DEVELOPMENT OF FUNCTIONAL COMPOSITE EDIBLE PACKAGING MATERIALS FOR CONTROLLED RELEASE OF BIOACTIVE SUBSTANCES

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ABSTRACT

DEVELOPMENT OF FUNCTIONAL COMPOSITE EDIBLE PACKAGING MATERIALS FOR CONTROLLED RELEASE OF BIOACTIVE SUBSTANCES

The aim of this study was to control the release of bioactive agents by modification of hydrophobicity and morphology of zein films using composite and blend film making methods. The bioactive agents incorporated into zein based films were lysozyme and phenolic compounds. The incorporation of beeswax, carnauba or candelilla wax into films gave composite films containing amorphous wax particles, while the incorporation of oleic, lauric or linoleic acid into films caused formation of blend films containing many spherical zein capsules within their matrix. The release profiles of phenolic compounds from zein films were successfully altered by the development of composite and blend films. The composites and blends can show 2.5 to 17 fold lower lysozyme release rates than the controls. The lysozyme release rates of composites reduced as the melting point of waxes increased. The chain length and the concentration of fatty acid used in blend films could also have affected the release rates of lysozyme. The phenolic antioxidants, catechin, gallic acid, p-hydroxy benzoic acid and ferulic acid are effective plasticizers of zein films. These phenolic compounds eliminate the classical brittleness problem of zein films and increase their flexibility considerably (up to 196%). Furthermore, catechin contributed to controlled release properties of films by reducing the film porosity. The phenolic compounds also provided antioxidant activity to films (up to 86 μmol Trolox/cm^2). The blends and composites of zein films containing phenolic compounds or lysozyme and phenolic compounds showed antimicrobial activity on critical food pathogenic bacteria or indicator microorganisms including *Escherchia coli* O157:H7, *Listeria monocytogenes*, *Listeria innocua* and *Campylobacter jejuni*. This work showed the possibility of obtaining advanced edible films having flexibility, antimicrobial and antioxidant activity and controlled release properties.

ÖZET

BİYOAKTİF BİLEŞİKLERİN KONTROLLÜ SALIMI İÇİN FONKSİYONEL KOMPOZİT YENEBİLİR AMBALAJ MATERYALLERİ GELİŞTİRİLMESİ

Bu çalışmanın amacı, zein filmlerin hidrofobisite ve morfolojisinde kompozit ve karışım film üretme metotlarını kullanarak farklılaştırmak ve bu yolla içerdikleri biyoaktif maddelerin kontrollü salımını sağlamaktır. Zein temelli filmlerin yapısında biyoaktif ajan olarak lisozim ve fenolik bileşikler kullanılmıştır. Filmlere balmumu, karnauba veya kandelilla gibi mumların ilavesi yapılarında amorf mum parçacıkları içeren kompozit filmler oluştururken; oleik, laurik veya linoleik asit gibi yağ asitleri ilavesi yapılarında küresel zein kapsülleri içeren karışım filmler oluşmaktadır. Fenolik bileşiklerin ve lisozimin karışım ve kompozit zein filmlerden salım profilleri incelendiği zaman bu filmlerin kontrollere göre çok daha düşük salım hızları gösterdiği görülmektedir. Örneğin lisozimin kompozit ve karışım filmlerden salım hızının kontrollere göre 2.5-17 kat daha düşük olduğu hesaplanmıştır. Kompozit filmlerden lisozim salım hızı film yapısında kullanılan mumların erime noktası arttıkça azalmaktadır. Karışım film yapısında kullanılan yağ asitlerinin zincir uzunluğu ve konsantrasyonu da lisozimin salım hızını etkilemektedir. Kateşin, gallik asit, p-hidroksi benzoik asit, ve frulik asit gibi antioksidant fenolik bileşiklerin ilavesi zein filmlerin klasikleşmiş kırılganlık problemini çözerek filmleri elastikiyetini belirgin bir şekilde arttırmıştır (% 196 a kadar). Ayrıca, bu fenolik bileşiklerden kateşin filmlerin gözenekliliğini de azaltarak kontrollü salım özelliklerini de etkilemektedir. Fenolik bileşikler beklendiği gibi geliştirilen filmlerin antioksidan potansiyellerini (86 μmol Trolox/cm^2 'ye kadar) de arttırmaktadır. Fenolik bileşikler veya lisozim ve fenolik bileşikleri bir arada içeren karışım ve kompozit zein filmler *Escherchia coli* O157:H7, *Listeria monocytogenes, Listeria innocua* ve *Campylobacter jejuni* gibi kritik gıda patojenlerine veya indikatör mikroorganizmalara karşı antimikrobiyel etki göstermektedirler. Gerçekleştirilen bu çalışma kontrollü salım özelliğine sahip, antimikrobiyel ve antioksidan aktivite gösteren elastik yenebilir filmlerin üretilebileceğinin göstermesi açısından önem taşımaktadır.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

CHAPTER 1

INTRODUCTION

Packaging is one of the most important processes to preserve the quality of food products during storage, transportation, distribution, sale, and end-use. The fundamental functions of packaging are protection, description of the contents, and convenience of transportation. Increase in consumer demand for minimally processed foods, changes in retail and distribution practices, and increased strictness of regulations concerning consumer health and safety are the major driving forces for innovation in food packaging technology (Suppakul, Miltz, Sonneveld, & Bigger, 2003). In the last two decades, extensive studies have been conducted to promote the functional properties of packaging materials and to improve the quality of packed foods. As a result of these studies, new packaging systems such as active packaging, modified atmosphere packaging (MAP) and edible films/coatings have been developed (Han, 2005b).

Active packaging incorporating antimicrobials and/or antioxidants is one of the most promising areas since the application of these technologies can improve safety of foods by inhibiting pathogenic bacteria or controlling spoilage flora, or prevent the quality loss of foods based on oxidation. Although different natural and chemical active agents have successfully been incorporated into plastic, biodegradable and/or edible packaging materials, health concerns of the consumers and environmental problems caused a particular interest on using natural compounds in edible packaging materials (Appendini & Hotchkiss, 2002; Han, 2005a; Lee, 2005). The natural antimicrobial agents such as antimicrobial enzymes, bacteriocins, essential oils and phenolic compounds are frequently employed in active packaging applications (Appendini & Hotchkiss, 2002; Han, 2005a; Joerger, 2007), while tocopherols, natural phenolic compounds, and ascorbic acid are used in active packaging applications as natural antioxidants (Lee, 2005). Lysozyme obtained from hen egg white is one of the most potential candidates for antimicrobial packaging since it has a GRAS status and it shows good stability and activity in different films and food systems under refrigerated storage temperatures (Mecitoglu et al., 2006; Ünalan, Korel, & Yemenicioğlu, 2011). Thus, lysozyme has recently been tested extensively in different plastic materials such as

cellulose acetate, nylon, and PVOH (Gemili, Yemenicioglu, & Altinkaya, 2009; Joerger, 2007) and biopolymeric materials from zein, soy protein, carrageenan, whey protein, chitosan, alginate, and pullulan (Joerger, 2007; Mendes de Souza, Fernández, López-Carballo, Gavara, & Hernández-Muñoz, 2010). This enzyme shows antimicrobial activity mainly on Gram-positive bacteria by splitting the bonds between N-acetylmuramic acid and N-acetylglucosamine of the peptidoglycan in their cell walls. On the other hand, the use of natural phenolic compounds in food packaging is particularly encouraged since they improve food oxidative and microbial status and show many different benefits on human health (Coma, 2008; Crespy & Williamson, 2004).

In traditional preservation methods, antimicrobial or antioxidant additives are mixed into initial food formulations to control microbial growth, oxidation and quality changes, and to extend shelf-life of foods. However, too much antimicrobials or antioxidants are needed since the critical effective concentration of these agents at the food surface should be reached also in bulk of the food. In addition to that, neutralization of active agents may occur in reactions and/or interactions in complex food systems. The dipping of foods into antimicrobial or antioxidant solutions is also not a promising solution since diffusion of the active agents from food surface to interior parts of food cause dilution of their critical concentration at the food surface very rapidly (Han, 2005a). On the other hand, active packaging containing antimicrobials and/or antioxidants enables extension of food shelf-life or increase in food quality by using minimum amounts of active compounds. However, rapid and uncontrolled release of active compounds from the packaging materials to food is the most important problem for active packaging technologies. In the case of a rapid and uncontrolled release, active compounds diffuse through the inner part of the foods (Buonocore, Conte, Corbo, Sinigaglia, & Del Nobile, 2005; Han & Floros, 1998); therefore, the surface of the food becomes microbiologically and oxidatively susceptible after a very short time and the effectiveness of the active packaging is greatly decreased (Appendini & Hotchkiss, 2002; Coma, 2008). On the other hand, very slow release of the active component from packaging materials to food is also a problem. In that case, the concentration of the active compound on the surface is below the critical level at which the active compound is effective. Therefore, a sufficient antimicrobial / antioxidant effect can not be achieved unless the release rate of active compounds from the packaging materials to food surface could be adjusted considering the physical and

chemical properties of food, growth kinetics of target pathogenic or spoilage microorganisms, and the expected food shelf life (Han, 2005a). In this way, bioactive agents can be used in active packaging more effectively since most of the natural substances are susceptible to complex interactions with food components (Quintero-Salazar, Vernon-Carter, Guerrero-Legarreta, & Ponce-Alquicira, 2005; Rose, Palcic, Sporns, & McMullen, 2002). Therefore, the use of active packaging that employs controlled release technologies enables maintaining the effective critical concentrations of antimicrobials and antioxidants at the food surface for longer time periods at which microbiological and chemical changes occur most intensively (Appendini & Hotchkiss, 2002).

Controlled release technology is widely used in the pharmacetiucal industry to develop matrices for controlled drug delivery. However, limited number of studies in the food industry has been made to develope active packaging materials which were able to retain the active agent in polymeric network and control its release rate. Controlled release technology was applied to synthetic polymers at first (Buonocore et al., 2003a; Chung, Papadakis, & Yam, 2001; Han & Floros, 1998). On the other hand, due to environmental concerns and high waste disposal costs of plastic packaging materials, the use of edible and biodegradable films in active packaging have also become very popular among food scientists. Therefore, in recent years, several studies has been done by using natural biodegradable and/or edible polymers for development of packaging materials with controlled release properties (Mastromatteo, Barbuzzi, Conte, & Del Nobile, 2009a; Ouattara, Simard, Piette, Begin, & Holley, 2000; Sebti, Carnet, Blanc, Saurel, & Coma, 2003). In contrast to synthetic polymers, environmentally friendly biodegradable and/or edible packaging materials produced at relatively mild conditions are readily accepted by the consumers without any health concerns and are quite compatible with most of the bioactive substances. Zein, a water insoluble hydrophobic storage protein found in corn and maize, attracts a particular interest as a biopolymer since it has excellent film forming and gas barrier properties; it is one of the rare proteins soluble in various organic solvents including ethanol and it is the major co-product of the oil and rapidly growing bioethanol industries (Manley & Evans, 1943; Selling, Woods, Sessa, & Biswas, 2008; Shukla & Cheryan, 2001; Wang et al., 2007; Zhang, Luo, & Wang, 2011a). Thus, a particular interest has been focused on the use of zein in active food packaging by incorporation of different natural antimicrobials including lysozyme (Gucbilmez, Yemenicioglu, & Arslanoglu, 2007;

Mecitoglu et al., 2006; Padgett, Han, & Dawson, 1998). However, the classical brittleness, and flexibility problems of zein films is a great limitation for their use as a free standing film and more widespread application as an active coating material. Moreover, to increase the potential application of active zein based films in food industry, further studies are needed to develop smart controlled release mechanisms for different natural antimicrobial agents used in active packaging.

The main objective of this study is to develop zein-wax composite and zein-fatty acid blend edible films for controlled release of lysozyme and phenolic compounds. In order to control the release of active compounds, different waxes and lipids were dispersed in the film matrix to change the hydrophilic/hydrophobic balance of films and/or morphology of the film matrix. This study brings a novel approach by showing the possibility of creating flexible active packaging with controlled release properties using an edible biopolymer.

CHAPTER 2

ACTIVE PACKAGING TECHNOLOGIES

2.1. Active Packaging

There is a long-standing interest to promote the functional properties of packaging materials and to improve the quality of packed foods since the consumer demands and market trends changes very rapidly during the last two decades. An innovative packaging concept which depends on the interactions between packaging materials or packaging components, food and the gas atmosphere in the package to provide fresh-like and safe products with high quality and long shelf-life has been developed to meet the consumer demands (Ozdemir & Floros, 2004; Suppakul et al., 2003; Vermeiren, Devlieghere, van Beest, de Kruijf, & Debevere, 1999). Labuza used active packaging term for the first time in 1987 (Rooney, 2005). Different definitions have been used since then, but now active packaging is d**e**fined as "a type of packaging that changes the condition of the packaging to extend shelf-life or improve safety or sensory properties while maintaining the quality of the food" (Quintavalla & Vicini, 2002). Many different active packaging technologies have been developed to extend shelf-life and provide better quality of the packed foods. Some important examples of active packaging such as oxygen scavengers, carbon dioxide emitters/absorbers, moisture absorbers, ethylene absorbers, ethanol emitters, flavor releasing/absorbing systems, time-temperature indicators, and antimicrobial containing films are listed in Table 2.1.

2.1.1. Antimicrobial Packaging System

The most promising version of the active packaging technology is the antimicrobial packaging systems. Microbial contamination and growth reduces the quality of foods and may cause foodborne diseases (Han, 2005a). In antimicrobial packaging systems, using different mechanisms, the growth rate of the microorganisms is reduced. The undesirable growth of microorganisms, especially on the surface of the

Table 2.1. Some important active packaging systems

(Source: Ozdemir & Floros, 2004)

Type of active packaging system	Substances used and mode of action
Oxygen absorbing	**Enzymatic systems** (glucose oxidase-glucose, alcohol oxidase, ethanol vapor) **Chemical systems** (powdered iron oxide, catechol, ferrous carbonate, photosensitive dye oxidation, ascorbic acid oxidation, catalytic conversion of oxygen by platinum catalyst)
Carbon dioxide absorbing / emitting	Iron powder-calcium hydroxide, ferrous carbonate-metal halide
Moisture absorbing	Silica gel, propylene glycol, polyvinyl alcohol, diatomaceous earth
Ethylene absorbing	Activated charcoal, silica gel-potassium permanganate, Kieselguhr, bentonite, Fuller's earth, silicon dioxide powder, zeolite, ozone
Ethanol emitting	Encapsulated ethanol
Antimicrobial releasing system	Sorbates, benzoates, propionates, ethanol, ozone, peroxide, sulfur dioxide, antibiotics, silver-zeolite, quaternary ammonium salts
Antioxidant releasing system	BHA, BHT, TBHQ, ascorbic acid, tocopherols
Flavor absorbing	Baking soda, active charcoal
Flavor releasing	Many food flavors
Color containing	Various food colors
Anti-fogging and anti-sticking	Biaxially oriented vinylon, compression rolled oriented HDPE
Light absorbing / regulating	UV blocking agents, hydroxybenzophenone
Monitoring	Time-temperature indicators
Temperature controlling	Non-woven microperforated plastic
Gas permeable / breathable	Surface treated, perforated or microporous films
Microwave susceptors	Metalized hermoplastics
Insect repellant	Low toxicity fumigants (pyrethrins, permethrin)

food, can be controlled by antimicrobial packaging; as a result, food quality and safety are assured throughout the self-life.

Food packaging is generally classified in two systems: package/food system and package/headspace/food system. Therefore, antimicrobial packaging is designed according to these systems. In package/food systems, package directly contacts with the solid, low viscosity, or liquid food without a headspace. In this system, initially incorporated antimicrobial agent diffuses through food and partitions at the interface (Figure 2.1A). Only nonvolatile substances can be used in package/food system. In package/headspace/food system, volatile substances migrate to food by evaporation through headspace and air gaps between food and package materials (Figure 2.1B). Diffusion of active substances is also a part of the migration process in package/headspace/food system.

Ionic or covalent immobilization of antimicrobial substances into the package material is another method used in antimicrobial packaging (Appendini & Hotchkiss, 2002). With immobilization, the growth of the microorganisms on the surface can be controlled without mass transfer. Furthermore, antimicrobial packages can be developed by using antimicrobial polymers such as chitosan and poly-L-lysine for film or coating (Appendini & Hotchkiss, 2002). Different researchers reported that surface modifications of packaging materials could also be used for producing antimicrobial packaging systems (Suppakul et al., 2003). This method is based on the introduction of functional groups to packaging materials by chemical methods or irradiation.

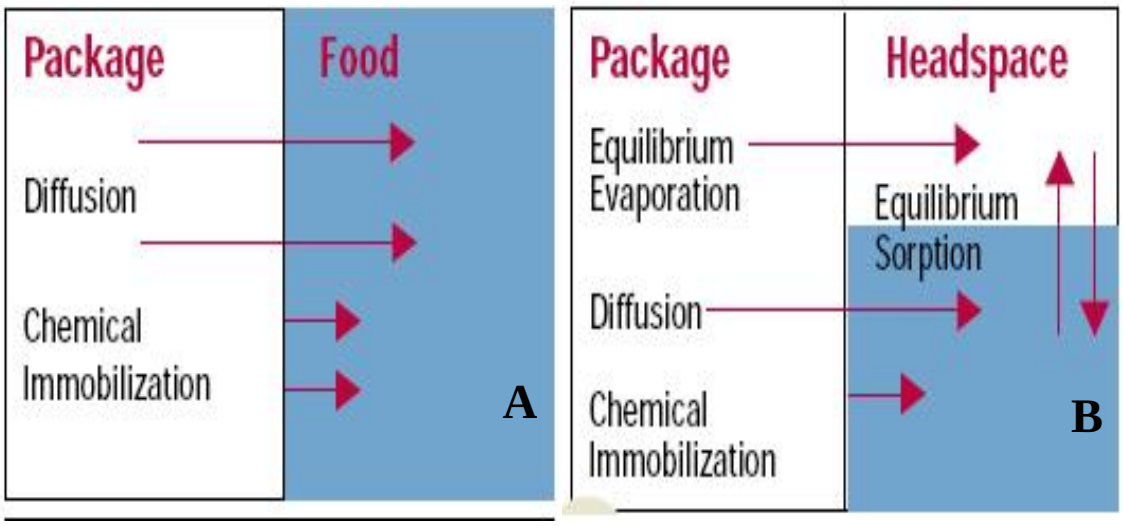


Figure 2.1. Package/food and package/headspace/food systems

(Source: Han, 2000)

2.1.1.1. Antimicrobial Films and Coatings

In antimicrobial packaging systems, sachets and films containing antimicrobial agents can effectively suppress surface microbial growth and increase shelf-life. Nowadays, especially films/coatings have become popular and many researches focus on developing antimicrobial films/coatings and their applications. The efficiency of antimicrobial films containing both chemical and natural antimicrobial compounds and systems were investigated by different researchers. Antimicrobial compounds used in antimicrobial films and coatings are summarized in Table 2.2.

Table 2.2. Antimicrobial agents used in antimicrobial films and coatings (Source: Appendini & Hotchkiss, 2002; Joerger, 2007; Quintavalla & Vicini, 2002)

Organic acids	Acetic acid, benzoic acid, citric acid, malic acid, propionic acid, sorbic acid, tartaric acid, organic acid mixture
Acid salts	Potassium sorbate, sodium benzoate
Acid anhydrides	Sorbic acidanhydride, benzoic anhydride
Parabens	Propylparaben, ethylparaben, methylparaben
Alcohol	Ethanol
Bacteriocins	Nisin, pediocin, lacticin, sakacin, bavaricin
Fatty acids	Lauric acid, palmitoleic acid
Chelating agents	EDTA, citrate, lactoferrin, conalbumin, polyphosphate
Enzymes	Lysozyme, lactoperoxidase, glucose oxidase, chitinase, β-gluconase, ethanoloxidase
Metals	Silver, copper, zirconium
Antioxidants	BHA, BHT, TBHQ, iron salts, α-tocopherol, ascorbic acid, plant extracts
Fungucides	Benomyl, imazalil, sulfur dioxide
Phenolic compounds	Catechin, p-cresol, hydroquinone, cinnamic acid, caffeic acid, p-coumaric acid
Plant extracts	Grape seed extract, Grapefruit seed extract, hop beta acid, rosemary oil, oregano oil, rheum palmatum, hinokitol
Probiotics	Lactic acid bacteria

Suppakul et al. (2003) classified antimicrobial films in two groups: (1) contains antimicrobial agent that migrates to the surface of the food, and (2) effects microorganism growth on the surface without migration. Figure 2.2 shows possible application ways of antimicrobial packaging systems. Films and coatings can be converted into antimicrobial packages by using different strategies.

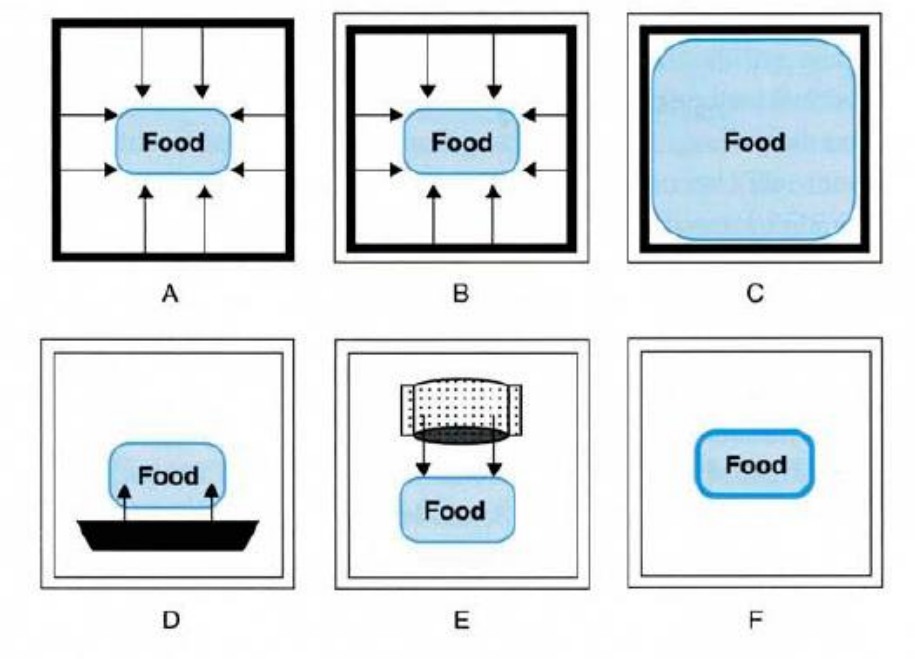


Figure 2.2. Possible application ways of antimicrobial food packaging systems: use of antimicrobial packaging materials (A); antimicrobial coating and conventional package material (B); immobilization of antimicrobial agents to polymeric packaging materials (C); antimicrobial tray or pads; (D) sachets with volatile antimicrobial agents (E); antimicrobial edible coatings on foods (F) (Source: Han, 2005a)

2.1.1.2. Incorporation of Antimicrobial Agents Directly into Films

Two strategies are used for the incorporation of antimicrobials into polymers: (1) addition of the antimicrobial agent into the melt form of polymer which are produced by thermal polymer processes, (2) mixing of the antimicrobial agent and polymer in the same solvent (solvent compounding). Thermally stable antimicrobials can be used with polymers which are thermally processed; otherwise, thermal processing methods such as extrusion and injection molding may denature heat sensitive compounds. Therefore,

mostly thermally stable chemical preservatives have been used in film making. On the other hand, solvent compounding may be preferred for the incorporation of heat-sensitive antimicrobials like proteins, peptides, and enzymes into polymers. In solvent compounding process, the solvent for both the antimicrobial compound and the polymer need to be the same. Generally, biopolymers such as methylcellulose, hydroxypropylmethylcellulose, carrageenan, chitosan, and zein are used in solvent compounding techniques with heat sensitive antimicrobial agents (Han & Gennadios, 2005). Recent studies used for the incorporation of antimicrobial agents into plastic or edible films are summarized in Table 2.3.

The migration of the non-volatile antimicrobial agents incorporated into packaging materials may be achieved by direct contact of packaging materials to food (Figure 2.3), therefore diffusion and partitioning of the active agent through packaging materials and foods are the main migration phenomena involved in this system (Quintavalla & Vicini, 2002). So the efficiency of the antimicrobial packaging systems is greatly affected by diffusion kinetics of active agents within the package material and the food. On the other hand, evaporation of the active agents is the the main migration phenomenon when the active agent is a volatile compound. The active agents migrate through the headspace by evaporation and then diffuse into food (Quintavalla & Vicini, 2002)

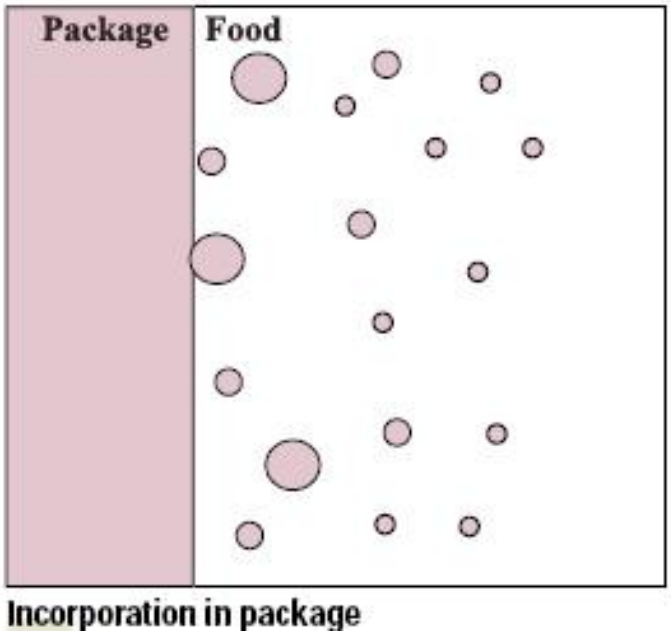


Figure 2.3. Diffusion of antimicrobial agents from package to food

(Source: Han, 2000)

Table 2.3. Antimicrobial agents used in plastic or edible packaging or coating materials

Antimicrobial agent	**Packaging material**	**References**
Naturally derived antimicrobials (linalool, carvacrol, and thymol)	LDPE	(Cran, Rupika, Sonneveld, Miltz, & Bigger, 2010)
Nisin, EDTA, sodium benzoate, and potassium sorbate	PLA	(Jin, Zhang, & Boyd, 2010)
Lysozyme, thymol, and lemon extract	LDPE, PDA, and PCL	(Del Nobile et al., 2009)
Nisin	PLA	(Jin & Zhang, 2008)
Fatty acids	HPMC	(Sebti, Ham-Pichavant, & Coma, 2002)
Potassium sorbate, chitosan	Sweet potato starch	(Shen, Wu, Chen, & Zhao, 2010)
Essential oils (thyme, clove and cinnamon)	Chitosan	(Hosseini, Razavi, & Mousavi, 2009)
Grape seed extract	Pea starch	(Corrales, Han, & Tauscher, 2009)
Lactoferrin	Chitosan	(Brown, Wang, & Oh, 2008)
Thymol	Zein	(Del Nobile, Conte, Incoronato, & Panza, 2008)
Na Lactate, K Sorbate, and Nisin	Sodium caseinate	(Kristo, Koutsoumanis, & Biliaderis, 2008)
Lysozyme, EDTA	Zein	(Gucbilmez et al., 2007)
Potassium sorbate	Topioca starch film	(Flores, Haedo, Campos, & Gerschenson, 2007)
Lactic acid and calcium propionate	Kafirin	(Petersson, Hagstrom, Nilsson, & Stading, 2007)
Potassium sorbate	WPI	(Sadikoglu, Sen, & Ozdemir, 2006)
Essential oils	WPI	(Seydim & Sarikus, 2006)
Grape seed extract	Ca alginate κ-carrageenan	(Cha, Choi, Chinnan, & Park, 2002)

2.1.1.3. Immobilization of Antimicrobial Agents to Polymers

Antimicrobial packaging materials can be produced by ionic or covalent linkages of antimicrobial agents to packaging polymers. The immobilized antimicrobial agent shows its activity only on the contact surface of food with package material (Figure 2.4). This technique requires both polymers and antimicrobials have some functional groups that are able to interact or form bonds (Table 2.4). The antimicrobial agent may immobilize to package materials with extensive H bonding, hydrophobic attraction, or ionic interaction. Peptides, enzymes, polyamines, and organic acids are examples that have functional groups. The antimicrobial agent may also immobilize to package materials with spacer arms which provide more freedom of motion to antimicrobial agents. Although immobilization processes have the risk of loss of activity of the antimicrobial agent, some successful immobilization studies have been conducted such as those showed in Table 2.4.

