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POST-HARVEST AS A TOOL TO IMPROVE BERRY FRUIT QUALITY

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Greta Peretto

January 31st, 2014

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ABSTRACT

The purpose of this thesis was to research some innovative post-harvest techniques with potential to extend the shelf-life of berry fruit during storage and enhance their overall quality. Emphasis was given to different packaging system, in particular microperforated film, edible film, and electrostatic spraying of antimicrobial coating. The berry fruits studied were blueberry and strawberry. These fruits were selected because of their increasing worldwide consumption, among the many different types of berries.

The application of microperforated polypropylene film to store freshly harvested blueberries, demonstrated the importance of selecting a proper packaging to increase the storage period and maintain the quality attributes of the fruit. The number of microperforations made to the film (1, 10, and 30) created a passive modified atmosphere within the package that led to improvement of firmness and antioxidant activity of the fruit, throughout their storage period. Equilibrium atmosphere within the packaging was achieved after two days of storage and the concentration of gases remained constant for the rest of the storage period. This stable atmospheric condition resulted in an extension of the shelf-life of blueberry for up to 16 days, at 4°C.

Strawberry shelf-life extension and quality improvements were obtained by storing the fruit in clamshells that contained strawberry puree edible films infused with carvacrol and methyl cinnamate. The strawberry puree edible films served as matrix for the controlled release of natural antimicrobial's (Carvacrol and methyl cinnamate) vapors, over time. Fresh strawberries packed in clamshells had an extended shelf life of 10 days, at 10°C and 90% RH. The released vapors from the strawberry puree edible film extended the strawberry shelf-life by delaying spoilage of the fruit and improved

main quality-related attributes, such as firmness and brightness. The natural antimicrobial vapors also increased the total soluble phenolic content and antioxidant activity of the stored strawberry.

Besides microbial spoilage, that limits strawberry shelf-life, the presence of foodborne pathogens bacteria that can be carried by the fruit as result of contamination, is another important issue that can lead to serious outbreaks. Antimicrobial edible coatings can be an effective post-harvest technique to assure microbial safety and, at the same time, retain overall quality of the fruit. Antimicrobial alginate coating was developed and optimized using response surface methodology. Antimicrobial activity against *Escherichia coli* O157:H7 and *Botrytis cinerea*, as well as physical properties such as viscosity, turbidity and whitish index of the coating were also optimized based on carvacrol and methyl cinnamate concentration.

After the optimization, the resulted antimicrobial coating solution was applied on the surface of freshly harvested strawberry fruit using an electrostatic spraying technology. This technology presented unique advantage with regard to transfer efficiency and evenness application of the antimicrobial coating solution. The antimicrobial coating application led to a significant increase on strawberry shelf-life. Additionally, the strawberry coated using the electrostatic spraying technology presented a significant reduction of visible decay of four days compared to not-coated fruit, and of one day compared to fruit coated with conventional spray method. Moreover, the firmness and color of the strawberries were improved by coating the fruit using the electrostatic spraying technology. Therefore, electrostatic spraying could be considered a potential technology for the commercial application of liquid coating to

extend the shelf-life and improve the post-harvest quality of strawberry and other perishable fruits.

*I dedicate this thesis to my family and José
for their constant help and love.*

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PUBLICATIONS

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1 LITERATURE REVIEW

Berries are botanically recognized as a heterogeneous group of small fleshy fruits produced from a single ovary. Based on scientific definition, many fruit commonly known as berries are not actual berries. For instance, blackberries and raspberries are considered false berries, or epigynous berries, as they are aggregate fruit composed of small drupelets with a fleshy mesocarp containing seeds from different ovaries of a single flower. Blueberry is also considered a false berry because it is derived from an inferior ovary rather than a superior ovary. Strawberries, on the other hand, are accessory fruits where the flesh is developed from the receptacle and the achenes are the actual fruit.

Among all berry crops grown worldwide, strawberries (*Fragaria x ananassa*) and blueberries (*Vaccinium*) are the two major crops of greatest economical importance in terms of volume and value. These berries had a world production that exceeded 4.5 million tons in 2011, with an increasing production of 6.6% since 2007 (FAO, 2013).

The increasing berry demand and consumption are due foremost to the latest healthy trend, which is driving the consumer attention. Berries are rich sources of minerals, vitamins, and phytochemicals. All these compounds relate to consumer health as they are associated to antioxidant properties that determine their health-promoting activity. Due to the presence of these compounds in berries, they are defined as natural functional foods which have great potential impact on health beyond basic nutrition that may reduce the risk of diseases or promote optimal health (Kaur and Kapoor, 2001). In addition to the indicated health promoting compounds, berries also contain other compounds such as carbohydrates, organic acids, pigments, and volatile compounds that

relate to their organoleptic and sensory properties, which contribute directly to the overall quality of the fruit.

However, all these health promoting and sensory desirable compounds may undergo significant changes during the post-harvest storage of berries that may significantly reduce their concentration and value over time. Berries are among the most perishable fruits, due to high physiological post-harvest activities that compromise their sensory and health-promoting characteristics. The fresh berries market has changed in the past few years, shifting from a small direct market to a wholesale market of fresh berries available throughout the year and they are required to meet high quality standards. Therefore, post-harvest technologies and techniques have gained a central role for extending the shelf life of berries while maintaining their quality and preserving their health promoting compounds.

Reduction of berries physiological deterioration and fungal decay is commonly achieved by rapid cooling after harvest and keeping temperatures to values close to zero during post harvest storage. Low temperature storage slow down the respiration rate of berries and retard their ripening process, which limits negative textural and color changes, and the loss of flavor. Nevertheless, this traditional preservation storage method has been limited to extend the shelf life of berries, as well as to maintain their healthy and sensory attributes. Therefore, the industry has been looking for new potential post-harvest technologies that can provide not only shelf life extension but also improve the visual and sensory quality of berries and preserve the healthy components that characterized these fruits.

In the present research work three new technologies were investigated for having the potential to improve the overall quality of berries. These novel technologies

included the use of microperforated polypropylene film, edible films and coating enriched with natural antimicrobial compounds, and a state-of-the-art electrostatic spraying technology for coating application. The effectiveness of such technologies was tested using blueberry and strawberry fruits as model system, because of the continue increasing production and economic importance of these berry fruits.

1.1 QUALITY OF BERRY FRUIT

Quality of berry fruit is a complex set of factors that can be ascribed to different intrinsic characteristics and components of the fruit that are directly measurable. The consumer perceives these factors as quality attributes in terms of appearance, flavor, aroma, texture, as well as nutritional and healthy aspects. This perception of quality by the consumer, who is considered the driving economical force that mainly influence the market, is the only relevant aspect that determines purchasing behavior based on the physical, nutritional and healthy aspects of the fruit.

1.1.1 Chemical composition and sensory quality of berry fruit

The quality of berries depends on their flavor (determined by amounts of sugars, organic acids and specific aroma compounds), texture, and appearance, since they have a high impact on consumer preferences and expectations. The chemical composition of berries relates to the sensory properties of the fruit. However, the

identification of the chemical factors affecting the sensory quality of the fruit is difficult to achieve. Since the large number of chemical substances (carbohydrate, proteins, minerals, organic acids, among others), which determine the quality of berries, can be contained in different concentrations and react with each other in different ways (Starast et al., 2007), providing different flavor and appearance to the berries.

Flavor is one of the main characteristic related to sensory quality of ripe berries that is generated by the combined effect of concentration and ratio among soluble sugars and organic acids present in the fruit, which are responsible for the typical sweet taste of berries. The sugars occurring on ripe berries are generally an equal mix of glucose and fructose. Whereas sucrose content can vary based on the ripening stage and post-harvest storage conditions, sucrose generally dominates in early development stage of berries and it is converted to invert sugars (glucose and fructose) as maturation progresses by hydrolysis from invertase (Sturm et al., 2003). The proportions of fructose, glucose, and sucrose are important in the perception of fruit quality as fructose is 1.8 times sweeter than sucrose, whereas glucose presents only 60% the sweetness of sucrose (Wang et al., 2008a). For strawberry these three sugars account for more than 99% of the total sugars in the ripe fruit, with sorbitol, xylitol, and xylose occurring in traces amounts (Montero et al., 1996). Sugar content in fruits is commonly associated with measurement of their soluble solids content (SSC). Therefore, the determination of SSC is considered an indicator of berry sweetness. However, the quantification of individual sugars requires HPLC analysis.

As previously mentioned, the sweet taste of berries is not only given by the high sugars content, but rather by the ratio of sugars to organic acids. The sugar/acid ratio, which is defined as the total sugar content compared to the total acid level, has a

major effect on taste, fruit ripeness, and represents an index of consumer acceptability. Citric and malic acids are the major organic acids in berry fruits, where the proportion of citric acid can range from 30% to 95% of the total organic acids. Citric acid is widely used also in the pharmaceutical industry as antioxidant, preservative and acidulant, and because of these properties it may contribute to maintain quality and nutritive value of berries. Small amount of tartaric acid is also reported to be present in several fruits of the Ericaceae family, except in the highbush blueberry that is characterized by a sweeter taste compare to other species of the same family, probably due to the absence of this organic acid. In addition to tartaric acid, glycolic and shikimic acids were also determined in strawberry but in lower quantities. Organic acids can be measured by titration. However, since titratable acidity (TA) is not an accurate measure of total acidity (defined as the total sum of acids present as free acids or combined with cations), direct measurement of individual organic acids by HPLC is used to obtain an accurately value of total acidity (Ulrich, 1970). Additionally, soluble solids content/ titratable acidity (SSC/TA) relate better to sourness and acidity than TA itself. Therefore, SSC/TA is generally used as an important tool to assess consumer acceptability and quality of berry fruit. For example Wozniak et al. (1997) and Pelayo-Zaldivar et al. (2005) showed a good correlation between the overall sensory quality of some strawberry cultivars and the SSC/TA in the fruit, during fruit ripening. Moreover, they reported a higher consumer preference for strawberries with higher sugar and volatile contents. Sugar/organic acid ratio may also be used as an index for the commercial selection of berries as low SSC/TA values indicate sour taste; therefore those berries would be better suitable for processing them into products like juice, jam, and wine, rather than use them for fresh consumption.

In addition to the sugar to acid ratio, other constituents as volatile compounds and polyphenols have also a great effect on berries flavor. The term flavor indicates the combination of taste and odor; therefore, it may not be an objective qualitative trait even though the presence of specific volatile compounds can be representative of typical berries flavor.

In addition to taste and flavor, the quality of berry fruit has been traditionally based on characteristics of external appearance, such as absence of defects, size, as well as color and texture, which are critical parameters influencing not only consumer acceptance of the berries, but also their commercial sale.

Texture encompasses a wide range of properties such as hardness, cohesiveness, and juiciness that tend to change rapidly after harvest, and is a main factor for determining the acceptability of the fruit. However, to the consumer, firmness and juiciness are the textural factors that most influence the perception of acceptability of the fruit and these parameters are strictly related to the physical anatomy of the tissue, chemical composition of cell wall components, as well as the turgid status of the cells. For example, a fruit tissue that presents small cells tend to have a greater content of cell walls with low amount of intercellular air spaces, which make the tissue firmer and less juicy (Toivonen and Brummell, 2008). Pectin, cellulose, hemicelluloses, glycoproteins, and in smaller amount esterified polyphenols, are the primary cell wall components that affect berries texture and quality as they are prone to modification during fruit ripening and post-harvest storage. Pectins, which are found mainly in the primary cell wall, where they provide rigidity and strength, represent also a rich part of the constituents of the middle lamella that serves as prime determinant of intercellular adhesion by holding cells together. These cell wall polysaccharides, due to enzymatic degradation, undergo

depolymerization during berries development, ripening, and post-harvest storage that increase water soluble components, which result in the textural softening of berries.

Appearance, in addition to texture, is an important quality attribute that is closely related to tissue components of berry fruits and is commonly used as a measure of freshness and quality of the fruits. Berries are worldwide appreciated also for their typical desirable intense colors that range from red and blue to purple, that attracts consumer's attention. The colors in berry fruits are mainly due to the presence of natural water soluble pigments called anthocyanins. These compounds belong to the highly distributed class of phenolic molecules known as flavonoids, and their presence and concentration in berries may vary based on different factors such as genetic, environmental factors, and/or storage conditions. There are chemical differences between anthocyanins that, even if they share a common basic chemical structure (C6–C3–C6), are characterized by the different number of hydroxyl groups in molecule, as well as the different types of sugars (glucose, galactose, arabinose, xylose, and rhamnose) and acids attached to the molecule (Kong et al., 2003). Indeed, the anthocyanins pigments consist of two or, sometimes, three portions represented by the aglycone base (anthocyanidin), sugars, and acylating groups. Although 19 different anthocyanidins have been detected, only 6 are commonly found in berries: cyanidin, pelargonidin, peonidin, delphinidin, petunidin, and malvidin. Furthermore, since anthocyanins are unstable molecules, they occur mainly in their glycosylated forms which made them more stable and soluble molecules, because of the attached sugars (Giusti and Wrolstad, 2003). The anthocyanin composition differs among berry species, but they are quite similar within the same cultivar. For this reason, anthocyanin profiles have been regarded as fingerprints for specific berries (Giusti and Jing, 2007). For

instance, in some varieties of strawberry, pelargonidin-3-glucoside was found to be the major anthocyanin that induced a more intense pigmentation in the fruit (Cerezo et al., 2010). Anthocyanins are the main compounds responsible for the colors of berries, however other compounds such as carotenoids, flavonoids, chlorophylls, and betalains are also present in smaller amount.

However, even if color is the basis for sorting berries into commercial grades since it directly relates to consumer perception, many other factors can influence berries appearance, including wound-related effects and molds contamination that contribute to provide an unattractive, low quality fruit and fruit products.

1.1.2 Phytochemical compounds and their health promoting properties

Berry fruit have additional health benefits, besides those imparted by the presence of micronutrients, as they are very rich source of bioactive compounds known as phytochemicals, which refer to those natural food components that provide benefits beyond the prevention of dietary deficiencies (Goldman et al., 1999). Phytochemicals in berries are mainly represented by polyphenols such as flavonoids (anthocyanins, flavanols, flavonols), phenolic acids, and tannins (Szajde and Borowska, 2008) that exhibit a wide range of biological effects including antioxidant activity. These compounds are believed to perform health-promoting properties, because of their ability to trap free radicals and reactive oxygen species (ROS) (Wang and Jiao, 2000) that may oxidize different biological system such as proteins, lipids or even DNA that lead to degenerative diseases. Free radicals are reactive molecules arising normally during

metabolism as byproduct that, having odd number of electrons, react with other compounds in order to capture electrons needed to gain stability. In this way, the nearest molecule that has been attacked loses its stability and become a free radical itself, beginning a chain reaction that lead to serious cellular damages. Antioxidant compounds are able to stabilize free radicals action by donating electrons, without becoming free radicals themselves, since they are stable in either form.

Antioxidation is, however, only one of the many mechanisms through which polyphenols can exert their action. Indeed, polyphenols have been reported to demonstrate antibacterial, antiviral, antimutagenic, anticarcinogenic, anti-inflammatory, antiproliferative, and vasodilatory actions (Seeram et al., 2003).

Thus, considering the high amount of phytochemicals in berries, their consumption can be inversely related to the incidence of dietary lifestyle induced diseases including cardiovascular disorders, neurodegenerative diseases, diabetes, and cancer (Kaur and Kapoor, 2001).

The concentration of antioxidants in fruits reported in various studies depend on many factors including genetic and environmental differences, as well as maturity stage of the fruit, harvesting time, storage time and conditions (Prior et al. 1998). Moreover, different analytical methods for their quantification may also provide different results that make comparison difficult. A common method for phenolic identification is based on the use of Folin Ciocalteu reagent, with the total amount of phenolics expressed as gallic acid equivalents; while for the evaluation of antioxidant activity, various methods are used. The most common methods reported in the literature for the evaluating the antioxidant activity in plant tissues are based on the measurement of the radical scavenging activity of the antioxidants against free radicals like the 1,1-

diphenyl-2-picrylhydrazyl (DPPH method). Other assays are the ferric reducing ability of plasma (FRAP), the Trolox equivalent antioxidant capacity method (TAEC), and the oxygen radical absorbance capacity (ORAC).

Based in the results of different studies, the levels of total phenolics in blueberry and strawberry were in the range of 106 to 495 mg/100g and 43 to 273 mg/100g of fresh weight, respectively (Howard and Hager, 2007).

Among berries antioxidants, anthocyanins are the major phenolic subgroup and therefore their intake results to be much higher than other antioxidant compounds. For this reason, anthocyanins have received special attention from the scientific community, especially in those berries that present a particularly high amount of these antioxidants as blueberry. In blueberry, 25 different anthocyanins have been identified, showing an overall content that range from 25 to 435 mg/100 g (Wu and Prior, 2005). The anthocyanins commonly detected in blueberry are the monoglycoside: glucosides, galactosides, and arabinosides) of delphinidin, cyanidin, petunidin, peonidin, and malvidin. These anthocyanins are usually present in the skin and the tissue directly beneath it; and some species, they are present in little amount also in the flesh. However, their composition and content can vary among different species because of genetic factors.

Numerous studies reported that the wide range of biological properties that characterize berry fruits result from the synergistic effect of multiple phytochemicals, rather than a single compound. Indeed, although anthocyanins are the major constituents, berries contain other flavonoids as well as phenolic acids that contribute to the to the overall antioxidant activity.

Flavonoids, belong to a large and heterogeneous group of biologically active compounds that include quercetin, myricetin, and kaempferol, which are the most common flavonols in berries. They possess the same C₁₅ (C₆-C₃-C₆) flavone nucleus, two benzene rings, a hydroxyl group at C₃, and a ketone group but they differ in number and position of hydroxyl groups (Macheix et al., 1990). Numerous *in vitro* studies have indicated that these compounds have great potential to reduce the risk to certain diseases, especially quercetin that has been proved to inhibit cyclooxygenase and lipoxygenase activities, two enzymes involved in the release of arachidonic acid that is the initiator of a general inflammatory response (Beattie et al., 2005). Among most common berries, blueberry and strawberry are particularly rich in flavonols which are quantified as the aglycone after acid or enzyme hydrolysis to remove sugar residues. Using this approach, the content of flavonols in strawberry were higher than 200 mg/100g (fresh weight) and they were located in the achenes, where they were four-fold higher than in the flesh (Aaby et al., 2005). Whereas, fourteen different flavonols in blueberry were found predominantly in the skin, with small amounts found in the seeds and none detected in the flesh. The accumulation of phenolic compounds is commonly greater in the external tissues of fruit than in the internal tissue since their formation depends on light, with the only exception for anthocyanins that may be present throughout the fruit (Mojer et al., 2002).

Another group of compounds generally found in berries that are implicated as active antioxidant is represented by the phenolic acids. The predominant phenolic acids identified are hydroxylated derivatives of benzoic and cinnamic acid that are commonly found in conjugated forms as esters and glycosides, rather than free acids. In blueberry, glycosides and esters account for 56.7% and 40.7%, respectively, of total phenolic

acids, while free acids account for only 2.6%. Among hydroxycinnamic acid ester, chlorogenic acid is the most abundant detected in blueberry; whereas in strawberry, the major compound is the glucose ester of *p*-coumaric, which is uniformly distributed throughout the skin and flesh (Gil et al., 1997, Taruscio et al., 2004).

Ellagic acid is the dominant acid in strawberry where it accounts for 51% of all phenolic acids (Skupi n and Oszmiański, 2004). Although it is considered a hydroxybenzoic acid, most of the ellagic acid in berries is present in forms known as ellagitannins, which constitute a separate class of phenolics (hydrolysable tannins). According to Skupi n and Oszmianski (2004), ellagic acid in strawberry is present as an ellagitannin esterified with glucose and its total content, determined after acid hydrolysis, range from 25.01 to 56.35 mg/100 g (fresh weight). These reported values are approximately three times more than those determined in other fruits. Ellagitannins and ellagic acid derivatives have not been identified in blueberries that apparently lack the genetic capacity to synthesize such compounds. As for the other polyphenols, ellagic acid has been indicated to have beneficial effects on health as antioxidants and anticarcinogens (H kkinen and T rr nen, 2000). Resveratrol is another naturally-occurring polyphenolic compound touted for its antioxidant, anti-cancer, anti-aging, anti-inflammatory and cardioprotective potential. Berries of the *Vaccinum* species, including blueberries, bilberries and cranberries, contain resveratrol, but to a lesser extent than grapes. Resveratrol is the most abundant naturally occurring of the polyphenols that belongs to the stilbenes class and its biological and pharmacological activities are thought to be due to its strong antioxidant property, which has been shown in a number of studies (Rimando et al., 2004).

Considering the wide range of antioxidant compounds, it is interesting to observe how they contribute to the total antioxidant activity in a different extent, based on their chemical structure, in particular on the number of hydroxyl groups in their molecule. Based on this, it has been demonstrated that antioxidant activity is generally more strongly correlated to the total content of polyphenols, especially flavonoids (Wang and Linn, 2000).

Other naturally occurring compounds such as vitamins have been received lot of interest because of their biological activities that promote or contribute to health. Many types of berries contain high level of vitamin C (ascorbic acid) especially strawberry that contain in average 60 mg/100mg (fresh weight) of vitamin C; therefore, strawberry is considered one of the richest sources of ascorbic acid in fruits (Cordenunsi et al., 2005). Ascorbic acid acts mainly as antioxidant as well as cofactor in hydroxylation reactions, which are required for collagen synthesis. Moreover, ascorbic acid has a role in hormone synthesis, the immune system, iron absorption, platelet aggregation, thrombus formation and may have a role in preventing heart disease, osteoporosis and a range of cancers (Lee and Kader, 2000). The stability of ascorbic acid is known to be influenced by numerous factors, including temperature, light exposure, atmosphere, fruit damage, food processing, and ascorbic acid oxidase. Berries provide good concentrations of ascorbic acid and other vitamins as part of an overall balanced diet.

1.2 QUALITY OF BERRIES DURING POST-HARVEST

Berries are well known for their nutritional, health promoting and sensory properties impart by their chemical composition and phytochemicals content. However, these characteristics diminish over time during post-harvest. They are highly perishable fruit with a very short shelf life, susceptible to mechanical damage, physiological deterioration, water loss and decay that lead to a fast depletion of nutrients and sensory properties, limiting therefore their marketing. These losses are mainly caused by the high respiration rate that provides the compounds that determine the rate of metabolic process directly related to quality parameters such as firmness, sugar content, and flavor. However, losses can be reduced by adequate storage methods capable of delaying respiration rate and other metabolic reaction associated with quality retention in order to prolong shelf-life, preserve high quality and retain the health benefits of the fruit. New approaches and technique are continuously developed.

1.2.1 Quality modification during post-harvest

Berry fruits are characterized by a high respiration rate that limits their shelf-life and accelerate senescence after harvest. Respiration is a metabolic process by which stored organic materials (generally sugars) are broke down into simple end products releasing energy needed for the cellular biochemical process of the fruit. O₂ in the air is used in this process and intermediate compounds, CO₂ and water are produced (Mathooko, 1996). Respiration process leads to decrease of fruit value and loss of flavor

because of reduction of the carbohydrates amount used as substrate for the process. . In general, storage life of perishable foods such as berries are inversely related with their rate of respiration, therefore its reduction is one of the main goal of post-harvest technologies in order to extend shelf life of the product while maintaining optimum quality when they reach the consumer. Fruits are classified according to their respiration rate and berries are considered fruit with high respiration rate that ranges from 10 – 20 mg/kg/hr for blueberry to 20 – 40 mg/kg/hr for strawberry at 4 °C, which reflects 10 and 5 days of storage life, respectively. Moreover, considering that berries have a fragile morphology due to soft texture and lack of protective rid (Atress et al., 2010), they can easily be subjected to mechanical injury during harvest, handling, storage and market, causing an increase of respiration rate as a general response to stress caused by disruption of cellular structure. Because of this fact, senescence process of the fruit accelerates and quality attributes tend to change rapidly leading to softening, browning and quality loss.

Among all changes that affect berries quality in post harvest, water loss is the main cause of deterioration as resulting in loss of salable weight as well as losses in appearance (shriveling) and turgidity, color, texture, and nutritional value (Kader, 2002). Water loss is caused by transpiration process of the fruit that is influenced by internal (morphological characteristics and maturity stage) and external factors (temperature and relative humidity). Transpiration of moisture occurs because of the difference in water vapor pressure between the fruit surface and the surrounding environment, therefore when the relative humidity of the environment is lower than 95%, moisture moves from the inside of fruit to the outside air, through the skin.

However, transpiration, high respiration and metabolic activities are not the only factors responsible for quality loss in post harvest, indeed berry fruit are very susceptible to spoilage caused by fungal decay (Nadas et al., 2003), especially when leakage of juice occurs due to physical damage. Berry fruit are a suitable substrate for fungal growth as they contain high levels of sugars and other nutrients, as well as an ideal water activity and low pH, which is adequate for fungal spoilage. In many cases, post-harvest decay results from preharvest quiescent infections caused by plant pathogens fungi that start the spoilage from the field, while other infections are due to the fungi proliferation after harvest when the main plant defenses are reduced.

Botrytis cinerea (grey mold) is one of the most common pathogens found in berries after harvest (Robbins and Fellman, 1993) that may cause severe loss as being a psychrotropic mold that is capable of surviving even in common refrigerated storage conditions. The spores of *B. cinerea* are able to survive in a dormant state within the berry until the sugar concentration is sufficient to support their growth that usually occurs during post harvest storage. In this way post-harvest handling can spread the fungus, which may continue to grow even at cold storage. Besides *B. cinerea*, strawberry fruit is usually attacked also by *Rhizopus* sp., that shorten its storage life, while blueberry cultivars are generally subjected to post-harvest decay caused by *Colletotrichum acutatum* and *Colletotrichum gloeosporioides* (Smith et al., 1996). The presence of these molds are clearly visible on the fruit's surface because of the grayish - brownish appearance that lead to an immediate loss of quality and marketability however, in addition molds commonly produce enzymes that degrade carbohydrates, fats, and proteins causing softening of fruit and flavor and aroma deterioration.

Rapid tissue degradation accompanied by loss of firmness are common in berries during post-harvest as a natural process of senescence due to high respiration and metabolic rates. Berries softening is a consequence of changes in physical and mechanical properties of the tissues based on changes in the chemical structure of the cell wall polysaccharides (pectins and hemicelluloses) that undergo degradation by means of enzymatic action. Enzymes are able to depolymerize and convert water insoluble polymers into water soluble components that result in fruit softening (Fischer and Bennet, 1991). Since berry cell walls contain high concentrations of pectic substances, polygalactouronase is the main cell wall degrading enzyme relate to ripening and post-harvest shelf-life by directly influencing fruit texture.

However, polysaccharide degradation can occur either from the activation of cell wall bound enzymes or derived from fungal pathogens that can further promote changes in texture and contribute to reduce fruit shelf-life (Cordenunsi et al., 2003). Depolymerization of cell wall components during storage may occur despite low amount of endogenous enzymes, indeed as fruit matures, textural changes are also influenced by water uptake, cell wall volume expansion, and the presence of metals such calcium (Manning, 1993).

Textural changes are not the only modifications affecting the quality of berries. Color is the other important sensory attribute related to visual appearance that tends to change during post-harvest, affecting fruit quality and consumer's perception. In order to ensure color quality during storage, degradation of anthocyanins should be minimize. Indeed berry color, which is impart mainly by the presence of anthocyanins, can be influenced by numerous degradative reactions involving anthocyanins, which include enzymatic action, pH, the presence of ascorbic acid, as well as elevated CO₂

concentration in storage environment. However, although synthesis of anthocyanins continues even after harvest (Holcroft and Kader, 1999) leading a potential more intense color of berries, discoloration of the fruits during post-harvest is commonly observed and it may be attributed to several causes such as the action of endogenous and exogenous enzyme-catalyzed reactions.

Once fruit is damaged and cell compartmentation broke down, free radical-induced or enzymes as polyphenol oxidase (PPO) contribute to overall color loss in the fruit because of the formation of condensation products (Kader et al., 1999). PPO and the endogenous phenolic compounds that serve as substrate for PPO (catechin and phenolic acids) will interact and, with time, lead to brown discoloration, which is common mainly in strawberry fruit. However, the formation of brown or colorless compounds can also be determined by the action of exogenous fungal enzymes such as β -D-glucosidase, which is described being responsible for inducing loss of color by breaking the linkage between the glucose and the anthocyanidin moieties (Wrolstad et al., 1994; Wightman and Wrolstad, 1996). A considerable loss of anthocyanin pigments, and the formation of brown compounds, may also due to non-enzymatic browning reaction. The presence of ascorbic acid in berries can affect color stability by its own breakdown. Indeed, in the presence of heavy metals and oxygen, ascorbic acid is oxidized to dehydro-ascorbic acid with the production of hydrogen peroxide and certain peroxi radicals that can degrade anthocyanins directly (Talcott et al., 2003).

Color change in berries is also attributed to modification in the acidity of the fruit since anthocyanin pigments exist in different forms depending on the pH of the medium and the importance of this phenomenon is that each of the forms is associated with a different color expression. The pH of the fruit has a profound effect of

anthocyanins stability since the red flavylium cation remains stable only in acid conditions. It was observed that as pH increases above 4, the hydroxyl group can lose a proton and form the blue quinonoidal base, while increases in pH above 7 can result in greenish-yellow compounds (Brouillard et al., 1997). Holcroft and Kader (1999) hypothesized that controlled atmosphere enriched with CO₂, which is a common storage technique employed to extend berries shelf life, can affect pH of the fruit by dissolution of CO₂ gas and therefore, affecting the color.

Flavor changes (soluble sugars and organic acids) in post harvest, along with color and texture, contribute to the final quality of ripe fruit. Cordenunsi et al. (2003) reported that total soluble solids (TSS) of strawberry during cold storage increased probably because of the water loss (dehydration process) and cell wall pectins degradation, rather than conversion of starch to sugars, since strawberries accumulate very little starch.

