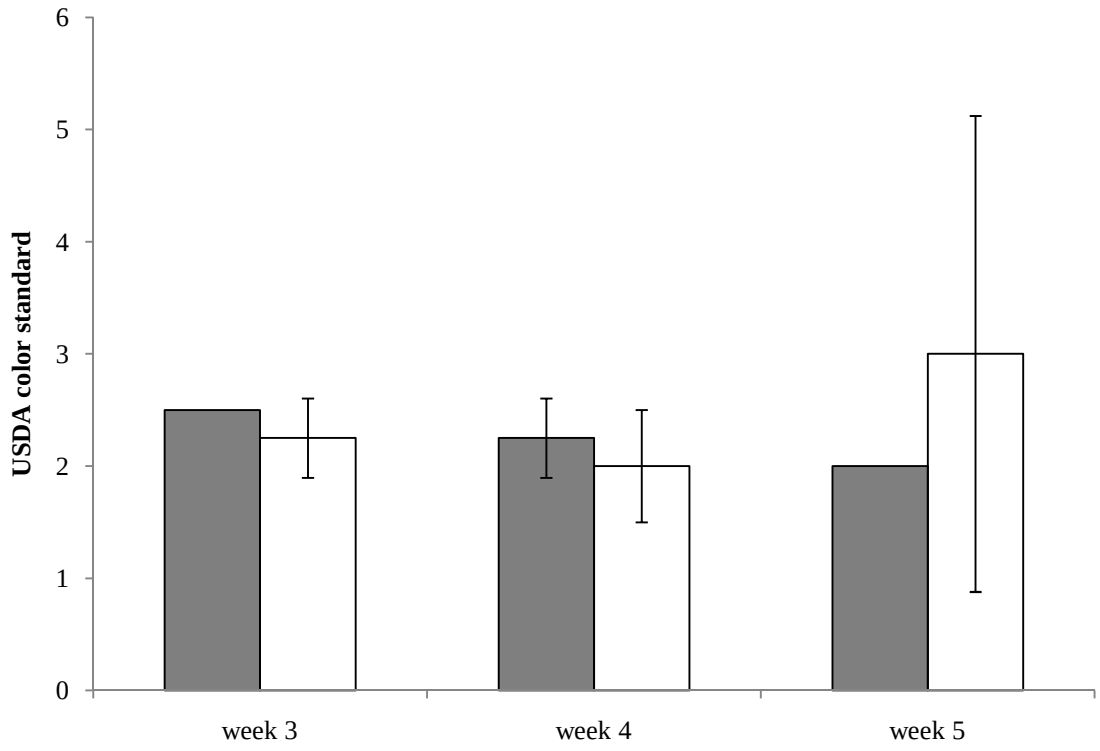

Figure 4.15b. - USDA color standard for juice processed at ATM and MO stored at 25 ℃. ■ - ATM, □ - MO.

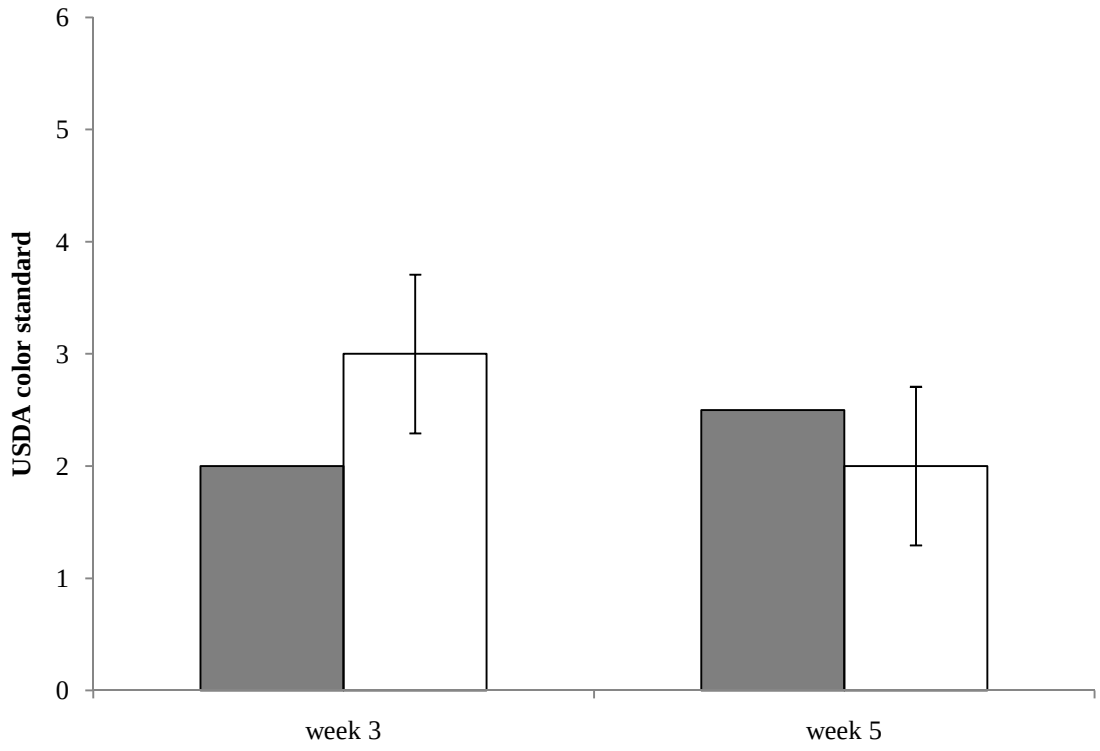

Figure 4.15c. - USDA color standard for juice processed at ATM and MO stored at 4 ℃. ■ - ATM, □ - MO.

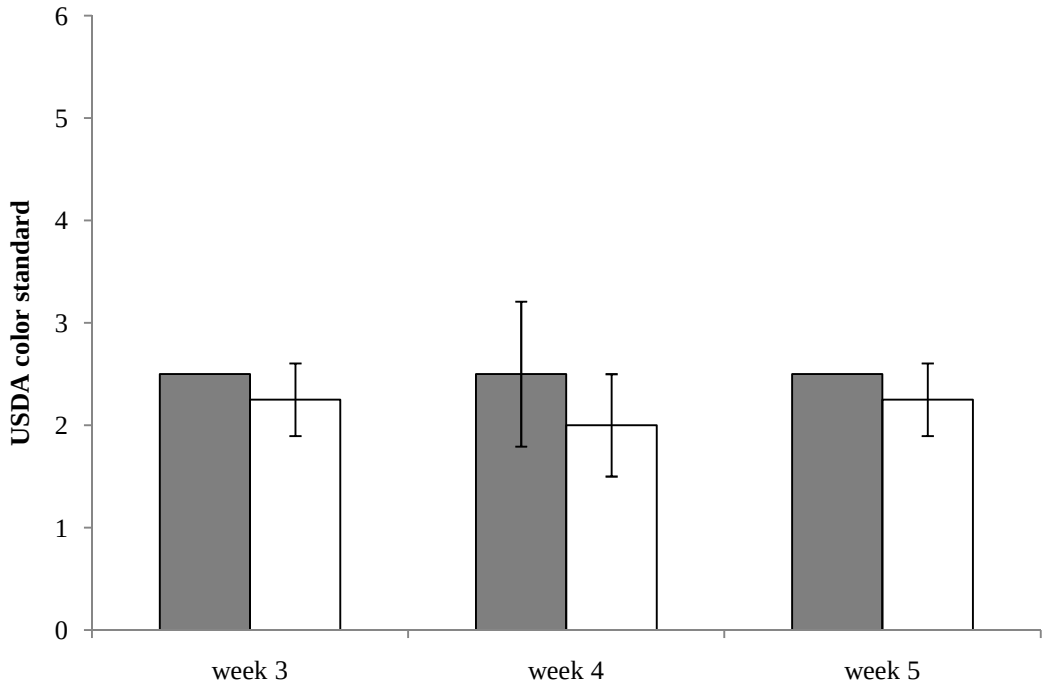

Figure 4.15d. - USDA color standard for juice processed at ATM and MO stored at 0 ℃. ■ - ATM, □ - MO.

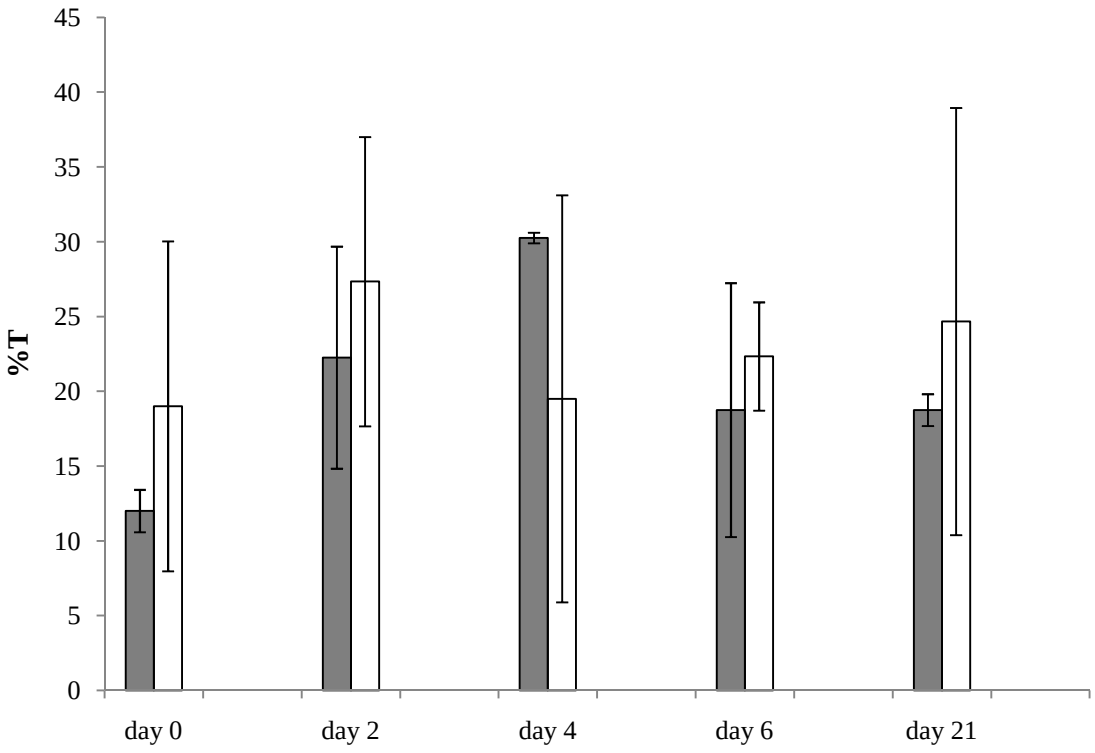

Figure 4.16a. - Percent transmittance over time for juice processed at ATM and MO conditions and stored at 50 °C. ■ - ATM, □ - MO.

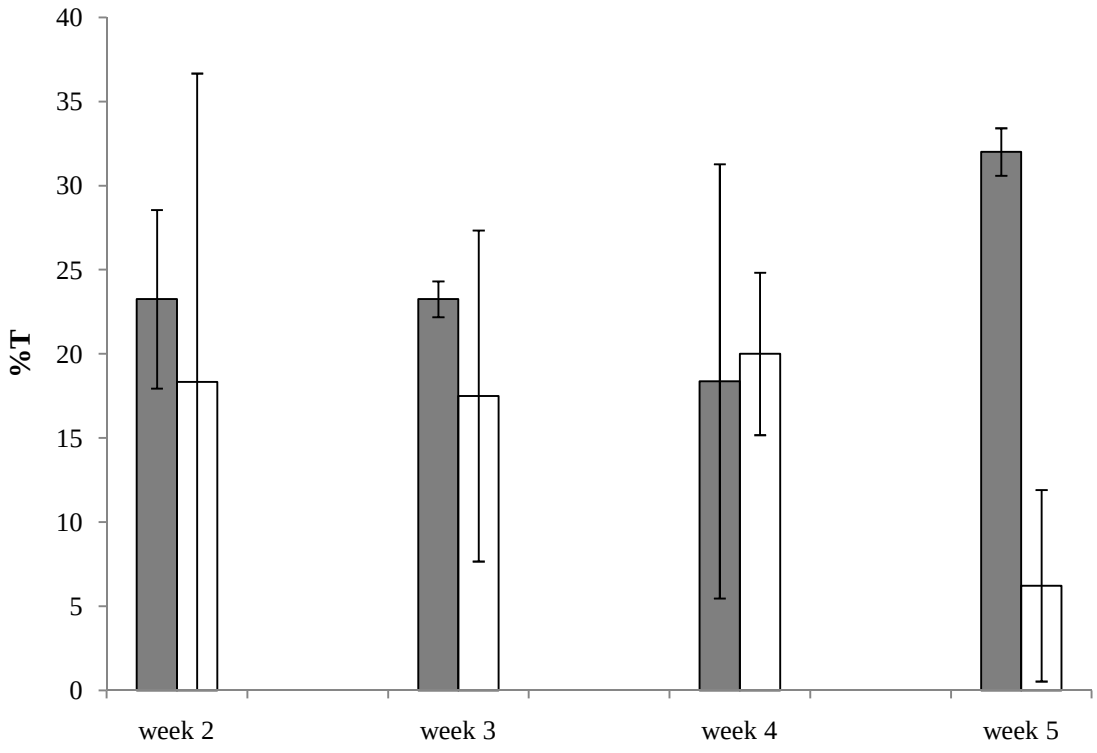

Figure 4.16b. - Percent transmittance over time for juice processed at ATM and MO conditions and stored at 25 °C. ■ - ATM, □ - MO.

