5-1-2018

APPLICATION OF PLANT BASED EDIBLE COATINGS FOR MAINTAINING POST HARVEST QUALITY AND EXTENDING SHELF LIFE OF STRAWBERRIES

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Rajiv Dhital, M. Sc. in Food Microbiology

Tribhuvan University, 2013

A Thesis

Submitted in Partial Fulfillment of the Requirements for the

Master of Science

Department of Plant Soil and Agricultural systems

In the Graduate School

Southern Illinois University Carbondale

May 2018

THESIS APPROVAL

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By

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A Thesis Submitted in Partial

Fulfillment of the Requirements

For the Degree of

Master of Science

In the field of Plant, Soil and Agricultural Systems

Approved by:

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December 18, 2017

AN ABSTRACT OF THE THESIS OF

Rajiv Dhital, for the Master of Science degree in PLANT, SOIL AND AGRICULTURAL SYSTEMS, Presented on * December 18, 2017, at Southern Illinois University Carbondale.

TITLE:

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THESIS ABSTRACT

Strawberries are a popular fruit with a pleasing color and flavor. However, its delicate tissue and high sugar content makes it highly perishable with visible mold. In this study, we have attempted to test feasibility of different edible coatings for extending shelf life of 'Chandler' strawberries subjected to simulated vibrations of local transportation. Six types of coatings were compared based on the quality of treated berries. Curcumin and limonene were used as natural antimicrobials and coatings were prepared from their liposomes and were over-coated with methyl cellulose. One set of each coating type were subjected to the simulated vibration of local transportation. The vibrated samples had lower shelf life than non-vibrated samples, indicating a robust coating which remains intact during road vibrations is required. Based on the number of berries with visible mold, limonene liposomes showed significantly lower fungal growth compared to the control on the 14^{th} day of storage. Titratable acidity and total phenolic contents were also found to be higher in limonene-coated strawberries compared to other coatings. From the findings, further study of liposome coatings of limonene with different particle size and

concentration of the lipid bilayer was necessary to characterize the liposome for an effective application in strawberries. To this regard, another study was done with the aim to develop and characterize alginate and limonene liposomes as edible coating materials and to determine their efficacy in shelf life extension and maintaining quality parameters of 'Chandler' strawberries. Alginate solution (1.5% w/v) and limonene liposomes prepared from 80% lecithin and 20% PDA were used as edible coating materials. Fungal decay percentage, total yeast and mold counts, headspace atmosphere analysis, total soluble solids, pH, titratable acidity, total anthocyanin content and total phenolics were analyzed to assess fruit quality during 14 days at 4ºC of storage. Days of storage were found to be significant in maintaining the quality of the strawberries. Among the coating types, strawberries coated limonene liposomes were found to be significantly effective in maintaining the lesser respiration rate, lower the change in pH (3.9), and had higher total anthyocyanin (43.849) content during storage. Thus, limonene liposomes were found to be useful for extending the shelf life and maintaining quality of strawberry fruits.

DEDICATION

This thesis is dedicated to my family, (Mother: Gita Sharma, Father: Kamal Raj Sharma, Brother: Rabin Dhital and Wife: Kamana Acharya).

ACKNOWLEDGEMENTS

Firstly, I am thankful to my advisor Dr. Choudhary for his constant support, encouragement and his patience in guiding me. This work would not be possible without guidance from him. I would like to express my sincere thanks to my committee members (Dr. Watson, Dr. Kohli) for their valuable support and guidance throughout my period of study. I am also thankful to my colleagues for being supportive during my research.

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CHAPTER 1

INTRODUCTION

Fruits and vegetables are considered as good sources of vitamins, minerals, fibers and phytochemicals typically antioxidants, which are beneficial in promoting human health. Several studies suggest that increase in consumption of fresh fruits and vegetables can be associated with the decrease risk for mortality caused by cancer and cardiovascular diseases (Hjartåker et al. 2015; Oyebode et al. 2014; Wang et al. 2014). Phytochemicals available in fruits and vegetables shown protective effects against chronic and degenerative diseases (Record et al. 2001). Phenolic compounds such as flavonoids, carotenoids and ascorbic acids present in fresh fruits and vegetables have also shown to have antioxidative property (Ames et al.1993).

Strawberry (*Fragaria x ananassa*) are widely consumed fruits consumed both in fresh forms and as process food products such as preserves, jams, yogurts and ice creams. They are a good source of antioxidants, minerals and vitamins that have antioxidant, anticancer, anti-inflammatory and anti-neurodegenerative biological properties (Hannum, 2004). Being non-climacteric fruits, strawberries are harvested at full maturity stage to get good sensorial properties related to flavor and color. Strawberries are perishable fruits with high respiration rate, soft texture and sensitive to temperature changes, which makes them susceptible to the mechanical damage, physiological damage, water loss and decay (Sanz et al. 1999). They have very short post- harvest shelf life and the loss can reach 40% during storage (Satin, 1996).

Attempts to reduce the post-harvest loss and increase the shelf life of fruits have been extensive. The methods to preserve fruits and vegetables include physical-based preservation, bio preservation and chemical-based preservation and technologies. The processes are focused to

extend the shelf life by adjusting the storage temperatures, humidity, pressure and gas composition (Krasaekoopt & Bhandari, 2010). One of the most commonly used method is cold storage. Biopreservation includes the rational use of antimicrobial capacity of microorganisms having a long history of safe use for the extension of shelf life and safety of foods (Stiles, 1996). Chemical preservation refers to the application of natural or synthetic chemical compounds as preservatives or antimicrobial compounds for the extension of shelf life of foods (Meireles, et al. 2016).

Recently, there is an increasing trend of application of plant based bioactive compounds as edible coatings in the preservation and shelf life extension of fruits and vegetables. Edible coatings are the thin layers of edible materials applied on foods as food coatings or placed between food components (Del-Valle et al. 2005). Edible films are mainly composed of hydrocolloids (proteins, polysaccharides and alginate), lipids (fatty acids, acylgycerol, waxes) and composites (Dhall, 2013) The purpose of their application in foods is to extend the shelf life of food products and provide barrier against hazards. They are capable to prevent loss of water and volatile compounds, reduce the respiration rate and maintain the physiochemical properties of foods. They show an excellent barrier to fats and oils and have high selective gas permeability (Gontard et al. 1996).

Plant based essential oils and phenolic compounds have been studied for their ability to protect food against pathogenic bacteria (Friedman et al. 2004; Burt, 2004; Seydim & Sarikus, 2006). Further, these compounds are designated as generally regarded as safe (GRAS) (Burt, 2004) in food applications. Further, plant based essential oils can be incorporated with coatings to prevent microbial growth, extend the shelf life and preserve nutritional qualities of foods (Vargas et al.2009).

Nanotechnology have been applied in various fields like engineering, communications, medicine and food industries. For food processing industries, nanotechnology has gained importance in food packaging and maintaining food safety. Liposomes are vesicle, having the capacity of self-assembly, that consists of one or several bilayer membrane of phospholipids capable of encapsulating a volume of aqueous media. Limited studied have been done in the application of antimicrobials encapsulated in nano liposomes as food packaging materials (Mekkerdchoo et al. 2009). They have been used in food industries for the encapsulation of antimicrobial, flavor compounds and enzymes (Gortzi et al. 2008; Makwana et al. 2014).

1.1 Objectives

The aim of this study is to develop edible coatings from different natural compounds, application of these edible coatings on strawberries and compare their efficacy on the extension of shelf life and maintaining post-harvest quality of during the storage.

CHAPTER 2

LITERATURE REVIEW

Consumption of fruits and vegetables have gained significant global attention in recent years. The phytochemicals and antioxidant compounds ascorbic acid (AA), lycopene, β-carotene, and phenolics extracted from fruits and vegetables contribute to the nutritional value fruit and vegetables (Wettasinghe et al., 2002; Hu and Jiang, 2007).Research suggests that consumption of flavonoids, such as anthocyanins, flavonols, isoflavonoids, and tannins, a group of phenolic compounds present in fruits and vegetables might help in reducing risk of pancreatic cancer especially in smokers (Kristal, 2007). Flavonoids are also found to be effective in the inhibition of allergic reactions by inhibiting production of histamine (Berg and Daniel 1988).

In fresh fruits and vegetables, metabolic activities such as respiration produce ethylene, which acts as a biocatalyst to accelerate the process of ripening and senescence and another process called transpiration that results in loss of water vapor from intracellular spaces to the environment from fresh produce will occur (Wills et al. 1981). The rate of occurrence of these two activities have a significant effect on post-harvest shelf -life of fruits and vegetables. Higher the rate of respiration and transpiration, lower the shelf-life of product and vice-versa (Rojas-Graü et al. 2009).

Recently, because of rising public concern regarding human health issues and environmental protection, minimally processed produce has underlined food processors for maintaining postharvest quality and shelf life extension. Edible coatings extend the shelf life of foods by protecting foods from mechanical, physical, chemical and mechanical damage (Baldwin et al., 2011). Edible coatings have shown to regulate the transfer of moisture, oxygen, carbon dioxide and preserve organoleptic properties of these foods. These materials have proved to be beneficial

for the improvement of structural integrity of frozen fruits and vegetables (Baldwin and Baker 2002). Furthermore, some edible coatings can carry functional ingredients such as antioxidants and nutrients that contribute to enhance safety and stability of foods (Min and Krochta 2005). Edible coatings are composed of biopolymers such as polysaccharides, proteins, lipids and resins, which are used alone, or in combinations (Valencia-Chamorro et al., 2010; Dhall, 2013). Due to their natural origin, biodegradability and edibility, edible coatings have gained interest in their applications and research too. With all these properties, edible coatings would replace the currently used commercial synthetic waxes, composed mainly of oxidized polyethylene (Embuscado and Huber, 2009; Valencia-Chamorro et al., 2010; Dhall, 2013). The physical and chemical properties of edible coatings vastly influence the function of the coatings (Sothornvit and Krochta 2000). Coating materials are generally selected on their solubility in water i.e. either hydrophilic or hydrophobic, ease off application on fruits and sensory properties (Lin and Zhao 2007).

Types of edible coatings

Edible coatings can play a vital role for the improvement of post-harvest quality and shelf life of minimally processed fruits and vegetables by; inhibiting growth of microorganisms, providing a barrier to moisture and gas exchange, improve the mechanical handling properties, and retain flavor compounds. In addition, edible coatings can serve as carriers for other compounds which are generally recognized as safe (GRAS), such as preservatives and other functional food ingredients from natural sources (Baldwin et al., 1995; Olivas and Barbosa-Canovas, 2005; Vargas et al., 2008). The commonly used coating materials along with the recently emerged coatings are:

2.1 Polysaccharide coatings

The U.S. Food and Drug Administration (FDA) either classifies these compounds as food additives or generally recognized as safe (GRAS) substances. Polysaccharides such as starch and its derivatives, cellulose and its derivatives, alginates, chitosan, pectins, plant and microbial gums etc. are used for coatings and film formation (Krochta and Mulder-Johnston (1997); Debeaufort et al .1998). The hydrophobic nature of these materials provide evidence that they exhibit gas barrier properties rather than moisture barrier. They have low permeability towards oxygen and selective permeability to carbon dioxide. These coatings applied to the surface of fruits and vegetables tend to modify the food's internal atmosphere, which eventually leads to reduce respiration rate in these commodities (Nisperos-Carriedo et al. 1990). In addition, they also provide a partial barrier to moisture, improve mechanical handling properties of fresh produce, hold and control production of volatile compounds (Olivas and Barbosa-Canovas, 2005).Certain water-soluble gel-forming polysaccharides can be used in the form of films for extension of shelf life of food products which can prevent moisture loss of food (Kester and Fennema 1986). Apart from moisture barrier properties, these compounds serve as gel forming, adhesive and mouth-feel agents in foods. (Whistler and Daniel, 1990). As edible coating materials, polysaccharides act as integral part of food products. Use of various polysaccharides as edible coating materials have gained serious attention because they are nontoxic, readily available, and cheaper.

2.2 Proteins and derivatives

Protein coating materials derived from plant source (e.g. zein, soy protein and wheat gluten) and animal source (e.g. milk protein) exhibit excellent barrier properties towards oxygen, carbon dioxide and lipids particularly at low relative humidity (Gennadios et al. 1994; Baldwin and

Baker 2002). Zein and soy proteins are the most studied plant proteins for their application as edible coating materials in fruits and vegetables.

2.3 Bilayer and emulsion coatings

Recently, there has been increasing interest in the development of composite or bilayer coatings by the integration of proteins, polysaccharides, and /or lipids together for the improvement in functional quality of coatings. The principle behind the coatings is because each individual coating material has its unique but limited function. The functionality can be enhanced when two different types of coating materials are applied together (Lin and Zhao, 2007). Usually, composite/film or coating is categorized as a bilayer or a stable emulsion. In a general bilayer composite/ coatings, lipid forms an additional layer over the polysaccharide or protein layer, while in the emulsion form of lipid is dispersed and entrapped in the matrix of protein or polysaccharide (Callegarin et al. 1997). Researchers have developed composite films prepared from corn zein and corn starch, gelatin and fatty acid, soy protein isolate and polylactic acid (Kamper and Fennema, 1984). In a study by Poverenov et al., in 2014, application of layer-by-layer edible coatings of alginate and chitosan significantly enhanced physiological and microbiological quality of fresh-cut melons. Similarly, the layer-by layer coatings of polysaccharides CMC and chitosan exhibited improved physiological qualities for mandarin fruits (Arnon et al. 2015). Bilayer based on wheat gluten with lipids when applied to strawberries, significantly retained firmness and reduced weight loss (Tanada-Palmu and Grosso, 2005). An emulsion coating with CMC and paraffin wax, beeswax extended the shelf life and reduced weight loss of peaches and pears (To˘grul and Arslan 2004).

2.4 Additives in Films and Coatings

Additives such as plasticizers, antimicrobial agents, anti-browning compounds, texture enhancers, nutrients, probiotics and flavors have been widely used in films and coatings (Nayik et al.2015). Plasticizers are one of the major additives used in films and coatings formulations to improve or modify their mechanical properties (Xu et al. 2001). Use of compounds such as glycerol, acetylated monogycerides, polyethylene glycol, sucrose, fructose, glucose and mannose as plasticizers in various foods have been studied (Sothornvit and Krochta, 2005; Zhang and Han, 2006).

Incorporation of antimicrobial compounds into edible films and coatings provides an innovative approach to improve microbial safety and shelf life of foods (Cagri et al., 2004). Starch based edible coatings containing potassium sorbate were applied on the surface of fresh strawberries for the reduction of microbial growth and extension of shelf life (Garcia et al., 1998). A bilayer edible coating made from plant based antimicrobial compounds, limonene and curcumin were applied in combination with methylcellulose (MC) for improving of post-harvest quality of fresh strawberries (Dhital et al., 2017).

Antioxidants like ascorbic acids incorporated with edible coatings have proved to reduce enzymatic browning of fruits and vegetables (Baldwin et al., 1996). Similarly, α-tocopherol mixed with xanthan gum coatings enhanced the nutritional and sensory properties of baby carrots (Mei et al. 2002). Further, Carrageenan and whey protein coatings mixed with ascorbic acid and citric acid prolonged the shelf life of apple slices (Lee et al., 2003). A blend of chitosan- based coatings and α-tocopherol acetate was found to be significant to delay the color change of fresh and frozen strawberries (Han et al., 2004b). Xanthan gum was used to carry high concentration of calcium and vitamin E for the prevention of moisture loss and surface whitening along with

high concentrations of calcium and vitamin E in carrots (Mei et al. 2002). Fruits and vegetables can be fortified by the development of edible coatings containing high concentrations of essential micronutrients like Zinc, Calcium, Vitamin E, this approach can provide an alternative to fortify foods (Park and Zhao, 2004).

