PEROXIDATION OF MEMBRANE LIPIDS IN MINIMALLY PROCESSED CUCUMBERS PACKAGED UNDER MODIFIED ATMOSPHERES

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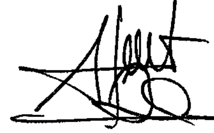

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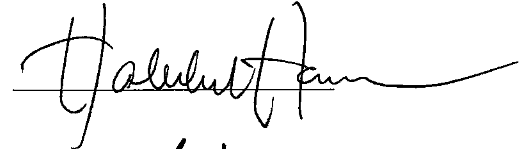
Prof. Dr. Haluk Hamamcı 

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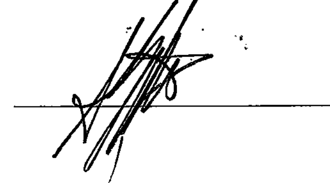
Assoc. Prof. Dr. Alev Bayındırlı 

ABSTRACT

PEROXIDATION OF MEMBRANE LIPIDS IN MINIMALLY PROCESSED CUCUMBERS PACKAGED UNDER MODIFIED ATMOSPHERES

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Peroxidation of membrane lipids is an inherent feature of ripening and senescence of plant tissues. Increased lipid peroxidation has also been observed in the development of chilling injury in susceptible tissues. In this study, the effect of storage conditions and physical tissue damage on membrane peroxidation of minimally processed cucumber (Cucumis sativus, L.) was investigated. Model modified atmospheres were achieved passively by storage of the minimally processed cucumbers at 4°C and 20°C in hermetically sealed jars. Treatments such as oxygen flushing of the packages and bruising of the fruit previous to sealing resulted in different atmospheric compositions and respiration rates. Lipid peroxidation in samples packaged under these conditions of modified atmosphere packaging (MAP) and also in covered petri plates (non-MAP) were determined by the FOX2 method. The TBARS assay was also carried out as an alternative assessment of lipid peroxidation. The initial level of lipid hydroperoxides and TBARS were found as 48-59 μmoles H_2O_2 equivalents and 0.11-0.20 nmoles MDA equivalents per gram. The results of the two assays correlated significantly for nonbruised samples. The levels of lipid

hydroperoxides increased by 2-5 fold in chilled non-MAP tissues and MAP was effective in reducing the generation of lipid hydroperoxides and TBARS. The effect of dipping solutions containing $CaCl_2$ and/or ascorbic acid on the development of lipid hydroperoxides investigated in non-MAP tissues had varying results. Tissue hardness of MAP samples increased for the first 3 days for all tissues but chilled tissues started to soften on the 6th day.

Keywords: Peroxidation; Membrane Lipids; Minimal Processing; Cucumber; Modified Atmospheres

ÖZ

MİNİMAL İŞLEM GÖRMÜŞ VE MODİFİYE ATMOSFERDE PAKETLENMİŞ SALATALIKLARDA MEMBRAN LİPİDLERİNİN PEROKSİDASYONU

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Membran lipidlerinin peroksidasyonu, olgunlaşan ve yaşlanan bitki dokularında doğal bir olaydır. Hassas dokularda, soğuk zararının gelişimesiyle lipid peroksidasyonunun arttığı gözlenmiştir. Bu çalışmada, saklama koşulları ve fiziksel doku hasarının minimal işlem görmüş (MİG) salatatalıkta (Cucumis sativus, L.) membran lipidlerinin peroksidasyonuna etkisi araştırılmıştır. MİG salatalıkların sızdırmaz kavanozlarda 4 vaya 20°C'de saklanmasıyla model modifiye atmosferler pasif halde oluşturulmuştur. Kavanozlar kapatılmadan içlerine oksijen basılması ve salatalıkların berelenmesi gibi uygulamalarda solunum hızları ve model modifiye atmosfer bileşimleri farklılık göstermiştir. Bu koşullarda, modifiye atmosfer paketleme (MAP) ile ve kapaklı petri kaplarında (MAP'siz) saklanan salatalıklarda lipid peroksidasyonunun gelişimi FOX2 yöntemiyle belirlenmiştir. Alternatif yöntem olarak, TBARM tayin edilmiştir. Lipid hidroperoksitleri ve TBARM başlangıç seviyeleri, gramda, sırasıyla, 48-59 µmol H_2O_2 eşdeğeri ve 0.11-0.20 nmol MDA eşdeğeri olarak bulunmuştur. Berelenmemiş örneklerde, iki yöntemin sonuçları arasında dikkate

değer korelasyon saptanmıştır. MAP'siz soğukta saklanan örneklerde, lipid hidroperoksitleri 2-5 kat artarken, MAP lipid hidroperoksitleri ve TBARM oluşumunu önlemekte etkili olmuştur. $CaCl_2$ ve/veya askorbik asit çözeltilerine daldırma MAP'siz saklanan dokularda, lipid hidroperoksitlerinin oluşumunda değişik etkiler göstermiştir. MAP'le muhafaza edilen ürünlerde, sertlik ilk günlerde artmış, soğukta saklanan örneklerde altıncı günden sonra yumuşama gözlenmiştir.

Anahtar Kelimeler: Peroksidasyon; Membran Lipidleri; Minimal İşlem; Salatalık; Modifiye Atmosfer

To My Parents

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ABBREVIATIONS

BHT	Butylated Hydroxytoluene
CI	Chilling Injury
FI	Fermentative Index
FOX2	Ferrous Oxidation Xylenol-orange version II
GC-MS	Gas Chromatography-Mass Spectrometry
HPLC	High Performance Liquid Chromatography
LC-MS	Liquid Chromatography-Mass Spectrometry
LOX	Lipoxygenase
MAP	Modified Atmosphere Packaging
MDA	Malondialdehyde
MPR	Minimally Processed Refrigerated
PUFA	Polyunsaturated Fatty Acid
Rco_2	Respiration rate determined as the rate of CO_2 evolution
Ro_2	Respiration rate determined as the rate of O_2 consumption
ROOH	Lipid Hydroperoxide
RQ	Respiratory Quotient
RR	Respiration Rate
TBA	Thiobarbituric Acid
TBARS	Thiobarbituric Acid Reactive Substances

CHAPTER 1

LITERATURE REVIEW

1.1 Minimally Processed Refrigerated Fruits and Vegetables

Minimally processed refrigerated (MPR) fruits and vegetables are defined as those prepared by a single or any number of appropriate unit operations such as peeling, slicing, shredding, juicing, etc. given a partial but not end-point preservation treatment including use of minimal heat, a preservative, or radiation. (Wiley, 1994) These methods of preparation are applied to achieve a low microbial load product which retains the fresh-like state during its shelf-life. Thus these products are living tissue with active enzyme systems.

Minimal processing of raw fruits and vegetables has two purposes. First it is important to keep the produce fresh, but convenient without losing its nutritional quality. Second, the product should have a shelf-life sufficient to make distribution feasible within the region of consumption. (Ahvenainen, 2000)

Other terms used to refer to minimally processed products are "lightly processed", "partially processed", "fresh processed", and "pre-prepared". Especially when the products are fresh horticultural produce, they may also be referred to as "fresh cut", "ready-to-use" or "ready-to-eat". (Schlimme, 1995)

Consumers are increasingly demanding convenient, ready-to-use and ready-to-eat fruit and vegetables with fresh-like quality, and containing only natural ingredients. With regard to rationalisation of production and the

utilisation of peeling waste, it is reasonable to aim for centralised peeling and minimal processing of fruit and vegetables. (Ahvenainen, 1996)

A systems approach to processing and distribution of MPR fruits and vegetables is essential to optimise storage and handling conditions for individual crops. Processing and distribution systems for MPR fruits and vegetables include such issues as processing at the location of production versus at the location of consumption, large versus small processing plants, bulk transportation versus packaged shipment. Other issues include controlled atmosphere/modified atmosphere/vacuum/air packaging, storage at the location of production or in the region of consumption of a single commodity versus a multiple fruit and vegetable processing plant. (Yıldız, 1994) All of these factors have a defining effect on the shelflife and quality of the final product. Figure 1.1 illustrates stages in the processing of cucumbers for the production of a ready-to-eat salad mix.

Fresh-cut produce, such as salads is one of the fastest-growing areas of minimal processing. (Mermelstein, 1998)

1.1.1 Reasons for Quality Changes in Minimally Processed Produce

Fruits and vegetables are perishable products with active metabolism during the postharvest period. (Robertson, 1993). As such, both quality and shelf life of fresh fruit and vegetable products are dependent on the physiological maturity of the item at harvest, harvesting and handling conditions immediately after harvest and respiration rate during storage. (Shewfelt, 1994)

Any operation such as peeling, slicing, cutting or dicing that disrupts the cellular structure with subsequent decompartmentation of enzymes and substrates, results in an enhanced rate of physiological reactions. Moreover, wounded surfaces and repeated handling provide favourable conditions for microbiological growth. (Piga et al., 1999)

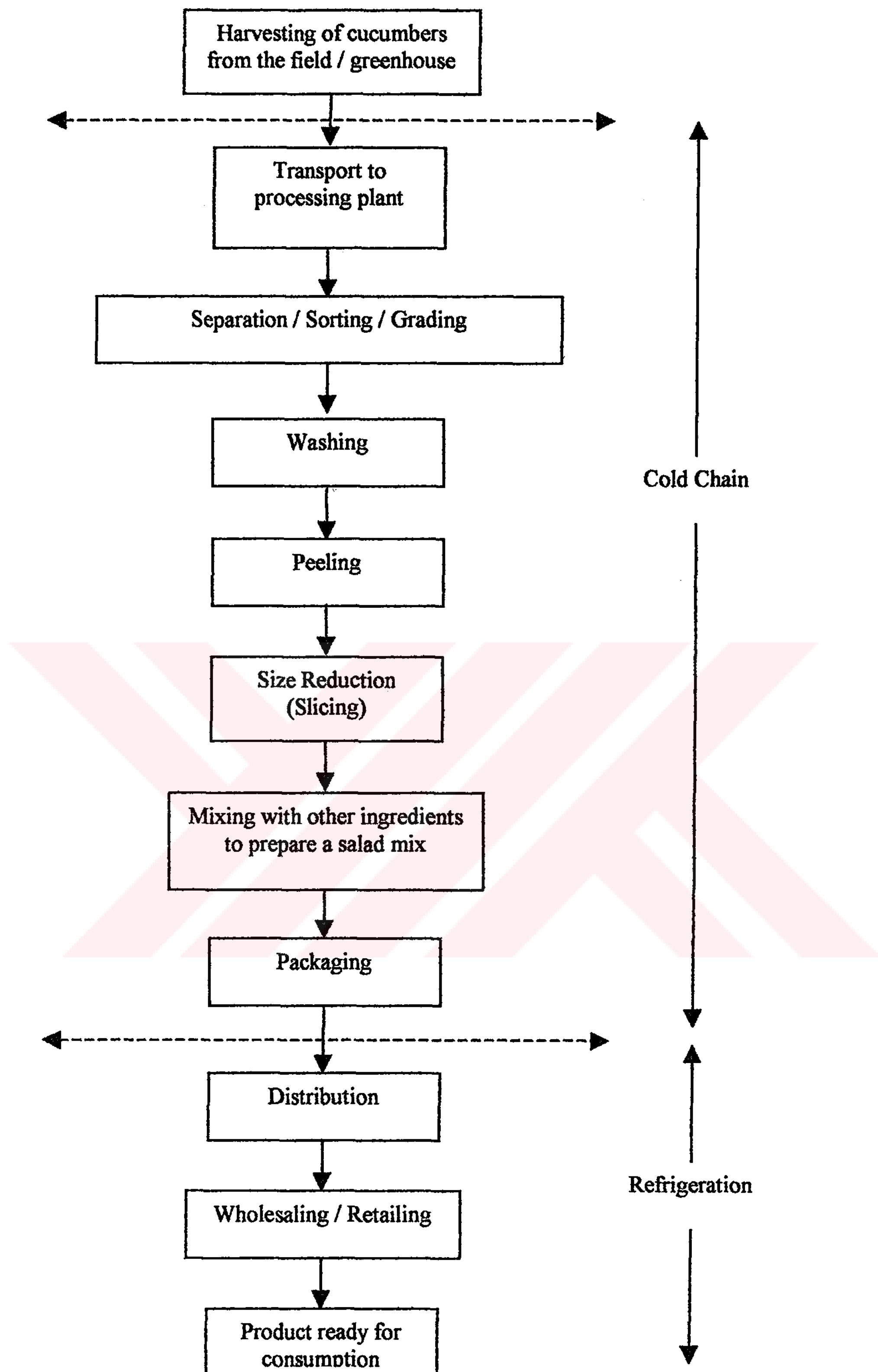


Figure 1.1 General flow diagram showing the processing steps for the preparation, handling and distribution of a minimally processed cucumber salad mix.

Fresh-cut products are highly perishable because a portion of their surface area is without epidermis, the outer protective layer of tissue (Watada et al., 1996)

Many examples of wound-induced ethylene production in fruit and vegetable tissues have been extensively reviewed. Because ethylene contributes to the neosynthesis of enzymes found in fruit maturation, it may play a part in physiological disorders of sliced fruits. (Voraquaux and Wiley, 1994)

Fungi and other microorganisms increase rates of respiration as do bruises and mechanical injuries. (Salunkhe et al., 1991)

1.1.2 Methods to Improve the Shelf Life and Safety of Minimally Processed Produce

The quality of the raw product needs to be excellent to ensure excellent quality fresh-cut product. Temperature, atmosphere, relative humidity and sanitation must be regulated to maintain quality of fresh-cuts. (Watada et al., 1996)

Light is a minor factor affecting the shelflife of fruits and vegetables. It can trigger the degradation of sensitive vitamins such as riboflavin, degradation of pigments and lipid oxidation. (Shewfelt, 1986)

1.1.2.1 Temperature Control

Temperature is a major, invisible, ever-present factor controlling respiratory, metabolic, and enzymatic activities, transpiration, and the growth of pests and microorganisms. Proper temperature management in the storage of MPR fruit and vegetable tissues can inactivate or retard the physiological defects. (Yıldız, 1994)

Respiration rates of fresh-cuts increases with temperature, and the degree of increase differs with the commodity. Since fresh cuts have been subjected to severe physical stress and are more perishable than intact products, they should probably be stored at temperatures lower than that recommended for the intact commodities. (Watada et al., 1996) For fruits and vegetables there is a great deal of variation in ideal refrigerated temperatures. (Wiley, 1994)

Decreasing temperature lowers metabolism, thus prolonging shelflife. Refrigeration and freezing are major tools used by food scientists to maintain fruit and vegetable quality but, unfortunately, some tissues are sensitive to low temperatures. Chilling injury (CI) is a physiological disorder induced by low, but not freezing temperatures. (Marangoni et al., 1996)

Chilling injury is a disorder observed in plant tissues, especially those of tropical and subtropical origin, resulting from the exposure of susceptible tissues to temperatures below 15 °C. the physical symptoms of chilling injury varies as does the lowest safe storage temperature of for some fruits and vegetables. (Salunkhe et al., 1991)

1.1.2.2 Relative Humidity

Water loss can be one of the main causes of deterioration since it not only results in direct quantitative losses (loss of salable weight), but also causes losses in appearance, (due to wilting and shrivelling) and texture (softening, flaccidity, limpness, loss of crispness and juiciness), and nutritional quality. (Kader, 1986)

These symptoms of water loss become objectionable when vegetables have lost 5 – 10 % of their weight due to transpiration. High relative humidity (over 95%) is highly essential for the maintenance of quality however at such high humidity prolific microbial growth may also take place. Therefore, an appropriate amount of relative humidity is very important. (Salunkhe and Wu, 1974)

Temperature and relative humidity are inextricably linked in the control of water loss. (Shewfelt, 1986) The amount of moisture the air can hold before it is saturated increases as the temperature rises.