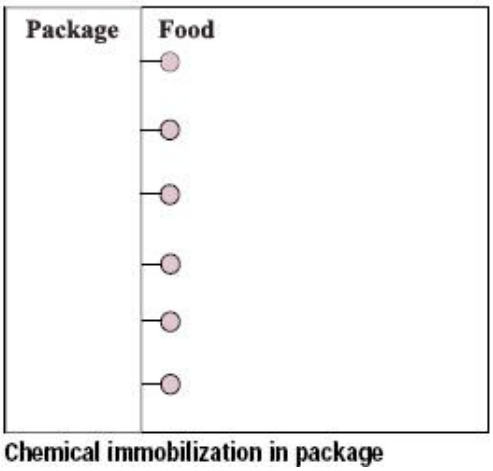


Figure 2.4. Immobilization of antimicrobial agent to package material (Source: Han, 2000)

2.1.1.4. Coating or Adsorbing Antimicrobial Agents onto Polymers

Incorporation of antimicrobial agents into packaging materials is often limited by the incompatibility of the agent with the polymer or by the inappropriate characteristics of the agent such as heat sensitivity to thermal polymer processing. A

novel method has been developed to overcome this problem: using a pre-cast polymeric film as the carrier of the antimicrobial agent and applying as coatings onto common

Table 2.4. Antimicrobials covalently/ionically immobilized in polymer support (Source: Appendini & Hotchkiss, 2002; Conte, Buonocore, Bevilacqua, Sinigaglia, & Del Nobile, 2006; Suppakul et al., 2003; Vartiainen, Ratto, & Paulussen, 2005)

Polymer support	**Antimicrobial agent***
Cellulose triacetate	Lysozyme
Polyvinylalcohol films	Lysozyme
Polyethylene films	Lysozyme
Polyethylene / polyamide film	Nisin
Polypropylene film	Glucose oxidase
Hydroxypropyl methylcellulose	Chitinase

synthetic plastics (Lee, Son, & Hong, 2008). A polymer-based solution coating is a successful method to achieve attachment of the antimicrobial agent to a plastic film and stability (Suppakul et al., 2003). Coatings may be applied to the food contact surface or to its outer surface. The diffusion of the antimicrobial agent may be affected significantly by location of coating (Figure 2.5).

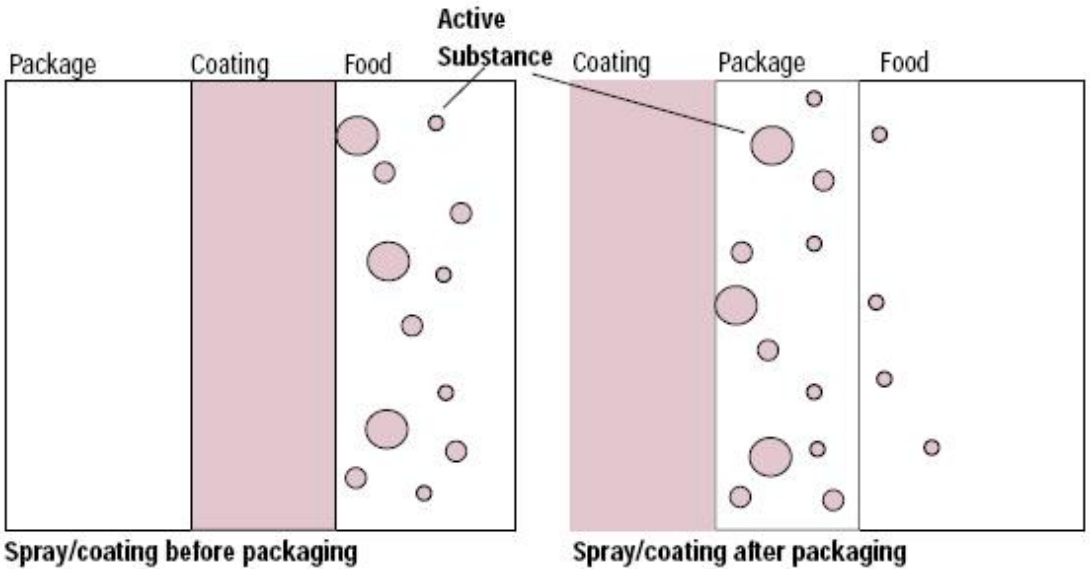


Figure 2.5. Different types of coatings

(Source: Han, 2000)

2.1.1.5. Use of Naturally Antimicrobial Polymers

Naturally antimicrobial polymers such as chitosan and poly-L-lysine may be used as films or coatings. Antimicrobial action of these polymers are based on the charged amine group in their structures (Appendini & Hotchkiss, 2002). The interaction between cationic polymers and negative charges at bacterial cell surface affects the membrane integrity negatively and causes leakage of the intercellular components of bacteria. Surface modifications may also create antimicrobial packaging materials (Suppakul et al., 2003).

2.1.2. Factors to be Considered during the Design of Antimicrobial Films or Coatings

Since interactions between antimicrobial agent, packaging material, and food determine the effectiveness of antimicrobial films, a number of factors should be considered during the design of antimicrobial film/coatings.

- *Compatibility of process conditions and the antimicrobial agent:* The activity of antimicrobial agents used for antimicrobial packaging can be affected by the film formation processes. The chemical stability of antimicrobial agents can be reduced by high temperature, shear forces, and pressures involved in extrusion processing (Suppakul et al., 2003). Therefore, thermal processes (extrusion) may reduce the residual activity of incorporated antimicrobial agents. Lamination, printing, drying operations, adhesives, and solvents used in film forming processes may also reduce the residual antimicrobial activity. In addition to chemical degradation, loss of volatile antimicrobial agents may reduce the residual antimicrobial activity of films, too (Han, 2000).
- *Interaction between the antimicrobial agent and film matrix:* Polarity, molecular weight, and ionic charges of antimicrobial agents are the main criteria for choosing suitable antimicrobial agent and polymeric material pair. For example, antimicrobials with low polarity are more suitable for non-polar plastic materials (Suppakul et al., 2003). Hydrophobic-hydrophilic interaction also plays a role in the formation of film matrix. Due to heterogeneous mixing of hydrophilic compounds

in hydrophobic plastics, some problems like loss of physical integrity, loss of transparency, hole creation, and powder blooming may arise during extrusion processes (Han, 2005a). On the other hand, anionic or cationic antimicrobial agents can bind to packaging materials through ionic interactions (Mascheroni et al., 2010). In a properly designed antimicrobial packaging system, the incorporation of the active agent should not affect the mechanical strength of polymeric materials negatively.

- *Interaction between the antimicrobial agent and food:* The compositions of food materials can show variations; thus, they have different chemical and physical properties such as water activity, acidity, and pH. Because of different characteristics of foods, the microbial flora in foods may be significantly different. Therefore, the antimicrobial agent should be chosen according to the microbial profile of the foods. Additionally, antimicrobial activity can be affected by biological and chemical characteristic of foods. For example, pH is an effective factor which controls or stimulates microbial growth in foods. Because of the association/dissociation properties of antimicrobial agents, their antimicrobial activity also changes with pH (Han, 2000). Water activity of foods may affect the diffusion of antimicrobial agents through packaging materials (Suppakul et al., 2003). Compatibility of antimicrobial agents with food components should be considered; otherwise, the residual antimicrobial activity will reduce due to inactive complex formation of antimicrobial agents with food components. In the case of nisin application in fresh meat, nisin-glutathione inactive complex formation reduced the effectiveness of the antimicrobial application (Rose, Sporns, Stiles, & McMullen, 1999).

- *Storage temperature:* Storage temperature of the packaged food can change the residual activity of the antimicrobial agent. At higher temperatures, the diffusion rate of the antimicrobial agent through package material will increase. Concentration of the active compound at the food surface become insufficient to suppress the microbial growth during shelf-life due to rapid diffusion of active compound and migration through inner parts of food. It was also reported that small amounts of antimicrobial agent may be more effective at low storage temperatures compared to high amounts of antimicrobial at high storage temperatures (Suppakul et al., 2003). As a part of the hurdle concept, antimicrobial packaging shows

synergistic effect with storage temperature. Therefore, in most of the studies, antimicrobial packaging is combined with refrigeration temperature.

- *Diffusion kinetics:* Effectiveness of the antimicrobial packaging system highly depends on the diffusion rates of the antimicrobial agent. Rapid and uncontrolled release of active compounds from the packaging material to food surface is the most important problem of active packaging. In contrast, the delivery of the active agents with controlled rate maintains the effective critical concentrations of active agents at the food surface and minimizes its neutralization due to complex interactions with food components (Han, 2005a). Therefore, the release rate of incorporated antimicrobial agent through food must be controlled. Controlled release systems can increase the efficiency of the antimicrobial packaging and overcome the quality and safety issues related to the use of active agents at too high or too low concentrations (Mastromatteo, Mastromatteo, Conte, & Del Nobile, 2010).

2.2. Antioxidant Packaging

Lipid oxidation is a major problem that affects the food quality. Thus, although most of the active packaging studies are related with incorporation of antimicrobial compounds, antioxidants can be incorporated into packaging materials to protect food against oxidation reactions. In early studies, synthetic antioxidants like butylated hydroxyanisol (Bhattacharya, Nagpure, & Gupta, 2007) and butylated hydrotluene (BHT) were successfully incorporated into packaging materials (Madhavi, Singhal, & Kulkarni, 1996; Moore et al., 2003). As an example, Jongjareonrak et al. (2008) used BHT incorporated fish gelatin based films to retard the oxidation level in lard. Due to significant health concerns about these synthetic antioxidants, consumer demands for natural antioxidants increased in recent years. As a result, researchers have focused on using natural antioxidants which are generally in GRASS status in packaging materials. Monophenols and phenolic acids, organic acids, plant extracts, Maillard reaction products, vitamin E, and vitamin C can be used as natural antioxidants for active packaging applications (Gucbilmez et al., 2007; Lee, 2005). Especially using natural antioxidants with biodegradable films have become a more promising subject in recent years. For example, gelatin films with tea polyphenols were used to retard fish oil

oxidation (Bao, Xu, & Wang, 2009). Similarly, Min and Krochta (2007) used ascorbic acid incorporated whey protein isolate (WPI) films to control lipid oxidation in peanuts. Some other studies in which natural antioxidants and biodegradable polymers used are presented in Table 2.5.

Table 2.5. Biodegradable polymers with natural antioxidants

Biodegradable polymer	Natural antioxidant	References
Alginate film	Ginseng extract	(Norajit, Kim, & Ryu, 2010)
Gelatin	Oregano and rosemary aqueous extracts	(Gomez-Estaca, Bravo, Gomez-Guillen, Aleman, & Montero, 2009)
Chitosan	Olive and rosemary oleoresin	(Ponce, Roura, del Valle, & Moreira, 2008)
Whey protein isolate	α-tocopherol	(Han & Krochta, 2007)
Chitosan	Green tea extract	(Siripatrawan & Harte, 2010)
Sunflower protein films	Essential oils	(Salgado, Lopez-Caballero, Gomez-Guillen, Mauri, & Montero, 2013)
Chitosan-Polyvinyl alcohol composite films	Mint extract, pomegranate peel extract	(Kanatt, Rao, Chawla, & Sharma, 2012)
Gelatin films containing chitosan nanoparticles	Tea polyphenols	(Bao et al., 2009)
Soybean protein isolate film	Bluberry extract	(Zhang et al., 2010)
Hydroxypropyl methylcellulose	Purple carrot extract, beetroot juice	(Akhtar et al., 2012)
Gelidium corneum films	Carvacrol	(Lim, Hong, & Song, 2010)
Red algae film	Grapefruit seed extract	(Shin, Song, Seo, & Song, 2012)
Tuna-skin and bovine-hide gelatin	Oregano and rosemary extracts	(Gomez-Estaca et al., 2009)

Phenolic compounds used in packaging materials are very effective in protecting foods against oxidation, but they generally show low compatibility with the color and flavor of the products. On the other hand, proteins, peptides, and amino acids are more compatible with foods when compared to phenolic compounds. Also, recent studies clearly showed their antioxidant capacity (Arcan & Yemenicioglu, 2007). Furthermore, proteins may have additional functional properties such as emulsifying. For example, using antioxidant chickpea protein extract not only increased the antioxidant capacity of zein film but also provided controlled release of lysozyme (Gucbilmez et al., 2007).

The rapid or slow release rate of the active agent is important for antioxidant incorporated films like antimicrobial ones, thus researchers focus on the control of diffusion rates in antioxidant incorporated films. For this purpose, Gemili et al. (2010) changed the polymeric structure of asymmetric cellulose acetate films to control the release rates of low molecular weight L-ascorbic acid, and L-tyrosine. Also, multilayer co-extruded films achieved gradual release of α-tocopherol (Granda-Restrepo, Peralta, Troncoso-Rojas, & Soto-Valdez, 2009). Mastromatteo et al. (2009a) developed a zein film that control the release rate of thymol by incorporating insoluble fibers into film matrix. Recently, nonoclays incorporated composite films were developed by Giménez, Gómez-Guillén, López-Caballero, Gómez-Estaca, and Montero (2012) to control the release rates of essential oils from gelatin–egg white films. On the other hand, Hwang et al. (2013) developed poly(L-lactic acid)/starch blend films of which resveratrol and α-tocopherol release profiles could be changed.

2.3. Bioactive Compounds Used in This Study

Although different natural and chemical preservatives have successfully been incorporated into plastic, biodegradable and/or edible packaging materials, health concerns of the consumers and environmental problems caused a particular interest in using natural antimicrobial compounds in edible packaging materials (Appendini & Hotchkiss, 2002; Han, 2005a). The natural antimicrobial agents frequently employed in active packaging include antimicrobial enzymes, bacteriocins, essential oils and phenolic compounds (Appendini & Hotchkiss, 2002; Joerger, 2007; Mastromatteo et al., 2010). In this study, natural bioactive compounds such as lysozyme and phenolic compounds were used as an antimicrobial and/or antioxidant agents. Lysozyme has only

antimicrobial effects while phenolic compounds have both antimicrobial and antioxidant effects (Table 2.6).

Table 2.6. Bioactive agents used for the controlled release experiments and their important characteristics

Bioactive agents	**Chemical class**	**Antimicrobial affects**	**Antioxidant affects**	**Reference(s)**
Lysozyme	Enzyme	G(+) bacteria	-	(Mecitoglu et al., 2006)
Catechin derivatives	Flavonoid	G(+) and G(-) bacteria	Free radical scavenging and metal chelating activity	(Ku, Hong, & Song, 2008b; Rajalakshmi & Narasimhan, 1996)
Gallic acid	Phenolic acid	G(-)	Free radical scavenging and metal chelating activity	(Belitz, Grosch, & Schieberle, 2009; Rauha et al., 2000)
Hydroxybenzoic acid	Phenolic acid	G(+) and G(-) bacteria	Free radical scavenging and metal chelating activity	(Gañan, Martínez-Rodríguez, & Carrascosa, 2009; von Gadow, Joubert, & Hansmann, 1997)
Fatty acids	Lipid	G(+) and G(-) bacteria	-	(Kabara, Swieczkowski, Conley, & Truant, 1972)

2.3.1. Lysozyme

Lysozyme is a natural antimicrobial enzyme, effective on many gram (+) food spoilage and pathogenic microorganisms. The antimicrobial activity depends on its lytic action on the bonds between N-acetylmuramic acid and N-acetylglucosamine of the peptidoglycan layer in the gram (+) bacteria (Duan, Park, Daeschel, & Zhao, 2007). On

the other hand, lysozyme does not show antibacterial activity against gram (-) bacteria due to their protective outer membrane (Min, Harris, & Krochta, 2005). However, the antimicrobial spectrum of lysozyme when it is combined with EDTA, a destabilizing agent for the outer membranes of gram-negative bacteria, increases significantly (Branen & Davidson, 2004; Padgett et al., 1998).

In food industry, lysozyme has been widely used in cheese manufacturing to prevent late blowing and off flavor formation caused by *Clostridium tyrobutyricum* (Roos, Walstra, & Geurts, 1998). In addition to that, the efficiency of lysozyme in preventing malolactic fermentation in winemaking has also been reported by (Gerbaux, Villa, Monamy, & Bertrand, 1997). Lysozyme as preservative is being used in some food products such as poultry, shrimp, sausage, and sake (Hughey & Johnson, 1987). Moreover, lysozyme has a great potential in food preservation due to its stability over a wide range of temperature and pH values (Gucbilmez et al., 2007; Ünalan et al., 2011). During the last two decades, lysozyme has been used very frequently in active packaging research by incorporating into different carrier matrices (Datta, Janes, Xue, Losso, & La Peyre, 2008; Duan et al., 2007; Mecitoglu et al., 2006; Min et al., 2005; Nam, Scanlon, Han, & Izydorczyk, 2007; Wu & Daeschel, 2007). Nowadays, researchers are focused on its controlled release from carrier matrices using different methods to increase its microbial growth control efficiency (Bezemer et al., 2000; Buonocore et al., 2005; Gemili et al., 2009; Gucbilmez et al., 2007)

2.3.2. Phenolic Compounds

Phenolic compounds are widely found in various types of edible plants, especially in vegetables, fruits, tea, and wine. They are divided into different categories according to their structural differences. Phenolic acids and flavonoids are the major classes of phenolic compounds. Phenolic compounds also exhibit a wide range of biological effects, including antioxidant and antimicrobial properties (Cowan, 1999). However, these compounds may show their antimicrobial activities based on different mechanisms. For example, phenolic acids and flavanoids may inhibit microbial growth with binding to adhesins, complexing with the cell wall, and inactivating some enzymes. On the other hand, tannins, an important polyphenol, may show antimicrobial activity by binding proteins, binding to adhesins, by inhibiting enzyme, substrate

deprivation, by forming complexes with cell wall, disrupting membrane, and complexing with meal ions (Cowan, 1999). Due to their antimicrobial activity, phenolic compounds have been used in antimicrobial coatings and films for a long time. Especially natural extracts such as grape fruit and seed, wasabi, green tea, and turmeric that contain various types of phenolic compounds were successfully incorporated into different film matrices (Joerger, 2007). On the other hand, Ku et al. (2008a) used only commercial catechin to produce antimicrobial and antioxidant edible films.

CHAPTER 3

EDIBLE FILMS

Edible films or coatings are thin layers of edible material which may be consumed with food. They can be placed on food or between foods. They can maintain or improve food quality during the storage period and extend shelf-life by providing a selective barrier to moisture, oxygen, aroma, flavor, and solute mass transfer (Choi, Kim, Hanna, Weller, & Kerr, 2003). Edible films are formed as stand-alone sheets of material while coatings are directly formed on the product by dipping or spraying. Edible films are considered not only a package material but also a food component, hence they should have some specifications such as high barrier and mechanical properties; biochemical, physicochemical, and microbial stabilities; compatibility with food regulations; being non-toxic and non-polluting; and low production cost (Cutter, 2006). Using edible films offers many advantages such as providing additional nutritional value, acting as carriers for antimicrobial or antioxidant agents, decreasing environmental pollution with biodegradable nature, preventing loss of volatile flavors/aromas, maintaining sensory qualities; and improving mechanical characteristics and handling of foods (Cutter, 2006; Debeaufort, Quezada-Gallo, & Voilley, 1998; Han & Gennadios, 2005; Hernandez-Izquierdo & Krochta, 2008; Lin & Zhao, 2007)

3.1. Compositions of Edible Films

Edible films are composed of two major components: (1) biodegradable and edible biopolymers; (2) food grade additives.

3.1.1. Film Forming Biopolymers

Biodegradable biopolymers or biomaterials are the main film forming materials used for edible films. Generally three different film forming materials are used (Table 3.1) which can be used alone or in combinations. The physical and chemical

characteristics of edible films are greatly affected by biopolymers or biomolecules used in matrix formation:

Polysaccharide based edible films: Polysaccharide-derived films can be used for the formation of desirable modified atmosphere with their selectively CO_2 and O_2 permeable properties (Cutter, 2006). So, they can be used to enhance the shelf-life of the products without creating anaerobic conditions by preventing oxidation, browning reactions, dehydration, and oil diffusion. However, due to their hydrophilic character, they exhibit very poor water vapor barrier properties. But this characteristic may be useful to remove water vapor that is formed during storage and prevent formation of condensed water in the package which accelerates microbial growth (Cha & Chinnan, 2004). Treatments or cross-linking that enhances protein-polysaccharide interactions can be used for improving functional properties of polysaccharide based films. Availability and low cost are important advantages of polysaccharides.

Table 3.1. Film forming materials
(Source: Han & Gennadios, 2005)

Proteins	Collagen, gelatin, casein, whey protein, corn zein, wheat gluten, soy protein, egg white protein, fish myofibrillar protein, sorghum protein, pea protein, rice bran proteip, cottonseed protein, peanut protein, and keratin.
Polysaccharides	Starch, modified starch, modified cellulose (CMC, MC, HPC, HPMC), alginate, carrageenan, pectin, pullulan, chitosan, gellan gum, and xanthan gum.
Lipids	Natural lipids, fatty acids, resins (shellac, terpene) and wax (beeswax, carnauba wax, candelilla wax, and rice bran wax)

Lipid based edible films: Lipid materials such as beeswax, candelilla wax, carnauba wax, triglycerides, acetylated monoglycerides, fatty acids, fatty alcohols, and sucrose fatty acid esters as well as resins such as shellac and terpene resin are used as edible film-forming materials. Due to their low polarity, lipids exhibit high barrier characteristics especially against water. Water vapor molecules cannot pass through tightly packed crystalline structure of lipids (Cutter, 2006). On the other hand, at higher storage temperatures, lipid based films may have low permeability to gases (oxygen,

carbon dioxide, and ethylene) and this characteristic limits the use of lipid-based films due to the formation of potentially anaerobic conditions which may cause some food safety problems. Other disadvantages of lipid based films are: poor adherence to hydrophilic surfaces, lack of structural integrity, greasy surface of the packed food, and rancidity development (Cutter, 2006; Rhim & Ng, 2007). The structure, degree of saturation, chain length, physical state, shape and dimension of crystals, and distribution of lipids into the film influence the functional properties of the film (Cha & Chinnan, 2004). Lipids and resins are not polymers; thus, they do not form cohesive stand-alone films. They generally are used as coating materials. Moreover, they provide a moisture-barrier component of composite films.

Protein based edible films: Although protein based films provide good barriers to oxygen and carbon dioxide, they exhibit very poor water barrier. Corn, wheat, soybeans, peanut, milk, gelatin or other animal/plant proteins can be used to produce protein-based edible films. Conformational changes, electrostatic charges, and amphiphilic nature of proteins are the important characteristic and differentiate them from other film forming materials (Han & Gennadios, 2005). With these characteristics, the physical and mechanical properties of edible films can be altered or controlled. Hydrophilic interactions and hydrogen bonding are also important for the formation of film matrix.

3.1.2. Plasticizers

Cohesive forces in film matrixes which are related to the molecular weight, polarity, and chain structure of polymers can result in undesirable brittleness problems in films. To overcome this problem, generally, low molecular weight substances called plasticizers are incorporated into the polymeric film forming materials. Plasticizers improve flexibility, elongation, toughness, process ability, and lower glass transition temperature (T_g) of the film (Han & Gennadios, 2005; Sothornvit & Krochta, 2005). The addition of plasticizers also affects the moisture sorption and gas barrier properties of the films. There are two theories to explain plasticizing effects for edible films: the gel theory and the free volume theory.

Gel theory: In gel theory, plasticizers attach to polymer chains and reduce polymer-polymer interaction forces. This increases the gel flexibility of the gel

structure. Additionally, unbound plasticizer molecules aggregate and form plasticizer domains which promote the movement of polymer molecules.

Free volume theory: Free volume is the volume difference in amorphous materials or polymers between a specific temperature and absolute zero temperature (Sothornvit & Krochta, 2005). Plasticizers increase the free volume of polymer structures by increasing motion of the polymers. Plasticizing action, changes in T_g, viscosity, cross-linking, diffusion, film drying, and film properties can be explained with free volume theory (Sothornvit & Krochta, 2005).

Glycerol, xylitol, sorbitol, mannitol, polyethylene glycol, sucrose, water, glucose, and fatty acids are some of the plasticizers commonly used in edible films and coatings (Sothornvit & Krochta, 2005). Some plasticizers react with polymer molecules (internal plasticizer) while others only interact with the polymers (external plasticizer). Internal plasticizers increase the space around the polymers and prevent them from approaching each other. Thus, they lower the T_g and reduce the elastic modulus of the films. On the other hand, external plasticizers interact with polymers and produce swelling. Effectiveness of the plasticizers depend on some properties such as the size and shape of plasticizer molecules, the number of oxygen atoms and their spatial distance within the structure of the plasticizers, and water-binding capacity (Han & Gennadios, 2005).

3.2. Physical and Mechanical Properties of Edible Films

Han and Gennadios (2005) defined edible film as " a dried and extensively interacting polymer network of a three-dimensional gel structure". Three-dimensional network structure can be formed by dry (extrusion casting) or wet (solvent casting) processing. In dry processing, like common plastic materials, biopolymers are heated above their T_g using an extruder. Edible films prepared from proteins and starch can be produced by extrusion, compression, or injection molding (Rhim & Ng, 2007). Thermoplastic behaviors of biopolymers become important at this process. Also, protein and starch should be plasticized before extrusion. The main advantage of dry processing over wet processing is production of films with low solubility due to the formation of highly cross-linked film matrices. Wet processing is composed of solubilization, casting, and drying steps and is based on drying of the film-forming solution. Film-

forming solution is prepared by solving the biopolymer in a solvent. Only water and ethanol can be used as a solvent in edible films. Properties of biopolymers (nature, type, possible interactions), and film forming conditions (drying temperature and drying rate, the moisture content, the solvent type, the plasticizer concentration, and the pH) directly affect the formation of three-dimensional network.

Edible films should meet the requirements of some specific functional properties such as moisture sorption, barrier properties (moisture, gas, aroma, oil permeability), and mechanical properties. There is a direct relationship between physical/chemical nature of polymers and barrier properties of films. Especially barrier properties are important for maintaining food quality during the storage period. The important barrier functions of edible films are water vapor, oxygen, and oil barrier properties. Barrier properties of films are generally measured in terms of permeability.

Water barrier: With controlling water activity, several reactions (browning, oxidation, degradation reactions, and enzyme activity) can be controlled in the food system. Water activity also affects the growth rate of microorganisms. Moreover, water causes texture changes in foods. Therefore, water vapor permeability (WVP) of edible films is important for the shelf-life and quality of foods. Polysaccharide and protein-based films provide poor barriers against water due to their hydrophilic nature. Lipid based films and coatings are good water barriers. Except for lipid-based materials, the water vapor permeability of most edible films is generally higher than that of common plastic films. To decrease the WVP of edible films, lipids can be used in polysaccharide and protein based films (Han & Gennadios, 2005). Type and the amount of plasticizer also affects the WVP.

Oxygen barrier: Oxygen is transferred from the environment through packaging material to foods. However, oxygen decreases the shelf-life and quality of foods by increasing deterioration of food components and microbial growth rates. Therefore, low oxygen permeability is required for packaging materials. Films can show oxygen barrier properties if they have groups that can associate through hydrogen or ionic bonding. In general, oxygen permeability of edible films is quite low. For example, films based on polysaccharides like alginate, cellulose ethers, chitosan, carrageenan, and pectin show good gas barrier properties (Lacroix, 2009). But especially non-ionic polysaccharide films have quite higher oxygen permeability than protein films. This may be due to the more polar nature and more linear structure of the proteins (Miller & Krochta, 1997). With increasing plasticizer amounts, because of the higher free volume in the film

matrix, oxygen permeability of films increases (Sothornvit & Krochta, 2005). Relative humidity also affects the oxygen permeability of edible films (Han & Gennadios, 2005).

Oil barrier: Aroma and oil migration also cause quality loss in foods. Polysaccharides and protein-based films are good barriers against oil at low to intermediate relative humidity (Sothornvit & Krochta, 2005).

In fact no polymer films, including edible films, are perfect barriers. However, each edible film is a barrier to some degree since it limits the permeability of water, oxygen, aroma, and oil. That is why composite films that contain hydrophobic materials such as fatty acids and waxes are sometimes required as edible films or coatings.

Besides their barrier functions, edible films and coatings protect food against physical shocks. Standardized mechanical tests of plastic materials are also applied to edible films and coating structures with some modifications. These tests include tensile strength, stress at break, offset yield stress, elastic modulus, offset yield, elongation at tensile strength, and elongation-at-break (Figure 3.1); but tensile strength, elongation-at-break, and elastic modulus tests are mostly used for measuring mechanical properties of edible films. The mechanical properties of edible films depend on the film forming polymeric material's structure, molecular length, geometry, molecular weight distribution, and position of lateral groups. In general, edible films have lower tensile strength than common plastic films while their elongation-at-break varies widely (Han & Gennadios, 2005). However, their elongation value is mainly dependent on plasticizer presence/absence and amount (Cuilbert & Contard, 2005). Mechanical properties of edible films are also affected by relative humidity. Absorbed water acts like a plasticizer in the film matrix.