Curcumin(1,7-bis(4-hydroxy-3-methoxyphenyl)-1,6-heptadiene- 3,5-dione), is a natural phenylpropanoid dimer, derived from the roots of Curcuma Longa and is yellowish orange in color (Luo et al. 2012) and is widely used for its antioxidant, antitumor, antibacterial properties in medicinal purposes and as an additive in foods (Parvathy et al. 2009). It is a lipophilic polyphenol and hence it is insoluble in water, but soluble in organic solvents such as dimethyl sulfoxide, acetone and ethanol (Aggarwal et al.2007). Recently, bioactive polymer composites developed by the incorporation of curcumin into biocompatible or hydrophilic polymers have gained special attention (Suwantong et al. 2010). Due to the insoluble nature of curcumin in water or aqueous solutions, applications of this chemical are limited. Many approaches have been developed to address this problem. The use of curcumin liposomes; development curcumin nano-particles are the most potent and modern approaches to address the solubility issues regarding curcumin (Luo et al. 2012).

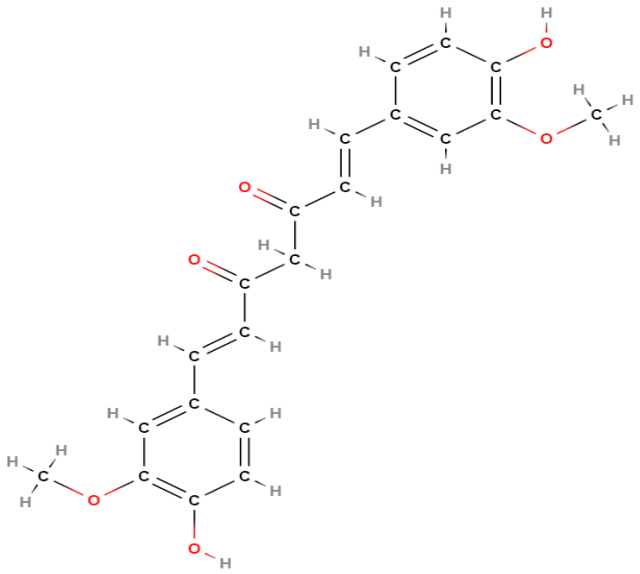


Figure 2.1: Chemical structure of Curcumin

Limonene (4-isopropenyl-1-methylcyclohexene) is a monocyclic terpene having a lemon-like odor. It is extensively present in citrus fruits like oranges, lemon, mandarins, lime and other citrus fruits (Sun, 2007). Limonene is listed as generally recognized as safe (GRAS) for its application in foods in Code of Federal Regulation (US FDA, 1991). Similarly, several studies on limonene have been done to explore its antimicrobial properties and antioxidant properties (Singh et al. 2010; Vigushin et al. 1998). Although, being a promising antimicrobial and antioxidant for application in foods, the use of limonene in foods is limited due to its properties of degradation in oxidative conditions and hydrophobicity (Li and Chiang, 2012). These issues can be addressed by a novel approach by the use of liposome-encapsulated limonene.

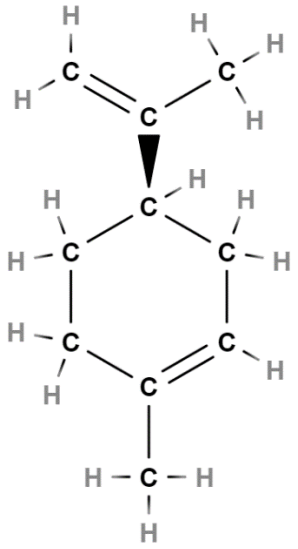


Figure 2.2 Chemical structure of d-limonene

2.5 Liposomes

Liposomes are self-assembled microscopic vesicles having one or several layers membranes formed usually by dispersion of phospholipid in water (Makwana et al. 2014). They are amphiphilic molecules having polar heads and hydrophobic hydrocarbon tails (Hasan et al. 2016). Studies have suggested that encapsulation of plant-based phytochemicals such as curcumin and limonene can be done for application as an edible coating material in foods (Makwana et al. 2014; Dogra et al. 2015; Umagiliyage et al. 2017).

CHAPTER 3

INTEGRITY OF EDIBLE NANO-COATINGS AND ITS EFFECTS ON QUALITY OF STRAWBERRIES SUBJECTED TO SIMULATED IN-TRANSIT VIBRATIONS

Abstract

Strawberries are a popular fruit with a pleasing color and flavor. However, its delicate tissue and high sugar content makes it highly perishable with visible mold. In this study, we have attempted to test feasibility of a new edible coating for extending shelf life of 'Chandler' strawberries subjected to simulated vibrations of local transportation. Six types of coatings were compared based on the quality of treated berries. Curcumin and limonene were used as natural antimicrobials and coatings were prepared from their liposomes and were over-coated with methyl cellulose. One set of each coating type were subjected to the simulated vibration of local transportation. The vibrated samples had lower shelf life than non-vibrated samples, indicating a robust coating which remains intact during road vibrations is required. Based on the number of berries with visible mold, limonene liposomes showed significantly lower fungal growth compared to the control on the 14^{th} day of storage. Titratable acidity and total phenolic contents were also found to be higher in limonene coated strawberries compared to other coatings. Further study is suggested to test liposome coatings of limonene with different particle size to improve integrity of the coatings when strawberries are subjected local transportation.

Keywords: Strawberry shelf life; edible coating; simulated local transportation; liposome; limonene; curcumin.

3.1. Introduction

Strawberries (*Fragaria x ananassa*) are a high demand fruit because of their pleasant aroma, brilliant color, and delicious taste. They are also a good source of natural antioxidants, vitamins, minerals and a significant amount of anthocyanins, flavonoids and phenolics (Rice-Evans and Miller 1996, Heinonen, Meyer et al. 1998). The berries are harvested at full maturity in order to maintain sensory (visual appearance, firmness, color) and nutritional (phytonutrients, minerals and vitamins) qualities (Hernandez-Munoz, Almenar et al. 2008) . One of the most important quality indicators in strawberries is the sugar to acid ratio, which characterizes degree of sweetness and depends on the maturity, cultivar and weather conditions of berries (Pineli, Moretti et al. 2011, Hu, Hu et al. 2012). Sugar to acid ratio in matured strawberries varies with the variety, usually within a range from 7:1 for fruits regarded as sweet and 6:1 for fruits regarded as acidic in taste (Wozniak et al. 1996). Due to its high respiration rate, soft texture and sensitivity to temperature and mechanical shocks and vibrations, strawberries have postharvest shelf life shorter than 1 week under ideal conditions at 0 ºC . This results in high degree of perishability to several pathogens, which would in turn cause changes in pH, titratable acidity, total soluble solids (TSS), loss in color, firmness and weight resulting in spoilage and, shortening the shelf life.

Several attempts have been made to increase the postharvest quality of fruits and vegetables. The most common method for maintaining quality and preventing decay is the use of low temperatures under refrigeration (Han and Nie 2004, Hernandez-Munoz, Almenar et al. 2006). Similarly, use of low temperatures and modified atmospheric packaging (MAP) in combination with increased concentration of carbon dioxide (Manning 1993) and paraffin-based active coatings by the use of essential oils for paper packaging of fruits and vegetables

(Rodriguez et al., 2007) are also in practice. These strategies, however, are expensive, time consuming, and cause change in visual appearance of fruits and develop off-flavor in fruits (Ke, Zhou et al. 1994). The need of alternative methods to minimize the risk of undesirable biological, physiochemical, and physiological changes of fruits and vegetables is desirable (Holcroft and Kader 1999).

There has been an increasing trend in use of natural bioactive edible coatings composed of polysaccharides, proteins, lipids, resins or of various composites in post-harvest preservation of fruits and vegetables (Valencia-Chamorro, Perez-Gago et al. 2010). Edible coatings such carnauba wax, whey proteins, gluten, shellac coatings, mucilage starch have exhibited beneficial roles in maintaining quality of fruits and vegetables. Apart from protection of products from mechanical and microbiological damage to the fruits, these compounds have shown to preserve post-harvest quality of fruits by preventing the loss of volatile compounds (Perez-Gago, Rojas et al. 2002). These coatings are developed from natural sources and are easily biodegradable, which meets the demand of consumers to have a safer food product (Pavlath and Orts 2009). These coatings are of interest as coating materials due to their low-cost, biodegradability, and solubility in water (Debeaufort, Quezada-Gallo et al. 1998).

Researchers have demonstrated that the use of plant based essential oils and phenolic compounds as coating materials can be done in order to increase the shelf life, prevent microbial growth, and to prevent nutrients loss from foods (Salmieri and Lacroix 2006). These compounds have shown strong antimicrobial and antifungal properties, which makes them a natural alternative for the prevention of pathogenic and spoilage organisms that may occur in foods (Lacroix 2007).

Limonene ((R)-(+)-para-Mentha-1,8-diene) is an essential oil extracted from lemon peels and other citrus fruits (Moufida and Marzouk 2003). It is commonly used as a food additive or flavoring agent and has a Generally Recognized as Safe (GRAS) status by US Food and Drug Administration (EPA 1994). Similarly, it has exhibited fungicidal activities against *Botrytis* and *Asperigillus niger*, the most common spoilage causing molds for fruit (Sharma and Tripathi 2008). A natural phenylpropanoid dimer Curcumin (1,7-bis(4-hydroxy-3-methoxyphenyl)-1,6-heptadiene-3,5-dione) is the principle curcuminoid of turmeric *Curcuma longa L.* which is used as a spice and traditional medicine in various parts of South and East Asia (Dogra, Choudhary et al. 2015). Several studies suggest curcumin to be an effective anti-proliferative, anti-oxidant and anti-inflammatory agent (Maheshwari, Singh et al. 2006). Like limonene, curcumin is also considered as "Generally Recognized as Safe" for application in food and pharmaceutical formulations by US Food and Drug Administration. Although having many food preservation qualities, the use of essential oils in food preservation is limited due to certain drawbacks. These include high costs and potential toxicity to the consumers. An approach to address these demerits while maintaining the efficacy of essential oils and decreasing their dose would be the incorporation of these chemicals in a formulation of edible coatings (Perdones, Escriche et al. 2016). A study by Sanchez-Gonzalez et al. (2011), in development of an antibacterial composite films of Hydroxypropylmethyl cellulose (HPMC) and chitosan mixed with different essential oils (Lemon, tea tree and bergamot) showed that the antibacterial activity of chitosan was enhanced when it was used with a mixture of the polymer and essential oil. Similarly, chitosan films incorporated with essential oils inhibited growth of the Gram negative and positive bacteria *Escherichia coli*, *Listeria monocytogenes* and *Staphylococcus aureus*.

Application of nanomaterials have shown to have a potential impact in a wide range of industries (Michael 2004, Wang, Liang et al. 2004). In food industries too, the application of nano-technology to enhance the quality of fruits has been used extensively (Yang, Li et al. 2010). Fruits and vegetables with nano-packing have shown better physiochemical, sensory physiological and preservation properties compared with normal packaging (Huang and Hu 2006, Li and Wang 2006, Li, Li et al. 2009). Liposomes are amphiphilic vesicles containing polar heads and hydrophobic carbon tails basically designed by dispersal of phospholipids in water (Bangham 1961). They can transport hydrophilic components by encapsulation in aqueous phase and hydrophobic components in stable state by inserting into hydrophobic domains (Brandl 2001, Shin, Chung et al. 2013). Size of the liposomes can be controlled to the order of nanometers, providing desirable properties to deliver essential chemicals of hydrophilic and hydrophobic nature. Their application in food preservation includes the encapsulation of nutrients, proteins, enzymes, antimicrobials and flavors and controlled release in the food environment to maintain food quality and prevent microbial spoilage (Makwana, Choudhary et al. 2015).

Mechanical injuries during transportations of fruits and vegetables between the chain of harvesting and consumption are one of the major causes of decay of fruits and vegetables (Barchi, Berardinelli et al. 2002). Strawberries are highly prone to in-transit vibration damage causing skin abrasion and bruising. From these abrasions and bruises on the tissues of berries, microbes are able to enter inside which in turn causes the degradation of berries and reduce the shelf life (Fischer, Craig et al. 1992). Significant losses due to damage caused by in-transit vibration has been recorded in strawberries (Pierson, Allen et al. 1982).

In this study, fresh ripened berries were picked up from the local farms in Southern Illinois. The berries were stored in a cold room at 4°C before any treatment and processing was done. Since the phenolic compounds, vitamin B and vitamin C are sensitive to the higher storage temperatures, refrigerated temperatures at 4 ^{0}C are considered as safe level for storage.

The berries were treated with various types of natural coatings and subjected to simulated shock and vibration. The effect of different natural edible coating formulations on shelf life of strawberries was compared. The effects of mechanical shocks and vibrations of simulated road transport on the quality of berries were analyzed. Physiochemical and nutritional quality of treated berries were evaluated by the measurement of visible mold growth, total soluble solids, pH, titratable acidity, and total phenolic content at different time intervals.

3.2. Materials and methods

Fresh ripened strawberries 'Chandler' were purchased from local farms located in southern Illinois. The berries were inspected for bruises, visual fungal growth, and decay. Uniform sized berries were selected and stored at 4°C prior to coating and mechanical vibration experiments. Steps for preparation of coating materials are shown in a flowchart form (Fig 8 and 9).

3.2.1. Preparation of phytochemical solutions

Curcumin solution with a concentration of 50mM was used as a coating material. Powdered curcumin was initially dissolved in 2-3 drops of ethanol. For example, for the preparation of 25 ml curcumin solution with concentration of 50mM, 0.00046gm curcumin powder was weighed and dissolved in ethanol followed by addition of 25 ml nano pure water.

Similarly, for the preparation of 25ml limonene solution with the concentration of 50 mM 0.01703 ml D-limonene was poured in a volumetric flask and desired volume was made by pouring 25ml Nano- pure water.

3.2.2. Preparation of Methyl cellulose solution

Methyl cellulose solution at a concentration of 1.5% (1.5gm in 100ml) was used as a coating material. Initially, powdered Methyl cellulose was dissolved in warm water at a temperature around 50ºC for the complete dissolution. Then, followed by addition of Nano-pure water to make up the desired volume.

3.2.3. Preparation of Curcumin and D- Limonene Liposomes

Thin film dehydration method was used for the preparation of lipid film. Briefly, a mixture of dimyristoylphosphatidylcholine (DMPC), Polydiacetylene (PDA; 10,12-Pentacosadiynoic acid) and N-hydroxysuccinimide (NHS) was dissolved in 25 mL of dichloromethane in a round bottom flask. The solution was then subjected to rotary evaporation for 1 hour to evaporate the solvent and bilayer film formation. The resulting film was dried overnight by placing the flask on a vacuum pump. The film was then hydrated by the addition of 50 mM concentration of Curcumin and 50 µM d-limonene prepared in Nano-pure water. The resulting solution was then left for sonication for 20 minutes for the complete detachment of the film. Further, the solution was placed in a probe sonicator (VCX 500, Vibra-cell, Newtown, CT) at 76 °C for 15 minutes to produce small vesicles with diameter less than 110 nm. The solution was then filtered through 0.45 µm nylon fiber to remove the lipid aggregates. Thus obtained liposomes were collected in a vial and covered with aluminum foil. Liposomes were polymerized by irradiation with a UV lamp emitting at 254 nm for approximately 2-5 min using a Pen Ray (UVGL-58, Mineralight, Upland, CA) UV source

(4.5 mW/cm^2) in air. The polymerized liposome solution was transferred into a dialysis membrane and dialysis was done for 48 hours changing the water every 4 hours. The dialyzed liposomes were stored at 4°C and stability was observed for 7 days. The liposomes were characterized and the results were presented in Dogra et al. (2015). A diagram representing the preparatory steps for the liposomes is given in figure 3.9.