1.1.2.3 Controlled/Modified Atmosphere

The primary factors in maintaining quality and extending the postharvest life of fresh fruits and vegetables are harvesting at optimum maturity, minimising mechanical injuries, using proper sanitation procedures and, providing the optimum temperature and relative humidity during all marketing steps. Secondary factors include the modification of O_2, CO_2 and/or C_2H_4 concentrations in the atmosphere surrounding the commodity to levels different from those in air. This is referred to as controlled atmosphere (CA) or modified atmosphere (MA). (Kader et al., 1989)

The principle behind control or modification of the atmosphere surrounding the product is to lower respiration and/or delay ripening. Striking the balance between O_2 and CO_2 is critical and an optimal ratio must be developed for each specific commodity. (Shewfelt, 1986)

Depleted O_2 and/or enriched CO_2 levels can also decrease ethylene production and sensitivity, retard textural softening, slow down compositional changes associated with ripening, reduce chlorophyll degradation and enzymatic browning, alleviate physiological disorders and chilling injury and maintain color and preserve vitamins of fresh produce, thereby resulting in an extended quality shelflife. (Day, 1993)

The ratio of CO_2 produced to O_2 consumed, known as the respiratory quotient (RQ), is normally assumed to be equal to 1.0 if the metabolic substrates are carbohydrates. If the substrate is a lipid the RQ is always lower than unity. The RQ is greater than unity if the substrate is an acid. The RQ is much greater than one when anaerobic respiration takes place. (Fonseca et al., 2002)

The respiration rate of fresh produce can be expressed as O_2 consumption rate and/or CO_2 production rate. In the closed (static) system for measuring respiration rate where M kg of produce is sealed in a hermetically sealed package with V m^3 of free volume is given by the equations below. (respiration rate as consumption or production, R in $m^3\ kg^{-1}s^{-1}$; volumetric concentration, Y as %v/v ; initial and final time, t_i and t_f in s)

$$R_{O_2} = \frac{\left(Y_{O_2}^{t_i} - Y_{O_2}^{t_f}\right) \times V}{100 \times M \times \left(t_f - t_i\right)} \quad \ldots\ldots(1)$$

$$R_{CO_2} = \frac{\left(Y_{CO_2}^{t_i} - Y_{CO_2}^{t_f}\right) \times V}{100 \times M \times \left(t_f - t_i\right)} \quad \ldots\ldots(2)$$

Studies have been performed on apple slices and mushrooms to determine the respiration rates, subsequent quality as affected by temperature and high oxygen partial pressures. (Gülden, 2002) The effect of noble gases on the anaerobic catabolism and quality attributes has also been determined. (Özdemir, 2001) Both of these studies were performed using a model closed system where the package atmospheres were evaluated using mathematical modelling. Another study performed using sweet cherries evaluated the suitability of some polymeric films to maintain the desirable modified atmosphere within the packages and the quality attributes as affected by the experimental conditions using active and passive packaging techniques. (Fincan, 1997)

The effects of superatmospheric oxygen levels on postharvest physiology and quality of fresh fruits and vegetables has recently been reviewed by Kader and Ben-Yoshua. It has been stated that superatmospheric oxygen may have different effects depending on the commodity, maturity and ripeness stage. (Kader and Ben-Yoshua, 2000)

1.2. Membranes and Senescence

1.2.1 Plant Cell Membranes – Structure and Function

The extensive membrane systems in plant cells constitute significant portions of their cellular material. They provide microenvironments essential for the characteristic chemical functions they perform. Thus membranes are not only viewed as impermeable structural entities but also as specific, selective, adaptable, and active mediators that regulate and maintain major cell functions. In addition to their permeability functions, membranes perform many chemical functions for the cell. (Benson and Jokela, 1976)

Biological membranes are composed of lipids and proteins, with smaller amounts of carbohydrate. The most abundant lipids in cell membranes are the phospholipids. Phospholipids are amphipathic molecules. They are diglycerides, with two fatty acids esterified to a glycerol molecule and a phosphate-containing polar group occupying the third position. In an aqueous environment, phospholipids spontaneously form a bilayer, with their hydrophobic tails buried in the interior and hydrophilic heads exposed to the water. (Hopkins, 1995) The fatty acids of phospholipid molecules may be saturated, mono- or polyunsaturated straight or branched-chain fatty acids.

The *fluid mosaic model*, proposed by Singer and Nicholson in 1972, is generally considered to be the most realistic membrane model currently available. This model depicts membranes as *fluid*, meaning that components can diffuse laterally and in the plane of the membrane, and as *mosaic*, meaning that the proteins are not spread uniformly over the outer polar surfaces but some of them are buried deeply and discretely into the membrane interior, in certain cases traversing the structure. (Stanley, 1991)

T.C. YÜKSEKÖĞRETİM KURULU DOKÜMANTASYON MERKEZİ

1.2.2 Plant Cell Senescence

Senescence in plants is usually viewed as an internally programmed deterioration leading to death. It is a developmental process that occurs in many different tissues and serves as different purposes. (Noodén et al., 1997)

Ripening fruit demonstrate many of the characteristics typically classified as senescence. During ripening a series of metabolic changes occur within the fruit or vegetable. However, in many respects, the ripened fruit represents only a stage of senescence, and the fruit undergoes further deteriorative changes (senescence) following harvest. (Beevers, 1976)

Food and horticultural scientists are interested in determining the mechanism of deterioration in fresh produce so that treatments can be found to that will allow shelf life to be prolonged. It has been suggested that deterioration of fruits and vegetables, seeds and other plant materials, either by natural senescence or by aging due to physiological damage, share a common mechanism. The focus of this process is thought to be alterations in the membranes, which in turn lead to alterations in the cellular metabolism and accelerated cell death. (Marangoni et al., 1996)

1.2.3 Membrane effects in postharvest physiology

Senescence is accompanied by a decline in the structural and functional integrity of cellular membranes. This is manifested in part by the onset of leakiness and loss of intracellular compartmentation. (Collins and Marangoni, 2000; Thompson et al., 1998) Losses in biological membrane functionality are observed not only during senescence but also during environmental stresses.

1.3 Lipid Peroxidation in Plant Tissue

Lipids are those substances soluble in solvents such as petroleum ether, hexane and other more polar solvents and are present in relatively small

amounts in vegetables (ranging from about 0.1 to 1.3 %, fresh weight basis). The lipids are mostly polar and consist primarily of membrane phospholipids and glycolipids. (Collins and Marangoni, 2000)

Cell membranes have distinct and specific lipid compositions that contribute to their identity. Of the classes of lipids the glycerolipids are generally the most abundant. In the plasma membrane phosphatidylethanolamine and phosphatidylcholine respectively make up to 30-40 % and 25-45 % of the total glycerolipids. (Moreau et al., 1998)

Phospholipids are the dominant lipids in microsomal membranes from young tissue whereas, the same membranes from corresponding senescing tissue contain phospholipids as well as steryl and wax esters and free fatty acids. These lipid metabolites which accumulate in senescing membranes give rise to a mixture of lipid phases in the bilayer and ensuing membrane leakiness. (Thompson et al., 1998)

Peorxidized lipids are released from membranes by blebbing of lipid-protein particles from the membrane surface. Lipid and protein catabolism is a normal feature of membrane turnover, and any significant accumulation of the catabolites in the bilayer could have a disruptive effect on membrane structure. Impairment of this blebbing process with advancing senescence appears to result in an accumulation of lipid catabolites in senescing membranes that causes bilayer destabilization and loss of membrane function. (Hudak et al, 1995, Thompson et al., 1998)

Lipid peroxidation is involved in normal developmental processes, including production of flavor and odor volatiles, formation of growth-regulator-like activities, as well as senescence. Characteristic flavors and aromas, such as those associated with cucumber (Cucumis sativus, L) and tomato (Lycopersicon esculentum Mill.) are due in part to the various enzymes in the respective lipoxygenase pathways. It has also been shown that induced defenses, including the hypersensitive response associated with resistance to

pathogens and herbivore defences involving protease inhibitors also rely in part on oxidation of polyunsaturated fatty acids (PUFAs). Lipid peroxidation via free-radical-mediated reactions has been implicated by abiotic stresses such as temperature stress, and exposure to gaseous pollutants. (Anderson, 1995)

Antioxidants such as β-carotene, lycopene, and α-tocopherol, as well as reducing agents such as ascorbic acid and glutathione, help limit peroxidative damage. Enzymes important in protecting membranes from lipid peroxidation include catalase, peroxidase, and superoxide dismutase. (Shewfelt and Rosario, 2000)

Ascorbate and tocopherol function together to protect the membrane lipids from damage. Each tocopherol can donate two electrons as a chain breaking antioxidant, and then it is "consumed". To be an effective antioxidant each oxidized tocopherol must be recycled. The current thinking is that ascorbate recycles tocopherol via the relatively stable tocopheroxyl radical formed, producing the ascorbate radical. (Buettner, 1993)

Membrane lipid peroxidation and "repair" is illustrated in Figure 1.2. In this figure only one leaflet of the bilayer is represented. A phospholipid molecule has been depicted as an oval representing the phosphate end, esterified to two linoleic acids. The peroxidation process is initiated by an oxidizing radical $X^{\bullet}$, by abstraction of a bis allylic hydrogen, thereby forming a pentadienyl radical (Figure 1.2a). This is oxygenated to form a peroxyl radical and a conjugated diene (Figure 1.2b). The peroxyl radical moiety partitions to the water-membrane interface where it is poised for repair by tocopherol (Figure 1.2c). The peroxyl radical is then converted to a lipid hydroperoxide, and the resulting ascorbate radical can be repaired by ascorbate (Figure 1.2d). After the tocopherol has been recycled by ascorbate, the resulting ascorbate radical can be recycled by enzyme systems (Figure 1.2e). The enzymes phospholipase A_2 (PLA_2), phospholipid hydroperoxide glutathione peroxidase ($PH\text{-}GP_x$), glutathione peroxidase (GP_x) and fatty acyl-coenzyme A (FA-CoA), cooperate to detoxify and repair the fatty acid chain of the phospholipid.

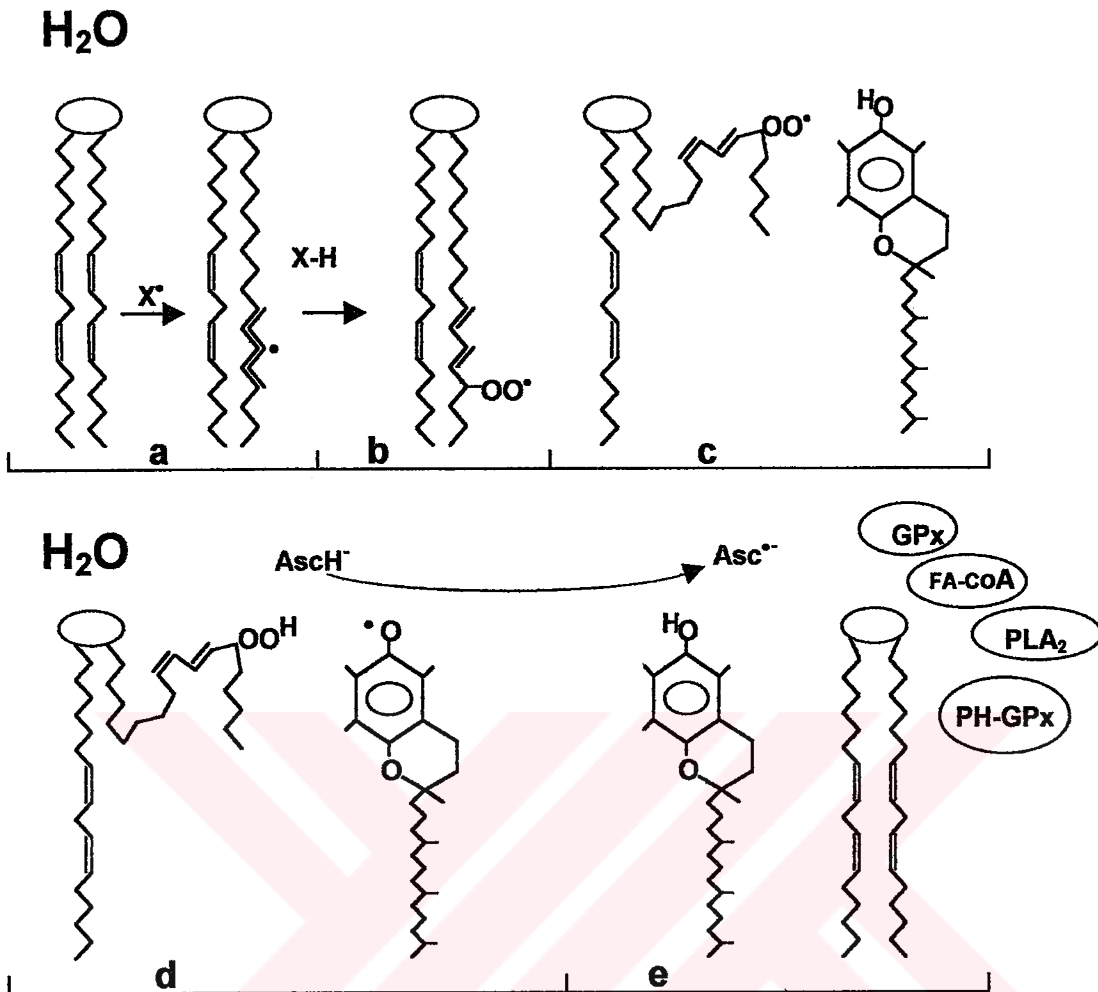


Figure 1.2. Membrane lipid peroxidation. (Buettner, 1993)

1.3.1 Mechanisms Involved in Lipid Peroxidation in Plant Tissue

Phospholipid autoxidation proceeds by the same mechanism as the fatty acid process. The primary products formed from autoxidation of linoleic acid, its methyl ester or its esters of glycerophospholipids, are the hydroperoxides substituted at the 9 and 13 positions. Thus the autoxidation of free fatty acids in bulk, methyl esters in solution or phospholipids in aqueous emulsion follow essentially the same course. (Porter, 1985)

The reaction of oxygen with unsaturated lipids (RH) involves free radical initiation, propagation and termination processes. Initiation takes place

by loss of a hydrogen radical in the presence of trace metals, light or heat (Eq.3). The resulting lipid free radicals (R•) react with oxygen to form (ROO•) (Eq.4). In this propagation process, ROO• react with more LH to form lipid hydroperoxides (ROOH), which are the fundamental primary products of autoxidation (Eq.5). (Frankel, 1984)

$$RH \rightarrow R\bullet + H^{+} \quad \ldots\ldots\ldots\ldots\ldots (3)$$

$$R\bullet + O_2 \rightarrow ROO\bullet \quad \ldots\ldots\ldots\ldots\ldots (4)$$

$$ROO\bullet + RH \rightarrow ROOH + R\bullet \quad \ldots\ldots\ldots\ldots (5)$$

The free radicals produced can combine with each other, or more likely with protein molecules and end the chain reaction.