Mechanical properties of films can be improved with physical, chemical, or enzymatic modifications. For example, particle size reduction with homogenization of whey protein based films resulted in a stronger film with lower WVP by increasing their interfacial area (Lacroix, 2009).

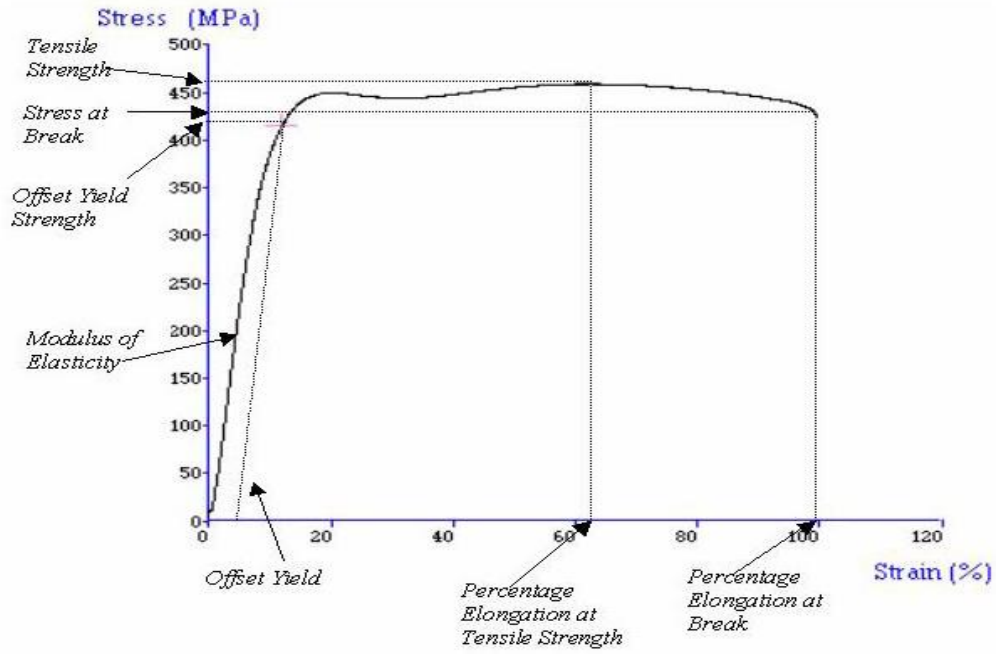


Figure 3.1. Stress-strain curve for tensile measurements

(Source: TA.XTplus, 2007)

3.3. Composite Films

Composite films are developed in order to take advantage of the complementary functional properties of different constituent materials and to overcome their respective disadvantages. Composite films are generally defined as a heterogeneous structure that is composed of a continuous matrix with some additives (Dutta, Tripathi, Mehrotra, & Dutta, 2009). Composite edible films can be produced with using different techniques (Figure 3.2):

1. *Emulsion type composite films:* Lipid globules or solid particles can be incorporated into hydrocolloid film forming solutions prepared from proteins, starches or celluloses, and their derivatives.
2. *Bi-layer or multilayer type composite films:* Bi-layer films are composed of several layers and can be produced using two different techniques: (1) coating technique; (2) emulsion technique (Figure 3.3). In coating technique, a second film matrix is layered onto a previously formed polysaccharide or protein film. On the other hand, lipids are dispersed into the film forming solution prior to film casting in emulsion technique. Continuous phase cannot stabilize the

emulsion and phase separation occurs during drying. At the end, a bi-layer film forms.

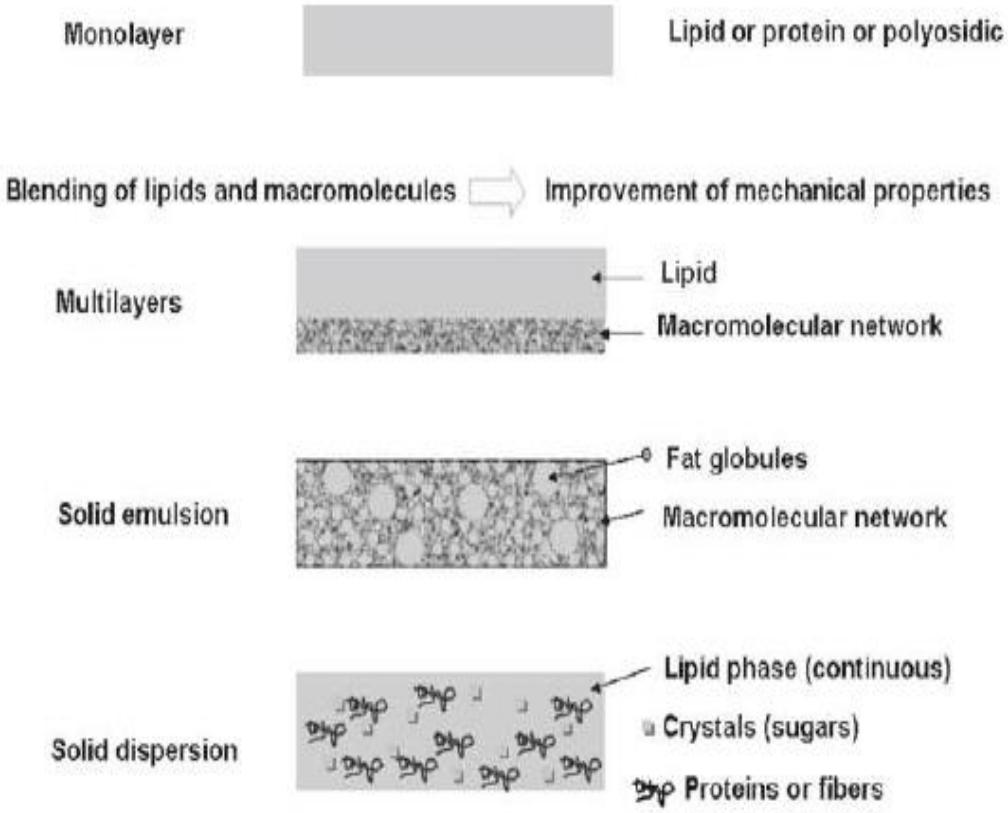


Figure 3.2. Composite film formation

(Source: Debeaufort & Voilley, 2009)

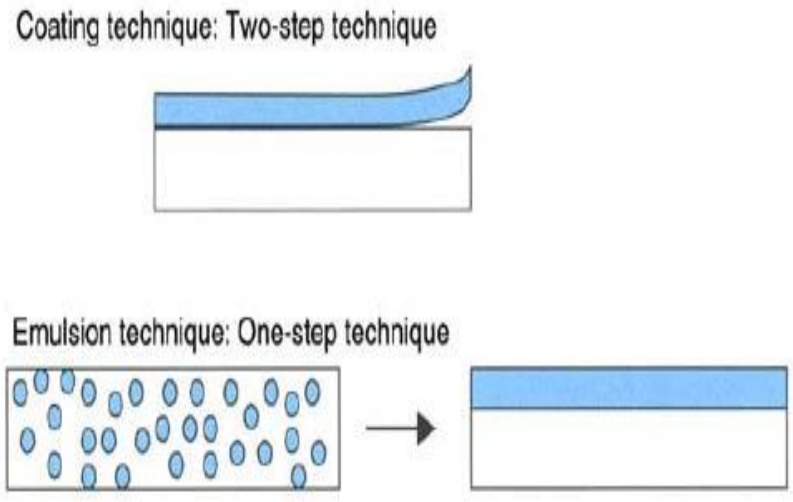


Figure 3.3. Bi-layer film formation techniques

(Source: Perez-Gago & Krochta, 2005)

Although bi-layer or multilayer films provide better mechanical and barrier properties than emulsion based films, their usage in an industrial plan does not seem very practical because their manufacturing requires more steps due to spreading or lamination and drying steps for each layer (Debeaufort et al., 1998). On the other hand, emulsion based films can be produced in only one step.

Cellulose ethers, pectinate, chitosan, starch, alginates, and carrageenan have been used in other to produce composite films and are generally combined with stearic or palmitic acids, beeswax, acetylated monoglycerides, and lecithin (Debeaufort & Voilley, 2009). Although composite film studies started with the incorporation of lipolytic substances into edible protein or polysaccharide based films, composite films including two or more biopolymers in one film matrix have recently been produced. Some composite edible film studies are summarized in Table 3.2. Other substances are also added to film and coating formulations in order to improve their functional properties such as enhancing film-forming ability of solutions, suspensions, or emulsions; promoting adherence of coatings to the support; or controlling flow and spread properties of coating solutions, suspensions, and emulsions. Besides organic compounds such as starches, chemically modified starches, dextran, microcrystalline cellulose, and insoluble cellulose derivatives; inorganic compounds such as food grade talc, titanium dioxide, silicon dioxide, single silicates, clay materials, insoluble carbonates, and phosphates may also be used to create a dispersed system (Bourlieu, Guillard, Valles-Pamies, Guilbert, & Gontard, 2009). Emulsifiers and texturizing agents can be used to stabilize this dispersed system. Additionally, acid and alkaline substances can be used to improve protein solubilization. Moreover, tanning agents such as sulfides, aldehydes, and ascorbic acid can be used to increase the networking within polymers. Some incorporated antioxidants, antimicrobial agents, or enzymes (e.g. transglutaminase) can stimulate intra- or intermolecular bond formation. Polymer solubilization can be promoted by using some enzymes, and induced polymer gelation can be achieved by using some salts (Debeaufort & Voilley, 2009).

3.4. Biopolymers and Natural Compounds Used in This Study

Different biopolymers and natural compounds were used as film-forming polymers, plasticizers, emulsifiers, and composite or blend film components.

Table 3.2. Some composite film studies from literature

Biopolymer(s)	Additive(s)	References
WPI	Mesquite gum	(Osés et al., 2009)
WPI	Spelt bran	(Mastromatteo et al., 2009b)
WPI	Soya oil and glycerol	(Shaw, Monahan, O'Riordan, & O'Sullivan, 2002)
WPI	Clay	(Sothornvit, Hong, An, & Rhim, 2010)
WPI, gelatin and sodium alginate	-	(Wang, Auty, & Kerry, 2010)
Soy protein isolate and gelatin	NaCl	(Cao, Fu, & He, 2007)
Soy protein and corn zein	-	(Cho, Lee, & Rhee, 2010)
Gelatin and resin	Stearic and palmitic acid	(Bertan, Tanada-Palmu, Siani, & Grosso, 2005)
Gelatin and gellan	NaCl	(Lee, Shim, & Lee, 2004)
Sodium caseinate	Glycerol, sorbitol, oleic acid, and beeswax	(Fabra, Talens, & Chiralt, 2008)
Caseinate and pullulan	Beeswax	(Kristo, Biliaderis, & Zampraka, 2007)
Caseinate and pullulan	Water/sorbitol	(Kristo & Biliaderis, 2006)
Hydroxypropyl Methylcellulose	Beeswax, shellac, stearic acid, mineral salts, organic acid salts, parabens, and EDTA	(Valencia-Chamorro, Palou, Del Rio, & Gago, 2008)
Konjac glucomannan, and carboxymethyl cellulose	Palm oil	(Cheng, Abd Karim, & Seow, 2008)
Chitosan	Oleic acid	(Vargas, Albors, Chiralt, & González-Martínez, 2009)
Chitosan	Tween 60, Tween 80, PEG, sorbitol, and glycerol	(Miranda, Garnica, Lara-Sagahon, & Cardenas, 2004)
Chitosan and methylcellulose	-	(Pinotti, García, Martino, & Zaritzky, 2007)
Chitosan and starch	-	(Xu, Kim, Hanna, & Nag, 2005)
Starch and alginate	Stearic acid, tocopherol	(Wu, Weller, Hamouz, Cuppett, & Schnepf, 2001)
Wheat gluten films	Spelt and wheat bran	(Mastromatteo et al., 2008)
Zein film	Carnauba wax	(Alkan et al., 2011)

3.4.1. Zein

Zein, the major storage protein of corn is a water-insoluble prolamine protein. Nearly 45-50% of the corn proteins is zein. Although zein is particularly rich in glutamic acid (21-26%), leucine (20%), proline (10%), and alanine (10%), it has low amounts of basic and acidic amino acids. High amount of nonpolar amino acid residues makes zein insoluble in water. Zein can only dissolve in organic solvents such as ethanol. Zein has a GRAS (Generally Recognized as Safe) status; therefore, it can be used safely in the food industry. Zein can be used both in coating and in film applications. Zein coatings are used as oxygen, lipid, and moisture barriers for nuts, candies, confectionery, and other foods (Buffo & Han, 2005). Zein films can be produced by solvent casting and it forms glossy, hydrophobic, grease-proof films that are resistant to microbial attack. Zein film is formed through the development of hydrophobic, hydrogen and limited disulfide bonds between zein chains.

Zein attracts a particular interest as a biopolymer since it has excellent film forming and gas barrier properties; it is one of the rare proteins soluble in various organic solvents including ethanol and it is the major co-product of the oil and rapidly growing bioethanol industries (Manley & Evans, 1943; Selling et al., 2008; Shukla & Cheryan, 2001; Wang et al., 2007; Zhang et al., 2011a). Thus, a particular interest has been focused on use of zein in active food packaging by incorporation of different natural antimicrobials. Several authors have conducted researches on the effectiveness of zein coatings and films containing and delivering antimicrobial agents such as nisin and lysozyme (Gucbilmez et al., 2007; Han, 2005a; Padgett et al., 1998; Teerakarn, Hirt, Acton, Rieck, & Dawson, 2002). Controlled release of the active compound from zein films has also been investigated by different researchers (Del Nobile et al., 2008; Gucbilmez et al., 2007; Mastromatteo et al., 2009a).

Although zein has excellent film forming and gas barrier properties, the classical brittleness, and flexibility problems of zein films is a great limitation for their use as a free standing film and more widespread application as a coating material. Many studies have been conducted to plasticize zein films and improve their flexibility and mechanical properties by addition of different ingredients such as organic acids, sugars, alcohols, fatty acids and different synthetic polymers, cross-linkers or plasticizers (Ghanbarzadeh et al., 2006; Kim, Sessa, & Lawton, 2004; Lai & Padua, 1997; Lawton,

2004; Sessa, Mohamed, & Byars, 2008; Woods, Selling, & Cooke, 2009). However, none of these studies provided an effective solution to flexibility and brittleness problems by use of natural bioactive compounds.

3.4.2. Natural Compounds

Natural compounds were used in this study: glycerol, soybean lecithin, waxes (candelilla wax, carnauba wax, and beeswax), fatty acids (oleic acid, lauric acid, and linoleic acid). The using purposes of these biomolecules are summarized in Table 3.3.

Table 3.3. Natural compounds used for the production of composite or blend films and their important characteristics

Natural compounds	Using purposes	Characteristics	Reference(s)
Glycerol	Plasticizer	Alcohol	(Kim, No, & Prinyawiwatkul, 2008)
Soybean lecithin	Emulsifier	Anionic and cationic surface active lipid	(McClements, Decker, & Weiss, 2007)
Beeswax, Carnauba wax, Candelilla wax	Increase tortuosity and hydrophobicity of film matrix	High molecular weight lipids	(Ozdemir & Floros, 2003)
Oleic acid, Lauric acid, Linoleic acid	Increase hydrophobicity of film matrix	Fatty acids	(Belitz et al., 2009)

3.4.2.1. Glycerol

Glycerol is a high boiling point point, water soluble, polar, nonvolatile, and protein miscible plasticizer. These properties make glycerol a suitable plasticizer for use with a compatible water-soluble polymer (Gounga, Xu, & Wang, 2007). Therefore it

has been incorporated into most hydrocolloid films such as zein, whey protein isolate, soybean protein isolate, and gluten (Miller & Krochta, 1997).

3.4.2.2. Lecithin

Lecithin, a surface active agent, has an important role in production of emulsions. Moreover, it can be used in food industry as aerating agent, viscosity modifier, dispersant and lubricant. Molecular structure of lecithin makes it an effective emulsifier especially in water in oil emulsions. It was also used as a plasticizer in multilayer films composed of ethylcellulose / hydroxypropylmethylcellulose / ethylcellulose (Guiga et al., 2010).

3.4.2.3. Waxes

Waxes are higher alcohols esterified with long chain fatty acids. Waxes are the oldest known edible film components, and widely used as a coating materials especially on fruits. Wax coatings forms excellent water and moisture barriers since they are extremely hydrophobic components. Wax based coatings are also used to prevent weight loss, to control aerobic respiration, and to improve the visual appeal of fruits and vegetables providing gloss (When & Shellhammer, 2005). On the other hand, the incorporation of the waxes into film structure could change the hydrophobicity, tortuasity and morphology of the films (Alkan et al., 2011; Ozdemir & Floros, 2003). Most of the waxes are natural origin such as beeswax, carnauba wax, candelilla wax. Carnauba wax, has GRAS status, is commonly added to edible coating formulation for fruits and vegetables (Weller, Gennadios, & Saraiva, 1998). But some synthetic acetylated monoglycerides shows similar characteristics with natural wax.

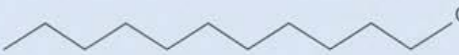
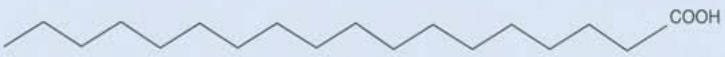
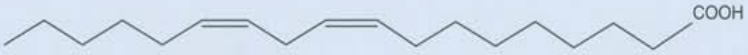
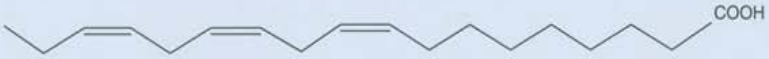
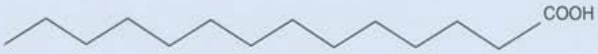
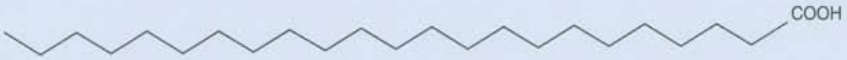
3.4.2.4. Fatty acid

Fatty acids are the major components of the lipids. They contain an aliphatic chain with carboxylic acid group. Especially fatty acid esters and salts are used emulsifiers in food systems. Fatty acids have also been used as plasticizers in edible films and coatings (Table 3.4). Some fatty acids also showed good antimicrobial

potentials against both Gram-positive and –negative microorganisms (Kabara et al., 1972).

Table 3.4. Fatty acid used as plasticizer in film structure

(Source: Sothornvit & Krochta, 2005)

Type of fatty acid	M_w	Chemical structure
Lauric acid	$C_{12}H_{24}O_2$ 200	
Stearic acid	$C_{18}H_{36}O_2$ 284	
Palmitic acid	$C_{16}H_{32}O_2$ 256	
Linoleic acid	$C_{18}H_{32}O_2$ 280	
Linolenic acid	$C_{18}H_{30}O_2$ 278	
Myristic acid	$C_{14}H_{28}O_2$ 228	
Behenic acid	$C_{22}H_{44}O_2$ 340	
Oleic acid	$C_{18}H_{34}O_2$ 282	
Arachidic acid	$C_{20}H_{40}O_2$ 312	

CHAPTER 4

CONTROLLED RELEASE

4.1. Controlled Release Theory

Traditionally, antimicrobial or antioxidant additives are mixed into initial food formulations to control microbial growth, oxidation and quality changes and to extend shelf-life of foods. In this method, too much antimicrobials or antioxidants are needed since the critical effective concentration of these agents at the food surface should be reached also in bulk of the food. The dipping of foods into antimicrobial or antioxidant solutions is also not a promising solution since diffusion of the active agents from food surface to interior parts of food cause dilution of their critical concentration at the food surface very rapidly (Han, 2005a). On the other hand, active packaging enables extension of food shelf-life or increase of food quality by using minimum amounts of active compounds. However, rapid and uncontrolled release of active compounds from the packaging materials to food is the most important problem for active packaging technologies. In the case of a rapid and uncontrolled release, active compounds diffuse through the inner part of the foods (Buonocore et al., 2005; Han & Floros, 1998); therefore, the surface of the food becomes microbiologically and oxidatively susceptible after a very short time and the effectiveness of the active packaging is greatly decreased (Appendini & Hotchkiss, 2002; Coma, 2008; LaCoste, Schaich, Zumbrunnen, & Yam, 2005) (Figure 4.1).

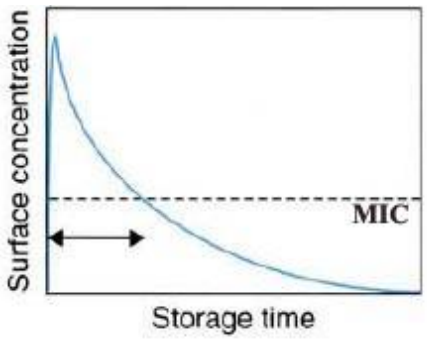


Figure 4.1. Free diffusion of antimicrobial agent from packaging material (Source: Han, 2005a)

On the contrary, a very slow release of the active component from packaging materials to food is also a problem. In that case, the concentration of the active compound on the surface is below the critical level at which the active compound is effective. Therefore, gradual diffusion of active compounds to food surface at a controlled rate (controlled release) based on shelf-life of food is critically important for active packaging technologies. The use of active packaging that employs controlled release technologies enables maintaining the effective critical concentrations of antimicrobials and antioxidants at the food surface for longer time periods (Figure 4.2). Moreover, controlled release prevents the neutralization of antimicrobials and antioxidants due to complex interactions with food components (Quintero-Salazar et al., 2005; Rose et al., 2002; Rose et al., 1999). In this way, bioactive agents can be used in active packaging more effectively since most of the natural substances are susceptible to complex interactions with food components.

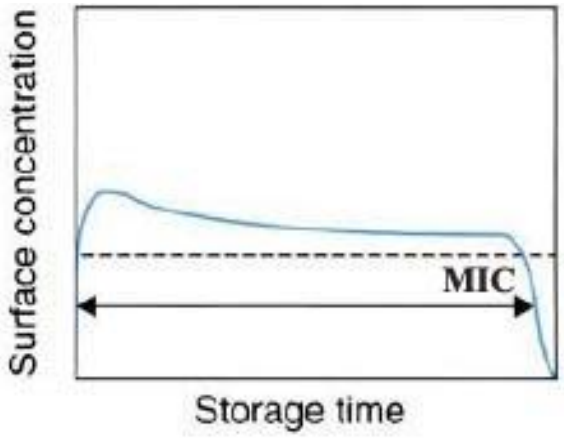


Figure 4.2. Controlled release of the antimicrobial agent from packaging material (in case of constant flux) (Source: Han, 2005a).

4.2. Controlled Release Strategies

Although controlled release technology have been widely used for pharmaceutical applications containing bioactive drugs, studies using controlled released in packaging technology became popular in the last two decades. Controlled release technology was applied to plastic packaging materials at first. One of the first studies in this area was conducted by Han and Floros (1998) and they recommended composite multilayer plastic films for controlling the diffusion rate of the potassium

sorbate. Multilayer systems were then used to control the release of lysozyme from polyvinyl alcohol matrix (Buonocore et al., 2005). A multilayer system is composed of a control layer, an active matrix layer, and a barrier layer (Figure 4.3). The outer layer is a barrier layer which prevents loss of active substances to the environment, the matrix layer contains the active substance and has a very fast diffusion, and the control layer is the key layer to control the flux of penetration (Buonocore et al., 2005). A multilayer control system was also applied by Guiga et al. (2010) to control nisin diffusion from renewable cellulosic derivatives (ethylcellulose / hydroxypropylmethylcellulose / ethylcellulose). LaCoste et al. (2005) presented a new technique for the controlled release of active compounds from packaging materials called "smart blending". In this technique, two or more polymers and sometimes fillers can be blended by a computer controlled system to alter the morphology of the films which could directly affect the release of the active agent. Additionally, release rates of the antioxidant and tocopherols were adjusted in LDPE/PP films by selection of the blend morphology with the smart blender (Jin, Zumbrunnen, Balasubramanian, & Yam, 2009).

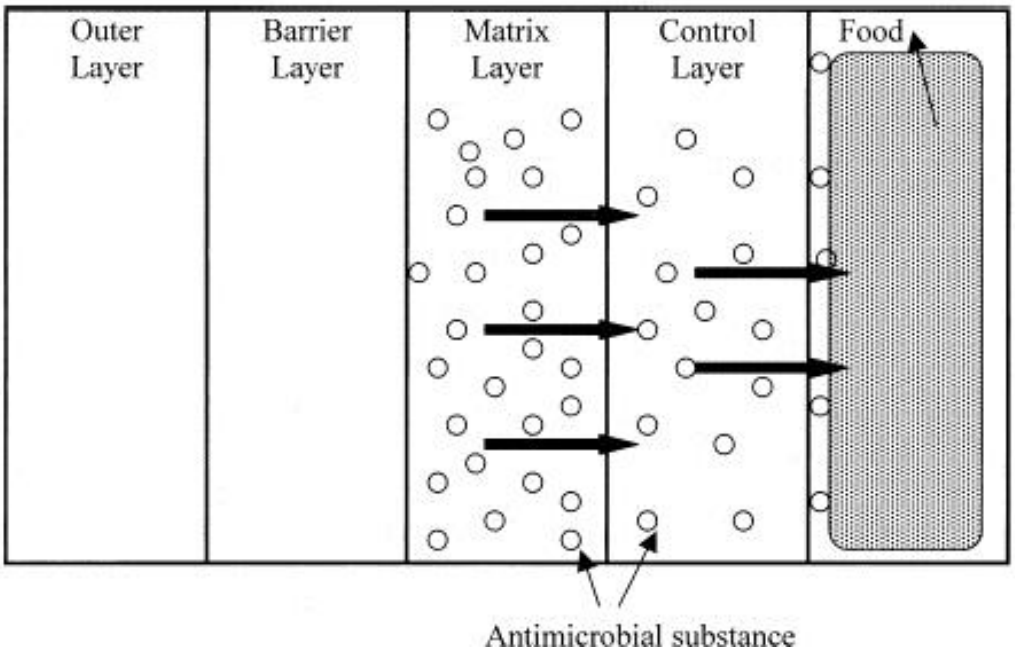


Figure 4.3. A multilayer packaging system
(Source: Ozdemir & Floros, 2004)

In literature, there are limited numbers of studies related to the controlled release of different active substances from edible films (Del Nobile et al., 2008; Gucbilmez et al., 2007; Ouattara et al., 2000; Ozdemir & Floros, 2003; Teerakarn et al., 2002; Zactiti

& Kieckbusch, 2006). Also, in most of the controlled release studies, the effect of only one strategy was investigated to control the release rates of active compounds. The strategies used in controlled release studies mentioned above are clearly discussed in the following paragraphs.

Increasing the hydrophobicity of the film matrices, in other words, changing the hydrophobic/hydrophilic balance of the films is one of the most important strategies for controlling the release rates of active compounds. By changing the hydrophobic/hydrophilic balance, the interaction levels of the film matrix with water, lipid, and active compounds changes; therefore, controlled release can be achieved. In order to change the hydrophobic/hydrophilic balance of the films, biopolymers or biomolecules that are more hydrophobic or more hydrophilic than film matrices can be introduced to the film structure with different ratios. The addition of hydrophobic components to the hydrophilic film forming solution results in a greater permeability decrease. Redl et al. (1996) used this strategy to control the release rates of active compounds for the first time. They used beeswax and acetylated monoglycerides as lipid compounds to control the diffusion of sorbic acid from edible wheat gluten films. The researchers claimed that the lipid components increased tortuosity in film matrix, thus the diffusion pathway for the hydrophilic active compound increased. Additionally, lauric acid and essential oils were successfully incorporated into film matrix to control the diffusion of acetic acid and propionic acid from chitosan films (Ouattara et al., 2000). On the other hand, Ozdemir and Floros (2003) used beeswax to control the diffusion of potassium sorbate from whey protein films. In addition to the tortuosity affects of beeswax molecules, diffusion rate was also affected by strong interactions between protein and beeswax, and potassium sorbate and beeswax. Therefore, physicochemical interactions between a macromolecular system and a diffusing molecule affect its diffusion, thus hydrophobic or hydrophilic nature of the active compound is also effective in this process. For example, it was observed that nisin, a hydrophobic nature compound, diffused much slower through hydrophobic zein films when compared to hydrophilic gluten protein films (Teerakarn et al., 2002). Researchers explained these results with the high affinity of hydrophobic nisin to hydrophobic film matrix due to hydrophobic interactions. As a result, hydrophobic interactions between film matrix and active compounds can slow down the release rate of active compounds. However, it should still be considered that water diffusion in hydrophobic films is slow. Especially in hydrophilic films, water diffuses very rapidly into film structures and this

is followed by rapid diffusion of active compounds out of the film matrices. On the other hand, water diffuses more slowly in hydrophobic films; therefore, diffusion of active compounds in hydrophobic films occurs more slowly. Alternatively, natural fibers such as spelt or wheat bran can be advantageously used to alter the mass transport properties of edible films (Mastromatteo et al., 2009a).