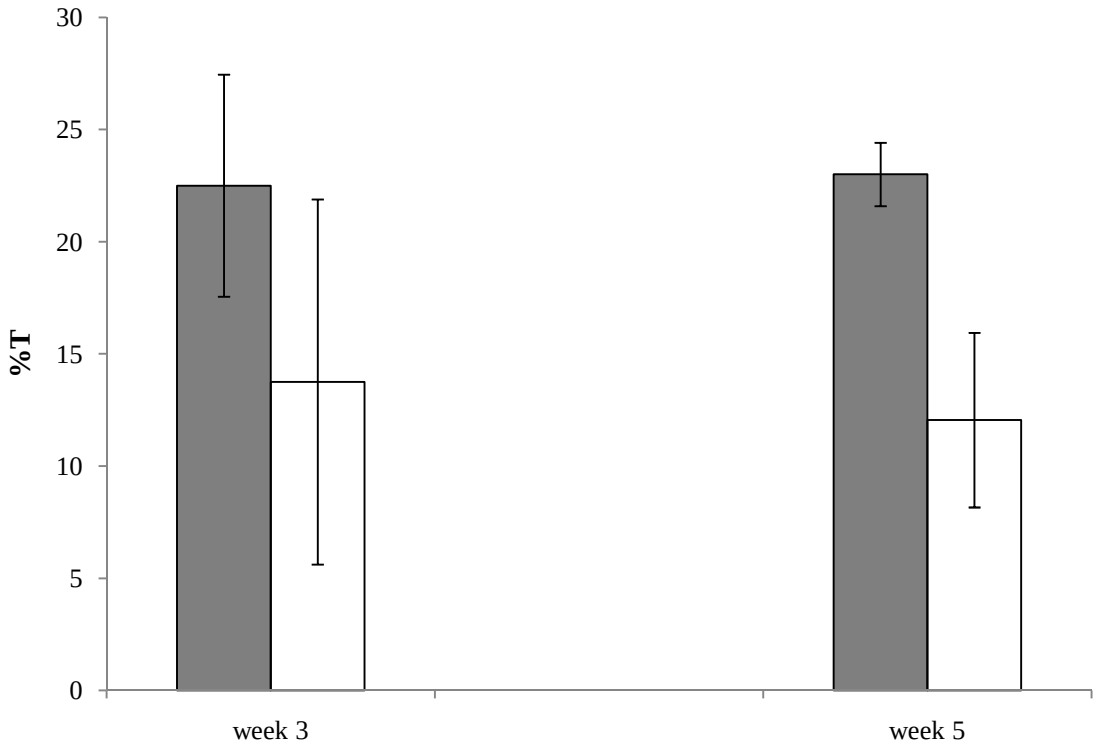

Figure 4.16c. - Percent transmittance over time for juice processed at ATM and MO conditions and stored at 4 ℃. ■ - ATM, □ - MO.

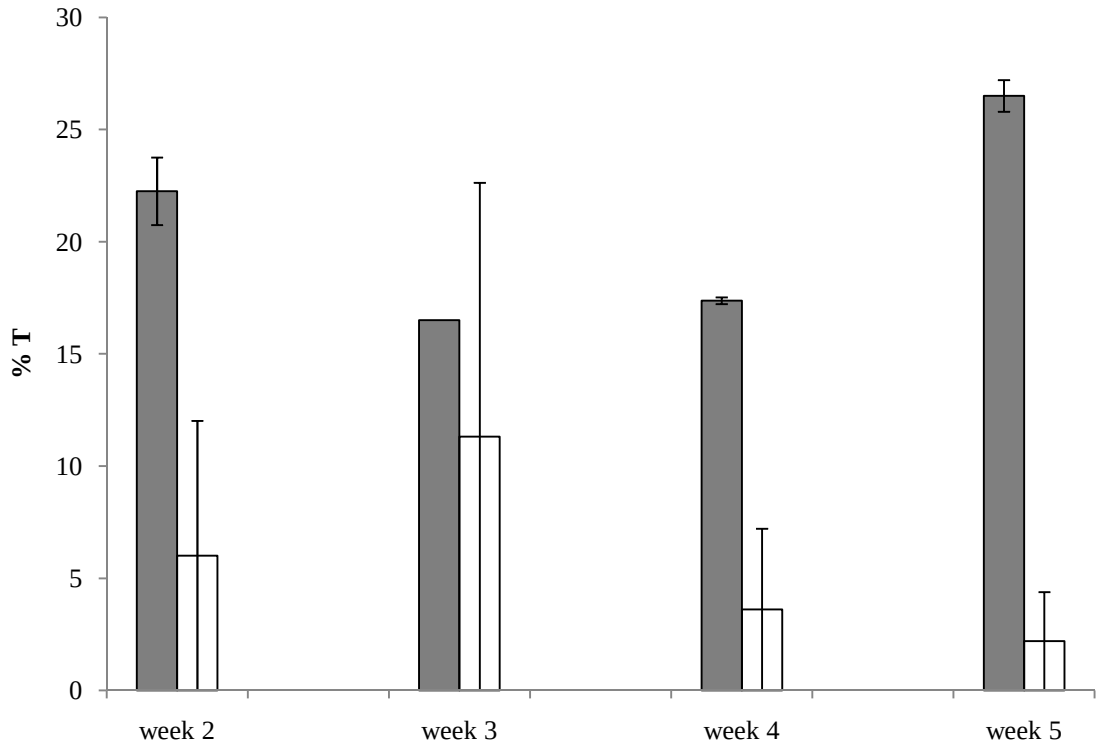

Figure 4.16d. - Percent transmittance over time for juice processed at ATM and MO conditions and stored at 0 °C. ■ - ATM, □ - MO.

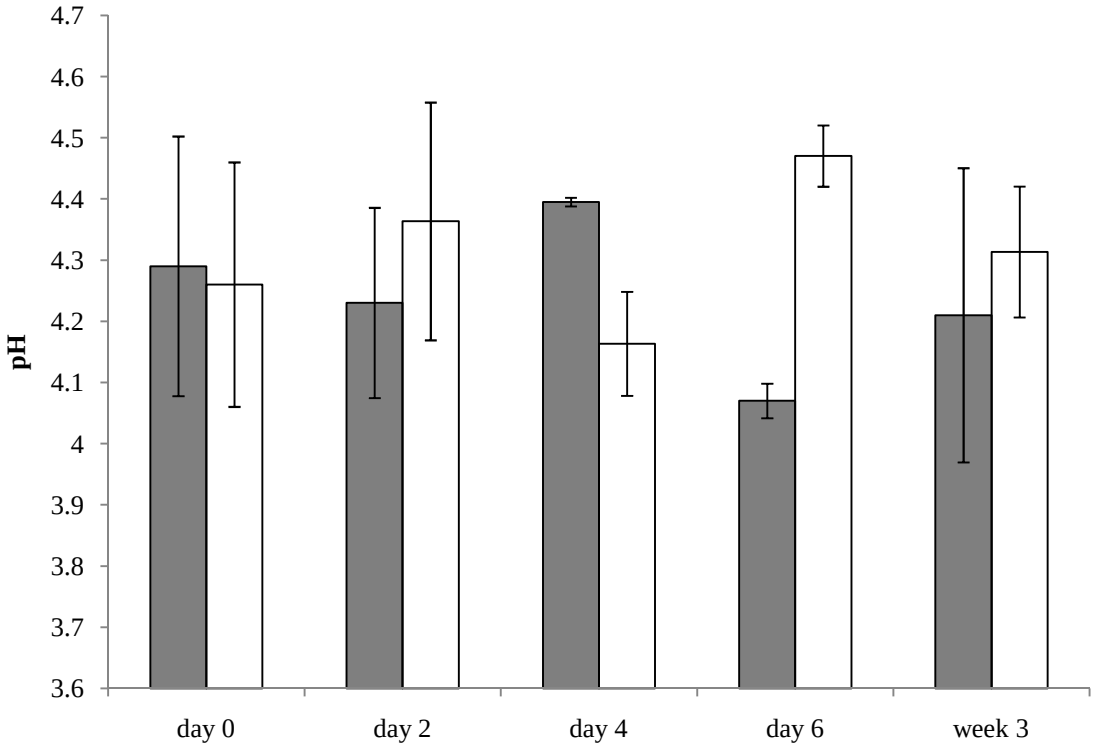

Figure 4.17a. - pH values for juice processed at ATM and MO stored at 50 ℃. ■ - ATM, □ - MO.

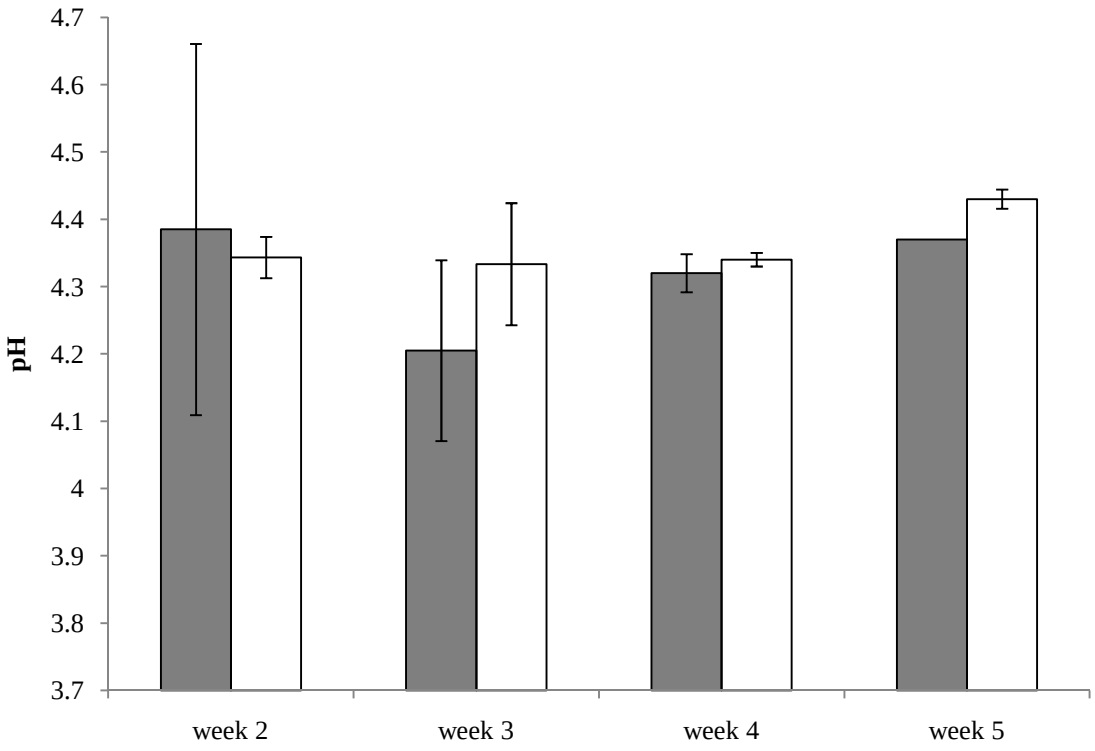

Figure 4.17b. - pH values for juice processed at ATM and MO stored at 25 ℃. . ■ - ATM, □ - MO.