3. 2.4. Application of coating materials

For each coating material types, six sterile clam shell boxes each containing 20 uniform sized berries were selected. The non-coated sample was used as a control. Two sets of coatings were prepared for the analysis of quality parameters. The first set consists of coatings in which curcumin was used, namely; Curcumin only, Curcumin liposomes, Methyl cellulose, bilayer coating of Curcumin followed by Methyl cellulose and non-coated as control. The second set consists of coatings in which D-limonene was used, namely; D-limonene only, D-Limonene liposomes, Methyl cellulose, bilayer coating of D-Limonene followed by Methyl cellulose and non-coated as control.

Berries were dipped in the solutions of coating materials for 10 minutes, air-dried in a UV sterilized cabinet drier for 1 hour at 20 ºC, transferred into clamp shell boxes, and stored at 4ºC. For the coatings in which bilayer of phytochemicals and Methyl cellulose were used, the berries were initially dipped in the phytochemicals for 10 minutes, dried, dipped in Methyl cellulose solution and stored at 4ºC.

3.2.5. Vibration tests

For each coating material, three boxes of coated strawberries were subjected to simulated vibration tests immediately after drying, while the other three boxes were not subjected to vibration tests. The system used to simulate the vibrations during local transportations

consists of a vibration shaker (Modal Shop 2060E) with a test platform, a digital signal generator, and an amplifier (Fig. 3.1). A sweep sine signal controlled by the signal generator and the amplifier was fed to the shaker. Based on the frequency components of the measured vibrations in a truck when transporting strawberries locally, each sinusoidal signal was set to sweep from 2 to 80 Hz at an acceleration level of 0.4 g (1 g = 9.8 m/s^2). A piezoelectric accelerometer placed on the test platform and a National Instrument data acquisition system were used to monitor the vibration levels during the test. Each box of strawberry sample was subjected to vibrations for 2 hours at room temperature. The non-vibrated samples were also kept in the same conditions of temperature for 2 hours. The vibrated and non-vibrated strawberries were again stored at 4ºC after the vibration tests.

Figure 3.1. Experimental setup showing clamshell containing strawberry placed on the vibration test apparatus.

3.2.6. Evaluation of fungal decay on strawberries

The stored strawberries were visually inspected for fungal decay for 14 days at different time intervals (0, 2, 5, 9 and 14 days) after coating and vibration tests were performed. Day 0 corresponded to the day of treatment and vibration. Fungal decay percentage on the berries was calculated. Fungal decay percentage is defined as the percentage (%) of strawberries which showed the visual presence of one or more colonies of molds on their surface during storage to the total number of berries.

3.2.7. Total soluble solids (TSSs), Titratable acidity (TA) and pH determinations

The quality parameters TSS, TA and pH of strawberries were measured at different time intervals (0, 2, 5, 9 and 14 days). One fruit from each triplicate were taken and wrapped in a sterile cheesecloth and squeezed with hands. The TSSs of the resulting juice were measured at 20ºC by a Brix refractometer (r^2 mini, Reichert Analytical Instruments, Depew, NY). Similarly, pH of the juice was measured by a pH meter (Corning pH/ion analyzer 350). TA was determined by titrating the diluted juice (5ml juice diluted in 95ml distilled water) up to pH 8.2 using 0.1N NaOH.

3.2.8. Total phenolic compound analysis

Fruit samples at different days of storage (0, 2, 5, 9 and 14 days) after coating and vibration tests were taken. Briefly, a 1.5 g strawberry sample grinded in a mortar and pestle was weighted and extracted with 20ml mixture of acetone, water and acetic acid (70:29.5:0.5 v/v). The samples were vortexed for 1 hour at room temperature for complete extraction, followed by centrifugation at 1640 g for 15 minutes at 20ºC. The supernatant was then filtered and allowed to stand at room temperature for evaporation of solvent. The residue was then dissolved in distilled water to a volume of 20 ml.

Total phenolic content of the extracted juice were determined by the use of Folin-Ciocalteu reagent as per the method of Slinkard and Singleton (1977). The standard calibration curve was prepared by using Gallic acid as a standard. The result was expressed as milligrams per liter of Gallic acid equivalents (GAE) per 100 gm fresh weight.

3.2.9. Statistical analysis

Significant differences among different coatings, vibration, and days of storage on different quality parameters were analyzed with one-way ANOVAs. The coatings and vibration treatments were compared on the basis of fungal decay percentage, pH, total polyphenol content, total soluble solids content and titratable acidity. The level of significance for all the analysis was chosen to be 0.05. The means for different treatments were compared with least significant difference (LSD) test. All statistical analyses were performed using JMP software package for windows (JMP®, Version 12.2. SAS Institute Inc., Cary, NC, 2016).

3.3. Result and discussion

3.3.1. Fungal decay

Due to their high physiological activities, strawberries are highly perishable fruits with a short shelf-life. From the observations made up to 14 days, fungal decay percentage was found to increase with the number of days of storage in each types of coatings and control. The effect of days of storage showed significant effect ($p < 0.05$) on fungal decay % of strawberries. A gray mold, *Botrytis cinerea*, is the most predominant mold that causes decay of strawberries (Harvey and Pentzer 1960). The organism primarily infects the flower causing rot or remains dormant. The dormant mold shows its activity when the concentration of sugars increases and favorable environmental conditions are available either before or after harvesting of berries (Ayala-Zavala, Wang et al. 2004)

Among the two sets of coatings designed, in Set 1, i.e. set of curcumin treated strawberries, effect of vibration on fungal decay % was also found to be significant ($p = 0.048$). The vibrated curcumin treated strawberries were found to have significantly higher fungal decay (45.5 %) compared to the non- vibrated strawberries (27.625 %). This effect was more pronounced om the 14^{th} day of storage (Fig. 3.10). For all coating treatments, significant difference was not observed between vibrated and non-vibrated until the 9^{th} days of storage. However, from 9^{th} days onwards all the vibrated samples were found to have significantly higher mold growth. The difference in fungal decay % between vibrated and non-vibrated can be attributed to the change in integrity of coatings by the simulated vibration. Mechanical damage due to vibration contributes to a significant loss in perishable fruits and vegetables. In a study by Fischer et al. (1992), major damage of strawberries due to simulated in-transit vibration of 5.0 to 10Hz was reported. Mechanical damages caused by vibrations can affect the integrity of plasma cells of skin and chemical contents of fruits resulting in change in bloom and tissue softening of fruits (Zhou, Su et al. 2007).

Among the berries coated with phytochemicals, curcumin coatings showed significantly lower fungal decay percentage compared to control and limonene coatings up to 2^{nd} day of storage (Fig. 3.2). However, there was no significant difference in the fungal decay % after the 2^{nd} day of storage. Curcumin coatings showed a lower fungal decay at the 14^{th} day of storage (Fig. 3.2), which was found to be significant compared to control.

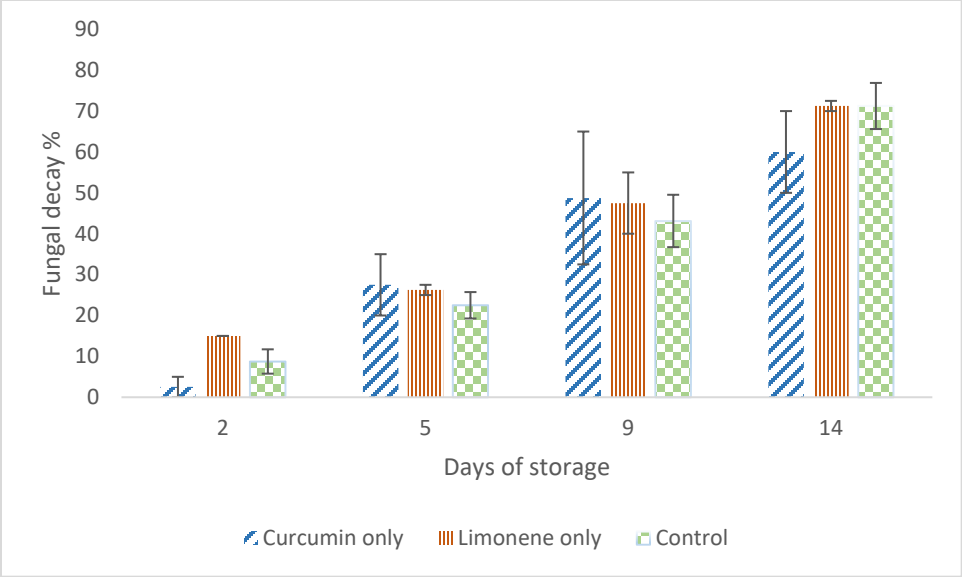


Figure 3.2 Fungal decay % varying with the days of storage among the phytochemical treatments only. Error bars represent ± Standard Error

Fungal decay % among the treatment formulations containing MC had higher mean value (40.78%) as compared with control samples (36.41%) (Fig. 3.3) which is in agreement with the study conducted by Perdones et al. (2016).

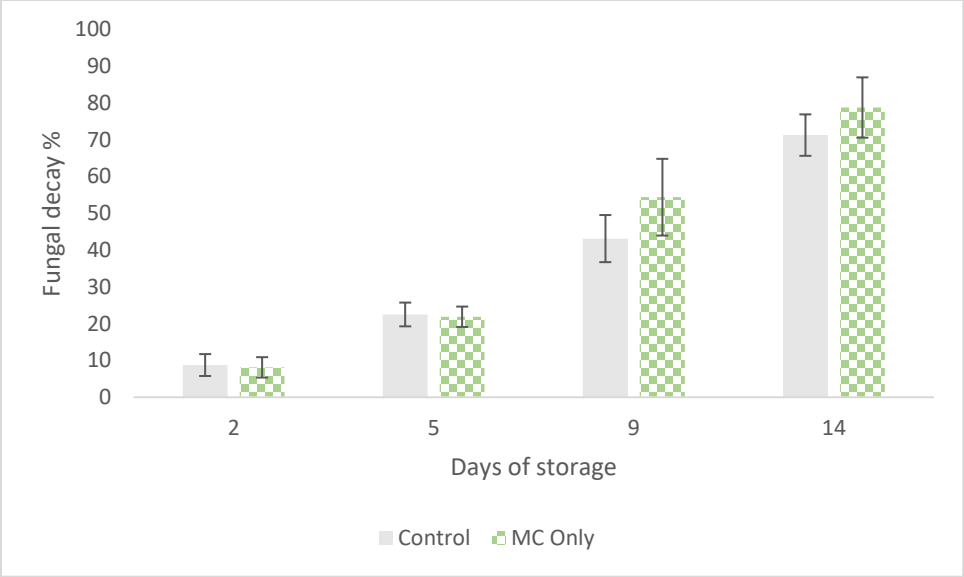


Figure 3.3. Fungal decay % varying with the days of storage among the strawberries coated with methyl cellulose (MC) compared to control. Error bars represent ± Standard Error.

The bilayer coating of phytochemicals and MC did not show any significant reductions in fungal decay percentage compared to control (Fig. 3.4). Liposome coated samples exhibited an elongated preservation effects, supported by significantly lower visual mold growth compared to control during the 14th day of storage onwards (Fig. 3.5). Curcumin liposome coated samples exhibited significant reduction in fungal decay in 2nd, 5th and 14th days of storage compared to control (Fig. 3.5). Meanwhile, on the 14th day of storage, limonene liposome coated samples were found to be significantly ($p = 0.0247$) effective in controlling fungal decay compared to control (Fig. 3.5). This indicates that, for further extension of shelf life, it is suggested to study the effects of liposome coatings.

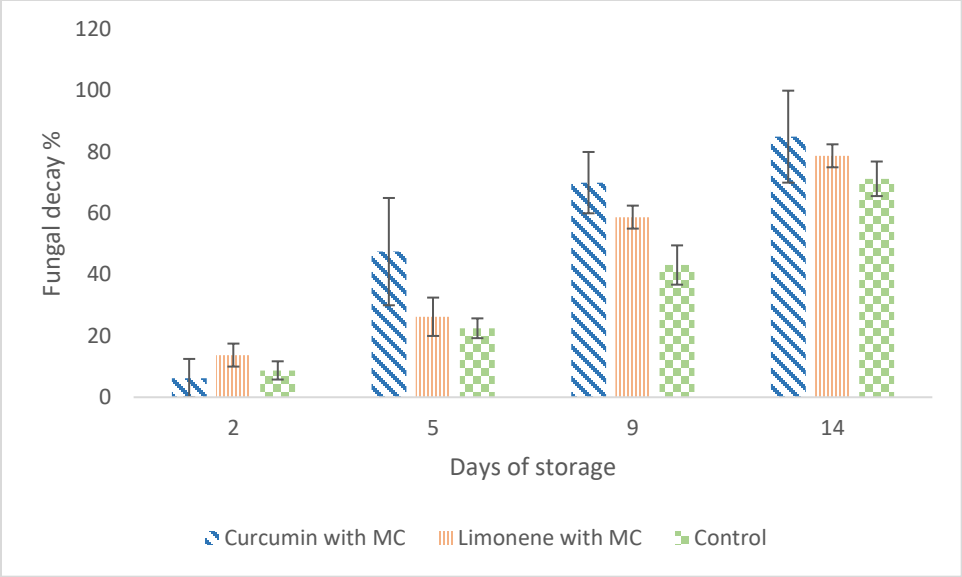


Figure 3.4. Fungal decay % varying with the days of storage among the coating treatments mixed with methyl cellulose (MC). Error bars represent ± Standard Error

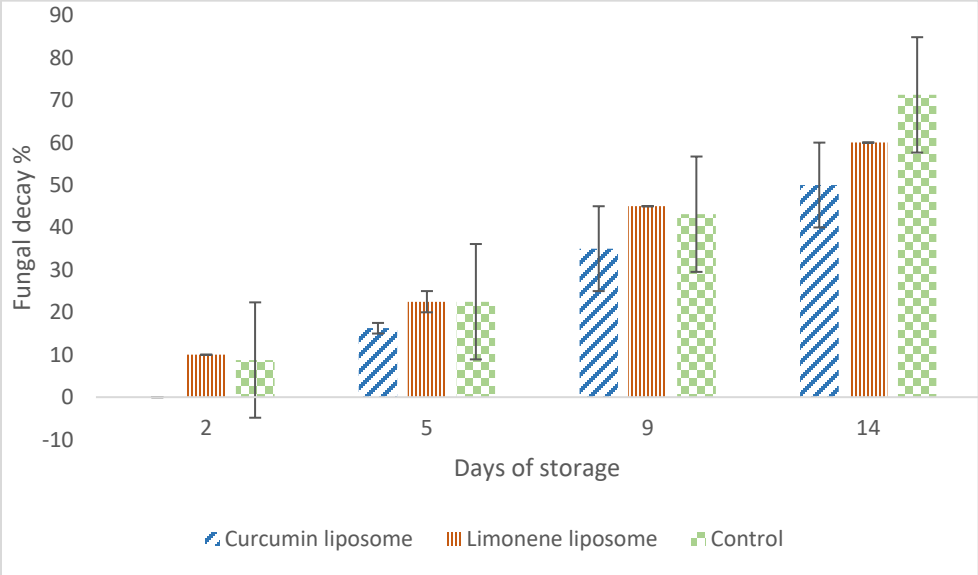


Figure 3.5. Fungal decay % varying with the days of storage among the liposome coating treatments compared to control. Error bars represent ± Standard Error

3.3.2. TSS

Total soluble solid was found to be significantly higher in the Set 2 limonene treated strawberries as compared to the Set 1 curcumin treated strawberries (p = 0.0301). Days of storage also showed significant effect (p = 0.0239) on the TSS on each set of coatings designed (Fig.3.6). No significant difference in TSS was observed between vibrated and non-vibrated strawberry samples. It was observed that there was an increase in TSS with days of storage. The change in TSS content might be due to the solubilization of the cell wall polyuronides and hemicelluloses in mature strawberries (Hernandez-Munoz, Almenar et al. 2008). TSS values of strawberries generally ranges from 7-12% depending upon the genotypic characteristic of the fruit (Galletta, Maas et al. 1995). High TSS values are generally accompanied by good flavor of strawberries (Kader 1991).