In the presence of light unsaturated fats can also form hydroperoxides by reacting with singlet oxygen produced by sensitised photoxidation, which is a non-free-radical process.

Lipid hydroperoxides are readily decomposed into a wide range of carbonyl compounds, hydrocarbons, ketones and other materials that contribute to flavour deterioration of foods. (Guillén-Sans and Guzmán-Chozas, 1998; Frankel, 1991)

Lipid hydroperoxides (ROOH) are also formed in plant tissue by lipoxygenase (LOX). Unlike the free radical processes described above, lipoxygenase, acting under aerobic conditions, does not produce lipid-free radicals. In addition, lipoxygenases in plant tissue either require or preferentially attack free fatty acid, usually acting in concert with hydrolytic enzymes. (Shewfelt and Purvis, 1995)

The LOX catalysed oxidative reactions are more complex than those of non-enzymatic hydroperoxides. An overview of the LOX or octadecanoid pathway of plants is shown in Figure 1.3. (Gardner, 1995)

Jasmonic acid is a senescence hormone biosynthesised as a product of hydroperoxide cyclase action in the LOX pathway. Other hormones related to senescence are also known to be synthesised by mechanisms involving the oxidation of linoleic acid by LOX. The LOX pathway is also responsible for the increase in levels of certain end products of the linoleic/linolenic acid cascade. Traumatin, a wound hormone, is similar in structure to traumatic acid, a direct product of the cascade. Other products of hydroperoxide lyase action, such as hexenal and hexanal, may also function in plant protection. (Gardner, 1985)

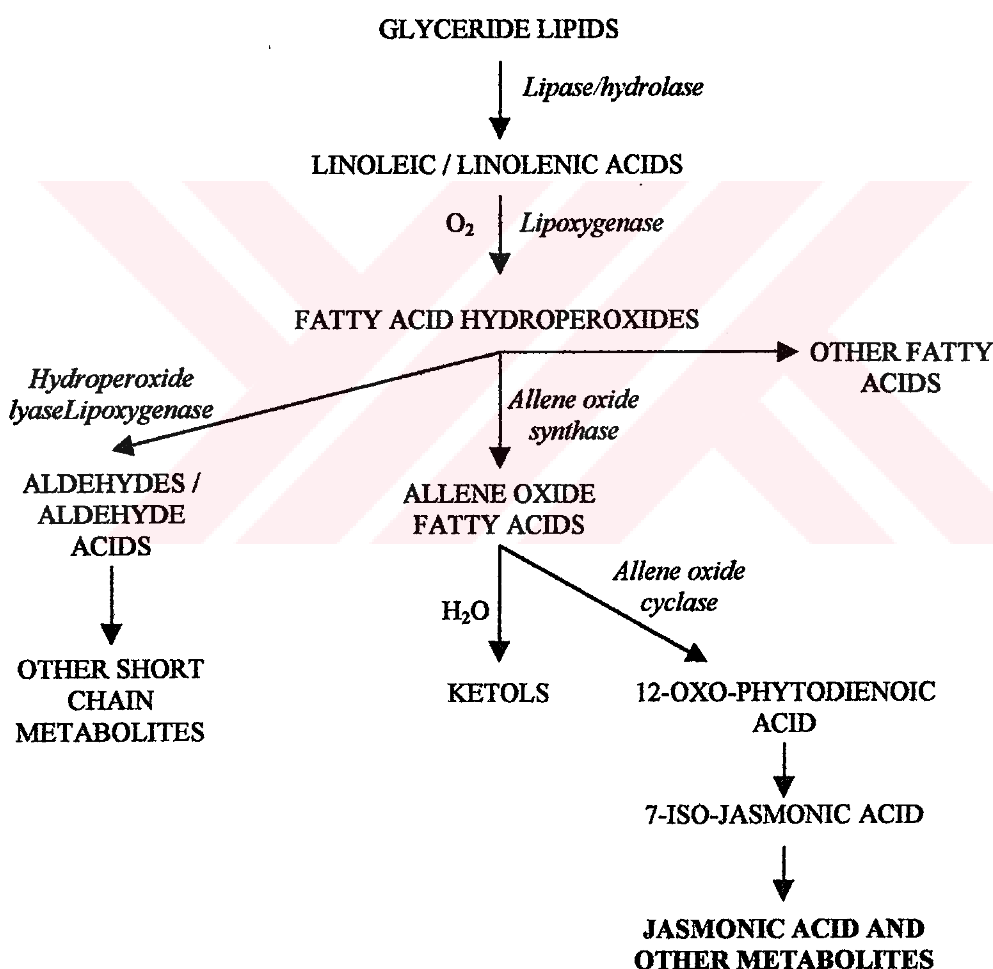


Figure 1.3. A summary of the major metabolic branches in the plant octadecanoid pathway.

Malondialdehyde (MDA) is claimed to be an important biological breakdown product expected from 5-membered cyclic peroxides of linoleate and linolenate because of its close linkability with amino groups of proteins, enzymes and DNA. Figure 1.4 explains the mechanism of cyclization of linolenate hydroperoxides and the formation of malonaldehyde. (Frankel, 1984)

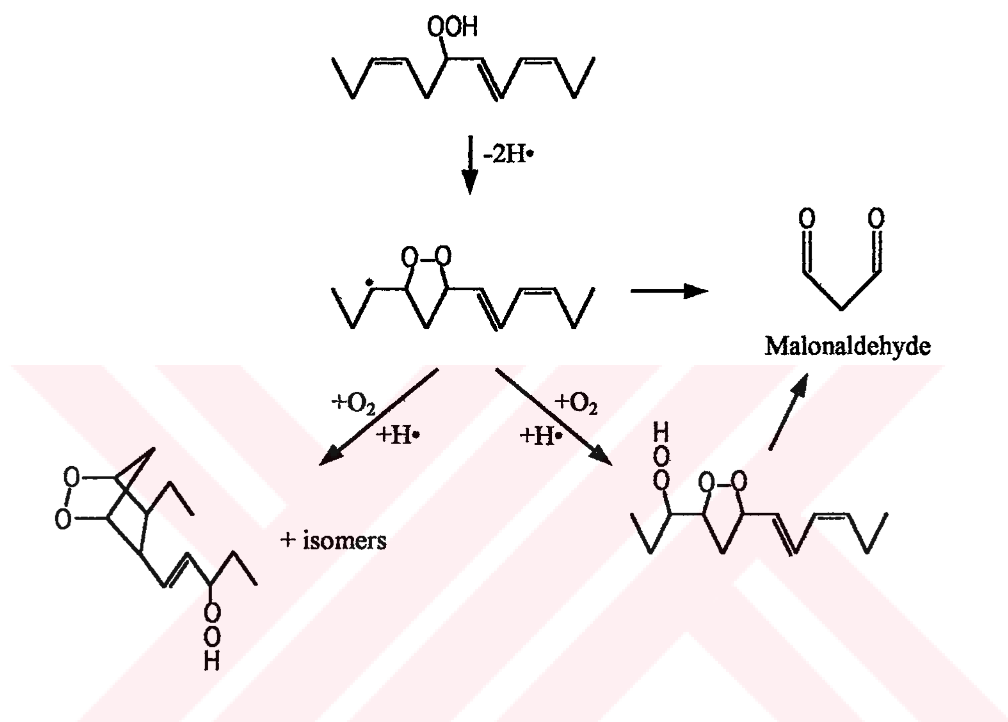


Figure 1.4 Mechanism of 1,3-cyclization of 12- and 13-hydroperoxides of linolenate and formation of malondialdehyde.

Shewfelt and Rosario (2000) argue that, lipid peroxidation, generally relegated to the status of a secondary effect of a primary event responsible for the degradation process, could actually be a critical, controllable event common to the mechanisms of many postharvest storage disorders. Potential candidates for the primary event include 1) a biophysical change in the membranes, 2) alteration of conformation of critical enzymes, 3) modification of the cytoskeleton and 4) major changes in the calcium balance.

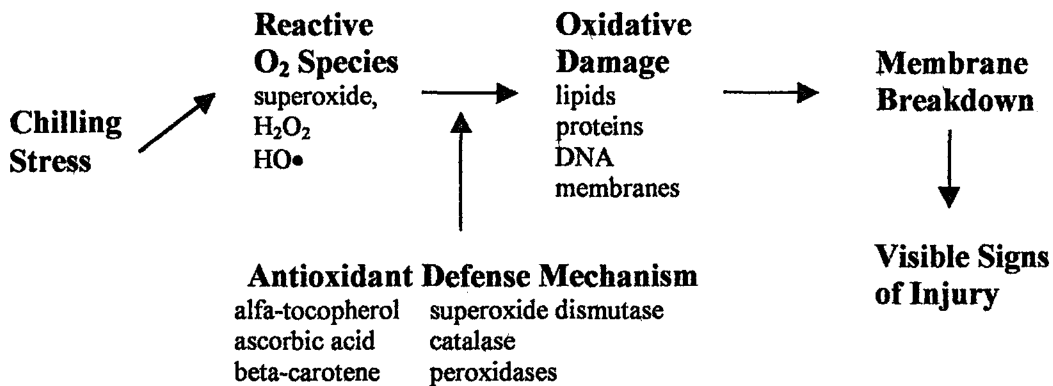


Figure 1.5 Schematic conceptual model proposed by Shewfelt and Rosario to explain the role of lipid peroxidation in chilling injury and other membrane disorders in plant tissue.

1.3.2 Determination of Peroxidation in Plant Tissues

Malondialdehyde, a major degradation product of lipid hydroperoxides, has attracted much attention as a marker for lipid peroxidation. The most common method for measuring MDA in food products and biological samples seems to be the thiobarbituric acid test which is based on a spectrophotometric quantitation of the pink complex formed after reaction of MDA with two molecules of 2-thiobarbituric acid (TBA). (Botsoglou et al., 1994) This test is also referred to as the thiobarbituric acid reactive substances (TBARS) assay.

Heath and Packer (1968) were among the first to use the TBARS assay for the determination of lipid peroxidation in plant tissues. They used isolated chloroplasts and concluded that upon illumination a cyclic peroxidation process was initiated and this photoperoxidation resulted in the destruction of the chlorophyll and PUFAs of the chloroplast membranes. Many studies on lipid peroxidation which followed made reference to this study.

There has been concern that non-MDA substances may inflate readings resulting in overestimation of the oxidation levels. Du and Bramlage (1992) identified sugars, namely sucrose, glucose and fructose, as the substances

causing major interference in the TBARS assay and modified the method of Heath and Packer (1968) by subtracting the sugar absorbance maximum at 440 nm from that at 532 nm. The assay accuracy was further increased when Hodges et al (1999) modified the method to correct for anthocyanin and other interfering substances. This was done by correcting for the compounds other than MDA which absorb at 532 nm by subtracting the absorbance at 532 nm of a solution containing plant extract incubated without TBA from an identical solution containing TBA. This study also makes a comparison with the two previous methods showing that they could be overestimating the MDA quantities by up to 189-499 %.

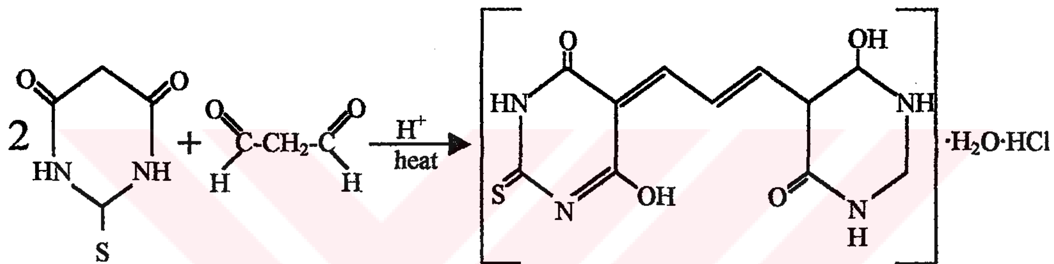


Figure 1.6 The reaction of thiobarbituric acid (TBA) with malondialdehyde (MDA)

MDA can only be formed from fatty acids with three double bonds. Hence the assay may also underestimate the extent of lipid peroxidation in plant tissues since plant tissues often contain high levels of $18{:}2(\Delta^{cis, 9, 12})$. (Griffiths et al., 2000)

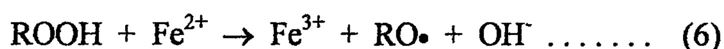
Flourescence spectra of peroxidized lipids extracted from fruit tissue have also been used as a method to determine lipid peroxidation. Schiff bases (with the structure: – N = C – C = C – N) formed by the reaction of aldehydes from peroxidized lipids with free amino acids have characteristic flourescence spectra that feature an excitation maximum at ≈360 nm and an emission maximum at ≈430 nm. (Hudak et al., 1995; Maguire and Haard, 1975) The

TBA-MDA adduct may also be detected by flourescence at an excitation maximum at ≈515 nm and an emission maximum at ≈543 nm.