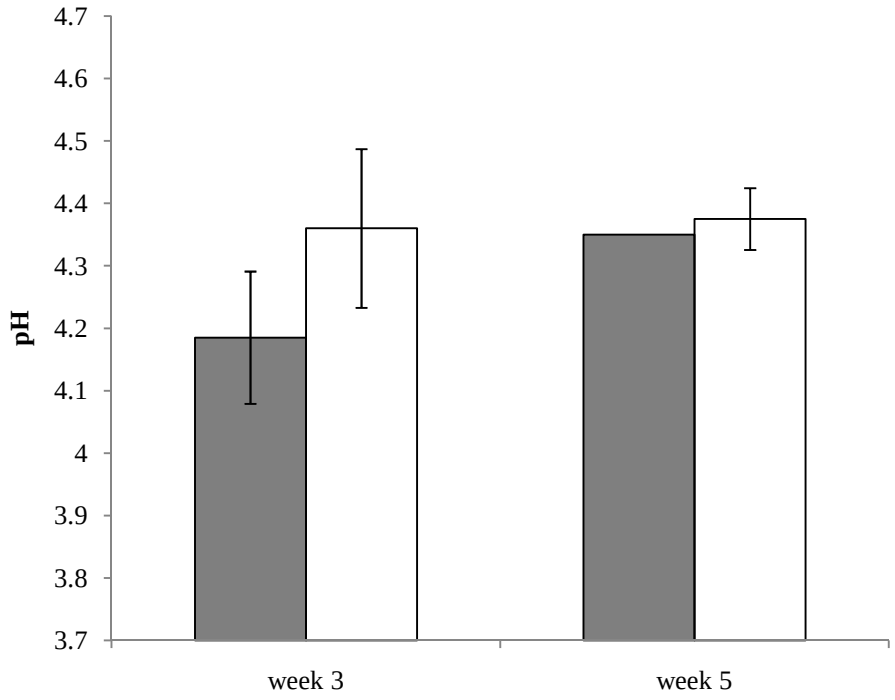

Figure 4.17c. - pH values for juice processed at ATM and MO stored at 4°C. ■ - ATM, □ - MO.

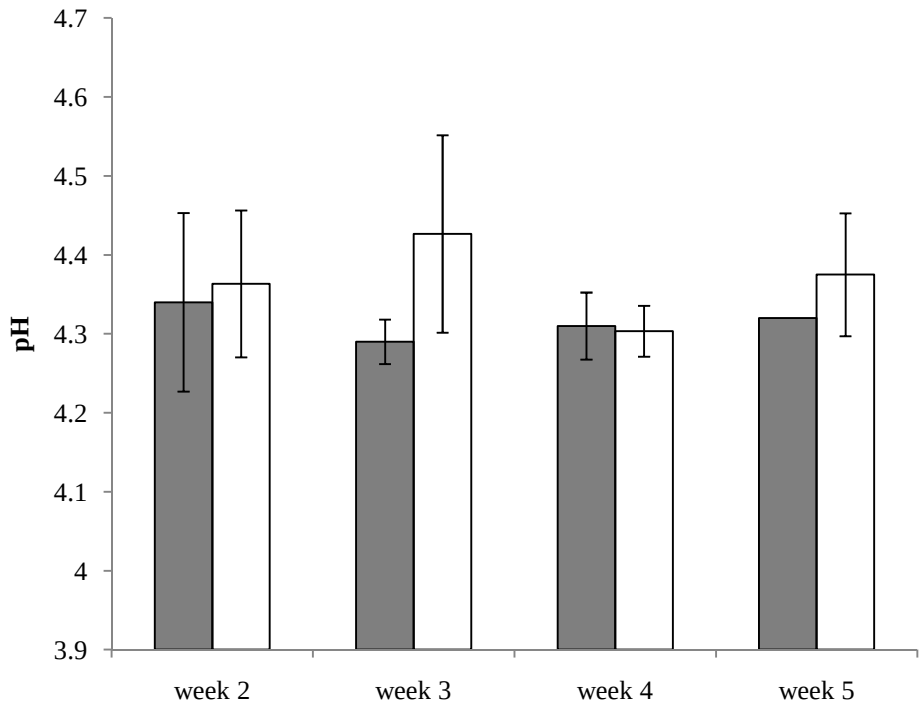

Figure 4.17d. - pH values for juice processed at ATM and MO stored at 0 ℃. ■ - ATM, □ - MO.

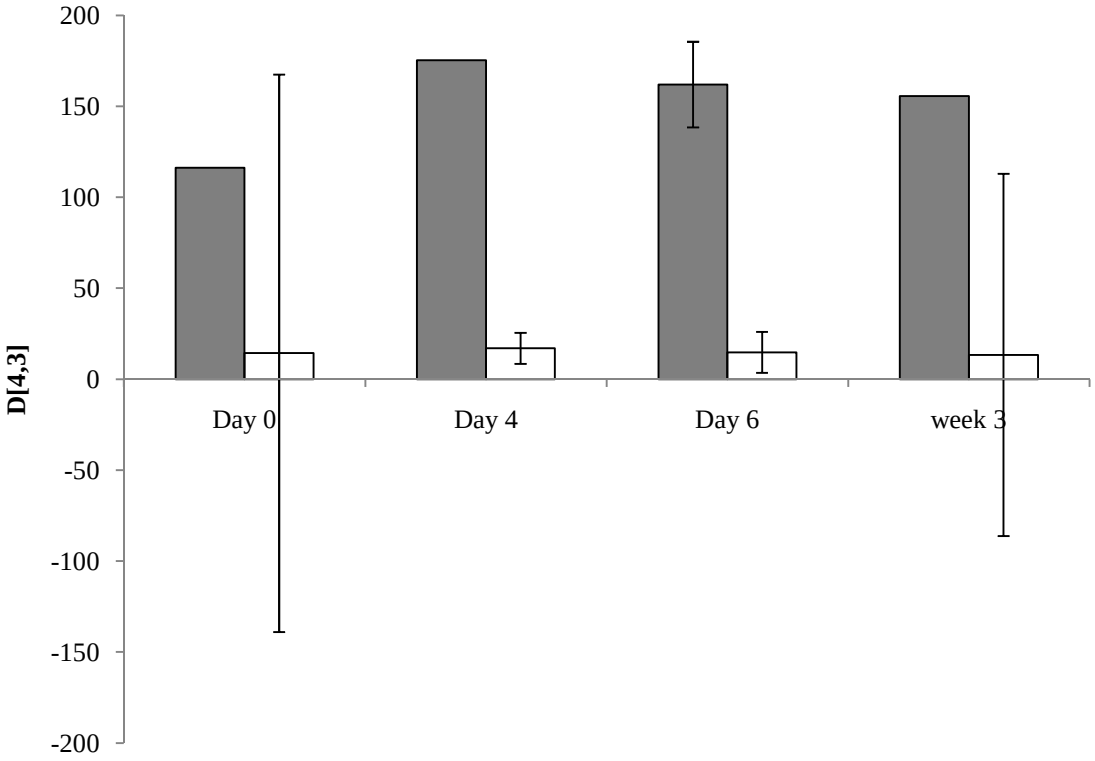

Figure 4.18a. - D[4,3] values for ATM and MO juice stored at 50 ℃ . ■ - ATM, □ - MO.

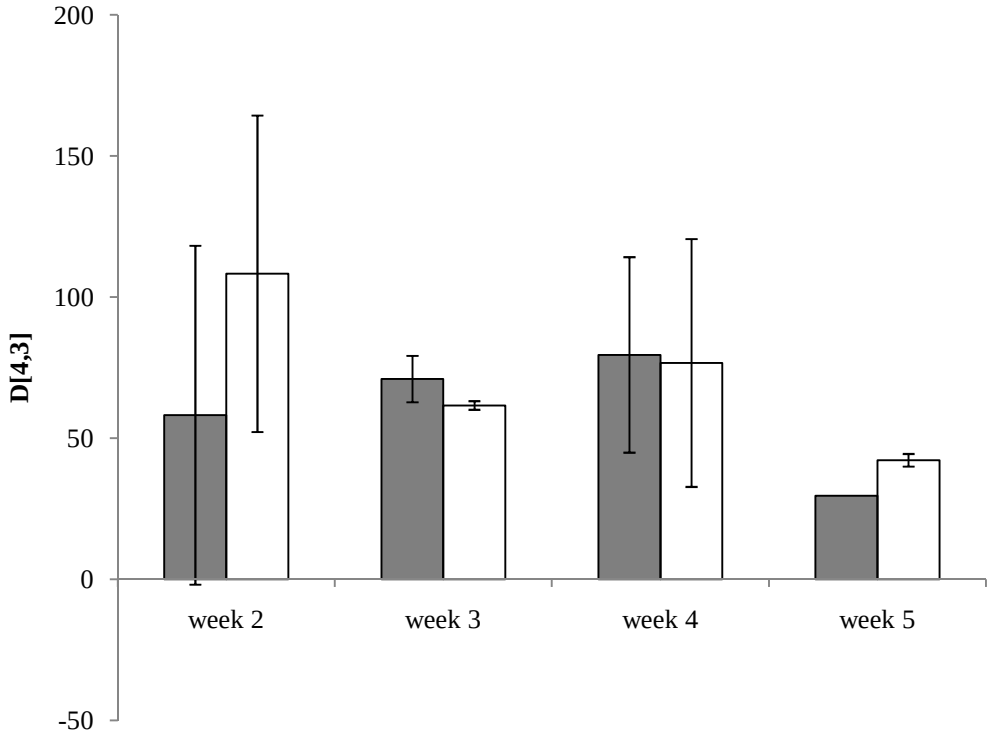

Figure 4.18b. - D[4,3] values for ATM and MO juice stored at 25℃. ■ - ATM, □ - MO.

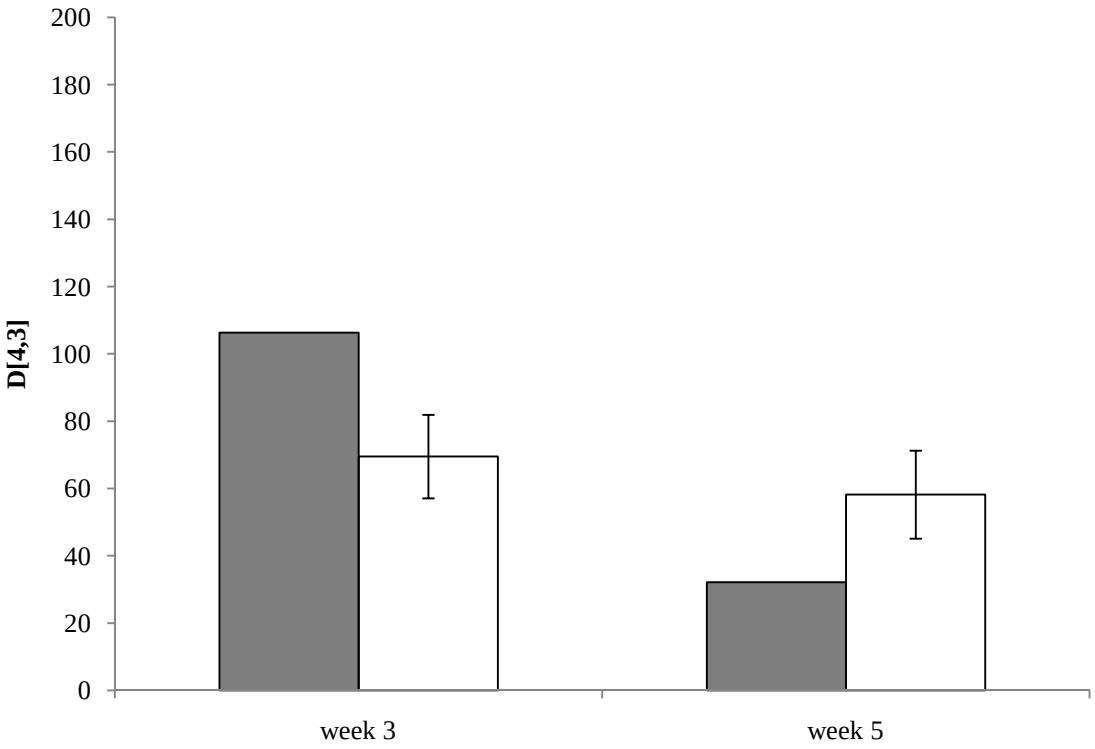

Figure 4.18c. - D[4,3] values for ATM and MO juice stored at 4 °C. ■ - ATM, □ - MO.