All of the film examples mentioned above have heterogeneous structures due to blending of some additives with continuous polymeric matrix. Thus, these films can be categorized as composite films. Composite films can be used to control the diffusion rate of active agents by changing the film compositions. This technique can also be used to modify the physical and mechanical properties of the edible films. For example, WVP of the protein based edible films decreased with incorporation of hydrophobic polysaccharides and lipids into film matrix (Gennadios, Hanna, & Kurth, 1997). During blending of these biopolymers or biomolecules with different hydrophobic or hydrophilic nature, to increase the interaction, emulsification processes need to be applied and surface active compounds need to be used. For example, release of the hydrophilic lysozyme was controlled by increasing the interaction between hydrophilic lysozyme and hydrophobic zein films by surface active compounds (Gucbilmez et al., 2007). In that study, surface active chickpea proteins were used to increase the interaction between lysozyme and zein film matrix. Using this approach, controlled release of lysozyme was achieved. In spite of that, the addition of bovine serum albumin (BSA) to film structure increased the release rate of lysozyme because of the very hydrophilic nature of BSA. Also, lecithin which is a surface active compound was used in the formation of multilayer composite films (Guiga et al., 2010).

Cross-linking is another strategy used for the controlled release of active agents. Buonocore et al. (2003b) investigated the affects of cross-linking degree of plastic films on diffusion of antimicrobial agents. Glyoxal was used as a cross-linker agent. Researchers reported that the release kinetic of an antimicrobial agent from a polymeric matrix can be controlled by only adjusting the degree of crosslink of the polymeric matrix since entrapment of the active compound occurs as the degree of crosslink of the polymeric matrix increases. Cross-linking degree can be modifying with changing the film composition or increasing the cross-linker concentrations. Cross-linking strategy can be successfully applied to one of the polymers in composite film structure for controlling release of active agents. For example, cross-linking of alginate or low methoxyl pectin in composite film structure with divalent cations (Ca^{+2}) can be used to

control the diffusion of active agents. Dong et al. (2006) applied cross-linking strategy to alginate/gelatin composite films for controlling the release of drug. They claimed that a higher degree of cross-linking formed in the matrix causing a delay in the kinetic of drug release. However, the applications of cross-linking strategy for achieving controlled release in edible films were very limited (Zactiti & Kieckbusch, 2006, 2009). Moreover, the release of lysozyme was blocked with calcium chloride cross-linking in sodium caseinate films (de Souza, Fernandez, Lopez-Carballo, Gavara, & Hernandez-Munoz, 2010). Polyphenols also can be used in especially protein based films for cross-linking. Haroun and El Toumy (2010) reported that polyphenols improved the mechanical properties of gelatin-based films by cross-linking of gelatin proteins.

Formation of bilayer or multilayer coatings can also be used for controlling the release of active compounds in composite films. For this purpose, positively or negatively charged polymers can be incorporated into the composite film structure. After formation of the composite film, a thin layer is formed by dipping the film into an oppositely charged biopolymer solution by electrostatic forces. This process can be used to form multilayer coatings of the films by repeating the dipping step using oppositely charged biopolymer solutions from the previous step (Figure 4.4). This technique can control the release of the active agent by forming barrier and control layers. On the other hand, hydrophobic interactions and/or hydrogen bonding were used for the formation of layers on the composite films (Weiss, Takhistov, & McClements, 2006). The researchers emphasized that using this technique for coating food surfaces and the incorporation of antioxidant and antimicrobial agents to layers could be a promising technology for maintaining or increasing food quality during the storage period. It was also reported that this technique could also be used for encapsulation and controlled release of lipophilic bioactive agents (McClements et al., 2007). Although the formation of multiple layers described above was used for controlling the release of micro- and nano- compounds in the drug industry, the studies related to using this techniques is at the beginning level in food packaging technology.

Alternative controlled release strategies were also proposed by some other researchers. The most promising one is the enzymatic erosion in PVA/starch based polymeric structures (Coluccio et al., 2006). It was shown that α-amylase hydrolyzed starch based polymeric matrix and increased the porosity of the matrix with time. Therefore, the amount of released drug concentration increased with the erosion process. However, this technique has not been applied to food packaging industry yet.

Changing the film composition is also another successful controlled release strategy. Gemili et al. (2009) changed the porosity of asymmetric cellulose acetate (CA) films by changing the film composition (increasing CA content), and achieved controlled release of lysozyme. The researchers also applied the same strategy for controlled release of low molecular weight L-ascorbic acid and L-tyrosine for the production of antioxidant packaging materials (Gemili et al., 2010).

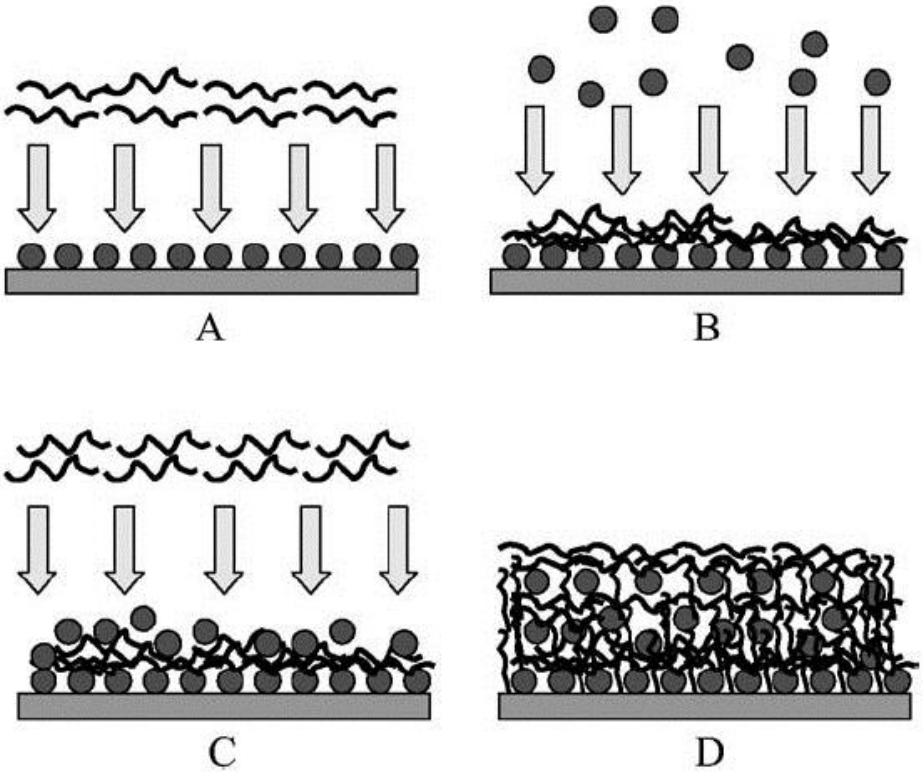


Figure 4.4. Multilayer film formation on the film surface
(Source: Rudra, Dave, & Haynie, 2006)

CHAPTER 5

MATERIALS AND METHODS

5.1. Materials

Maize zein (batch number: 058K0093), (+)-catechin hydrate (≥98%), gallic acid, commercial lysozyme (protein ≥90%, ≥40,000 units/mg protein), *Micrococcus lysodeikticus*, carnauba wax (No.1, refined), beeswax, candelilla wax, oleic acid (90 %), lauric acid, linoleic acid were from Sigma Chem. Co. (St. Louise, Mo, USA). Soybean L-α-lecithin and glycerol were from Merck (Darmsdadt, Germany). All other chemicals were reagent grade. Fresh hen eggs used in production of lysozyme were obtained from a supermarket in Izmir (Turkey). *Campylobacter jejuni* isolated from broiler chicken carcass was kindly provided by Prof. Dr. Halil İbrahim Atabay in Department of Food Engineering at Izmir Institute of Technology, Turkey.

5.2. Preparations of Films

Zein films were produced as described in Padgett et al. (1998). Briefly, 1.4 g zein was dissolved with 8.1 mL of ethanol (96 %) by mixing slowly with a magnetic stirrer for 25 min. Glycerol (0.4 mL) was then added to the medium as a plasticizer. The temperature of the mixture was then increased until it started to boil. The mixing was ceased and the solution was cooled to the room temperature after it had been boiled for 5 min. After that, the lysozyme (23.4 mg or 11.7 mg/g film forming solution) used as antimicrobial agent and/or phenolic compounds (50-100 mg/g film forming solution) used as an additional plasticizer for the zein and lecithin (5-10% (w/w) of zein) used as emulsifier (for wax and oleic acid containing films only) were added into film forming solutions and the mixtures were homogenized (Heidolph, Germany, rotor Φ=6.6 mm tip) at 10,000 rpm for 4 min. Then, 4.3 g portions of the homogenized film forming solutions were poured into the glass templates (W×L×H: 8.5×8.5×0.4 cm) and dried for 19 ±2 h at 25 °C in an incubator unless otherwise was stated (see section 5.9). This procedure was also used to obtain zein–wax composite films by adding waxes

(carnauba wax, candelilla wax and beeswax) and zein–fatty acid blend films by adding fatty acids (oleic acid, lauric acid, linoleic acid) into film forming solutions at 5-20% (w/w) of zein. The waxes were added just before initiation of heating to melt them during boiling and ease their homogenization, while fatty acids was added after the boiling step following cooling to room temperature.

5.3. Production of Partially Purified Lysozyme

Partially purified lysozyme was produced according to the method previously applied by Mecitoglu et al. (2006). For this purpose, carefully separated egg whites were diluted 3-fold with 0.05 M NaCl solution. To precipitate the egg white proteins other than the lysozyme, the pH of this mixture was set to 4.0 by adding several drops of 1 N acetic acid, and the solution was diluted with equal volume of 60 % (v/v) ethanol. After 6 h incubation at room temperature in the presence of 30 % ethanol, the mixture was centrifuged at 15.000 x *g* for 15 min at 4 °C and the precipitate was discarded. The supernatant containing lysozyme was first dialyzed for 21 h at 4 °C by three changes of 2000 mL distilled water and then lyophilized by using a freeze drier (Labconco, FreeZone, 6 liter, Kansas City, MO, USA). The lyophilized enzyme was stored at –18 °C until it was used in film making.

5.4. Release Profiles of Zein Films

5.4.1. Lysozyme Release Profiles of Films

The release tests of zein and zein–wax composite films were conducted in distilled water at 4 °C by applying shaking during the incubation period. Briefly, 4x4 cm pieces of films were placed into glass Petri dishes containing 50 mL of deionized water. The dishes were kept at 4 °C in an incubator and shaken with an orbital shaker working at 80 rpm. The release tests were conducted until equilibrium was reached for release of LYZ or an insignificant increase was observed in lysozyme activity. The lysozyme activity was monitored by taking 0.1 mL (x 3) aliquots from the release test medium at different time intervals.

The release tests of zein and zein–fatty acid blend films were also conducted in distilled water at 4 °C by applying shaking during the incubation period. But to provide the films especially the blends in fully sunk position during the release experiments, film samples were squeezed between two glass templates which contained a square hole (4 cm x 4 cm) at the centre. The corners of the glass templates were covered with paraffin film to prevent contact of water with the part of the films squeezed between glasses. The apparatus were then fixed on small glass stands, then placed into a 500 mL glass beaker containing 150 mL distilled water at 4 °C and kept in an orbital shaker working at 80 rpm and 4 °C. The release tests were conducted until equilibrium was reached for release of lysozyme or an insignificant increase was observed in lysozyme release. The lysozyme activity was monitored by taking 0.25 mL (x3) aliquots from the release test solution at different time intervals.

The enzyme activities in collected aliquots were determined spectrophotometrically at 660 nm by using Shimadzu spectrophotometer (Model 2450, Japan) equipped with a constant cell holder at 30°C. The reaction mixtures were formed by mixing 0.1 mL (0.25 mL) of release test medium with 2.4 mL (2.25 mL) of 0.26 mg/*mL Micrococcus lysodeicticus* solution prepared in 0.05M, pH 7.0 Na-phosphate buffer. The enzyme activities were calculated from the slopes of initial linear portions of absorbance vs. time curves and expressed as unit (U) which was defined as 0.001 changes in absorbance in 1 min. All calculations were corrected by considering the activity removed by collected aliquots during sampling. The total lysozyme activity released from each film corresponded to maximum units released per cm^2 of the films (U/cm^2) at the equilibrium. All activity measurements were conducted for three times. The release curves were formed by plotting calculated released activities (U/cm^2) *vs*. time (h). The initial release rates of lysozyme were determined from the slope of the initial linear portion of release curve (see Apendix A). The release rates were expressed as $U/cm^2/h$.

5.4.2. Phenolics Release Profiles of Films

To determine the phenolic release profiles of films, release tests were conducted in water as described at section 5.4.1. The soluble phenolic content was monitored in release medium taking 0.1 mL (x 3) aliquots from the release test medium at different

time intervals until the phenolic release reached an equilibrium. The phenolic acid and flavonoid content was determined spectrophotometrically according to the classical Folin-Chiocalteu method of Singleton and Rossi (1965) at 765 nm and the aluminium chloride colorimetric method given by Meyers, Watkins, Pritts, and Liu (2003) at 510 nm. The total soluble phenolic and flavonoid contents released from the films were expressed as mg gallic acid or mg catechin per cm^2 of the films (mg/cm^2) using the calibration curve prepared by gallic acid and catechin (see Apendices B-D). All concentration measurements were conducted for three times. The release curves were formed by plotting calculated released phenolic contents (mg/cm^2) *vs.* time (h).

5.5. Scanning Electron Microscopy (SEM) of Films

The cross-sectional morphology of selected films was determined by using SEM (Philips XL 30S FEG, FEI Company, Netherlands) under high vacuum mode at an operating voltage varying between 2 and 6 kV. Films were prepared for SEM by crashing, following freezing in liquid nitrogen. Then samples were gold coated with a sputter coater (Emitech K550X, Quorum Technologies Inc., UK) under 15mA for 60 sec. The thickness of the films was measured from SEM cross-sectional views of films by using Scandium software (Olympus Soft Imaging Solutions GmbH, Münster, Germany).

5.6. Fourier Transform Infrared (FTIR) Analyses of Films

For the FTIR analysis, the zein films were placed on the horizontal attenuated total reflectance sampling accessory (ZnSe crystal plate) of a FTIR spectrometer equipped with DTGS detector (Spectrum 100 Instrument, Perkin-Elmer Inc., Wellesley, MA). FTIR spectra of the samples were recorded between 4000 and 650 cm-1. Interferograms were averaged for 32 scans at 4 cm-1 resolution. The background spectrum was automatically subtracted from the spectra of the samples. For each film, the average spectrum of the nine different scans (three sets of experiments with 3 replicates). Spectrum software (Perkin-Elmer) was used for all data analysis. The spectra were interactively baselined from two arbitrarily selected points. Finally, the spectra were normalized in specific regions for comparison of the films.

5.7. Soluble Phenolic Content and Antioxidant Capacity of Films

The soluble catechin concentration in the release medium of zein–wax composite and zein–fatty acid blend films at the end of the release experiment described in section 5.4.1 was determined spectrophotometrically at 510 nm according to the colorimetric method of Meyers et al. (2003)developed for quantification of flavonoids. The total soluble catechin concentrations released from the films were expressed as catechin per cm^2 of the films (mg/cm^2) using the calibration curve prepared by catechin. All concentration measurements were conducted for three times.

The antioxidant capacity of the films was based on calculating the trolox equivalent antioxidant capacity (TEAC) of their soluble catechin content. The TEAC of catechin was determined by using spectrophotometric ABTS radical cation decolorization assay conducted at 734 nm (Re et al., 1999). The results were expressed as Trolox equivalents released per cm^2 of films ($\mu mol\ Trolox/cm^2$) (for Trolox standard curves see Apendices E-F).

Bound antioxidant capacity of films obtained from the release tests was determined by modifying the method described by Gucbilmez et al. (2007). Briefly, the films were washed two times with 100 ml of deionized water (2×50 ml) for 60 min by shaking to remove residual soluble phenolic compounds remained in films following release tests. Three pieces were cut from the films and placed into Petri dishes containing 50 ml of ABTS free radical solution. The reaction was conducted at 30 °C by shaking at 80 rpm and the percent inhibition of ABTS solution was monitored for 60 min at 734 nm. The antioxidant capacity was determined for 15 min inhibition period as $\mu mol\ trolox/cm^2$. Average of three measurements was used in calculations.

5.8. Antimicrobial Potential of Films

The antimicrobial potentials of the zein films plasticized with phenolic compounds were tested against *L. monocytogenes* (ATCC 7644) and *C. jejuni*. while the antimicrobial potential of the composite and blend films were tested against *Listeria innocua* (NRRL B-33314; supplied from USDA, Microbial Genomics and Bioprocessing Research Unit (Peoria, IL, USA) as a test microorganisms. For antimicrobial tests, 18 discs (1.3 cm in diameter) from each film were prepared by a

cork borer. Total of 15 discs were selected randomly and 3 discs were placed into each Petri dish containing nutrient agar, which had been previously inoculated with 0.1 mL cell culture. The inoculums of microorganisms were prepared in nutrient broth using an overnight culture at 37 ºC of *L. monocytogenes* at aerobic conditions, and 48 h culture of *C. jejuni* at microaerophilic conditions (microaerophilic conditions were achieved by jars of Anoxomat, Mart Microbiology, Holland). The bacterial counts of inoculums for *L. monocytogenes* and *C. jejuni* used in tests were 3.0×10^9 and 6×10^7 CFU/ml, respectively. The Petri dishes inoculated with *L. monocytogenes* and *C. jejuni* and containing film discs were incubated for 48 h at 37 °C. On the other hand the inocula of *L. innocua* were prepared in peptone water (0.1 %) by using 48 h culture growth in nutrient agar, and the cell concentration was set to 1.0 McFarland unit (corresponded to 13 x 10^7 CFU / mL). The Petri dishes were firstly incubated at +4 °C for 4 h to prevent rapid diffusion of the lysozyme and then secondly they were incubated at 37 °C for 36 h. The diameter of the zones formed was measured by using a calliper. The results were expressed as average zone areas (mm²).

5.9. Mechanical Properties of Films

Tensile strength at break, elongation at break, and elastic modulus were determined using a Texture Analyser TA-XT2 (Stable Microsystems, Godalming, UK) according to ASTM Standard Method D 882-02 (ASTM, 2002). For conditioning of the films used in mechanical testing, the standard drying period of 19 h was extended to 24 for zein films containing phenolic compounds and to 48 h for zein–wax composite and zein–fatty acid blend films at 25 °C. Moreover, for these films only, the drying was conducted at 50 % RH using a controlled test cabinet (TK 120, Nüve, Turkey). Films were cut into 5 mm wide and 80 mm length strips. The initial grip distance was 50 mm and crosshead speed was 50 mm/min. At least seven replicates of each film were tested.

Tensile strength at break is the stess at the point at which the film looses its structural integrity and breaks down. The elongation at break is the maximum % change in length of film before breaking. Elastic modulus (Young's modulus) is calculated from the slope of initial linear portion of stress-strain curve. Modulus is the ratio of stress per strain before elastic limit and gives information about the stiffness of the material.

5.10. Statistical Analysis

Analysis of variance (ANOVA) was applied using Minitab 15 (Minitab Inc., State College, PA, USA) to determine the effects of film compositions on antimicrobial potentials and mechanical properties of the films. Multiple comparisons of means were performed using Fisher's least significant difference (LSD) method with a significance coefficient of 5% ($P<0.05$).

CHAPTER 6

RESULTS AND DISCUSSIONS

6.1. Development of Zein-Wax Composite and Zein-Fatty Acid Blend Films for Controlled Release of Phenolic Compounds

6.1.1. Incorporating Phenolic Compounds into Zein Film

In order to select suitable phenolic compounds for using within zein films different phenolic acids (gallic acid, hydroxyl benzoic acid, and ferulic acid) and flavanoids (flavones, catechin, and quercetin) were incorporated into zein films (Figure 6.1). Phenolic compounds were determined according to the release profiles, and phenolics induced mechanical and morphology changes of the films.

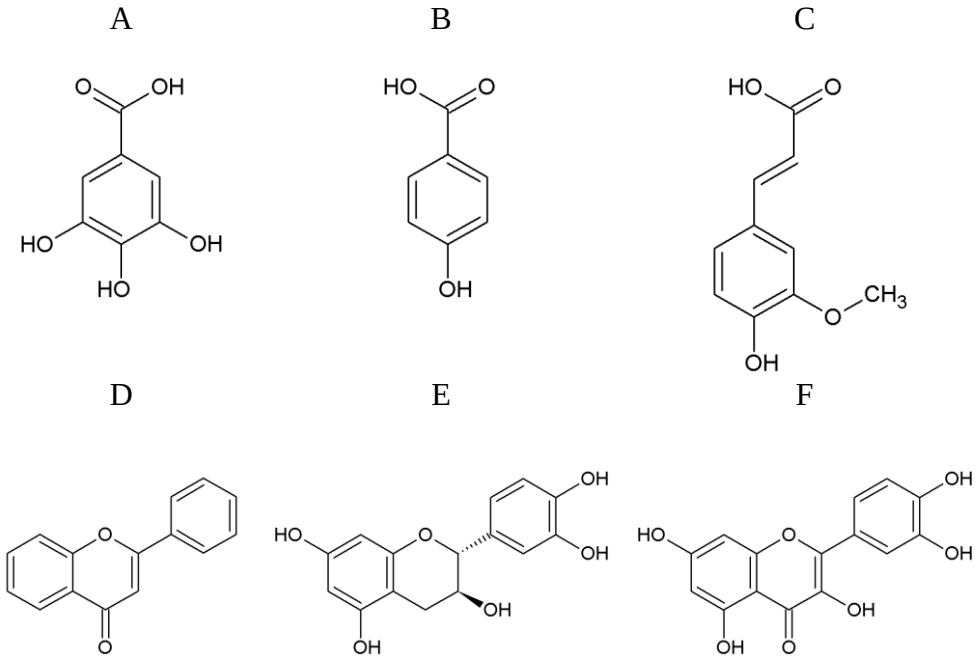


Figure 6.1. Chemical structures of different phenolic compounds used within zein films: gallic acid (A), hydroxyl benzoic acid (B), ferulic acid (C), flavone (D), (+)-catechin (E), and quercetin (F)

6.1.1.1. Effect of Phenolic Compounds on Mechanical Properties of the Films

The effects of different flavonoids, catechin, flavone and quercetin, and fenolic acids, gallic acid, ferulic acid and hydroxyl benzoic acid, on mechanical properties of zein films were tested at 3 mg/cm^2 concentration (Table 6.1). The thicknesses of films containing different phenolic compounds did not vary considerably with the exception of flavone containing films which were slightly thicker than the others. On the other hand, the addition of gallic acid, hydroxyl benzoic acid, ferulic acid, or catechin into zein films significantly increased the elongation of films (P<0.05). The elongation, tensile strength and Young's modulus values of ferulic acid and hydroxyl benzoic acid containing films were not statistically significantly different from those of gallic acid containing films (P<0.05). Moreover, similar to gallic acid containing films, ferulic acid and hydroxyl benzoic acid containing films showed lower tensile strength and Young's modulus than catechin containing films. The elongation of hydroxyl benzoic acid containing films was also significantly higher than that of catechin containing films, but ferulic acid containing films showed similar elongation with catechin containing films. On the other hand, during hydration tests conducted in distilled water, the films containing ferulic acid and hydroxyl benzoic acid showed rapid swelling and lost their structural integrity (their surface become quite rough), while catechin and gallic acid containing films maintained their structural integrity and smooth surface even after hydration. It is clear that the molecular properties of phenolic compounds affected the film morphology and strength of film matrix considerably. It seems that the extensive H bonding of catechin or gallic acid molecules which contained higher number of OH groups than ferulic acid and hydroxyl benzoic acid created a stronger network within film matrix and this prevented loss of film structural integrity following hydration. The films containing flavonoids, flavone and quercetin, on the other hand, showed completely different mechanical properties than using catechin and phenolic acids. The flavone was selected particularly to see the effect of a phenolic compound lacking OH groups, while quercetin was selected due to its same number of OH groups with catechin. However, these flavonoids did not cause significant changes in elongation of films (P>0.05). Moreover, flavone and quercetin gave most identical tensile strength and Young’s modulus values with the controls. The lack of any considerable effect for

flavone was expected, but the ineffectiveness of quercetin should be related with its insolubility within the films. Both flavone and quercetin showed limited solubility in ethanol and formed tiny crystals and granules within the films after drying.

Table 6.1. Mechanical properties of zein films containing different phenolic compounds

Phenolic compounds[a]	**Tensile strength at break (MPa)**	**Elongation at break (%)**	**Young's modulus (MPa)**	**Film thickness (μm)**
Control	10.19±0.83a[b]	3.34± 0.66c	528±39a	131.8±2.0
CAT[c]	1.16±0.16d	142.24±25.52b	45±14d	132.1±3.5
GA	0.48±0.12e	182.41±23.61a	12±4e	116.9±0.8
HBA	0.45±0.02e	188.64±25.10a	12±1e	120.1±0.7
FA	0.70±0.05e	135.05±50.21ab	24±3de	123.9±0.5
FLA	6.70±0.31b	2.21±0.26c	398±15c	143.4±1.4
QU	5.28±0.29c	1.23±0.18c	474±15b	125.9±3.5

[a] phenolic compound concentration of films: 3 mg/cm^2

[b] different letters in each column show significant difference at $P<0.05$

[c] phenolic compounds used in film composition: CAT: catechin, GA: gallic acid, HBA: hydroxyl benzoic acid, FA: ferulic acid, FLA: flavone, QU: quercetin

For further analysis the effects of phenolic compounds on mechanical properties of zein films, catechin (flavonoid ($C_6C_3C_6$)) and gallic acid (phenolic acid (C_6C_1)) were selected as main model phenolic compounds at concentration range between 0.75 and 3.0 mg per cm^2 of films (Figure 6.1).The average thicknesses of control films and films containing catechin or gallic acid at different concentrations (0.75, 1.5, 2.25 or 3 mg/cm^2) were 131.8±2.0, 127.1±5.3 and 129.0±12.2 μm, respectively. Thus, the addition of catechin or gallic acid did not affect the film thickness considerably. The glycerol used in all zein films did not show a considerable plasticizing effect, but it reduced the brittleness of zein films and enable conducting mechanical tests for highly brittle control films. In contrast, addition of catechin or gallic acid into zein films increased the flexibility of films and this increased the elongation of films in a

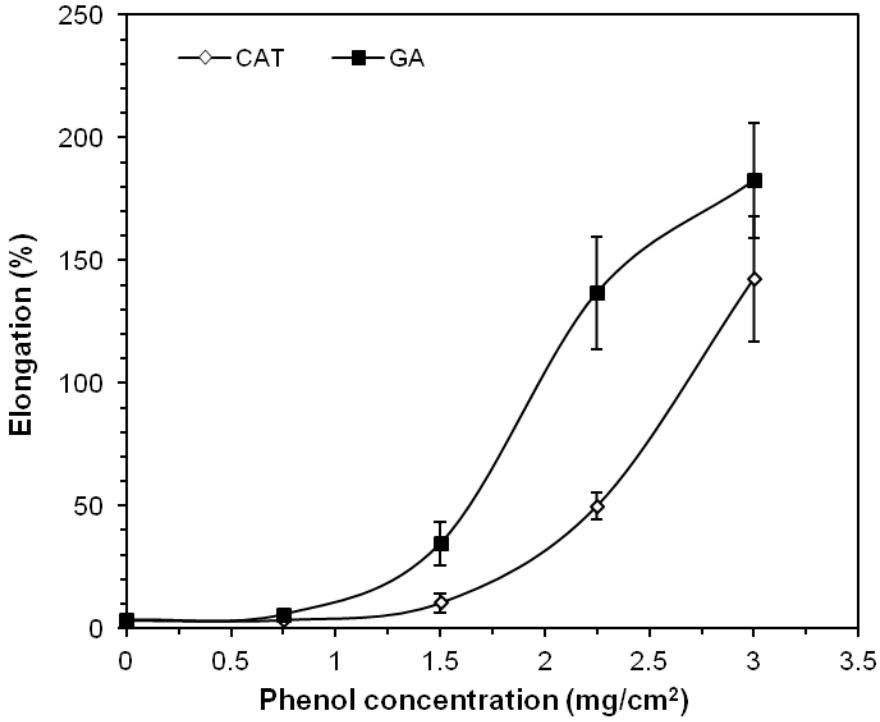


Figure 6.2. Effects of catechin and gallic acid concentrations on elongation of zein films (CAT: catechin; GA: gallic acid)

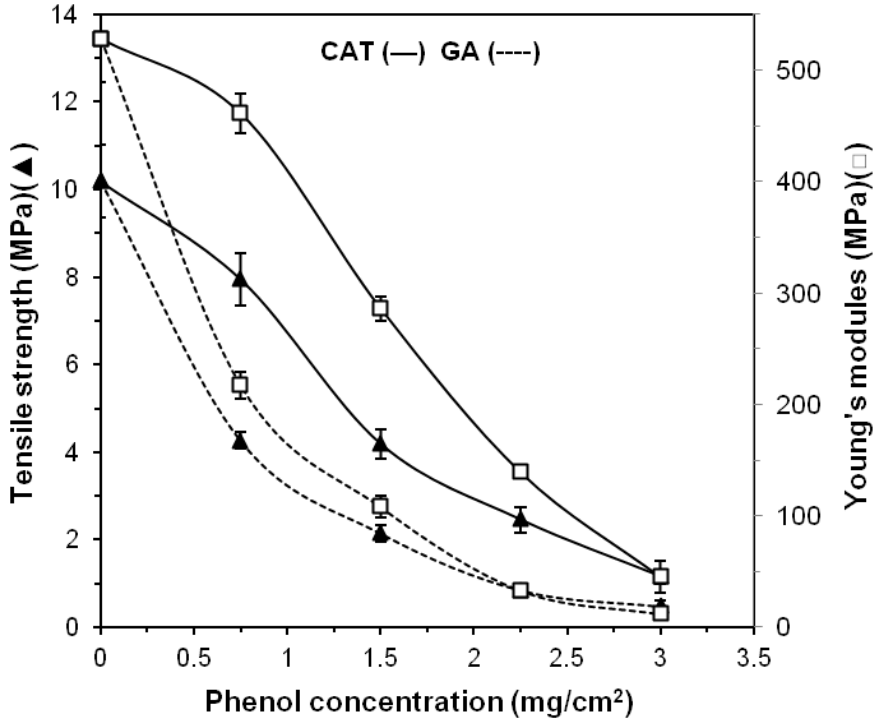


Figure 6.3. Effects of catechin and gallic acid concentrations on tensile strength and Young's modulus of zein films (CAT: catechin; GA: gallic acid)

concentration dependent manner (Figure 6.2; see Apendix G). The gallic acid is a particularly effective plasticizer for zein since it caused considerably higher film elongation than catechin at low phenolic concentrations. The effect of catechin and gallic acid on tensile strength and Young's modulus of zein films was also given in Figure 6.3. The tensile strength and Young's modulus of zein films were reduced as phenolic concentration in the films was increased. However, catechin containing films showed higher tensile strength and Young's modulus values than gallic acid containing films at all phenolic concentrations.