Berries quality is evaluated also in terms of nutritional value. Nutrient content can be lost between harvest and consumption and losses vary based on type of nutrient, presence of physical damage, and storage environment. It is well established that water soluble vitamins such as vitamin C are more susceptible to post-harvest losses as being very labile and, under adverse storage conditions, can also undergoes oxidation (due to the activity of ascorbic acid oxidase) even if oxidation of the active form of the vitamin (L-ascorbic acid) to dehydroascorbic acid (DHA) does not result in loss of biological activity since DHA is readily reconverted to L-ascorbic acid (Nunes et al., 1998). Most studies of vitamin loss in fruits and vegetables have focused on ascorbic acid since it is an essential nutrient that presents also reducing and antioxidant properties. The rate of post-harvest oxidation of ascorbic acid in berries has been reported to depend upon

several factors such as temperature, water content, pH, packaging, storage atmosphere, and storage time (Barth et al., 1993). Thus, being so unstable, it is taken as indicator of berries freshness. However, although continuous increments of ascorbic acid during strawberry maturation were reported by Montero et al., (1996) and Wang et al., (1996), where it accounted for about 15% of the total antioxidant capacity, overall nutritional content of berries can be adversely affected by improper handling and storage. For instance, in strawberry fruit, up to 50% of ascorbic acid was lost during one week of storage at 6 °C (Cordenunsi et al., 2003), and 42% of loss was observed in CO₂ enriched atmosphere (10% to 30%), where in the same conditions, only 15% of loss was observed in raspberry and black berry that resulted to be less sensitive (Agar et al., 1997).

In addition to vitamin C, post-harvest handle and storage can affect also phytonutrients such as phenolic compounds, which are the responsible for the positive biological effects on health. It is well known that controlled stress after harvest (wounding, phytohormone treatments, UV light exposure, other radiation treatments, controlled or modified atmosphere exposure, and water stress) may enhance the health benefits of fruit by stimulating the formation of phenolic compounds. Indeed, phenolic compounds are the secondary metabolites of plant, produced before and after harvest, that are needed to protect the species against adverse factors which threaten its survival in a adverse environment, such as drought, UV radiation, infections or physical damage (Kähkönen et al., 1999). Currently, different results of phenolic compounds content have been reported in different post-harvest studies where their amount, in some cases decreased because of extreme or improper storage conditions, but in others increased because of moderate stresses. As an example, anthocyanin synthesis, which continues

after harvest and also at low temperature storage, is inhibited in fruits stored in high CO₂ concentrations. Holcroft and Kader (1999) found a negative effect of the atmosphere on the anthocyanins concentration and on the activities of the key enzyme of the anthocyanin synthesis pathway (phenylalanine ammonia lyase). Nunes et al. (2006), instead, showed that anthocyanin content in strawberries and blueberries was much lower in fruit that ripened in cold storage instead of in the field. Opposite result was obtained by Conner et al. (2002) where the antioxidant activity of nine blueberry cultivars, kept at 5 °C, remained for 3 weeks at the same value registered at harvest time. Moreover, another study reported the positive effect of high temperature on anthocyanins content in blueberry fruit, where 1.2- fold increase was observed during 8 days of storage at 20 °C, while storage at 0 °C, and 10 °C did not result in significant changes (Kalt et al., 1999).

In general is possible to state that low storage temperature is an effective way to extend the shelf-life of perishable fruit as berry, however some ripening-related quality aspects of the fruit may increase with higher temperature, reflecting high sensorial, nutritional and healthy values. Identification of proper storage temperature must be combined with other post-harvest techniques in order to obtain the highest quality of the product in terms of shelf-life extension and quality attributes.

1.2.2 Microbial safety

The increasing consumption of fresh berries, due to their health-promoting properties, has brought up an important concern related to their microbial safety. Berries are among the most susceptible fruits to cause potential health problems in terms of food safety, because they do not receive any treatments during their production, packaging and distribution. Therefore, in each of these handling phases they are exposed to a high degree of contamination and become a vehicle for the transmission of bacteria such as *Listeria monocytogenes*, *Salmonella* sp., *E. coli* O157:H7 (Abadias et al., 2008), parasitic, and virus pathogens capable of causing human illness. Moreover, the berries' sensitive/vulnerable morphology and the lack of natural protective covering make them even more prone to microbial attack and proliferation. In recent years, the increase in incidence of foodborne outbreaks associated with the consumption of berry fruits has been reported and several cases were due to the presence of *Cyclospora cayetanensis* in raw raspberry and strawberry. The natural host for this parasite has not been identified; however, the use of contaminated water for irrigation and poor hygiene practices during harvesting have been suggested as the most likely routes that led to contamination (Daeschel and Udompijitkul, 2007).

From this perspective, effective post-harvest technologies and good sanitation practices are needed to prevent pathogens contamination in berries and/or remove them, in order to ensure that a safe and healthy product reach the consumer.

1.3 POST-HARVEST TECHNIQUES TO IMPROVE BERRY FRUIT QUALITY

The main purposes for applying suitable post-harvest techniques are to maintain the safety, sensory and nutritional quality of berries, and reduce their losses between harvest and consumption, to meet the consumer's quality expectations in relation to their needs. Post-harvest technologies can be defined as integrative processes that must include scientific and technological knowledge aiming to assure the safety and quality of fruits, by extending their shelf-life, eliminating avoidable losses, and maintaining or enhancing their nutritive and quality properties to make them readily marketable. In the last decade, numerous studies have been concentrated in trying to find new approaches that can provide the best compromise between extended shelf-life and the maintenance of nutritional value of berries.

Cooling applied immediately after harvest and throughout the storage period is the most common and effective technology used to achieve this goal as cooling has a direct effect on slowing berry's respiration rate, decreasing enzymatic activity that leads to fruit softening, and delaying decay caused by spoilage microorganisms.

The shelf-life of berry fruits varies inversely with their rate of respiration, which is affected by a wide range of environmental and storage factors that include light, chemical and water stress, pathogens attack, temperature and atmospheric composition (Mathooko, 1996). Among the environmental factors, storage temperature is the most important factor affecting the shelf-life of berries, since temperature has a profound effect on the rates of biological reactions. High storage temperature storage accelerates the respiration rate of berries and shortens their shelf-life period, which are in

turn associated with the loss of berries' quality (Shin et al., 2007). As an example, it was found that strawberries stored at 5 °C developed more decay and had a shorter shelf-life than those stored at 0 °C (Ke et al., 1991). The effect of low storage temperature, on the content of phytochemical compounds present in berries may be beneficial or detrimental, based on the specific degree of temperatures used. Significant fluctuations and even increases in anthocyanin, phenolic and flavonoid concentrations were observed in different types of berries stored at higher temperatures than at lower temperatures after several days of storage (Ayala-Zavala et al., 2004; Kevers et al., 2007). Moreover, Kalt et al. (1999) observed that the antioxidant capacity of strawberry and raspberry increased during storage temperature higher than 0 °C, and no losses in ascorbate content were observed during 8 days of storage at temperatures of 10, 20 and 30 °C. This increment on antioxidant capacity in berries stored under higher storage temperature than 0 °C, may be explained by the formation of compounds with enhanced antioxidant activity, even at the point when the fruit attributes are already deteriorated due to the higher storage temperature. Conversely, it is of common knowledge that nutrients can be lost without detectable changes in flavor and texture, depending on storage temperature conditions. In general, post-harvest storage operations that maintain sensory characteristics of the fruit also reduce the losses of nutritional compounds (Clydesdale, 1988).

Along with refrigeration storage, where recommended temperatures vary according to different chilling susceptibilities of berries, relative humidity (RH) must be maintained at 90 to 95% as berries are very sensitive to water loss, which results in shriveling and loss of gloss (Kader, 1991). Water loss, commonly evaluated as weight loss, is reported to be greatly affected by low RH values, and to have an effect on the

quality, firmness and color of strawberry (Shin et al., 2008). In order to minimize water losses due to transpiration, as and increase quality and shelf-life, berries must be stored at low temperature and high RH environment. However, the elevated RH needed during refrigerated storage of berries to maintain their shape and volume, increases their susceptibility to decay caused by microbial spoilage organisms. Therefore, in addition to proper storage temperature and RH conditions, other techniques must be used to reduce the respiration rate, transpiration and spoilage of berries, as well as to extend their shelf-life and maintain their overall quality.

Modification of O₂ and CO₂ concentrations in the atmosphere surrounding the fruit, to levels different from those in the air, are referred as controlled and modified atmosphere. These post-harvest technologies are widely used to preserve the quality of berry fruits, especially during long storage periods. Controlled atmosphere (CA) storage involves a constant monitoring and adjustment of the atmosphere composition within the storage environment, as it takes into account the respiration of the fruit that constantly changes the atmosphere. The gasses are therefore measured periodically and adjusted to the predetermined level by the introduction of new gas mixtures. Usually low concentration of O₂ and high CO₂ levels used in CA are reported to have great beneficial effect on extending the shelf-life of berries, because of the decrease in respiration rate and preservation of texture, due to reduced enzymatic action on cellular membranes (Smith, 1992). Moreover, CA storage decreases the rate of decay of berries by inhibiting aerobic bacteria and fungi. However, incorrect control of CO₂ and O₂ concentrations or overextended CA storage can have detrimental effect on berries, causing tissue discoloration (bleaching and loss of typical pigmentation) and off-flavor production (ethanol, acetaldehyde, ethyl acetate), as well as reduction of vitamin C

content and titratable acidity (Agar et al., 1997). Kader (1995) showed that elevated CO₂ concentration (greater than 50%) resulted in less decay of strawberries stored at 5 °C. However, the resulting disturbance in the respiratory enzyme system led to an increased deterioration of the berries after 8 days of storage and caused discoloration of the fruits.

Modified atmosphere packaging (MAP) of fresh fruits refers to the post-harvest technique of sealing actively respiring produce in polymeric film packages to modify the O₂ and CO₂ levels within the package atmosphere (Beaudry, 2000). At difference from CA, where there is a continuous infusion of gasses, MAP involves only one adjustment of the atmosphere since its effect is maximize by the utilization of a proper packaging material. Moreover, besides the positive effects on shelf-life extension and moisture retention, packaging isolates berry fruits from the external environment and helps to reduce their exposure to pathogens and contaminants. However, MAP has also the potential to induce undesirable effects on berries such as fermentation and off-flavors development, when O₂ levels are too low to sustain the normal aerobic respiration of the fruit. Similarly, injuries occur when CO₂ exceeds tolerable levels, which are highly dependent on the type of fruit species and varieties. Critical levels of O₂ and CO₂ that may cause damage to berry fruits have been reported to be lower than 2.0 KPa for O₂ and higher than 25 KPa for CO₂. Kim et al. (1995) examined the influence of different CO₂ and O₂ levels on shelf-life and several qualitative traits of blueberry and they found that the optimal storage conditions were obtained at 17-18% CO₂ and 9% O₂. Higher levels of CO₂ decreased the ratio of soluble solids to titratable acidity, leading to loss of fruit flavor. Moreover, Van der Steen et al. (2002) reported that MAP conditions of 15 to 20% carbon dioxide and 5 to 10% oxygen reduced the

growth of *Botrytis cinerea* and reduced the respiration and softening rates of blueberries, raspberries and blackberries, thereby extending their post-harvest life.

The composition of the atmosphere within a package, which provide the effectiveness of MAP, results from the interaction of a number of factors that include the permeability characteristics of the package, the respiratory behavior of the fruit, and the environment. Considering that berries tend to lose moisture during storage, the characteristics of the film package (permeability, thickness, perforations) must be selected based on the characteristics of the fruit (respiration, transpiration, mass), in order to be able to contain the desired atmosphere throughout the storage period and therefore extend the shelf-life and retain the quality attributes of the berries. During the respiration process the fruit reduces O₂ and increases the CO₂ levels within the package, creating a gradient across the film that provides the driving force for gas movements into and out of the package. Two strategies to create film barriers are commonly used; one is the utilization of continuous films while the other is based on the control movements of gasses through microperforations made in the film.

1.3.1 Microperforated packaging

Control of atmosphere within MAP is not precise as being a function of product respiration rates, film permeability, and external factors such as temperature. The limits for the development of MAP must consider the fruit damages that can occur due to very high CO₂ or very low O₂, resulting from temperature fluctuations during

storage. The key to successful MAP of fresh fruits is to use a packaging film of correct intermediary permeability, where a desirable equilibrium modified atmosphere is established when the rate of O₂ and CO₂ transmission through the pack balances the product's respiration rate (Caner et al., 2008). One strategy to decrease the risk for developing injurious gas concentrations is to use microperforated films. These types of films are able to promote rapid development of adequate O₂ and CO₂ levels in the package atmosphere to extend fruit shelf-life. They also provide a desirable oxygen concentration to ensure proper fruit respiration, and at the same time, maintain an adequate humidity within the film to minimize weight loss.

They are commonly used in MAP of high respiration fresh fruits such as berries, which tolerate simultaneously low O₂ and high CO₂ levels (Fonseca et al. 2000), since the rate of gas exchange is greater than in other type of films (Fishman et al., 1996). It has been proved that the diffusion of O₂ and CO₂ through air is 8.5 and 1.5 million times greater, respectively, than through low density polyethylene films (González et al., 2008). Microperforated films retain many of the good results of sealing such as reduction of water loss and alleviation of water stress without the possible deleterious effects of anaerobiosis such as off-flavors, fermentation or CO₂ damage (Mangaraj et al., 2009).

The number and dimension of the microperforations, which are made using laser beam, control the gas exchange rate of microperforated films. Indeed, by altering the number and size of the perforations, packaging films with specific flow rates can be adjusted for storing a specific fruit. The high respiration rate of fresh fruits such strawberries requires much greater permeability than that provided by non-perforated films.

The size of the perforations normally used in MAP is between 50 and 200 μm in diameter (González-Buesa et al., 2009). Microperforated films have been used successfully for extending strawberry shelf-life. Sanz et al. (2002) showed that strawberry quality was preserved for 10 days at 2 °C by using a microperforated film with different perforation areas ranging from 1.57 mm² to 4.71 mm². Almenar et al. (2007) studied the chemical, physical and sensory quality of strawberries (weight loss, pH and acidity, solid content, color, firmness, fungal decay and development of off-flavors). They reported that fruit's properties were maintained for 6 days at 10 °C using films with one and three microperforations. The central importance of using microperforated films for maintaining strawberry quality during storage was proved also by Kartal et al. (2012). They demonstrated how microperforated films with 7 and 9 holes (90 μm) produced an internal atmosphere of 15 kPa CO₂/5 kPa O₂ at 4 °C that helped to maintain pH, total soluble solids, electrical conductivity, color, texture and the sensory properties of strawberry fruits during storage.

Common plastic films used in MAP are low-density polyethylene (LDPE), linear low-density polyethylene (LLDPE), high-density polyethylene (HDPE), polypropylene (PP), polyvinyl chloride (PVC), and polyester, i.e. polyethylene terephthalate (PET), polyvinylidene chloride (PVDC), and polyamide (Nylon). Although an increasing choices of packaging materials are available to the MAP industry, most packagings are still constructed from four basic polymers: polyvinyl chloride (PVC), polyethylene terephthalate (PET), polypropylene (PP) and polyethylene (PE), for the packaging of fruits and vegetables (Mangaraj et al., 2009). The desirable characteristics of a polymeric film for MAP depend on the respiration rate of the fruits, storage temperature, and optimum O₂ and CO₂ concentrations for the fruits that will

result in optimum MA conditions within a definite time period. For most fruits, PP may be a suitable solution as it is more permeable to CO₂ than to O₂, has good resistance to chemicals, and is effective at barring water vapor.

1.3.2 Edible films and coatings

One of the most effective and innovative ways to extend the storability of berry fruits is the application of edible films and coatings on their surface, followed by cold storage.

By definition, edible films and coatings are any type of material used to coat or wrap food products to extend their shelf-life, improve their quality characteristics, and that may be eaten together with the food (Pavlath and Orts, 2009). Generally, an edible coating is a thin layer of edible material formed on a food; whereas, an edible film is a preformed thin layer of edible material placed on or between food components (Krochta and De Mulder-Johnston 1997).

Both edible films and coatings provide numerous advantages on fruit quality as they can create semipermeable barriers to gases and water vapor to maintain the quality of the product. Overall, the main purpose of using edible films and coatings for the preservation of perishable fruit is to retard the transfer of gasses, vapor, and volatiles compounds, providing the fruits with a sort of modified atmosphere storage that decreases their respiration rate and senescence, reduces flavor loss, retains moisture, and delays color changes during the storage period. They also play an important role on food conservation and marketing by protecting the product from mechanical damage,

physical, chemical and microbiological activities. Moreover, edible films and coatings can also decrease the amount of conventional synthetic and disposable packaging materials.

The major components used in edible films and coatings are polysaccharides, proteins and lipids that, as general rule, are used to control oxygen and other gas transmission, provide mechanical stability, and reduce water transmission.

Polysaccharides are the most extensively used components in edible films and coatings for fruits (Kester and Fennema, 1986) as they are effective gas barriers, although they are highly hydrophilic. Nevertheless, they are a poor barrier to water vapor. However, their poor water vapor barrier characteristic may provide some benefits as it allows movement of water vapor across the film, thus preventing water condensation, which is a potential source of microbial spoilage. The main polysaccharides included in edible films and coatings are starch and starch derivatives, cellulose derivatives, alginate, carrageenan, chitosan, pectin, and several gums.

Although there is a very wide range of natural macromolecules that can be used in the formulation of edible films and coatings, their use is limited by conditions such as cost, availability, and functional attributes. The properties that these compounds provide to films and coatings such as mechanical properties (flexibility, tension, viscosity), optical properties (opacity and transparency), the barrier effect against gases flow, structural resistance to water and microorganisms, and sensory acceptability are also important and must be taken into account for developing a proper edible packaging. Moreover, edible films and coatings must be formulated according to the properties of the target fruit they are to be applied to, such as respiration and transpiration rate (Falguera et al., 2011).

Thus, the optimization of an edible packaging composition that convey suitable mechanical and physical properties as well as consumers acceptability is a complex process that requires proper knowledge and expertise on different technical aspects related to both the packaging and food product properties. In order to optimize the formulation of an edible packaging, the design of response surface methodology (RSM) is a useful tool that has been implemented in many works since it determines the optimal mix of components that allowed to exploit the features of the added substances (Ozdemir and Floros, 2008). RSM is a collection of mathematical and statistical technique that simultaneously optimizes the levels of the components (variables) to attain the best system performance. It is applied when a response or a set of responses of interest are influenced by several variables (Bezerra et al., 2008). For instance, Tapia et al. (2008) used RSM to determine the optimum alginate- and gellan-based coating composition on fresh-cut papaya based on their ability to improve water vapor resistance.

Several examples of the most common polysaccharides used for fresh berries storage are presented here: Chitosan, a not water soluble polysaccharide derivate of chitin obtained from marine invertebrates, was used as coating on strawberries and raspberries for improving their quality and storability (Han et al., 2004). Chitosan films or coatings can increase shelf-life and preserve quality of the berries by decreasing their respiration rates, inhibiting microbial development, and delaying ripening. They have been used on berry fruits with good results because of their excellent film-forming ability and physicochemical properties, such as biodegradability and null toxicity. In addition, they showed antimicrobial activity against a wide range of bacteria and other microorganisms such as *Botrytis cinerea* (Romanazzi et al., 2002, No et al., 2007).

Another polysaccharide used for the preservation of berries is starch, which is an inexpensive polysaccharide obtained from cereals, legumes, and tubers. It is composed of two macromolecules, amylose and amylopectin, highly branched and their ratio varies with the starch source. Films formed with starch are often very brittle and have poor mechanical properties (Peressini et al. 2003). Therefore, to overcome this problem, starches are usually blended with other compounds such as plasticizers that reduce intermolecular forces and increase the mobility of polymer chains. Usually, hydrophilic compounds such as polyols (glycerol, sorbitol, polyethylene glycols) and lactic acid are used. García et al. (1998) proved that starch-based coatings were able to extend storage life of strawberries, decreased water losses, and improved fruit quality. Moreover, color changes were delayed, and weight and firmness losses were lower in the coated fruits than in the control fruits (non-coated).

Alginate is another biopolymer that is used for edible films and coatings because of its unique colloidal properties and its ability to form strong gels or insoluble polymers upon reaction with multivalent metal cations like calcium. Alginate, a polysaccharide derived from marine brown algae (Phaeophyceae), is a binary copolymer composed of β -D mannuronic (M block) and α -L guluronic (G block) residues. The proportion, distribution, and length of these blocks determine the chemical and physical properties of the alginate molecule. While G-block provides gel-forming capacity, MM and MG units provide flexibility. Because of these properties, alginate is finding increasing use in the food industry as texturizing and gelling agent (Rojas-Graü et al., 2007a). As a texturizing agent, viscosity of alginate solution is the most important characteristic to be considered. It depends on the alginate concentration, length of the molecule, and the number of monomer units in the chains, with longer

chains resulting in higher viscosity. Another attribute of alginate is the possibility to form films that, along with plasticizer, are proved to provide strong oxygen barriers (Miller and Krochta, 1997). In regard to physical properties, sodium alginate forms a strong film, despite the negative charge on the molecule. However, removing the negative charge by cross-linking the alginate with calcium increased the tensile strength of the resulting film (Nieto, 2009). The water vapor permeability of the film (WVP) is also affected by the composition of alginate (M:G ratio). Alginate–Ca²⁺ films with higher concentrations of G have lower WVP than films with higher concentrations of M due to greater ability of G to form intermolecular cross-links via calcium salt-bridges. Although properties of alginate films are influenced by surrounding RH, alginate–Ca²⁺ films retain their strength, even at high RH values (Olivas and Barbosa-Cánovas 2008). Alginate–Ca²⁺ coatings have been used successfully to prolong the shelf life of fresh-cut Gala apples without causing undesirable anaerobic respiration. These coatings minimized weight loss and browning, and preserved firmness during storage (Rojas-Graü et al., 2007b).

A new trend in this field is characterized by the utilization of fruit puree that have been shown to be a promising tool for improving quality and extending shelf-life of fruits (McHugh et al., 1996; McHugh and Senesi, 2000). Apple-based edible film was the first film made from fruit purees and it showed to be an excellent oxygen barriers, particularly at low to moderate relative humidity, but was not a very good moisture barrier. In this case, the addition of lipids was necessary to improve the water barrier properties of the film.

The use of additives in film and coating forming solution is a common practice that helps to obtain higher performance of the system. Plasticizers are the main

additives used in films and coatings to improve their mechanical properties. Indeed, without plasticizers, most films and coatings are brittle, and it is difficult to form a homogenous coating. Therefore, combining plasticizers with the main component of the film leads to move the component's chains apart, and thus reduces rigidity of the structure (Guilbert and Biquet, 1996). They also attract water molecules around it, which reduces intermolecular interactions of the main component (Ke and Sun, 2001).

The functionality of edible films and coating can be expanded by incorporation of several active ingredients such as antibrowning agents, antimicrobials, colorants, flavors, nutrients, and spices (Tapia et al., 2008) that enhance the safety and even the nutritional and sensory attributes of the fruits. Among all, one of the important functions of edible films and coatings is their use as carriers of antimicrobials and antifungal agents to increase the shelf-life of perishable fruits and enhanced their safety. Edible coatings and films have been studied as antimicrobial carriers since they can act as an active packaging system and control the release of the active compounds that, in this way, can migrate selectively and gradually from the package to the surface of the fruit where they are retained at high concentration throughout the storage period.

1.3.2.1 Antimicrobial activity of essential oils and their utilization in edible films and coatings

As it has been previously mentioned, the use of edible films and coatings for fresh fruits is of interest because they can serve as carriers for a wide range of beneficial food additives, including plant-derived safe antimicrobials (Pranoto et al., 2005). In this

sense, natural essential oils (EOs) derived from plants in combination with structural polymers can be considered a promising treatment, because of their effectiveness as antimicrobial compounds. Moreover they have gained interest because of consumer awareness and concern regarding use of synthetic chemical additives.

The antimicrobial components are commonly referred to a number of small terpenoids and phenolic compounds in the essential oil fractions that are recognized to have a wide spectrum of antimicrobial activity against foodborne pathogens and spoilage bacteria (Gutierrez et al., 2009). Because of the great number of constituents, essential oils (Eos) seem to have no specific cellular targets. Nevertheless, Burt (2004) reported that their hydrophobicity is an important characteristic of EOs that make them able to pass through the cell wall and cytoplasmic membrane, disrupt the structure of their different layers of polysaccharides, fatty acids and phospholipids, thus rendering the membranes more permeable.

Recent studies have shown that EOs of oregano, thyme, cinnamon, lemongrass, and clove are among the most active against different types of microbes, including foodborne pathogens (Friedman et al., 2002). Among all these EOs, carvacrol is considered one of the most important components of certain EOs that exerts antimicrobial activity not only because of its high abundance in some oils, where it can reach levels of 75%, but also because of its high specific activity as compared to other EOs components. Studies performed on the antimicrobial activity of carvacrol in buffers (Friedman et al., 2002), apple juice (Friedman et al., 2004), and wines (Friedman et al., 2006) have shown that it has a broad spectrum against almost every Gram-positive and Gram-negative bacteria tested.

Besides antibacterial activity, carvacrol has been described as antifungal (Chami et al., 2005), antitoxigenic (Ultee and Smid, 2001), insecticidal, and antiparasitic agent.

Because of all these properties, carvacrol has been incorporated into several edible films and coatings where it showed to be highly effective against pathogenic bacteria and fungi. The effectiveness is generally evaluated using an agar diffusion method that evaluates the antimicrobial activity of films and coatings by measuring the inhibitory zone around the film placed on direct contact with the contaminated surface. Rojas-Graü et al. (2007c) evaluated the antimicrobial activities against *E. coli* O157:H7 of several EOs (oregano, cinnamon, and lemongrass) and oil compounds (carvacrol, cinnamaldehyde, and citral) incorporated in alginate–apple puree edible film. The bactericidal activity was determined for film-forming solution and the results showed that carvacrol exhibited the strongest antimicrobial activity. In line with this study, Du et al. (2008) showed that carvacrol-containing tomato-based edible films inactivated *E. coli* O157:H7 and its inactivation was related to carvacrol levels in the film. Several studies tested the efficacy of carvacrol also on food product, mainly on fresh cut apple where their shelf-life was extended because of the antimicrobial effect of carvacrol that reduced population of *Listeria innocua* (Rojas-Graü et al., 2007b) and *E. coli* (Raybaudi-Massilia et al., 2008).

However, all these methods for testing the antimicrobial activity of active compounds require their direct contact with the microorganism and in some cases is not relevant to commercial fruits, such as small berry fruits in which only a small portion is in direct contact with the packaging material. One advantage of EOs is their bioactivity in the vapor phase that allows the vapor to be used as fumigants. Indeed, EOs have a

relatively high vapor pressure and are capable of reaching an organism through the gas phase (Du et al., 2009). In a study concerning the antifungal activity of some esters, aldehydes, alcohols, and terpenes, characteristic of apple flavor, Caccioni et al. (1997) proposed that the effectiveness against *Botrytis cinerea* of the considered molecules was dependent on their actual vapor pressure rather than on their whole concentration in the system. Therefore, they can be incorporated also in edible film that are not wrapped around the product and exert antimicrobial activity through their release from the film as vapors. In a study related to the efficiency of several EOs in the vapor phase, Du et al. (2009) demonstrated that the concentration of oregano and allspice oils in tomato films needed to be effective against three pathogenic bacteria (*E. coli*, *L. monocytogenes*, and *Salmonella enterica*) were lower than those used in a direct contact test (overlay test). This observation suggested that volatile components diffused more efficiently through the air than through the agar media. Ayala-Zavala et al. (2005) proved that vapor of methyl jasmonate, a naturally occurring compound, reduced microbial contamination and inhibited grey mold infection in strawberry fruits, and the same outcome was obtained also by Wang et al. (2007) using thymol, eugenol, and menthol vapors, which derived from plant sources. Blueberry shelf-life and antioxidant activity was also increased using several naturally occurring essential oils including carvacrol, anethole, cinnamaldehyde, cinnamic acid, perillaldehyde, linalool, and p-cymene as vapors (Wang et al., 2008).

One of the most imitating factors for using EOs into edible films and coatings is related to their sensory compatibility with the coated product. Indeed, EOs-enriched edible films and coatings are generally intended to be consumed with the product and therefore should not affect consumer acceptance. However EOs, as being volatile

compounds and characterized by a strong smell, could be perceived even if the film is not directly applied on the fruit. This issue is of particular interest to berry fruits as they have a unique and highly desirable fruity aroma that is one of the most attractive attributes to consumers and the loss or alteration of their typical aroma and the development of objectionable odors may severely reduce their quality and marketability. The incorporation of naturally occurring berries aroma compounds into edible packaging could represent a valid and innovative approach to overcome undesirable odor imparted by EOs that are generally characterized by a strong herbaceous odor. Some of these compounds have also been proved to exert antimicrobial and antifungal properties that may enhance the effectiveness of the antimicrobial coating.

Methyl cinnamate is a methyl ester of cinnamic acid and is one of the major volatile components of strawberry aroma produced and released during fruit maturation (Lunkenbein et al., 2006). Because of the sweet fruity flavor, it is commonly used in many applications as ingredient in decorative cosmetics and fragrances (Ali et al., 2010) and, since it is generally recognized as safe (GRAS), it may also be potentially used as food additive (Huang et al., 2009). Since methyl cinnamate has been proven to have antifungal activity against phytopathogenic fungi under vitro conditions (Vaughn et al., 1993; Rahmani et al., 2010), it has the potential to be used in fruit preservation in conjunction with edible packages. Moreover, because of its compatible aroma with berry fruit, it can also act as a flavor enhancer.

1.3.2.2 Electrostatic spraying technology for edible coating application

The use of edible coatings has become popular among different post-harvest techniques to protect berry fruits during their storage period as it can fortify the natural layers of the fruit to prevent moisture losses, control the exchange of gases involved in the respiration process, and improve mechanical and handling properties. It can also be used as a vehicle for incorporating antimicrobial EOs. Furthermore, the application of coating directly on the fruit surface makes their use more advantageous for practical application as it requires a minimal process. Edible coatings can be applied by different methods such as panning, fluidized bed, dipping, and spraying. All these techniques exhibit several advantages and disadvantages and their performance depends primarily on the characteristics of the fruit to be coated and the physical properties of the coating (viscosity, density, surface tension) (Andrade et al., 2012). Among all, spray coating is the most commonly used technique as it offers many advantages including uniform surface coating, the possibility to increase the surface area of the liquid over the fruit surface, and thickness control.