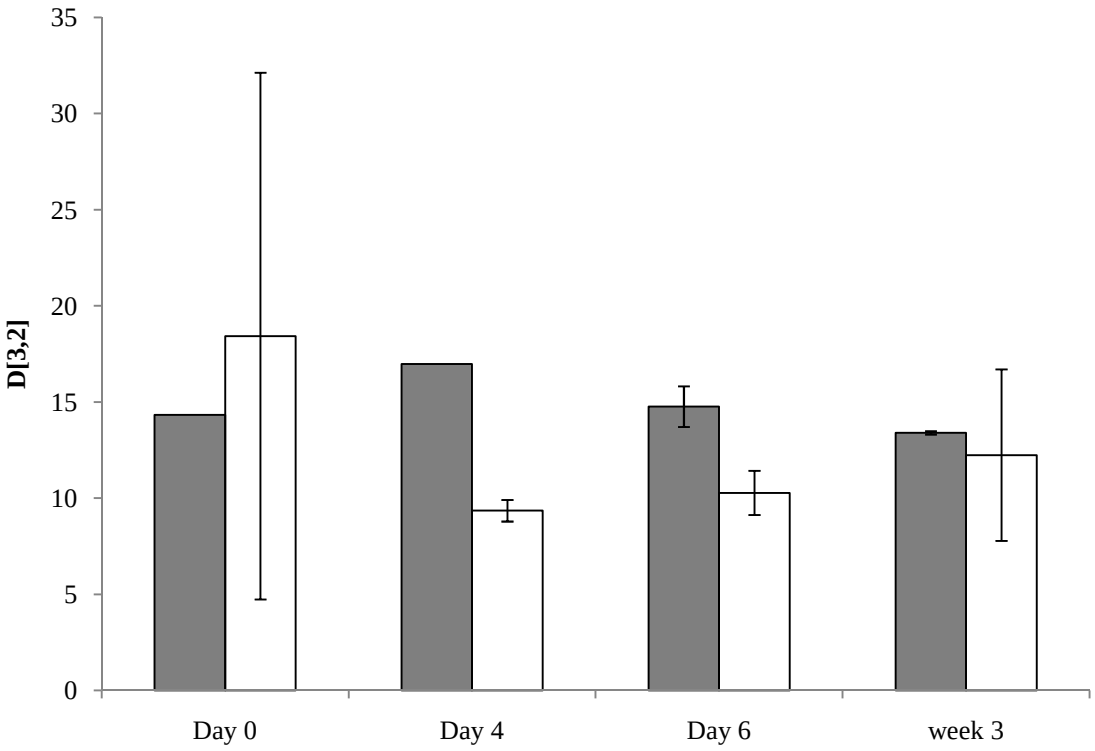

Figure 4.19a. - D[3,2] values for ATM and MO juice stored at 50 °C. ▩ - ATM, ⊐ - MO.

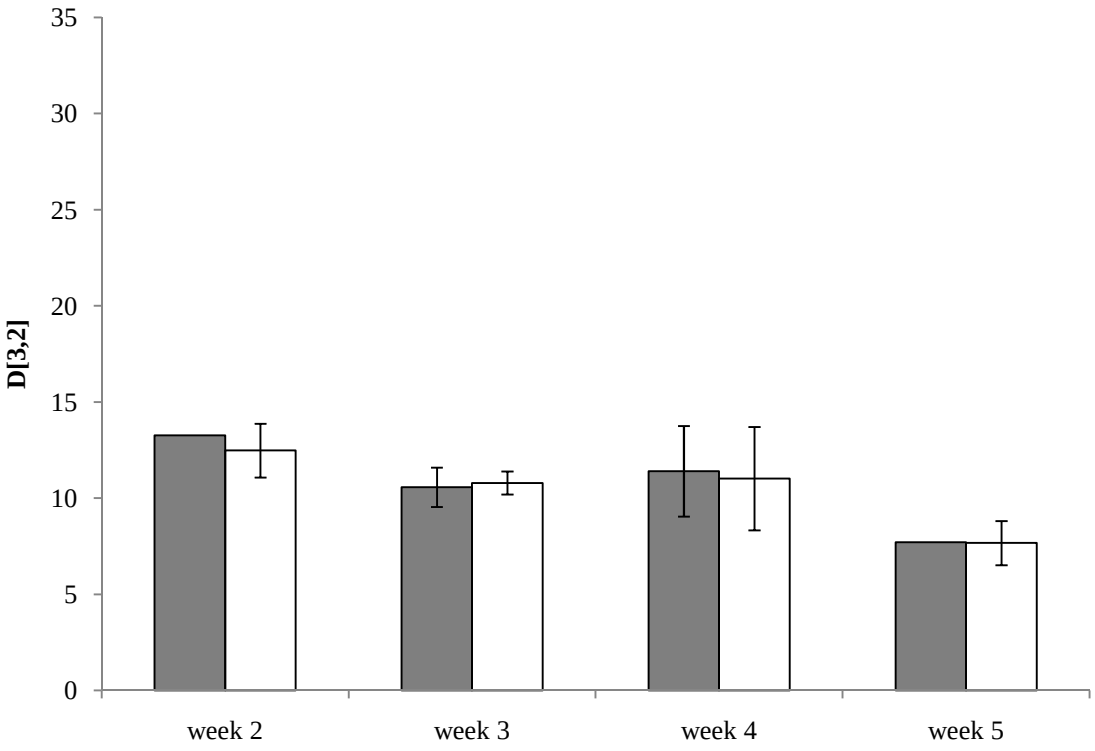

Figure 4.19b. - D[3,2] values for ATM and MO juice stored at 25℃. ■ - ATM, □ - MO.

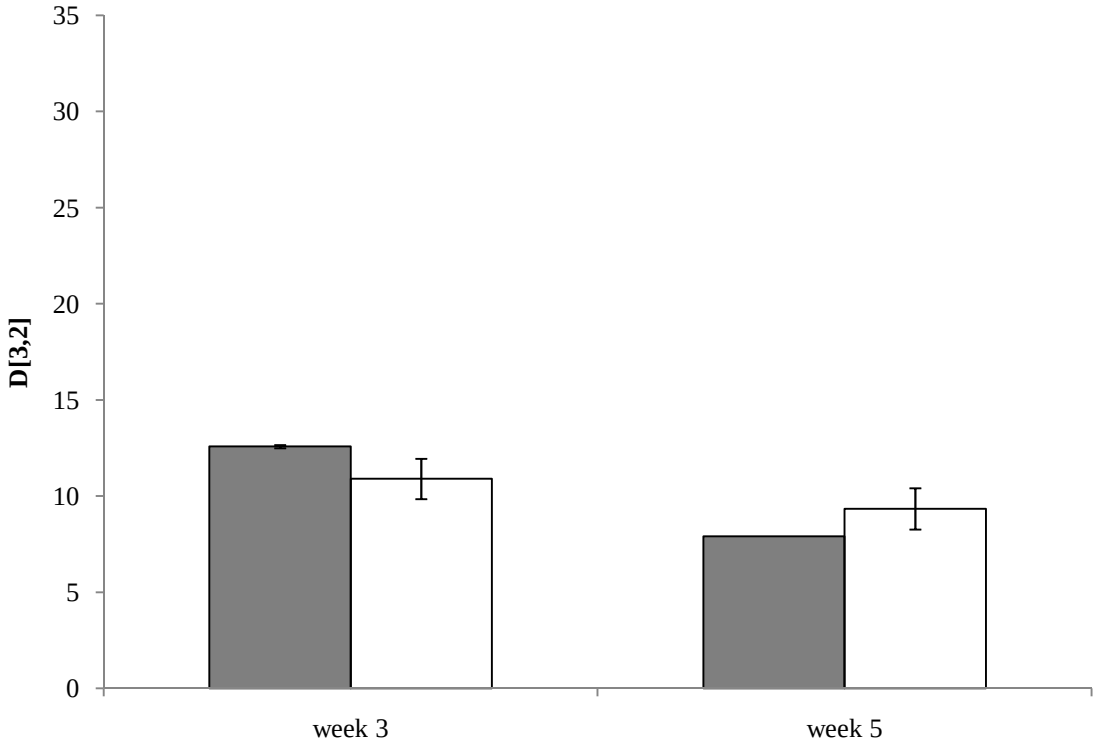

Figure 4.19c. - D[3,2] values for ATM and MO juice stored at 4°C. ■ - ATM, □ - MO.

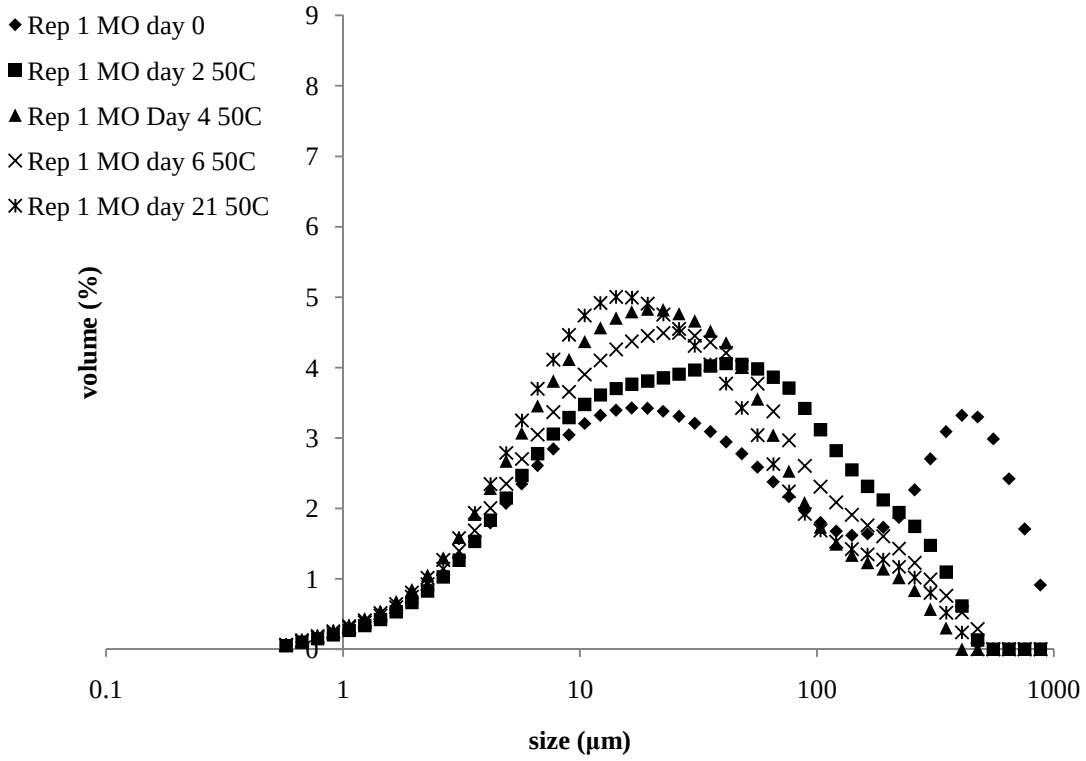

Figure 4.20a. - Particle size for replication 1 at MO conditions and stored at 50°C

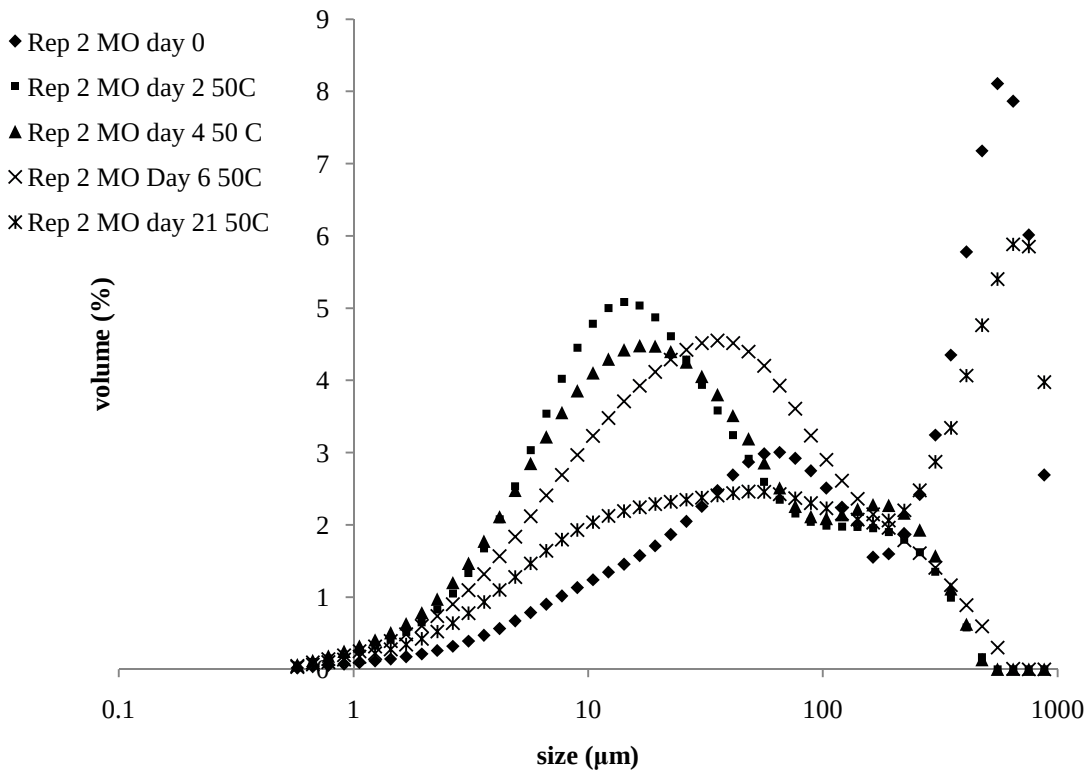

Figure 4.20b. - Particle size for replication 2 at MO conditions and stored at 50°C