More advanced chromatographic and spectrometric techniques based on the TBARS assay have also been made available. The LC-MS technique has recently been applied by Jardine et al. (2002). The results of studies performed comparing the GC-MS method with the basic TBARS assay concludes that the latter method gives two to six-fold higher results, however also indicates its usefulness because of its sensitivity and simplicity, if one clearly understands its nonspecificity. (Liu et al., 1997) The HPLC method provides high sensitivity reproducibility and specificity. The results, when compared to the spectrophotometric methods, were much lower. (Bergamo et al., 1998) These advanced methods are however time consuming and costly and often require strict control of ambient oxygen levels.

In recent years another method has been developed that is based upon the oxidation of ferrous (Fe^{2+}) to ferric (Fe^{3+}) ions by ROOHs with the subsequent binding of the Fe^{3+} ion to the ferric-sensitive dye xylenol orange. (Nourooz-Zadeh, 1999) Known as the ferrous oxidation-xylenol (FOX) orange assay (versions I and II), the technique is sensitive (nanomole to micromole levels of ROOHs), inexpensive and not affected by ambient oxygen concentrations.

The FOX2 assay is based on the oxidation of ferrous to ferric ions by ROOHs in suspension preparations. (Eq. 6) Undesirable chain oxidation is prevented (Eq. 7-9) by inclusion of the lipid-soluble chain breaking antioxidant butylated hydroxytoluene (BHT), which repairs the alkyl radicals produced by the reaction of alkoxyl radicals with unsaturated lipids (Eq. 10).



$$ROOH + Fe^{2+} \rightarrow Fe^{3+} + RO\bullet + OH^- \; .... \; (6)$$

$$RO\bullet + RH \rightarrow ROH + R\bullet \; .... \; (7)$$

$$R\bullet + O_2 \rightarrow ROO\bullet \; .... \; (8)$$

$ROO\bullet + RH \rightarrow ROOH + R\bullet$ .... (9)

$R\bullet + BHT \rightarrow RH + BH\bullet$ .... (10)

The FOX assay has recently been reviewed by DeLong et al. (2002) and adapted to more accurately detect the presence of LOOHs as a measure of incipient lipid peroxidation in plant tissues.

The iodometric assay was used to verify the presence of LOOHs in reconsructed LOOH systems and plant tissue extracts. In this method, the LOOHs are reduced to alcohols by iodide ($2I^-$) resulting in the conversion of $2I^-$ to the triiodide (I_3^-), which is measured spectrophotometrically at 290 or 360 nm. The iodometric method could not measure LOOH levels at low concentrations in plant extracts ($<11\mu M.H_2O_2$ Equiv.). This method also requires working with deoxygenated reagents because the reactions are highly sensitive to O_2. (DeLong, 2002)

Measurement of ethane evolution has been used as a non-invasive indicator of lipid peroxidation in many biological tissues. Ethane is a product of linoleic acid peroxidation which is not metabolized further. (Kuo and Parkin, 1989)

1.4 Cucumbers

Cucumbers are the unripe fruit of the species Cucumis sativus, a climbing plant of the Cucurbitacea family. The cucumber plant most likely originated in India. Of the 1341 accessions of Cucumis sativus L. 238 have been introduced from India and 174 from Turkey. (Anon,1996)

Varieties of cucumber include both the slicing or the fresh salad type and the pickling type. Almost all cucumbers grown in greenhouses are the long, seedless type which grow on gynecious type vines (plant carrying only female flowers). (Papadopoulos, 1994)

Washed waxed, intact cucumbers have been tentatively excluded from the classification of MPR fruits and vegetables. Whereas, precut or sliced cucumbers which have a relatively short shelflife are considered MPR foods. (Wiley, 1994)

Chilling induced biochemical and physiological changes in cucumber have been well documented and include increased electrolyte leakage, enhanced rates of respiration, and enhancement of ethylene evolution following short durations of chilling, followed by impairment of ethylene evolution with longer durations of chilling. Immersion of cucumbers in heated water (25-42 °C for 30 min) enhanced chilling tolerance of the fruit. (McCollum et al., 1995)

Cucumbers are chilling sensitive and are injured if held at temperatures less than 10 °C for more than 3 days. Pitting and increased decay are the two visible symptoms of CI in cucumber fruit. Crops can develop symptoms during storage at chilled temperatures or subsequently during storage at nonchilled temperatures. Intermittent warming treatments has been shown to decrease the physiological responses of increased ethylene production and ion leakage. (Cabrera and Salveit, 1990)

Cucumbers packaged in perforated or sealed low density polyethlene (LDPE) bags were found to have less severe CI than nonwrapped fruit in storage at 5 °C and 90-95 % relative humidity. Fruit in the sealed bags, where a passive modified atmosphere was allowed to develop, had the least decay. (Wang and Qi, 1997)

Ethane evolution was measured as an indicator of *in situ* lipid peroxidation in whole cucumber fruit in association with the development of chilling injury. Results indicated that the potentiation of lipid peroxidation associated with the onset of irreversible chilling injury. (Kuo and Parkin, 1989)

CHAPTER 2

MATERIALS AND METHODS

2.1 Materials

2.1.1 Cucumbers

Greenhouse cucumbers (Cucumis sativus, L) harvested in a mature condition within 24 hours were purchased from a local supplier to be used in the experiments. The fruit were sorted to obtain a lot of similar size and shape and free of deformities. The cucumber fruit were stored at 10 °C prior to the initiation of the experiments. Determinations at day 0 were initiated after the cucumbers had been equilibrated to the storage temperatures.

2.2 Methods

2.2.1 Experimental Plan

An outline of the experimental procedure is given in Figure 2.1.

2.2.2 Preparation of the Samples

2.2.2.1 Preparation of the Samples for Modified Atmosphere Packaging

The cucumbers were washed with cool water (6-8 °C), peeled with a fruit peeler and cut into slices of about 1cm thickness. The treatment of the fruit for bruised samples was achieved by dropping a weight from the same height

onto each slice (800 g from ca 10 cm height). This application resulted in a bruised surface with visible change of appearance of the tissue up to ca 1 mm depth. 100 grams of the prepared fruit were weighed into 720 ml glass jars. Three samples were prepared for each treatment to be stored at 4 °C and 20 °C. The treatments consisted of packaging under normal atmosphere or under high partial pressure (superatmospheric) oxygen in combination with bruising of the fruit for some of the samples (Table 2.1). The superatmospheric oxygen packaging was achieved by flushing the jars with oxygen gas previous to sealing the samples with hermetic lids. The rest of the samples were sealed directly for normal atmosphere packaging, where a modified atmosphere develops passively over time. The lids were previously fitted with self sealing septa for the removal of gas samples for analysis. The 720 ml jars containing 100 g samples of sliced and peeled cucumbers had approximately 600 ml of headspace which corresponds to a headspace to fruit weight ratio of 6 ml/g. For the lipid peroxidation determinations, sufficient number of jars were prepared to allow for sampling every three days of storage.

Table 2.1 Sample codes.

	4 ° C	**20 ° C**
A- Normal Atmosphere Packaging	A4.1, A4.2, A4.3	A20.1, A20.2, A20.3
B- O_2 Flushing Treatment	B4.1, B4.2, B4.3	B20.1, B20.2, B20.3
C- Bruising Susceptibility	C4.1, C4.2, C4.3	C20.1, C20.2, C20.3
D- Bruising + O_2 Flushing	D4.1, D4.2, D4.3	D20.1, D20.2, D20.3

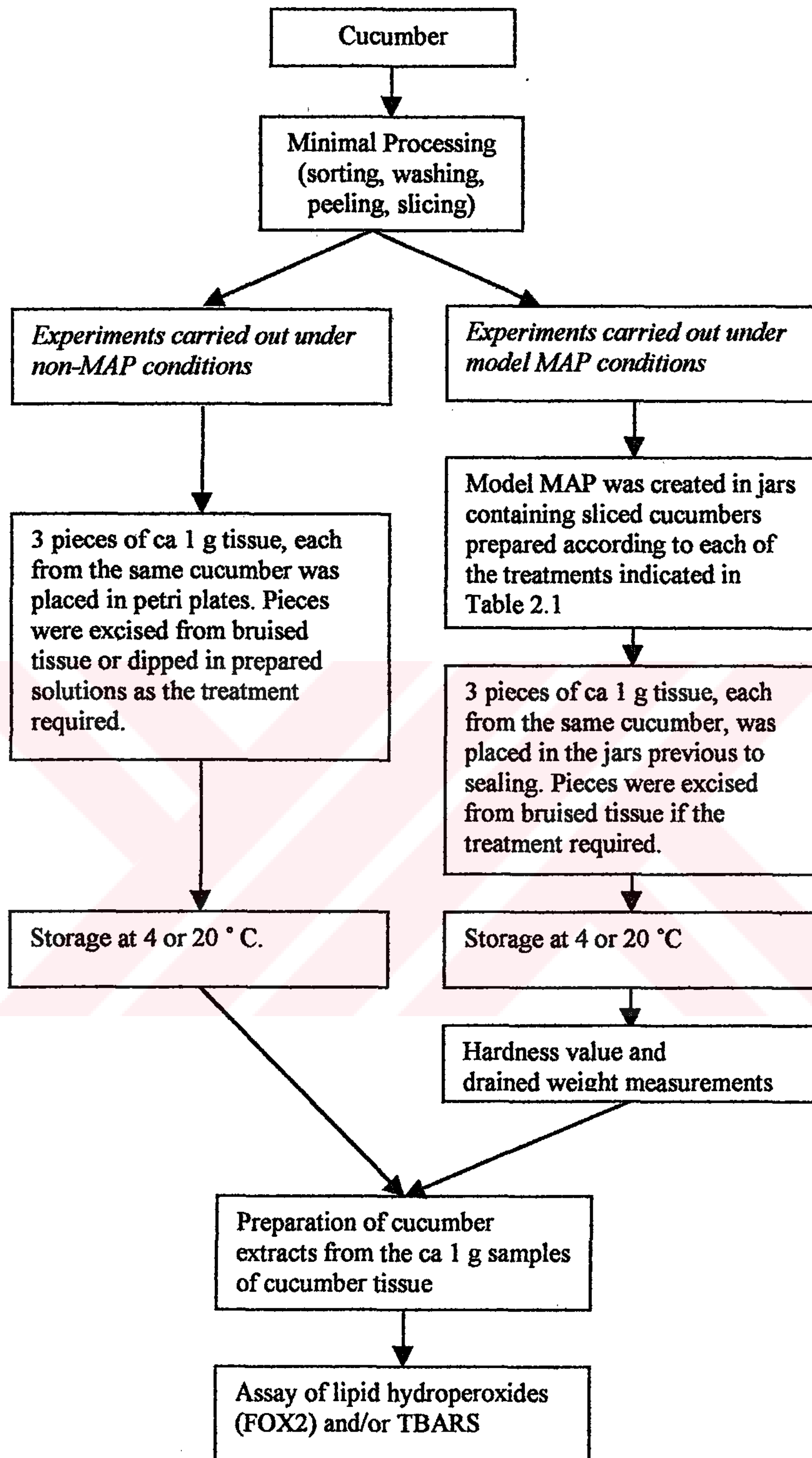


Figure 2.1 Outline of the experimental procedures.

2.2.2.2 Preparation of samples for the determination of the effect of several dipping solutions on lipid peroxidation

Cucumbers were washed and peeled as previously described. 0.5 – 1.0 gram pieces excised from the mesocarp tissue were placed in petri plates after their exact weights being recorded. Enough fruit pieces were prepared from each fruit to allow for the preparation of fruit extracts to be used in the lipid hydroperoxides and TBA reactive substances assays every two days. Three different solutions were used in the dipping applications. First solution contained 1% (w/v) ascorbic acid, second solution contained 0.2 % $CaCl_2$ and the third solution contained both 1% ascorbic acid and 0.2% $CaCl_2$. These concentrations have previously been applied for the inhibition of browning of fresh-cut pear (Sapers and Miller, 1998). Also 1 and 2.5 % $CaCl_2$ dipping solutions imparted greater structural integrity to minimally processed cantaloupe (Lunà-Guzman and Barrett, 2000). The control fruit were not dipped into any solution. The samples were placed in petri plates so that each plate contained 3 paralleled samples of fruit prepared with the different dipping applications and the control. The plates were placed in the refrigerator for storage at 4 °C.

2.2.3 Hardness Value Measurements

Hardness values of the fruit slices were obtained previous to packaging and for the samples in the jars opened every 3 days. A fruit hardness tester (Everwell Corporation, Model FT011, Japan) with a 10 mm conical probe was used for the determinations. Five hardness values (one from each slice) for each of the samples were determined by positioning the tester perpendicular to the slice plane at the centerline of the mesocarp tissue.

2.2.4 Headspace gas composition measurements

Analysis of the headspace gas composition was carried out on a gas chromatograph (Model CRA4, Shimadzu, Japan) equipped with a computerised integrator (Chromatopac, Shimadzu, Japan) and a thermal conductivity detector.

A gas tight syringe was used for the withdrawal of 0.2 ml gas samples from the self sealing septa on the lids of the jars. A Propak Q column at 40 °C was used for the detection of CO_2 and N_2 in the gas samples. The gas flow rate was adjusted to 22 ml/min and the injection and detector temperatures were set at 50 °C and 60 °C respectively. A Molecular Sieve 5A column at 35 °C was used to determine the O_2 and CO_2 in the samples. The gas flow rate was adjusted to 14 ml/min and the injection and detector temperatures were set at 50 °C and 70 °C respectively.

2.2.4.1 Determination of Respiration Rate (RR)

The RR was determined by the glass jar technique (Fonseca et al., 2002; Özdemir 2001). 300 g of peeled and sliced cucumber fruit were enclosed in 720 ml hermetically sealed jars with septa fitted to the lids for gas sampling. Approximately 400 ml of headspace remained in the jars. Sampling and gas composition determination was carried out after the first 6 hours and 18 hours of packaging. This method was applied for the determination of the RR of non-bruised and bruised cucumber slices at 4 °C and at 20 °C.