Electrostatic spraying technique is a promising new technology for coating application that needs to be studied to determine its potential in this field of application. Electrostatic spray technology has been utilized by the painting and agricultural industries. Most recently, there has been a growing effort to adapt this technology for food coating applications, since it provides greater retention and efficient distribution of the liquid onto the food surface. The general principle of this technology is based on the application of a charge to the liquid droplets as they are sprayed through a nozzle. Outside the nozzle, the droplets carry the charge to the nearest grounded surface, which

magnetically attracts the charged particles and allowed them to entirely cover the target product. In this way, electrostatic spray applications increase the transfer efficiency and evenness of the coating process and therefore may improve the overall product quality (Bailey, 1998).

The versatility of electrostatic spraying technology for different type of food materials application have allowed the food industries to use this technique for powder coating on different foodstuff products, mainly for seasoning and flavors, to improve the taste and appearance of foods, which increase consumer acceptability. Other reasons to apply powder coatings are to increase the nutritional value of the products, provide anticaking properties, and apply antimicrobial agents (Khan et al., 2012). Different products have been electrostatically coated, such as French fries coated with glucose powder and smoke extract, which had more uniform color and texture compared to fries coated with traditional methods. Additionally other foods that have been electrostatically coated are cheese coated with antimycotic powder, which led to improved functionality and increased shelf-life (Amefia et al., 2006), and potato chips coated with spices that showed more even coating and color (Ratanatriwong et al., 2003). Khan et al. (2012) reported that powder coating technique can be improved by using electrostatic spraying through higher transfer efficiency, better adhesion, low energy usage, less waste production, and air borne dust (values ranging from 40% to 84% have been reported by different sources depending on the particle size), making this method cost effective.

Electrospraying has also received significant attention as a novel technology for the application of liquid coatings. It has been used in agriculture for the application of pesticides where increased the deposition of the liquid onto blueberry plants from two to seven fold (Scherm et al., 2007). I have been also used in cabbage, in which the

overall leaf coverage was significantly better when electrostatic sprayer was used compare to conventional spray application (Perez et al., 1995). The success of liquid electrostatic coating for pesticides application has increased the interest for using this technology in the food industry as sanitizer, for example, by using electrolyzed water to eliminate foodborne pathogens in chicken carcass and eggshell (Russell, 2003). Similarly, electrostatically sprayed organic acids demonstrated to be highly effective against foodborne pathogens on spinach (Ganesh et al., 2010) and iceberg lettuce (Ganesh et al., 2012).

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2 EFFECTS OF MICROPERFORATED PACKAGING AND STORAGE TEMPERATURE ON BLUEBERRY (*VACCINIUM CORYMBOSUM* L.) QUALITY

2.1 ABSTRACT

The effects of three levels of microperforations (MPF) (1, 10 and 30) in polypropylene film (PP) and three storage temperatures (4 °C, 10 °C and 18 °C) on quality attributes in highbush blueberry (*Vaccinium corymbosum* L. cv. Duke) were investigated. Physicochemical properties: total soluble solids, sugars, pH, firmness, color, dry matter, respiration and nutritional values such as vitamin C, antioxidant activity, total phenols and phenolic compounds composition were evaluated. Blueberries were hand-harvested and packaged in clamshells, then wrapped in PP film with a specific oxygen transmission rate of $1450 \pm 250 \text{ cm}^3/\text{m}^2/24\text{h}$. All berries were evaluated immediately after harvest (SP0 = day0) and during storage at 2 (SP1), 5 (SP2) and 8 (SP3) days, while the last evaluation (SP4) was carried out after 4, 6 and 8 additional days at 18 °C, 10 °C and 4 °C, respectively, until the fruit visually spoiled. Even if the shelf life of blueberries from perforated packaging was not extended compared to the control (non-packaged clamshell), antioxidant activity, total phenols and firmness were improved by using microperforated PP film. Atmosphere equilibrium within packages was obtained for 10 and 30 MPF after 2 days, remaining constant for the remainder of the storage period. Refrigerated storage (4 °C) delayed ripening and extended blueberry shelf life up to 16 days compared to the other temperatures.

Blueberry should be packaged with microperforated PP film and stored at low temperature in order to extend the shelf life and preserve quality and nutritional value.

2.2 INTRODUCTION

Research into retention of berry fruits nutritional compounds has been stimulated by the increased interest in such fruits as having a chemical composition, as well as a high level of biologically active components, which provide health benefits beyond basic nutrition. According to numerous studies it is believed that phytochemicals, which have exhibited a wide range of biological effects such as antioxidant, anti-microbial and anti-inflammatory activity, are the compounds largely responsible for the potential protection of human health from different lifestyle diseases (Szajdek and Borowska, 2008; Bomser et al., 1996; Heinonen et al., 1998).

Many berries also contain high concentrations of vitamins, minerals and dietary fibers that contribute to promote human health (Zhao, 2007). Of these, blueberries are among the most appreciated by the consumer for their sweetness, aromatic flavor and high antioxidant content (Prior et al., 1998). However, the shelf life and qualitative traits are limited by changes in color, texture, weight loss and microbial growth which are affected by pre and postharvest activities and handling, as well as storage temperature and packaging (Wang, 2006; Kader, 1988; Goldman et al., 1999).

Wills et al. (1998) stated that blueberries are one of the most perishable fruits due to the soft texture and high water content (83%) that negatively affect shelf life, so careful handling and proper storage conditions are necessary in order to avoid bruises and mechanical damage that can lead to an accelerated physiological deterioration.

An experiment conducted at a storage temperature of 0 °C with 90 to 95% relative humidity, extended blueberry shelf life up to 2 weeks (Salunkhe and Desay, 1984) preserving the qualitative characteristics.

Moreover, it has also been demonstrated that low storage temperature, combined with a rapid post-harvest cooling, prolong blueberry conservation by decreasing the respiration rate and therefore slowing the ripening process (Cappellini et al., 1972; Ballinger et al., 1978). In order to prolong the shelf life and retain qualitative traits, proper packaging and storage technique are also necessary. In the last few years, new alternatives such as the use of controlled atmosphere (CA) have been adopted to achieve this goal. Indeed, using CA for blueberry conservation, Beaudry et al. (1992) reported an extension of the shelf life by using different combinations of high CO₂ and reduced O₂ concentration in the storage environment. As an alternative to CA, edible films and coatings have been developed to reduce moisture loss and quality changes (Fisk et al., 2008). Active packaging could be considered another effective approach for improving food safety, while maintaining quality, by using different substances to absorb oxygen, ethylene and moisture that could compromise fruit shelf life. However, although all these treatments have been proved to be highly effective in preserving fruit quality, consumer demand is oriented towards fresh products that are not subjected to any process that may convey an idea of artificiality. Post-harvest research has thus

focused on studying technologies that meet the specific needs of natural storage techniques.

With this aim, passive modified atmosphere packaging has been developed by using perforated film to naturally modify the atmosphere surrounding the product by consuming O₂ and producing CO₂ (passive MAP). More rapid gas fluxes are thus created, increasing the film's oxygen transmission rate (OTR) to the desired level for commodities, such as blueberries, with a high respiration rate (Kader et al., 1989; Renault et al., 1994; Fishman et al., 1996; Sanz et al., 1999). The purpose of the present work was to investigate the quality and nutritional value of blueberries packaged in microperforated polypropylene film to simulate hypothetical market situations of ideal (4 °C), suboptimal (10 °C) and retail (18 °C) temperature. The study aimed to investigate a postharvest field of research where little is still known, achieving promising outcomes by using modified atmosphere created by the fruits own respiration.

The number of microperforations used for this experiment was chosen based on the results obtained from a preliminary study (Peretto et al., 2011) carried out in our lab using 9 different numbers of microperforations on blueberry kept at the same storage temperatures as those used for the current study (data not shown).

2.3 MATERIALS AND METHODS

The experiment was conducted in autumn 2011 in the North-East of Italy using “Duke” blueberry cultivar grown in the Trentino area (46° 3’ N; 11° 14’ E). Late in the harvest season, approximately 6 kg of ripe berries of uniform size and free of physical damage and fungal infection were hand-harvested by trained pickers and packed in polyethylene terephthalate (PET) clamshell cups. Each clamshell (100x145x55 mm with 28 holes 8 mm wide) was filled with 125 g of blueberries and kept at 4 °C for 24 hours until the experiment began. The clamshells were then wrapped in microperforated PP film “Oxy” (Mach Flexopackaging - Italy, thickness 35 µm), having an O₂ transmission rate of 1450±250 cm³/m²/24h (23 °C, 0% RH) and vapor transmission rate of 4.5±1 g/m²/24h (38 °C, 90% RH), which was hermetically heat-sealed. 1, 10, and 30 microperforations (0.1 mm wide), made using laser technology, were tested and compared with common retail clamshell packages (NP, non-packaged with PP film) used as control. Samples were stored at 3 temperatures (4 °C, 10 °C and 18 °C) to simulate different market storage conditions at 80% controlled relative humidity (RH). The first sampling point for qualitative analysis was carried out before packaging (SP0) and after 2 (SP1), 5 (SP2), and 8 (SP3) days of storage. The last evaluation (SP4) was carried out after 4, 6 and 8 additional days at 18 °C, 10 °C and 4 °C, respectively, when 10% on average of fruit were visually spoiled. A total of 144 clamshells were tested, evaluating 3 replications.

Double rubber patches were placed on the surface of the film for sampling O₂ and CO₂ within the package. Ten-mL samples of the headspace gases were analyzed

immediately after packaging and every hour throughout the experiment by a PBI Dansensor CheckPoint O₂/CO₂, using a gas-tight syringe.

Dry matter of blueberries, calculated at different storage times, was obtained in a PID System ventilated oven (model M80-VF; Instruments s.r.l.; Bernareggio, MI) set at 65 °C for 72 hours.

For each sampling day, ten fruits per replication (four measurements per fruit) were subjected to compression force for structural analysis. Skin and flesh texture were measured at room temperature by using a Stable Micro Systems – TA.XT.plus Texture Analyzer equipped with a 5 kg flat probe, using a 2 mm/s speed and 5 mm/s target mode.

Approximately 60-70 g of blueberries, discarding any that exhibited signs of decay, from each clamshell (per sampling day) was homogenized in a stainless steel blender for pH, TSS and color evaluation. pH was measured on pureed berries at room temperature using a portable pH-meter (Hanna Instruments, HI 255). Color was measured using a hand-held tristimulus colorimeter (Minolta Chroma meter, model CR-300) that provided L*, a*, b* values used to calculate the chroma [$C^* = (a^{*2} + b^{*2})^{1/2}$], which indicates the intensity of color saturation (Francis, 1980). Three determinations were carried out on blueberry puree at harvest time and every sampling point during storage. Total soluble solids expressed as Brix° were analyzed from berry juice using a portable refractometer (Hanna Instruments, HI 96801).

Acetic acid (glacial) and sodium carbonate anhydrous were purchased from Riedel-de Haën (Hanover, Germany). Gallic acid monohydrate was obtained from Fluka (Sigma-Aldrich, Italy); methanol from VWR Prolabo (France), Folin-Ciocalteu's reagent from Labochimica (Padova, Italy). Chlorogenic acid hemihydrates, ferulic acid,

D-(+)-glucose and D-(-)-fructose were purchased from Aldrich Chemical Company (Sigma-Aldrich, Italy); *p*-cumaric acid, formic acid and caffeic acid from Sigma (Sigma-Aldrich, Italy); methanol from Carlo Erba (Milan, Italy). Deionized water (18 Ω A) was prepared with ultrapure water using a mod. Arium® pro purification system (Sartorius, Italy); quercetin 3-gal, cyanidin 3-glu, cyaniding 3-rut, epicatechin, hydroxybenzoic acid, ellagic acid and resveratrol were purchased from Extrasynthese (Genay, France). All reagents and standards were analytical and HPLC grades.

Five g of fruits from each treatment were extracted with 20 mL of methanol using an Ultra Turrax T25 model at 13500 rpm until uniform consistency. Samples were filtered (filter paper, 589 Schleicher) and TP content was determined by FC assay (with gallic acid as calibration standard) using a Shimadzu UV-1800 spectrophotometer (Columbia, MD, USA). The FC assay was carried out by pipetting 200 μ L of blueberry extract into a 10 mL PP tube. This step was followed by the addition of 1 mL of Folin-Ciocalteu's reagent. The mixture was vortexed for 20-30 s and 800 μ L of filtered 20% sodium carbonate solution was added after 1 min and before 8 min of addition of the FC reagent. This was recorded as time zero. The mixture was then vortexed for 20-30 s after addition of sodium carbonate. After 2 h at room temperature, the absorbance of the colored reaction product was measured at 765 nm. The TP content in the extracts was calculated from a standard calibration curve, built with different concentrations of gallic acid, ranging from 0 to 600 μ g mL⁻¹ (Correlation coefficient: $R^2 = 0.9994$). Results were expressed on the basis of mg of Gallic Acid Equivalent per kg (mg GAE kg⁻¹) on fresh weight.

The assay was based on the methodology of Benzie and Strain (1996). The Ferric Reducing Antioxidant Power (FRAP) reagent was prepared fresh containing

1mM 2,4,6-tripyridyl-2-triazine (TPTZ) and 2 mM ferric chloride in 0.25 M sodium acetate at pH 3.6. 100 μ L of the methanol extract prepared as above was added to 1900 μ L of FRAP reagent, and accurately mixed. After leaving the mixture at 20 °C for 4 min, the absorbance at 593 nm was determined by a Shimadzu UV-1800 spectrophotometer (Columbia, MD, USA). A standard curve (0-1200 μ g mL⁻¹ ferrous ion) obtained by the addition of freshly prepared ammonium ferrous sulfate, was employed for calibration. FRAP values were calculated as μ g mL⁻¹ ferrous ion (ferric reducing power) from three determinations and are presented as g kg⁻¹ of Fe²⁺E (ferrous ion equivalent) on fresh weight.

Five g of samples were homogenized until uniform consistency in a 20 mL meta-phosphoric and acetic acid solution. Ascorbic acid was determined following the ISO 6557-2 method.

High-performance liquid chromatography (HPLC) was used to separate and determine individual phenolic compounds in berry samples. After TP extraction, homogenized samples from the methanol extracts were filtered through cellulose acetate syringe filters (0.45 μ m). For each treatment, triplicate extractions and analyses were done. *P*-cumaric, chlorogenic, caffeic, ferulic and ellagic acid were separated and quantified using HPLC-DAD constituted by Jasco X-LC system, consisting of a PU-2080 model pump, multiwavelength detector (mod. MD-2015), autosampler (mod. AS-2055) and column oven (mod. CO-2060). ChromNAV Chromatography Data System software was used for result analyses. The separation of phenolic acids was achieved on a Tracer Extrasil OSD2 column (5 μ m, 250 x 4.6 mm), operating at 35 °C, at 1 mL/min flow rate. The mobile phase consisted of two solvents: 0.1% formic acid (A) and methanol (B). Gradient elution was as follows: 0-100% B over 50 min and held at 100%

B for an additional 10 min to clean up the column. Six wavelengths (250, 280, 310, 325, 370 and 510 nm) were used to detect eluent composition. HPLC analysis at 250 nm was used for hydroxybenzoic acid quantification; 280 nm for cinnamic and gallic acid and flavanols (catechin and epicatechin); 310 nm for *p*-cumaric acid and resveratrol; 325 nm for chlorogenic, caffeic and ferulic acid, 370 nm for ellagic acid and flavonols (quercetin-3-galattoside); 510 for anthocyanins (cyanidin-3-glucoside, cyanidin-3-rutinoside).

Five g of fresh sample were homogenized in distilled water (20 mL) with an Ultra Turrax T25 model at 13500 rpm. Samples were filtered (filter paper, 589 Schleicher) and appropriate aliquots of extracts were assayed by a Jasco X.LC HPLC system equipped with a PU-2080 pump, RI-2031 refractive index detector, AS-2055 autosample and CO-2060 column. ChromNAV Chromatography Data System was used as software. Sugars separation was achieved with a HyperRez XP Carbohydrate Ca⁺⁺ analytical column (7.7 mm x 300 mm, ThermoScientific), operating at 80 °C. Isocratic elution was effected using water at 0.6 mL min⁻¹ flow rate. Quantification of D-(+)-glucose and D-(-)-fructose was based on a calibration method. All standards utilized in the experiments were accurately weighed, dissolved in water and the calibration curves were generated with concentrations ranging from 100 mg L⁻¹ to 1000 mg L⁻¹ of standards.

Data obtained from the combination of 4 microperforations (NP, 0, 10 and 30 MPF) x 3 storage temperatures (4 °C, 10 °C and 18 °C) x 3 replications were statistically processed by ANOVA and means were separated by HSD Tukey Test at the significance level of $P \leq 0.05$. Statistical analysis was conducted within the same

sampling point. Letters are reported after the values only for significant differences. The results from the samples evaluated before storage (SP0) are reported as a reference.

2.4 RESULTS

Blueberry fruits have a high respiration rate (around 30 mg CO₂ kg⁻¹ h⁻¹ at 5 °C) (Salveit, 2004) that along with the transpiration process, limits shelf life and causes changes in quality attributes during storage. Regarding this, passive modified atmosphere packaging has the potential to control O₂/CO₂ rate throughout the marketing chain, slowing the metabolic activity of the fruit. During storage at 4 °C (Fig. 1), the atmosphere within packages averaged 20.07% O₂, 1.14% CO₂ and 20.05%, 1.22% for 10 MPF and 30 MPF respectively. For 1 MPF only 18.9% O₂ and 2.51% CO₂ were detected, resulting from a more intense respiration and lower exchange rate with the outside atmosphere. During storage, blueberries in 1 MPF film were surrounded by little O₂ because of the specific film oxygen permeability and the single perforation that limited O₂ diffusion through the film from the external atmosphere. As temperature strongly affects fruit respiration, 1 MPF pattern increased along with higher temperatures, probably because the respiration rate did not change proportionally with the amount of oxygen that passed through the film (oxygen transmission rate), even with 30 MPF. A decrease of 2 and 4 days of blueberry shelf life (for 10 °C and 18 °C respectively), compared to the standard storage temperature (4 °C), was reported as a consequence of temperature effect. In spite of this, blueberry shelf life at 18 °C was extended up to 12 days by using microperforated film, which created a more suitable

atmosphere within the package with an oxygen level around 17% (on average) and CO₂ level close to 4%. The selective permeability to O₂ and CO₂ of the film over storage time, combined with the physiological activity of the fruits, allowed the system to achieve atmosphere equilibrium that slightly differed among the samples based on the number of perforations. 1 MPF reached the highest O₂ consumption/CO₂ production (15.5%/6.38% on average) two days after packaging, resulting as very unstable during the storage period at all temperatures. However, even if 1 MPF created a very low gas permeability environment by increasing CO₂ content, anaerobic condition was not achieved (Kader et al., 1989). At 18 °C storage temperature, O₂ level reduced to 14% in the semi-closed package with only 1 MPF as a result of the film's specific barrier to O₂. However, little has been published on developing MA packaging that can cope with large increases in temperature.

Microperforations-related differences were only observed on dry matter content (DM%) at 10 °C (Fig. 2) and 18 °C, since the lowest temperature (4 °C), by decreasing fruit respiration rate and slowing the ripening process, did not show any significant differences, even if DM value increased during 16 days of storage. At both 4 °C and 18 °C, the control packages (NP) exhibited higher DM% than the microperforated samples and this could be attributed to a quite long storage period in the former case and to an increase of fruit metabolic activity, that was more accentuated in the final stage of the storage period, which led to a higher water loss in the latter. Therefore at SP4, DM content in NP fruits had increased by 25.3% (4 °C) and 8.66% (18 °C) since the beginning of the storage period, whereas MPF samples reached only 15.6% and 0.38% on average. By the end of storage period at 18 °C (12 days), all packaged treatments showed an apparent increase in DM%, highlighting the strong

effect of high temperature on water loss content, especially for blueberries with a higher respiration rate (NP and 30 MP).

Figure 3 shows the effect of different MPF and storage temperatures on pH and TSS content during blueberry storage. The pH value detected at the beginning of the storage period (2.93), which was in agreement with the finding of Kim et al. (1995) for ‘Coville’ blueberry, increased after 8 days of storage at 4 °C for all the samples, decreasing in the remainder of the period. Berries stored at 10 °C showed similar behavior, which was expressed as a steeper values decrease in the first five days and a consequent increase after three more days that led to significant differences ($P \leq 0.05$) among the samples at the end of the storage period. Thus, after 12 days, blueberries from 10 MPF showed the least acidic profile (3.03), which was 9.63% higher than both the control and 1 MPF. At the same temperature, considering the whole storage period, blueberries from the control showed lower pH values compared to those from microperforated packaging. This behavior could be supported by the fact that they were exposed to lower CO₂ concentration than the packaged fruits (atmosphere surrounding the control package was similar to air, with only 0.003% CO₂). Therefore the gas did not dissolve into the fruit cells, raising the pH as a direct consequence (Kader, 1999).

Previous findings (Zhao, 2007) showed that blueberries continue to respire after harvest with a consequent change in TSS composition. TSS in 10 MPF fruits were negatively affected by high temperature, showing the lowest amount at 18 °C (9.17° Brix) compared to the other temperatures (11.3° Brix on average). Moreover, the final soluble solids value recorded at 18 °C was 10.7% lower than the initial one and also 11.6% and 10.7% lower than 4 °C and 10 °C, respectively. As mentioned earlier, high storage temperature strongly affected fruit respiration rate, leading to sugars being

consumed as substrate. Sugar content in ripe blueberry is an equal mixture of glucose and fructose that continues to be produced by sucrose conversion during postharvest storage by hydrolysis from invertase activity (Kader et al., 1993). Generally TSS content and the ratio between organic acids are considered the best marker for consumer preference but, since fructose is sweeter than glucose, a higher amount is preferred in berry fruits. Even if no significant changes were detected during storage, the initial fructose value was 46 mg kg⁻¹ f.w. and it changed slightly; neither the storage temperature nor the microperforations had a significant effect on the concentration (data not reported). The initial glucose content (44.8 mg kg⁻¹ f.w. on fresh weight) was slightly less than fructose, but was instead higher ($P \leq 0.05$) for 10 MPF compared to the other samples after 14 days of storage at 10 °C. Moreover, microperforations at 4 °C affected glucose concentration, showing 54.5 mg kg⁻¹ f.w. for 1 MP compared to 43.8 mg kg⁻¹ f.w. for 30 MP.

Blueberry firmness showed an apparent increase during storage at 4 °C, from 27.2 N to above 40 N (data not reported). Significant differences were detected among microperforations ($P \leq 0.05$) at the same temperature. As firmness is also related to atmosphere gas composition, it has been demonstrated that fruits exposed to high levels of CO₂ increased in softness (Harb and Streif, 2004). In this regard, considering that CO₂ level for 30 MPF at 4 °C was lower than the other microperforated packaging, the retention of firmness can be explained by the creation of a suitable atmosphere within the packaging. With storage at 18 °C firmness was negatively affected by the clamshell package (NP) (even if no significant differences were detected) because of the high temperature that led to an increased ripening process and consequently the hydrolysis of starch to sugar and the degradation of cell wall components (Thompson, 1996). Storage

temperature therefore had a significant effect on fruit firmness and, although berries at 4 °C showed two days longer shelf life than those held at 10 °C, the plumpness of the latter resulted as being the highest. This outcome could be partly explained by hardening due to water loss as reported by Duan et al. (2010).

No significant differences were found between the control and blueberries from microperforated packaging in terms of L* value. Only temperature affected the brightness, which was significantly lower at 4 °C in NP samples. At the same temperature, the opposite behavior was detected for berries from 30 MPF which appeared much paler than those from the other two temperatures (data not shown). Blueberry color saturation (chroma) changed among microperforated packaging, showing more intense color for 1 MPF and NP fruits at 4 °C and 10 °C respectively (data not shown).

The values of blueberry antioxidant activity (AOA), as measured by the FRAP method, is presented in figure 4. Significant differences were shown only among microperforations at 18 °C, in which berries from 30 MPF had higher values than those from 10 MPF. Regarding storage temperature, all the samples were characterized by a similar pattern with a slight increase within 5 days (SP2), followed by a decline and a consequent stabilization of the value around the initial one detected immediately after harvest (7.79 g Fe²⁺E kg⁻¹ f.w.). The observation regarding the apparent stability of AOA during storage was in accordance with the findings of Connor et al. (2002) for 'Elliot' blueberry held at 5 °C, which indicated that blueberries benefits could be retained for a certain period of time after harvest. Higher temperatures facilitated blueberry spoilage, whereas shelf life extension and retention of AOA were obtained at

4 °C, probably because the low temperature prevented post-harvest biosynthesis and/or degradation of phenolic compounds, avoiding changes in antioxidant activity during storage (Jin et al., 2011). Moreover, the prolonged storage time and the higher amount of water loss might have facilitated the accumulation of antioxidant compounds.

It is widely known that blueberries are an important source of natural antioxidant compounds and for this reason, they have been included in a special category of functional food (Mazza et al., 2002). Nevertheless, the amount of total phenols can be strongly affected by the selection of post-harvest technique that might modify fruits metabolism and consequently the qualitative, nutritional and organoleptic aspects related to phenols concentrations. In the present work, microperforated films partially influenced total phenolic content of blueberry stored at 18 °C (Fig. 5), in which berries from 30 MPF showed the highest phenolic content (947 mg GAE kg⁻¹ f.w.) after 5 days (29.6% more than NP blueberries). A high level was reached by NP blueberries throughout the storage period, even if no significant differences were detected. Zheng et al. (2003) reported that several works have shown high levels of total phenolic compounds during post-harvest storage under elevated O₂ concentration in different berry fruits. In our case, the atmosphere was not enriched with O₂, but both NP and 30 MPF fruits were exposed to a higher oxygen level than the other samples and this could partly explain the phenolic pattern. Temperature is also a basic factor in preserving berry quality during storage and a significant difference in total phenols content was detected in fruits stored at higher temperatures which showed lower phenols content than those at 4 °C, possibly because of the spoilage process that, starting earlier, interfered with the accumulation process.

Ascorbic acid (AA) is quite susceptible to post-harvest losses, which are generally accelerated by longer storage times and high temperatures (Lee and Kader, 2000), so its quantification can be considered a valid indicator of fruit freshness. Indeed, storage temperature had a significant impact on AA concentration among the different levels of perforation, especially at the beginning of the storage period (Fig. 6), even if the outcomes of the present study disagreed with the finding reported by Lee and Kader (2000). Fruits from control and microperforated packaging, with the exception of 10 MPF, showed higher vitamin C content at 10 and 18 °C (68.2 mg 100 g⁻¹ f.w. on average) than those stored at 4 °C (62.5 mg 100 g⁻¹ f.w. on average). For all samples a sharp decrease was detected at day 8 (SP3), followed by an increase in the remaining storage time. Significant differences in AA content based on the effect of storage temperature were also detected for blueberries from 10 and 30 MPF at the final sampling point, in which vitamin C losses were promoted at higher temperatures. The higher AA content for blueberries stored at 4 °C could also be explained by the fact that these fruits had a longer shelf life and therefore a considerable water loss that caused the consequent AA concentration. Considering the effect of the different levels of perforations on AA, the only significant evidence of higher AA content was detected for wrapped fruits (data not shown), probably because of the utilization of PP film that reduced water stress and consequently the loss of ascorbic acid (Nunes et al., 1998).

HPLC analysis identified and quantified the phenolic compounds listed in tables 1 and 2, belonging to the group of flavonols, anthocyanins, flavanols and phenolic acids. Ellagic acid and resveratrol were also detected. Polyphenols account for the majority of antioxidant activity in fruits when compared with ascorbic acid, and their antioxidant properties are mainly due to the redox properties, which allow them to

act as reducing agents, hydrogen donators and singlet oxygen quenchers (Kaur et al., 2001). Among all the anthocyanins contained in blueberries, cyanidin 3-rutinoside and cyanidin 3-glucoside (the most commonly occurring anthocyanins pigment in nature) were predominant, with the former having an initial value of 310 mg kg⁻¹ on fresh weight that tended to increase within the storage period at all temperatures (Tab. 1). As reported in other studies (Kalt et al., 1996; Kalt et al., 2003), anthocyanins concentration increased during blueberry ripening, showing the highest value at advanced stage of ripeness. Both anthocyanins were affected more by temperature than by microperforations, even if the latter had some effect on both anthocyanins.

In the middle of storage period, within 5 and 8 days, significant differences were observed at 10 °C, when the highest cyanidin 3-glucoside content was recorded in berries from the control and 30 MPF, whereas cyanidin 3-rutinoside showed the highest value for 10 MPF. The lowest amount detected for berries from 1 MPF could be explained by the effect of the relatively high CO₂ concentration, which inhibited anthocyanins biosynthesis and accumulation. Studies on strawberry (Gil et al., 1997; Holcroft and Kader, 1999) reported that relatively high CO₂ levels promoted anthocyanins loss during storage and this finding was in agreement with the lowest amount of cyanidin 3-glucoside observed for blueberries packaged with only 1 MPF, in which the CO₂ level reached 3.89% (compared to 0.03%, 1.72% and 1.54% for NP, 10 and 30 MPF, respectively). Storage temperature did not have any significant effect on the control, whereas all blueberries from microperforated packages showed a higher amount of anthocyanins at 10 °C. Controlled stress, as a storage temperature of 10 °C, could explain such enhancement of anthocyanins which is also confirmed by Kalt et al. (1999), who showed that berries stored at temperatures above 0 °C had a higher amount

of phenolic compounds. The same result was not obtained at 4 °C and 18 °C, possibly because temperatures negatively affected post-harvest phenolic synthesis. Besides anthocyanins, flavonols are the other class of phenolic compounds that strongly characterize blueberry nutritional composition. Myricetin and quercetin, are predominant, followed by small amounts of other compounds such as chlorogenic acid (hydroxycinnamate) and stilbenes (Rimando et al., 2004). Quercetin 3-galactoside, located predominantly in the skin, showed some significant differences among temperatures, sharply dropping at 18 °C at the end of the storage period and confirming the negative impact of high temperature on berry nutritional content. Considering the distinct levels of perforation, different results have been obtained based on temperature and sampling day. For instance, at 18 °C the highest amount was recorded for the control, 1 and 10 MPF two days after the harvest, whereas at the end of storage the same pattern was achieved with NP and 30 MPF. The hydroxibenzoic, caffeic, coumaric and ferulic acids detected in the study, have mostly been affected by temperature (Tab. 2). At 18 °C the final value of caffeic acid was 3.21 mg kg⁻¹ f.w. and was significantly lower than the 6.09 mg kg⁻¹ f.w. and 6.16 mg kg⁻¹ f.w. detected in SP4 at 4 °C and 10 °C, after 16 and 14 days respectively. It is widely known that flavonoids biosynthesis is closely associated with fruit development stages and this might explain the considerable changes that took place especially at the end of the storage period. Regarding this, the finding of two phenol-decomposing microorganisms (*Bacterium album* and *Pseudomonas aeruginosa*) by Ermolaev et al. (1975) could provide a further explanation for the phenolic degradation at high temperature, even if no bacteria detection was carried out in the present study. Moreover, it has been demonstrated that *Pseudomonas* spp., whose growth is accelerated by high temperature, have a primary

role in the process of fruit spoilage since they occur naturally in soil, water, and consequently on fruit and vegetable peel (Franzetti et al., 2006). As regards resveratrol, this is considered one of the most valuable natural compounds in fruits as it has a strong antioxidant activity. Blueberries are a very rich source of this stilbene (20.3 mg kg⁻¹ f.w. on average were detected in the present study), which can be strongly compromised by inappropriate storage conditions. However, different treatments or stress such as high temperature, UV irradiation, and application of abiotic stresses can be useful in order to maintain its concentration during the storage period. In the current project, temperatures affected resveratrol concentration, showing the higher value at 4 °C and 10 °C but only for the packaged samples (1, 10 and 30 MPF).