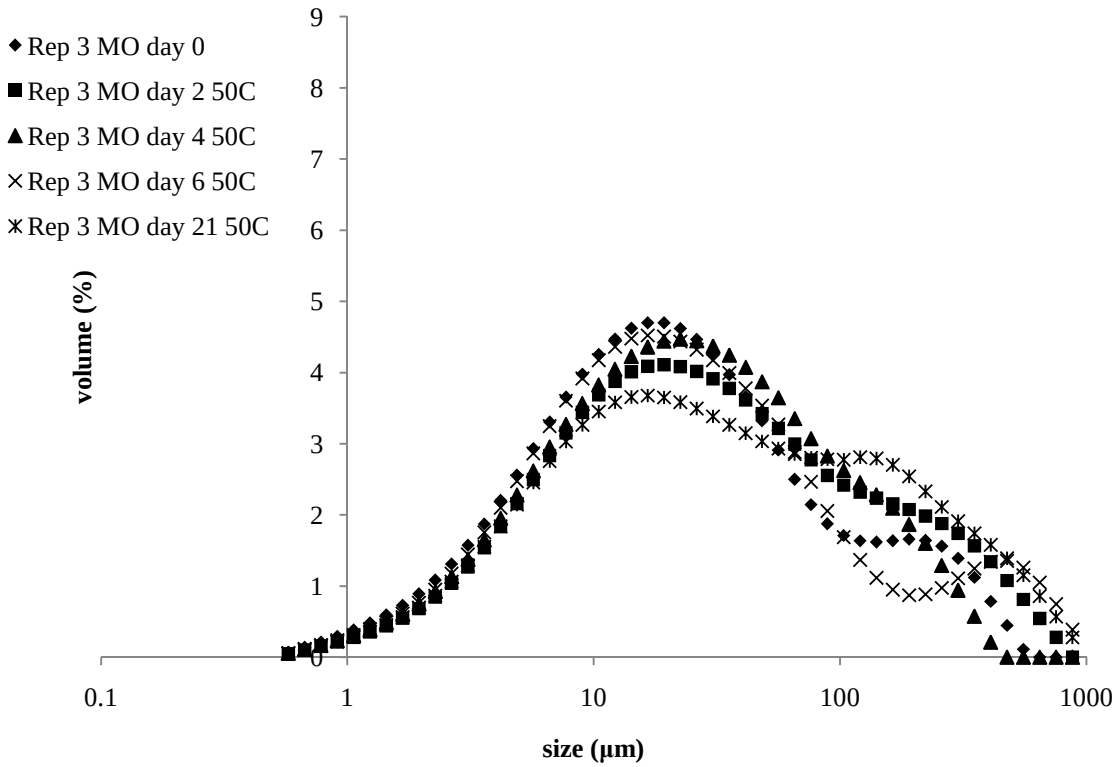

Figure 4.20c. - Particle size for replication 3 at MO conditions and stored at 50℃

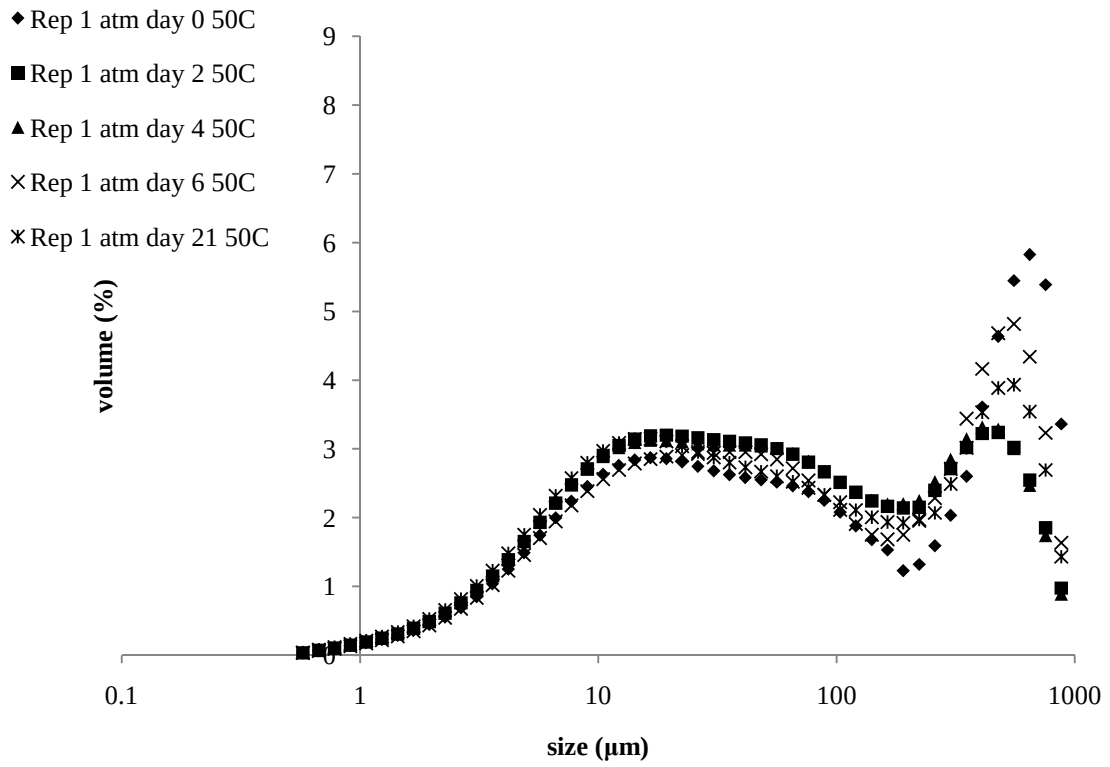

Figure 4.20d. - Particle size for replication 1 at ATM conditions and stored at 50℃

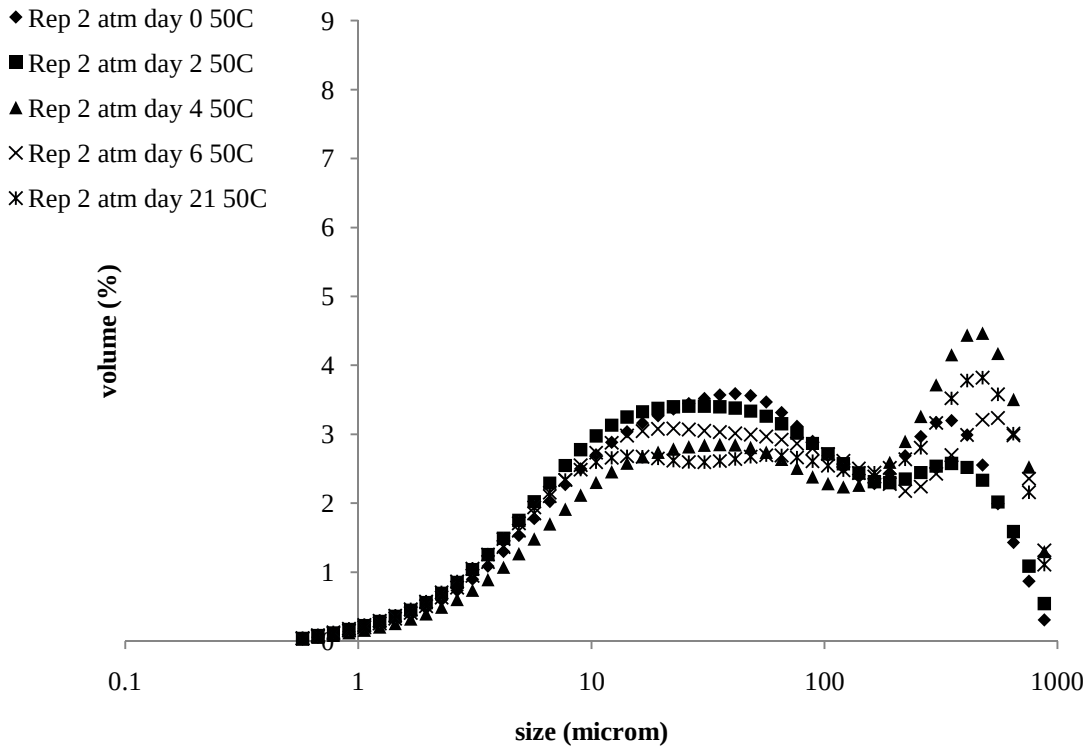

Figure 4.20e. - Particle size for replication 2 at ATM conditions and stored at 50°C

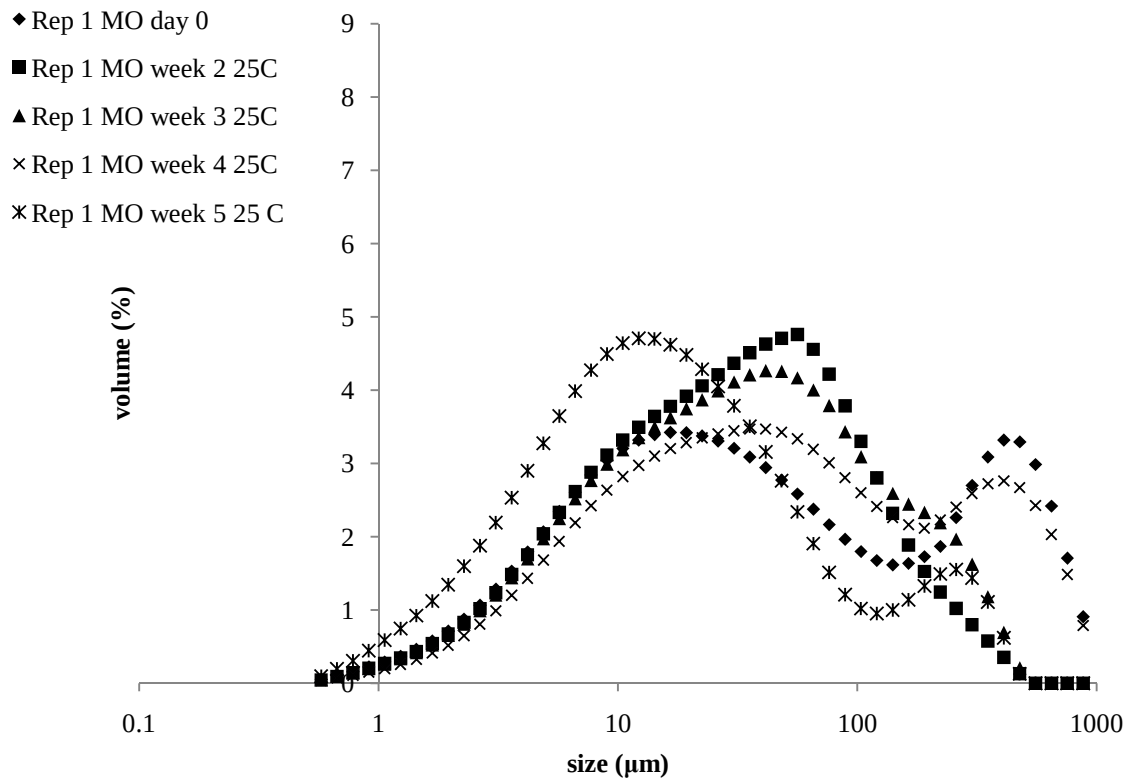

Figure 4.21a. - Particle size for replication 1 at MO conditions and stored at 25℃

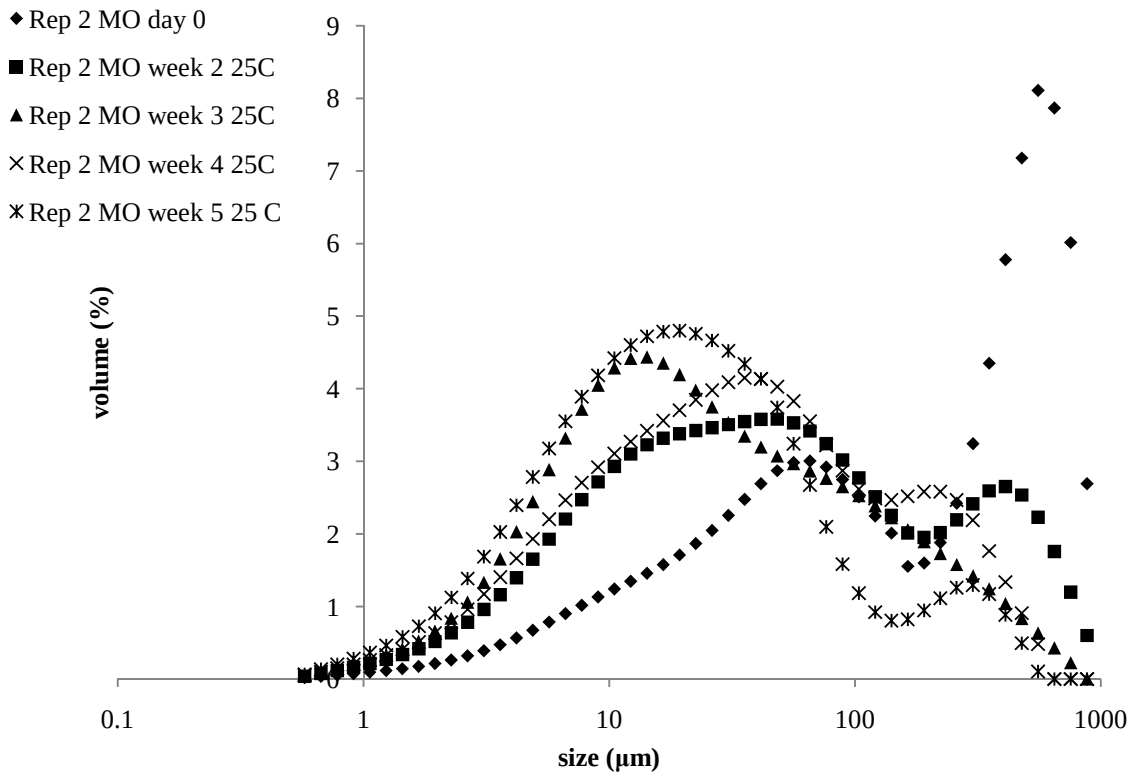

Figure 4.21b. - Particle size for replication 2 at MO conditions and stored at 25°C