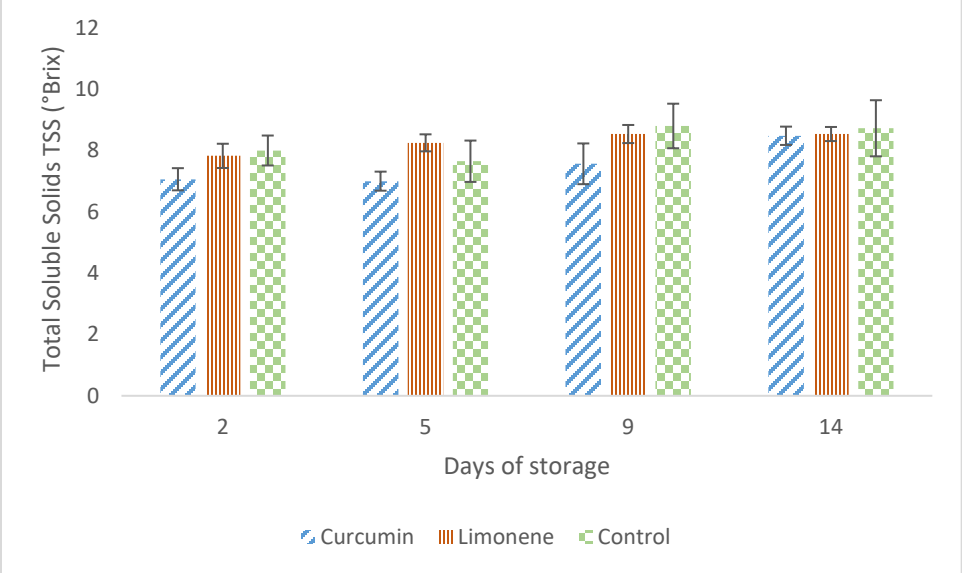


Figure 3.6. Mean total soluble solids for two sets of coatings. Error bars represent ± Standard Error

3.3.3. pH

The limonene coated sample set was found to have significantly higher pH than curcumin coated samples ($p < 0.0001$) and the control ($p = 0.0132$) (Fig. 3.7). The days of storage was shown to have significant effect ($p < 0.05$) on the pH among the curcumin treated strawberries whereas the effect was not apparent among the limonene treated and control strawberries. The pH of limonene coated berries, decreased up to 5 days of storage but there is no significant increase on the 14th day. The obtained results are in agreement with similar research conducted by Montero (1996) who observed initial decreases followed by increases of pH during storage of strawberries.

However, for both vibrated and non-vibrated curcumin coated and control berries, the pH increased throughout storage. A similar study conducted by Zheng et al. (2007) found an increase in pH during storage, which can be related to effects of increased O_2 on the respiration rate of fruits.

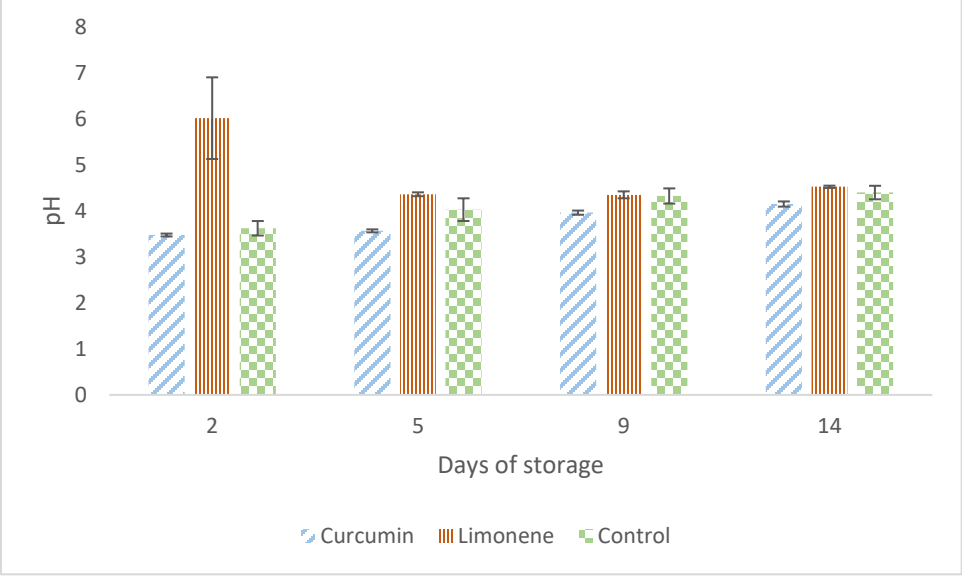


Figure 3.7. Mean pH for two set of coatings. Error bars represent ± Standard Error

3.3.4. Titratable acidity (TA)

Days of storage was shown to have significant effect ($p = 0.0284$) on TA content among the set of both vibrated and non-vibrated limonene coated strawberries. There was an increasing trend observed up to the 9th day of storage, and a non-significant decrease in the value at 14 days of storage. No pronounced effect was observed in TA among the set of both vibrated and non-vibrated curcumin coated and control strawberries. Titratable acidity content was found to be significantly higher ($p < 0.0001$) in limonene as compared to curcumin treated strawberries (Fig. 3.8). This observation indicates a decrease in the fruit respiration metabolism resulting an increase in the TA (de Oliveira, Magnani et al. 2014). TA is defined as the percentage of citric acid per strawberry wet weight. The differences in TA content observed between the curcumin coated and uncoated samples compared with limonene coated samples can be attributed to the increased water loss (Hernandez-Munoz, Almenar et al. 2008). Decreased TA content can be related to the decline in the organoleptic quality of the strawberries (Yang, Li et al. 2010).

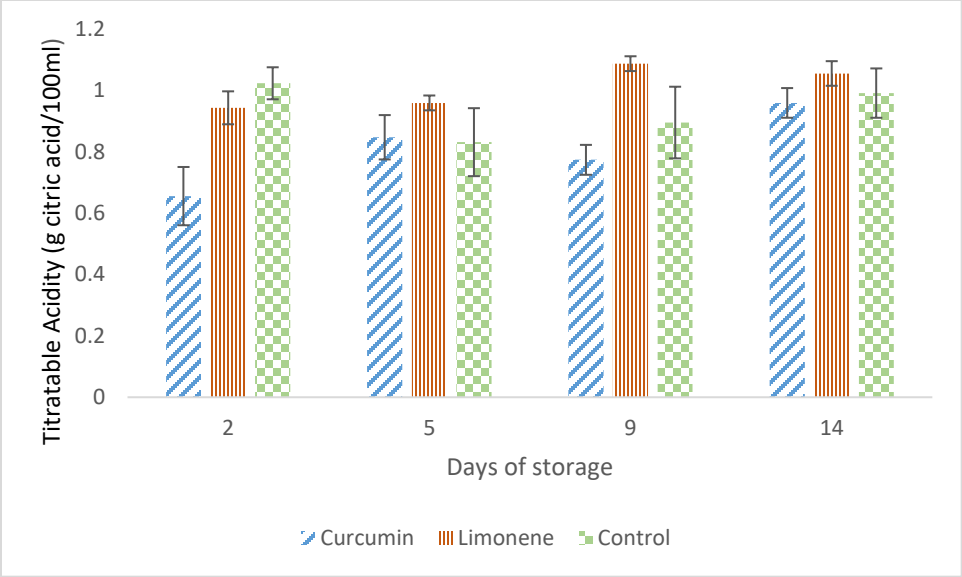


Figure 3.8. Mean titratable acidity for two sets of coatings. Error bars represent ± Standard Error

3.3.5. Total phenolic content (TPC)

Limonene coated strawberries were found to have significantly higher (p = 0.0023) TPC as compared to curcumin coated and control samples (Fig 3.9). There was an increasing trend in the TPC content of the limonene coated berries up to 14 days of storage. The change in the concentrations of phenolic compounds in fruits during storage can be attributed to cell structure breakdown. Meanwhile, in curcumin coated and control samples, there was no significant increase in the TPC content from 2nd day to 5th day of storage, however significant increase was observed 5nd to 9th days of storage (Fig 3.9). The days of storage was found to have a significant effect (p < 0.0001) on TPC content of curcumin treated strawberries whereas the effect was not seen in limonene treated strawberries. These observations concurred with the findings by Ayala-Zavala et al. (2004).

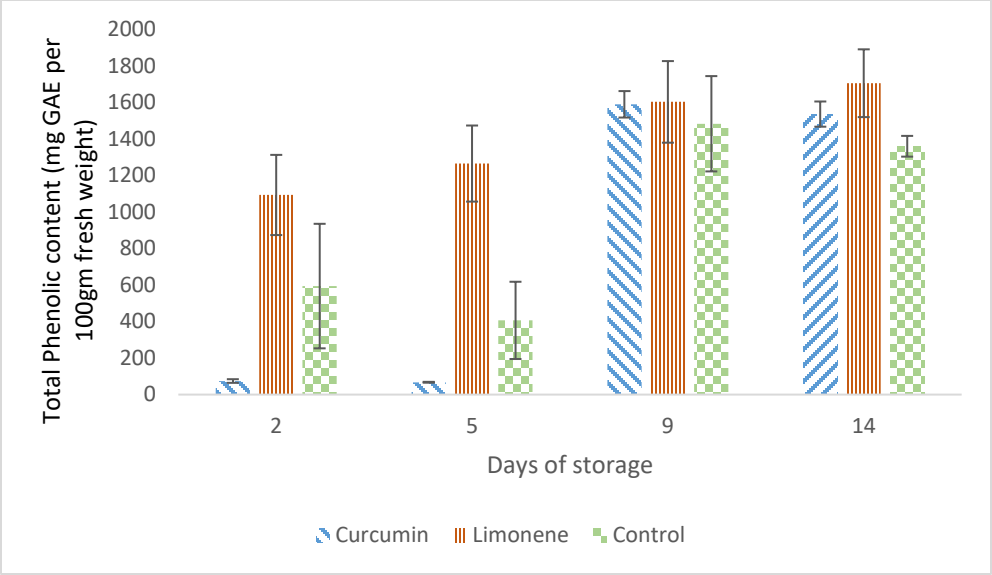


Figure 3.9. Mean total phenolic content for two set of coatings. Error bars represent ± Standard Error

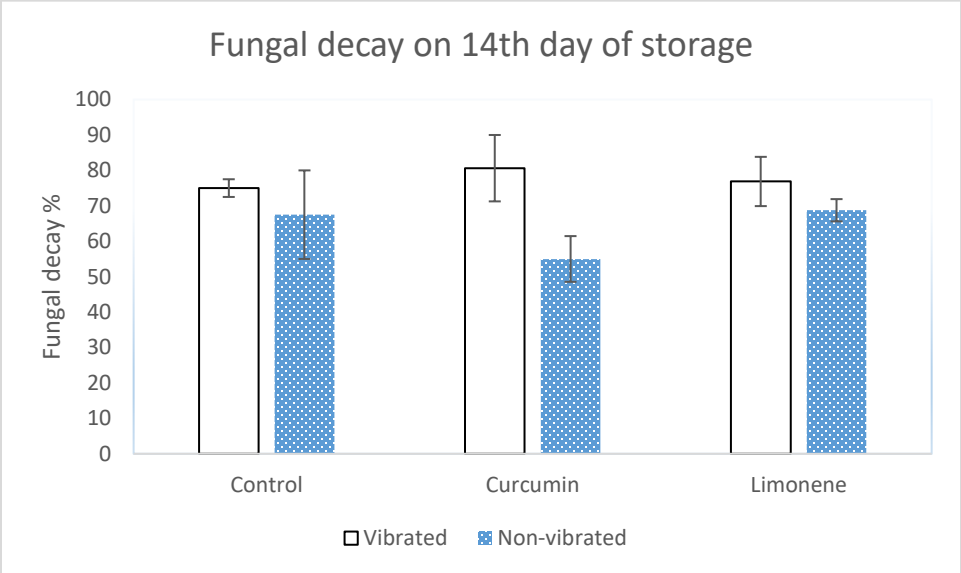


Figure 3.10. Fungal decay percentage among vibrated and non-vibrated samples. Error bars represent ± Standard Error