To understand the possible mechanism of plasticization with phenolics, the zein film structure should be discussed in more details. It has been recently shown that the zein films consist of a meshwork which is composed of doughnut structures formed by asymmetric rods joined to each other (Guo, Liu, An, Li, & Hu, 2005). It is the hydrophobic interactions that keep the zein rots together and maintain film integrity (Guo et al., 2005), but these interactions are also responsible for the brittleness and lack of flexibility in zein films. It was assumed that the formation of hydrogen bonds between the hydroxyl groups of phenolics and the carbonyl group of zein protein formed a week but an elastic film network. Moreover, the increased number of phenolic hydroxyl groups provided with free phenolics increased the hydrophilicity of the films. Thus, the hydrophobic interactions are weakened and elastic networks with more mobile zein molecules are formed. And tensile strength and elastic modulus of films reduced while the elongation value increased, a behavior expected for a plasticizied materials. The reduction in elastic moduls while the elongation of the films increased was also reported by Ghanbarzadeh et al. (2006) when zein films plasticized with glycerol, mannitol, and sorbitol. Moreover, according to Sothornvit and Krochta (2005), similar changes in tensile strength, elastic modulus, and elongation values was also observed for polysaccharide based films when different plasticizer type and concentration used in film composition.

In the literature, reports about plasticizing effect of pure phenolic acids and flavonoids on protein based films scarce. In fact, it is only Ou, Wang, Tang, Huang, and Jackson (2005) who reported a limited increase in elongation of soy protein films by addition of ferulic acid. In contrast, Emmambux, Stading, and Taylor (2004) incorporated condensed tannins like tannic acid into films from sorghumkafirin, a zein like prolamin, reported an antiplasticizing effect of this phenolic compound after determining a reduced elongation, but increased tensile strength and Young's modulus

of the films. The antiplasticizing effect could be due to molecular properties of tannic acid, since this phenolic compound contains too many hydroxyl groups and it binds proteins very tightly to reduce their mobility within the film matrix. Emmambux et al. (2004) applied extensive heating (at 55 to 75 °C range) to zein–tannic acid mixtures during both film preparation and drying. Thus, it is also possible that the antiplasticizing effect was due to oxidation of tannic acid which could cause covalent crosslinking of proteins (Thalmann & Lötzbeyer, 2002). Another study which employed a pure phenolic compound during edible film production came from Ku et al. (2008b), but these workers incorporated the phenolic compound catechin into a carbohydrate film from agar extracted from Gelidium corneum and did not determine any plasticizing effect of catechin. On the other hand, there are many different studies in the literature related to plasticization of zein films with different compounds. For example, Lawton (2004) obtained cast zein films and tested different plasticizers including triethylene glycol, dibuthyl tartrate, levulinic acid, polyethylene glycerol 300, glycerol and oleic acid. In this study, any plasticizing effect of glycerol and oleic acid was not determine, but other compounds showed considerable plasticizing effect when films were stored 1 week at relative humidity values exceeding 60%. However, Lawton (2004) attributed the obtained plasticizing effect to water molecules absorbed by the films during storage, but not to the applied plasticizers. On the other hand, although oleic acid was not a good plasticizer for cast zein films, it showed some plasticizing effect (with 12% elongation) when films were obtained from stretched resins formed by zein–oleic acid emulsions (Lai & Padua, 1997). Although Xu, Chai, and Zhang (2012) recently reported the synergistic effect of glycerol and oleic acid on zein film plasticization but cast zein films did not showed elongation higher than 3%. Kim et al. (2004) reduced the brittleness of zein films and increased their tensile strengths by using some chemical crosslinkers such as 1-[3-dimethylaminopropyl]-3-ethylcarbodiimide hydrochloride and N-hydroxysuccinimide, but this process did not increase the film elongation considerably (increased only from 2.23 to 3.6%). Selling et al. (2008) improved the tensile strength, elongation and Young's modulus of zein films by glutaraldehyde crosslinking, but the films lost their edible nature due to toxicity of this compound. On the other hand, Shi, Huang, Yu, Lee, and Huang (2010) used lauryl chloride for chemical modification of zein, and eliminate the brittleness problem by increasing the elongation of cast zein film to 300%.

6.1.1.2. FTIR Analysis of the Films

FTIR spectroscopy was employed to determine the possible hydrogen bond formation in zein films by the effect of plasticizer catechin and gallic acid (for whole spectrum of the developed films see Apendix H). It is accepted that the specific regions of the FTIR spectrum representing the characteristic protein bands consisting of amide A (3600–3100 cm^{-1}) originated mainly from N–H stretching, while amide I (1750–1600 cm−1) originated mainly from C=O stretching (Barth, 2007). Figure 6.4 and 6.5 shows that the bandwidth of the amide A, and I regions broadened as the catechin or gallic acid concentration in the film structure increased. In the literature, there are increasing number of reports that the broadening of amide A, and I bands at the indicated regions was due to hydrogen bond formation between protein and phenolic compounds (Alkan et al., 2011; He et al., 2011; Mohammed-Ziegler & Billes, 2002; Zou, Li, Percival, Bonard, & Gu, 2012). Zou et al. (2012) who investigated the interactions of zein with procyanidins attributed the band broadening in amide I regions to hydrogen bond formation between zein and the phenolic compounds. He et al. (2011) who worked with collagen films containing procyanidins also reported that band broadening at amide A, and amide I of film spectra suggested H bond formation between the collagen and the phenolic compound. On the other hand, band shifting at amide A towards lower wave-numbers could also indicate the hydrogen bond formation (Hasni et al., 2011; Mohammed-Ziegler & Billes, 2002). In this study, the peak point of amide A region at 3288 cm^{-1} for the control film shifted down to 3287, 3286, and 3285 cm^{-1} with the addition of catechin at 1.5, 3.0, and 4.5 mg/cm^2, respectively. In addition to that, the band at 3288.14 cm^{-1} for control film shifted down to 3287.98 cm^{-1} with the addition of 0.25 mg/cm^2 gallic acid and down to 3287.51 cm^{-1} with the addition of 2.5 mg/cm^2 gallic acid into films. These results obtained by FTIR analyses suggested the potential roles of H-bonds formed between zein and phenolics in dramatic morphological and mechanical changes in catechin or gallic acid plasticized films. However, further studies are needed with model protein and phenolic compounds in less complex systems than films to fully understand the individual contribution of H-bonds and other confounding factors in band shifting and broadening. The studies on possible roles of phenolic -OH groups in band broadening are particularly needed since phenolic compounds might also

give peaks at the amide A, amide I and II regions (Ramos-Tejada et al., 2002; Robb, Geldart, Seelenbinder, & Brown, 2002).

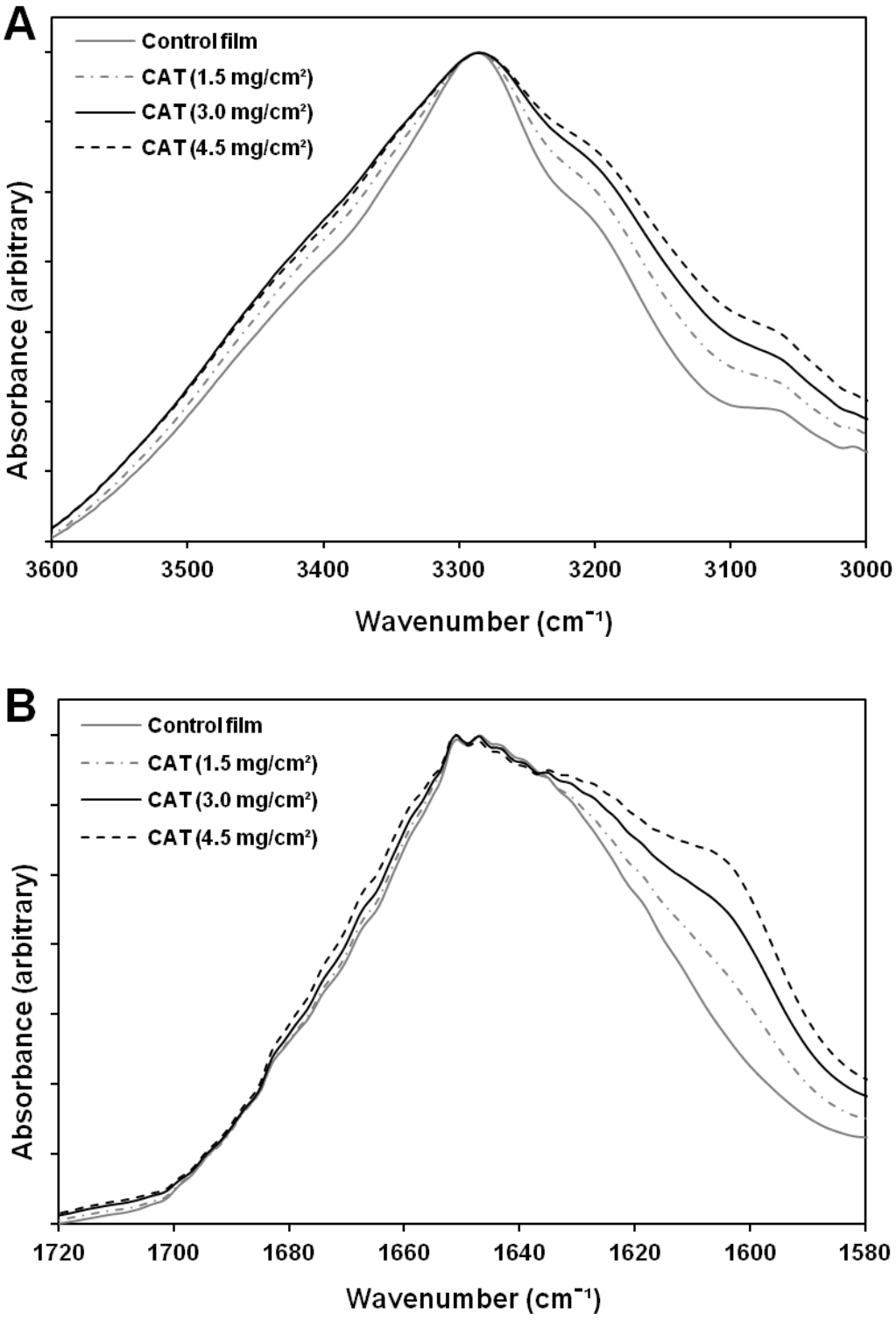


Figure 6.4. FTIR spectrum of zein films incorporated with catechin at different concentrations at amide A (A), and amide I (B) spectral regions

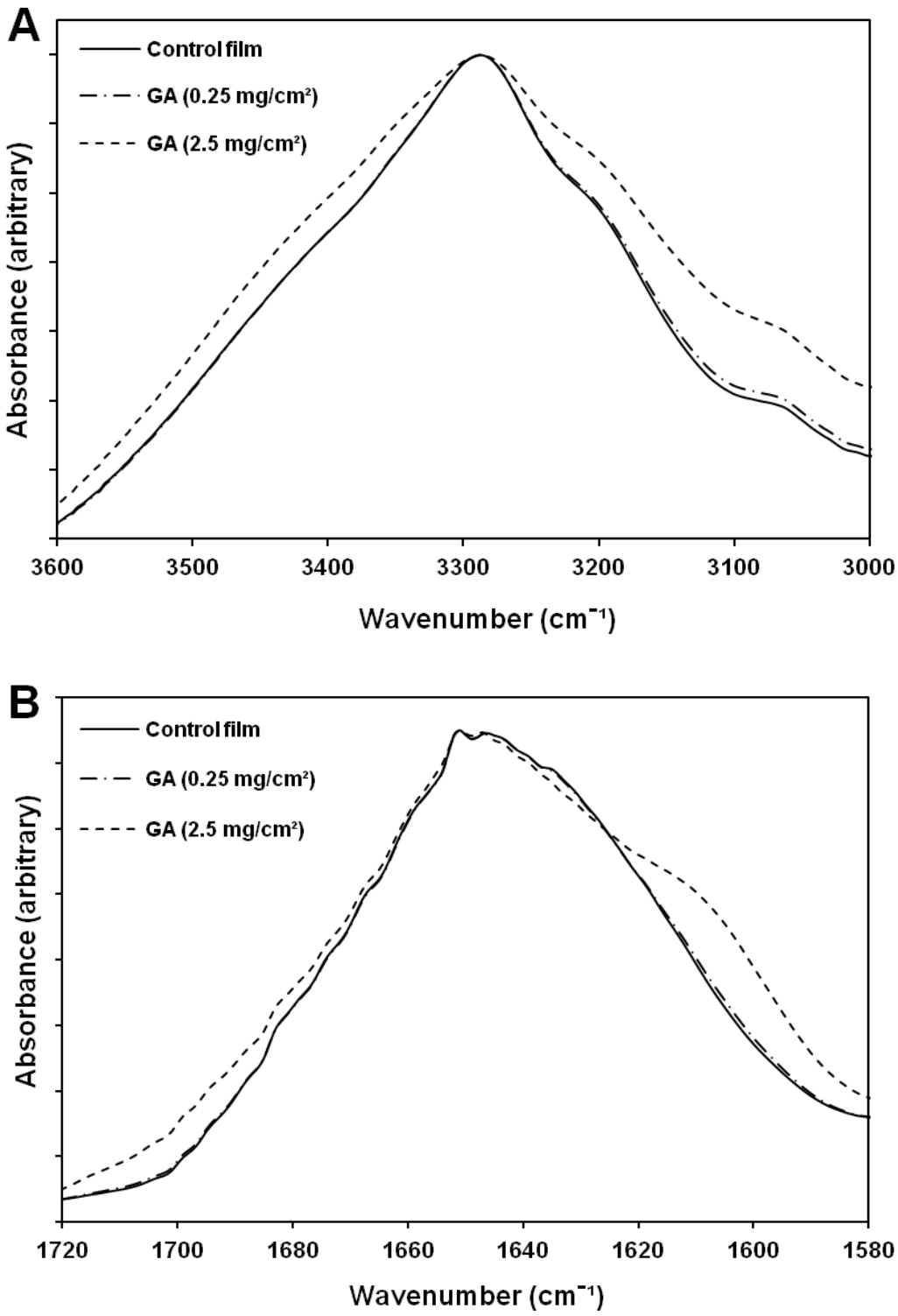


Figure 6.5. FTIR spectrum of zein films incorporated with gallic acid at different concentrations at amide A (A), and amide I (B) spectral regions

6.1.1.3. SEM Analysis of the Films

The SEM images of the developed films were obtained to understand the morphological changes in films occurred by plasticization with phenolics. Figure 6.6 shows the cross-sectional views of control films and films containing different phenolic compounds at 3 mg/cm^2. As seen in Figure 6.6A, control zein film without any additives had very porous structure. The incorporation of different phenolic compounds caused formation of different film structures. For example, the incorporation of catechin reduced the porosity of films, while incorporation of gallic acid increased film porosity (Figure 6.6B and C). On the other hand, the high number of large pores observed in hydroxyl benzoic acid and ferulic acid containing films could be related with rapid swelling and loss of integrity of these films when they were incubated in distilled water (Figure 6.6D and E). The SEM photos of flavone and quercetin containing films also showed the presence of different shapes and forms of aggregates and this supported our observations about solubility problems of these phenolic compounds in films (Figure 6.6F and G).

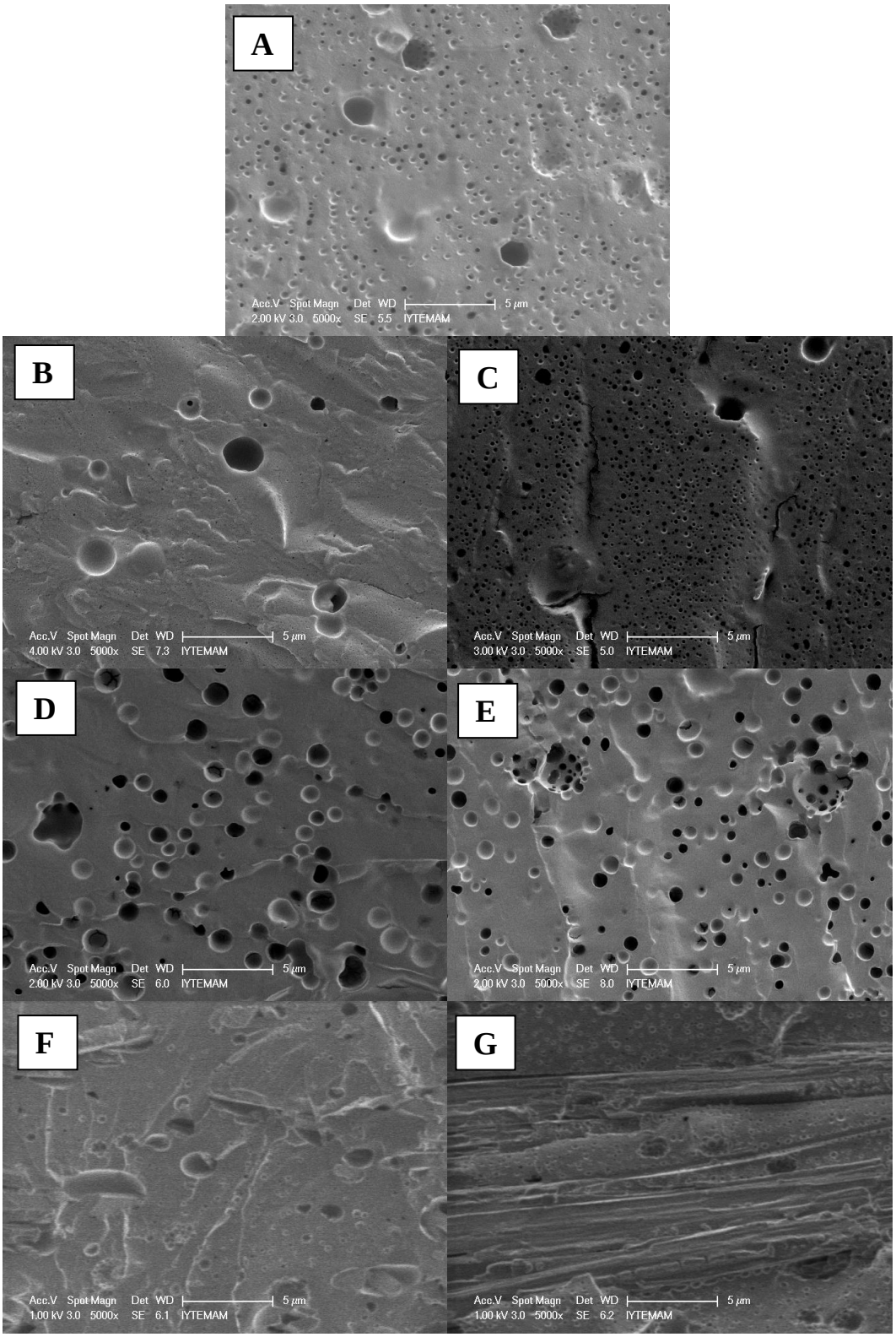


Figure 6.6. SEM images of zein films incorporated with different phenolic compounds (phenolic concentration of films: 3.0 mg/cm2; control (A); catechin (B); gallic acid (C); hydroxyl benzoic acid (D); ferulic acid (E); quercetin (F); and flavones (G))

6.1.1.4. Catechin and Gallic Acid Release Profiles of Zein Films

The release profiles of gallic acid and catechin from zein films incubated at 4 °C were given in Figure 6.7. In 1.5 and 3.0 mg/cm^2 phenolic containing films, total gallic acid released was 1.6 and 1.9 fold higher than total catechin released, respectively (Table 6.2). Thus, the average soluble catechin and gallic acid contents were 49% and 88% of total catechin and gallic acid incorporated into films, respectively. The TEAC of gallic acid (34.6 μmol trolox/g) is also 2.1 fold higher than that of catechin (16.1 μmol trolox/g). Therefore, the antioxidant potential of total gallic acid released from 1.5 to 3.0 mg/cm^2 phenolic containing films were 3.6 and 4.1 fold higher than those of total catechin released from corresponding films, respectively.

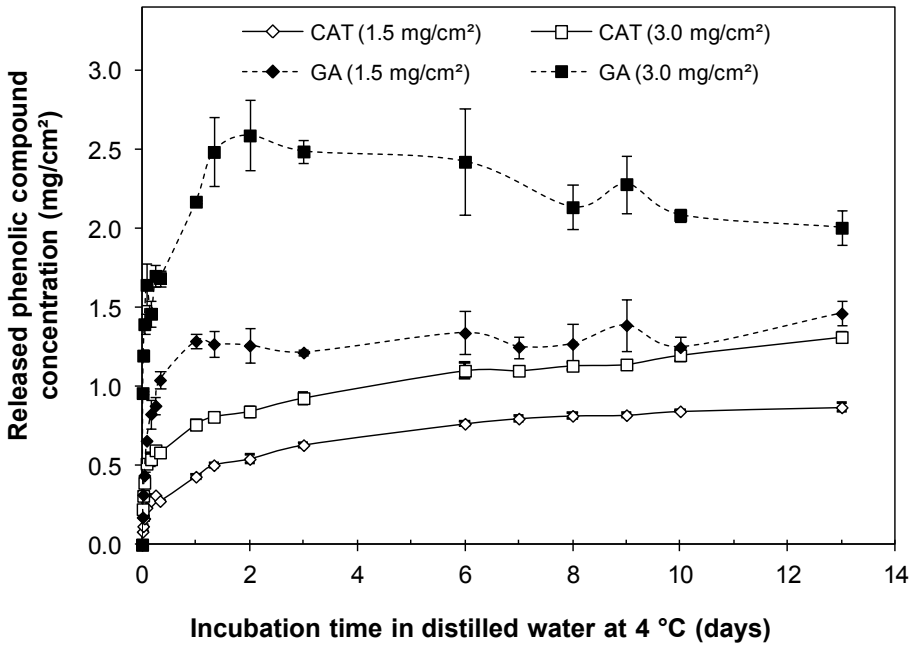


Figure 6.7. Release profiles of different phenolic compounds from zein films (CAT: catechin; GA: gallic acid)

It was assume that, greater amounts of gallic acid released from zein film than that of catechin, since the incorporation of gallic acid increased the film porosity, while the incorporation of catechin reduced the film porosity. Moreover, catechin (MW: 290.3) have greater molecular weight and more H bonding –OH groups (OH) than gallic acid (MW: 170.1). Thus, greater trapping of catechin within the films might also be arised due to its molecular size which limited its diffusion from the films. In both catechin and

gallic acid containing films, the trapped phenolic compounds showed some free radical scavenging activity (bound antioxidant activity). However, for both phenolic compounds the TEAC of bound antioxidant activity changed between 1.3 and 2.6% of soluble TEAC. Although, greater amounts of catechin than gallic acid trapped within zein films, the gallic acid containing films showed 3.5 to 7 fold higher bound TEAC than the catechin containing films. The higher bound antioxidant activity of gallic acid containing films should be related with higher free radical scavenging activity of gallic acid than catechin and morphological properties of gallic acid containing films which enable better contact of bound gallic acid with free radical solution. The use of antioxidant phenolic compounds in edible films is quite beneficial to improve oxidative stability of packed foods. For example, Ku et al. (2008b) successfully controlled oxidative changes in sausages packed with agar based films containing catechin. Moreover, the gallic acid and catechin consumed with food could also contribute to human health since these potent antioxidants have protective effects against cardiovascular diseases and anticarcinogenic activity (Madlener et al., 2007; Shahrzad, Aoyagi, Winter, Koyama, & Bitsch, 2001; Yilmaz & Toledo, 2004).

Table 6.2. Soluble phenolic concentration and free radical scavenging activity of different zein films

Film composition[a]		**Total released phenolics**	**Antioxidant potential**	**Bound antioxidant activity**
CAT (mg/cm^2)	**GA (mg/cm^2)**	**(mg/cm^2)**	**(μmol Trolox/cm^2)**	**(μmol Trolox/cm^2)**
1.5	-	0.9 ±0.03 (58%)[b]	14.0 ±0.5	0.18 ±0.04
3.0	-	1.3 ±0.03 (43%)	21.0 ±0.5	0.41 ±0.04
4.5	-	2.0 ±0.02 (44%)	32.0 ±0.3	-[c]
6.0	-	3.1 ±0.08 (52%)	49.0 ±1.3	-
-	1.5	1.4 ±0.08 (93%)	50.5 ±2.8	1.32 ±0.02
-	3.0	2.5 ±0.22 (83%)	86.2 ±7.6	1.49 ±0.00

[a] phenolic compounds: CAT: catechin, GA: gallic acid

[b] percentage of soluble phenolic content in the films

[c] not determined

6.1.1.5. Antimicrobial Potential of the Catechin or Gallic Acid Containing Zein Films

The films containing 3 mg/cm^2 catechin did not show any antimicrobial activity on *L. monocytogenes* and *C. jejuni* used in microbial tests. In contrast, films containing gallic acid at the same concentration showed good antimicrobial activity on both *L. monocytogenes* and *C. jejuni* and formed 207±34 mm^2 and 166±36 cm^2 clear zones around tested discs, respectively. The effectiveness of gallic acid on *L. monocytogenes* and *C. jejuni*, but lack of antimicrobial activity of catechin on *C. jejuni* showed parallelism with reports of previous workers who tested solutions of these compounds on indicated bacteria (Gañan et al., 2009; Vaquero, Alberto, & de Nadra, 2007b). However, our results showing lack of antimicrobial activity of catechin on *L.monocytogenes* contradicted with those of Vaquero et al. (2007b) and Ku et al. (2008b) who found this flavonoid effective against this bacteria in agar well diffusion test and in a food packaging application conducted with inoculated sausages, respectively. Due to differences in the antimicrobial test methods, it is hard to compare the effectiveness of catechin concentrations in our study with those of indicated workers. However, it seems that the catechin concentration which gave optimal mechanical properties (elongation over 100% without formation of sticky film structure) in our study is less than the critical inhibitory concentration necessary to form detectable clear zones during the applied zone inhibition method.