2.5 CONCLUSION

The results from this study demonstrated the importance of selecting the proper packaging and storage conditions in order to preserve the quality and freshness of blueberry fruits. The creation of a suitable environment for the fruits, which retained nutritional value and qualitative aspects, was achieved by the use of polypropylene film with different levels of microperforations. Passive modified atmosphere packaging can be considered a good and low-cost alternative to the traditional packaging for blueberry preservation, since a slight improvement on firmness, antioxidant activity and total phenols has been detected. Moreover, since fruit respiration is considered the most important aspect to take in account when a new packaging is developed, in this study the atmosphere equilibrium achieved within the packaging played a key role in

determining the more suitable packaging for these perishable fruits. In this regard, fruits from microperforated packaging reached equilibrium after only 2 days of storage and the gas concentration was maintained constant for the remainder of the period. Although PP films maintained fruits qualitative aspects, demonstrating that blueberries continued to satisfy consumer demand for fresh highly-nutritional fruit, temperature was the basic factor affecting the shelf life. The lowest temperature extended blueberry shelf life up to 16 days, while at 10 °C and 18 °C the shelf life was limited to 14 and 12 days, respectively. Even if positive results have been achieved by using microperforated film, further investigation could be necessary to improve the use of this technology in post-harvest handling for fresh commodities.

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2.7 FIGURES AND TABLES

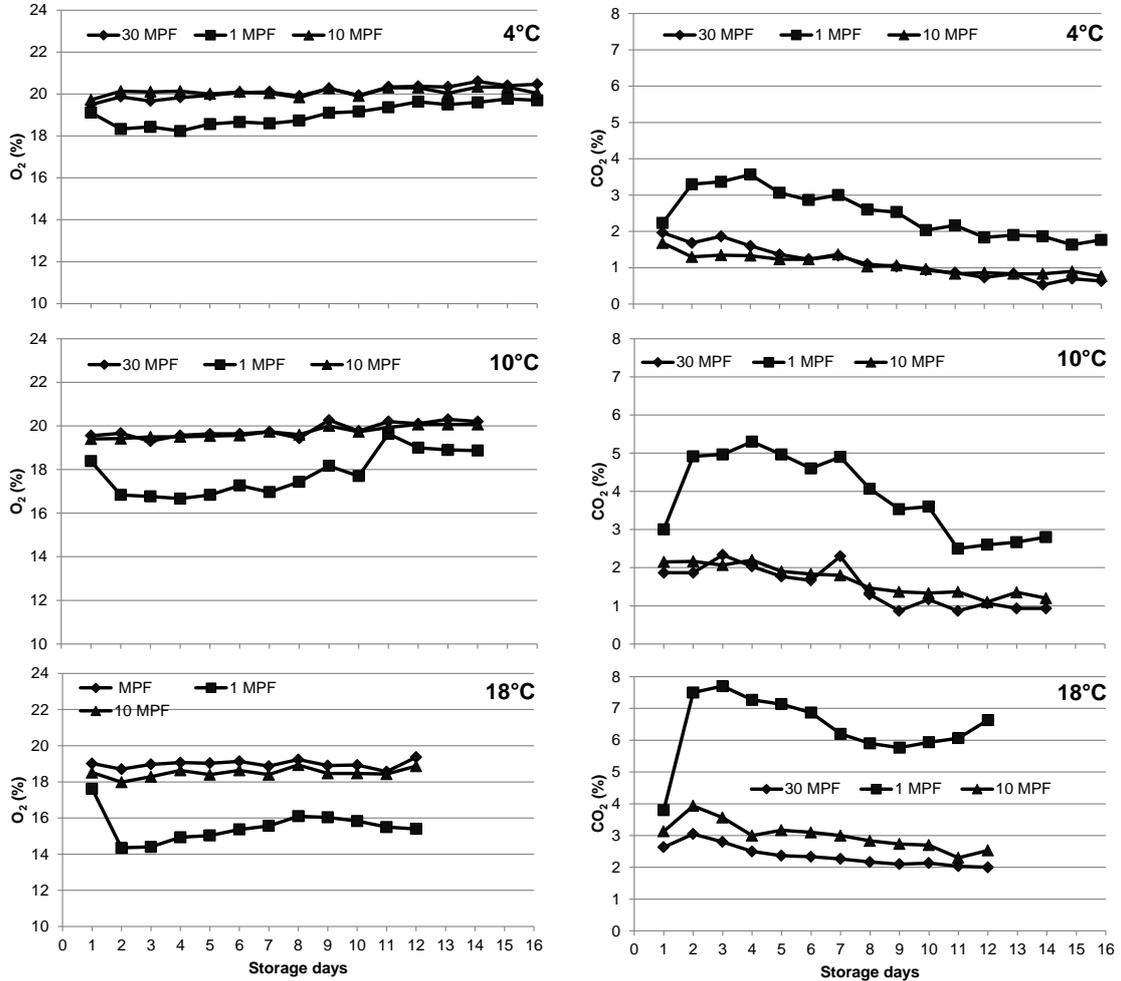


Figure 1. Effect of microperforations (1, 10 and 30 MPF) on percentage O₂ and CO₂ in blueberries stored at different temperatures (4 °C, 10 °C and 18 °C). Within the same sampling point, treatments with no letter in common differ significantly at $p \leq 0.05$ (Tukey HSD test).

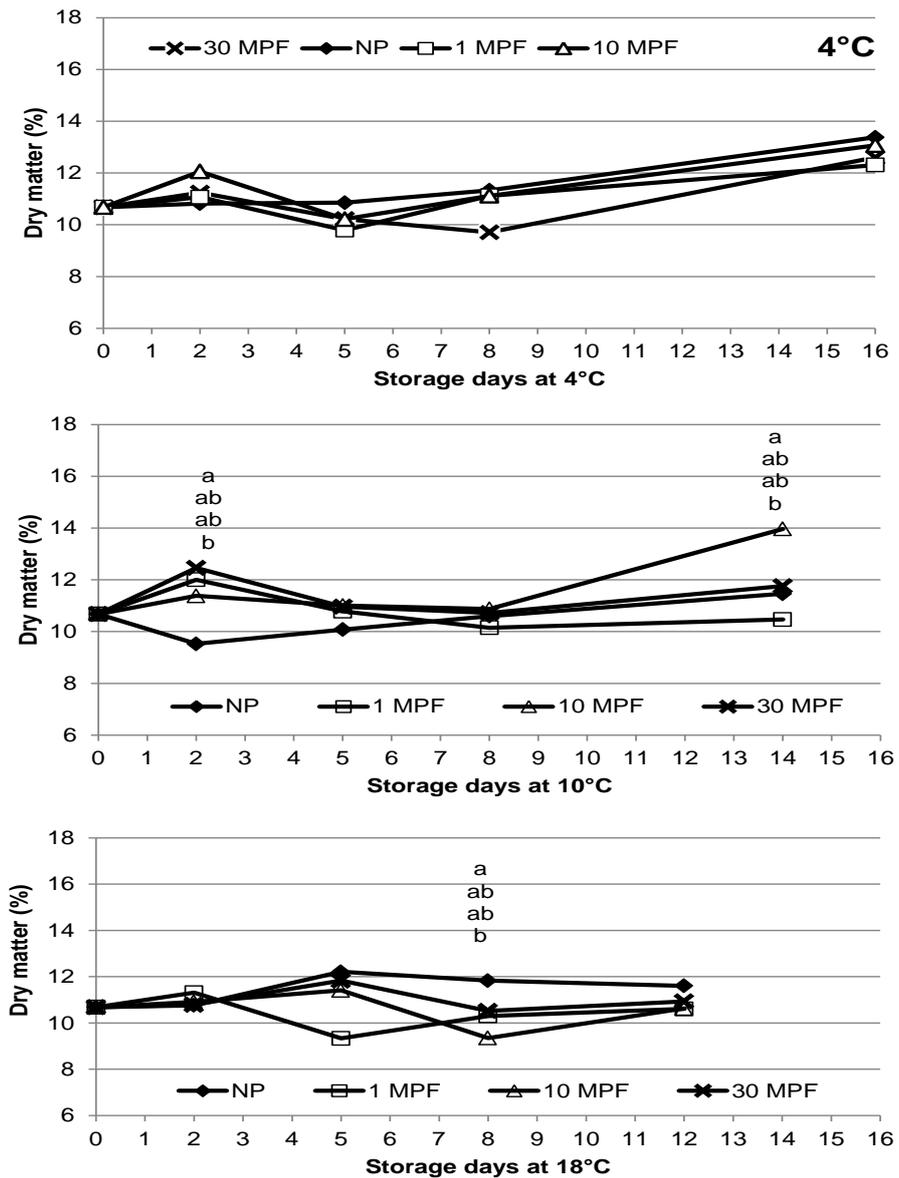
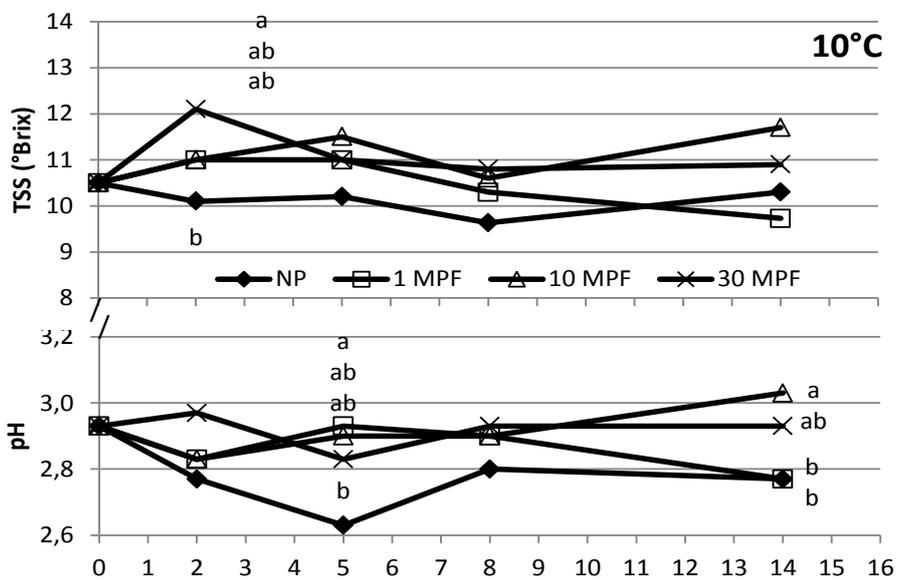
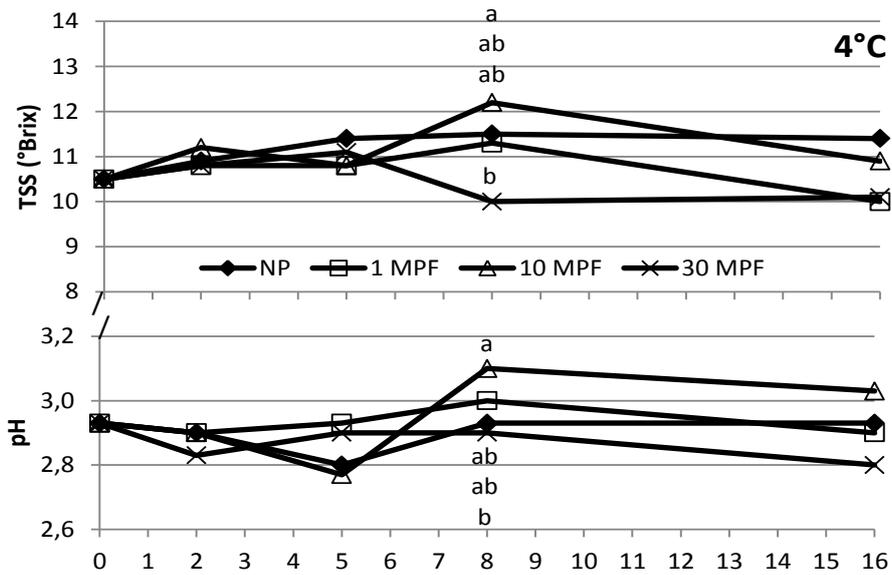


Figure 2. Effect of microperforations (1, 10 and 30 MPF) on dry matter content (DM%) in blueberry compared to non-packaged-clamshell control fruits (NP) stored at different temperatures (4 °C, 10 °C and 18 °C). Within the same sampling point, treatments with no letter in common differ significantly at $p \leq 0.05$ (Tukey HSD test).



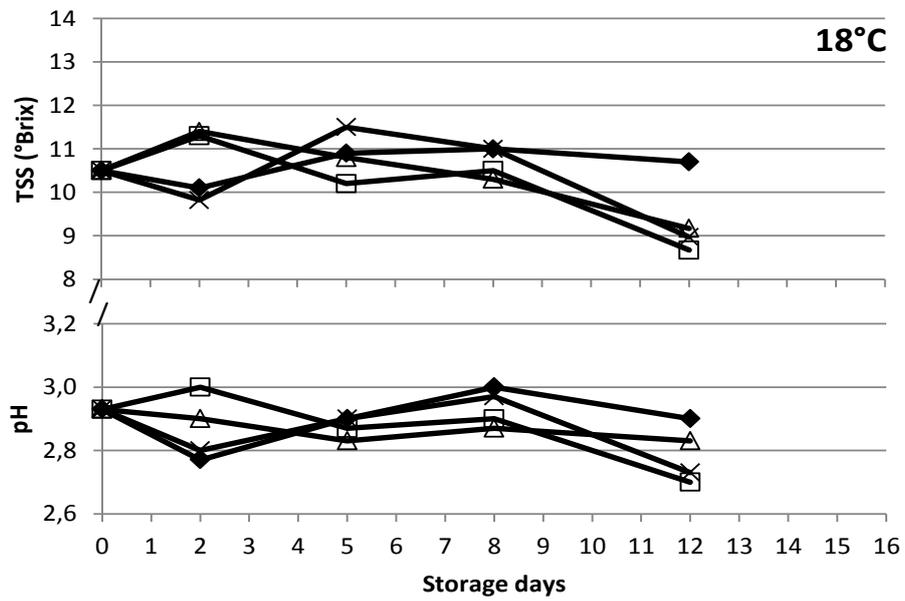


Figure 3. Effect of microperforations (1, 10 and 30 MPF) on blueberry pH and total soluble solids compared to non-packaged-clamshell control fruits (NP) at different storage temperatures (4 °C, 10 °C and 18 °C). Within the same sampling point, treatments with no letter in common differ significantly at $p \leq 0.05$ (Tukey HSD test).

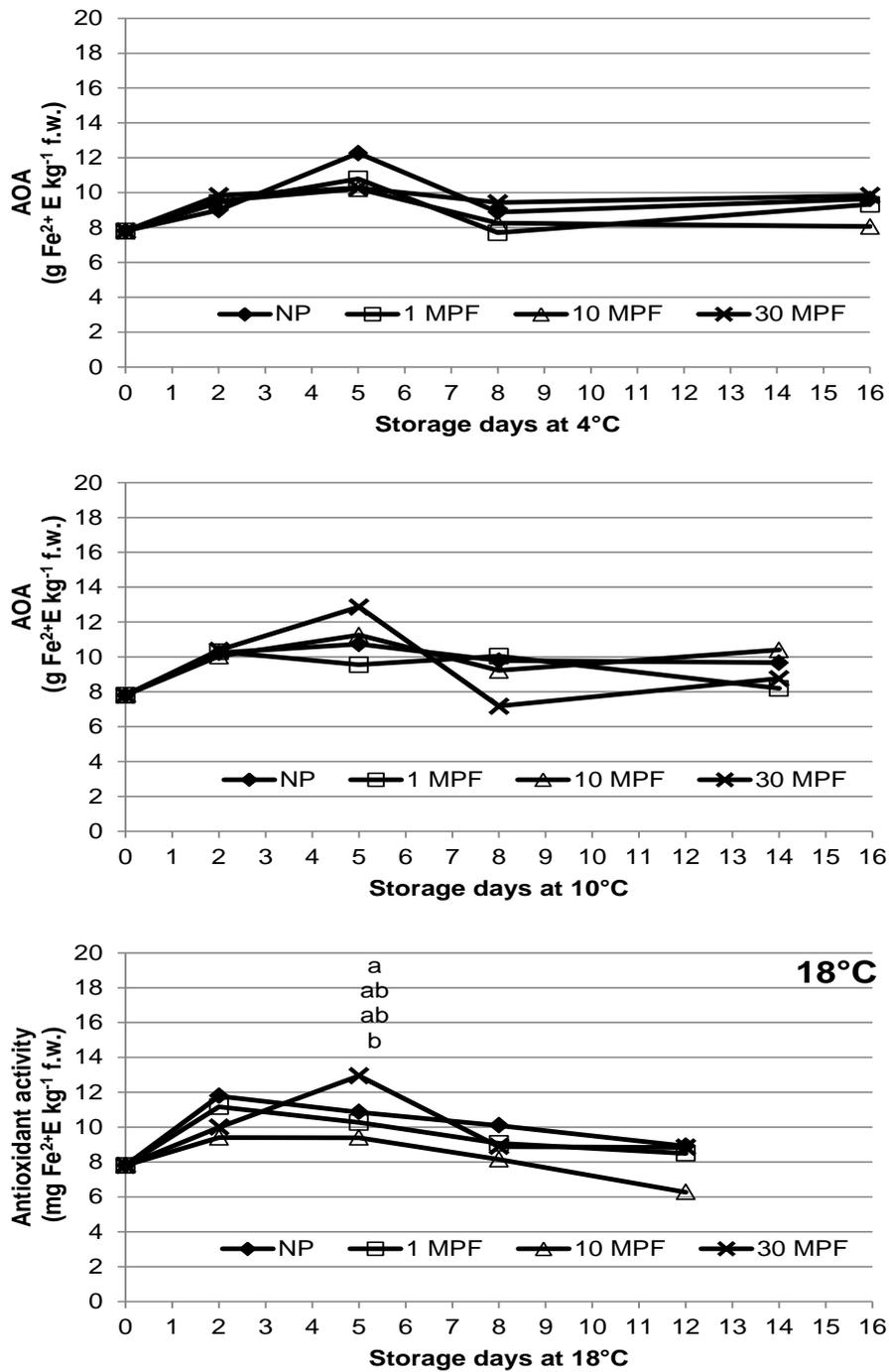


Figure 4. Effect of microperforations (1, 10 and 30 MPF) on blueberry antioxidant activity compared to non-packaged-clamshell control fruits (NP) at different storage temperatures (4 °C, 10 °C and 18 °C). Within the same sampling point, treatments with no letter in common differ significantly at $p \leq 0.05$ (Tukey HSD test).

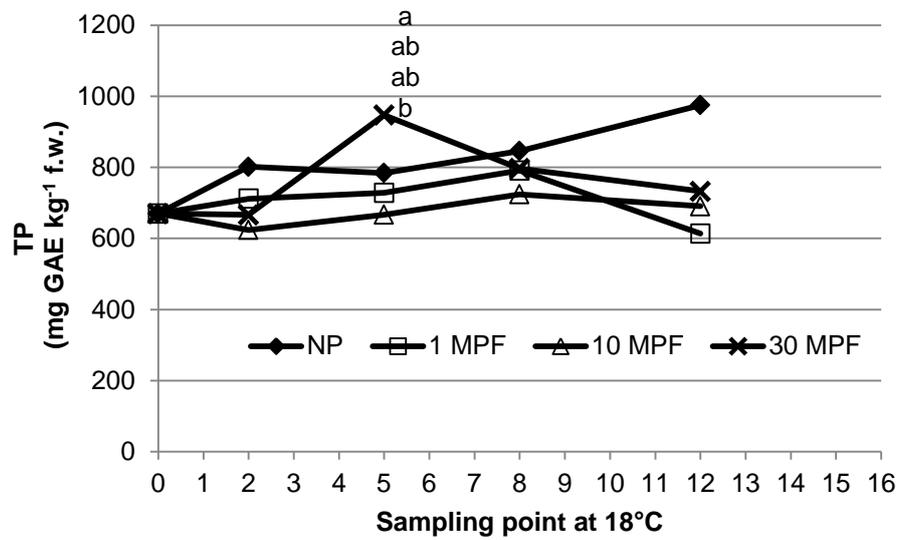


Figure 5. Effect of microperforations (MPF) at 18 °C in blueberry total phenols compared to non-packaged-clamshell control fruits (NP) during 12 days of storage. Within the same sampling point, treatments with no letter in common differ significantly at $p \leq 0.05$ (Tukey HSD test).

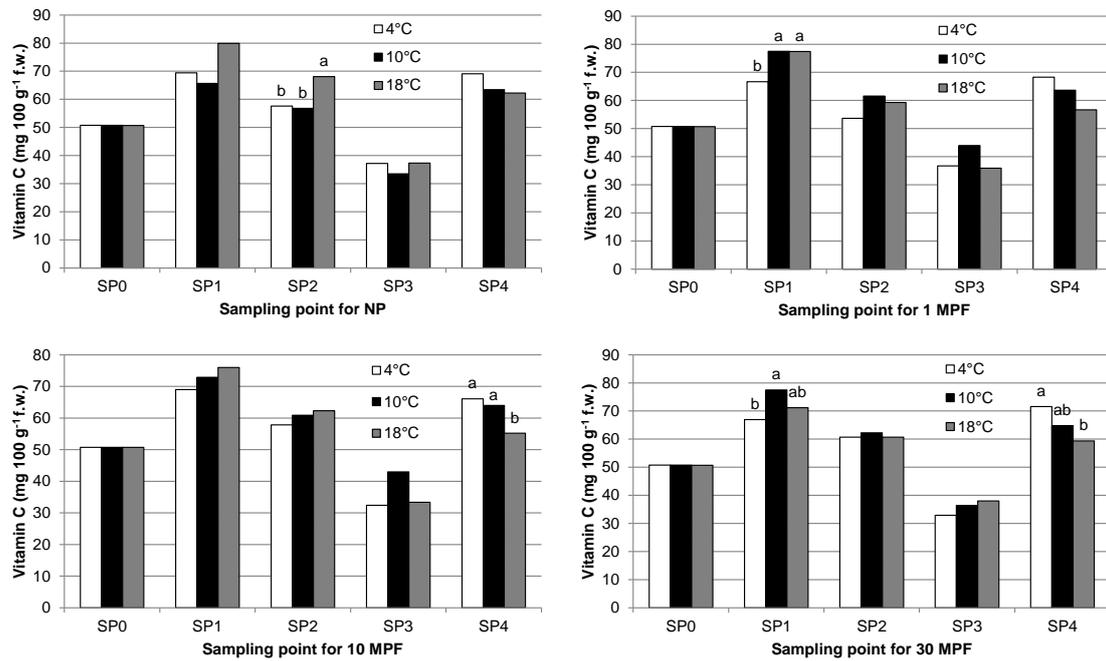


Figure 6. Effect of temperature on vitamin C content in non-packaged clamshell control (A), 1 MPF (B), 10 MPF (C), and 30 MPF (D) blueberry during storage period. SP0 = day 0 (before storage); SP1 = 2 days of storage; SP2 = 5 days; SP3 = 8 days. SP4 at 4 °C = 16 days, SP4 at 10 °C = 14 days, SP4 at 18 °C = 12 days. Within the same sampling point, treatments with no letter in common differ significantly at $p \leq 0.05$ (Tukey HSD test).

Table 1. Effect of microperforations (1, 10 and 30 MPF) on quercetin 3-galactoside, anthocyanins and epicatechin content in blueberry compared to non-packaged-clamshell control fruits (NP). Values were obtained before storage (SP0), and after 2 (SP1), 5 (SP2), and 8 (SP3) days for all samples. SP4 was carried out after 8, 6 and 4 additional days at 4°C, 10°C, and 18°C, respectively.

		4°C				10°C				18°C				
MP		Storage period												
		0	1	2	3	4	1	2	3	4	1	2	3	4
Querc 3-gal	0	2.86	1.73	2.12	2.94	3.00	1.92 ^{ab}	2.76	2.36	2.12 ^a	2.49	2.88	2.98 ^a	
	1	2.36	2.58	2.75	2.63	2.90	1.37 ^b	1.40	3.11	2.60 ^a	2.26	1.51	1.66 ^b	
	10	2.52	2.34	2.67	2.03	4.37	2.55 ^a	2.60	2.47	2.38 ^a	1.67	2.15	0.23 ^c	
	30	2.62	2.59	2.07	1.80	2.15	2.53 ^b	1.89	2.09	0.46 ^b	0.24	0.51	2.59 ^a	
Cyan 3-glu	0	115	110	138	125	115	90 ^{bc}	118	119	112	126	135	103	
	1	117	115	114	118	137	84.8 ^c	101	139	139	110	96.1	70.1	
	10	123	121	109	102	108	115 ^{ab}	118	132	116	100	94.0	41.3	
	30	96.2	131	101	130	105	117 ^a	103	138	130	100	110	88.1	
Cyan 3-rut	0	312	319	343	368	451	290	326 ^a	325	312	380	373	313 ^a	
	1	344	322	342	347	362	199	281 ^{ab}	372	398	318	285	226 ^{ab}	
	10	366	339	280	268	327	329	325 ^a	379	355	262	288	120 ^b	
	30	337	383	336	342	312	342	243 ^b	399	330	196	322	350 ^a	
Epic	0	36.0	36.7	43.7	55.1 ^a	42.1	34.3	46.7	36.2	38.2	42.6	23.4	35.3	
	1	28.9	29.1	27.3	44.1 ^{ab}	33.8	36.2	42.6	36.2	39.0	35.0	25.8	30.0	
	10	31.2	32.4	27.7	33.0 ^{ab}	28.9	28.6	35.8	41.6	36.7	30.8	36.2	16.0	
	30	28.4	30.6	42.7	28.0 ^b	30.8	25.5	34.2	40.9	24.8	19.5	13.2	35.6	

Within the same sampling point, treatments with no letter in common differ significantly at $p \leq 0.05$ (Tukey HSD test).

Table 2 - Effect of microperforations (1, 10 and 30 MPF) on phenolic acids, ellagic acid, and resveratrol content in blueberry compared to non-packaged-clamshell control fruits (NP). Values were obtained before storage (SP0), and after 2 (SP1), 5 (SP2), and 8 (SP3) days for all samples. SP4 was carried out after 8, 6 and 4 additional days at 4°C, 10°C, and 18°C, respectively.

Parameter (mg kg ⁻¹ f.w.) MP		4°C				10°C				18°C				
		Storage period												
		0	1	2	3	4	1	2	3	4	1	2	3	4
Hydroxybc acid	0	0.60	0.51	0.69	0.27	0.74	0.46	0.64	0.72	0.51 ^b	0.56 ^b	0.71	0.76	
	1	0.59	0.81	0.62	0.78	0.52	0.80	0.26	0.59	1.04	0.70 ^b	0.61 ^b	0.48	0.73
	10		0.67	0.50	0.75	0.69	0.54	0.70	0.67	0.56	0.42 ^b	0.56 ^b	0.48	0.23
	30		0.42	0.61	0.66	0.77	0.43	0.53	0.57	0.93	1.35 ^a	0.97 ^a	1.24	0.76
Ferulic acid	0	5.76 ^a	4.91	5.10	5.50	3.77	3.14	5.66	4.41	4.98	4.74	5.52	4.08	
	1	4.40	5.32 ^{ab}	3.19	4.52	4.15	5.59	3.30	4.44	5.05	5.30	4.29	5.83	3.36
	10		3.51 ^{bc}	3.74	4.03	3.92	4.71	4.80	4.75	4.69	4.21	3.94	4.54	1.88
	30		2.93 ^c	4.14	3.94	3.92	2.78	4.79	4.08	6.56	5.91	3.23	4.49	4.16
Ellagic acid	0	3.43 ^b	11.9	19.6	15.4	7.22	9.34	16.1	13.1	11.6 ^a	13.1	16.8	13.5	
	1	9.38	10.4 ^a	10.5	15.5	12.0	13.5	8.97	14.0	14.1	13.1 ^a	10.1	9.74	7.31
	10		10.8 ^a	10.6	17.6	10.1	9.03	9.44	16.9	14.0	10.5 ^a	11.9	13.9	5.08
	30		8.18 ^{ab}	11.1	14.4	12.4	7.96	11.8	15.5	16.7	2.89 ^b	10.8	11.7	7.17
Resveratrol	0	16.3	17.8	19.0	18.5	24.6	16.0	20.1	19.5	18.6	21.3	16.7	20.8 ^a	
	1	20.3	22.4	20.9	24.8	19.8	23.3	12.1	16.1	29.0	22.6	18.7	14.7	12.1 ^{ab}
	10		20.4	20.0	21.4	19.5	19.9	21.2	19.4	19.2	19.5	15.4	14.7	6.31 ^b
	30		17.2	20.1	20.1	21.7	17.5	18.0	16.1	23.0	20.1	13.7	19.6	14.9 ^{ab}

Within the same sampling point, treatments with no letter in common differ significantly at $p \leq 0.05$ (Tukey HSD test).