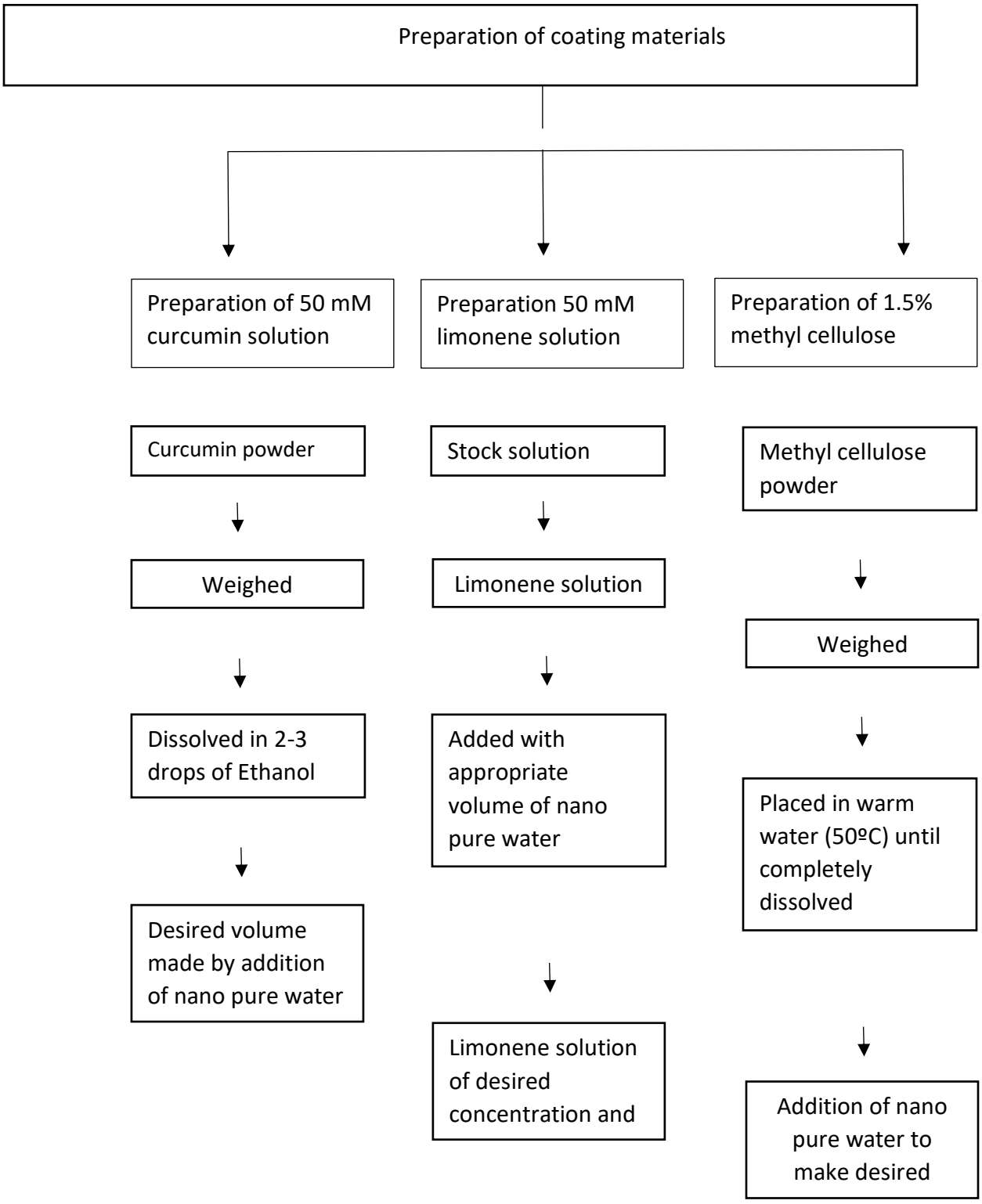


Figure 3.11. Flowchart representing steps in preparation of coating materials

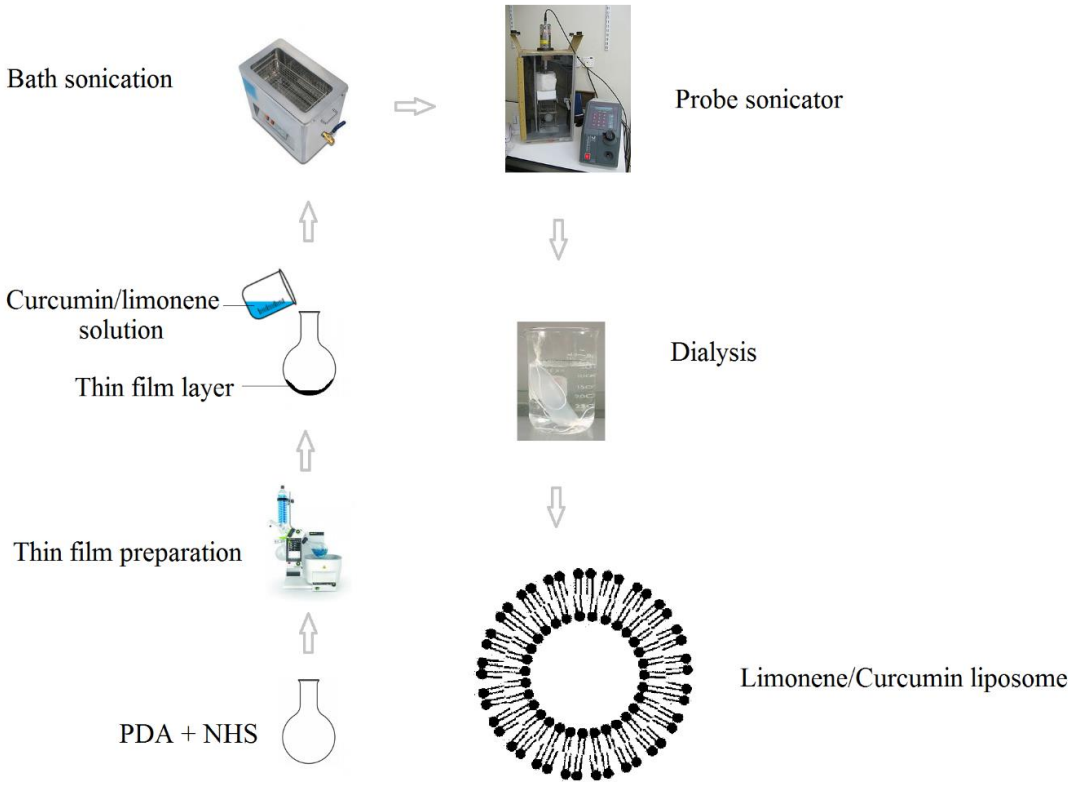


Figure 3.12. Pictorial representation of liposome preparation

3.4. Conclusion

In summary, the results obtained in this paper indicate that storage time significantly affects the quality parameters of coated and non-coated strawberries. Vibration was also found to have a significant effect on fungal decay and total soluble solids among all the coating treatments. Among different coating types, liposomes were found to be the most effective for the preservation of strawberry quality and the limonene liposome was found to be effective in controlling fungal decay on strawberries for a prolonged period of storage. Similarly, titratable acidity and total phenolic contents were also found to be higher in limonene coated strawberries compared to other coatings.

Acknowledgement

Partial funding for this project came from Research Grant No. US-4680-13C from BARD The United States - Israel Binational Agricultural Research and Development Fund; USDA-NIFA Food Safety Outreach grant; National Institutes of Health; and the Elevating Research grant from SIU, Carbondale, IL. Arosha L. Umagiliyage provided assistance with total phenolic assay, April Vigardt provided trucks to measure vibrations during local produce transportation, and Derek J. Fisher provide critical review of the manuscript.

CHAPTER 4

EFFICACY OF LIMONENE NANO COATINGS ON POST-HARVEST SHELF LIFE OF STRAWBERRIES

Abstract

Strawberries are highly demanded fruits because of their color, nutritional values and appearance. The aim of this study was to develop and characterize alginate and limonene liposomes as edible coating materials and to determine their efficacy in shelf life extension and maintaining quality parameters of 'Chandler' strawberries. Alginate solution (1.5% w/v) and Limonene liposomes prepared from 80% lecithin and 20% PDA were used as edible coating materials. Fungal decay percentage, total yeast and mold counts, headspace atmosphere analysis, total soluble solids, pH, titratable acidity, total anthocyanin content and total phenolics were analyzed to assess fruit quality during 14 days at 4ºC of storage. Days of storage was found to be significant in maintaining the quality of the strawberries. Among the coating types, strawberries coated limonene liposomes were found to have found significantly effective in maintaining the lesser respiration rate, lower the change in pH (3.9), and had higher total anthyocyanin (43.85) content during storage. Thus limonene liposomes were found to be useful for extending the shelf life and maintaining quality of strawberry fruits.

Keywords:

Strawberry, Edible coatings, Shelf life, Quality, Liposomes

4.1. Introduction

Consumption of fruits and vegetables have gained significant global attention in recent years. Fruits and vegetables are the source of nutrients such as proteins, vitamins, minerals, fibers, and phytochemicals that are essential to improve human nutrition and health (Li, 2008). The phytochemicals contribute to the normal functioning of the human body (Wettasinghe et al. 2002). The antioxidant compounds, such as ascorbic acid (AA), lycopene, β-carotene, and phenolics contribute to nutritional content of fruit and vegetables. These compounds are known to prevent oxidation caused by reactive oxygen species that lead to damage the cells and DNA, and cause some degenerative diseases (Hu and Jiang, 2007).

Strawberries (*Fragaria x ananassa),* with their characteristic appearance, color and nutritional values, are highly consumed fruits (Almenar et al. 2007). They are considered as a good source of nutrients, anthocyanins, flavonoids and phenolic compounds (Heinonen et al.1998). United states is the largest producer of strawberries in the world (Wu et al. 2012) and per capita consumption was 7.9 pounds in 2013 (USDA Report 2014). Of the total strawberry production, 81 percent comes from fresh market (NASS 2015).

Strawberries are one of the most susceptible foods prone to physical injuries and fungal spoilage (Park et al. 2005). These results in the change of several physiochemical properties, fungal growth that results in short shelf life and causes a significant postharvest loss. The goal of the research is to develop edible nano-coatings from plant based antimicrobials, which would maintain the postharvest quality of strawberries and extend their shelf life. In our preliminary studies, edible nano-coatings prepared by the nanoencapsulation of curcumin and limonene in liposomes when applied on the surface of strawberries were effective in extending the shelf life and maintain the quality (Dhital et al. 2017).

Application of plant based essential oils compounds as coating materials in foods have shown to prevent microbial growth and loss of nutrients; and increase the shelf life of foods (Salmieri & Lacroix, 2006). Essential oils of oregano, thyme, cinnamon, lemongrass and clove exhibit antimicrobial activity against strains of *E. coli* (Smith-Palmer et al. 1998; Hammer et al. 1999; Friedman et al. 2002). Some plant essential oils and their components are responsible for increasing the sensory attributes of fruits and preventing the microbial growth. Terpene citral, a citrus essential oil is known to have antimicrobial properties and contribute for sensory properties of foods (Rodov et al. 1995).

Limonene ((R)-(+)-para-Mentha-1,8-diene) is obtained from essential oil of citrus fruits i.e. orange, lemon, mandarin, lime, grapefruit (Moufida and Marzouk 2003). It is a colorless liquid hydrocarbon regarded as safe used largely by cosmetic, food and pharmaceutical industries and has Generally Recognized as Safe (GRAS) status by US Food and Drug Administration (EPA, 1994). Limonene has a very strong antifungal property and is effective against food spoilage fungal species. It shows an antibacterial properties effective against pathogenic bacteria like; *Staphylococcus aureus, L.monocytogenes, Salmonella enterica* (Sharma and Tripathi 2008; Alonso-Gutierrez et al. 2013). Due to hydrophobic nature and tendency to degrade under oxidative conditions, limonene possess a challenge during its application as an edible coating material because of poor dispersion in water (Li and Chiang, 2012). To address this issue, low concentrations of limonene are used for dispersion in water, which in turn reduces its antimicrobial activity. The problem of hydrophobic nature and use of low concentrations of limonene limits its efficacy while using as a coating material. A new approach of encapsulation of phytochemicals in liposome that has both hydrophobic tails and hydrophilic heads through nanotechnology (Umagiliyage et al. 2017).

Incorporation of antimicrobial compounds into edible films and coatings provides an innovative approach to improve microbial safety and shelf life of foods (Cagri et al. 2004). Some of the most commonly used antimicrobial agents in food are; benzoic acid, sodium benzoate, sorbic acid, potassium sorbate and propionic acid which can be incorporated into edible films and coatings (Cruz-Romero et al. 2013). Starch based edible coatings containing potassium sorbate applied on the surface of fresh strawberries reduced the microbial growth and extended the shelf life (Garcia et al. 1998). A bilayer edible coating made from plant based antimicrobial compounds, limonene and curcumin were applied in combination with methylcellulose (MC) for improving of post-harvest quality of fresh strawberries (Dhital et al. 2017). Edible films containing organic acids, protein and glycerol have shown to inhibit the growth of pathogenic organisms including *L. monocytogene*s, *S. gaminara* and *E. coli* 0157:H7 (Hettiarachchy and Satchithanandam, 2007).

Nano-technology has been extensively used to enhance the quality of fruits and vegetables (Yang et al; 2010). Encapsulation of antimicrobial compounds using the approaches of nanotechnology can address the problems of microbial degradation and hence improve the quality of fruits. Liposomes are used in the encapsulation of nutrients, proteins, enzymes, antimicrobial and flavors and their controlled release in food environment to delay the microbial spoilage and maintain the food quality (Makwana et al. 2015). An improved antimicrobial activity of nano-encapsulated eugenol was reported by Shah et al. (2012b) against *E. coli* 0157:H7 and *Listeria monocytogenes* in bovine milk. Limonene encapsulated in Nanoemulsion exhibited antimicrobial activities towards *Escherichia coli, Lactobacillus delbrueckii* and *Saccharomyces cerevisiae (Donsi et al. 2012).*

Alginate is a generic term for the salts and derivatives of alginic acid. Alginates are commercially produced from brown algae *Macrocystis pyrifera,, Laminaria hyberborea, Laminaria digitata, Ascophyllum nodosum, Laminaria japonica, Edonia maxima, Lessonia nigrescens, Durvillea Antarctica,* and *Sargassum* spp. (Draget, 2005). These compounds have good film-forming properties. The alginate films are typically uniform, transparent and water-soluble. Upon addition of calcium ions, alginate undergoes conformational changes resulting in the formation of calcium alginate (Moe, 1995). These compounds when applied as coating materials improved the quality of fruits and vegetables by reducing the shrinkage, moisture migration, oxidative rancidity, oil absorption , holding volatile compounds, improvement in sensory properties of products (Hershko and Nussinovitch, 1998). Alginate has wide range of application in industrial sectors due to their ability to retain water, film-forming, gelling, viscosifying and stabilizing properties. Their film forming properties make them useful in food processing industries. In addition, alginate coatings have shown good oxygen barrier properties (Conca and Yang, 1993) that eventually can retard lipid oxidation in fruits and vegetables (Kester and Fennema 1986). Alginate based coatings applied to fresh cut 'Fuji' apples showed that these coatings could carry antioxidants, which are responsible for the maintaining color of cut fruits during storage (Rojas-Grau et al. 2007 b)

Mechanical injuries due to vibration can cause in a significant loss of fruits and vegetables. A study by Singh and Xu (1993) reported that about 80% of apples could be damaged by simulated transportation by truck. Damage due to vibration during the transportation was noted on different fruits and vegetables that include peaches, apricots, potatoes, tomatoes (Barchi et al. 2002). In strawberries, In-transit vibration causes skin abrasion and bruising which makes easier for microbes to enter inside the berries and cause degradation (Fischer et al. 1992).

The goal of this study was to develop novel nanocoating treatments prepared from plant-based antimicrobials encapsulated in nano-liposomes. The objectives in this study were: (1) Preparation and characterization of the edible coating materials, (2) application of edible coating materials on strawberry fruits and analyze the quality parameter of strawberries treated with edible coatings during storage and 3) compare the efficacy of coating materials based on quality parameters.

4.2 Materials and methods

Fresh strawberries of 'Chandler' variety purchased from local farms located in southern Illinois. Berries were visually inspected for bruises, visual fungal growth, and decay. Uniform sized berries were selected and stored at 4°C prior to coating application.

4.2.1. Preparation of D- Limonene Liposomes

Thin film dehydration method was for the preparation of lipid film (Figure 1). Briefly, a mixture of soy-based lecithin and Polydiacetylene (PDA; 10,12-Pentacosadiynoic acid) at different proportions (100 % lecithin , 80% lecithin, 60 % and 50 % lecithin) was dissolved in 25 mL of dichloromethane in a round bottom flask. The solution was then subjected to rotary evaporation for 1 hour to evaporate the solvent and bilayer film formation. The resulting film was dried overnight by placing the flask on a vacuum pump followed by film hydration by the addition of 50 µM D- limonene prepared in Nano-pure water. The resulting solution was then left for sonication for 20 minutes for the complete detachment of the film. Further, the solution was placed in a probe sonicator (VCX 500, Vibra-cell, Newtown, CT) at 76 °C for 15 minutes. The solution was then filtered through 0.45 µm nylon fiber to remove the lipid aggregates. Thus, obtained liposome solution was collected in a vial and covered with aluminum foil. Liposomes were polymerized by irradiation with a UV lamp emitting at 254 nm for approximately 2-5 min using a Pen Ray (UVGL-58, Mineralight, Upland, CA) UV source (4.5 mW/cm^2) in air. The

polymerized liposome solution was dialyzed in a dialysis membrane Spectra/Por® Biotech Cellulose Ester (CE) membrane (MWCO: 100,000) for 48 hours changing the water every 4 hours. Thus obtained liposome solution was collected and stored at 4°C for further studies.

4.2.2. Characterization of Liposomes

UV−vis absorption spectra of all the dialyzed non-polymerized and polymerized liposomes prepared with varying concentration of soy-based lecithin and PDA were recorded at room temperature using a PerkinElmer Lambda 25 (spectral slit width 1 nm) UV/vis spectrometer using a cuvette of 1 cm path length. Sterile distilled water used as blank and to calibrate the spectrometer at 400-800 nm. Liposome samples were placed and cuvettes readings were noted.

4.2.3. Alginate solution preparation

Sodium alginate powder was dissolved in double distilled water to prepare alginate solution. Briefly, Sodium alginate was dissolved in 500 ml of water upon stirring at 70 °C for 2 hours on a hot plate to obtain a 1.5 % (w/v) solution.

4.2.3.1. Characterization of alginate coatings

4.2.3.1.1 Fluorescent imaging

In order to determine homogeneity of the coating, on strawberries using fluorescence microscopy,we labelled alginate with pyrene molecule. Strawberries were dipped in the solution of pyrene alginate and freeze- dried at -80 ºC for 24 hours. The freeze- dried strawberries were cut in pieces of 5 mm by 10 mm. A Leica inverted fluorescent microscope was used for fluorescent alginate coated strawberries imaging. A UV lamp source was used to excite the fluorescent molecules. A long pass band UV filter was used to select the excitation wavelengths. The emission was collected from 420 nm to 500 nm.

4.2.3.1.2 Scanning electron microscopy

The SEM images of the alginate-coated berries were taken for the characterization of alginate coatings. Briefly, alginate coatings (1.5% w/v) were applied on the berries, followed by freeze-drying (-80 ºC) for 24 hours. The freeze-dried samples were cut using a razor blade in small pieces (5 mm by 5 mm). They were sputter coated for 4 minutes with a layer of Ag-Pd using a DESK II, DENTON VACUUM sputter. The edges of the samples were grounded using a thin layer of silver paint (SPI, USA). The samples were imaged using a scanning electron microscope QUANTA FEG 450 (FEI), the acceleration voltage was 5 kV using ETD detector at high vacuum.