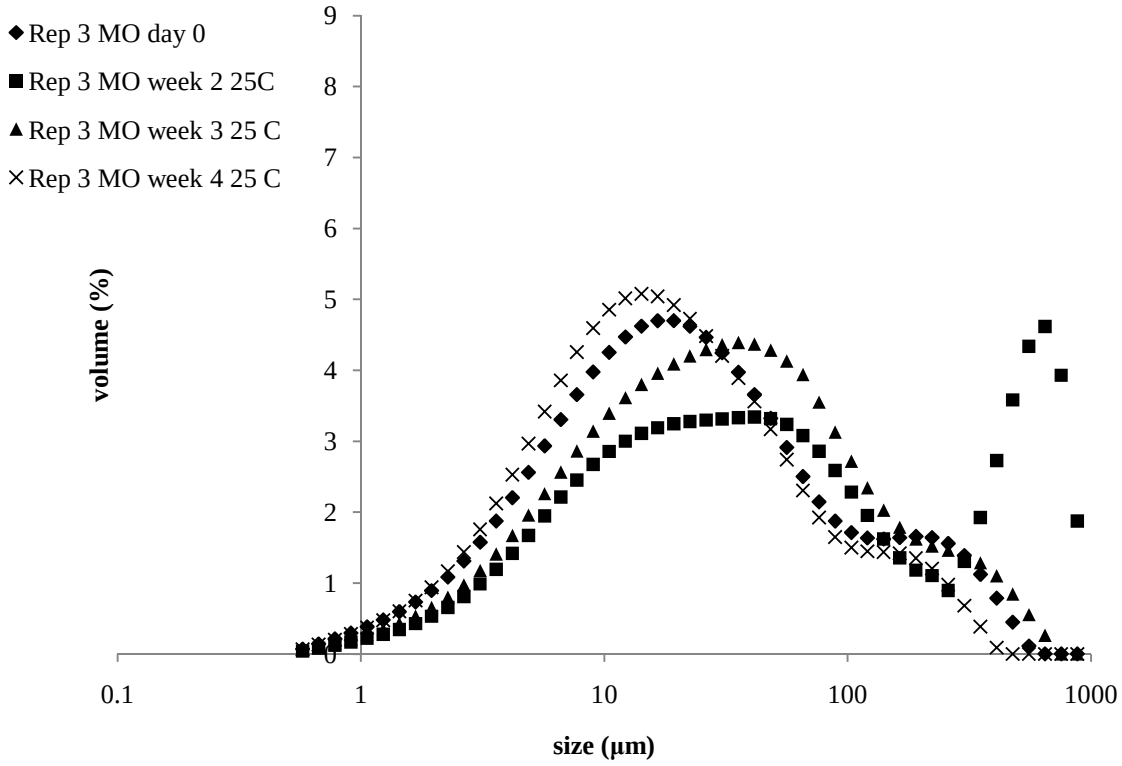

Figure 4.21c. - Particle size for replication 3 at MO conditions and stored at 25℃

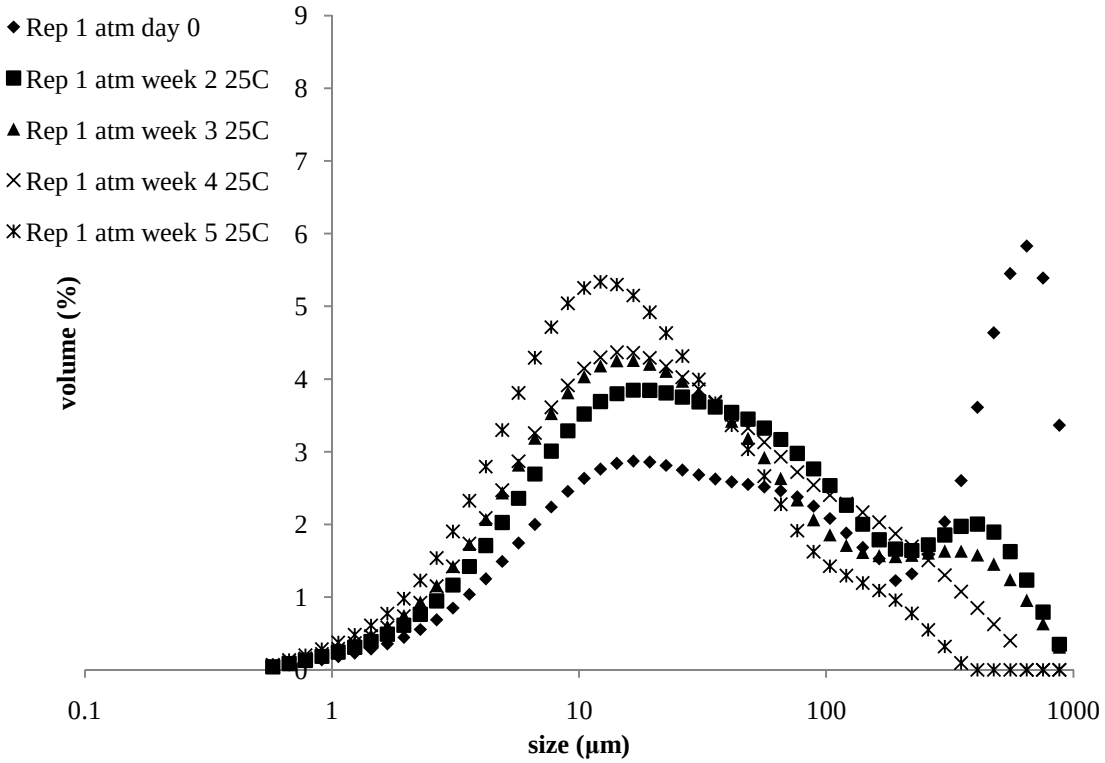

Figure 4.21d. - Particle size for replication 1 at ATM conditions and stored at 25℃

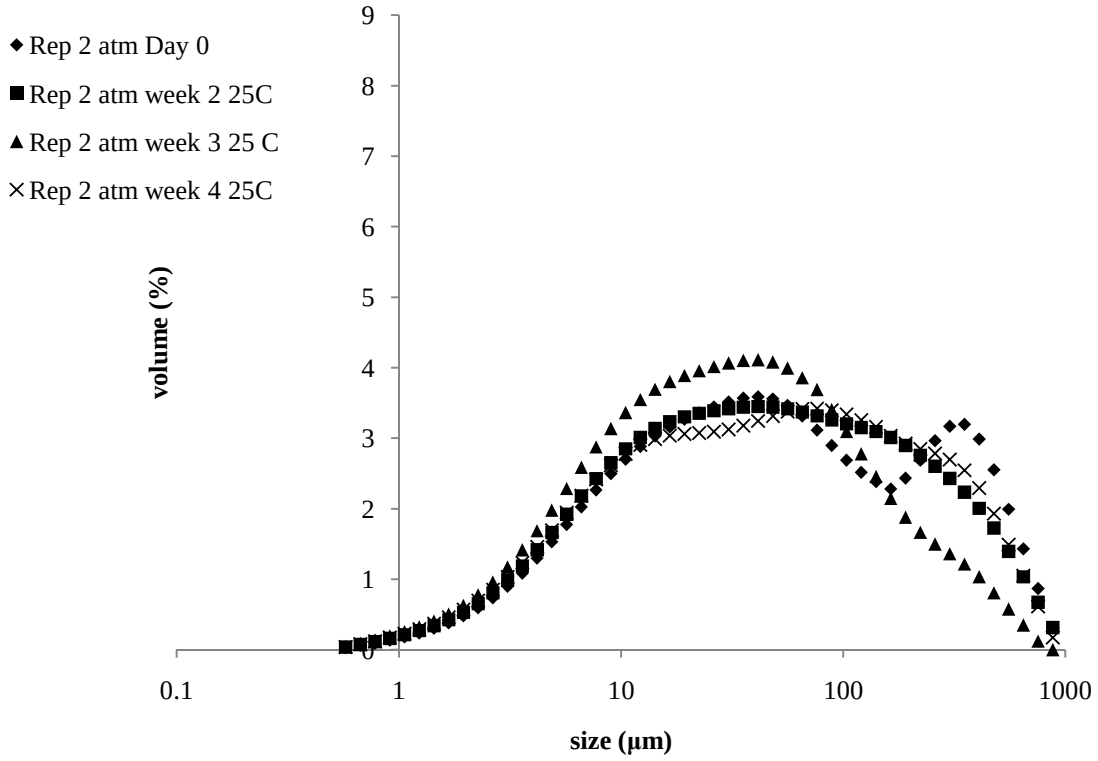

Figure 4.21e. - Particle size for replication 2 at ATM conditions and stored at 25°C

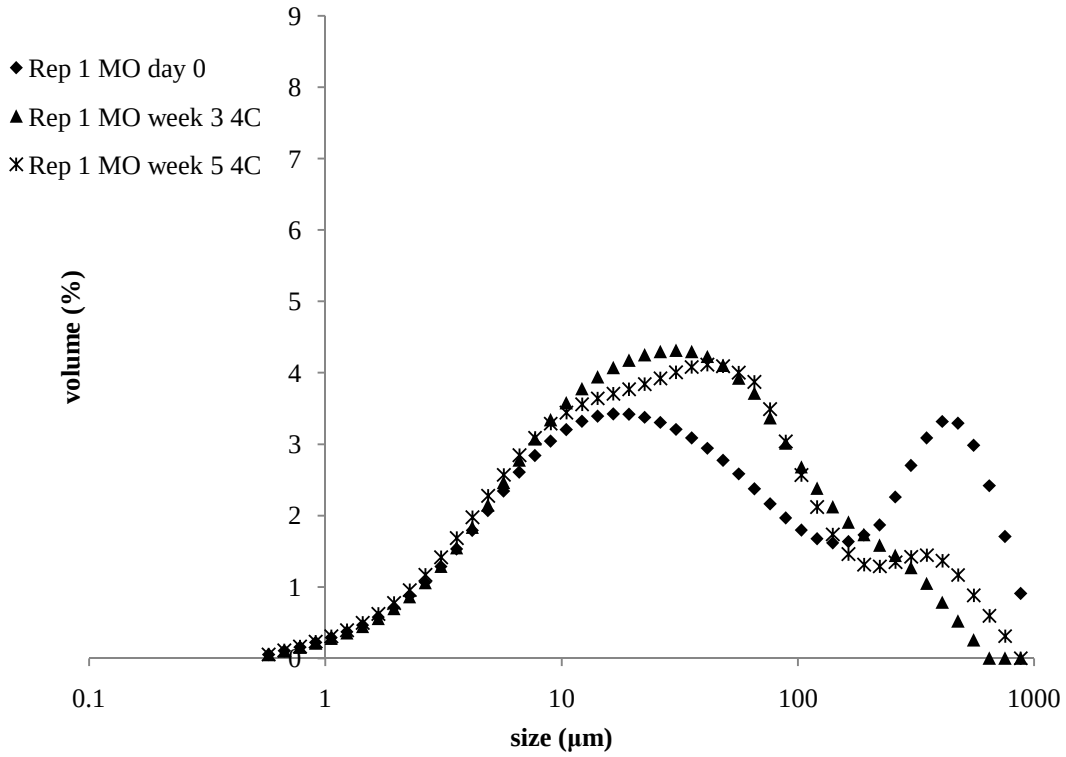

Figure 4.22a. - Particle size for replication 1 at MO conditions and stored at 4°C