2.2.4.2 Determination of Fermentative Index (FI)

The FI, which is the CO_2 production rate (R_{CO_2}) of the tissues under anaerobic conditions, for the characterisation of the tissues was determined by the glass jar technique described above. The headspaces of the jars were initially flushed with N_2 gas and the gas composition within the jars were then determined with gas chromatography. The initial concentration of the N_2 gas was at least 99%.

2.2.5 Preparation of Fruit Extracts

Previously weighed cucumber flesh was hand ground with a precooled mortar and pestle on ice using 0.5 g of inert sand in 8 ml of 95% ethanol containing 0.1% (w/v) BHT to arrest any further oxidation and 1 ml of pure

water. Weight loss of the flesh due to dehydration was made up for with water. Each sample was then centrifuged (Model K-30, Sigma Laboratory Centrifuges, Germany) at 4°C and 3000 g for 10 minutes with supernatant aliquots being used for the TBARS and lipid hydroperoxides assays. The blanks were processed identically except for the presence of the tissue segments.

2.2.6 Determination of Lipid Hydroperoxides (FOX2)

Lipid hydroperoxides were determined using the FOX version II (FOX2) assay (DeLong et al., 2002 and Nourooz-Zadeh, 1999). For each 1000 ml volume, the FOX reagent consisted of 90 % HPLC grade methanol (v/v), 10 % 250 mM H_2SO_4 (v/v) (25 mM final concentration), 880 mg of butylated hydroxytoluene (BHT, 4 mM), 98 mg of ferrous ammonium sulfate hexahydrate (250 μM), and 76 mg of xylenol orange (100 μM). A working reagent was prepared by mixing a solution of ammonium ferrous sulphate and xylenol orange in an aqueous solution of sulfuric acid and subsequently adding the methanolic solution of BHT. This solution is stable for 1 month at 4 °C in the dark. The pure, double distilled water was generated by a Millipore water purification system (Millipore, Danvers, MA). All glassware and plastic tubes used for the chemical assays were rinsed with double distilled water.

For some samples, 100 μl of the plant extract was combined with 100 μl of 10 mM triphenylphosphine (TPP) in methanol. The mixture was momentarily stirred with a vortex stirrer and then incubated for 30 minutes to allow for the complete reduction of any present hydroxides by TPP (+TPP). Samples without TPP (-TPP) addition were treated identically except that the TPP aliquot was substituted with methanol. Following the 30 minute TPP incubation, 2000 μl of FOX2 reagent was added to each sample with the absorbance at 560 nm being recorded exactly 10 minutes after reagent addition on a UV-VIS Spectrophotometer (Model UV-1202, Shimadzu, Japan). The absorbance difference between the samples without and with TPP indicated the presence of

lipid hydroperoxides. Hydroperoxide values were then expressed as micromolar H_2O_2 equivalents using a standard curve spanning 0-20 μM H_2O_2 range.

2.2.7 Determination of TBA Reactive Substances (TBARS)

A modified TBARS assay (Hodges, et al., 1999) was used as an alternative assessment of lipid oxidation. The TBARS reagent consisted of 20 % (w/v) trichloracetic acid (TCA), 0.65 % (w/v) thiobarbituric acid (TBA) and 0.01 % (w/v) BHT in double distilled H_2O. Two hundred microliters of the fruit extract were combined with 800 μl of water and either 1000 μl of reagent with TBA added (+TBA) or 1000 μl of reagent without TBA (-TBA). The samples were then mixed vigorously, incubated at 95 °C for 30 minutes, cooled under running tap water and centrifuged at 3000 g for 10 minutes. Sample absorbances were measured spectrophotometrically at 532, 600 and 440 nm. The MDA equivalents are calculated using Eq. 11 - 13.

$$[(A532_{+TBA} - A600_{+TBA}) - (A532_{-TBA} - A600_{-TBA})] = A \quad (11)$$

$$[(A440_{+TBA} - A600_{+TBA}) \times 0.0571] = B \quad (12)$$

$$\text{MDA equivalents } (\text{nmol.ml}^{-1}) = (A - B / 157000) \times 10^6 \quad (13)$$

2.2.8 Statistical Analysis

Correlation analysis was performed on the FOX2 and TBARS results. Analysis of variance (ANOVA) was performed by statistical data analysis tool-pack of MS Excel and followed by least significant difference (LSD) where appropriate. Significance of results was defined as $p \leq 0.05$. All assays and GC determinations were performed on triplicate set of samples except the hardness measurements where 5 sets of data were collected for each condition.

CHAPTER 3

RESULTS AND DISCUSSIONS

3.1 Effect of Temperature and Bruising on Lipid Peroxidation

Cucumbers are chilling sensitive fruits. Chilling sensitivity in whole cucumber fruit and the physical and/or physiological changes that are induced by exposure to chilling has been well documented and it has been concluded that 7 - 10 days of continuous chilling is required to impart irreversible injury (Kuo and Parkin, 1989). Furthermore membranes have been targeted as being the site of chilling injury due to peroxidation of the lipids. Detected by ethane evolution, Kuo and Parkin (1989) observed the increase in lipid peroxidation after 8 days of chilling of whole cucumbers. Our experiments spanned the first eight days of storage of minimally processed cucumber fruit.

Some tissues (such as those of pears and melons) accelerate their ethylene production during chilling, but cucumbers do not until they are transferred to warmer temperatures (Wang and Adams, 1982). Elevated ethylene production is also observed due to wounding of the tissues. Wounding of plant tissues takes place in the course of preparation of the fruit by slicing or peeling operations or by bruising. Although cucumbers are non-climacteric fruit, the increase in ethylene levels could cause accelerated respiration, deterioration and senescence.

The degree of lipid peroxidation was monitored by assaying lipid hydroperoxides and TBA reactive substances for bruised and non-bruised cucumber pieces stored at 4 and 20 °C at 48-hour intervals. Figure 3.1 shows the

results of the FOX assay used to determine μM H_2O_2 equivalents of lipid hydroperoxides per gram of cucumber fruit. The standard curve used for the calculation of the lipid hydroperoxide levels can be found in the Appendices section (Figure A.1). The results of the TBARS assays have been depicted in Figure 3.2. FOX and TBARS data revealed that the two assays correlated significantly for normal samples at 4 and 20 °C and bruised samples at 4 °C ($p \leq 0.05$).

The two methods employed detect different groups of lipid peroxidation products but it is evident that the trends are similar for the two methods and the levels of these indicators of lipid peroxidation increase as time progresses. The storage temperature affected the lipid peroxidation significantly. The bruising treatment however, did not have significantly different effects in any of the experiments involving storage at non-modified atmosphere conditions.

Although there is a generally continual increase in lipid peroxidation products over the ten-day period, less obvious trends may also be considered during the first few days. Looking at the TBARS results of the fruit stressed by chilling and/or bruising for the first four days of the experiment indicates an increase followed by a slight decrease. These "peaks" could be explained as a reaction of the tissue to the stress imposed on it. During the first two days the stress factor causes an increase in TBARS accumulation and in the following two days the tissue tries to acclimate itself to the situation by decreasing the amount of secondary lipid peroxidation products formed. This may be possible by the activation of enzyme systems which aid in the decomposition of the lipid peroxidation products as well as prevention of MDA formation by repair of the peroxidized fatty acid molecules.

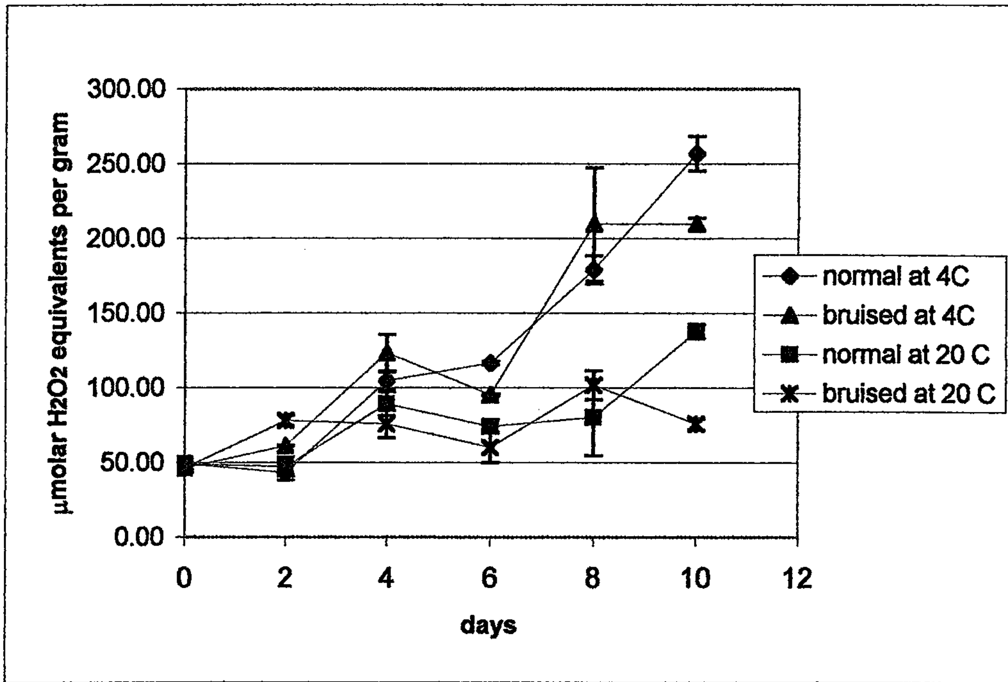


Figure 3.1 Lipid hydroperoxide levels in cucumber mesocarp tissue stored at 4 and 20 °C, expressed as µM H_2O_2 equivalents per gram.

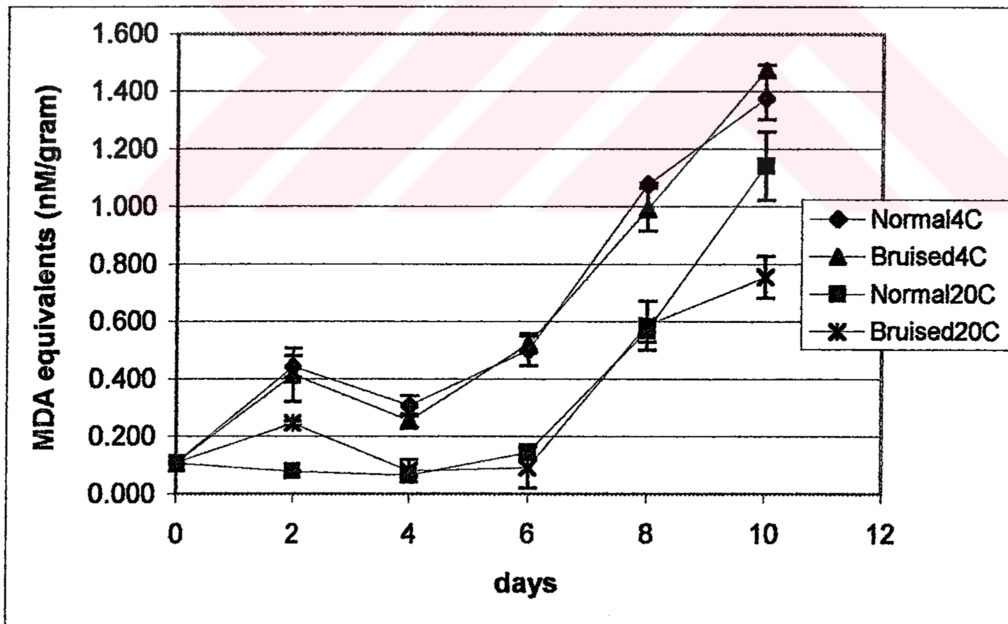


Figure 3.2 TBARS levels in cucumber mesocarp tissue stored at 4 and 20 °C, expressed as nmoles MDA equivalents per gram fresh weight.

3.2 Effect of Several Dipping Solutions on Lipid Peroxidation

Calcium and ascorbic acid dips are commonly used in the fruit and vegetable industries to increase the shelflife of the products. Their salts may be incorporated into dipping mixtures used to delay enzymatic browning of minimally processed fruits.

It is well known that calcium plays an important role in maintaining the quality of fruits and vegetables. Calcium is essential for the structure and function of the cell walls and membranes. It is important in maintaining cell permeability and compartmentation. Addition of calcium to calcium deficient tissues decreases respiration and suppresses ethylene production. Postharvest dipping in calcium chloride solution is effective in increasing the calcium content of the fruit (Sapers and Miller, 1998; Lunà-Guzman and Barrett, 2000).

Cucumbers are one of the fruits containing the lowest amounts of vitamin C or ascorbic acid. The ascorbic acid content of vegetables and fruits vary greatly from being as low as 4.7 mg/100g fresh weight for cucumbers to being as high as 93.2 mg/100 g fresh weight for broccoli (Collins and Marangoni, 2000). The nutritional composition of cucumber slices are given in Table A1 in the Appendices section.

Ascorbic acid plays a vital role in sustaining the lipid peroxidation repair mechanism of the membranes. It has been suggested that chill-induced injury is due to lipid peroxidation and that naturally occurring antioxidants may serve as protectors against this injury (Rollé and Chism, 1987). Therefore ascorbic acid dipping treatments were applied to cucumber mesocarp pieces to increase the amount of ascorbic acid content in cucumber tissue.

Three different dipping treatments were applied to cucumber mesocarp pieces before they were placed in storage. The control fruit pieces were not subjected to dipping. The effect of these treatments on the generation of lipid hydroperoxides in refrigerated cucumber tissue for the duration of six days is

illustrated in Figure 3.3. The standard curve used for the calculation of the lipid hydroperoxide levels can be found in the Appendices section (Figure A.2).