6.1.2. Development of Zein–Wax Composite Films for Controlled Release of Catechin

The catechin and its derivatives are potent natural antimicrobial and antioxidants that can be utilized in place of chemical antioxidants and antimicrobials to increase quality and shelf-life of meats, poultry, fish and their products (Almajano, Carbó, Jiménez, & Gordon, 2008; Ku et al., 2008b; O'Grady, Maher, Troy, Moloney, & Kerry, 2006; Saucier & Waterhouse, 1999; Vaquero, Alberto, & de Nadra, 2007a; Vaquero et al., 2007b; Yilmaz, 2006). In fact, recently, catechin has been successfully used as antimicrobial and antioxidant for bioactive packaging (Ku et al., 2008b). In this study, bioactive zein films have been developed by incorporation of (+)-catechin, a basic

catechin, into zein films. It is well known that the release mechanism of many films are effected from polymer swelling occurred as a result of diffusion of water molecules into the polymeric film matrix (Mastromatteo et al., 2010). Therefore, it is commonly accepted that the incorporation of hydrophobic compounds into films retards their hydration and subsequent diffusion of active agents from their film matrix (Ouattara et al., 2000; Ozdemir & Floros, 2003). Thus, to slow down the release rate of catechin the hydrophobicity of zein films was increased by incorporating carnauba wax into film forming solutions by means of homogenization in presence of lecithin emulsifier. The distribution of hydrophobic wax particles within the film matrix aimed not only to increase hydrophobicity of the films but also to increase the film tortuosity (Ozdemir & Floros, 2003). In this work, wax was added in presence of a GRAS status surface active compound soy lecithin. This aimed not only to form and distribute small wax particles within film matrix but also to maintain stability of reduced sized of wax particles during film drying. Lecithin is an amphiphilic molecule and holds on hydrophobic globules through its hydrophobic tail and forms negatively charged films on surfaces of the hydrophobic globules with its charged carboxyl group. Therefore the charged polar sites at hydrophobic surfaces prevent the coalescence of newly formed hydrophobic droplets with electrostatic repulsion forces and also increase the interactions between hydrophilic and hydrophobic molecules in the film structure.

6.1.2.1. Catechin Release Profiles from Zein–Wax Composite Films

Release profile of catechin from zein films incorporated with wax and/or lecithin was investigated (Figure 6.8). The addition of lecithin (5% (w/w) of zein) had no effect on release profiles of catechin. To slow down the release of catechin by developing composite structure, carnauba wax was added into film composition at 5% (w/w) of zein. However, composite structure had only limited effect on catechin release. On the other hand, using carnauba wax and lecithin together slowed down the catechin release from zein films compared to that of zein–carnauba wax (zein–CAR) composite film lacking lecithin. Moreover it is worth to report that the total released catechin from zein–CAR composite film was higher than that of zein–CAR composite film lacking lecithin. As a matter of fact, catechin release from zein-CAR composite films continued after 4^{th} days of release experiment while it was almost stopped after same period of

time from zein-CAR composite films lacking lecithin. Thus the differences in release profiles of zein and zein–CAR composite films could be related to changes in film morphoplogy or differences in their swelling characteristics. Moreover, it seemed that using lecithin in composite film composition increased the effects of wax on catechin release. Lecithin is an amphiphilic molecule; therefore it interacts with hydrophobic molecules through their hydrophobic tail, and with hydrophilic molecules through their charged carboxyl groups. Lecithin may hold on hydrophobic wax globules through its hydrophobic tail and form negatively charged films on surfaces of the hydrophobic wax globules with its charged carboxyl group. This may increase the ability of hydrophobic wax globules to interact with zein or catechin. As it is well known, physiochemical interactions between a macromolecular system and a diffusing molecule affect its diffusion (Ozdemir & Floros, 2003). Moreover, the formation of negatively charged films on the wax surfaces may also prevent the aggregation of the little wax globules formed by homogenization during film drying; therefore, maintain the film homogeneity.

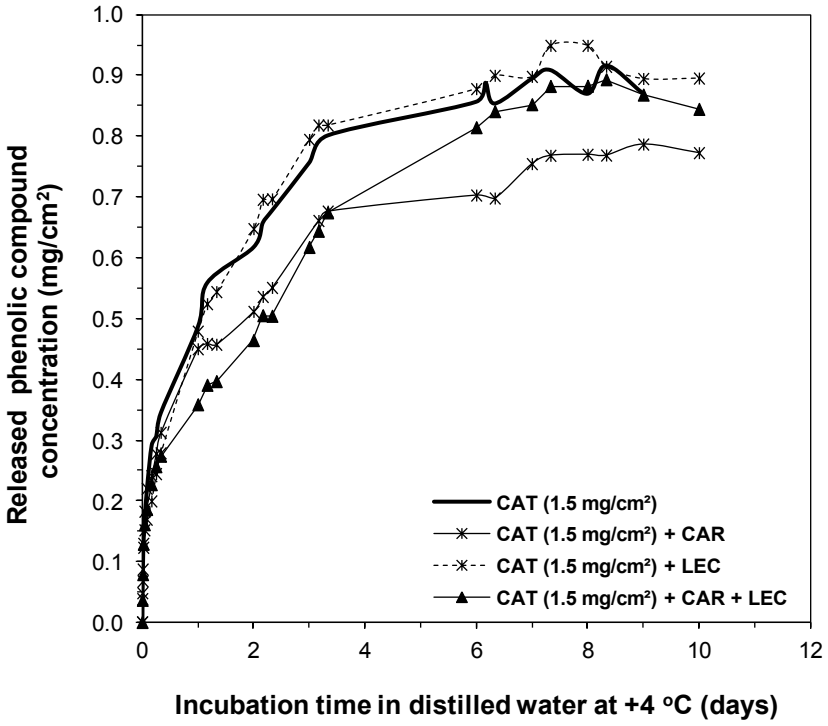


Figure 6.8. Release profiles of catechin from zein and zein–CAR composite films (wax and lecithin concentrations: 5% (w/w) of zein; CAT: catechin, CAR: carnauba wax, LEC: lecithin)

The effects of phenolic compound concentration on the release profiles of catechin from composite films were also investigated in this study. The release tests in distilled water with zein and zein-wax composite films showed that films containing 0.75 mg/cm^2 catechin showed similar release profiles (Figure 6.9). On the other hand, catechin in zein–CAR composite films released more slowly than the catechin in zein films containing 1.50, 2.25, or 3.00 mg/cm^2 catechin. It is clear that the controlled release properties obtained by composite making affected from catechin concentration. Catechin (contained 5 OH groups) can form extensive H–bonding in zein matrix to form a network since phenolic hydroxyl groups are capable to form H–bonding with peptide carbonyl groups of proteins (Damodaran, 1996). Thus, it appears that slow release of catechin is a result of increased hydrophobicity of films by carnauba wax and the network formed by catechin itself. The H–bonding put part of the catechin into insoluble form, but most of the catechin was soluble and released from the films.

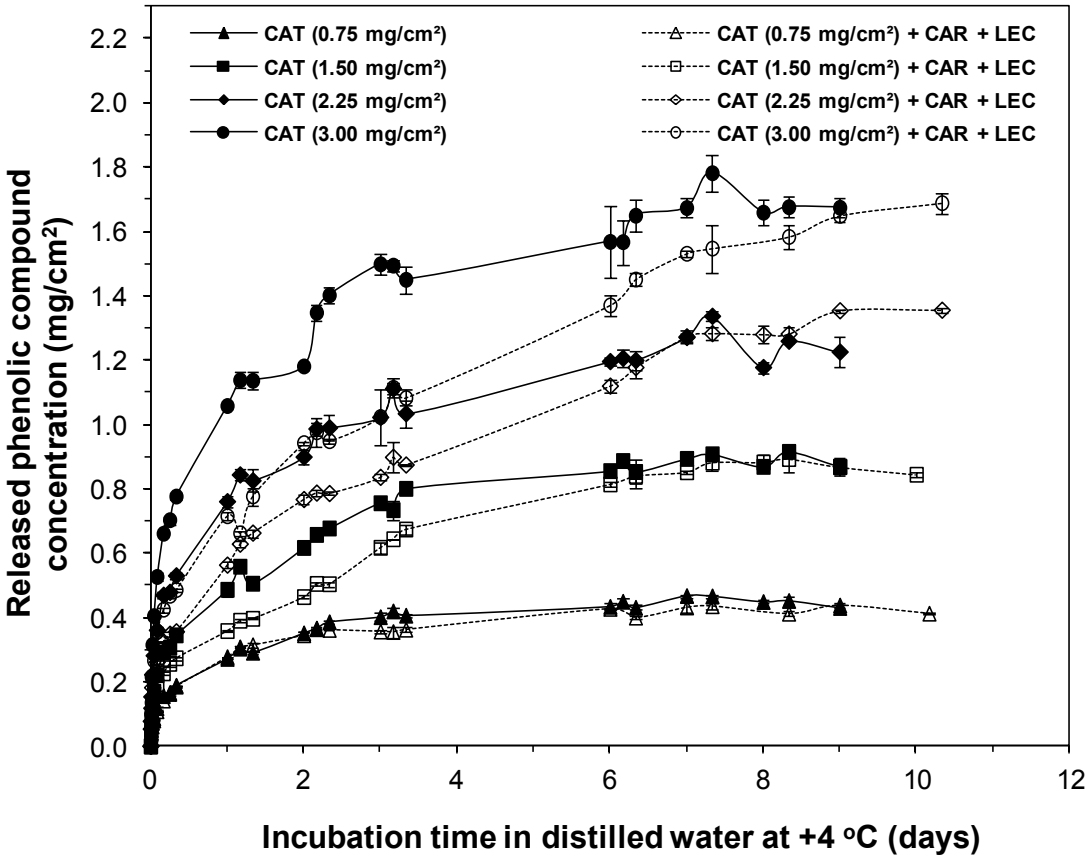


Figure 6.9. Release profiles of CAT from zein and zein-CAR composite films (wax and lecithin concentrations: 5% (w/w) of zein; CAT: catechin, CAR: carnauba wax, LEC: lecithin).

6.1.2.2 SEM Analysis of the Films

The SEM images of the developed films were obtained to understand the morphological changes in films occurred by addition of catechin and formation of composite structures (Figure 6.10). As seen in Figure 6.10A and B, control zein films without any additives have very porous structure. But the incorporation of catechin into zein films reduced the porosity of films and gave denser films (Figure 6.10C and D).

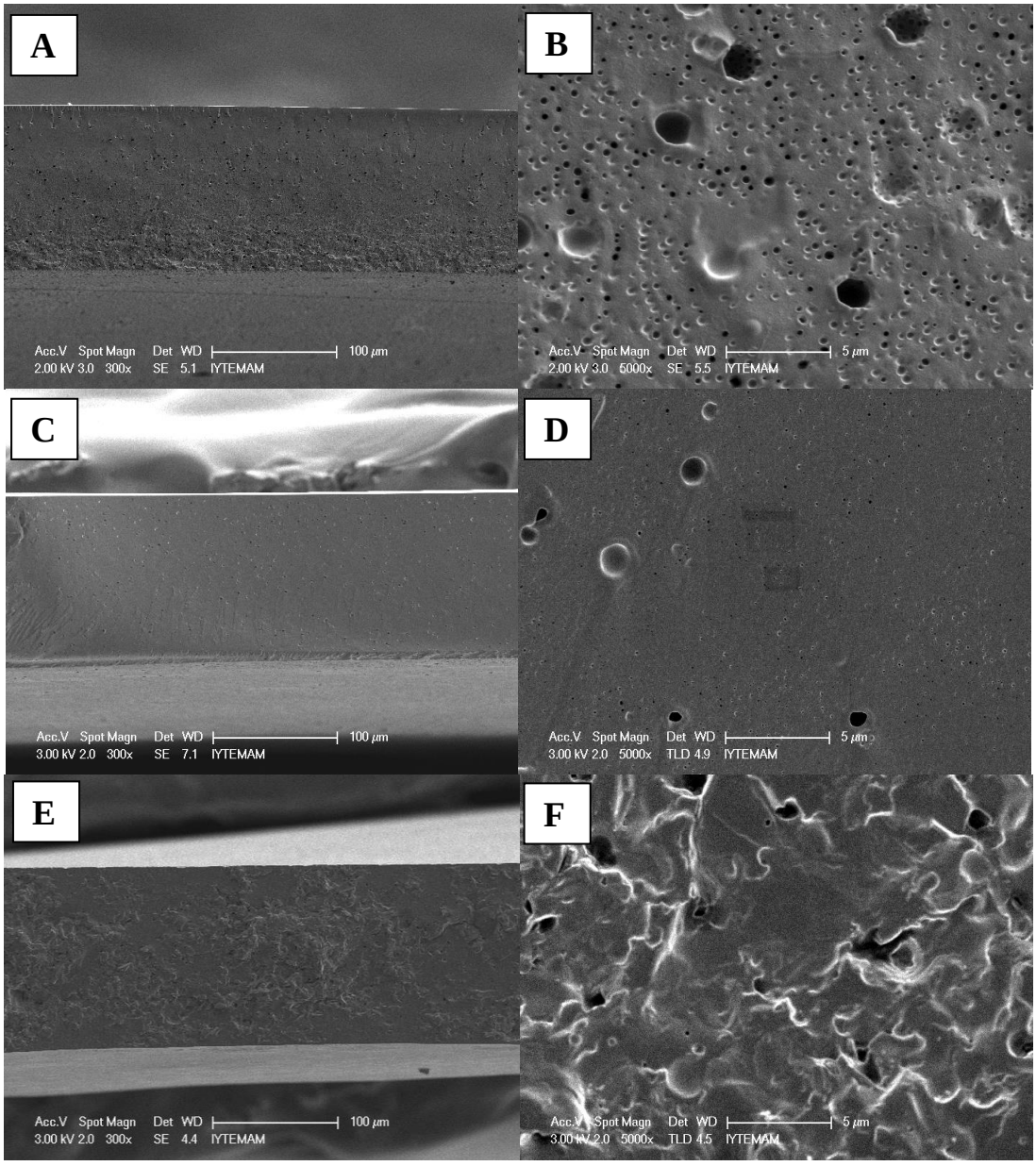


Figure 6.10. Cross-sectional SEM images of developed films: Control zein film (A, B); zein film containing 3.00 mg/cm^2 CAT (C, D); zein-CAR composite film containing 3.00 mg/cm^2 CAT

On the other hand, the addition of wax into zein films caused dramatic changes in film morphology and formed some amorphous wax aggregates within the film matrix (Figure 6.10D and E). Evaluation of these SEM results together with the release test results suggests that the distribution of wax particles within zein film is beneficial to increase film tortuosity and to manipulate the release profiles of catechin from the composite films.

6.1.2.3. Antioxidant Potential of Catechin Containing Zein–Wax Composite Films

The antioxidant capacity of the films was based on calculating the TEAC of their soluble catechin content. On the other hand the bound antioxidant activity method was designed to measure the antioxidant potential of insoluble part of the film components. In zein films incorporated with 0.75 to 3 mg/cm^2 catechin 59 to 62% of the catechin existed free and solubilized during the release tests (Table 6.3). Thus, the release of the considerable portion of catechin from films to food surface is an advantage to improve antioxidant and bioactive status of packed foods. On the other hand, the remaining catechin within the films was expected to be bound to the film matrix by H bonds. The H bond formation between zein and catechin has been explained in the Section 6.1.1.2. As expected the antioxidant potential of the films increased as the catechin concentration increased. The incorporation of waxes did not change the soluble catechin content since extremely hydrophobic waxes did not contain hydrogen bonding groups to interact with catechin. Therefore, both antioxidant potential and bound antioxidant activity of zein and zein-wax composite films were found almost equal.

6.1.2.4. Antimicrobial Potential of Catechin Containing Zein–Wax Composite Films

The antimicrobial effects of the developed films were tested on *L. innocua* by using the classical disc diffusion method. The control zein and zein–CAR composite films without catechin did not form any inhibition zones (Figure 6.11). Moreover, an extensive bacterial growth was observed below control film discs when they were

Table 6.3. Soluble phenolic concentration and free radical scavenging activity of zein and zein–CAR composite films

Film composition[a]			Total released phenolics (mg/cm^2)	Antioxidant potential (μmol Trolox/cm^2)	Bound antioxidant activity (μmol Trolox/cm^2)
CAT (mg/cm^2)	CAR (%)[b]	LEC (%)[b]			
0.75	-	-	0.47 ± 0.01	7.5 ± 0.1	0.17
1.5	-	-	0.92 ± 0.01	14.8 ± 0.2	0.27
2.25	-	-	1.37 ± 0.01	22.0 ± 0.2	0.34
3.0	-	-	1.78 ± 0.06	28.7 ± 1.0	0.31
0.75	5	5	0.44 ± 0.01	7.1 ± 0.1	0.15
1.5	5	5	0.89 ± 0.04	14.4 ± 0.6	0.20
2.25	5	5	1.36 ± 0.01	21.9 ± 0.1	0.26
3.0	5	5	1.69 ± 0.32	27.2 ± 5.2	0.33

[a] CAT: catechin; CAR: carnauba wax; LEC: lecithin

[b] Concentrations of wax and lecithin as % of zein (w/w).

removed carefully from the agar. Although the antimicrobial potential of catechin against pathogenic species of *Listeria* was reported previously (Ku et al., 2008b), no inhibition zones were observed for the films containing 3.0 mg/cm^2 catechin. However, no bacterial growth was observed below catechin containing disks and this indicated a limited antilisterial effect of this phenolic compound at the studied concentration. These results were in line with the previous findings that reported at Section 6.1.1.5. On the other hand slight zone formation around the tested disks was observed for the zein–CAR composite films containing catechin. However quantitative results could not be given because of the partial formation of the zones. Moreover, due to the melting of the wax in film structure at the incubation temperature, it could not be determined whether the test bacteria grow under the disks or not. These results showed the limited antimicrobial potentials of composite films containing catechin at the tested concentration.

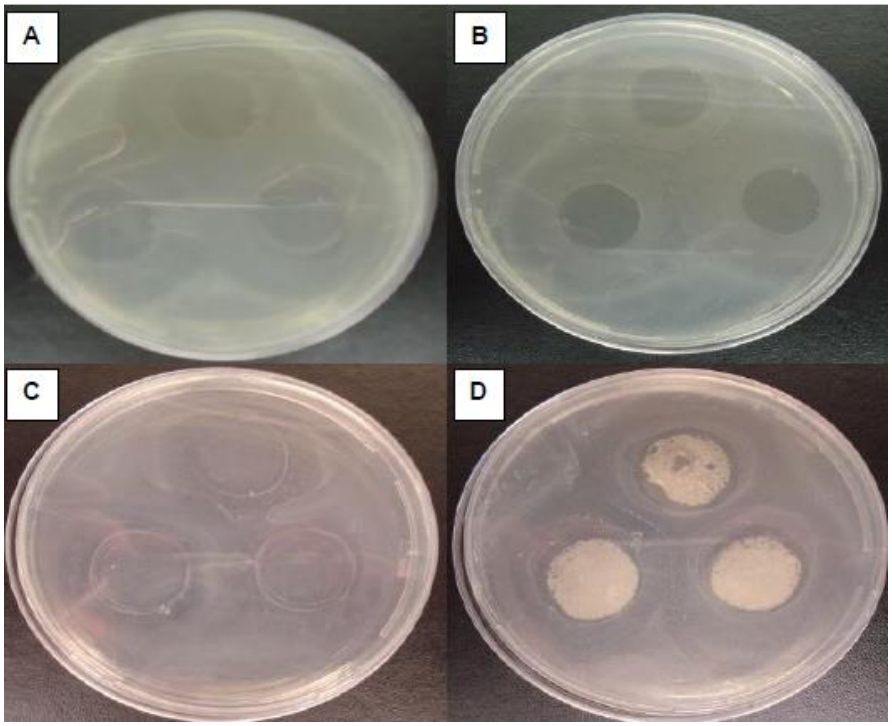


Figure 6.11. Antimicrobial potential of zein based composite films against *L.innocua* (Control zein film (A); zein film containing 3.00 mg/cm^2 catechin (B); control zein–CAR composite film (C); zein–CAR composite film containing 3.00 mg/cm^2 catechin (D)

6.1.2.5. Mechanical Properties of Catechin Containing Zein–Wax Composite Films

In order to analyze their mechanical properties tensile strength at break, elongation at break, and Young's modulus values of films were determined (Table 6.5). In this section the effects of wax addition into zein films were investigated. Table 6.4 shows that, the control zein film lacking catechin showed very little elongation, but the tensile strength value of the control films at the breaking point (10.2 MPa) was significantly higher than those of the catechin plasticized films (1.8 MPa) and composite and blend films (1.5 MPa) (P<0.05). It has been showed in Section 6.1.1.1 that, the addition of catechin into zein films effectively plasticized zein films and improved their elongation significantly. But the formation of zein–CAR composites containing catechin caused a significant reduction in film elongation (P<0.05). However, the composites film is still much more flexible than the controls. This result confirmed the recent findings of Alkan et al. (2011) who tested mechanical properties of

zein and zein–wax composites containing gallic acid. Fabra et al. (2008) also reported reduction in elongation of wax containing caseinate films and attributed this effect to discontinuities in the polymer network by the incorporated waxes. On the other hand, no significant change occurred in tensile strength and Young's modulus values of the films by addition of waxes to form a composite or blend film structure (P>0.05). These results contradicted with Sohail, Wang, Biswas, and Oh (2006) who incorporated paraffin wax into casein–zein hydrolizate films and reported a reduced tensile strength for these composite films. The effects of waxes on tensile strengths of zein films were also different than casein films which showed an increase in their tensile strengths by addition of carnauba or candelilla waxes (Chick & Hernandez, 2002). These reports showed that the mechanical changes in protein–wax composite structures are complex. It appears that the mechanical changes in composite systems could be highly variable depending on amounts and molecular properties of each constituent in the mixture and degree of their compatibility and interactions within the films.

Table 6.4. Mechanical properties of zein and zein–CAR composite films

Film composition[a]			Tensile strength	Elongation	Young's	Film
CAT (mg/cm^2)	CAR (%)[b]	LEC (%)[b]	at break (MPa)	at break (%)	modulus (MPa)	thickness (μm)
-	-	-	10.2 ± 0.8a[c]	3.3 ± 0.6b	528 ± 39a	131.8 ± 2.0
3.0	-	-	1.8 ± 0.2b	136.8 ± 27.4a	86 ± 14b	123.2 ± 0.4
3.0	5	5	1.5 ± 0.2b	30.8 ± 11.7b	84 ± 20b	140.3 ± 0.9

[a] CAT: catechin; CAR: carnauba wax: LEC: lecithin

[b] concentrations of wax and lecithin as % of zein (w/w).

[c] different letters in each column show significant difference at P<0.05.

6.1.3. Development of Zein–Fatty Acid Blend Films for Controlled Release of Catechin

Not only waxes but also fatty acids, acetylated monoglycerides and essential oils were used to modify hydrophobicity and morphology of films and change their release profiles for the active compounds (Ouattara et al., 2000). One of the first studies in this area was conducted by Redl et al. (1996) and they reduced the diffusion rate of sorbic

acid from gluten based films by incorporating acetylated monoglyceride. Ouattara et al. (2000) developed chitosan based films of which acetic acid and propionic acid diffusion could be changed by incorporating lauric acid and essential oils into film compositions. Thus, to slow down the release rate of catechin the hydrophobicity of zein films was increased by incorporating oleic acid into film forming solutions by means of homogenization in presence of lecithin emulsifier. As a matter of fact, Wang, Filho, Geil, and Padua (2005) reported that using emulsifying agent in zein–oleic acid (zein–OLA) system improved the dispersion of oleic acid in film forming solution and enabled to develop more uniform films. Moreover, emulsifying agents also enhanced the interaction of oleic acid with zein (Wang et al., 2005).

6.1.3.1. Catechin Release Profiles from Zein–Oleic Acid Blend Films

As seen in Figure 6.12, the addition of oleic acid (10 to 60% (w/w) of zein) with lecithin (5% (w/w) of zein) effectively slowed down the release of catechin. The catechin release profiles from blend films did not change considerably with the increasing oleic acid concentration from 10 to 40% (w/w) of zein film. Although the effects of fatty acid on catechin release slightly decreased when oleic acid concentration increased to 60%, the released of catechin was still much slower than that of control zein film. It appeared that the addition of oleic acid caused some morphological changes in films or differences in their swelling characteristics which resulted different release profiles. On the other hand, the total released catechin amount from blend films and related to this antioxidant potentials of the blend films increased as the oleic acid concentration in film composition increased (Table 6.5). The addition of oleic acid could limit the interactions between zein and catechin which decreased the bound catechin amount to film matrix. The release tests also showed that zein–oleic acid (zein–OLA) blend films were more effective than zein–CAR composite films to slow down catechin release at the single plasticizer concentration of 3 mg/cm^2. Moreover, the soluble catechin content of zein–OLA blend films was 1.2 to 1.6 fold higher than those of zein and zein–CAR composite films.

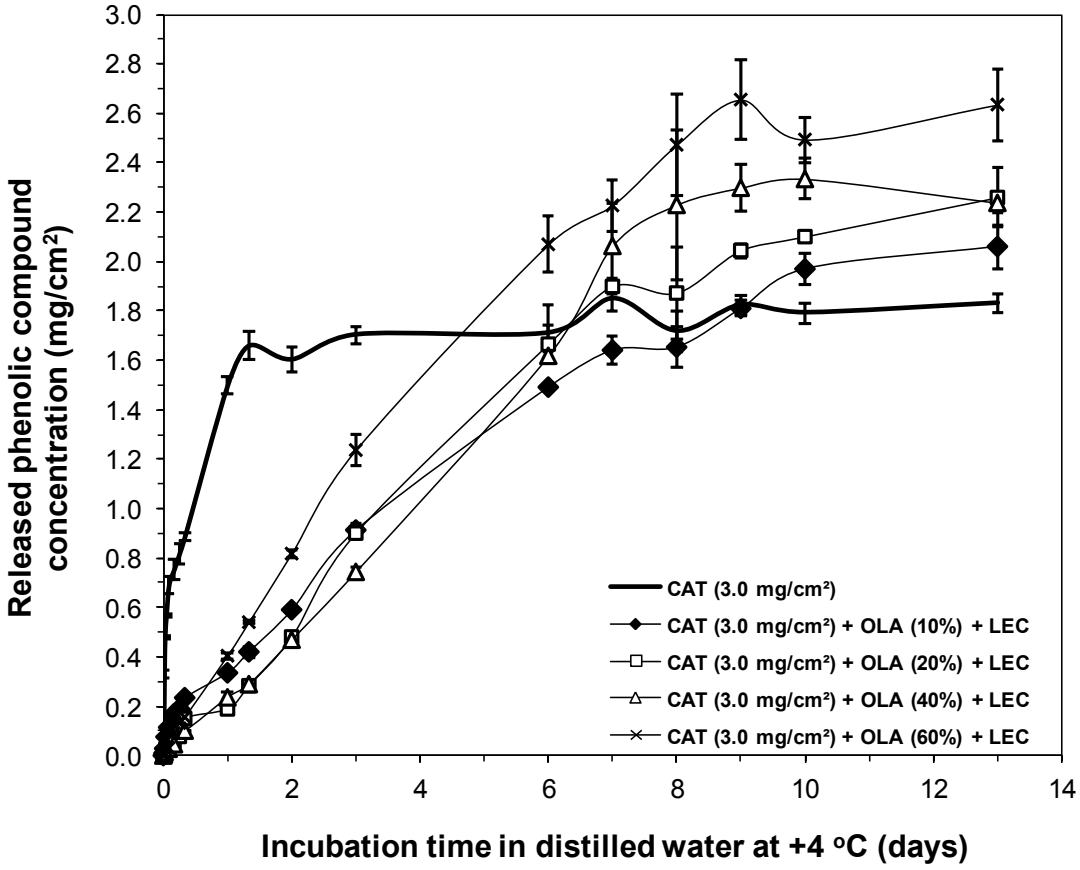


Figure 6.12. Release profiles of catechin from zein and zein–OLA blend films (lecithin concentrations: 5% (w/w) of zein; CAT: catechin, OLA: oleic acid, LEC: lecithin).