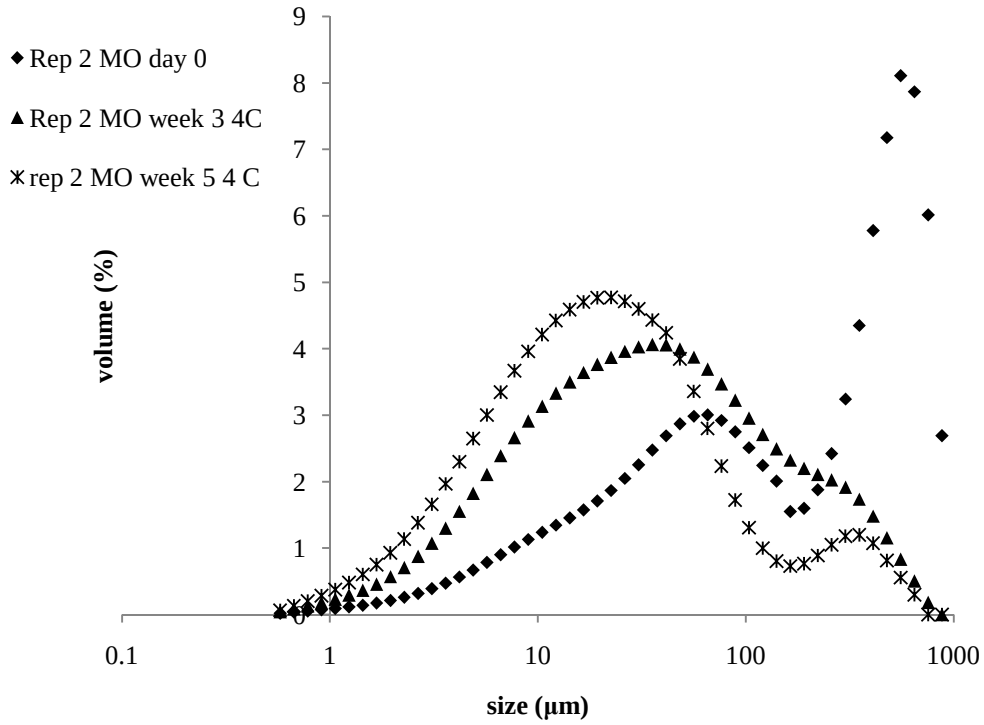

Figure 4.22b. - Particle size for replication 2 at MO conditions and stored at 4°C

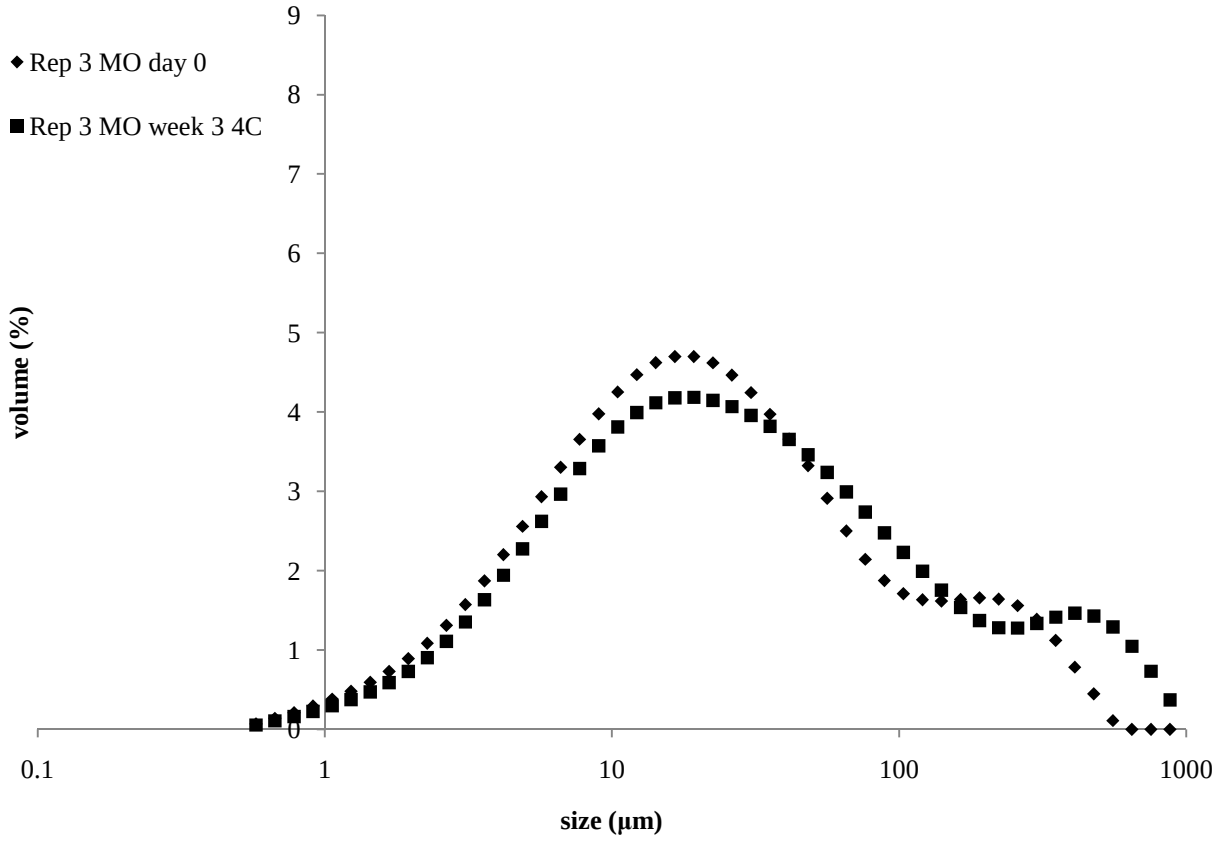

Figure 4.22c. - Particle size for replication 3 at MO conditions and stored at 4℃

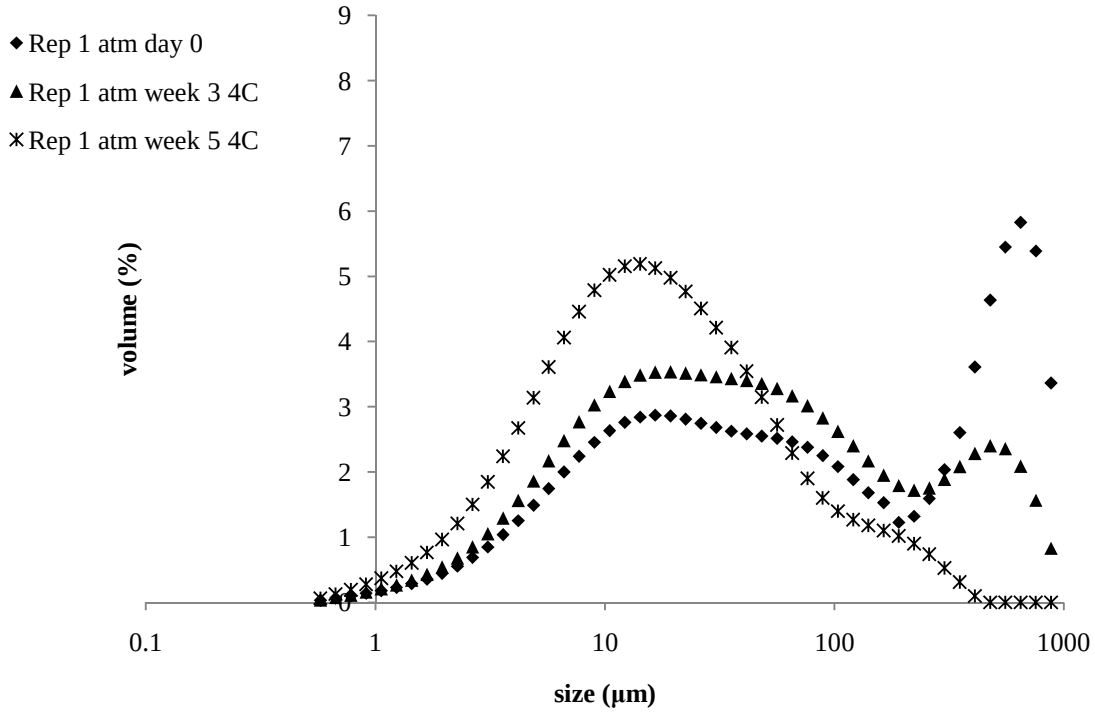

Figure 4.22d. - Particle size for replication 1 at ATM conditions and stored at 4℃

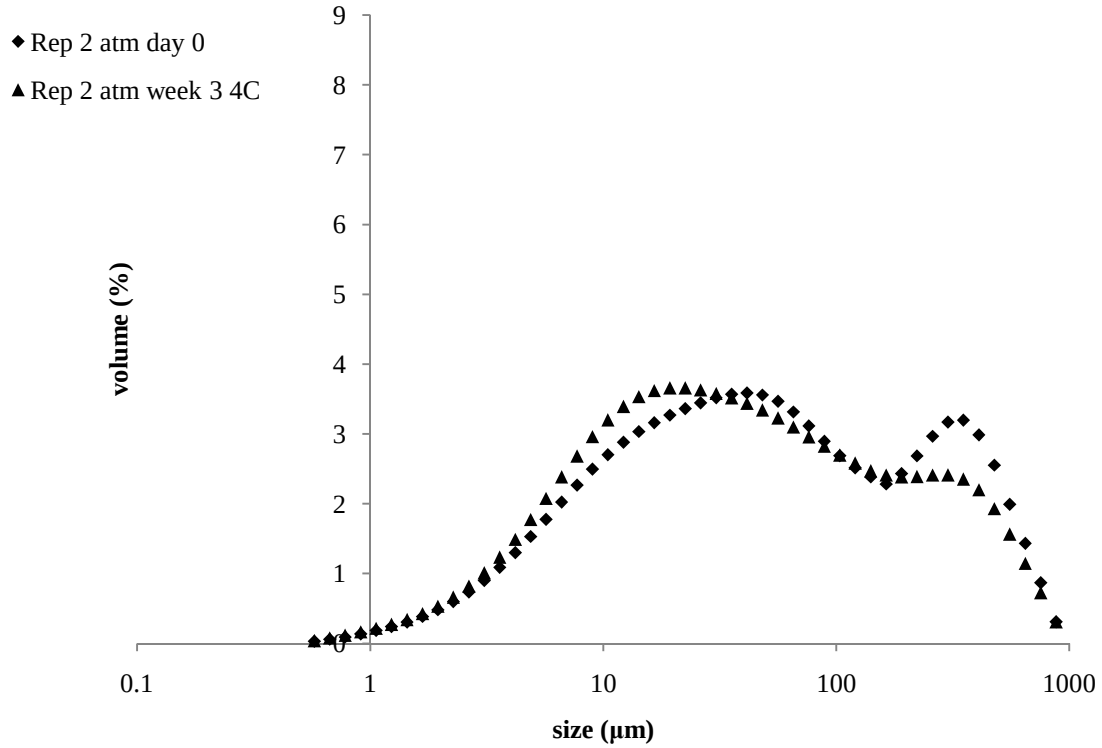

Figure 4.22e. - Particle size for replication 2 at ATM conditions and stored at 4°C

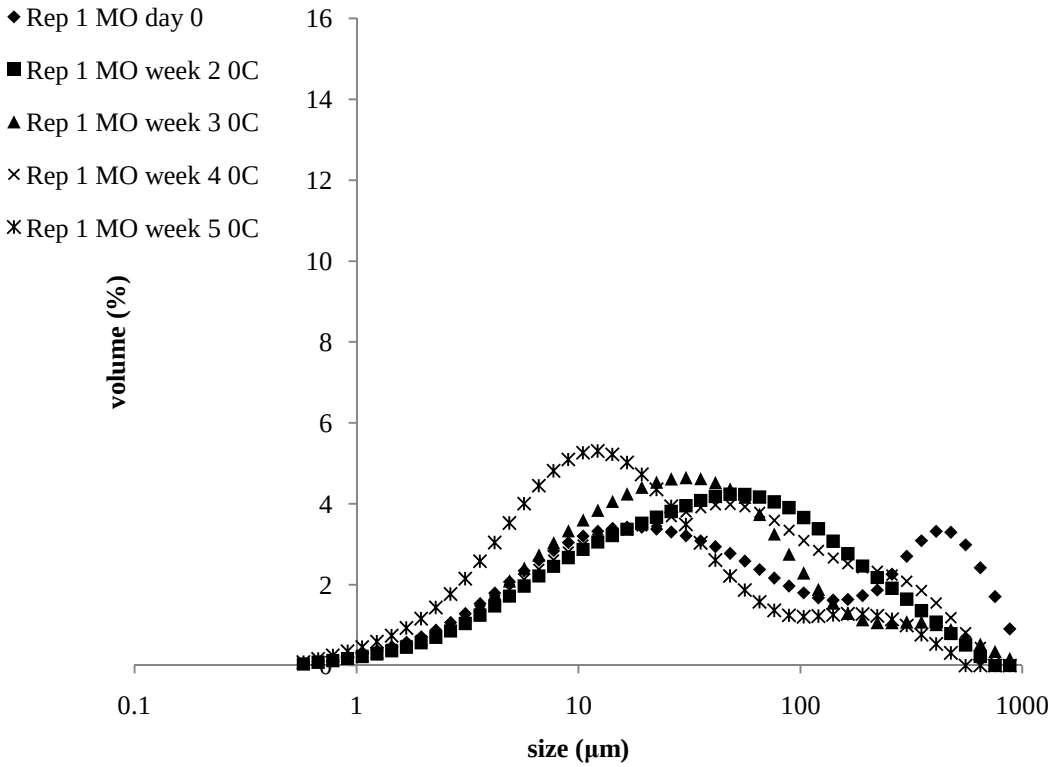

Figure 4.23a. - Particle size for replication 1 at MO conditions and stored at 0℃