4.2.4. Application of coating materials

The summary of application is represented in Figure 3.1. Briefly, berries were randomly selected and divided into four groups depending upon coating treatment types; limonene liposome, alginate and non-coated control. Each treatment was performed in triplicate and each replicate had 20 berries. Berries were dipped in the solutions of 50 µM liposome solutions for 10 min. For Alginate coatings, 1.5 % alginate solution was cooled to room temperature and strawberries were dipped in the alginate solution for 3 minutes. The berries were then immersed in 5% w/v aqueous solution of $CaCl_2$ for 2 min. For the control samples, the berries were rinsed with sterile distilled water. All the treated berries were air dried at room temperature in an UV sterilized cabinet drier for 2 hours and packed in sterile clamshell box stored at 4º C.

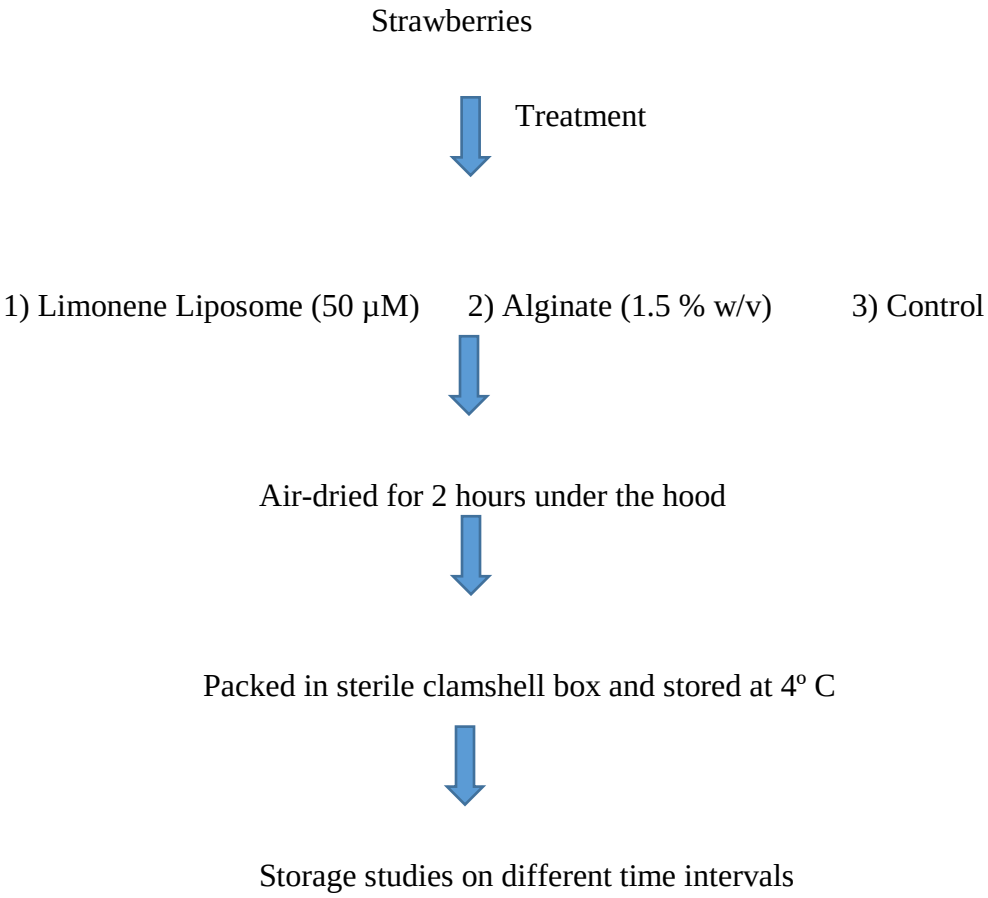


Figure 4.1: Flowchart for the coating treatments

4.2.5 Fungal decay percentage

Berries were visually evaluated for the presence of visible mold growth during the experiment. Any berry with visible growth was considered to be decayed. Fungal decay percentage was calculated by using the formula:

Fungal decay % = (the number of decayed fruits / total number of fruits) ×100

4.2.6. Total Yeast and mold count

Total yeast and mold count on the berry surface was performed by serial dilutions followed by spread plating over the surface of sterile DRBC plate method as recommended by the International Standard Organization (ISO 21527-1, 2008) with slight modifications. Briefly, berries from each treatment and untreated control were stirred (in 150 mL Erlenmeyer flask) at 150 rpm in 20 mL of 0.1% (w/v) sterile peptone water for 30 min. The resulting suspension was then serially diluted from 1:10 to $1:10^6$ dilutions. Then, 0.1 ml inoculum of each dilutions was used for plating and spread evenly over the plates. The plates were incubated at 25 ºC for 5 days. Results were expressed as log colony forming units per ml (CFU/ml) based on average count of triplicate set.

4.2.7. Headspace atmosphere analysis

In hermetically sealed 500 ml glass jars, each jar containing 5 berries coated with treatments were placed in and sealed. The jars were kept at 4ºC for 1 hour. Head space Carbon dioxide concentrations in the sealed jars were determined using an OXYBABY 6.0 gas analyzer (WITT-GASETECHNIK GmbH & Co KG, Witten, Germany) comprising an electro-chemical cell for oxygen analysis and an IR-absorption cell for carbon dioxide analysis. The experiment was performed in triplicate.

4.2.8. Fruit weight loss

Strawberries just after coating and air drying were weighed. Twenty berries corresponding to each coating treatment were used and the experiment was performed in triplicate. Weights of the berries were measured at 2, 5, 9 and 14 days after coatings. Weight loss was estimated as the percentage loss of initial weight.

Weight loss % = (Initial weight- final weight/ Initial weight) X 100

4.2.9. Determination of Total soluble solids (TSSs), pH, Titratable acidity (TA)

TSS, TA and pH of strawberries was measured at different time intervals 2, 5, 9 and 14 days after coating treatment is done. Fruit from each coating treatments were crushed with the help of sterile mortar and pestle and juice will be collected. Sampling was done triplicate.

The TSSs of the resulting juice was measured at 20ºC by a Brix refractometer (r^2 mini, Reichert Analytical Instruments, Depew, NY). Similarly, pH of the juice was measured by a pH meter (Corning pH/ion analyzer 350). TA was determined by titrating the diluted juice (5ml juice diluted in 95ml distilled water) up to pH 8.2 using 0.1N NaOH.

4.2.10. Analysis of total anthocyanin content

Analysis of total anthocyanin content was performed at intervals of 2,5,9 and 14 days after coating treatment is done. Strawberry sample (2g) was crushed with 20 ml of methanol in 1% HCl with mortar and pestle. Then, the mixture was centrifuged at 1000×*g* for 20 min. The supernatant was collected and absorbance was noted at 530 nm. Absorbance readings was converted to milligrams of pelargonidin-3-glucoside per 100 g of fruit fresh weight, using a molar absorption coefficient of 36000 (Cordenunsi et al. 2003).

4.2.11. Analysis of total Phenolic compounds content

Fruit samples treated with different coatings and stored at different time intervals (2, 5, 9 and 14 days) were selected. Briefly, a 1.5 g strawberry sample grinded in a mortar and pestle was used and extracted with 20ml mixture of acetone, water and acetic acid (70:29.5:0.5 v/v). The samples were vortexed for 1 hour at room temperature for complete extraction, followed by centrifugation at 1640 g for 15 minutes at 20ºC. The supernatant was filtered and allowed to stand at room temperature for evaporation of solvent. The residue was then dissolved in distilled

water to a volume of 20 ml. The experiment was done in triplicate. Total phenolic content of the extracted juice was determined by the use of Folin-Ciocalteu reagent as per the method of Slinkard and Singleton (1977). The standard calibration curve was prepared by using Gallic acid as a standard. The result was expressed as milligrams per liter of Gallic acid equivalents (GAE) per 100 gm fresh weight.

4.2.12. Statistical analysis

The tests conducted in triplicate for each sample and simple random sampling for each tests. Generalized linear mixed model analysis were carried out to determine the effect of coatings and days of storage on different quality parameters. The coating treatments were compared on the basis of fungal decay percentage, total yeasts and mold counts, weight loss, pH, total soluble solids content and titratable acidity, total phenolic content, and total anthocyanin content. Treatment means were separated using Fisher's protected least square mean separation at $P \leq 0.05$.Data were analyzed using SAS 9.4 version (SAS Institute, Inc., Cary, NC).

4.3 Result and discussion

4.3.1. Characterization of coating materials

4.3.1.1. Characterization of liposomes

Liposomes prepared with a mixture of different concentrations of lecithin and PDA were characterized by UV/Vis spectroscopy. Figure 4.2 shows the absorption spectrum of Lecithin: PDA nanovesicles. The yellow line corresponds to unpolymerized liposomes, the lack of absorption peaks demonstrates the absence of conjugation in PDA backbone. On the other hand, the blue line shows the absorption spectra of liposomes prepared from the mixture of 80% lecithin and 20% PDA after photo polymerization. From the absorption spectra after UV light

irradiation, the peak present at 655 nm along with its narrow shoulder at 593 nm corresponds to π- π* electronic transitions (Li et al. 2008; Day and Ringsdorf, 1978). The low absorbance value at lower wavelengths shows low scattering that indicates low polydispersity of nanovesicles (Tomaszewska et al. 2013).

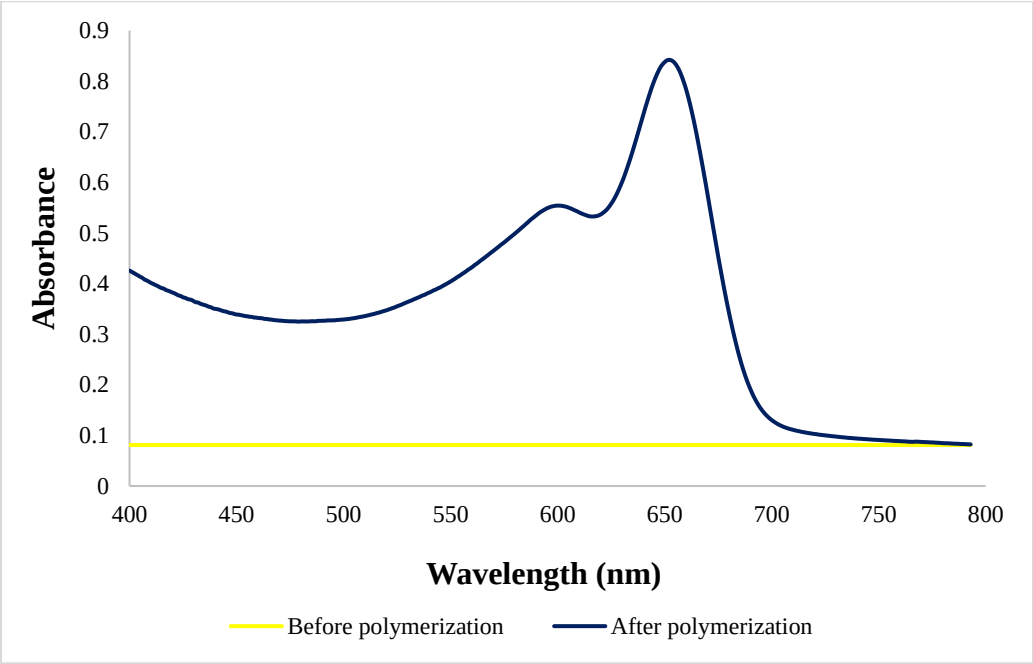


Figure 4.2: UV-Vis spectra for polymerized and non-polymerized 80% lecithin liposomes

4.3.1.2. Characterization of alginate coatings

4.3.1.2.1. Fluorescence imaging of fluorescent alginate coated berries

The alginate concentration for this particular experiment was roughly ten times than the concentration of alginate originally used for the coating. As shown in the fluorescent micrographs (Figure 4.3), the coating extends along the berry and the thickness of the coatings was about 10 µm. The blue intense emission was due to the presence of pyrene molecules chemically bound to alginate polymer (Srivastava et al. 2009).

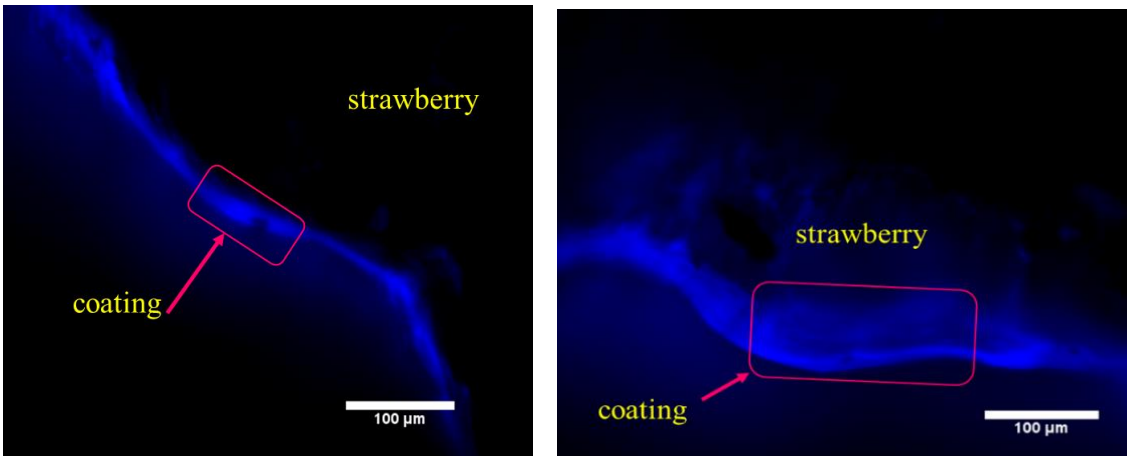


Figure 4.3: Fluorescence micrographs of strawberries coated with pyrene alginate at 100 µm. The blue color represents the presence of pyrene molecules.

Alginate was labeled with amino pyrene in order to image the presence and homogeneity of the coating. Pyrene was selected due to the emission wavelength range and long lifetime excited state. The emission intensity was recorded from 420 to 500 nm in a Nuance fluorescence camera. The figure 4.4 corresponds to the fluorescent micrograph spectrum, a maximum emission peak was observed at 490 nm. As it is well known that pyrene emission wavelength shifts due to the presence of stacking of pyrene molecules. This peak is showing the presence of pyrene excimer in the alginate coating (Uddin and Azam, 2013). This indicates self-association of pyrene molecules due to its hydrophobic character.