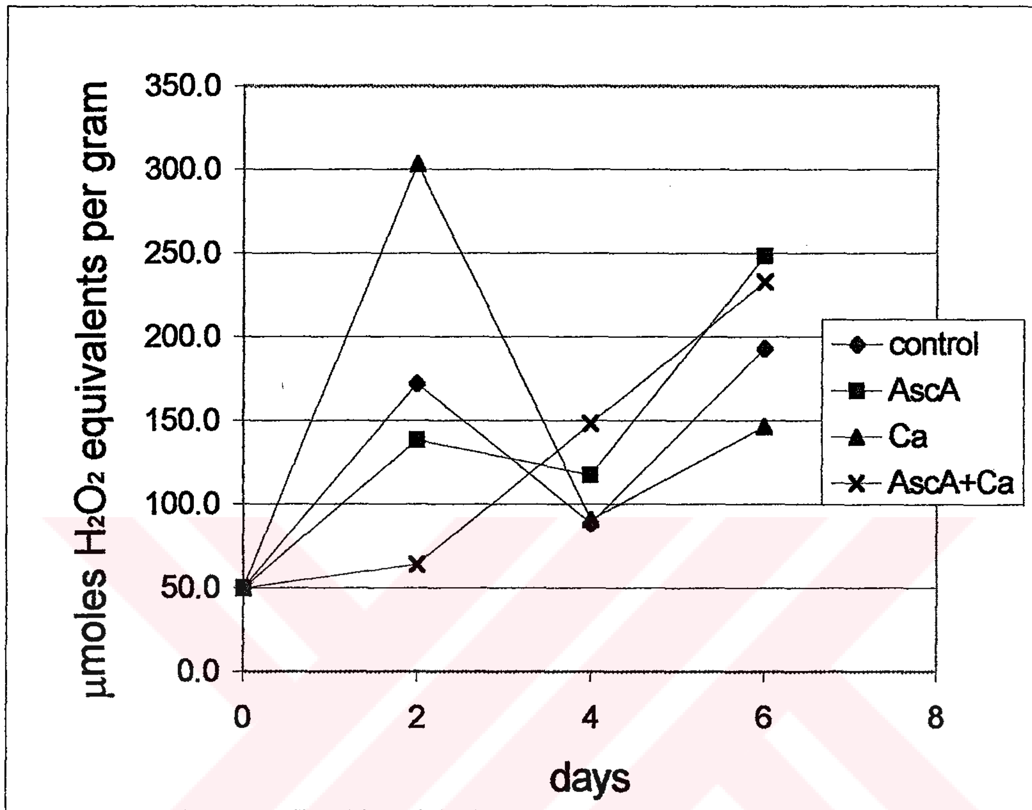


Figure 3.3 Effect of various dipping solutions on the level of lipid hydroperoxides. In cucumber tissue stored at 4 °C. AscA: solution containing 1.0 % ascorbic acid; Ca: solution containing 0.2 % $CaCl_2$; AscA+Ca; solution containing 1.0 % ascorbic acid and 0.2 % $CaCl_2$

The solution containing only $CaCl_2$ was not effective in reducing lipid peroxidation during the first two days but the levels were much lower when compared with those of other treatments in the days that followed. Ascorbic acid, on the other hand, effectively reduced lipid hydroperoxide development. Although the levels of peroxides increased for other treatments, the samples dipped in the solution containing both ascorbic acid and $CaCl_2$ did not show a sharp rise in the second day. Therefore ascorbic acid and calcium dips may be

applicable to minimally processed cucumber to reduce chilling injury. Further research may be carried out with dips composed of various concentrations. The $CaCl_2$ used in this application was a nonchelated form and possibly chelated $CaCl_2$ or calcium lactate could effect the degree of peroxide generation differently. However, it should also be noted that dipping solutions containing calcium resulted in hardening of the tissues. This effect is due to the interaction of calcium ions with the cell wall components. Calcium reacts with pectic acid to form calcium pectate. Calcium ions also impact tissue firmness by contributing to an increased membrane integrity and the consequent maintenance or increase of cell turgor pressure (Luna-Guzmàn and Barett, 2000).

3.3 Modified Atmosphere Packaging Applications for Minimally Processed Cucumber Fruit

Retail sale demands that the ready-to-use commodities be packaged, and as a consequence the atmospheric composition within the pack changes due to the respiration of living tissues. This change can be detrimental or beneficial to the overall quality of the commodity. (Varoquaux and Wiley, 1996) Equilibrium modified atmospheres (EMA) may be achieved using polymer films with permeabilities of oxygen and carbondioxide matching the respiration rate of the packaged fresh-cut produce resulting in a decreased O_2 concentration and increased CO_2 concentration established inside the package. Packaging also allows for the maintenance of a high relative humidity surrounding the products and thus prevents weight loss to a great extent.

It has been demonstrated that modified atmosphere packaging increases the chilling tolerance of cucumber fruits (Wang and Qi, 1997). This can be attributed to the retention of the fruit weight by high RH and decreasing of the respiration rate through modification of the atmosphere within the package.

In this research, the effect of modified atmosphere packaging was investigated in relation to the development of lipid hydroperoxides in cucumber

mesocarp tissue. For this purpose, model modified atmospheres were created passively within hermetically sealed jars. Direct packaging of the fruit, bruising of the fruit, storage at different temperatures and O_2 flush sealing allowed for different atmospheric conditions to develop within the packages. Package atmospheric composition, fruit respiration rates and fermentative index were determined through the analysis of the headspace gas compositions. Hardness values and drained weight were also determined to observe some of the physical changes that occurred during storage of the product.

3.3.1 Atmospheric Composition

The headspace composition of the normal atmospheric packaging treatment was the same as ambient air at the instant the jars containing processed fruit was sealed. The initial composition of oxygen flushed packages were about 70 % oxygen (v/v). At the end of 5 days the compositions changed due to the respiration of the product. Results of the headspace analyses are presented in Figures 3.4 and 3.5.

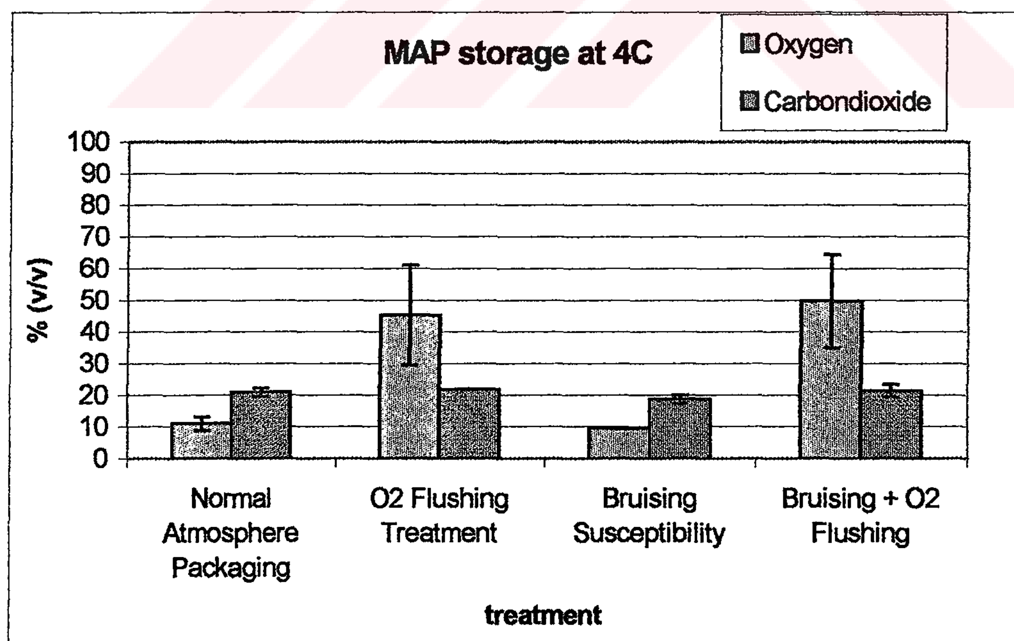


Figure 3.4 Atmospheric composition of MAP cucumber at the end of a 5 day storage period at 4 °C.

T.C. YÜKSEKÖĞRETİM KURULU DOKÜMANTASYON MERKEZİ

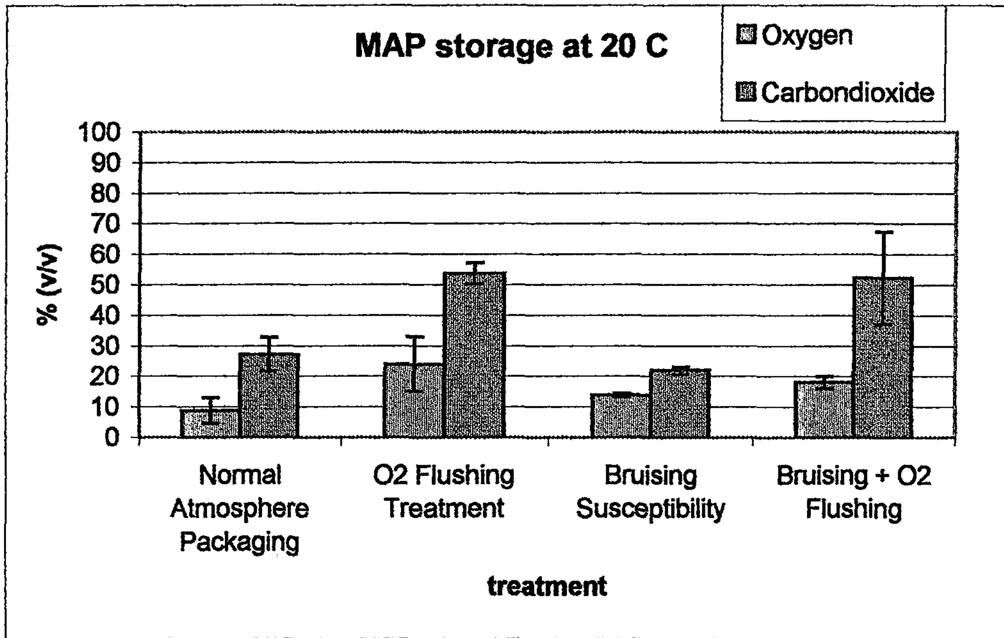


Figure 3.5 Atmospheric composition of MAP cucumber at the end of a 5 day storage period at 20 °C.

3.3.2 Respiration Rates

Cucumbers have been classified as immature fruit with low respiration with an intensity of 10-20 mg CO_2 $kg^{-1}hr^{-1}$ (43.1-86.2 ml CO_2 $kg^{-1}hr^{-1}$) at 10°C (Gordon, 1993; Collins and Marangoni, 2000). More precise respiration rates have been presented as 4.3 and 15 mg CO_2 $kg^{-1}hr^{-1}$ (18.5 and 64.7 ml CO_2 $kg^{-1}hr^{-1}$) at 5 and 20°C respectively (Watada et al, 1996). Respiration rates of fresh-cuts are generally higher than intact products. Watada et al report that slicing increased RR of cucumbers by 30 % at 5 °C and 200 % at 20 °C determined using a flow through system.

The results of respiration rate determinations are presented in Figure 3.6 and Figure 3.7. The respiration rates decreased sharply after the first six hours of packaging and were higher at 20 °C .

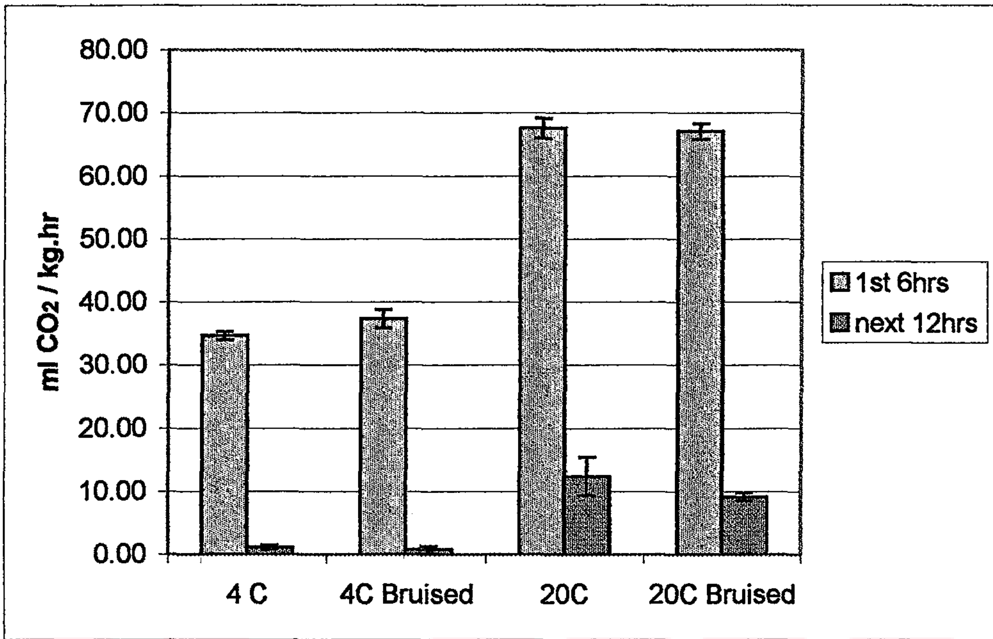


Figure 3.6 Respiration rate expressed as CO_2 production rate (*Rco_2*)

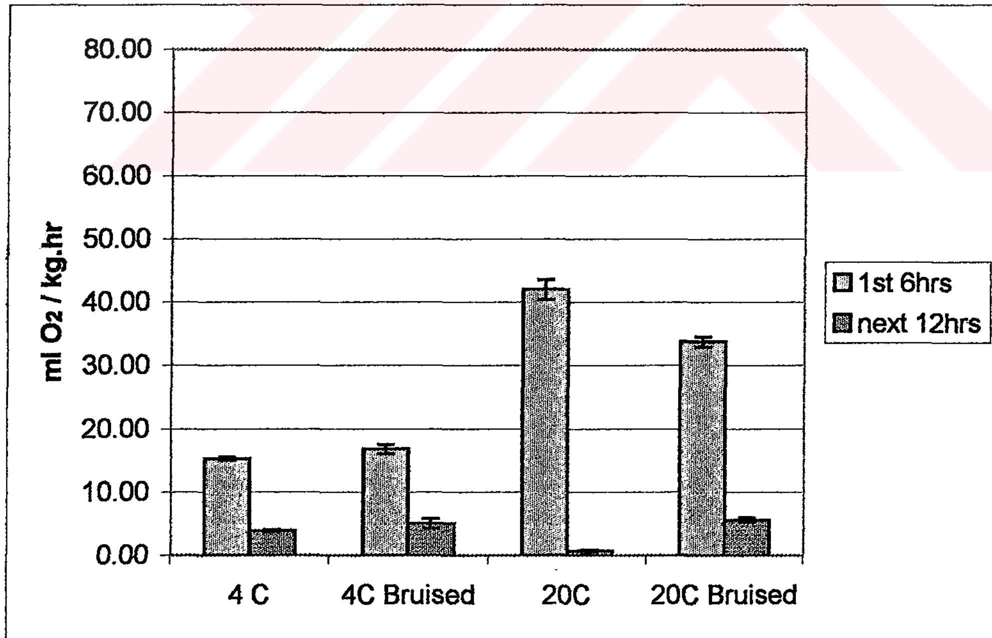


Figure 3.7 Respiration rate expressed as O_2 consumption rate (*Ro_2*)

3.3.3 Anaerobic Respiration

Anaerobic respiration within the packages has two sources. The primary source being the anaerobic respiration of the tissue cells. The second source of CO_2 development can be attributed to the proliferation of anaerobic bacteria. It is thought that the latter could be very effective at 20 °C especially for bruised samples where the tissue cells have partly lost their integrity. The CO_2 compositions along with the fermentative indexes calculated from them are given in Figure 3.8.