Table 6.5. Soluble phenolic concentration and free radical scavenging activity of different zein films

Film composition[a]			Total released phenolics (mg/cm^2)	Antioxidant potential ($\mu mol\ Trolox/cm^2$)
CAT (mg/cm^2)	OLE (%)[b]	LEC (%)[b]		
3	-	-	1.83 ± 0.04	30 ± 1
3	10	5	2.06 ± 0.09	33 ± 2
3	20	5	2.26 ± 0.12	36 ± 2
3	40	5	2.33 ± 0.08	38 ± 1
3	60	5	2.66 ± 0.16	43 ± 3

[a] CAT: catechin; OLA: oleic acid; LEC: lecithin

[b] concentrations of oleic acid and lecithin as % of zein (w/w).

6.1.4. Development of Zein–Fatty Acid Blend Films for Controlled Release of Catechin and Gallic Acid

The previous section shows that zein–OLA blend films could be effectively used for controlled release of catechin. However, catechin containing zein based films did not show adequate antimicrobial activity against *Listeria* species (Sections 6.1.1.5 and 6.1.2.4). In contrast, films containing gallic acid at the same concentration showed good antimicrobial activity against both *L. monocytogenes* and *C. jejuni*. Therefore, to increase the antimicrobial potential of the films and to develop alternative films with controlled release properties, catechin and gallic acid were used together in zein–OLA blend films. Gallic acid has lower molecular weight (MW: 170.1 g/mol) than catechin (290.3 g/mol). Moreover, the addition of gallic acid into zein films increased the film porosity (Figure 6.6 C). Therefore, it's hard to control the release rate of gallic acid from zein films. Alkan et al. (2011) used zein–wax composite films to decrease the release rate of gallic acid, but composite structure increased the release rate of gallic acid especially at higher gallic acid concentrations. On the other hand, the incorporation of catechin decreased the porosity of zein and zein–CAR composite films. In addition to that the release of catechin can be slowed down by developing zein–CAR composite and zein–OLA blend films (Sections 6.1.2 and 6.1.3). Thus, by using two phenolic compounds, it was aimed that to develop packaging materials with higher antimicrobial potential and controlled release properties.

6.1.4.1. Phenolic Compound Release Profiles from Zein–Oleic Acid Blend Films

Figure 6.13 A and B shows the release profiles of phenolic compounds from zein-OLA blend films with different oleic acid (10-40% (w/w) of zein) and lecithin (5-10% (w/w) of zein) concentrations. Phenolic compound release from zein-OLA blend films containing oleic acid (10%) and lecithin (5%) was much slower than that of control zein films. But, at the same lecithin concentration level, phenolic compounds released more rapidly from blend films when the oleic acid concentrations increased to 20 and 40%.

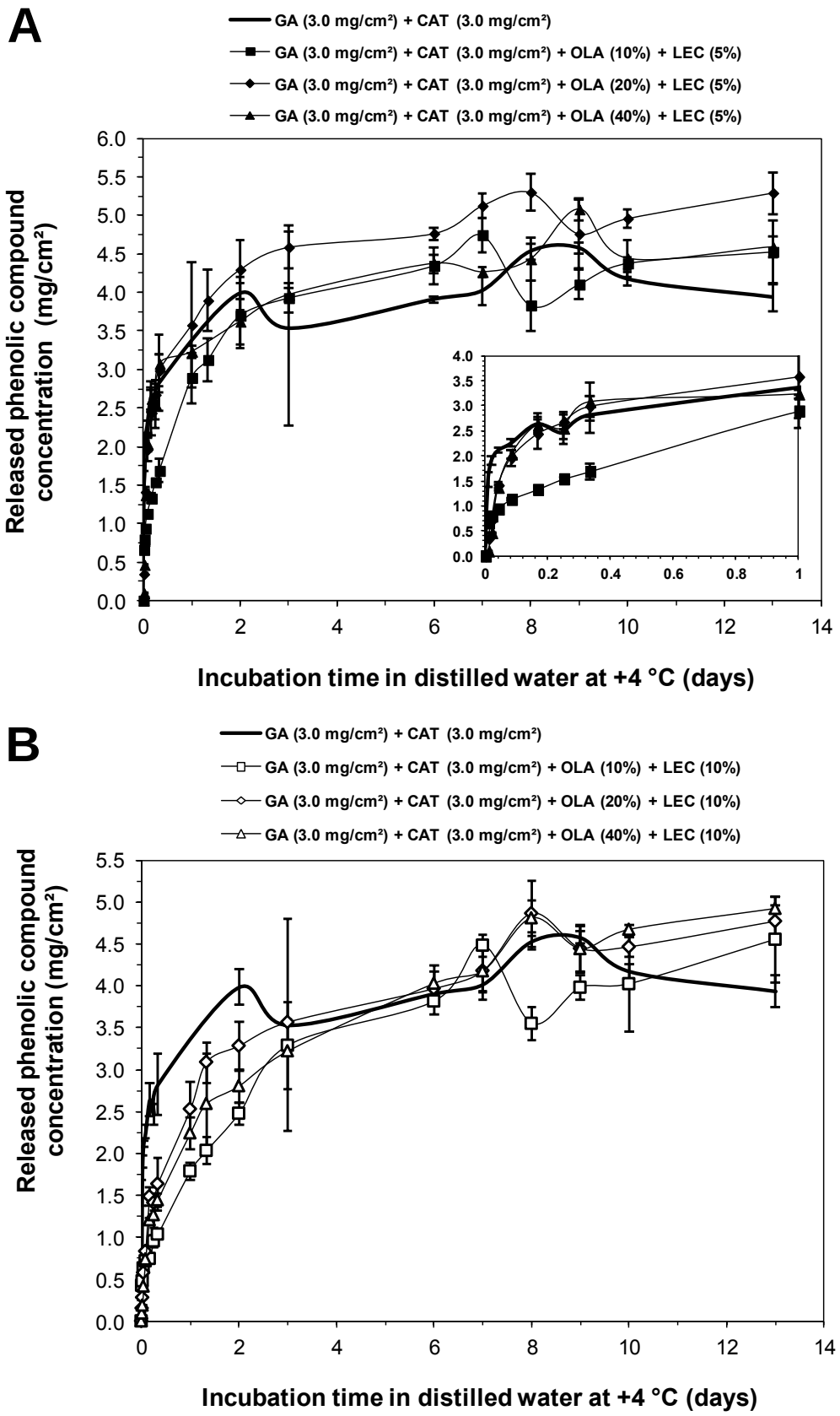


Figure 6.13. Release profiles of phenolic compounds from zein and zein–OLA blend films (GA: gallic acid; CAT: catechin, OLA: oleic acid, LEC: lecithin)

On the other hand, increasing the lecithin concentration to 10% slowed down the release of phenolic compounds from blend films. Especially, the phenolic compounds release from blend film containing oleic acid and lecithin at 10% was much slower than those of control zein film and other blend films. In fact, the amount of released phenolic compounds from the blend film corresponded to 55% of that released from control film at the end of 1 day release tests. It was clear that the homogenization of film making solution in presence of lecithin helped better distribution of hydrophobic lipids within film matrix. Phenolic compounds in zein-OLA blend films released more rapidly when the oleic acid in film composition increased to 20 and 40%. But it should also report that, phenolic compound release from blend films was still much slower than that of control zein film. This was evident from release of 21-30% less phenolic compound from blend films than the control film in 1 day.

The release test results showed that, in order to obtain controlled release of phenolic compounds from zein films containing gallic acid and catechin together, more lecithin needed to get better distribution of oleic acid in film matrix compared to zein films containing only catechin. In addition to that, oleic acid concentration was critical for controlled release of phenolic compounds, and effects of oleic acid on release of phenolic compounds was decreased as the concentration of oleic acid increased in film composition. It was assume that, at high oleic acid concentration the film integrity was distributed which lead the release of phenolic compounds more rapidly.

6.1.4.2. SEM Analysis of the Films

Figure 6.14 shows the morphological changes occurs in zein-OLA blend films containing different oleic acid concentrations. Zein film plasticized with gallic acid and catechin had porous structure (Figure 6.14A). On the other hand, Figure 6.14B-C show that the addition of oleic acid into zein films caused formation of many spherical capsules within films. Moreover, as the oleic acid concentration increased within film composition numbers and size of the spheres were increased. The morphological changes of zein structure when it is mixed with oleic acid without use of lecithin emulsifier was recently explained by Wang, Yin, and Padua (2008). According to these authors the morphological changes in zein–OLA system occurred at three steps; (1) formation of large numbers of oleic acid coated zein spheres, (2) partial melting of the

spheres by means of oleic acid, and (3) transformation of a sponge like morphology by interconnection of spheres with channels and tunnels. The spheres observed by SEM of zein–oleic acid films in this work lacked the interconnections specified by Wang et al. (2008). Thus, it seemed that the lecithin emulsifier used in this work stabilized the oleic acid coating formed around zein spheres. It appears that the repulsion formed by negative charges of lecithin at the oleic acid coating of formed zein spheres prevented the interaction and melting down of these spheres which formed the sponge-like structure described by Wang et al. (2008). Thus, it appeared that the reduced release rates of phenolic compounds in gallic acid and catechin containing zein–OLA blend

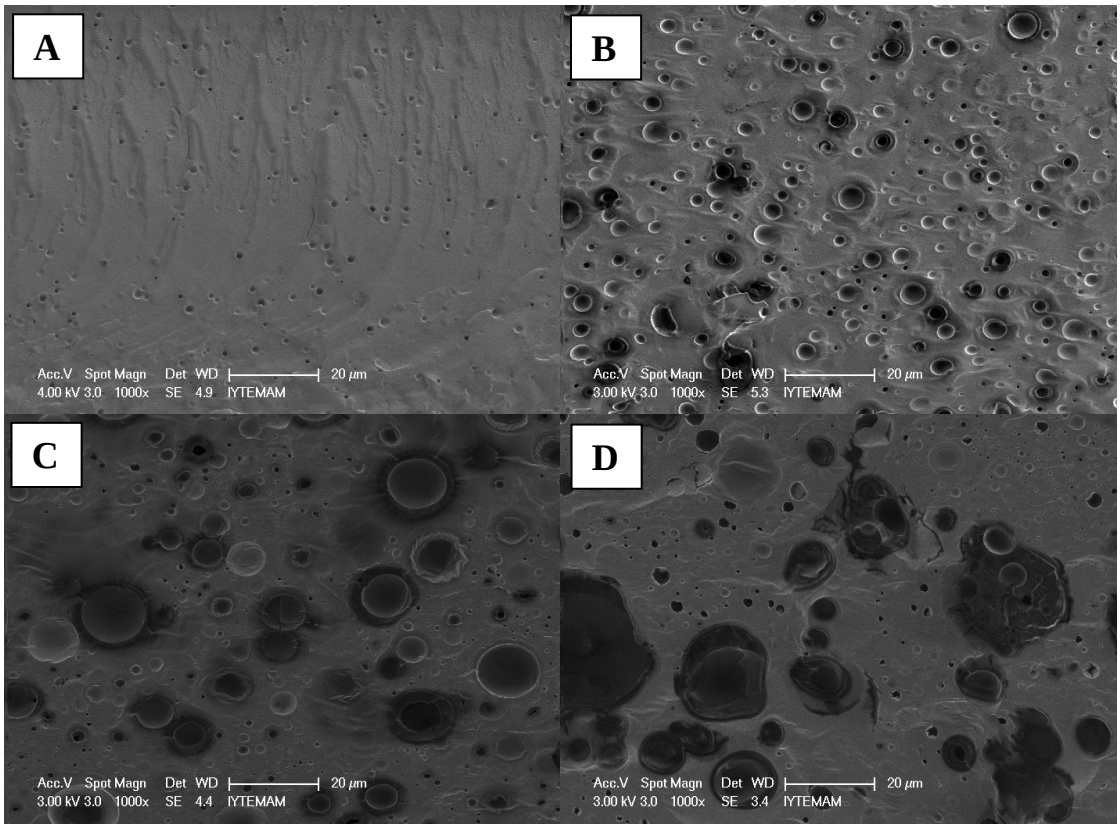


Figure 6.14. Cross-sectional SEM images of developed films: Zein film containing GA and CAT (A); zein–OLA blend film containing GA, CAT, 10% OLA and 10% LEC (B); zein–OLA blend film containing GA, CAT, 20% OLA and 10% LEC (C) zein–OLA blend film containing GA, CAT, 40% OLA and 10% LEC (D) (Phenolic compound concentrations in film: 3.0 mg/cm^2; concentrations of oleic acid and lecithin given as % of zein (w/w); GA: gallic acid, CAT: catechin, OLA: oleic acid, LEC: lecithin)

films are in part due to the entrapment of phenolics within spherical capsules which increased the barriers against phenolic compounds diffusion. In blend film containing oleic acid at 40%, bigger zein aggregates with non uniform shape were observed. The reduction in lecithin/oleic acid ratio could be reason for that which also explained the relatively rapid release of phenolic compounds from blend film containing 40% oleic acid than that of other blend films.

6.1.4.3. Antioxidant Potential of Gallic Acid and Catechin Containing Zein–Oleic Acid Blend Films

The test of total phenolic compound concentrations in release mediums at different intervals showed the soluble phenolic contents of different films (Table 6.6). In zein film incorporated with 3 mg/cm^2 gallic acid and catechin 76% of the phenolic compounds existed free and solubilized during the release tests. On the other hand, the remaining phenolic compound within the films was expected to be bound to the film matrix by H bonds. This hypothesis has been explained in the previous sections and the interactions between zein and phenolic compounds through hydrogen bonds have been showed by using FTIR analysis. On the other hand, the incorporation of oleic acid increased the soluble phenolic content except the blend film containing 10% oleic acid and lecithin in film composition. This result suggested that the formation of coating layer of oleic acid on the zein proteins which lead to spherical zein capsules could limit the interaction with between zein and phenolic compounds. Similar results have been reported for blend films containing only catechin (section 6.1.3.1). Moreover, blend films containing more lecithin, had less soluble phenolic content than that of other blend films containing same amount of oleic acid. Lecithin prevented the melting down of oleic acid coated zein spheres, therefore the immobilized portion of catechin within the zein–OLA film matrix which contained many spherical capsules slightly increased.

The highest antioxidant potential originated from free soluble catechin for zein–OLA blend films containing 5% ((w/w) of zein) lecithin in film composition fallowed by zein–OLA blend films containing 10% ((w/w) of zein) lecithin. It is clear that the use of phenolics is highly beneficial since the release of phenolics may improve the antioxidant and bioactive status of foods.

Table 6.6. Soluble phenolic concentration and free radical scavenging activity of zein and zein-oleic acid blend films

Film composition[a]				Total released phenolics (mg/cm^2)	Antioxidant potential ($\mu mol\ Trolox/cm^2$)
GA (mg/cm^2)	CAT (mg/cm^2)	OLA (%)[b]	LEC (%)[b]		
3	3	-	-	4.58 ± 0.09	74 ± 1
3	3	10	5	4.75 ± 0.22	77 ± 4
3	3	20	5	5.30 ± 0.24	85 ± 4
3	3	40	5	5.08 ± 0.14	82 ± 2
3	3	10	10	4.55 ± 0.51	73 ± 8
3	3	20	10	4.86 ± 0.39	78 ± 6
3	3	40	10	4.92 ± 0.15	79 ± 2

[a] GA: gallic acid; CAT: catechin; OLA: oleic acid; LEC: lecithin

[b] concentrations of oleic acid and lecithin as % of zein (w/w).

6.1.4.4. Mechanical Properties of Gallic Acid and Catechin Containing Zein–Oleic Acid Blend Films

As expected, the control zein film was highly brittle and showed very little elongations (Table 6.7). The addition of catechin effectively plasticized zein films and improved their elongation significantly ($P<0.05$). The plasticizing effects of phenolic compounds has already reported and explained in the previous sections. On the other hand, the addition of oleic acid at 10% did not significantly change the elongation value of zein film containing phenolic compounds ($P>0.05$). However using oleic acid at 20% in film composition improved the elongation value of zein film containing phenolic compounds significantly ($P<0.05$). But further increase in oleic acid concentration cause also a significant reduction in elongation of phenolics plasticized zein films ($P<0.05$). However, the blend film is still much more flexible than the control.

There are many different studies in the literature related to plasticization of zein films with oleic acid. However researchers reported that oleic acid has no or limited plasticizing effects on cast zein films or resins (Lai & Padua, 1997; Lawton, 2004; Xu et al., 2012). But results obtained in this study showed that the mechanical changes in protein–OLA blend structures are complex. It appears that the mechanical changes in

blend systems could be highly variable depending on amounts and molecular properties of each constituent in the mixture and degree of their compatibility and interactions within the films.

Table 6.7. Mechanical properties of zein based blend films containing gallic acid and catechin

Film composition[a]				Tensile strength at break (MPa)	Elongation at break (%)	Young's modulus (MPa)	Film thickness (μm)
GA (mg/cm^2)	CAT (mg/cm^2)	OLA (%)[b]	LEC (%)[b]				
-	-	-	-	17.67 ± 0.93a[c]	4 ± 1d	775 ± 44a	113 ± 1
3.0	3.0	-	-	0.51 ± 0.14b	196 ± 15b	17 ± 8b	175 ± 2
3.0	3.0	10	10	0.19 ± 0.02b	209 ± 28b	5 ± 1b	204 ± 3
3.0	3.0	20	10	0.20 ± 0.01b	352 ± 24a	5 ± 1b	145 ± 4
3.0	3.0	40	10	0.15 ± 0.04b	151 ± 51c	3 ± 1b	222 ±9

[a]GA: gallic acid; CAT: catechin; OLA: oleic acid; LEC: lecithin

[b] concentrations of oleic acid and lecithin as % of zein (w/w).

[c] different letters in each column show significant difference at $P<0.05$.

6.2. Development of Zein-Wax Composite and Zein-Fatty Acid Blend Films for Controlled Release of Lysozyme

Lysozyme obtained from hen egg white is one of the most potential candidates for antimicrobial packaging since it has a GRAS status and it shows good stability and activity in different films and food systems under refrigerated storage temperatures (Mecitoglu et al., 2006; Ünalan et al., 2011). Thus, lysozyme has recently been tested extensively in different plastic materials such as cellulose acetate, nylon, and PVOH (Gemili et al., 2009; Joerger, 2007) and biopolymeric materials from zein, soy protein, carrageenan, whey protein, chitosan, alginate and pullulan (Joerger, 2007; Mendes de Souza et al., 2010). But studies to develop smart controlled release mechanisms for lysozyme containing zein films are scarce. In this part of the study, bioactive zein films have been developed by incorporation of lysozyme. In order to control the release rate of lysozyme the zein film morphology was modified by incorporating wax and fatty

acid into film forming solutions by means of homogenization in presence of lecithin emulsifier. The incorporation of waxes into films gave composite films while incorporation of fatty acids into films caused formation of blend films.

6.2.1. Development of Zein-Wax Composite Films for Controlled Release of Lysozyme

It is well known that the release mechanism of many films are effected from polymer swelling occurred as a result of diffusion of water molecules into the polymeric film matrix (Mastromatteo et al., 2010). Therefore, it is commonly accepted that the incorporation of hydrophobic compounds into films retards their hydration and subsequent diffusion of active agents from their film matrix (Ouattara et al., 2000; Ozdemir & Floros, 2003). Thus, to slow down the release rate of lysozyme the hydrophobicity of zein films was increased by incorporating waxes into film forming solutions by means of homogenization in presence of lecithin emulsifier. The distribution of hydrophobic wax particles within the film matrix aimed not only to increase hydrophobicity of the films but also to increase the film tortuosity (Ozdemir & Floros, 2003). In this work, wax was added in presence of a GRAS status surface active compound soy lecithin (LEC). This aimed not only to form and distribute small wax particles within film matrix but also to maintain stability of reduced sized of wax particles during film drying.

6.2.1.1. Effects of Plasticizer Catechin on Lysozyme Release Profiles of Films

It has been reported in previous section that some phenolic compounds including catechin can be used effectively as natural plasticizer for zein films which has commercialization problems as self-standing films due to their brittleness and lack of flexibility. The interaction of hydroxyl groups of phenolic compounds with carbonyl groups of zein biopolymer creates a plasticizing effect and causes modifications in film morphology depending on the molecular properties of phenolic compounds (Alkan et al., 2011). The major morphological change caused by catechin in zein films is the reduced film porosity. Thus, effects of catechin induced morphological changes in

lysozyme release profiles of zein films was determined before evaluating the lysozyme release profiles of developed composite blend films. The release tests were conducted by using films with 0.7 mg/cm^2 lysozyme since zein films containing the indicated amount of enzyme produced by the same method had been effective on different bacteria and successfully used in a food application (Gucbilmez et al., 2007; Ünalan et al., 2011). Figure 6.15 shows that the lysozyme release from catechin plasticized zein films was much slower than those of control zein films.

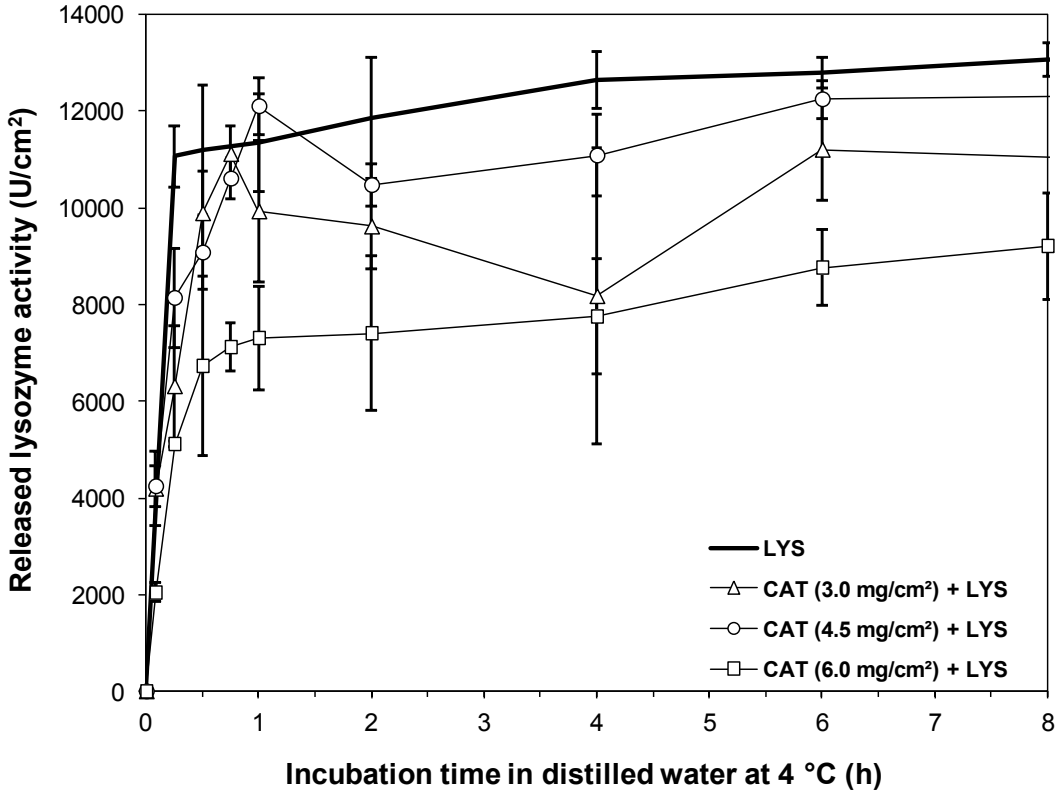


Figure 6.15. Release profiles of lysozyme from catechin plasticized zein (lysozyme concentration: 0.7 mg/cm^2; CAT: catechin, LYS: lysozyme).

The initial release rates of 3 and 4.5 mg/cm^2 catechin containing films were almost 2.5 fold lower than those of control films, while films containing catechin at 6 mg/cm^2 showed almost 4 fold lower initial release rate than the control films (Table 6.8). These results clearly showed the effect of catechin on lysozyme release profiles and supported the previous findings of this study in which reported reduced porosity of zein films by incorporation of catechin. On the other hand, the similar total amounts of lysozyme activity released from control films and different catechin containing films at

equilibrium showed the lack of enzyme trapping by possible catechin induced morphological changes (Table 6.8). This result also showed the lack of any considerable modifications in lysozyme activity due to its possible interactions with the catechin.

Table 6.8. Total soluble lysozyme activity and some kinetic parameters determined from release curves of catechin plasticized zein films

Film composition		**Initial lysozyme release rate**	**Total released lysozyme activity**
Catechin (mg/cm^2)	**Lysozyme (mg/cm^2)**	**($U/cm^2/h$)**	**(U/cm^2)**
-	0.7	44260 (0-0.25)[a]	12622±2098a[b] (72)[c]
3.0	0.7	17242 (0-0.75)	11408±2282a (72)
4.5	0.7	16873 (0-0.75)	13481±418a (72)
6.0	0.7	11517 (0-0.75)	12030±1171a (72)

[a] time periods (h) of data used in best fitting curves. r^2 of curves were between 0.6188 and 1.

[b] different letters in each column show significant difference at $P<0.05$

[c] time (h) at which the equilibrium was reached for lysozyme release

6.2.1.2. Lysozyme Release Profiles of Zein–Wax Composite Films

It is well known that the release mechanism of many films are effected from polymer swelling occurred as a result of diffusion of water molecules into the polymeric film matrix (Mastromatteo et al., 2010). Therefore, it is commonly accepted that the incorporation of hydrophobic compounds into films retards their hydration and subsequent diffusion of active agents from their film matrix (Ouattara et al., 2000; Ozdemir & Floros, 2003). Thus, to further slow down the release rate of lysozyme the hydrophobicity of catechin plasticized films was increased by incorporating carnauba wax (CAR) into film forming solutions by means of homogenization in presence of

lecithin emulsifier. The distribution of hydrophobic wax particles within the film matrix aimed not only to increase hydrophobicity of the films but also to increase the film tortuosity (Ozdemir & Floros, 2003). Figure 6.16 shows that the lysozyme release from catechin incorporated zein–CAR composite films was much slower than those of catechin plasticized control zein films. In fact, for 6 mg/cm^2 catechin containing zein–CAR composite films, the initial release rate of lysozyme is almost 4 and 17 fold lower than those of similar amount of catechin containing zein film and control zein film, respectively (Table 6.9). Similar to the catechin containing zein films, the increase of catechin concentration in zein–CAR composites reduced the lysozyme release rate effectively.

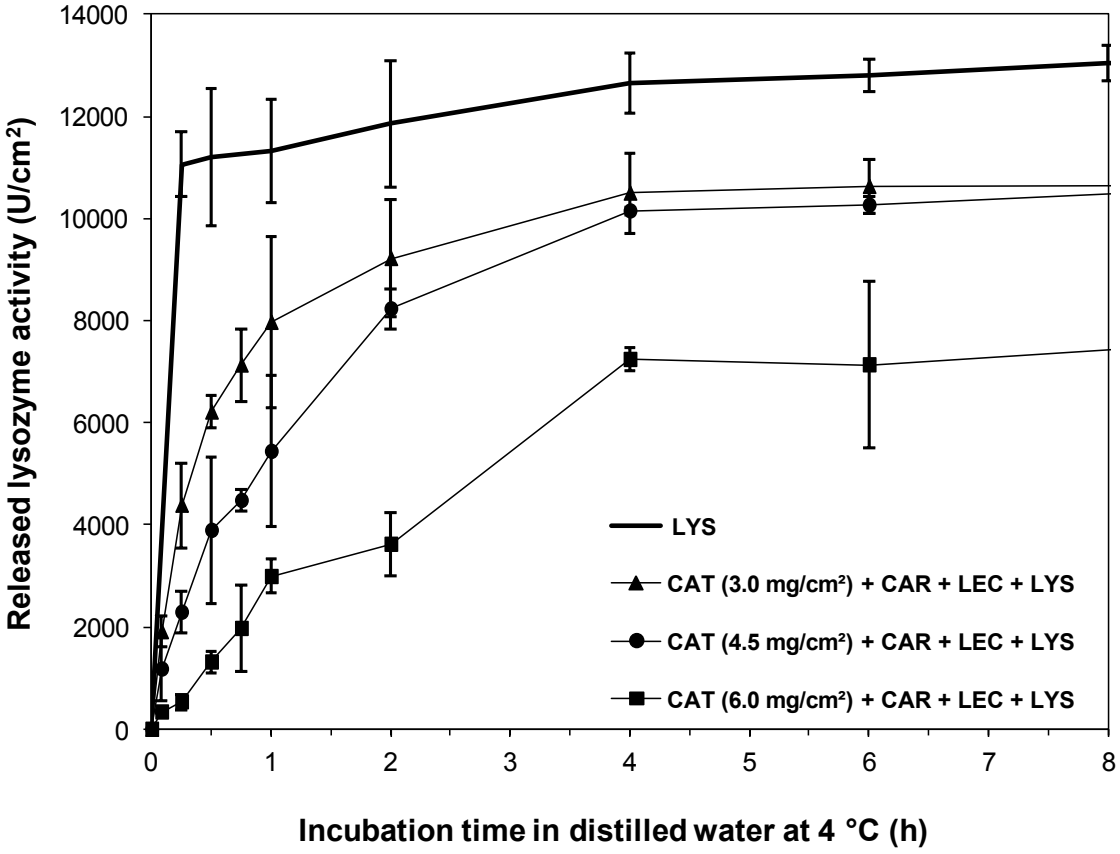


Figure 6.16. Release profiles of lysozyme from catechin plasticized zein–CAR composite films. (Lysozyme concentration: 0.7 mg/cm^2; wax and lecithin concentrations: 5% (w/w) of zein; CAT: catechin, LYS: lysozyme, CAR: carnauba wax, LEC: lecithin).