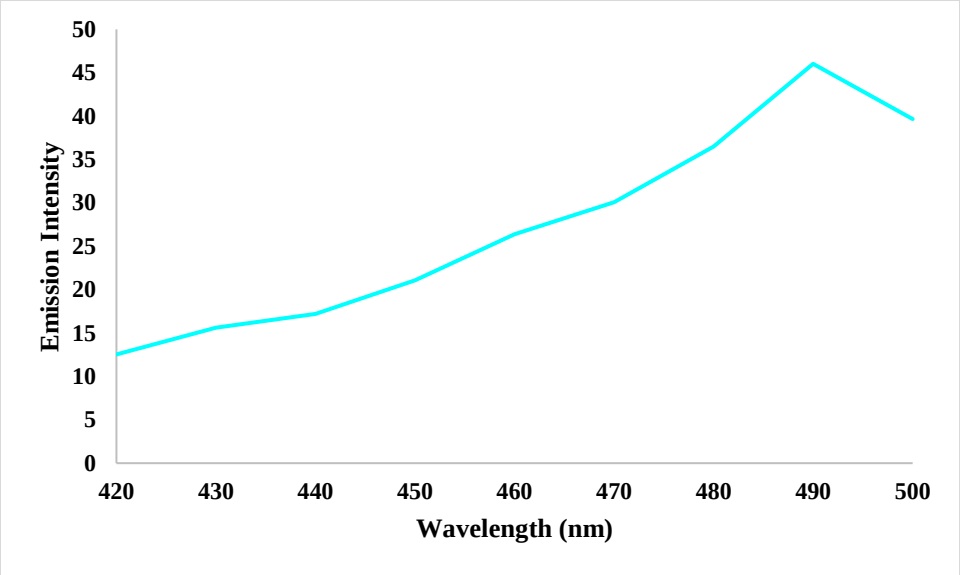
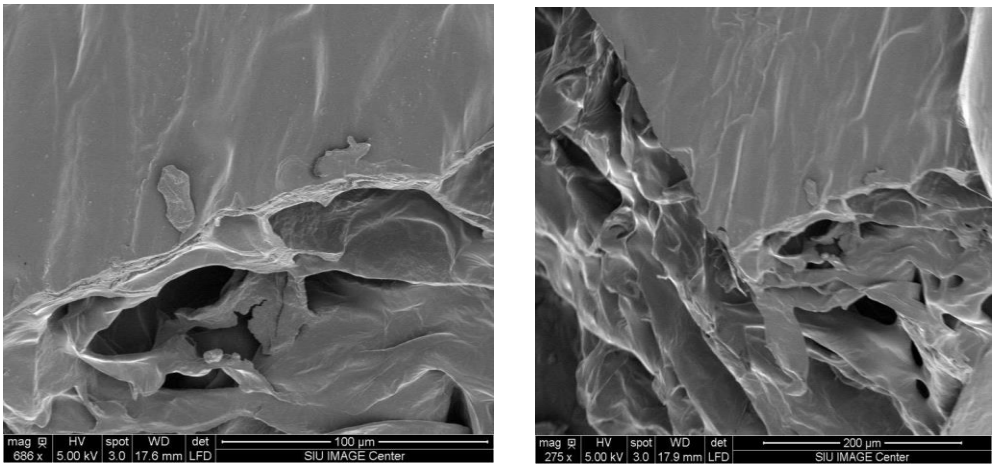


Figure 4.4: Fluorescence spectrum of pyrene alginate on coated strawberry

4.3.1.2.2. SEM of alginate coated berries

Strawberries coated with 1.5 % alginate were subjected to Electron imaging, in order to determine the thickness of the coated layer. From the electron micrographs showed in Figure 4.5, we could determine that the alginate layer was about 180 nm ± 40.

A) B)



C) D)

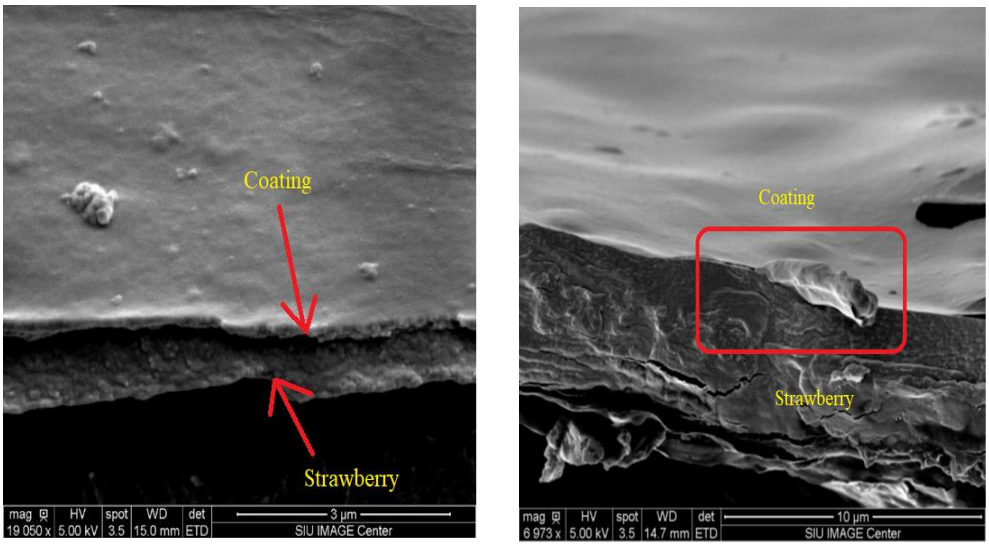


Figure 4.5: SEM images of cross section from: A) non-coated berries at 100 µm B) non-coated berries at 200 µm C) Alginate coated berries at 3 µm D) Alginate coated berries at 10 µm.

4.3.2 Headspace atmosphere analysis

The composition of gases present in the headspace atmosphere is dependent on the physiological activity of the fruits and by the microbial metabolism (Poverenov et al. 2014). There was a significant change in CO_2 concentration during the storage time ($p < 0.05$) (Figure 4.6).

Concentration of CO_2 at both 5th and 9th days of storage was significantly lower than that of 2nd day, but there was a significant increase observed in the CO_2 concentration after 9 days of storage. The increase in CO_2 concentration at after 9 day can be related with the damage in fruits and fungal decay (Hernández-Muñoz et al. 2006).

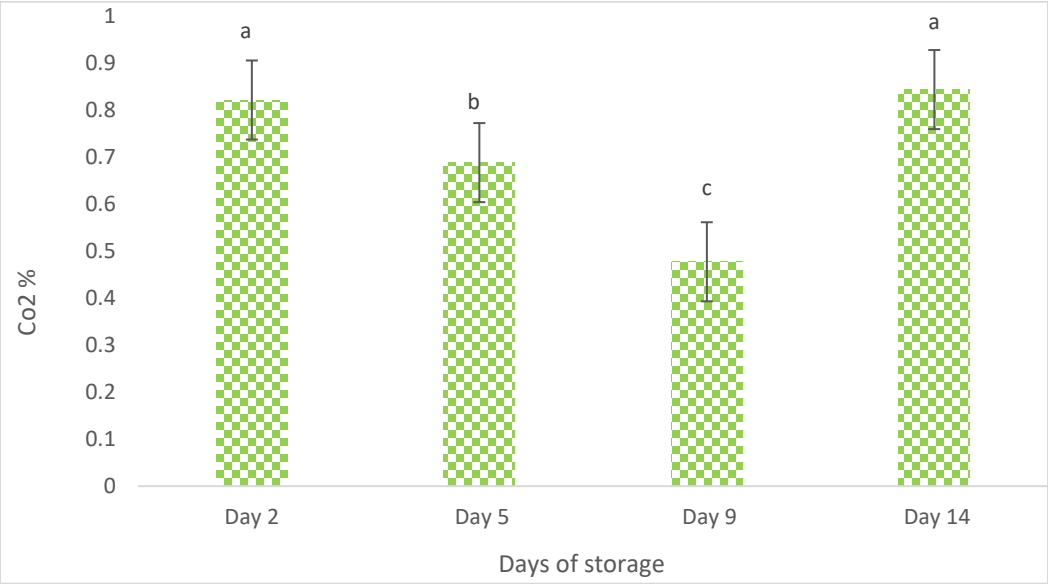


Figure 4.6: Concentration of CO_2 on various days of storage.LS-means with the same letter are not significantly different

A significant difference was observed in the concentration of CO_2 in liposome treated strawberries compared to those treated with alginate and non- treated control ($p< 0.05$) (Figure 4.7). The liposome treated berries showed lower concentration of CO_2 up to 14 days of storage. These results provide an evidence to the antimicrobial characteristics of limonene against spoilage microbes in fruits during storage (Vu et al. 2011). The increased concentration CO_2 among alginate treated strawberries can be attributed to their lower gas exchange properties (Poverenov et al. 2014). Permeability of the edible coatings is one of the major factors which tend effect the headspace composition of fruits and vegetables. If the coatings is not permeable

enough, normal gases exchange is stopped which results in hypoxic contditions inside fruit tisse. This is indicated by generation of off- flavor and enhanced production of CO_2 (Baldwin et al. 1999; Han, 2005). The increase in the CO_2 composition in control and alginate treated berries can be attributed to the production of CO_2, ethanol, organic acids produced by spoilage microbes (Jacxsens et al. 2003).

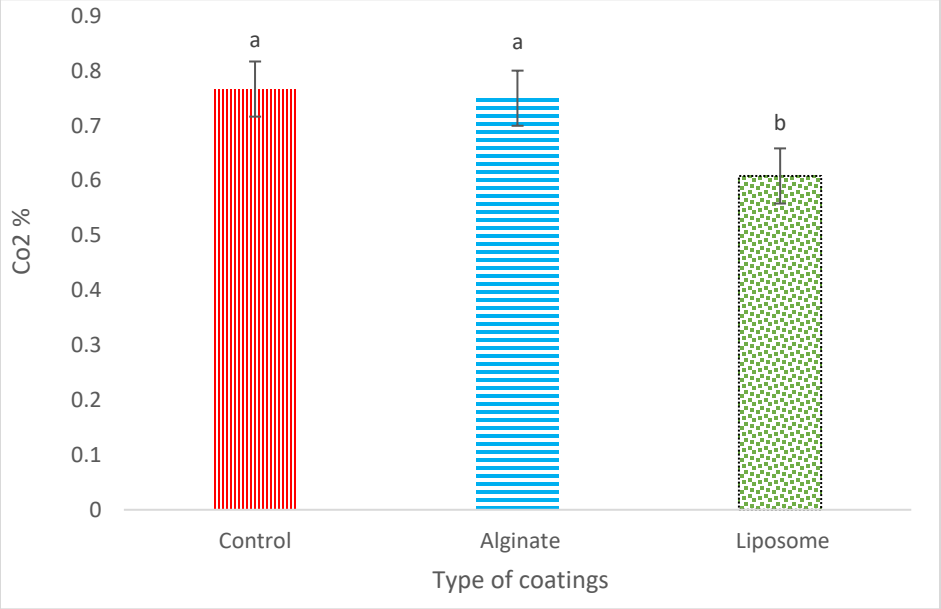


Figure 4.7: Concentration of CO_2 on various coatings. LS-means with the same letter are not significantly different

4.3.3. Fruit weight loss

The loss of weight in fruits is associated with respiration rate and evaporation of moisture through the skin. The rapid loss of water from the skin is one of the major factor that contributes to the perishability of strawberry fruits (Aharoni and Barkai-Golan, 1987). This leads to the dehydration of fruits and ultimately to shrinkage and deterioration. Edible coatings were found to prevent water transfer, protect the fruits skin from mechanical injuries resulting in delaying water loss (Ali et al.2011; Chien et al. 2007). In our study, no any significant difference was observed

between the coating types. However, there was a significant difference noticed in between the days of storage of the coated berries (Figure 4.8) with the highest weight loss observed in 14 days of storage.

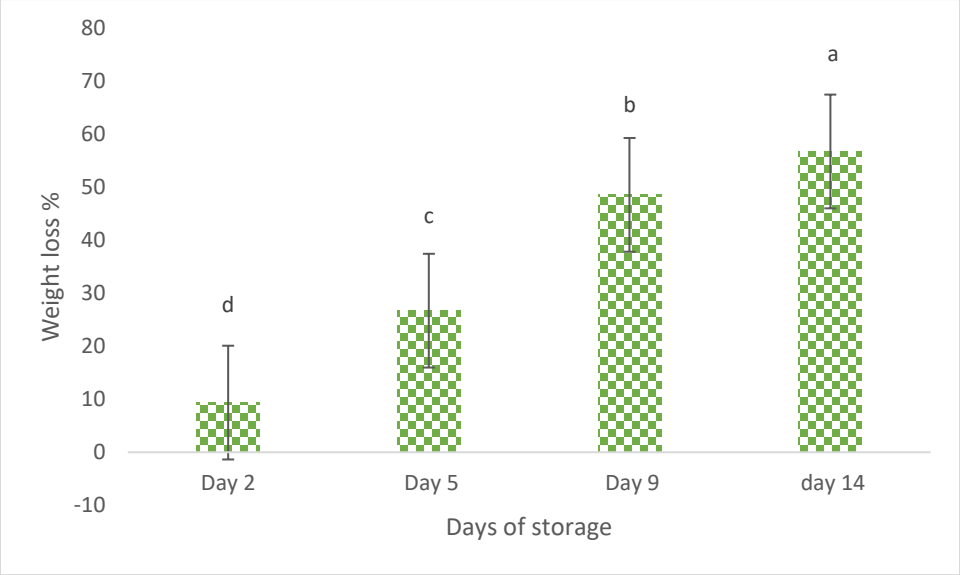


Figure 4.8: Percentage weight loss on days of storage. LS-means with the same letter are not significantly different

4.3.4. pH

There was a significant difference in pH of the berries between 2nd and 5th days of storage (Figure 4.9). The pH tend to rise significantly ($p < 0.05$) from 2nd to the 5th days of storage and there was a significant ($p < 0.05$) decrease in pH in the 9th day compared to the 5th day, however the difference was not significant among 2nd day and 9th day. Further, the pH of the berries increased on the 14th day but it was only significantly higher ($p < 0.05$) than 2nd day of storage. These results are in agreement with similar research conducted by Holocroft and Kader (1999) who observed increase in pH with the increase in storage days. The increase in pH during the

storage can be related to the effects of respiration rates of fruits due to the increased level of oxygen (Zheng et al. 2007).

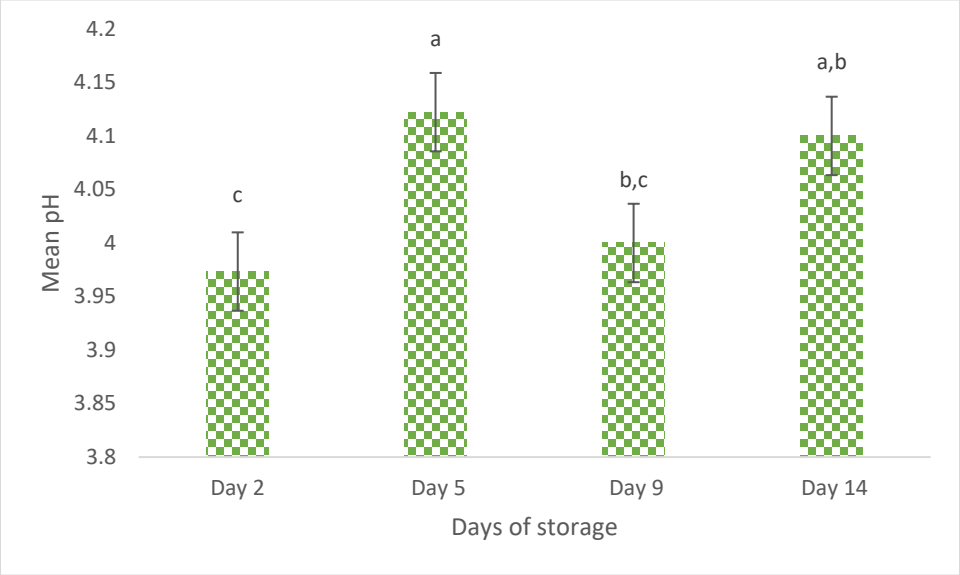


Figure 4.9: Mean pH on days of storage. LS-means with the same letter are not significantly different

Limonene liposome treated strawberries were found to have significantly lower pH values as compared to control ($p < 0.05$) (Fig 4.10.). Whereas, no significant differences were found between the liposome treated and alginate treated berries. Similarly, no any significant difference was observed among control and alginate treated berries.