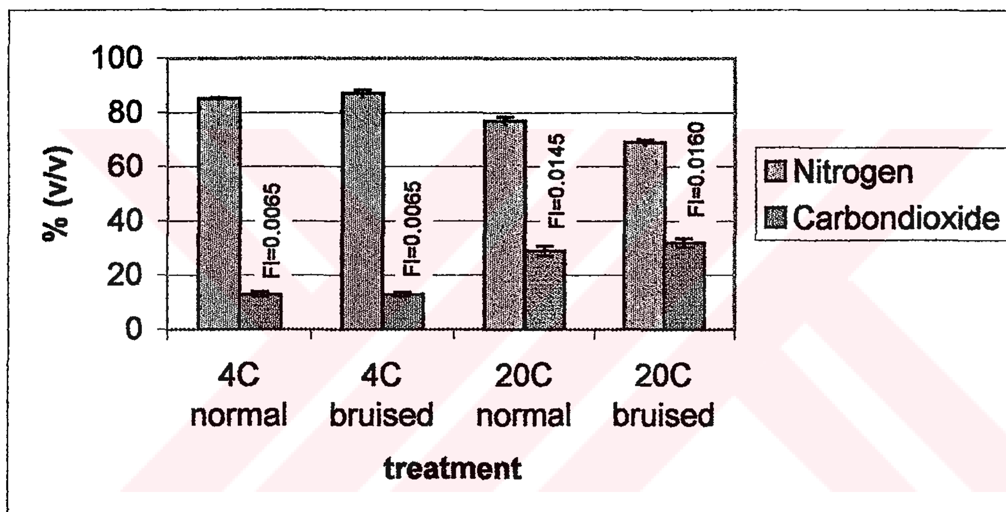


Figure 3.8 Headspace gas composition of jars on the 5th day after sealing with 99% N_2 gas. The fermentative indexes calculated for each treatment is also presented and have the units of ml CO_2 $kg^{-1}h^{-1}$.

3.3.4 Determination of Lipid Peroxidation

Figure 3.9 illustrates the detected levels of lipid hydroperoxides in MAP cucumber mesocarp tissues stored at 4 °C. The standard curve used for the calculation of the lipid hydroperoxide levels can be found in the Appendices section (Figure A.3). Lipid hydroperoxides levels show continual increase in the

samples subjected to bruising. Whereas, the samples which have not been bruised have decreasing levels of lipid hydroperoxides after the 3rd day.

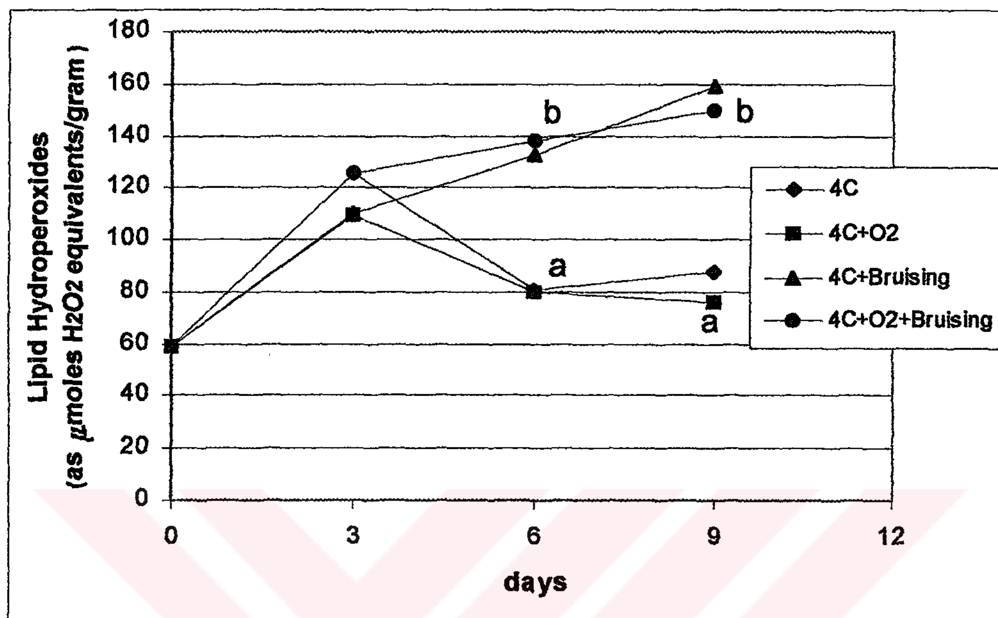


Figure 3.9 Results of the FOX assay showing the levels of lipid hydroperoxides in cucumber tissue stored at 4 °C under MAP. Means having different letters are significantly different ($p \leq 0.05$).

When the processed cucumbers were packaged and stored at 20 °C, the determinations of lipid hydroperoxides levels were within the 60-140 μmoles/gram of fresh weight (Figure 3.10). All treatments resulted in an increased levels for the first three days. It should be noted that the bruised samples were subjected to microbial decay at 20 °C and this microbial proliferation may have effected the results of the determinations.

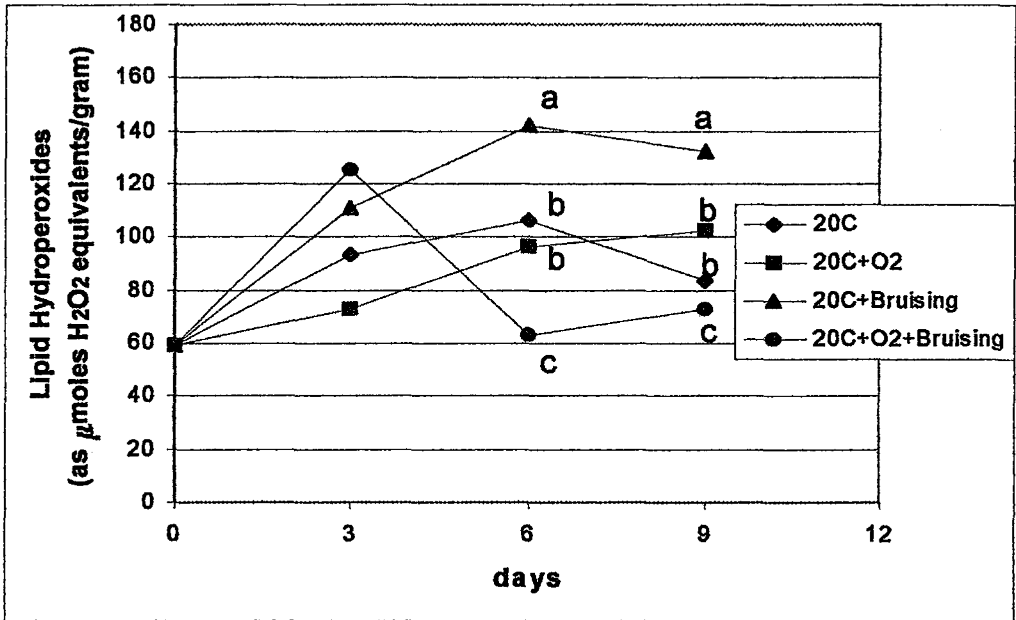


Figure 3.10 Results of the FOX assay showing the levels of lipid hydroperoxides in cucumber tissue stored at 20 °C under MAP. Means having different letters are significantly different ($p \leq 0.05$).

The results of the TBARS assays for MAP cucumber samples are presented in Figures 3.11 and 3.12. At 4°C , the levels of MDA equivalents did generally show a slight increase up to day 6 however, this increase did not follow the same pattern as the levels of lipid hydroperoxides. On day 9, the levels of TBARS for bruised samples stored at 4°C were significantly higher than those of non-bruised samples. When stored at 20°C the levels of TBARS for bruised samples immediately showed a significant increase and the levels of MDA equivalents were maintained above 600 nmoles on days 3-9.

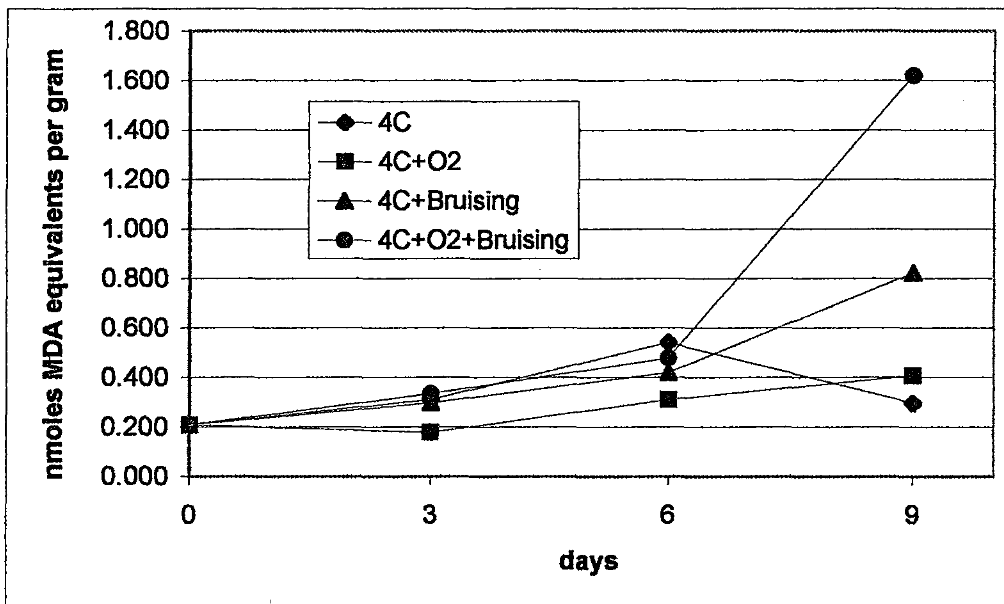


Figure 3.11 Results of the TBARS assay showing the levels of lipid hydroperoxides in cucumber tissue stored at 4 °C under MAP.

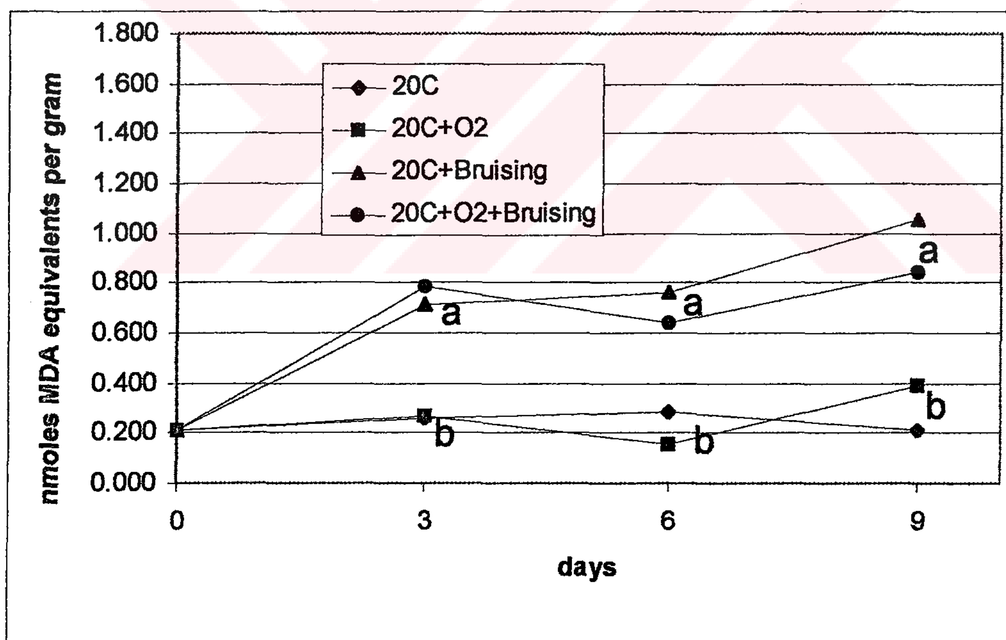


Figure 3.12 Results of the TBARS assay showing the levels of lipid hydroperoxides in cucumber tissue stored at 20 °C under MAP. Means having different letters are significantly different ($p \leq 0.05$).

water droplets formed inside the jars due to condensation of transpiration which accounted for the 1-2 % weight loss from the fruit slices. The exceptions however were the bruised samples stored at 20°C where there was considerable leakage of water and cellular contents. The amount of liquid accumulated inside the jars reached up to 11.4 % for bruised and 12.2 % for the bruised and O_2 flushing treatments. This indicated a severe disruption and deterioration of the cellular membranes.

3.3.5 Hardness Value Determinations

The initial response of cucumber to wounding is the production of a clear, sticky exudate which covers the entire wound surface, forming thick droplets in places and eventually drying to form a presumably protective layer over the wound. This exudate appears to provide a moisture barrier and facilitate rapid cork initiation. Cork formation is attributed to the deposition aldehydes associated with the formation of lignin. This lignification has been observed in up to two parenchyma cell layers. (Walter et al, 1990).

In this study, the wounding of the tissues in the course of preparation of the product for storage caused clearly visible exudate formation. Upon storage, nonbruised tissue became lighter in color, and obtained a dry corky texture. The hardness values of the fruit are presented in Figure 3.13. All samples showed significant increases in hardness values during the first few days of storage. Samples stored at 4 °C softened following the 6th day of storage.