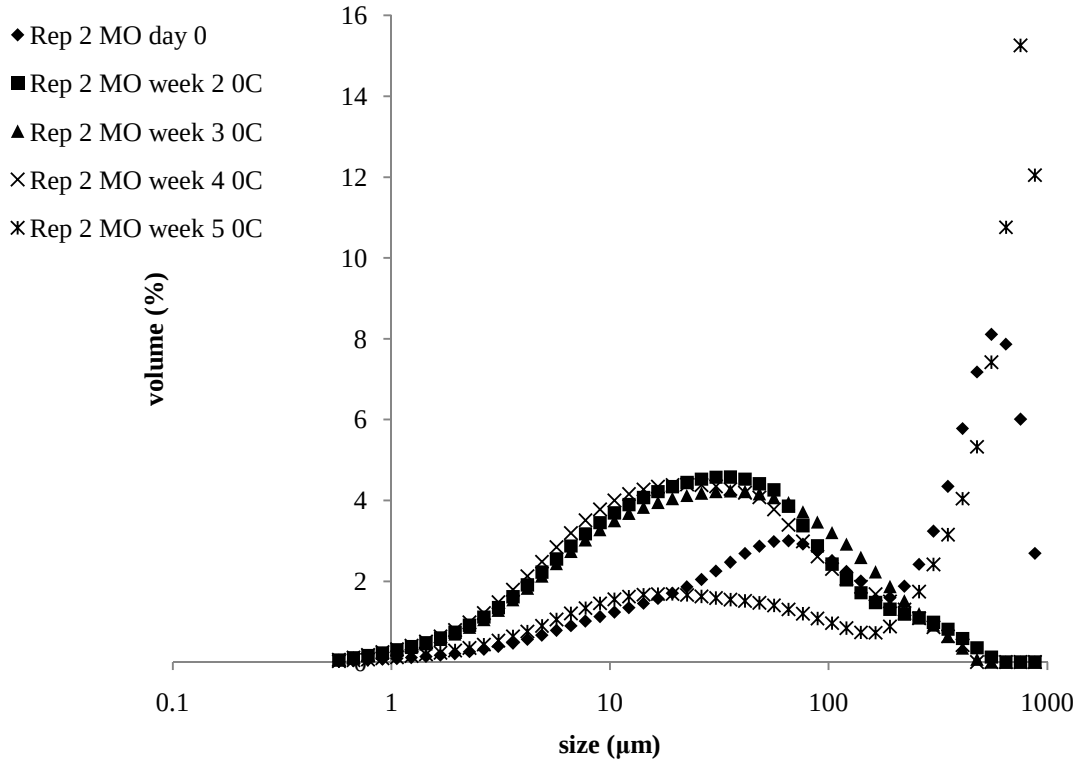

Figure 4.23b. - Particle size for replication 2 at MO conditions and stored at 0°C

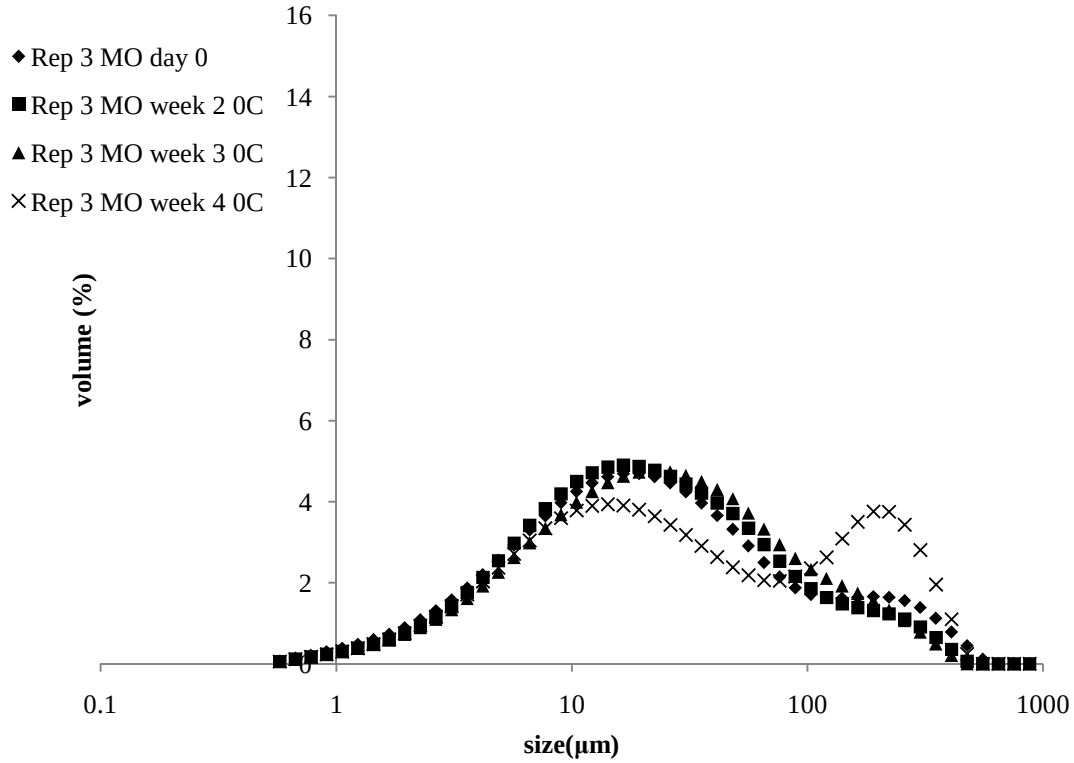

Figure 4.23c. - Particle size for replication 3 at MO conditions and stored at 0℃

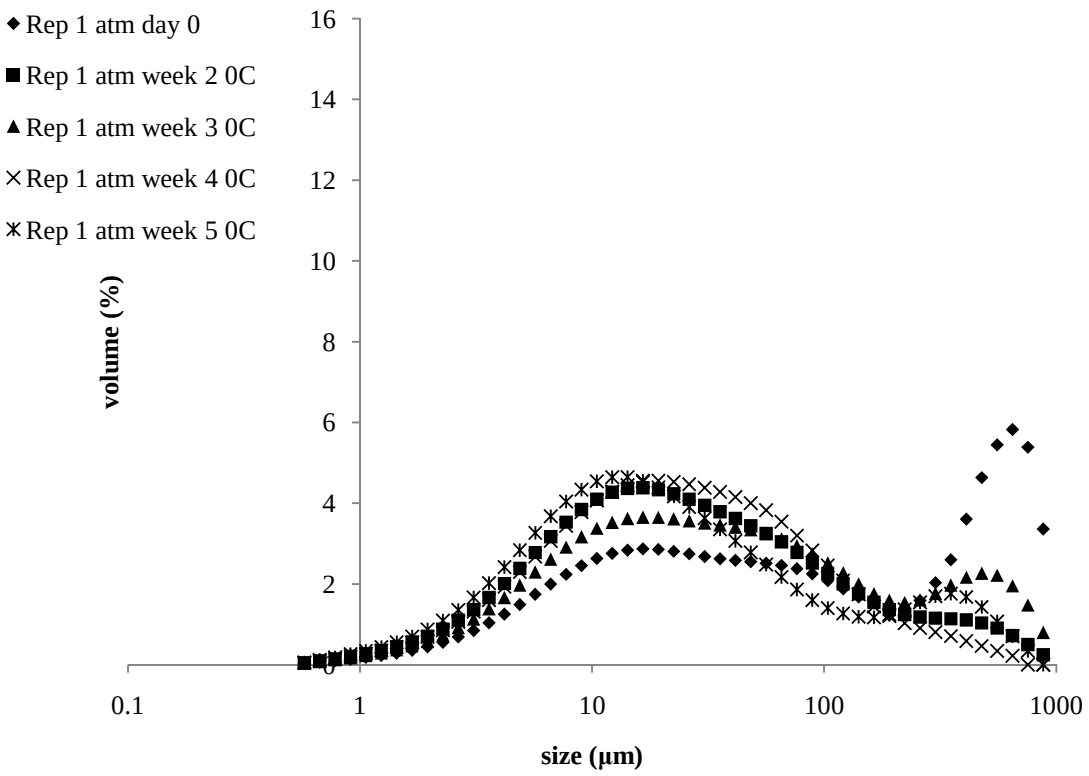

Figure 4.23d. - Particle size for replication 1 at ATM conditions and stored at 0℃

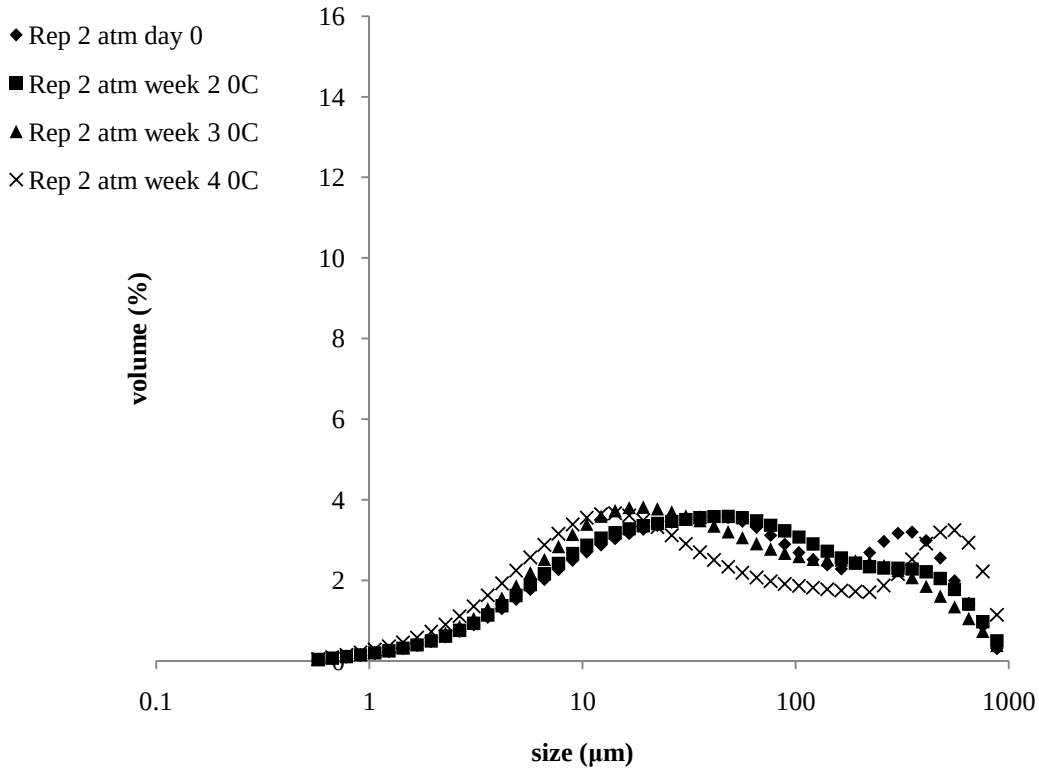

Figure 4.23e. - Particle size for replication 2 at ATM conditions and stored at 0°C

CHAPTER 5

CONCLUSIONS

This study shows that micro-oxygen levels, as low as 8ppm, decrease PPO activity but does not eliminate it. It has been suggested that dissolved oxygen on the banana tissue as well as mechanisms of reaction of the enzyme may play a role in PPO activity. Browning reaction by PPO is neither first nor zero-order.

Results show that Raman may represent an effective way to quantify enzymatic activity, since it allows the identification and quantification of compounds that are being formed throughout the reaction. In addition, the sample preparation time necessary for the absorbance assay, may be reduced, eliminating the possibility of catechol changes before the activity is measured.

Micro-oxygen processing decreased dissolved oxygen in orange juice, however no significant differences in vitamin C retention and browning were observed between samples processed under micro-oxygen and atmospheric conditions. In addition, dissolved oxygen concentrations of 34ppb may be enough to initiate oxidative reactions. Also, sample preparation for shelf stability studies required exposure of the samples to atmosphere, which may have been an important factor that affected the results.