3 INCREASING STRAWBERRY SHELF-LIFE WITH CARVACROL AND METHYL CINNAMATE ANTIMICROBIAL VAPORS RELEASED FROM EDIBLE FILMS

3.1. ABSTRACT

The effect of carvacrol and methyl cinnamate vapors incorporated into strawberry puree edible films on the post-harvest quality of strawberry fruit (*Fragaria x ananassa*) was investigated. Fresh strawberries were packed in clamshell and kept at 10 °C for 10 days with 90% relative humidity. Strawberry puree edible films, applied in the clamshell, served as carriers for the controlled release of the natural antimicrobial compounds without the direct contact with the fruit. Changes in weight loss, visible decay, firmness, surface color, total soluble solids content, total soluble phenolic content and antioxidant capacity of strawberries during storage were evaluated. A significant delay and reduction in the severity of visible decay was observed in fruit exposed to antimicrobial vapors. Carvacrol and methyl cinnamate vapors released from the films helped to maintain firmness and brightness of strawberries as compare to the not-treated strawberries. The natural antimicrobial vapors also increased the total soluble phenolic content and antioxidant activity of fruit at the end of the storage period.

3.2. INTRODUCTION

Strawberry is a perishable fruit characterized by high respiration and metabolic rates that limit its shelf-life (Atress et al., 2010). Rapid deterioration and post-harvest losses are mainly caused by improper storage temperature and microbial spoilage. Fungi are ubiquitous microorganisms with a great capacity to colonize many kinds of substrates and to proliferate under common environmental storage conditions, such as low temperature (El-Shiekh et al., 2012). Among all, grey mold caused by *Botrytis cinerea* is considered the most common disease affecting strawberries (Wang, 2003). Therefore, reducing microbial spoilage plays a key role to prolong the shelf-life of fresh strawberries as well as preserve their quality attributes during storage. Even though rapid cooling after harvest and low storage temperatures are usually applied because of their effects to reduce the rates of biological reactions and microbial growth (Kader and Saltveit, 2002), other techniques must be combined with refrigeration in order to maintain quality and delay strawberry decay. A wide range of different approaches, mainly based on the application of modify atmosphere packaging (MAP) has been developed. However, traditional MAP is not enough to ensure final product quality and safety (Serrano et al., 2008). Among the various alternatives, edible films made from fruit and vegetables may be considered a valid and effective way to preserve quality of fresh fruit and vegetables since they act as a selective barrier to moisture transfer, limiting therefore water loss, and protecting fresh fruit to external hazards. Edible films may also retard loss of volatile compounds, reduce respiration rate, and delay changes on fruit physical properties. The ability of edible films to extend the shelf-life of fresh

food products may be further improved by including antimicrobial plant essential oils (EOs) for controlling pathogenic microorganisms.

Methyl cinnamate is a methyl ester of cinnamic acid and is one of the major volatile components of strawberry aroma produced and released during fruit maturation (Lunkenbein et al., 2006). Because of the sweet fruity flavor, methyl cinnamate is commonly used in many applications as ingredient in decorative cosmetics and fragrances (Ali et al., 2010) and, since it is generally recognized as safe (GRAS), it may also be potentially used as food additive (Huang et al., 2009). Methyl cinnamate has been proven to have antifungal activity against phytopathogenic fungi under vitro conditions (Vaughn et al., 1993; Rahmani et al., 2010); nevertheless its utilization on shelf-life studies of fresh products has never been investigated. In this study, it was used in combination with carvacrol (the major component of essential oils from oregano and thyme) because of its well known powerful antimicrobial properties (Lambert et al., 2001; Burt and Reinders, 2003).

EOs, and their aromatic volatile components, have been largely investigated for their antimicrobial properties in vitro as vapors (Du et al., 2009; Avila-Sosa et al., 2012; Kloucek et al., 2012) as well as on direct contact with food product (Hammer et al., 1999; Friedman et al., 2002; Burt, 2004; Holley and Patel, 2004). Few studies reported the beneficial effects of essential oil treatments on strawberry quality (Reddy et al., 1999; Tzortzakis, 2007; Wang et al., 2007). However, to the best of our knowledge, this is the first study to investigate the effect of essential oil vapors released from edible films on shelf-life and quality of strawberries without direct contact with the fruit. Furthermore, since most of the volatiles are characterized by a strong flavor that clashes with the natural characteristic of the fruit, the selection of strawberry puree edible film

and methyl cinnamate was made on the basis of the organoleptic compatibility with strawberry fruit.

Considering the potential use of volatile compounds as fumigants for the storage of fresh product, the aim of this study was to determine the effectiveness of a new approach based on the use of strawberry puree edible films for the controlled release of antimicrobial carvacrol and methyl cinnamate vapors during storage on strawberry shelf-life and overall quality.

3.3. MATERIAL AND METHODS

Seedless strawberry puree (Sabroso Co., Medford, OR, USA) was used as primary ingredient in strawberry puree edible films. High methoxyl pectin 1400 (TIC Gums, Belcamp, Md, USA) was added to increase films strength, create a semi-permeable film, and facilitate the release from cast surface. Vegetable glycerine (Starwest Botanicals Inc., Rancho Cordova, CA, USA) was used as a plasticizer agent. Carvacrol and methyl cinnamate were the active compounds tested and, along with Folin-Ciocalteu phenol reagent, sodium carbonate anhydrous, 1-diphenyl-2-picrylhydrazyl (DPPH), and Trolox, were purchased from Sigma-Aldrich (St. Louis, MO, USA). Gallic acid monohydrate and methanol were obtained from Fisher Scientific (Pittsburgh, PA, USA) and ethyl alcohol from Pharmco-Aaper (Oakland, CA, USA). Strawberry puree solution was obtained by combining 490 g of strawberry puree (49% w/w) with 500 g of 3% w/w pectin solution and 10 g (1% w/w) of glycerine in a mixer bowl at slow speed for 30 min according to McHugh and Senesi (2000). C and MC

were then incorporated at 0.75% (w/w) and homogenized for 15 min at 20000 rpm using a Polytron 3000 homogenizer (Kinematica, Luzern, Switzerland). Methyl cinnamate, because of its insolubility in water, was previously dissolved in ethanol (50% w/w) for 15 min at 200 rpm on a stirring plate. The solutions were degassed under vacuum for almost one hour to remove bubbles and then used for film casting. The concentration of volatile compounds was chosen based on the results obtained from preliminary studies (data not shown) in which different concentrations of carvacrol and methyl cinnamate were tested on visual appearance, decay, and weight loss of strawberries. Incorporation of both carvacrol and methyl cinnamate in strawberry puree film forming solution at 0.75% (w/w) showed the most promising results and therefore they were used for this experiment.

Films were cast on 29 x 29 cm glass plates covered with polyester film to facilitate the removal of dry films after ~15 h at room temperature. A 35 mil (1 mil = 0.0254 mm) gap draw down stainless steel bar was used to spread 55 g of strawberry puree solution on each plate. The quantity of the solution poured on the plate was chosen based on previous experiment in order to obtain a constant thickness of the film in the whole surface. Films were then cut into 14.5 x 8 cm patches and used for the treatments. Some of the films were stored on layers of aluminum foil in zip plastic bags at 4 °C and 65% RH until physical-chemical and mechanical properties were evaluated. Two film patches were then taped on the top and bottom of PET clamshell. The clamshells were previously modified by taping the holes and placing a second PET layer (with 14 holes; 0.6 cm in diameter) inside the clamshell at 2 cm from the bottom. This arrangement was made to allow the release of vapors from the films without touching the fruit.

3.3.1. Physical properties of strawberry puree edible film

Film thickness was measured with a micrometer IP 65 (Mitutoyo Manufacturing, Tokyo, JAPAN) to the nearest 0.00254 mm (0.0001 in.) at five random positions around the film. Mean value was used to calculate water vapor permeability (WVP) and tensile strength.

The gravimetric Modified Cup Method (McHugh et al., 1993) based on standard method E96-80 (ASTM, 1989) was used to determine WVP. A cabinet with a variable speed fan was used to test film WVP. Cabinet temperature of 25 ± 1 °C was maintained in a Forma Scientific reach-in incubator (Thermo Electron Corp., Waltham, MA). Fan speeds were set to achieve air velocities of 152 m/min to ensure uniform relative humidity throughout the cabinets. Cabinets were pre-equilibrated to 0% relative humidity (RH) using anhydrous calcium sulphate (W.A. Hammond Drierite, Xenia, OH). Circular test cups made from polymethylmethacrylate (Plexiglas TM) were used. A film was sealed to the cup base with a ring containing a 19.6 cm² opening using 4 screws symmetrically located around the cup circumference. Both sides of the cup contacting the film were coated with silicon sealant. Distilled water (6 mL) was placed in the bottom of the test cups to expose the film to a high percentage RH inside the test cups. Average stagnant air gap heights between the water surface and the film were measured. Test cups holding films were then inserted into the pre-equilibrated 0% RH desiccator cabinets. Steady state of water vapor transmission rate was achieved within 2 h. Each cup was weighed 8 times at 2 h intervals. Eight replicates of each film were tested. Relative humidity at the film undersides and WVPs were calculated using the WVP Correction Method (McHugh et al., 1993). The WVP of the films was calculated

by multiplying the steady state water vapor transmission rate by the average film thickness determined as described above and dividing by the water vapor partial pressure difference across the films: $WVP = (WVTR * thickness) / (p_{A1} - p_{A2})$, where WVTR is water vapor transmission rate, p_{A1} and p_{A2} are water vapor partial pressure inside and outside the cup, respectively. Units for WVP were $g\ mm/kPa\ h\ m^2$.

In the present work, mechanical properties of strawberry puree edible films incorporated with volatile compounds were tested and compared with strawberry puree film to evaluate the effects of carvacrol and methyl cinnamate may have on physical-mechanical properties of films. Standard method D882-97 (ASTM, 1997) was used to measure tensile properties of films. Films were cut into strips with a test dimension of 165 mm x 19 mm according to standard method D638-02a (ASTM, 2002). All films were conditioned for 48 h at $23 \pm 2\ ^\circ C$ and $50 \pm 2\%$ RH before testing by a saturated salt solution of magnesium nitrate (Fisher Scientific, Fair Lawn, NJ, USA). The ends of the equilibrated strips were mounted and clamped with pneumatic grips on an Instron Model 55R4502 Universal Testing Machine (Instron, Canton, MA, USA) with a 100 N load cell. The initial gauge length was set to 100 mm and films were stretched using a crosshead speed of 7.5 mm/min. Tensile properties were calculated from the plot of stress (tensile force/initial cross-sectional area) vs. strain (extension as a fraction of original length), using Series IX Automated Materials Testing System Software (Instron, Canton, MA, USA). Thirteen specimens of each type of film were evaluated. Color of strawberry puree films with and without the addition of antimicrobial agents was determined to study the effect of such components on films characteristics. Color was measured using a Konica Minolta spectrophotometer (CM508D, Konica–Minolta Inc., Ramsey, NJ, USA) under a standard white reflector plate. CIE - L^* , a^* , b^* color

coordinates were obtained from the reflection spectra of the samples using a D65 illuminant/10 ° - observer angle. The colorimeter was calibrated using a standard white plate. Ten films were evaluated for both strawberry puree edible films with and without EOs and fifteen readings were made for each film by changing the position of the detector over the film. The films were allowed to reach room temperature before color determination.

3.3.2. Strawberry preparation, storage, and qualitative analysis

Fresh strawberries were harvested by California Giant (Watsonville, CA, USA) and sent by overnight shipping to a local wholesale distributor (Fuji Melon, Oakland, CA, USA). Strawberries were picked up from the distributor earlier in the morning and kept at 2 °C walk in cold room until treatments. Berries with uniformity in size, color, and appearance were selected for treatments. An amount of 360 selected berries without signs of mechanical damage and fungal decay were randomized before being used for treatments. For each treatment and storage time, fifteen strawberries were placed in polyethylene terephthalate (PET) clamshells. Clamshells with antimicrobial edible films were used for strawberry treatment, whereas strawberries in clamshells without edible film were used as controls. Both control and treated strawberry fruit were kept at 10 °C and 80 - 90% RH for 10 days. Physico-chemical properties of fruit were evaluated at day 0, 3, 7, and 10 of storage, and all the measurements were conducted in triplicate.

Each clamshell was inspected after 3, 7, and 10 days of storage. Fruit were considered infected when a visible lesion, characterized as brown spots and softening of

the wounded zone, was observed. The results were expressed as fruit infection percentage.

Weight loss was measured by recording the weight of three clamshells, the same ones throughout the storage time, at the beginning (day 0) and after 3, 7, and 10 days. Weight losses were expressed as percentage loss of the initial weight.

L*, a*, and b* colorimetric values were obtained using a CM508D spectrophotometer (Konica – Minolta Inc., Ramsey, NJ, USA) and used to calculate the chroma value [$C^* = (a^{*2} + b^{*2})^{1/2}$], which indicates the intensity of color saturation (Francis, 1980). Five measurements were carried out on the surface of each berry; eight berries were used for each clamshell.

Strawberry firmness was determined in eight fruit (the same fruit used for color determination) from each clamshell using a TA – XT2 Texture Analyzer (Stable Micro Sistem Ltd., UK) by measuring the force required for a 2 mm probe to penetrate 7 mm into strawberry flesh at a rate of 2 mm/s.. Berries were cut into two pieces alongside and texture was measured in both sides at the highest elevation closer to the stem end. Measurements were taken at day 0, 3, 7, and 10.

Total soluble solids content (%) were analyzed from strawberry juice using a digital refractometer LR01 (Maselli Measurements, Parma, IT). Juice was obtained by homogenizing the berries in a stainless steel blender (Waring Commercial, Torrington, CT, USA), the homogenates were then filtered with cheese cloth. Seven fruit from each clamshell were tested at day 0, 3, 7, and 10. The same homogenate was also used for total soluble phenolics and antioxidant capacity analysis.

An adaptation of the DPPH method (Brand–Williams et al., 1995) was used to estimate the AC. Five g of homogenized sample (the same used for TSS determination

and obtained from seven fruit per clamshell) were extracted with 20 mL of methanol using polytetrafluoroethylene (PTFE) tubes, tubes were capped, vortexed for 15 s, and stored for 48 h at 4 °C. Homogenates were then centrifuged (rotor SA-600, SORVALL RC 5C Plus, Kendro Laboratory Products, Newtown, CT, USA) at 29.000 x g for 15 min at 4°C. Sample aliquots of 50 µL were taken from the clear supernatant (equivalent methanol volume as control) and reacted with 2950 µL of DPPH reagent, obtained by dissolving 0.047 g/L in methanol. Solutions were kept in a covered shaker at room temperature until steady state conditions were reached (no significant decrease in absorbance was experienced as compared with the control, 20–22 h). The spectrophotometer was blanked with methanol, the solutions were placed in 4.5 mL disposable cuvettes, and the absorbance at 515 nm was recorded using a spectrophotometer UV-1700 (Shimadzu scientific instruments, Inc., Columbia, MD, USA). Antioxidant capacity was calculated by measuring the decrease in absorbance of samples as compared with the methanol samples and quantifying as µg Trolox equivalent from a standard curve developed with Trolox (0–750 µg/mL) and expressed as mg Trolox per g fresh weight.

The same methanol extract as for antioxidant capacity was also used for total soluble phenols analysis. The assay was conducted according to Swain and Hillis (1959) method with some modifications. A 150 µL aliquot of methanol extract was taken from the clear supernatant, diluted with 2400 µL of nanopure water, followed by 150 µL of 0.25N Folin-Ciocalteu's reagent, and incubated for 3 min at room temperature. The reaction was stopped by adding 300 µL of 1N sodium carbonate and the mixture was incubated for 25 min. The standard curve was developed with different concentration of

gallic acid, ranging from 0 to 0.375 mg/mL. Results obtained from the reading of absorbance at 765 nm were expressed as mg of gallic acid per 100 g fresh weight.

3.3.3. Fungal identification

Polymerase chain reaction-based method (PCR) and DNA sequencing were used in the present work for the rapid identification of major fungal species from strawberries. Isolation of fungal strains from berries were carried out by slicing surfaces of infected strawberries and placing them on DRBC (Dichloran Rose Bengal Chloramphenicol) agar plates. The plates were then incubated at room temperature for 4 days to allow fungal growth. Fungal colonies were purified by serial transfer 2 to 3 times to a fresh DRBC plates. Pure fungal isolates were maintained on PDA (Potato Dextrose Agar) plates until they were transferred in YPD (Yeast extract Peptone Dextrose) medium for 18 to 24 hrs to allow them to grow. Mycelia were harvested by centrifugation to remove the supernatant, grinded with a pestle and used for DNA extraction. A MasterPure™ Yeast DNA Purification Kit (Epicentre® Biotechnologies, Madison, WI, USA) was used to extract fungal DNA. Concentrations of DNA were determined by using a NanoDrop Spectrophotome and PCR (Polymerase chain reaction) was used to amplify the DNA. The divergent D1/D2 domain at the 28S rDNA genes of fungi were amplified with primers NL-1 (5' –GCATATCAATAAGCGGAGGAAAG) and NL4 (5' -GGTCCGTGTTTCAAGACGG) in a Qiagen kit (Fast Cycling PCR kit, Qiagen, Hilden, Germany). Reactions were carried out in a DNA Engine (Bio-Rad, Hercules, CA, USA) programmed as follows: an initial denaturation step at 95 °C for 5

min, 35 cycles of 96 °C for 5 sec, 57 °C for 5 sec, 68 °C for 30 sec, and final extension at 72 °C for 1 min. PCR fragments were subjected to gel electrophoresis in 1% agarose, stained with ethidium bromide and viewed on UV transilluminator (Bio-Rad, Hercules, CA, USA). Purified PCR products (DNA Clean & Concentrator -5 Kit, Zymo Research, Irvine, CA, USA, Cat. No. D4004) were used for DNA sequencing and final identification of the fungus. Sequence reactions were carried out using ABI Prism BigDye™ Terminator v3.1 Cycle Sequencing Kit (Life Technologies, Grand Island, NY, USA). DNA sequences were determined by using ABI Prism 3100 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA). The nucleotide sequences obtained were Blast searched using GeneBank database (<http://www.ncbi.nlm.nih.gov/BLAST/>) as well as DNA data libraries (DDBJ/EMBL).

The data were statistically processed by ANOVA using the CoStat 6.4 (CoHort software, USA) program. Means were separated by HSD Tukey Test at the significance level of $p \leq 0.05$.

3.4. RESULTS

3.4.1. Physical properties of film

WVP of strawberry puree edible films was significantly higher ($p \leq 0.05$) than those with EOs (Table 1), resulting in a potential higher passage of water vapor through the film. Several studies reported that edible films made from fruit puree have poor

water vapor barrier properties because of the hydrophilic nature of their constituents (Kester and Fennema, 1986; Rojas-Graü et al., 2006). The addition of glycerol and pectin, to improve film extensibility and flexibility, might further compromise their vapor barrier properties. McHugh et al. (1996) demonstrated that the addition of essential oils, being highly hydrophobic mixtures, decreased WVP as the water vapor transference occurs through the hydrophilic portion of the film. However, composition, structure, as well as functional groups of the oils play an important role on moisture transfer mechanism and thus in the barrier properties of films (Morillon et al., 2002). In this sense, carvacrol has been proved to create a good barrier because of its chemical structure (Du et al., 2011) and this could explain the lower WVP value of films containing it in the present study.

Tensile strength, percent elongation, and elastic modulus are common indicators used to describe how mechanical properties of films are related to their chemical structure. Table 1 shows that tensile strength, as a measurement of the maximum stress developed in a film during tensile testing, did not significantly ($p \leq 0.05$) change when carvacrol and methyl cinnamate compounds were added into the film, even if a negative tendency occurred in strawberry puree films with volatile compounds. The presence of micro-bubbles in the specimens might have affected tensile strength of the film. Indeed, it is likely that considering both the high amount of water in the film-forming solution (48.5%) and the hydrophobic nature of carvacrol and methyl cinnamate, air bubbles might have formed and entrapped when the active compounds were incorporated into the solution. Our findings were in agreement with those obtained by Rojas-Graü et al. (2007), who studied the effect of plant essential oils on alginate-apple puree edible films. Percent elongation, described as the measure of the

film's stretch ability prior to breakage (Krochta and DeMulder-Johnson, 1997), and elastic modulus (a measure of the stiffness of the film) had the tendency to be lower for films enriched with volatile compounds than the control film. Changes in mechanical properties as affected by EOs incorporation into films were also observed on other biopolymeric films such as chitosan (Zivanovic et al., 2005), alginate (Pranoto et al., 2005), and hydroxypropylmethylcellulose (Sánchez-González et al., 2009), reflecting different results, probably because of the different interactions between film matrix and natural active compounds.

The addition of carvacrol and methyl cinnamate showed significant effects ($p \leq 0.05$) on films color parameters (Table 2). Films with volatile compounds was significantly darker (L^*), more red (+ a^*) and yellow (+ b^*) than control. Du et al. (2009) observed that apple films obtained darker color when allspice, cinnamon, and clove bud oils were added to the film forming solutions. Changes on color characteristics of films enriched with volatile compounds could be explained by some alteration of the macromolecular structure which may have occurred when volatile compounds were added; however, more analyses are necessary to confirm this explanation.

3.4.2. Quality parameters of strawberry

Delay on fruit spoilage in vapor-treated strawberry was extended up to three days compare to the control and the severity of decay was significantly ($p \leq 0.05$) reduced (Fig. 1A). Indeed, while control samples started to show signs of mold growth

by day 7, strawberries treated with volatile compounds-enriched films did not develop any fungal decay until 10 days, exhibiting 57% less spoilage than the control. Visual appearance and microbiological safety are considered the major factors contributing to the marketability of fresh product. Natural volatile compounds, in addition to proper storage temperature, could be used to delay senescence of the fruit, maintain quality attributes, as well as to avoid the use of chemicals as a means of preservation. Many studies have been reported on the antifungal activities of essential oils, mainly against food storage fungi, proving their potential use as natural fumigants in controlling fungal deterioration of some foods during storage (Daouk et al., 1995; Vázquez et al., 2001). Among all natural essential oils, carvacrol has gained lot of attention because of its great antibacterial and antifungal activity (López et al., 2007; Du et al., 2008), especially against *Botrytis cinerea*. Martínez-Romero et al. (2007) reported the high effectiveness of carvacrol in reducing the growth of *B. cinerea* in grapes, showing that a 97% of inhibition was obtained for 1 mL/L of carvacrol at the vapor phase.

The weight loss of fruit significantly ($p \leq 0.05$) increased throughout storage time for both control and treated strawberries (Fig. 1B). However, no significant difference in weight loss was found between control and treated strawberries during storage. During the first week, changes on weight had a tendency to be more pronounced for treated berries than the control, whereas in the second week of storage control samples exhibited the highest weight loss (11.6 ± 2.51). This acceleration of weight loss for control fruit in the final phase of storage may be explained by an increase of fruit's metabolic activity as well as a rapid microbial growth as previously reported, which led to a faster degradation of the fruit (Sánchez-González et al., 2011).

Our findings were consistent with previous studies in which fruit exposed to essential oil vapors presented very low weight loss (Tzortzakis, 2007).

Firmness of strawberry during post-harvest storage is generally affected by physicochemical changes due to ripening process that continues even after harvest, leading to softening of the fruit. These modifications are mainly attributed to the action of pectolytic enzymes on the solubilization of pectin and other cell wall components (Gayosso-García Sanchio et al., 2010). Texture of strawberry (Fig. 2A) significantly ($p \leq 0.05$) decreased as a function of storage time for both control and treated fruit and, since the amount of loss of firmness during storage is one of the main factors used to determine strawberry quality after harvest, the 19.6% loss recorded at the end of storage time for vapor-treated strawberries is a promising result especially if compare to the 34.7% registered for the control. This outcome was in accordance with a previous work of Tzortzakis (2007) in which strawberries exposed to eucalyptus and cinnamon volatile oil compounds presented higher firmness value than the no treated fruit. However, there is not report in the literature with regard to the reason of the positive effect that EOs might have on strawberry firmness. Here we hypothesized that the antimicrobial effect of EOs might have indirectly decreased the amount of extracellular pectinase (the main hydrolytic enzyme responsible for pectin breakdown) produced by *Botrytis cinerea* (Aguilar and Huitrón, 1993; Walton, 1994). Therefore, the higher firmness observed for strawberries exposed to antimicrobial vapors might be related to the less number of infected fruit due to the antimicrobial vapors released from the films.

Storage time had a significant ($p \leq 0.05$) effect on increasing total soluble solids in carvacrol and methyl cinnamate vapor - treated strawberry (Fig. 2B), evolving from the initial $10.3 \pm 0.48\%$ to $13.3 \pm 1.25\%$, whereas no significant differences were

detected for control samples in the same period. During the first 7 days of storage, control samples showed higher total soluble solids content than those treated with vapors enriched films; however, since the difference was not statistically significant they might be due to a higher sugars synthesis in the fruit (Tanada-Palmu and Grosso, 2004). At the end of the storage period, there was a significant difference between the samples; treated strawberries presented higher total soluble solids content than the control (+ 22.6%), that could be partially explained by the conversion of starch to sugars but, since strawberries accumulate very little starch, it may be mainly because of an increase in anthocyanins which contribute to soluble solids (Mitcham, 2007).

Color is one of the main desirable characteristic that might determine consumer acceptance of the product (Sivarooban et al., 2008). Lightness (L^*) of vapor-treated strawberries (Fig. 3) was significantly ($p \leq 0.05$) affected by storage time reflecting an increase of the value in the period ranging from 3 to 7 days, and a consequent decrease in the final day (day 10). However, L^* value registered at the end of storage period was no significant different from the initial one, showing that no drastic changes occurred throughout storage. At day 3 and 7, vapor-treated strawberries were also significantly brighter than control samples (28.0 ± 1.28 vs 26.4 ± 1.21 and 27.43 ± 2.05 vs 25.6 ± 2.58 , respectively), which might be considered a positive outcome considering that decrease on brightness is usually due to the formation of dark tissues and brown spots, which might be related to no proper storage conditions as well as fungus infection (Lacroix and Ouattara, 2000).

Major changes on strawberry color may be noticed by the assessment of a^* value (Fig. 3), being a measure of redness. The a^* value of all strawberry samples slightly decreased over time, showing the tendency to have a less saturated red color.

However, significant reductions were detected after 3 and 7 days, respectively for control and treated fruit. Carvacrol and methyl cinnamate vapors had a significant effect on red color of strawberries only after 3 days of storage, showing more saturated color than control samples (+ 8.63%). Explanation for this result may find basis in the relation existing among red color, anthocyanin, and fungus on strawberry fruit. Anthocyanins are considered the compounds responsible for red color of strawberry (Abby et al., 2007) and discoloration of the fruit may be due to anthocyanins degradation by the action of hydrolytic enzyme that, by breaking down the linkage of the glycosidic substituent in the moieties, lead to loss of color during post-harvest storage (Manzanares et al., 2000; Oey et al., 2008). β -glucosidase is the enzyme responsible for color degradation and it is synthesized by several type of fungus such as *Botrytis cinerea* (Gueguen et al., 1994) which was identified in the present study. Thus, the higher severity of decay mainly due to *B. cinerea* recorded for non treated berries might be associated with discoloration of the fruit. Measurements of b^* value, as representing the chromaticity of blue (negative value) and yellow (positive value) color, reflected a significant ($p \leq 0.05$) reduction toward negative b^* value for treated strawberry which ranged from the initial value of 20.2 ± 3.82 to 17.0 ± 3.17 at the end of storage time. Chroma values were also determined and it hardly changed during storage for control samples. Indeed no significant differences were detected from the beginning to the end of storage time, although fruit developed a significant less vivid coloration, as evidenced by lower values of chroma after three days.

Strawberries are good sources of natural antioxidants and nutrients (Wang and Lin, 2000); however, unsuitable storage conditions of the fruit may deplete their amount. In this study we found that antioxidant capacity (Fig. 4A) significantly ($p \leq$

0.05) increased for both, control (19.8%) and vapor-treated berries (28.7%) throughout the storage period, as was previously reported also by Aiala-Zavala et al. (2005) for strawberry treated with methyl jasmonate and ethanol vapors. A sharp significant increase was observed especially in treated strawberry in the last day of storage. As showed on Fig. 4A, the effect of vapors on antioxidant capacity reflected a lower value on day 7 compare to the control. Indeed, antioxidant capacity of vapor-treated berries was 12.4% lower than that registered for control fruit.

Total soluble phenols content (Fig. 4B) of berries showed a significant increase throughout storage period for both control and treated fruit. Whereas total soluble phenols content of vapor-treated strawberries gradually increased during storage time, the amount of total phenols detected in control (non-treated) fruit only sharply increased during the first three days (from 141 to 167 mg GA 100 g⁻¹ f.w.) then remained steady afterward. Vapor treatments significantly decreased total phenols content of strawberry fruit during storage, with the only exception for the last day where vapor-treated strawberries presented 9.69% higher value than control. Total phenols are the major antioxidants in plant and fruit tissues, and are produced as secondary metabolites in response to abiotic and biotic stresses to protect cellular constituents (Cisneros-Zevallos, 2003). Therefore, the constant exposure of strawberries to antimicrobial vapors, which can be considered a controlled stress, might have caused such enhancement at the end of storage time. Results reported by Tzortzakis (2007) showed that oil vapor treatment tended to decrease the total phenolic content of strawberry fruit during exposure. This outcome could be associated with the different interaction between constituents of essential oils and food matrix.

3.4.3. Fungus identification

Results from PCR-based method for fungal identification showed the presence of *Trichoderma* sp., *Cladosporium silenes* and *Botrytis cinerea* on strawberry fruit surface. As previously reported in section 1, *Botrytis cinerea* is the pathogen responsible for gray mold which is considered to be the most important disease of strawberry that can cause 80-90% losses of fruit in wet seasons (Bower, 2007). For this reason, natural volatile compounds as alternative to chemicals may have potential as post-harvest fumigants against fungal activity. Incorporation of selected bioactive compounds into storage environment and packaging, at appropriate levels, has been proven to inhibit or even kill post-harvest pathogenic fungi (Isman, 2000; Bouchra et al., 2003; Daferera et al., 2003), and therefore reducing post-harvest product loss.

3.5 CONCLUSION

Strawberry edible film as carrier matrix for the controlled release of antimicrobial vapors was designed to solve different issues related to strawberry deterioration during storage. Based on our results, carvacrol and methyl cinnamate vapors released from strawberry puree edible film extended strawberry shelf-life by delaying spoilage of the fruit and improved fruit quality-related attributes. These findings might have feasible commercial relevance, since only small amounts of active compounds are needed as they were gradually released over time from the film. Furthermore, organoleptic issues arising from the direct contact of the enriched edible

films with food product could be avoided by using the film as carriers for antimicrobial vapors. However, further studies are necessary in order to determine the amount of vapors released during time as well as the sensory effect of carvacrol and methyl cinnamate on the sensory quality of the fruit.