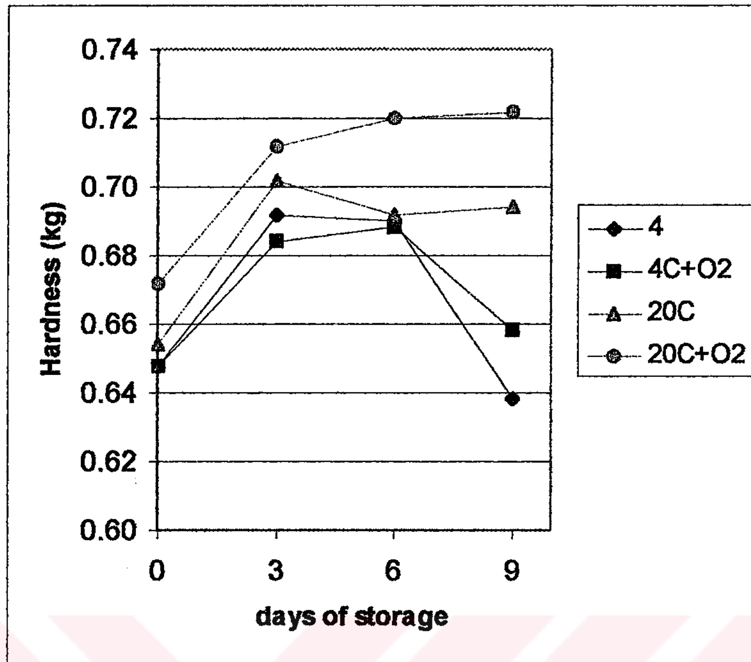


Figure 3.13 Hardness values of non-bruised cucumber slices stored at 4 and 20 °C under MAP. Data points represent the mean of 5 samples.

CHAPTER 4

CONCLUSIONS AND RECOMMENDATIONS

The initial phase of the experiments performed using cucumber mesocarp tissue stored in petri plates revealed that the levels of lipid hydroperoxides and MDA (secondary end products of lipid peroxidation) increased as the days of storage progressed. Lipid peroxidation determined as such was significantly greater in chilled tissue (4 °C) than non-chilled tissue stored at 20 °C. These results indicate that chilling causes an almost immediate response of the tissue and decreases its ability to suppress or alleviate lipid peroxidation. Even though bruising of the tissues disrupts cell structure and causes the release of enzymes which take part in the oxidation mechanisms, it was not the determining factor of lipid peroxidation under non-modified atmosphere storage. The concentration of lipid hydroperoxides in fresh cucumbers averaged at about 48 μmoles per gram fresh weight and after ten days, increased by more than 2-fold at 20 °C and by 5-fold at 4 °C. The TBARS assay confirmed the increase in peroxidation and the values for treatments without bruising correlated significantly with the corresponding results of the FOX2 assay.

Dipping treatments had varying effects on the progress of lipid hydroperoxides formation. Further analyses are recommended to confirm that a dipping solution of a certain composition is effective in decreasing the accumulation of peroxidation products in chilled tissues.

The model modified atmosphere packaging applied in the experiments resulted in different headspace compositions depending on the respiration rates

and the initial headspace compositions of the jars. The respiration rates, increased by the minimal processing of the cucumbers, decreased as the modified atmospheres were established within the packages.

Under refrigeration at 4 °C and MAP, lipid hydroperoxides increased from initial values of about 59 µmoles per gram fresh weight to above 110 µmoles within the first three days. In the days that followed, the levels continued to increase for bruised samples whereas lipid hydroperoxides in non-bruised fruit decreased to levels below 90 µmoles per gram fresh weight. The initial level of TBARS in cucumber mesocarp tissue was about 0.2 nmoles MDA equivalents per gram. After 9 days at 4 °C and MAP, bruised fruit maintained TBARS levels below 0.5 nmoles MDA equivalents per gram. Therefore we can conclude that MAP decreases the lipid peroxidation in chilled cucumber tissue. By controlling lipid peroxidation it is possible to extend shelflife and keeping quality of chilling sensitive produce. This is especially significant in the case of minimally processed fruit where chilled and refrigerated conditions are essential during processing, transportation, storage and display for sale.

The hardness of minimally processed tissues increased by 5.6-7.3 % due to lignification of the tissues subsequent to the peeling and slicing operations. The cucumber slices retained this hardness, except for the fruit stored at 4 °C. These, softened to their original hardness values after the 6th day of storage. This symptom of softening of the tissues can be attributed to chilling injury.

The FOX assay is a relatively new method for the measurement of lipid hydroperoxides. The assay is relatively simple, requires inexpensive chemicals, produces results rapidly, is not sensitive to ambient O_2 or light levels, and does not require heating or special reaction conditions, and the lipid components do not need to be separated from the rest of the cellular homogenate. Several methodological conditions in the carrying out of the FOX assay were experimented with during the initial phases of this study. The method of reagent preparation by Nourooz-Zadeh (1999) has been found to be superior to the

method described by DeLong et al. (2002). Also, the use of extra pure, analytical grade ethanol and HPLC grade methanol is advisable. It has been confirmed that using lesser quality alcohol (95 %) produces a measurable signal when tested against control reactions using HPLC grade methanol. It is possibly due to this modification that the calibration curves used in the study were linear beyond the 30-40 µmolar H_2O_2 range, whereas in other studies curvilinearity of the standard curve at about 30 µmolar H_2O_2 has been reported (Delong et al., 2002).

Current technological approaches to minimising storage disorders in fresh fruits and vegetables tend to focus on manipulation of storage conditions because there is no clear understanding of the biochemical control of senescence, chilling injury, and other associated disorders. The study of lipid peroxidation provides a potential linkage of these disorders and means of explaining interrelatedness (Shewfelt, 2000). The incorporation of membrane isolation techniques and the quantification of the levels of lipids, possibly by chromatographic techniques, into the methodology of future studies are highly recommended.

Preparation of minimally processed cucumbers in a salad mix containing other processed produce would make it more convenient for the consumers. A typical salad designed for consumption in the Turkish market may contain, along with cucumbers, diced tomatoes and sliced onions. Future studies could focus on the effect of packaging different produce as a mix. Contained in the same package, products will interact due to their respiration rates and production of hormones.

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APPENDICES

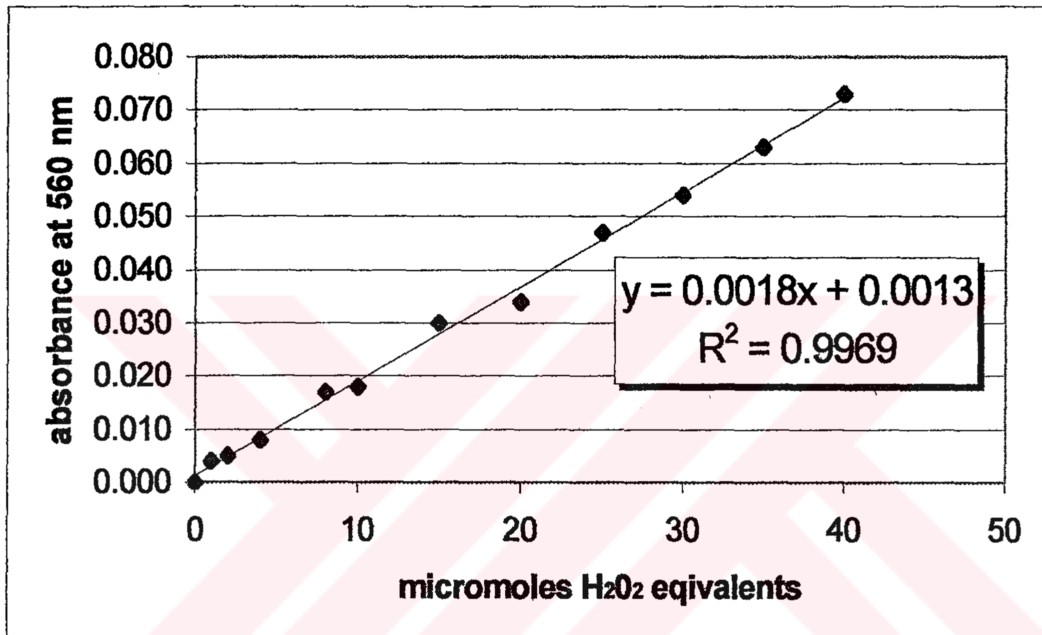

Figure A1. Calibration curve for the FOX assay for determining the lipid hydroperoxides content of bruised and non-bruised cucumber tissue stored at 4 and 20 °C.

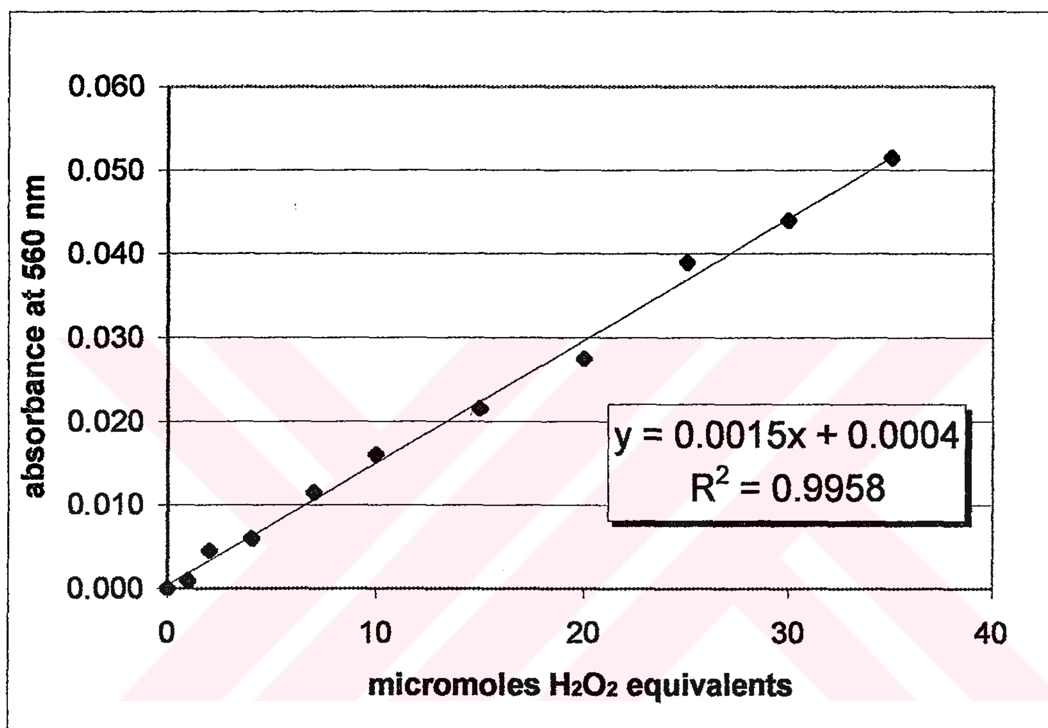

Figure A2. Calibration curve for the FOX assay used in the determination of lipid hydroperoxides content of cucumber tissue stored at 4 °C, treated with various dipping solutions.

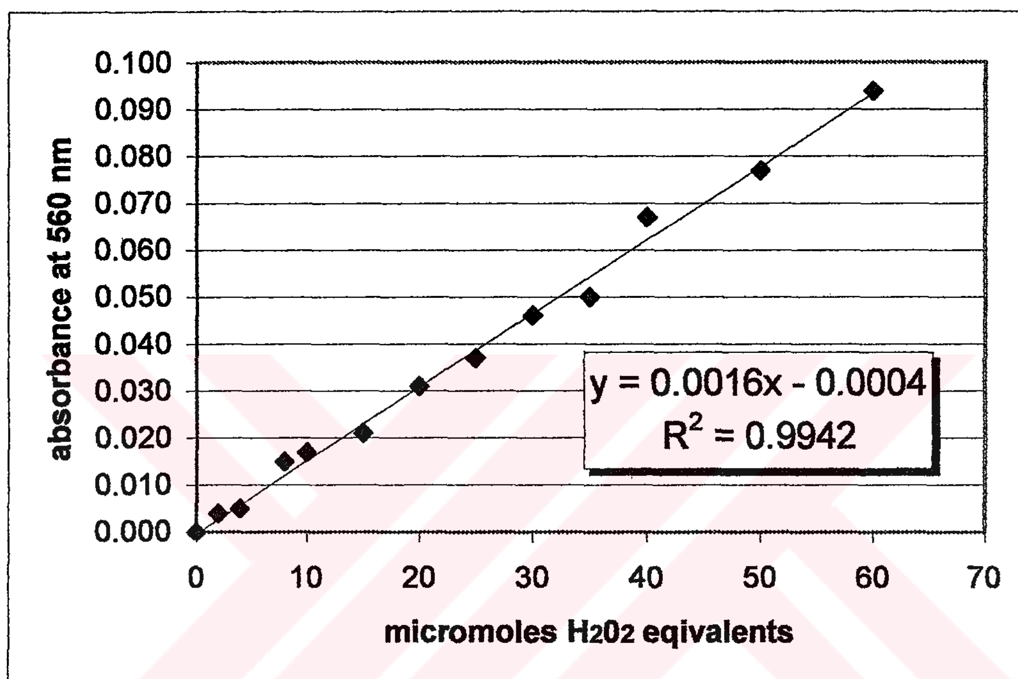

Figure A3. Calibration curve for the FOX assay used in the determination of lipid hydroperoxides content of cucumber tissue stored at 4 and 20 °C, under MAP.

Table A1. Nutritional composition of peeled sliced cucumber. (USDA Nutrient Database for standard reference. Release 12. March 1998)

Nutrient	Units	1 cup, sliced (119.000 g)
Proximates		
Water	g	114.823
Energy	kcal	14.280
Energy	kj	59.500
Protein	g	0.678
Total lipid (fat)	g	0.190
Carbohydrate, by difference	g	2.975
Fiber, total dietary	g	0.833
Ash	g	0.333
Minerals		
Calcium, Ca	mg	16.660
Iron, Fe	mg	0.190
Magnesium, Mg	mg	14.280
Phosphorus, P	mg	24.990
Potassium, K	mg	176.120
Sodium, Na	mg	2.380
Zinc, Zn	mg	0.167
Copper, Cu	mg	0.038
Manganese, Mn	mg	0.101
Vitamins		
Vitamin C, ascorbic acid	mg	3.332
Thiamin	mg	0.025
Riboflavin	mg	0.013
Niacin	mg	0.124
Pantothenic acid	mg	0.339
Vitamin B-6	mg	0.086
Folate	mcg	16.660
Vitamin B-12	mcg	0.000
Vitamin A, IU	IU	88.060
Vitamin A, RE	mcg_RE	8.330
Vitamin E	mg_ATE	0.094
Lipids		
Fatty acids, saturated	g	0.050
14:0	g	0.001
16:0	g	0.040
18:0	g	0.005
Fatty acids, monounsaturated	g	0.005
16:1	g	0.000
18:1	g	0.005
20:1	g	0.000
22:1	g	0.000
Fatty acids, polyunsaturated	g	0.077
18:2	g	0.032
18:3	g	0.044