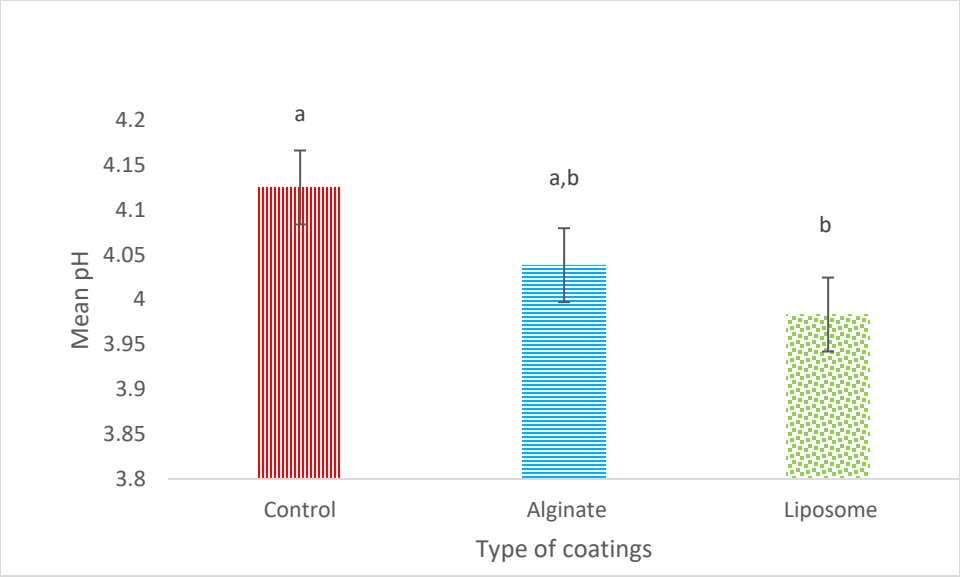


Figure 4.10: Mean pH on coating types. LS-means with the same letter are not significantly different

4.3.5. Titratable acidity (TA)

There was a non-significant difference between the coating materials. An increasing trend of TA was observed up to 9th days of storage among treated and untreated strawberries (Figure 4.11). There was a significant ($p < 0.05$) increase in the TA of the strawberries in 5th day of storage compared to the 2nd day. However, there was a not significant increase in the TA values in 9th days compared to 5th day of storage (Fig.) Further, there was a non-significant decrease in the values in the 14th days of storage. The decreased in TA content in the 14th day of storage can be attributed to the loss of water from fruits (Hernandez-Munoz, Almenar et al. 2008) due to the respiration and microbial growth.

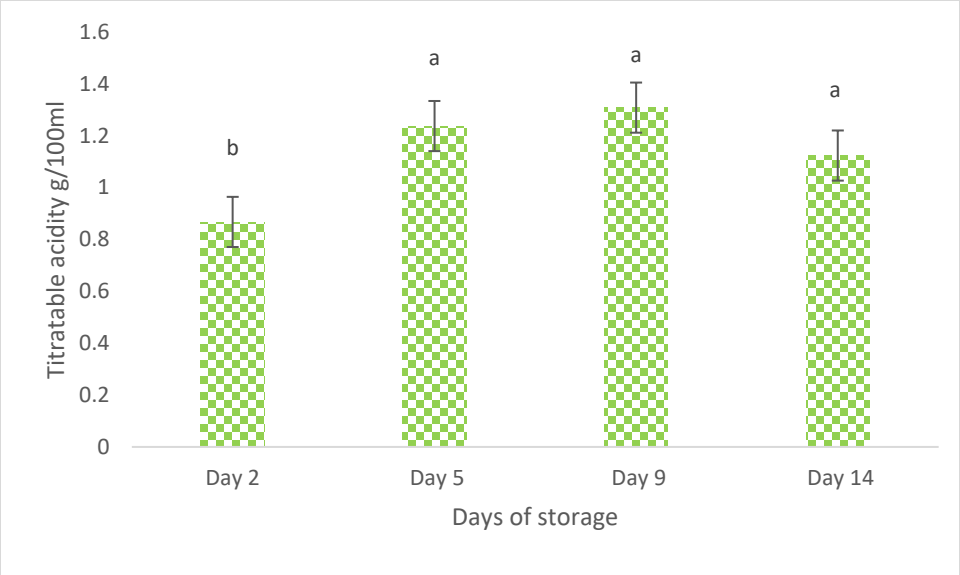


Figure 4.11: Mean Titratable acidity on days of storage. LS-means with the same letter are not significantly different

4.3.6. Total soluble solids (TSS)

There was no significant differences in the TSS level observed between the coating types. The mean TSS value was tend to increase significantly (($p < 0.05$) from the 2nd days of storage to 5th days of storage (Figure 4.12), whereas significantly ($p < 0.05$) reduced in the 9th days of storage compared to 5th days. There was no significant change observed from 9th day of storage onwards.

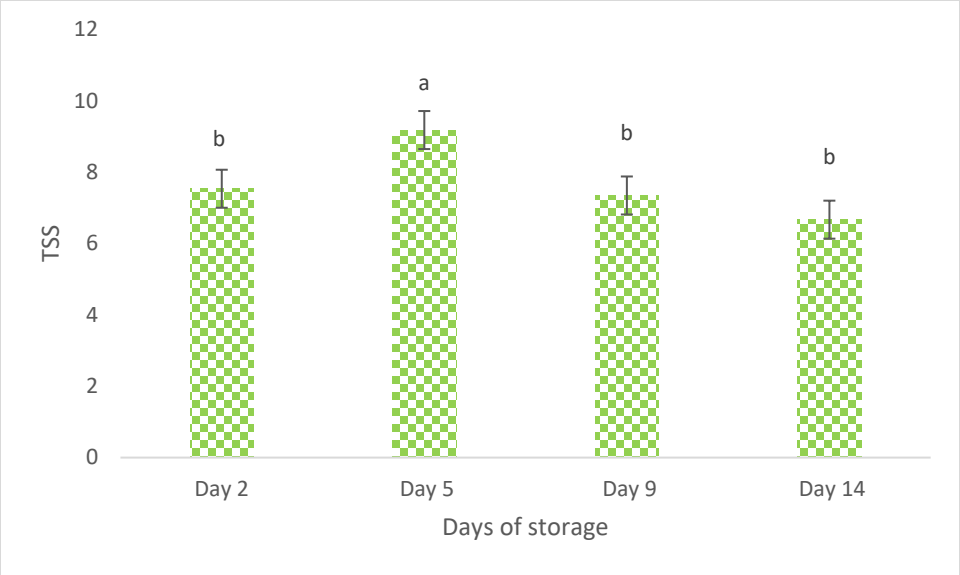


Figure 4.12: Mean TSS on days of storage. LS-means with the same letter are not significantly different

4.3.7. Total Phenolic Content (TPC)

There was no significant difference on total phenolic content observed among the coating types on days of storage. However, there was an increasing trend in the TPC up to 14 days of storage. There was a significant increase ($p < 0.05$) in the TPC content of strawberries from 2nd day to 5th day of storage but there was no significant increase from 5th day onwards to the 14th day of storage. (Fig. 4.13). These results concurred with the findings by (Nunes et al. 2006). The increase in the phenolic content of strawberries during storage can be attributed to the accumulation of anthocyanins and the development of its dark red-brownish color (Nunes et al. 1995; Montero et al.1996).

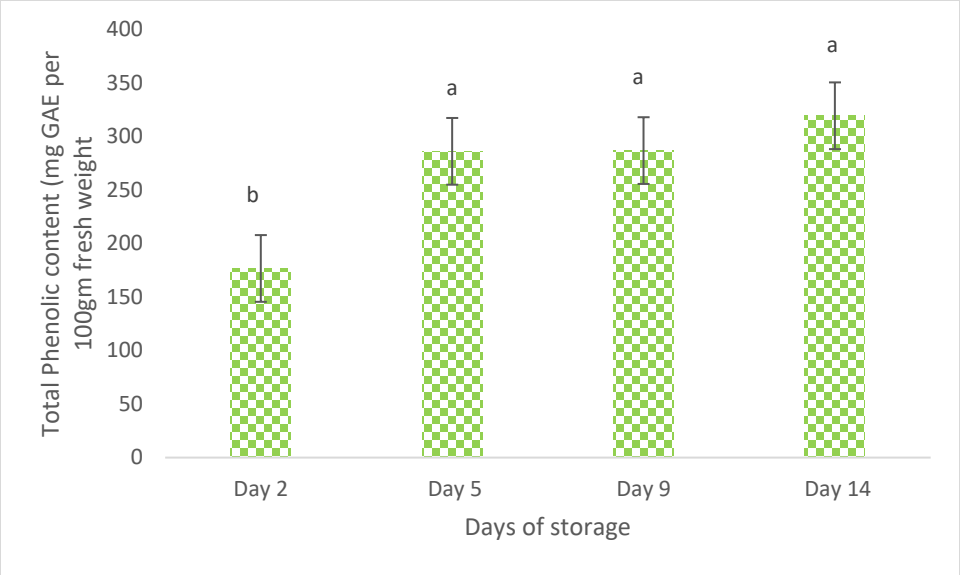


Figure 4.13: Mean TPC on days of storage. LS-means with the same letter are not significantly different

3.3.8. Total anthocyanin content

Similarly, there was a significant increase in total anthocyanin content of the strawberries during storage with the highest values observed in the 14th days of storage (Figure 4.15). These findings are in agreement with the studies done by Jiang and Joyce (2003) and Ayala-Zavala et al. (2004). Anthocyanins are responsible for the characteristic red color of ripe strawberries (Timberlake & Bridle). They are biologically significant for their antioxidant properties (Wang et al.1996). A regulatory enzyme, phenylalanine ammonia-lyase is responsible for the biosynthesis of anthocyanin in fruits and vegetables (Martinez et al. 1996).

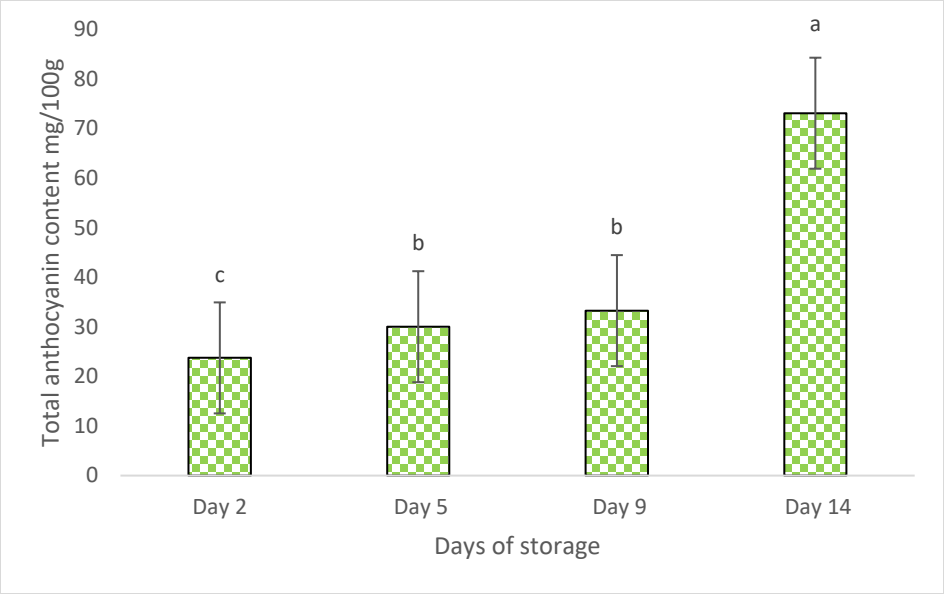


Figure 4.14: Mean Total anthocyanin on days of storage. LS-means with the same letter are not significantly different

There was significant difference in total anthocyanin content of strawberries among the coating types (Figure 4.15). The liposome treated strawberries showed significantly higher ($p < 0.05$) amount of anthocyanin content compared to the alginate treated and control strawberries. Similarly. Alginate treated strawberries also had significantly higher anthocyanin content compared to control.

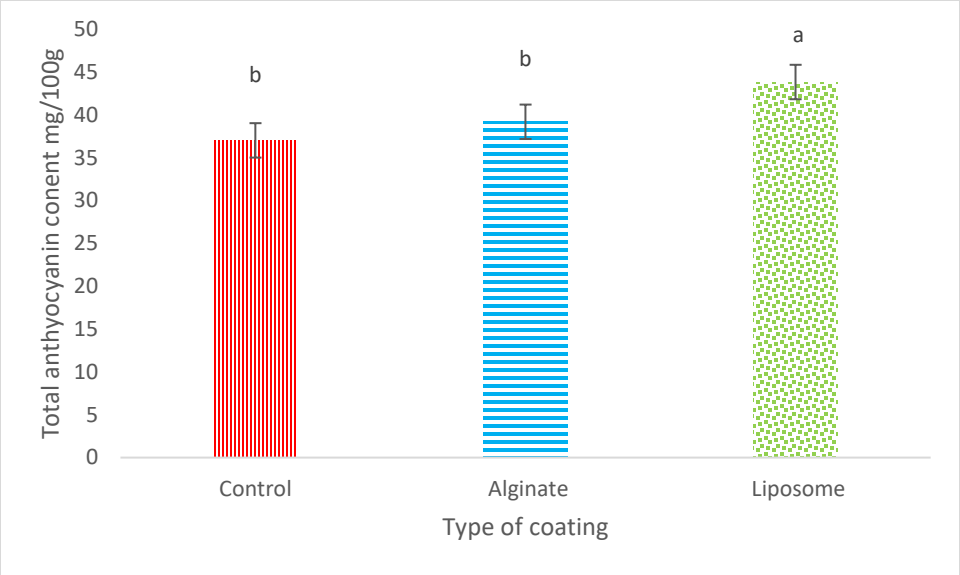


Figure 4.15: Mean Total anthocyanin on types of coatings. LS-means with the same letter are not significantly different

4.4. Conclusion

Limonene liposome was found to be an effective coating material for the shelf life extension and maintaining quality parameters of the strawberries. The result obtained in this study can be helpful to know that storage time significantly affects the quality of the treated and non- treated strawberries. The study has shown the possibility of development and application of antimicrobial phytochemicals encapsulated in liposomes. The edible coatings prepared with limonene liposomes were effective in the preservation of post-harvest quality of strawberries. The strawberries coated with limonene liposomes were shown to have lower respiration rates compared to control and alginate coatings. Similarly, the strawberries coated with liposomes had significantly lower pH values (3.9) and higher anthocyanin contents (43.849). These results suggests that limonene liposomes can be effective in maintain post-harvest quality of strawberries.

CHAPTER 5

CONCLUSIONS AND RECOMMENDATIONS

The results obtained in this research indicate that storage time significantly affects the quality parameters of coated and non-coated strawberries. In the first study, vibration was also found to have a significant effect on fungal decay and total soluble solids among all the coating treatments. However, vibration did not show any effects on other physio-chemical parameters that were analyzed. Among different coating types, liposomes were found to be the most effective for the preservation of strawberry quality and the limonene liposome was found to be effective in controlling fungal decay on strawberries for a prolonged period of storage. Similarly, titratable acidity and total phenolic contents were also found to be higher in strawberries coated with limonene compared to other coatings.

In the second study, the edible coatings prepared with limonene liposomes were effective in the preservation of post-harvest quality of strawberries. The strawberries coated with limonene liposomes were shown to have lower respiration rates compared to control and alginate coatings. Similarly, the strawberries coated with liposomes had significantly lower pH values (3.9) and higher anthocyanin contents (43.849). These results suggests that limonene liposomes can be effective in maintain post-harvest quality of strawberries.

Edible coatings prepared from d-limonene liposome was found to be an effective coating material for the shelf life extension and maintaining quality parameters of the strawberries for a prolonged storage period. The result obtained in this study can be helpful to know that storage time significantly affects the quality of the treated and non- treated strawberries.

FUTURE RESEARCH

The study has shown the possibility of development and application of antimicrobial phytochemicals encapsulated in liposomes. Studies can be done by the application of several nano encapsulated antimicrobial phytochemicals and compare their efficacy for maintenance of fruits quality. Determination of concentration volatile compounds through gas chromatography and mass spectroscopy could be helpful for the determination of stage of ripening and spoilage of the strawberries. Similarly, tests for color analysis, sensory evaluation, testing effects of vibration on coating materials in long hauls can be done in order to evaluate the efficacy of coatings.

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