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3.7 FIGURES AND TABLES

Table 1. Effect of volatile compounds (VC) on water vapor permeability (WVP) and tensile properties of strawberry puree edible films (SPEF); SPEF-VC: strawberry puree edible film with volatile compounds. Data shown are the means \pm standard deviation.

Physical properties	VC concentration of SPEF (% w/w)	
	Control (SPEF)	SPEF-VC
	0	1,5
Film thickness (mm)	0.054 \pm 0.006 ^{ns}	0.057 \pm 0.003
RH inside cup (%)	82.2 \pm 1.68 ^b	81.9 \pm 0.5 ^a
WVP (g mm/kPa h m ²)	0.92 \pm 0.17 ^a	0.61 \pm 0.05 ^b
Tensile strenght (MPa)	2.38 \pm 0.89 ^{ns}	2.07 \pm 0.41
Elongation (%)	56.7 \pm 6.93 ^{ns}	56.2 \pm 7.51
Elastic modulus (MPa)	5.18 \pm 1.91 ^{ns}	4.26 \pm 1.39

^{a-b}: different letters within a row indicate significant differences among films ($p \leq 0.05$); ^{ns}: not significantly different within the films ($p \leq 0.05$).

Table 2. Effect of volatile compounds (VC) on color parameters of Control (strawberry puree edible films); SPEF-VC: strawberry puree edible film with volatile compounds. Data shown are the means \pm standard deviation.

Film	VC concentration (% w/w)	L*	a*	b*
SPEF-VC	1.5	67.1 \pm 0.55 ^b	23.4 \pm 0.40 ^a	18 \pm 0.53 ^a

^{a-b}: different letters within a column indicate significant differences among films ($p \leq 0.05$).

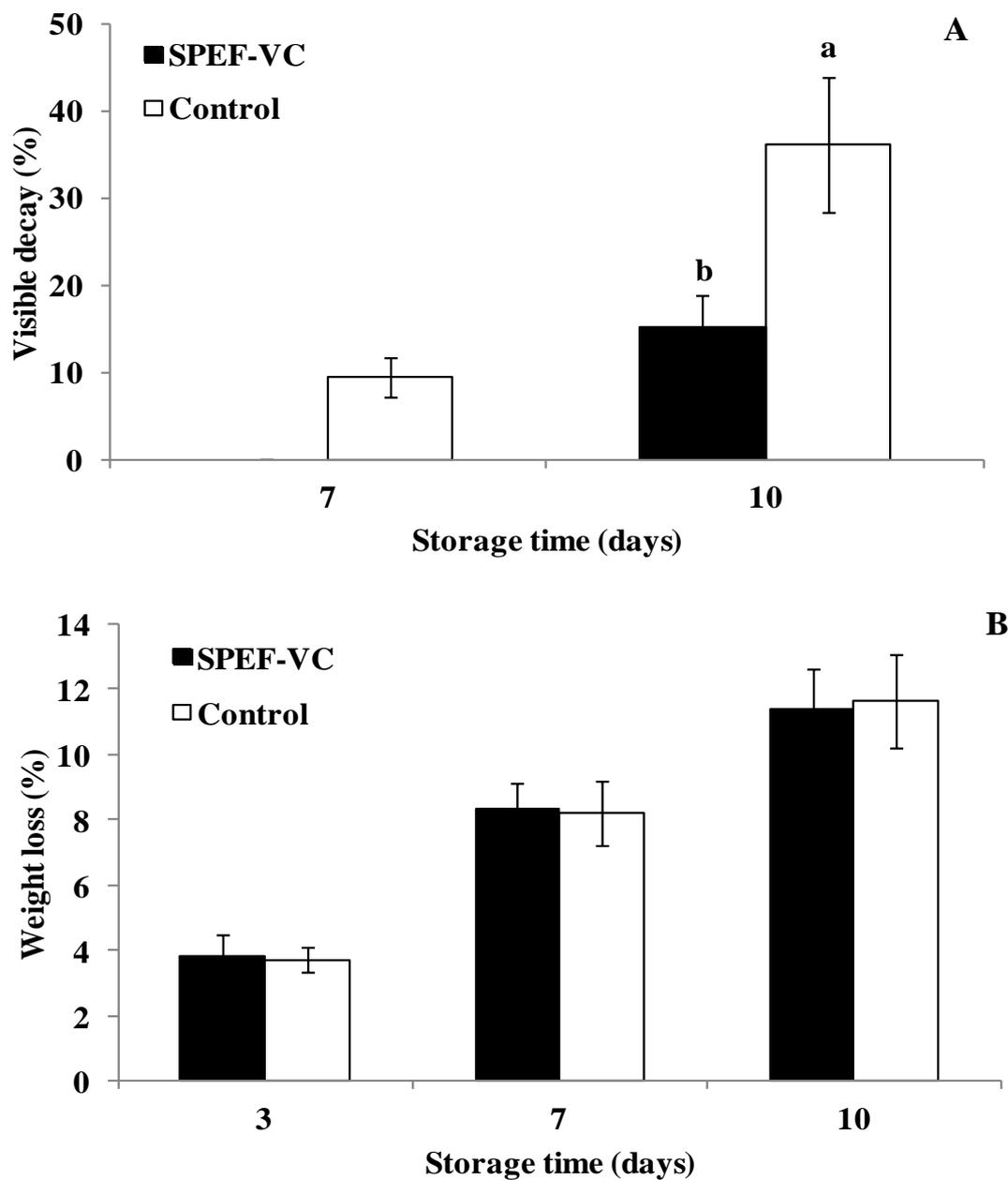


Figure 1. Effect of carvacrol and methyl cinnamate volatile compounds released from SPEF on: (A) fruit visible decay (%) and (B) weight loss (%) during 10 days of storage at 10 °C. Data shown are the means and bars indicate standard deviation. SPEF-VC: strawberry puree edible film with volatile compounds; Control: no films. a-b: different letters indicate significant differences among treatments ($p \leq 0.05$).

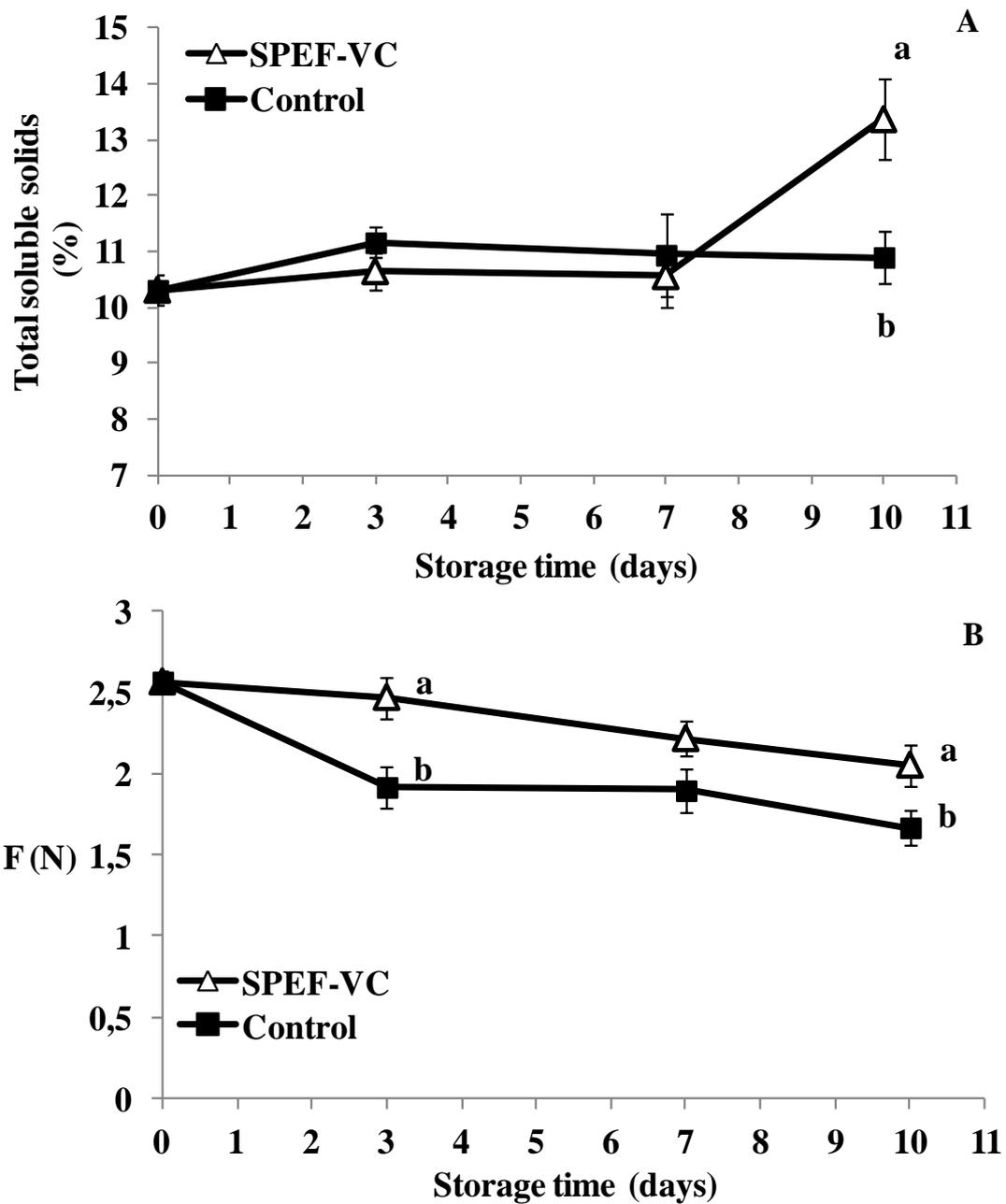


Figure 2. (A) Firmness (N) and (B) total soluble solids content (%) of strawberries during 10 days of storage at 10 °C. Data shown are the means and bars indicate standard deviation. SPEF-VC: strawberry puree edible film with volatile compounds; Control: no films. a-b: different letters indicate significant differences among treatments ($p \leq 0.05$).

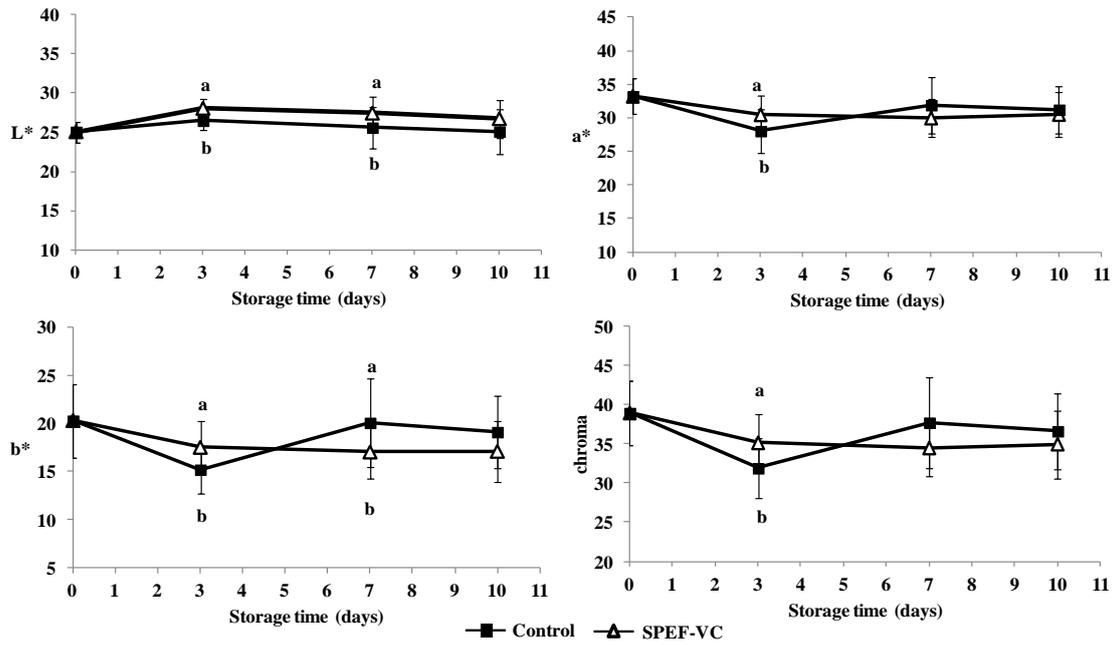


Figure 3. Color parameters (L^* , a^* , b^* , and chroma) of strawberries during 10 days of storage at 10 °C. Data shown are the means and bars indicate standard deviation. SPEF-VC: strawberry puree edible film with volatile compounds; Control: no films. a-b: different letters indicate significant differences among treatments ($p \leq 0.05$).

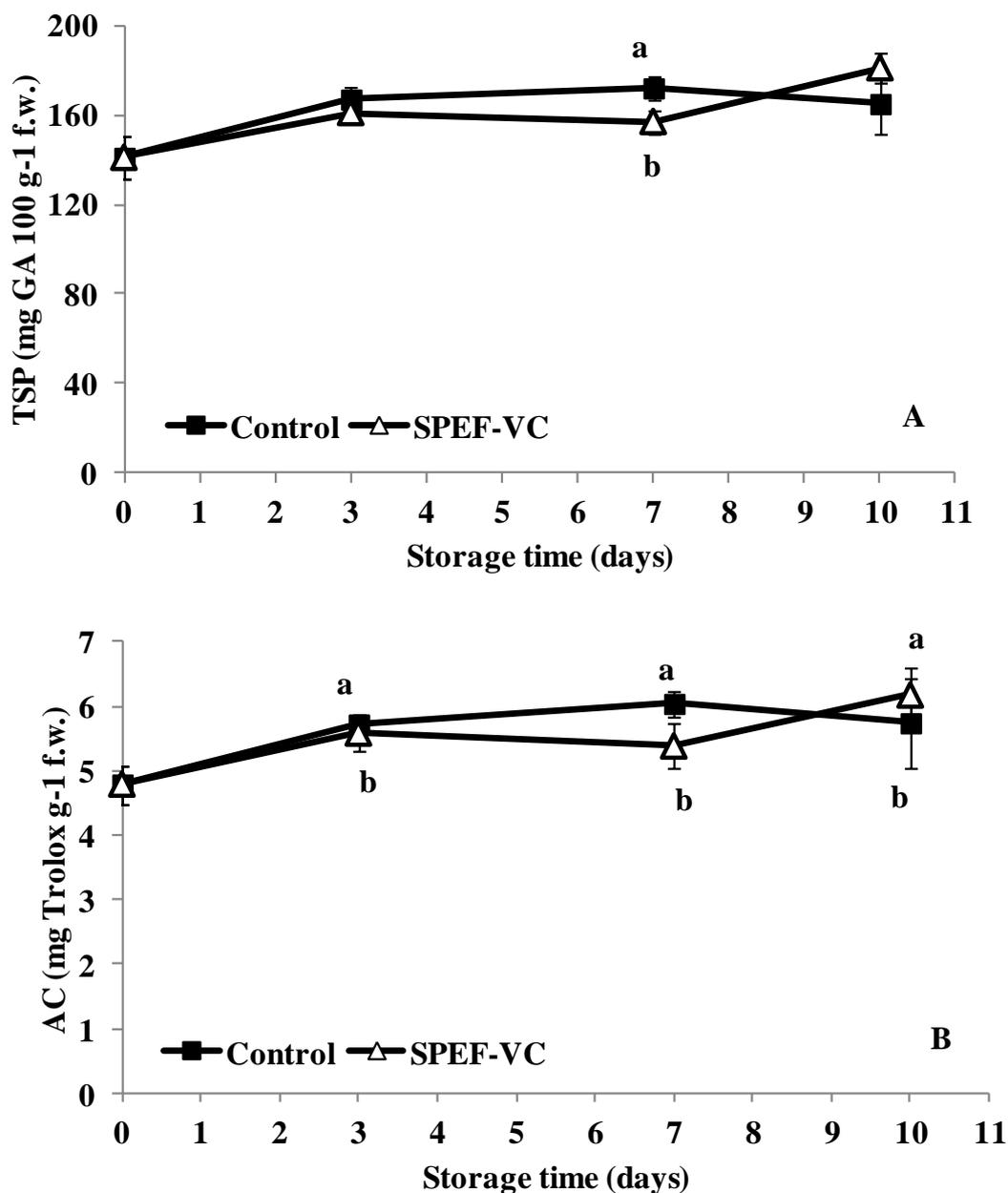


Figure 4. (A) antioxidant capacity (AC) and (B) total soluble phenols (TSP) of strawberry during 10 days of storage at 10 °C. Data shown are the means \pm standard deviation. SPEF-VC: strawberry puree edible film with volatile compounds; Control: no films. a-b: different letters indicate significant differences among treatments ($p \leq 0.05$).

4 OPTIMIZATION OF ANTIMICROBIAL AND PHYSICAL PROPERTIES OF ALGINATE COATINGS CONTAINING CARVACROL AND METHYL CINNAMATE FOR STRAWBERRY APPLICATION

4.1 ABSTRACT

Increasing strawberry consumption has led to a growing safety concern because they are not washed after harvest. An antimicrobial edible coating could be an effective postharvest technique to ensure microbial safety and, at the same time, retain overall quality of the fruits. Response surface methodology was used to optimize the antimicrobial activity against *Escherichia coli* O157:H7 and *Botrytis cinerea* and several physical properties (turbidity, viscosity, and whitish index) of an alginate coating. A full factorial design was used to select the concentrations of carvacrol and methyl cinnamate on the basis of on their effect against *E. coli* and *B. cinerea*. A central composite design was then performed to evaluate the effects/interactions of the two antimicrobials on the coating characteristics. The results from analysis of variance showed the significant fitting of all responses to the quadratic model. To attain the desirable responses, the optimal concentrations were at 0.98% (w/w) carvacrol and 1.45% (w/w) methyl cinnamate.

4.2 INTRODUCTION

Microbial contamination and limited shelf life are considered the main causes of loss of quality of fresh fruits. Strawberries are among the most perishable fruits characterized by an intense physiological post-harvest activity due to the high respiration rate and the presence of common storage spoilage microorganisms such as *Botrytis cinerea*. Moreover, because strawberries are not usually washed during production, harvest and handling, they are a potential source of foodborne pathogens, mainly *Escherichia coli* (serotype O157:H7), which has been implicated as the causative agent in gastroenteritis outbreaks resulting from the consumption of fresh strawberries (Lynch et al., 2009).

Application of edible coatings can be considered a potential approach to preserve strawberry quality by assuring microbial safety and stability while maintaining nutritional and sensory characteristics (Du et al., 2011). The barrier properties of edible coatings provide protection against spoilage by reducing moisture and gas transfer, as well as decreasing microbial growth, thus preventing not only quantitative loss but also losses in appearance and nutritional quality (Kader, 2002; Hao et al., 2004). In the past few years, new components have been used in edible coating formulations to satisfy increasing consumers' demand for natural high - quality products. Polysaccharide-based coatings, which have low oxygen permeability, have been widely used for extending the shelf life of strawberries. These coatings modify the internal atmosphere of the fruit by allowing enough gas exchange to prevent strawberries from going anaerobic, while at the same time retarding ripening and senescence (Krochta et al., 1997; Lacroix et al., 2005). Among them, starch and derivatives (Garcia et al., 1999; Ribeiro et al., 2007)

chitosan and hydroxypropyl methylcellulose (Park et al., 2005) have been proposed for coating strawberries to extend storage life, decrease water losses, and improve fruit quality.

Alginate, an anionic polysaccharide obtained from marine algae, can be considered a food ingredient with good potential to be used as a coating, because of its unique property to form strong gels with metal cations and create thick aqueous solutions (Roopa and Bhattacharya, 2008). The alginate molecule is characterized by a linear polymeric structure of 1,4-linked- β -D-mannuronic and α -L-guluronic residues (Azarakhsh et al. 2012), which may vary in composition and sequence. This composition determines the physical properties of alginates such as viscosity of solutions and gel strength (Gombotz and Wee, 1998).

Promising results have been achieved on fresh fruits coated with alginate solution (Oms-Oliu et al., 2008; Campos et al., 2011) and further improvements could be obtained by incorporating antimicrobial compounds into the solution to provide protection against microbial contamination, thus enhancing food safety and stability (Rojas-Graü et al., 2007; Ponce et al., 2008; Raybaudi-Massilia et al., 2008). There are many categories of antimicrobial agents such as organic acids and enzymes that have potential to be used into edible coating (Rojas-Graü et al., 2009). Most relevant, natural essential oils (EOs) appear to have received the most attention from researchers due to their strong antimicrobial activity against a wide range of microorganisms, including pathogens (Burt, 2004). Natural plant EOs are considered as Generally Recognized as Safe (GRAS) by the U.S. Food and Drug Administration (López et al., 2007) and therefore they represent a suitable alternative as chemical preservatives to be used on fruits and other food products.

Methyl cinnamate (MC), a methyl ester of cinnamic acid, is one of the major volatile components of strawberry aroma produced and released during fruit maturation (Lunkenbein et al., 2006; Ali et al., 2010). Its antimicrobial activity against common phytopathogenic fungi has been tested under *in vitro* conditions (Wannissorn et al., 2009; El-Shiekh et al., 2012). Most recently, the successful incorporation of MC into edible films has been reported (Peretto et al., 2014). In the present work MC was used in combination with carvacrol (C; the major component of EOs from oregano and thyme), for which antibacterial and antifungal properties have been widely studied. Therefore, the development of an alginate coating containing MC in combination with C has been proposed in this study to improve the antimicrobial activity of C as well as to overcome the negative impact of the strong odor of C on the sensory properties of strawberries. However, although EOs have a positive effect on extending fruit shelf - life, the concentrations needed in edible coatings to have an effective antimicrobial property may have a negative effect on barrier-mechanical and optical properties of the coating and therefore affect its performance and acceptability.

The aim of this work was to optimize the antimicrobial and physical properties of alginate coatings containing C and MC for their potential application on fresh strawberries and other perishable fruits. Response surface methodology (RSM) was used as an effective statistical technique for simultaneously investigating and optimizing the response variables.

4.3 MATERIALS AND METHODS

Food grade sodium alginate (Keltone[®] LV, ISP, San Diego, CA, USA) was the primary ingredient use in the edible coating formulations. Glycerol (Starwest Botanicals Inc., Rancho Cordova, CA, USA) was added as plasticizer to provide good flexibility to the coatings. C and MC were purchased from Sigma-Aldrich (St. Louis, MO, USA).

Coating solutions were prepared by dissolving sodium alginate powder (2% w/v) in distilled water while heating on a stirring hot plate for 15 min at 70 °C until the mixture became clear. Then, 1.5% (w/v) glycerol was added to the solution and stirred for 5 min. Finally, C and MC (active compounds) were incorporated into the solution a little at the time and homogenized with a Polytron 3000 (Kinematica, Luzern, Switzerland) for 10 min at 4000 rpm. Because of the insolubility of MC in water, MC was previously dissolved in ethanol (40% w/w) at 40 °C under stirring at 220 rpm for 5 min.

4.3.1 Antimicrobial properties of coating solutions

An overlay diffusion test (Du et al., 2008) was performed as a qualitative test for antimicrobial activity of coatings against *E. coli* and *B. cinerea*. Frozen cultures of *E. coli* O157:H7 (strain RM 1484, original designation SEA13B88), obtained from the U.S. Food and Drug Administration, were streaked on tryptic soy agar (TSA) plate and incubated overnight at 37 °C. One isolated colony was restreaked on TSA and then incubated at 37 °C for 24 h. This was followed by inoculating one isolated colony into 5

mL of trypticase soy broth (TSB) and incubating it at 37 °C for 24 h, under agitation. The microbial broth was then serially diluted (10 x) in 0.1% peptone water. Afterward, 100 µL of 10⁵ colony-forming units (CFU/mL) was uniformly spread onto TSA plates and left to dry for 5 min at room temperature. Plates were divided into three or four even areas on the basis of the compound concentrations, and a 10 mm diameter filter paper, aseptically cut in the shape of a disk, was placed at the center of each area. Then, 20 µL of each solution was placed on top of the filter paper. The plates were incubated at 37 °C for 24 h. The inhibition radius around the filter paper (colony-free perimeter) was measured in triplicate with a digital caliper (Neiko Tools, Ontario, CA, USA), and the inhibition area was then calculated in square millimeters.

Botrytis cinerea was isolated from small pieces of surface tissue cut from molded strawberries. The molded pieces were placed in a 500 mL flask, and 0.05% Tween 80 was added until the pieces were completely covered. The flask was cupped and shaken for 10 min; after that the solution was serially diluted (10 x) with 0.05% v/v Tween 80. Aliquot of 100 µL was spread onto potato dextrose agar (PDA, with 100 mg of chloramphenicol in 1 L media plates) plates and stored for 3 – 5 days at room temperature. Cultures were then restreaked into PDA plates and incubated at room temperature for 6 days. Spores from day 6 cultures were harvested in 10 mL of 0.05% Tween 80 solution, which was vortexed for several minutes until uniform spores suspension was obtained. The inoculum's concentration was adjusted to 10⁵ spore/mL using a hemocytometer. Then, 100 µL of the final inoculum were plated and evenly spread onto PDA plates and left to dry for 5 min. The plates, in triplicate, were divided into three and four areas; at the center of each area, 20 µL of each coating solution were placed on top of a 10 mm diameter filter paper. The inhibition radius and area around

the filter paper were measured and calculated, as those for the overlay test on *E. coli*, after 4 days at room temperature.

4.3.2 Physical properties

The viscosity of the solutions was measured using a Brookfield Digital Rheometer (model DV – III+, Brookfield Engineering Laboratories, Middleboro, MA, USA) with a SC4-21 spindle (0.66 mm diameter, 1.23 mm long) set at 125 rpm constant rotation speed. Eight milliliters of solution at 40 °C was used for the measurement, immediately after the incorporation of EOs. Five viscosity readings were made for each coating solution from 1 to 5 min at constant shear rate (116.25 1/s) and temperature (40 °C).

Turbidity studies of the solution, previously warmed at 40 °C, were determined with an HI 88703 turbidity meter (Hanna Instruments, Carrollton, TX, USA). Ten milliliters of each solution was placed into transparent glass cuvettes for this assay and measurements unit were expressed as nephelometric turbidity units (NTU)

The color of the alginate coating solution was measured using a Konica Minolta spectrophotometer (CM508D, Konica–Minolta Inc., Ramsey, NJ, USA) under a standard white reflector plate. CIE – L^* , a^* , b^* color coordinates, obtained from the reflection spectra of the samples using a D65 illuminant/ 10 ° - observer angle, were used to calculate the whitish index as $W_i = 100 - [(100 - L^*)^2 + a^{*2} + b^{*2}]^{1/2}$ (Avena-Bustillos and Krochta, 1994).

To optimize the antimicrobial and physical properties of alginate coating, a full factorial design (FFD) was used as first step to determine which independent variables, between C and MC, influenced the most the antimicrobial activity against *B. cinerea* and *E. coli* (responses). Levels of C and MC, ranging from 0.25% to 1.25% (w/w) (Table 1), were selected on the basis of preliminary tests on antimicrobial activity. A 2² factorial design was replicated three times to obtain more precise estimates of the effects as well as to analyze the variation at each treatment combination.

After the concentrations of the variables had been selected, on the basis of their effect on antimicrobial activity, a RSM was run for evaluating the antimicrobial activity against *E. coli* and *B. cinerea*, viscosity, turbidity, and whitish index (W_i) of the coating, to determine the concentrations of the independent factors that optimize coating characteristic for its potential application on fresh strawberries. A central composite design (CCD), characterized by 11 experimental points (4 star points, 4 cube points, and 3 central points), was selected for this purpose. Three replicates of each experimental condition were carried out, and the mean values were stated as observed responses. Experimental runs were randomized to minimize the effects of unexpected variability in the observed responses. It was assumed that a second-order response function was fitted for relating the responses to the independent variables:

$$Y = \beta_0 + \sum \beta_i x_i + \sum \beta_{ii} x_i^2 + \sum \beta_{ij} x_i x_j$$

Y is the dependent variable and, β_0 , β_i , β_{ii} , and β_{ij} are constant coefficients of intercept, linear, quadratic and interaction terms, respectively. X_i , X_i^2 and X_{ij} represent linear, quadratic, and interactive effects of independent factors, respectively. The coefficients of the independent variables in the model were estimated by multiple

regressions and evaluated by analysis of variance (ANOVA). ANOVA was used to compare the control (alginate-based coating without antimicrobial) with the coating formulations (with antimicrobials) generated by the CCD for viscosity, turbidity and W_i . Tukey's means comparison test was applied at a significance level of 0.05 to determine differences among treatments. The Minitab 14 statistical package was used to perform data analysis, experimental design matrix, and optimization procedure (Minitab Inc., USA).

After the elaboration of response surface models, a multicriteria methodology was used for the simultaneous optimization of the significant response variables. The Derringer function (desirability function) is the most currently used methodology to find optimal compromises between the total number of responses taken into account (Derringer and Suich, 1980; Murphy et al., 2005). Each estimated response variable was transformed into a dimensionless individual desirability value (d_i) using the desirability function of the statistical program. The desirability function included the minimum and maximum acceptable values of each response. The values of d_i vary in the interval $0 \leq d_i \leq 1$, increasing as the desirability of the corresponding response increases. This transformation makes it possible to combine the results obtained from responses localized in different regions because they were measured on different orders of magnitude.

The individual desirabilities were then combined to give an overall desirability (D) by using a geometric mean equation

$$D = \sqrt[m]{d_1 d_2 \dots d_m}$$

where m is the number of responses studied in the optimization process. The simultaneous optimization process aimed to find the levels of the factors that demonstrated the maximum overall desirability.

4.4 RESULTS

Five combinations of C and MC were generated from the FFD. A preliminary antifungal test against *B. cinerea* was made for the five solutions (containing the five combinations of active compounds) with and without the addition of calcium chloride. Indeed, as a coating agent, alginate is commonly used in combination with CaCl_2 because of its ability to form strong gels upon cross-linking reaction.

Results indicated that solutions with CaCl_2 had poor inhibition on fungus growth (Table 2). It was previously reported that the physical properties of the gel adversely affected the release of low molecular weight compounds (Seifert and Phillips, 1997). The calcium – alginate gel is characterized by a typical egg – box structure in which the guluronic acid of alginate molecule can be linked to a similar region in another alginate molecule by means of calcium ions (Liu et al., 2005). Therefore, the poor antifungal activity of alginate gels containing CaCl_2 could be related to the limited release of C and MC from the gel structure. On the basis of this result, a sodium alginate solution without CaCl_2 was selected as coating material for future studies.

Experimental data presented in Table 3 are the inhibition of *E. coli* and *B. cinerea* by combined concentrations of C and MC in alginate edible coatings.

The highest value (as the average of the three replicates) for antimicrobial activity against *E. coli* and *B. cinerea* was obtained by the combination of 1.25% (w/w) C and MC. It was also observed that the inhibition of both pathogens was mainly dependent on carvacrol concentration. The statistical analysis results of the FFD (Table 4) showed that only C had a significant ($p \leq 0.1$) effect on both microorganisms. These results also suggested that the MC range from 0.25 to 1.25 % (w/w) was probably too low to achieve significant antimicrobial activity. Therefore, in the following central composite design, higher concentrations of MC, ranging from 0.5 to 2.5% (w/w), were selected, whereas the concentrations of C remained the same in the range of 0.25 – 1.25% (w/w).

Table 5 shows the experimental design and the results obtained for the response variables. The ANOVA for coating antimicrobial activity against *E. coli* (Table 6) indicated that the quadratic model was found to adequately describe the experimental result without any significant lack of fit ($p > 0.05$). The main effect of C was found to be significant ($p < 0.05$), and the positive regression coefficient (Table 7) indicated that the antimicrobial activity of the coating increased when C was added into the solution. On the other hand, as in previous results, MC did not show any significant activity on *E. coli*. However, it showed to have a strong linear effect against *B. cinerea* ($p < 0.001$) (Table 6). On the basis of the regression coefficient terms (Table 7), the linear (499.34) and quadratic (94.92) terms of MC showed the largest antimicrobial effect against *B. cinerea*, followed by the linear effect of C (80.56). These results indicated that the increased concentration of MC in the coating significantly increased the antimicrobial activity of this compound, although no significant interaction between the two variables was observed (Table 6). The positive quadratic term of MC indicated

that the antimicrobial activity of the coating increased quadratically when this compound was incorporated. The significant p value of regression ($p < 0.001$) and the nonsignificant lack of fit ($p > 0.05$) in the ANOVA proved that the proposed second-order polynomial model was fitted to represent the relationship between the two variables and the experimental results on antimicrobial activity against *B. cinerea*. This relationship can be better understood by examining the surface plots depicted in Figure 1, in which the effects of the independent variables (C and MC) on the inactivation of selected microorganisms (*E. coli* and *B. cinerea*) were evaluated. Figure 1a shows that the inhibitory action of C on *E. coli* was very effective and directly proportional with its concentration, whereas MC did not exert a significant antimicrobial activity against this foodborne pathogen within the range of concentration studied. Conversely, in Figure 1b, we observe a highly linear increase in the inhibitory action of MC on *B. cinerea* growth, with an increase in MC concentration in the coating, whereas, C was not effective in deterring *B. cinerea* growth under the indicated concentrations range. The efficacy of C incorporated into edible films against *E. coli* O157:H7 was previously reported by Du et al. (2008) and Rojas-Grau et al. (2006). They indicated that the addition of carvacrol into tomato-based edible films and apple puree edible films caused inactivation of *E. coli* and that the inactivation was directly related to the increase of C levels in the films. Carvacrol is considered a broad-spectrum antimicrobial, because it is effective against bacteria, yeasts, and fungi (Sivropoulou et al., 1996). The biocidal mode of carvacrol on bacteria occurs *via* membrane damage resulting in an increase in membrane permeability to protons and potassium ions, depletion of the intracellular ATP pool, and disruption of the proton-motive force, ultimately leading to cell death (Kiskó and Roller, 2005). The antifungal activity of C was reported in previous works (Adam et

al., 1998; Martínez-Romero et al., 2007) where spore germination and mycelium growth of *B. cinerea* were reduced when exposed to carvacrol's vapor, and the reduction was significantly greater as carvacrol concentration in the vapor increased, showing a high potential to improve the shelf life and safety of perishable foods. The antifungal properties of C against a wide range of foodborne fungi have been previously documented (Suppakul et al., 2003; Jantan et al., 2008) and compared with those of other naturally occurring compounds, showing a high potential application against important phytopathogenic fungi affecting food products (Karam et al., 2012). The antifungal effectiveness of C could be further amplified by exploiting the high antifungal properties of MC. However, the combination of the two compounds did not show any synergistic effect on either foodborne pathogen (Table 6); the incorporation of C and MC exerted high antimicrobial activity. C (X_1) significantly ($p < 0.05$) inhibited *E. coli* and *B. cinerea*, whereas MC (X_2) significantly ($p < 0.001$) inhibited *B. cinerea*.

The results from ANOVA (Table 6) showed the significant fitting of the turbidity experimental data to the quadratic model presented a determination coefficient (R^2) of 0.985. This implied that 98.5% of the variations could be explained by the fitted model (Chen et al., 2012). The turbidity of the coating solutions was negatively affected ($p < 0.05$) by the addition of the antimicrobial compounds. A strong linear effect ($p < 0.001$) was observed for both antimicrobial compounds and their interaction ($p < 0.05$). However, a higher positive regression coefficient was observed for MC, which suggested that it had a stronger effect on the turbidity of the coating compared to C. This fact is further supported by the response surface shown in Figure 2, where an upsurge in turbidity with an increase in MC concentration was observed but only a slight rising trend was observed as C concentration increased. Alginate coating without

antimicrobial compounds served as control in the statistical comparison with the antimicrobial alginate solutions to determine if the addition of C and MC affected the turbidity of the coatings. The control alginate coating presented a significantly lower value ($p < 0.05$) of turbidity (144.3 ± 4.04) compared to all antimicrobial solutions containing C and/or MC. Turbidity is an optical property of liquids defined as the measurement of the scattered light that results from the interaction of incident light with suspended solids in the liquid (Gippel, 1995). The presence of particulate material in all antimicrobial solutions increased the turbidity values of the coating. Those particulates in the solutions were probably formed due to the water insoluble property of MC. Moreover, the production of oil in water emulsions, due to the presence of C in water-based alginate solution, could have also increased the turbidity of the coating (Han et al., 2008).

With regard to visual characteristics, an edible coating for fruit applications should improve the appearance of the product and impart a natural aspect at the same time. Color is an important property to be considered when a new coating is developed. In this study, W_i was calculated because the incorporation of antimicrobial compounds led to a change of the color from transparent to white, compared to the control (Figure 3). Table 6 shows the results of ANOVA, in which carvacrol presented the highest significant effect ($p < 0.001$), achieving a negative impact on coating transparency. Additionally, the data shows that the quadratic terms of C, linear and quadratic term of MC, and the linear interaction between the two active compounds were also significant ($p < 0.05$), even when the quadratic and interaction terms of the regression coefficient of the variables were negative (Table 7). The regression model was highly significant with a determination coefficient (R^2) of 0.990, which indicated that almost the total

variation was explained by the model. The value of the adjusted R^2 (0.977) confirmed that the model was highly significant on the W_i . The fitness of the quadratic model was further confirmed by the significant regression p value ($p < 0.05$) and no significant ($p > 0.05$) lack of fit (Table 6).

Alginate was used as primary ingredient in edible coating solution due to its unique property of increased viscosity upon hydration. Aqueous solutions of alginate are considered non-Newtonian fluids, being characterized by shear-thinning characteristic, meaning that the viscosity decreases as the shear rate increases (Storz et al., 2010). Therefore, in this study the shear rate was maintained constant at 125 rpm. The effect of the addition of antimicrobial compounds was studied on the viscosity of the fluid to identify whether their incorporation would affect the fluid characteristic of the coating solution and eventually compromise further coating process application. Considering the coating and strawberry physical characteristics, preliminary studies indicated a decrease in coating performance at viscosity values above 83 cP. Hence, viscosity values lower than 83 cP were used as a target for the optimization of the solution. Table 7 shows the results of ANOVA for the fitting model of the viscosity experimental data to a second order function, which was found to describe the experimental response influenced by the antimicrobial variables without any significant ($p > 0.05$) lack of fit (Table 6). Because, the full model was not able to predict viscosity responses based on antimicrobial variables, the model was reduced by excluding the nonsignificant linear and quadratic terms of MC, as well as the interaction between C and MC, to adequately correlate the quadratic relationship between the concentrations of the antimicrobial compounds and viscosity of the fluid. The results showed that only the linear and quadratic effect of C significantly ($p < 0.05$) affected the viscosity of the

coating solution (Table 6). However, the positive linear term (+ 6.20) and the negative quadratic term of the regression coefficient (-4.59) indicated that increasing C concentrations led to an increase in viscosity until a turning point was reached at 0.68% (w/w) C. Concentrations of C above this value tend to decrease the viscosity of the coating solution, probably due to the plasticizer action of C. It has been reported that C reduces the intermolecular forces in polymer chains, thus decreasing the viscosity of the coatings (Nostro et al., 2012). Similar results were observed when different concentrations of essential oils, above 1.5% (w/w), were added into apple and tomato edible films (Du et al., 2009a,b).

On the basis of the findings of each response surface model, an overall optimization study was performed to obtain an antimicrobial coating with physical properties suitable for strawberry application. The optimum condition for alginate coatings with the most desirable characteristics, obtained by the overall desirability function, was at 0.98% (w/w) carvacrol and 1.45% (w/w) methyl cinnamate concentrations.

4.5 CONCLUSIONS

The results of the ANOVA of central composite design in the response surface methodology showed the significant fitting of all responses to the quadratic model. Considering all the desirable responses (antimicrobial activity against *E. coli* and *B. cinerea*, viscosity, turbidity and W_i), they were optimized for 0.98% (w/w) carvacrol and 1.45% (w/w) methyl cinnamate concentration.

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4.7 FIGURES AND TABLES

Table 1. Levels of the variables used in FFD 2²

independent variables	levels % (w/w)		
	-1	0	+1
carvacrol	0.25	0.75	1.25
methyl cinnamate	0.25	0.75	1.25

Table 2. Antifungal activity of alginate coating solutions generated from the FFD with and without calcium chloride

compounds concentration % (w/w)		inhibitory zone on <i>B. cinerea</i> (mm ²)	
Carvacrol	methyl cinnamate	alginate coating with CaCl ₂	alginate coating without CaCl ₂
		0.25	0.25
1.25	0.25	2.56 ± 0.56 ^b	81.2 ± 13.5 ^a
0.25	1.25	0.00 ± 0.00 ^b	4.62 ± 0.85 ^a
1.25	1.25	6.88 ± 1.33 ^b	83.5 ± 14.1 ^a
0.75	0.75	0.00 ± 0.00 ^b	46.1 ± 10.6 ^a

Data shown are the means of three replicates ± standard deviation.

^{a, b}: different letters within a row indicate significant differences among the two coating solution ($p < 0.05$).

Table 3. Experimental design for antimicrobial alginate edible coating (FFD)

Run	variables (% w/w)		inhibitory zone (mm ²)	
	carvacrol	methyl cinnamate	<i>B. cinerea</i>	<i>E. coli</i>
1	0.25	0.25	0.00	8.81
2	1.25	1.25	73.0	60.9
3	0.25	1.25	5.37	13.0
4	0.75	0.75	42.2	31.8
5	0.25	1.25	5.54	13.1
6	1.25	1.25	85.9	50.1
7	0.25	0.25	0.00	3.75
8	0.25	0.25	0.00	10.1
9	1.25	0.25	71.8	61.1
10	1.25	0.25	85.2	52.9
11	1.25	1.25	91.5	70.2
12	0.25	1.25	2.96	16.7
13	1.25	0.25	86.6	55.0
14	0.75	0.75	49.98	29.44
15	0.75	0.75	51.25	31.85

Table 4. Statistical analysis and estimated effect of FFD

responses	variables			
	carvacrol		methyl cinnamate	
	effect	<i>p</i> value	effect	<i>p</i> value
<i>E. coli</i>	47.44	0.00 ^a	5.39	0.105
<i>B. cinerea</i>	80.07	0.00 ^a	3.43	0.349

^a: statistically significant at $p < 0.001$.

Table 5. Experimental design^a used to obtain different combinations of C and MC in alginate-based edible coating for antimicrobial activity and experimental results for response variables

	Concentration %(w/w)				Inhibitory zone (mm ²)			Responses		
	C	MC	Inhibitory zone (mm ²)		turbidity	Wi	viscosity			
			<i>E. coli</i>	<i>B. cinerea</i>						
1	0.25	0.50	0.00	4.0	1613	66.9	83.81			
2	1.25	0.50	20.9	11.2	6670	75.0	74.45			
3	0.25	2.50	0.00	1030	10380	71.6	50.64			
4	1.25	2.50	21.5	1241	25583	74.4	80.77			
5	0.04	1.50	0.00	253	2077	68.9	58.48			
6	1.46	1.50	41.9	508	16600	78.2	76.79			
7	0.75	0.09	8.69	70.5	490	69.8	71.87			
8	0.75	2.91	9.22	1347	26489	81.2	77.97			
9	0.75	1.50	10.8	480	10817	76.6	77.76			
10	0.75	1.50	3.78	525	1257	76.2	77.92			
11	0.75	1.50	18.9	617	9730	77.2	77.76			

Viscosity: cP; Turbidity: (NTU); W_i: whitish index, Inhibitory zone indicates the antimicrobial activity against *E. coli* and *B. cinerea*: mm².^aCentral composite design (CCD) with 11 experimental points (4 star points, 4 cube points, and 3 central points)

Table 6. Analysis of variance (ANOVA) for regression equation fitted to experimental responses value obtained from the optimization of the concentration of carvacrol and methyl cinnamate into alginate coating solution (p value)

Source	inhibitory zone (mm ²)				
	<i>E. coli</i>	<i>B. cinerea</i>	turbidity	W_i	viscosity
X_1 (C)	0.002 *	0.037 *	0.000 **	0.000 **	0.034 *
X_2 (MC)	0.939	0.000 **	0.000 **	0.026 *	
X_1^2	0.198	0.098	0.316	0.003 *	0.102 *
X_2^2	0.436	0.039 *	0.090	0.001 *	
X_1X_2	0.960	0.432	0.021 *	0.011 *	
Regression	0.021 *	0.000 **	0.000 **	0.000 **	0.003 *
R^2	0.886	0.985	0.985	0.990	0.859
R^2 (adjust)	0.771	0.970	0.970	0.977	0.812
Lack of fit	0.756	0.412	0.153	0.367	0.750

*Significant at $p < 0.05$. ** Significant at $p < 0.001$. C: carvacrol; MC: methyl cinnamate; Inhibitory zone indicates the antimicrobial activity against *E. coli* and *B. cinerea*: mm²; viscosity: cP; turbidity: NTU; W_i : whitish index.

Table 7. Regression coefficients for the model fitted to the experimental responses values.

model term	Coefficients				
	inhibitory zone (mm ²)				
	<i>E. coli</i>	<i>B. cinerea</i>	turbidity	W_i	viscosity
Constant	11.14	540	1.0601	76.66	76.54
X_1 (C)	12.71	80.56	5099	3.006	6.201
X_2 (MC)	0.1747	499.34	8054	0.9249	
X_1^2	3.81	-69.20	-719	-1.62	-4.592
X_2^2	-2.14	94.92	1354	-2.92	
X_1X_2	0.16	34.54	2536	-1.325	

C: carvacrol; MC: methyl cinnamate; Inhibitory zone indicates the antimicrobial activity against *E. coli* and *B. cinerea*: mm²; viscosity: cP; turbidity: NTU; W_i : whitish index.

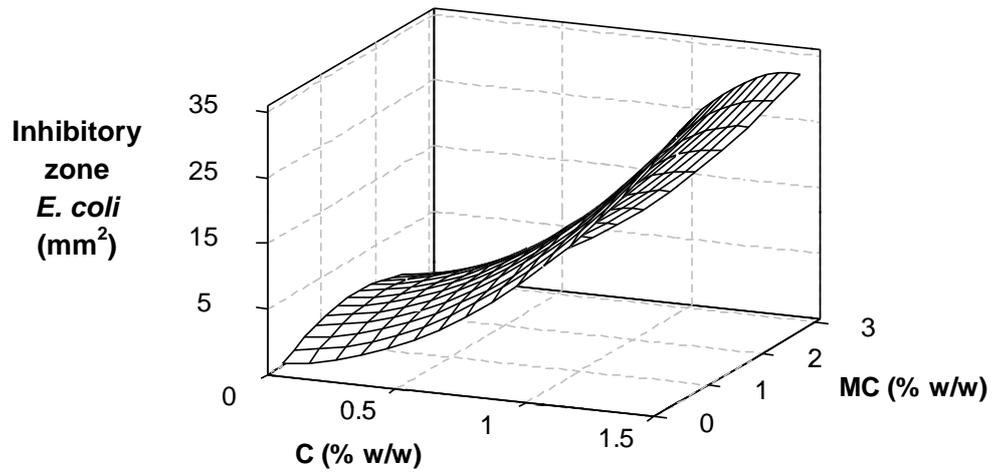


Figure 1a. Response surface of antimicrobial activity against, (a) *E. coli* as a function of carvacrol and methyl cinnamate (% w/w)

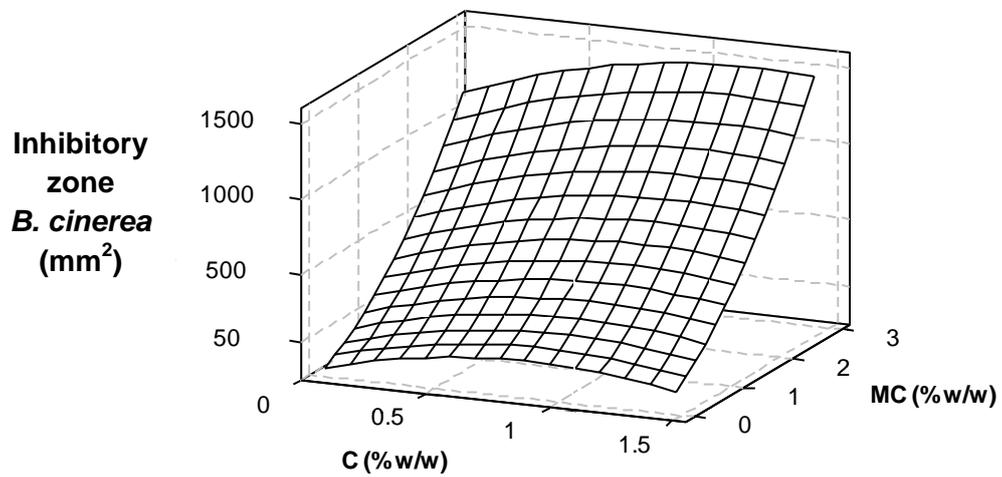


Figure 1b. Response surface of antimicrobial activity against (b) *B. cinerea*, as a function of carvacrol and methyl cinnamate (% w/w)

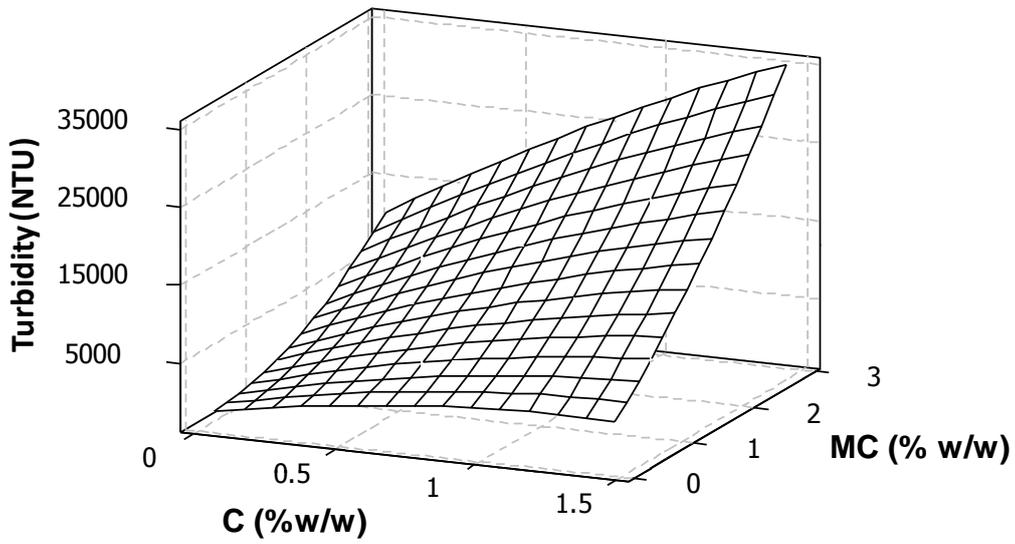


Figure 2. Response surface of turbidity (NTU) as a function of carvacrol and methyl cinnamate (% w/w)

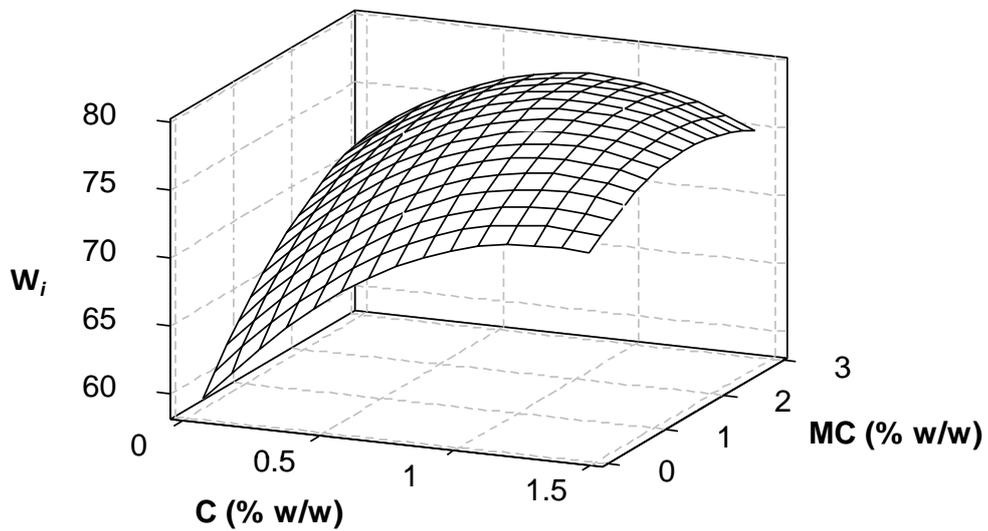


Figure 3. Response surface of whitish index (W_i) as a function of carvacrol and methyl cinnamate (% w/w)

5 ELECTROSTATIC SPRAYING OF ANTIMICROBIAL COATING TO IMPROVE STRAWBERRY QUALITY

5.1 ABSTRACT

Strawberries are perishable fruits characterized by a short shelf-life. Microbial contamination and mold growth are common causes of deterioration during storage. There is a growing need to improve storage technologies such as application of edible coating to extend the shelf-life as well as enhance the overall quality of perishable fruits. This paper aimed to use electrostatic spraying technology for the post-harvest application of alginate antimicrobial coating on strawberries. The effects of electrostatic alginate coating on weight loss, visible decay, firmness, surface color, total soluble phenolic content and antioxidant capacity of fresh strawberries stored at 7.5 °C for 13 days were evaluated. The higher transfer efficiency and evenness of electrostatic spraying coating led to a significant delay and reduction of visible decay compare to not-coated fruit (four days) and those coated with conventional spray method (one day). Firmness and color retention of strawberries were also improved using electrostatic spraying coating.

5.2 INTRODUCTION

The increasing consumer demand for fresh, safe, and high-quality fruit has lately gained interest on the development of new post-harvest method to prolong shelf-life, while ensuring food safety and maintaining nutritional and sensory quality. Applied a thin layer of edible coating on fruit surface has the potential to prevent moisture loss and control gasses exchange, thus improving quality attributes of perishable fruit (Andrade et al., 2012). Along with the development of new type of coating, different coating application technology have been developed to provide practical and efficient coating performance. Recently, there has been an increase on the utilization of electrostatic spraying technology for the application of powder and liquid coatings in the food industry (Khan et al., 2012a).

Electrospraying was first utilized in the paint industry due to several advantages it provided over conventional application methods such as production of more even, uniform and reproducible coatings with less overspray material (Amefia et al., 2006). In electrostatic coating, an intense electrical field is applied to the coating material that is forced out away from the electrode and is attracted to the nearest grounded target, allowing the charged particles to disperse over the whole surface. In this way, electrostatic spray applications increase transfer efficiency, evenness of the coating process and therefore may improve the overall product quality (Bailey, 1998). The versatility of electrostatic technology for different type of materials application allowed the food industries, especially those involving powder coating for seasoning and flavors, to use electrospraying for overcome problems related to uneven coating, excessive use of expensive additive, and release of dust to the surrounding environment

which decreasing additional cleaning needs and health hazards to the line operators (Ratanatriwong et al., 2009). Different food products have been coated using electrostatic powder coatings such as french fries, cheese (Amefia et al. 2006), and potato chips (Ratanatriwong et al., 2003), to obtain more uniform coating that led to improvements in overall quality and shelf life of the product as well as saving time and cost. Previous works also reported the application of electrostatic powder coatings on meat, crackers and bread (Khan et al., 2012b).

Electrospraying has also received significant attention as a novel technology for the application of liquid coatings, especially in agriculture for the applications of pesticides on different crops such as cabbage (Perez et al., 1995) and blueberry plants (Schermet al., 2007). These applications resulting in a better leaves coverage and less damage than conventional sprayer methods due to their increasing control over pests. Moreover, fine liquid antimicrobial coating has been applied with electrostatic spraying technology as potential sanitizer on different type of food product. Russell (2003) found that electrolyzed water was effective on eliminating pathogenic bacteria from eggs when used in conjunction with electrostatic spraying. Similarly, electrostatically sprayed organic acids demonstrated to be highly effective against foodborne pathogens on spinach (Ganesh et al., 2010) and iceberg lettuce (Ganesh et al., 2012). Other applications of liquid electrostatic coating included the application of chocolate (Gorty and Barringer, 2011), production of cocoa butter microcapsules, and impregnation of bread with vegetable oil (Bocanegra et al., 2005).

Even though the applications of liquid electrostatic coating on foodstuff as sanitizers has been widely studied, the utilization and effect of antimicrobial edible

coating on fruit using electrostatic spraying was not previously investigated. To the best of our knowledge no prior research has been reported on the application of electrostatic antimicrobial coatings for its potential to extend the shelf-life of the fruit by acting as selective barriers for fruit quality and microbial protection. In this study, emphasis was given to the electrostatic application of alginate coating enriched with carvacrol and methyl cinnamate, natural antimicrobial compounds, on fresh strawberries.

Strawberries are highly perishable fruits characterized by high physiological post-harvest activity that limits their shelf-life. Therefore, applications of alginate as polysaccharide-based coatings with low gas permeability have the potential to extend strawberry shelf-life by reducing respiration rate (Rojas-Graü et al., 2007). The incorporation of natural antimicrobial compounds into coating can inhibit microbial growth that can seriously degrade strawberry quality. Carvacrol, the major component of essential oils from oregano and thyme; and methyl cinnamate, a methyl ester of cinnamic acid that is also one of the major volatile components of strawberry aroma produced and released during fruit maturation, were added into alginate coating formulation for their well known powerful antimicrobial and antifungal properties (Lambert et al., 2001; Burt and Reinders, 2003).

Efficiency and quality of electrostatic spraying depends on several parameters related to the electrostatic processes and the coating materials. The effect of electrostatic alginate antimicrobial coating on quality-related attributes of strawberry during storage was evaluated in this study. In addition, the efficiency of electrostatic spraying was determined by transfer efficiency and evenness of coating.

5.3 MATERIALS AND METHODS

5.3.1 Alginate coating

Food grade sodium alginate (Keltone[®] LV, ISP, San Diego, CA, USA) was used as primary ingredient in edible coatings formulation. Coating solution was prepared by dissolving sodium alginate powder (2% w/v) in distilled water while heating on a stirring hot plate for 15 min at 70 °C until the mixture became clear. Then, 1.5% (w/v) glycerol (Starwest Botanicals Inc., Rancho Cordova, CA, USA) was added to the solution and stirred for 5 min to provide good flexibility to the coating. Antimicrobial coating was obtained by adding 0.98% (w/w) carvacrol and 1.45% (w/w) methyl cinnamate (Sigma-Aldrich, St. Louis, MO, USA) active compounds into the alginate solution a little bit at the time and homogenizing with a Polytron 3000 (Kinematica, Luzern, Switzerland) for 10 min at 4,000 rpm. Concentration of the active compounds used was based on the results of a preliminary study for the optimization of carvacrol and methyl cinnamate concentrations (Peretto et al., 2013a). Other parameters such as turbidity, viscosity and whitsh index were also considered in order to develop a suitable coating for strawberry application that could impart a natural aspect and improve appearance of the fruit. Considering the insolubility of methyl cinnamate in water, it was previously dissolved in ethanol (40 % w/w) at 40 °C under stirring at 220 rpm, for 5 min. Antimicrobial solutions were prepared fresh at the day of treatment and kept at 40 °C until spray application.

Resistivity of coating was determined using a Ransburg Multifunction Electrostatic Meter (ITW Ransburg, Toledo, Ohio) to ensure the value of the coating was suitable for electrostatic application (lower than 0.1 M Ω).

5.3.2 Strawberry preparation and storage conditions

Fresh strawberries were harvested by California Giant (Watsonville, CA, USA) and sent by overnight shipping to a local wholesale distributor (Fuji Melon, Oakland, CA, USA). Strawberries, were picked up from the distributor earlier in the morning, and kept at 2 °C until treatments. Berry samples selected for uniform size, color, weight and absence of physical and pathological defects were sprayed and stored on polystyrene weighing dishes at 7.5 °C (\pm 0.5 °C), 90% RH for 13 days, as average of common commercial storage conditions. Not coated control fruits were stored at the same conditions as the sprayed samples. All treatments (electrostatic and non electrostatic sprayed fruits) and controls were tested immediately after sprayed (day 0), 4, 8 and 13 days after storage. Three replicates for each treatment and storage time, consisting in a total of 36 fruits, were used for overall qualitative analysis.

5.3.3 Electrostatic spray of alginate coating

A waterborne electrostatic gun applicator (Vector Solo cordless 85kV, ITW Ransburg, Toledo, Ohio) was used to coat targets at 85kV, for electrostatic coating

(ES), and 0kV for non electrostatic coatings (NE). The coating equipment consisted of an isolated fluid system, an air line, applicator nozzle, fan air and fluid needle adjustment, and compensation valve.

The air supply line was set at 100 psi and flow rate of the coating in the fluid line was set at 70 g/min. Non-electrostatic coating was done in the same machine with the same settings except the voltage was zero. Four coating targets (strawberries) at a time were placed on top of a metallic rack (16 cm high) in order to ground the targets as well as to allowed the coating to cover the whole surface of the fruit. Strawberries were positioned in a square shape with a 2 cm space between them. The applicator was set in a vertical position at 60 cm away from the targets. The operating parameters and valve settings were tested and adjusted based on previous tests in order to obtain a short-round-spray pattern for uniform field coverage.

The weight of strawberries before and after coating was measured and used to calculate the transfer efficiency as $TE (\%) = [(W_f - W_i)/W_s] \times 100$, where W_f and W_i are the weight of targets after and before coating, respectively, and W_s is the weight of the material sprayed toward the targets during spraying period (6 seconds). Expressed mathematically, transfer efficiency is the net amount of material deposited on a target divided by the total material sprayed.

The evenness of coating was indirectly evaluated by comparing the blue color value b^* of water sensitive paper (Q. Instruments, Jena, Germany) applied on the back side of the fruits not directly exposed to the spray. One 6 mm diameter water sensitive paper disc was attached on each fruit and color parameter b^* of each disc was determined after spraying using a Konica Minolta spectrophotometer (CM508D,

Konica–Minolta Inc., Ramsey, NJ, USA) under a standard white reflector plate. The yellow moisture paper changed to blue when contact with aqueous drops, therefore the blueness ($-b^*$) to yellowness ($+b^*$) value was used to determine the coating efficiency of ES and NE spray on coated targets. The moisture sensitive papers were applied only on the back of the fruits based on the results of previous studies in which papers applied on top and sides of the fruits had the same b^* value for ES and NE spray as being completely covered by the coating solution. Three readings for each paper disc were recorded using a 3 mm diameter target mask.

5.3.4 Quality of strawberry

Strawberries were inspected daily and fruits were considered infected when a visible lesion, characterized as brown spots and softening of the infected area, was observed. The results were expressed as fruit infection percentage.

The same fruits for each treatment were weighed daily throughout the storage time. Weight loss was expressed as percentage loss of initial weight.

L^* , a^* , and b^* colorimetric values were obtained using a CM508D spectrophotometer (Konica – Minolta Inc., Ramsey, NJ, USA). Eighteen berries, for each treatment, were selected for color assessment and five measurements were made on the surface of each fruit by changing the position of the mask over the fruit.

Strawberry firmness was determined using a TA – XT2 Texture Analyzer (Stable Micro Sistem Ltd., UK) on the same eighteen fruits after used for color determination. The force required for a 2 mm probe to penetrate 7 mm into strawberry flesh at a rate of 2 mm/s was measured. Berries were cut into two pieces alongside and texture was measured in both sides at the highest elevation closer to the stem end. Measurements were taken at day 0, 4, 8, and 13.

An adaptation of the DPPH method (Brand-Williams et al., 1995) was used to estimate the AC. Five g of homogenized sample were extracted with 20 mL of methanol, collected in polytetrafluoroethylene (PTFE) tubes, and stored for 48 h at 4 °C. Homogenates were then centrifuged (rotor SA–600, SORVALL RC 5C Plus, Kendro Laboratory Products, Newtown, CT, USA) at 29.000 x g for 15 min at 4°C. Sample aliquots of 50 µL were taken from the clear supernatant (equivalent methanol volume as control) and reacted with 2950 µL of DPPH reagent (obtained by dissolving 0.047 g/L in methanol) in a covered shaker at room temperature until steady state conditions were reached (no significant decrease in absorbance was experienced as compared with the control, 20–22 h). The spectrophotometer was blanked with methanol, the solutions were placed in 4.5 mL disposable cuvettes, and the absorbance at 515 nm was recorded using a spectrophotometer UV–1700 (Shimadzu scientific instruments, Inc., Columbia, MD, USA). AC was calculated by measuring the decrease in absorbance of samples as compared with the methanol samples and quantifying as µg Trolox equivalent from a standard curve developed with Trolox (0–750 µg/mL) and expressed as mg Trolox per g fresh weight.

The same methanol extract as for AC was also used for TSP analysis. The assay was conducted according to Swain and Hillis (1959) method with some

modifications. A 150 μL aliquot of methanol extract was taken from the clear supernatant, diluted with 2400 μL of nanopure water, followed by 150 μL of 0.25N Folin–Ciocalteu’s reagent, and incubated for 3 min at room temperature. The reaction was stopped by adding 300 μL of 1N sodium carbonate and the mixture was incubated for 25 min. The standard curve was developed with different concentration of gallic acid, ranging from 0 to 0.375 mg/mL. Results obtained from the readings of absorbance at 765 nm were expressed as mg of gallic acid per 100 g fresh weight.

Triplicate samples were used for all measurements. Data were statistically processed by ANOVA using the CoStat 6.4 (CoHort software, USA) program. Means were separated by HSD Tukey Test at the significance level of $p \leq 0.05$.

5.4 RESULTS

5.4.1 Transfer efficiency and evenness of coating

The use of electrostatic spraying technology for the application of antimicrobial alginate coating led to a significant improvement on transfer efficiency compare to NE coating (+ 33.82%) (Fig. 1A). Transfer efficiency could be considered a measurement of process efficiency as it determines the amount of material reaching the targets compared to the portion of the material lost in the surrounding environment. Even though a small amount of coating was also delivered into the surrounding area, the transfer efficiency value of ES spraying coating reached approximately 60%. The

positive effect of electrostatic spraying on increasing coating deposition has been widely studied and reported in many works where food product were coated with seasonings (Amefia et al., 2006). Law (2001) reported that electrostatic spray of liquids, used either as potential sanitizers or pesticides, have been proved to increase transfer efficiency and provide an overall better coverage of crops, leading to identical appearance of all product sides, due to the self-dispersion of the charged particles across the whole target surface.

ES coated strawberries have more uniform coatings compare to NE sprayed fruits (Fig. 1B). The significant lower b^* value, a indicator of blue chromacity, indicated that the back side of ES coated fruit was reached by the coating droplets to a greater extent than those of the NE coated fruit (32.5 vs 50.1). The electrical field applied on the liquid coating induced electrostatic forces with sufficient intensity to overcome surface tension forces that atomizing the liquid surface into a spray of charged particles. Due to their charges, the droplets repelled each other and create a cloud of charged droplets that was attracted to the coating target which creating a uniform film of liquid coating. Therefore, ES spray can increase droplets depositions on targets and overcome common problems of uneven distribution and poor surface coverage obtained with conventional (NE) spray (Amefia et al., 2006).

5.4.2 Quality of strawberry

In order to determine the effectiveness of electrostatic antimicrobial coating for improving quality of fresh strawberries, visual decay and weight loss, as primary

determinants of quality, were monitored daily throughout the storage period and compare to non treated (control) and NE coated fruits. Fungal decay (Fig. 2) occurred rapidly for control fruits, with 2.7% of strawberries showing sign of infection after 7 days of storage and 8.3% after three additional days (day 10) of storage. NE sprayed fruit started to show first signs of mold growth at day 10. The electrostatic treatment provided superior postharvest disease control as the fruit did not develop any fungal decay until 11 days of storage, exhibiting 66% less spoilage than the control. This one day of delay recorded for ES coated strawberries over NE can have important economical implications as berries can last longer in the fresh market. Moreover, the severity of decay for ES treated fruits was significantly ($p < 0.05$) reduced compare to the control for the remaining storage period and beyond 13 days of storage. ES sprayed coating significantly inhibited strawberry decay with only 5.55% of infected fruits, compare to 16.6% and 8.33% for control and NE sprayed fruits after 13 days of storage, respectively. The application of electrostatic coating led to an inhibition of mold growth through the creation of an even coating on strawberry surface that allowed the antimicrobial properties of carvacrol and methyl cinnamate to perform in a greater extent than NE coating. Especially, methyl cinnamate has been proven to have antifungal activity against phytopathogenic fungi under in vitro conditions (Vaughn et al., 1993; Rahmani et al., 2010) and in practical application on strawberry when released as vapor from edible film (Peretto et al., 2014b). Strawberries are potential sources for foodborne pathogen and fungal infection as they are not washed before packaging, thus utilization of antimicrobial coating to prevent these infection has gained importance over other treatments. Similar positive outcomes were obtained in sanitation application using liquid electrostatic spraying technology in the food industry. Ganesh

et al., (2010) demonstrated that electrostatically sprayed food-grade acids were more effective in reducing *Salmonella typhimurium* on spinach compared to conventional spray system due to the more even distribution of the liquid antimicrobial solution on the whole sample surface. Similarly, Law and Cooper (2001) reported that air-assisted antimicrobial electrostatic spray provided better control of fungal infection on banana, over conventional hydraulic sprays.

The weight loss of strawberries increased with storage time in all coated and not-coated fruits. Even though no significant differences were detected, weight loss of control fruits had the tendency to have higher weight loss than the other samples, whereas ES coated fruit showed the lowest value during storage time. The higher effectiveness (TE and evenness) of the ES spraying system could have induced the charged alginate coating droplets to disperse over the whole fruits surface and, in this way, to control respiration rate of the fruit. Previous studies demonstrated that alginate coatings modify the internal atmosphere of the fruits by allowing enough gas exchange to prevent strawberries from going anaerobic, while at the same time, retarding ripening and senescence (Lacroix and LeTien, 2005).

The marketability of fresh strawberries is strongly influenced by sensory and visual appearance such as external color of the fruits. Table 1 shows that non-coated fruits exhibited significant changes on L* value during postharvest storage. Brightness of control fruits decreased significantly ($p \leq 0.05$) after four days of storage, probably due to the higher gas exchange and respiration rate that were not controlled by the barrier properties of the alginate coating, leading to higher metabolism rate and faster loss of lightness (brightness). Changes in fruit color are common over time in post-harvest, mainly influenced by improper storage conditions that accelerate the natural

ageing process, promote microbial growth, and induce loss of overall quality. Applying thin layer of antimicrobial coating can extend shelf life of food product as well as improve their qualitative attribute by creating a modify atmosphere (Olivas et al., 2006). In general, the use of antimicrobial alginate coating had a significant effect on L* color parameter compare to non-coated strawberries, showing higher values throughout storage period. The higher brightness observed for coated strawberries could be explained by the reflection of the light generated by the presence of shiny/glossy oil droplets of carvacrol into the coating formulation. Similarly, Vargas et al., (2006) reported an increase of luminosity of strawberries coated with chitosan-oleic acid, compare to non coated fruits.

Positive a* (+a*), a indicator of redness, was significantly higher in control strawberries compared to ES coated berries after four days of storage. This suggested that electrostatic spraying technology achieved more uniform coating on strawberry surface which led to a possible higher discoloration of fruit skin due to the phytotoxic activity of the EOs in the coating solution that directly contact with the fruits (Amiri et al., 2008). However, this slight discoloration on ES coated berries was not significant when compared to fresh (day 0) strawberries. The redness of ES coated fruits remained the same throughout the storage period, while the redness of non-coated and NE coated strawberries decreased as storage time increased. By the end of 13 day storage, non-coated and NE coated strawberries showed significant lower a* value compared to day 0, 4, and 8. In addition, no significant differences in redness were found among the treatments after day 8 and 13 of storage. All these results indicated that electrostatic spraying technology can provide a more uniform coating on the fruit surface and ES

coated berries could maintain the same redness as fresh (day 0) strawberries during 13 days of storage.

Color changes among fruits were also determined using b^* value during storage. A positive b^* value is a measure of yellowness, and a negative value of blueness. The b^* value was lower for control strawberries in comparison with coated fruits, indicating a loss of yellowness, which is common in mature fruits (Ribeiro et al., 2007). Although no significant differences were observed between ES and NE coated fruits, ES coating showed a better retention of blue color during storage time over NE. The color retention could be due to more uniform alginate coating achieved by ES spray.

Texture loss is one of the most important and visible changes that occurs during fruit maturation as a consequence of metabolic changes and water loss that can seriously compromise storage life of fresh fruits. The application of antimicrobial alginate coating on strawberries using electrostatic spraying technology showed significant higher fruit firmness and mechanical properties ($p \leq 0.05$) in comparison with control and NE, especially at the end of storage period (Fig. 3). Texture data indicated that although the maximum force required to penetrate the flesh of the fruits did not show a significant decrease during storage, not coated fruits showed higher texture loss (7.02%) after 13 days of storage, whereas the loss for coated fruit was less marked especially when ES was used (2.78%). Significant differences ($p < 0.05$) between firmness of ES and NE coated strawberries were observed during storage; mechanical properties seemed to be better preserved when electrostatic coating was used. Texture changes in strawberry fruit is related to the degradation of cell wall components mainly due to the action of extracellular pectolytic enzymes produced by

Botrytis cinerea (Aguilar and Huitrón, 1993). As reported in our previous study (Peretto et al., 2014b), the antimicrobial activity of C and MC could have indirectly decreased the amount of such enzymes, by inhibiting microbial growth. Furthermore, the good gas barrier properties exerted by the alginate coating could have reduced the metabolic activity of the fruits, therefore helping to maintain a better firmness. Many polysaccharide-based coatings have been developed for strawberries in order to prevent water loss and texture changes during post-harvest storage, for instance starch-based coating improved overall sensory conditions due to firmness retention and turgency (García et al., 1998).

The antioxidant capacity of strawberries did not change significantly throughout storage period. There was no significant difference between electrostatic and non-electrostatic coated berries (Table 2). However, control fruits showed a lower value in comparison with coated fruits, especially after four days of storage where not coated fruit showed 6.20 mg Trolox/g f.w vs 8.37 and 7.42 for ES and NE coated fruit, respectively. Strawberries are rich source of phytochemicals with antioxidant properties that have been proved to exert health-protecting benefits. The level of antioxidants in fresh strawberries can be influenced by the application of edible coatings as a consequence of passive modified atmosphere that controls gas exchanges (Falguera et al., 2001). Some studies suggested that edible coatings, as a tool to improve post-harvest storage of fresh products, could be considered a stress condition for the fruits and trigger high synthesis of phenolic compounds, which contribute to the antioxidant power (Oms-Oliu et al., 2008). Our results showed that the use of ES technology did not increase the antioxidant capacity and total soluble phenols of strawberries, although

higher values were obtained when the fruits were coated with the alginate antimicrobial coating.

5.5 CONCLUSION

Using electrostatic spraying technology for the application of liquid coating increased the functionality in terms of transfer efficiency and evenness. These improvements lead to significant increased on several quality aspects of strawberries during storage and extend the shelf-life of coated fruits. Therefore, considering these promising results, electrostatic spray could be considered a possible mean for liquid coating application of perishable fruits. This technology could provide greater retention and distribution of coating required to exert its beneficial effect on post-harvest quality of perishable fruits.

5.6 REFERENCES

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5.7 FIGURES AND TABLES

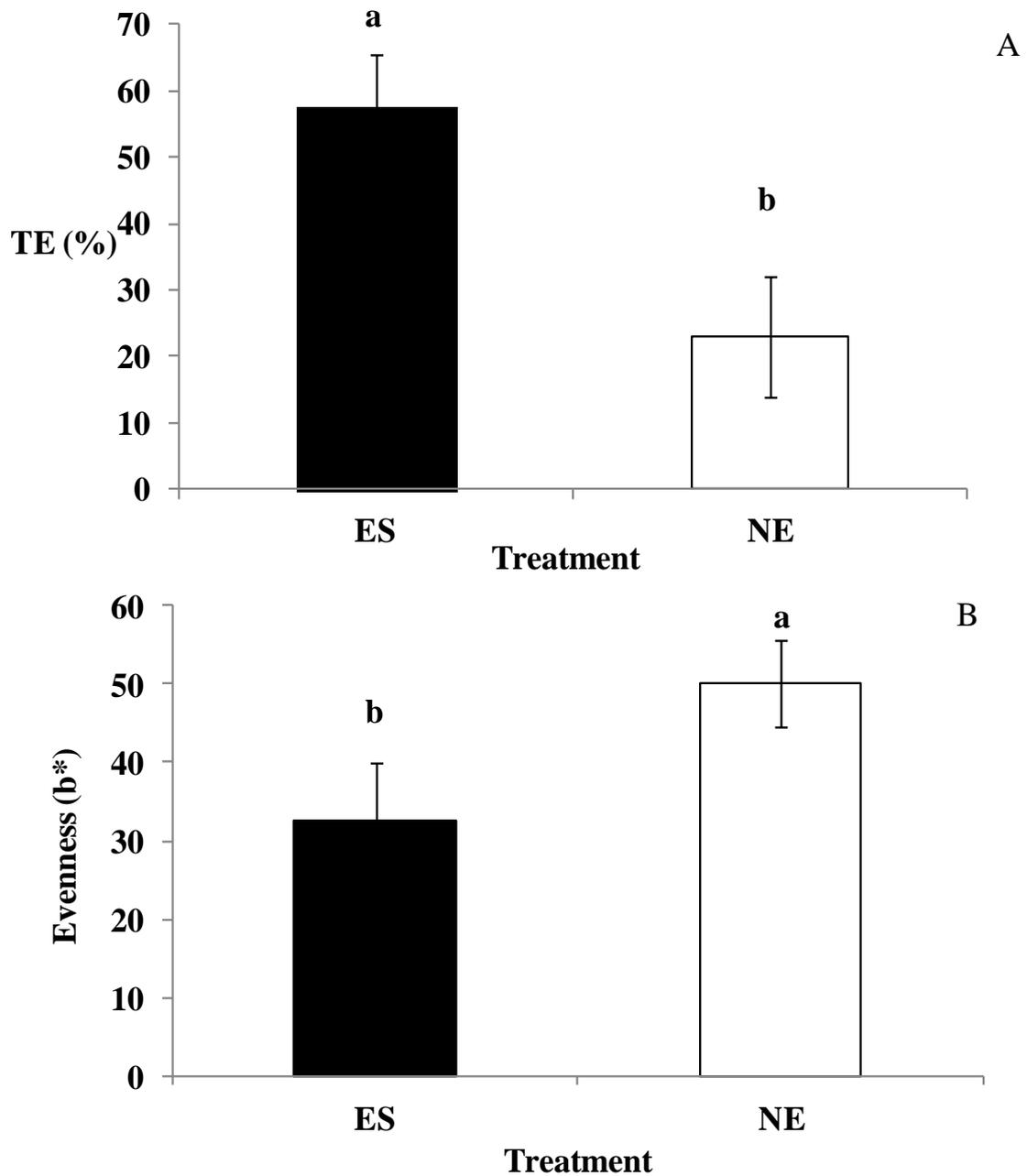


Figure 1 A: transfer efficiency (TE%) and **B:** coating evenness of electrostatic (ES) and non-electrostatic (NE) coating. Data shown are the means and bars indicate standard deviation.

a-b: different letters indicate significant differences among treatments ($p \leq 0.05$).

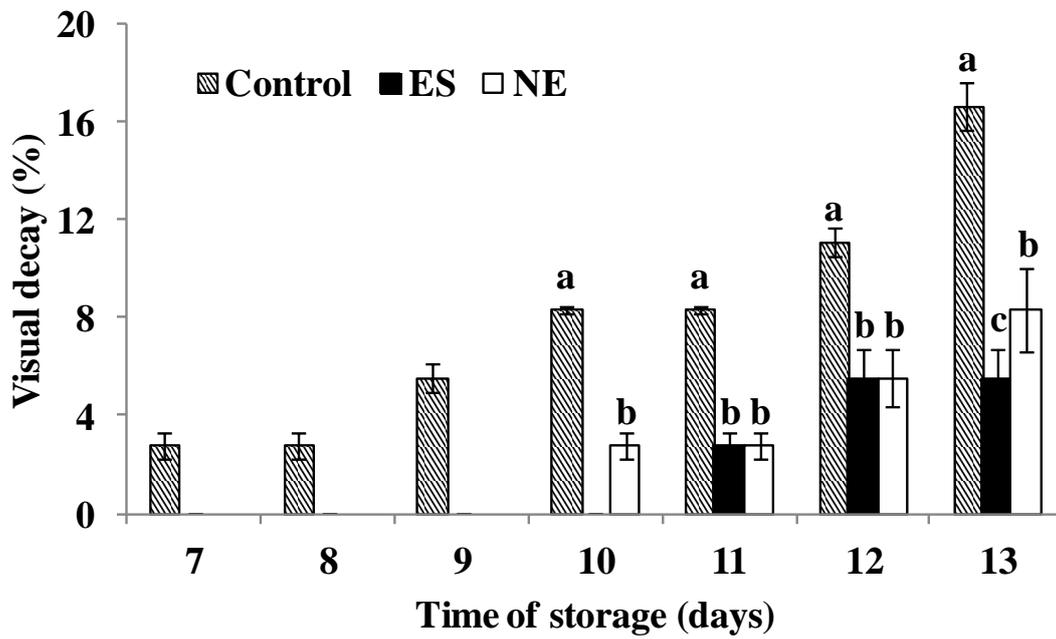


Figure 2. Visible decay of strawberries expressed as percentage of infected fruits during storage time for not sprayed fruits (control), electrostatic (ES) and non-electrostatic (NE) coating. Data shown are the means and bars indicate standard deviation. a-b: different letters indicate significant differences among treatments ($p \leq 0.05$).

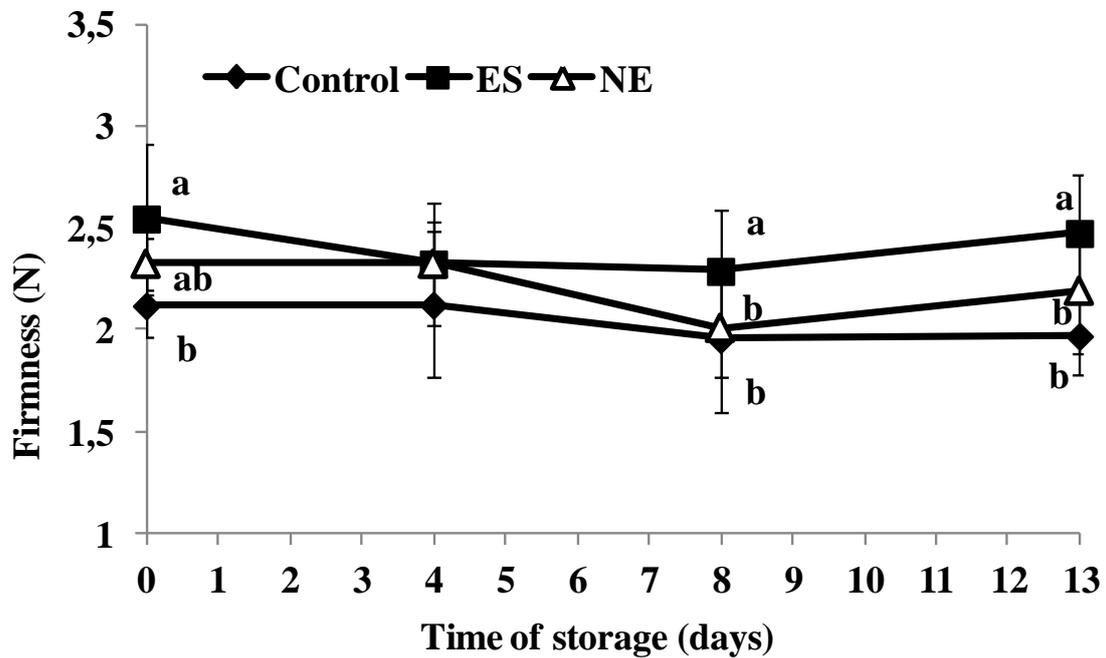


Figure 3. Firmness of not coated (control), electrostatic (ES) and non-electrostatic (NE) coated strawberries during storage time. Data shown are the means and bars indicate standard deviation. a-b: different letters indicate significant differences among treatments ($p \leq 0.05$).

Table 1. Color parameters (L*, a*, b*) of strawberries during 13 days of storage at 7.5 °C. Data shown are the means. Control: no coating; ES: electrostatic coating; NE: non-electrostatic coating

Treatment	Storage time			
	0	4	8	13
	L			
Control	27.60 ± 2.15 A	22.47 ± 3.53 ^b B	21.97 ± 2.46 ^b B	24.33 ± 3.07 ^b B
ES	26.30 ± 1.81	25.01 ± 1.29 ^a	25.89 ± 1.72 ^a	25.18 ± 3.31 ^{ab}
NE	26.41 ± 2.12 A	22.97 ± 2.49 ^{ab} B	24.44 ± 2.49 ^a B	27.10 ± 1.16 ^a A
	a*			
Control	26.79 ± 2.92 AB	29.95 ± 4.11 ^a A	27.93 ± 5.90 AB	25.64 ± 3.14 B
ES	27.95 ± 3.25	26.86 ± 2.59 ^b	27.47 ± 3.34	27.44 ± 4.38
NE	28.90 ± 3.52 A	28.73 ± 3.34 ^{ab} A	27.15 ± 4.16 AB	25.34 ± 2.08 B
	b*			
Control	14.63 ± 2.16 ^b AB	13.44 ± 1.26 ^b B	15.93 ± 3.31 A	14.90 ± 2.46 AB
ES	14.85 ± 2.16 ^a B	19.02 ± 3.55 ^a A	16.32 ± 2.63 B	15.41 ± 3.32 B
NE	16.79 ± 3.14 ^{ab} A	17.20 ± 3.39 ^a A	16.32 ± 3.58 AB	13.74 1.82 B

^{a-b}: different letters within a column indicate significant differences among treatments ($p \leq 0.05$)

A – B: different letters within a row indicate significant differences during storage time ($p \leq 0.05$)

Table 2. Total soluble phenols (TSP) and antioxidant activity (AC) of strawberries during 13 days of storage at 7.5 °C. Data shown are the means \pm standard deviation. Control: no coating; ES: electrostatic coating; NE: non-electrostatic coating

Parameter	Treatment	Storage time at 7.5 °C			
		0	4	8	13
TSP (mgGA/100g f.w.)	Control	170.1 \pm 15.07	133.6 \pm 18.39	113.1 \pm 8.08	126 \pm 6.93 b
	ES	171.1 \pm 28.13	131.8 \pm 9.71	118.8 \pm 14.5	138.5 \pm 12.43 a
	NE	155.3 \pm 27.33	127.2 \pm 25.43	120.8 \pm 9.73	144.5 \pm 7.88 a
AC (mgTrolox/g f.w.)	Control	5.8 \pm 0.49	6.2 \pm 0.65 b	6.2 \pm 1.17	5.6 \pm 0.38
	ES	6.0 \pm 0.65	8.37 \pm 1.17 a	6.61 \pm 0.62	5.70 \pm 0.58
	NE	5.9 \pm 0.35	7.42 \pm 0.95 a	6.26 \pm 0.74	5.65 \pm 0.41

a-b: different letters within a column indicate significant differences among treatments ($p \leq 0.05$).

6. FINAL CONCLUSIONS

Traditional post-harvest technologies such as cold refrigeration storage, control atmosphere (CA), and modified atmosphere packaging (MAP), have been used extensively by the food industry worldwide as a mean to extend the shelf life and maintain the quality of fruits. However, all these technologies have been limited to extend the shelf life of berries, as well as to maintain their healthy and sensory attributes. Moreover, they have the potential to induce undesirable effects on berries (and other fruits) such as fermentation, off-flavors development, and cell membrane damage, which depreciate fruit quality.

To overcome the limitations of the traditional post-harvest technologies, mentioned above, three new technologies were investigated for having the potential to improve the overall quality of berries, using blueberry and strawberry as model systems. These novel technologies included the use of microperforated polypropylene film, edible films and coating enriched with natural antimicrobial compounds, and a state-of-the-art electrostatic spraying technology for coating application.

The application of microperforated polypropylene film (1, 10, and 30 microperforations) to store freshly harvested blueberries, created a passive modified atmosphere within the package that led to improvement of antioxidant activity, total phenols and firmness of the berries, throughout their storage period. Equilibrium atmosphere within the packaging was achieved after two days of storage and the concentration of gases remained constant for the rest of the storage period. This stable atmospheric condition resulted in an extension of the shelf-life of blueberry for up to 16 days, at 4°C.

Fresh strawberries packed in clamshells containing strawberry puree edible films, infused with the antimicrobial compounds carvacrol and methyl cinnamate, exhibited a significant delay and reduction in the severity of visible decay after kept storing them for 10 days at 10 °C and 90% relative humidity. Additionally, the carvacrol and methyl cinnamate vapors released from the films helped to maintain firmness and brightness of strawberries as compare to the not-treated strawberries. The natural antimicrobial vapors also increased the total soluble phenolic content and antioxidant activity of fruit at the end of the storage period. Also, an alginate coating containing carvacrol and methyl cinnamate, as an effective post-harvest technique to assure microbial safety and at the same time retain overall quality of strawberry, was applied using a novel electrostatic spraying technology. Response surface methodology, followed by full factorial design, a central composite design, and desirable responses was used to optimize the antimicrobial activity of the coating against *Escherichia coli* O157:H7 and *Botrytis cinerea* and several physical properties (turbidity, viscosity and whitish index) of alginate coating, The results of the ANOVA of central composite design in the response surface methodology showed the significant fitting of all responses to the quadratic model. Considering all the desirable responses (antimicrobial activity against *E. coli* and *B. cinerea*, viscosity, turbidity and W_i), they were optimized for 0.98% (w/w) carvacrol and 1.45% (w/w) methyl cinnamate concentration.

The results of these studies demonstrated that microperforated polypropylene film promote rapid development of an adequate passive modified atmosphere to extend fruit shelf-life and quality of blueberries. By altering the size and density of the perforations, packaging films with specific flow rates can be adjusted for a specific fruits. Additionally, it was demonstrated that both edible films and coatings, containing

the antimicrobial compounds carvacrol and methyl cinnamate, provide numerous advantages on fruit quality as they can create semipermeable barriers to gases and water vapor maintaining the overall (physical, chemical and safety) quality of strawberries. Moreover, edible films and coatings are environmentally friendly technologies that can also decrease the amount of conventional synthetic and disposable packaging materials. Overall, the studied new potential post-harvest technologies provided not only shelf life extension, but also improved the visual and sensory quality of berries and preserved the healthy components that characterized these fruits. Further investigation could be necessary to improve the use of these technologies in post-harvest handling of other berries fruits and other fresh commodities.