This result clearly showed the effectiveness of barriers formed against lysozyme diffusion when carnauba wax and catechin were used in film making. It seemed that the increased hydrophobicity and tortuosity of films caused by carnauba wax and reduced porosity of films caused by catechin is responsible for the effective reduction in lysozyme release rates.

Table 6.9. Total soluble lysozyme activity and some kinetic parameters determined from release curves of catechin plasticized zein–CAR composite films

Film composition				**Initial lysozyme release rate ($U/cm^2/h$)**	**Total released lysozyme activity (U/cm^2)**
Catechin (mg/cm^2)	**Lysozyme (mg/cm^2)**	**Wax (%)[a]**	**Lecithin (%)[a]**		
-	0.7	-	-	44260 (0-0.25)[b]	12622±2098a[c] (72)[d]
3.0	0.7	5 (CAR)[e]	5	11013 (0-0.75)	12603±498a (48)
4.5	0.7	5 (CAR)	5	6781 (0-0.75)	12109±1089a (72)
6.0	0.7	5 (CAR)	5	2611 (0-0.75)	11551±488a (72)

[a] concentrations of wax and lecithin as % of zein (w/w).

[b] time periods (h) of data used in best fitting curves. r^2 of curves were between 0.8458 and 1.

[c] different letters in each column show significant difference at $P<0.05$.

[d] time (h) at which the equilibrium was reached for lysozyme release.

[e] CAR: carnauba wax

The use of waxes with different melting points (MP) was also tested to evaluate possibility of creating further modifications in morphology and change lysozyme release profiles of composite films (Figure 6.17). The formation of composite film by candelilla wax (MP: 68.5-72.5 ^oC) instead of carnauba wax (MP: 82-86 ^oC) did not considerably change the release profiles of lysozyme, but the initial lysozyme release rate of zein–candelilla (zein–CAN) films was almost 1.5 fold higher than those of zein–

CAR film (Table 6.10). Moreover, the lysozyme release rate also increased considerably when beeswax (MP: 62-66 ^{o}C) was used in composites instead of candelilla wax and carnauba wax. These results clearly showed the increased lysozyme release rates from zein-wax composite films as the MP of wax reduced. This was expected since lower MP of waxes increased the efficiency of mixing and homogenization of zein with the wax and this reduced the film tortuosity which is provided with the dispersed wax particles. On the other hand, it is worth to note that the formation of zein-wax composites did not cause a considerable lysozyme trapping within the films. In zein–CAR and zein–beeswax (zein–BW) composites the released activity at the equilibrium reached 92 and 89 % of that for control film, respectively while activity released from zein–CAN composite films reached 77 % of that for control film.

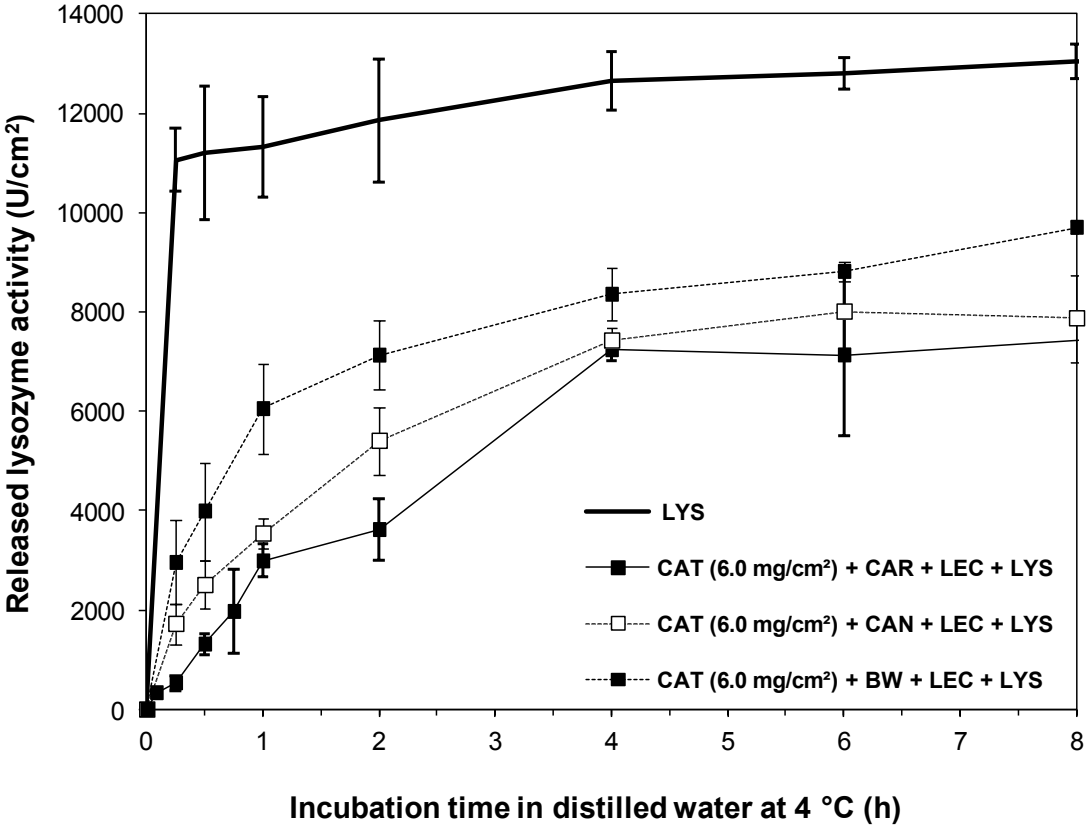


Figure 6.17. Release profiles of lysozyme from catechin plasticized zein–wax composite films. (Lysozyme concentration: 0.7 mg/cm^2; wax and lecithin concentrations: 5% (w/w) of zein; CAT: catechin, LYS: lysozyme, CAR: carnauba wax, CAN: candelilla wax, BW: beeswax, LEC: lecithin).

Table 6.10. Total soluble lysozyme activity and some kinetic parameters determined from release curves of catechin plasticized zein–wax composite films

Film composition				Initial lysozyme release rate ($U/cm^2/h$)	Total released lysozyme activity (U/cm^2)
Catechin (mg/cm^2)	Lysozyme (mg/cm^2)	Wax (%)[a]	Lecithin (%)[a]		
-	0.7	-	-	44260 (0-0.25)[b]	12622±2098a[c] (72)[d]
6.0	0.7	5 (CAR)[e]	5	2611 (0-0.75)	11551±488a (72)
6.0	0.7	5 (CAN)	5	3982 (0-1)	9751±709a (72)
6.0	0.7	5 (BW)	5	6702 (0-1)	11184±714a (72)

[a] concentrations of waxes and lecithin as % of zein (w/w).

[b] time periods (h) of data used in best fitting curves. r^2 of curves were between 0.8524 and 1.

[c] different letters in each column show significant difference at $P<0.05$.

[d] time (h) at which the equilibrium was reached for lysozyme release.

[e] CAR: carnauba wax, CAN: candelilla wax, BW: beeswax

6.2.1.3. SEM Analyses of Zein and Zein–Wax Composite Films

The SEM images of the developed films were obtained to understand the morphological changes in films occurred by plasticization with catechin and formation of composite structures. As seen in Figure 6.18 A to C, the incorporation of catechin into zein films reduced the porosity of films and gave denser films at a concentration dependant manner. These results clearly explained the reduced release rate of lysozyme in catechin containing films. It is also important to report the formation of some 1 to 3 μm sized particles and aggregates within films containing 6 mg/cm^2 catechin (Figure 6.18C). These particles and aggregates formed within the films increased when catechin at 6 mg/cm^2 was incorporated with lysozyme at 0.7 mg/cm^2 (Figure 6.18D). It seemed

that these are insoluble lysozyme aggregates, catechin crystals, or other insoluble complexes formed by interaction of different film components.

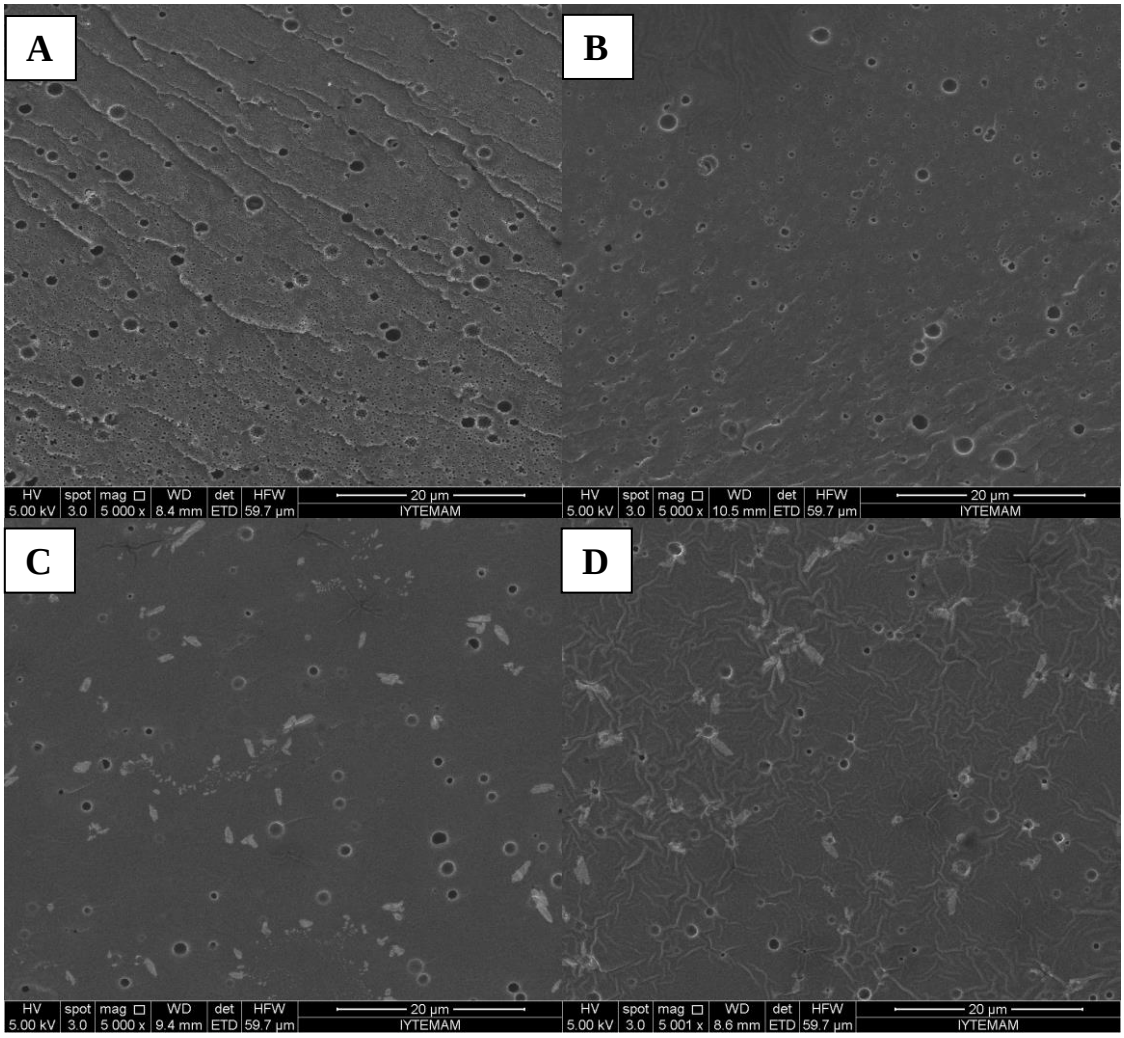


Figure 6.18. Cross-sectional SEM images of developed films: Control zein film (A); zein film containing 3 mg/cm^2 catechin (B); zein film containing 6 mg/cm^2 catechin (C); zein film containing 6 mg/cm^2 catechin and 0.7 mg/cm^2 lysozyme (D)

On the other hand, the morphological effects of composite making with different waxes are seen in Figure 6.19A to C. The addition of waxes into zein films caused dramatic changes in film morphology and formed some amorphous wax aggregates within the film matrix. These aggregates were observed most intensively in zein–CAR composites (Figure 6.19A). The wax aggregates within zein-CAN composites were less intensive than those in zein–CAR composites (Figure 6.19B), while beeswax caused the

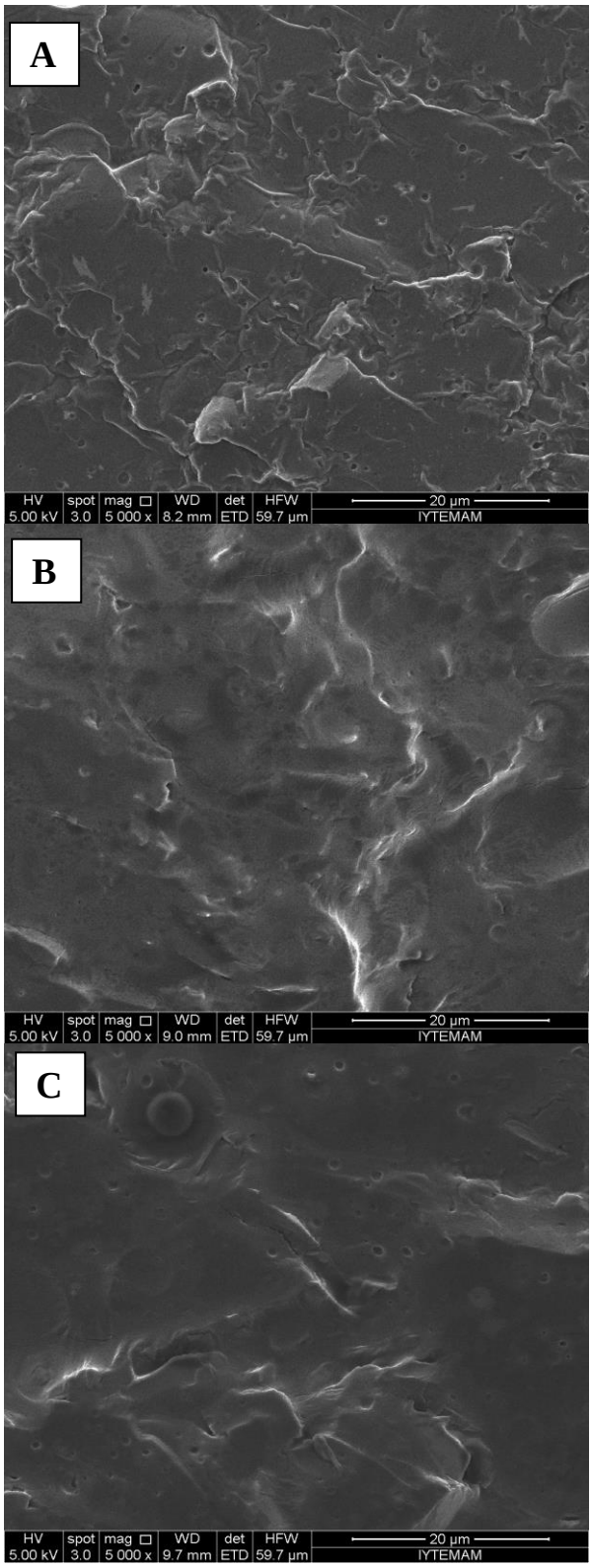


Figure 6.19. Cross-sectional SEM images of developed films: zein–CAR composite film containing 6 mg/cm^2 catechin and 0.7 mg/cm^2 lysozyme (A); zein–CAN composite film containing 6 mg/cm^2 catechin and 0.7 mg/cm^2 lysozyme (B); zein–BW composite film containing 6 mg/cm^2 catechin and 0.7 mg/cm^2 lysozyme (C)

formation of lowest amount of wax aggregates within the films (Figure 6.9C). These observations compared well with our release test results which suggested a relationship between MP of employed waxes and film tortuosity. As indicated at previous section, carnauba wax has the highest MP of all three waxes, thus more wax aggregate formation occurred within their composite films. In fact, the aggregation of carnauba wax within heated and cooled film forming solutions was clearly observed in this work during film making studies. However, the carnauba wax aggregates were distributed effectively by high speed homogenization in presence of lecithin. Due to their lower MP, candelilla and beeswax formed little and almost no aggregates following heating and cooling of film forming solutions, respectively. Thus, candelilla and beeswax were more easily distributed within the film making solutions by homogenization and formed less aggregates than carnauba wax within the matrix of final dried composite films. These results showed that the MP of waxes is a critical factor affecting the amount of insoluble wax aggregates within the matrix of composite films. Evaluation of these SEM results together with the release test results suggests that the increased intensity of wax particles is beneficial to increase film tortuosity and to reduce the release rates of lysozyme from the composite films.

6.2.1.4 Antioxidant Potential of Zein and Zein–Wax Composite Films

The test of total flavonoid concentrations in release mediums at different intervals showed the soluble catechin contents of different films (Table 6.11). In zein films incorporated with 3 to 6 mg/cm^2 catechin 59 to 64 % of the catechin existed free and solubilized during the release tests. Thus, the release of soluble catechin on food surface and a resulting antioxidant activity is expected during a potential food application. On the other hand, the remaining catechin within the films was expected to be bound to the film matrix by H bonds. This hypothesis compares well with previous findings of Alkan et al. (2011) who developped active zein films containing gallic acids and explained zein-gallic acid interaciton with H bonds formed between these components. The incorporation of waxes increased the soluble catechin content slightly since extremely hydrophobic waxes did not contain hydrogen bonding groups to interact with catechin. Thus, the highest antioxidant potential originated from free soluble catechin was calculated for zein-wax composites followed by zein films. Although the

release of the considerable portion of catechin from films is an advantage to improve antioxidant and bioactive status of packed foods, this might affect the flexibility of films during storage of actively packed foods. Further studies should be conducted to evaluate mechanical properties of developed films during food applications. However, it is clear that the use of catechin is highly beneficial to improve antioxidant and bioactive status of food and reduce problems associated with brittleness during production, storage and handling of pre-cast films before food application.

Table 6.11. Total soluble catechin concentrations and antioxidant potential of the zein and zein-wax composite films

Film composition				**Total released catechin (mg/cm^2)**	**Antioxidant potential (µmol Trolox / cm^2)**
Catechin (mg/cm^2)	**Lysozyme (mg/cm^2)**	**Wax (%)[a]**	**Lecithin (%)[a]**		
Zein films					
-	0.7	-	-	-	-
3.0	0.7	-	-	1.77±0.06a[b]	28.6
4.5	0.7	-	-	3.02±0.06c	48.7
6.0	0.7	-	-	3.83±0.08d	61.8
Zein–wax composite films					
3.0	0.7	5 (CAR)[c]	5	1.97±0.05b	31.8
4.5	0.7	5 (CAR)	5	3.19±0.02c	51.5
6.0	0.7	5 (CAR)	5	4.21±0.16e	67.9
6.0	0.7	5 (CAN)	5	4.29±0.08e	69.2
6.0	0.7	5 (BW)	5	4.28±0.09e	69.0

[a] concentrations of waxes and lecithin as % of zein (w/w).

[b] different letters in each column show significant difference at $P<0.05$

[c] CAR: carnauba wax; CAN: candelilla wax; BW: beeswax

6.2.1.5. Antimicrobial Potential of Zein and Zein–Wax Composite Films

The antimicrobial effects of the developed films were tested on *L. innocua* by using the classical disc diffusion method. The control zein film without lysozyme and catechin did not form any inhibition zones (Table 6.12). Moreover, an extensive bacterial growth was observed below control film discs when they were removed carefully from the agar. Although the antimicrobial potential of catechin against *L. monocytogenes* was reported previously (Ku et al., 2008b), no inhibition zones were observed for the films containing catechin. However, no bacterial growth was observed below catechin containing disks and this indicated a limited antilisterial effect of this phenolic compound at the studied concentration. In contrast, all other films containing lysozyme showed strong antimicrobial effect on *L. innocua* and formed clear zones around their discs. Although, there are slight differences among their zone area, no significant differences were determined among the antimicrobial performances of zein, and zein-wax composite films containing lysozyme or lysozyme and catechin ($P>0.05$). This result confirmed our release tests which did not indicate any significant trapping of lysozyme within the films by composite and blend formation.

Table 6.12. Antimicrobial potential of zein based composite and blend films

Film composition				Average zone area (mm^2)
Catechin (mg/cm^2)	**Lysozyme (mg/cm^2)**	**Wax (%)[a]**	**Lecithin (%)[a]**	
-	-	-	-	*bacterial growth under the disc
-	0.7	-	-	119 ± 26a[b]
6.0	-	-	-	*no bacterial growth under the disc
6.0	0.7	-	-	115 ± 23a
6.0	0.7	5 (CAR)[c]	5	89 ± 46a
6.0	0.7	5 (CAN)	5	90 ± 10a
6.0	0.7	5 (BW)	5	127 ± 25a

[a] concentrations of waxes and lecithin as % of zein (w/w).

[b] different letters in column show significant difference at $P<0.05$

[c] CAR: carnauba wax; CAN: candelilla wax; BW: beeswax

6.2.1.6. Mechanical Properties of Zein and Zein–Wax Composite Films

In order to analyze their mechanical properties tensile strength at break, elongation at break, and Young's modulus values of films were determined (Table 6.13). The control zein films lacking lysozyme and catechin showed very little elongation, but the tensile strength value of the control films at the breaking point (17.67 MPa) was significantly higher than those of the catechin plasticized films (0.93-3.23 MPa) and composite and blend films (0.77-1.11 MPa) (P<0.05). The addition of lysozyme alone reduced the tensile strength and Young's modulus of zein films significantly (P<0.05), but it did not cause any significant change in film elongation (P>0.05). In contrast, the addition of catechin effectively plasticized zein films and improved their elongation significantly. Similar to the previous findings, the plasticizing effect of catechin was concentration dependent since the increase of catechin concentration in zein films from 3 to 6 mg/cm^2 increased film elongation almost 5 fold. To understand the possible mechanism of plasticization with catechin, the zein film structure should be discussed in more details. It has been recently shown that the zein films consist of a meshwork which is composed of doughnut structures formed by asymmetric rods joined to each other (Guo et al., 2005). The hydrophobic interactions among asymmetric rods is the primary force which keep them together and maintain the film integrity (Guo et al., 2005), but these interactions are also responsible for the brittleness and lack of flexibility in zein films. It was assumed that the formation of hydrogen bonds between the hydroxyl groups of catechin and the carbonyl group of zein protein formed a week but an elastic film network. Moreover, the increased number of phenolic hydroxyl groups provided with free catechin increased the hydrophilicity of the films. Thus, the hydrophobic interactions are weakened and an elastic network with more mobile zein molecules is formed. This hypothesis is in line with that of previous findings which showed the plasticizing effect of phenolic compounds such as catechin, gallic acid, p-hydroxy benzoic acid, and ferulic acid on zein. On the other hand, the formation of zein-wax composites in presence of lysozyme and catechin caused a significant reduction in film elongation (P<0.05). However, the composites films are still much more flexible than the controls. This result confirmed the recent findings of Alkan et al. (2011) who tested mechanical properties of zein and zein-wax composites

containing gallic acid. Fabra et al. (2008) also reported reduction in elongation of wax containing caseinate films and attributted this effect to discontinuities in the polymer network by the incorporated waxes. However, it is important to note that the zein-CAR composites showed more elongation than zein-CAN and zein-BW composite films. On the other hand, no significant change occurred in tensile strength and Young's modulus values of the films by addition of waxes to form a composite or blend film structure (P>0.05). These results contradicted with Sohail et al. (2006) who incorporated paraffin wax into casein-zein hydrolizate films and reported a reduced tensile strength for these composite films. The effects of waxes on tensile strengths of zein films were also different than casein films which showed an increase in their tensile strengths by addition of carnauba or candelilla waxes (Chick & Hernandez, 2002). These reports showed that the mechanical changes in protein-wax composite structures are complex. It appears that the mechanical changes in composite systems could be highly variable depending on amounts and molecular properties of each constituent in the mixture and degree of their compatibility and interactions within the films.

Table 6.13. Mechanical properties of zein and zein–wax composite films

Film compositions[a]				**Tensile strength at break (MPa)**	**Elongation at break (%)**	**Young's modulus (MPa)**	**Film thickness (μm)**
CAT (mg/cm^2)	**LYS (mg/cm^2)**	**WAX (%)[b]**	**LEC (%)[b]**				
-	-	-	-	17.67 ± 0.93a[c]	4 ± 1f	775 ± 44a	113 ± 1
3.0	-	-	-	3.23 ± 0.58c	33 ± 5e	167 ± 40c	191 ± 3
6.0	-	-	-	0.93 ± 0.20d	172 ± 23a	31 ± 7d	186 ± 3
-	0.7	-	-	13.80 ± 1.26b	3 ± 1f	670 ± 45b	138 ± 1
6.0	0.7	-	-	1.02 ± 0.21d	136 ± 26b	42 ± 8d	189 ± 2
6.0	0.7	5(CAR)[d]	5	1.11 ± 0.08d	95 ± 7c	66 ± 6d	176 ± 2
6.0	0.7	5(CAN)	5	0.89 ± 0.08d	65 ± 15d	56 ± 6d	179 ± 2
6.0	0.7	5(BW)	5	0.77 ± 0.07d	62 ± 18d	39 ± 9d	172 ± 2

[a] CAT: catechin; LYS: lysozyme; LEC: lecithin

[b] concentrations of waxes, and lecithin as % of zein (w/w).

[c] different letters in each column show significant difference at P<0.05

[d] CAR: carnauba wax; CAN: candelilla wax; BW: beeswax

6.2.2. Development of Zein–Fatty Acid Blend Films for Controlled Release of Lysozyme

Not only waxes but also fatty acids, acetylated monoglycerides and essential oils were used to modify hydrophobicity and morphology of films and change their release profiles for the active compounds (Ouattara et al., 2000). In section 6.1.3, it was showed that catechin release could be manipulated by development of zein-oleic acid (zein–OLE) blend films. The obtained controlled release properties were attributed to multiple factors including increased hydrophobicity of films, and morphological changes in films formed by blend film making. The morphological changes affecting the release properties of zein–OLE blend films were related to the formation of extensive spherical zein capsules which increased the diffusion barriers for catechin. Thus, in this part of the study the lysozyme release profiles of blends formed by homogenization of zein with fatty acids in the presence of lecithin emulsifier were investigated. To evaluate the potential of blend films as an antimicrobial packaging material with controlled release properties two different lysozyme with different purity level were used in film making. Commercial lysozyme (C-lysozyme) has very high purity level with high in vitro activity (71875 U/mg powder), while partially purified lysozyme (PP-lysozyme) has low in vitro activity (12548 U/mg powder) because of the possible presence of impurities.

6.2.2.1. Effects of Oleic Acid and Lecithin on Partially Purified Lysozyme Release Properties of Zein–Fatty Acid Blend Films

In order to understand the effects of catechin and oleic acid on initial lysozyme release rates, the percentages of these ingredients were changed while the lecithin concentration was kept constant. Similar to the zein-wax composite films, the release experiments were started with using zein films containing 0.7 mg/cm^2 PP-lysozyme. The Figure 6.20 shows that lysozyme release from catechin plasticized blend films are much slower than those of control zein film. The initial lysozyme release rates of zein–OLE blends plasticized with 3.0 and 6 mg/cm^2 catechin were 2-4 and 7-14 folds lower than those of unplasticized control zein film, respectively. Similar to the previous finding, the increase– of catechin concentration in zein–OLE reduced the lysozyme

release rate effectively while the oleic acid content of the film was kept constant. This result revealed that the catechin has a concentration dependent critical role in formation of morphological changes important for the controlled release properties of blend films. On the other hand, in films containing catechin at 3 or 6 mg/cm^2, increasing the oleic acid content of the films from 5% to 10% reduced the initial lysozyme release rates of blend films too (Table 6.14). However, the release tests also showed that increasing the oleic acid content of the films was less effective on decreasing the initial lysozyme release rate than increasing the catechin content of the films since increasing the oleic acid content of the blend films caused almost 2 fold reduction in initial lysozyme release rate while increasing catechin content of the blends caused more than 3 fold reduction. But using higher concentration of catechin or oleic acid increased the lysozyme trapping and gave significantly lower soluble enzyme activity (P>0.05).

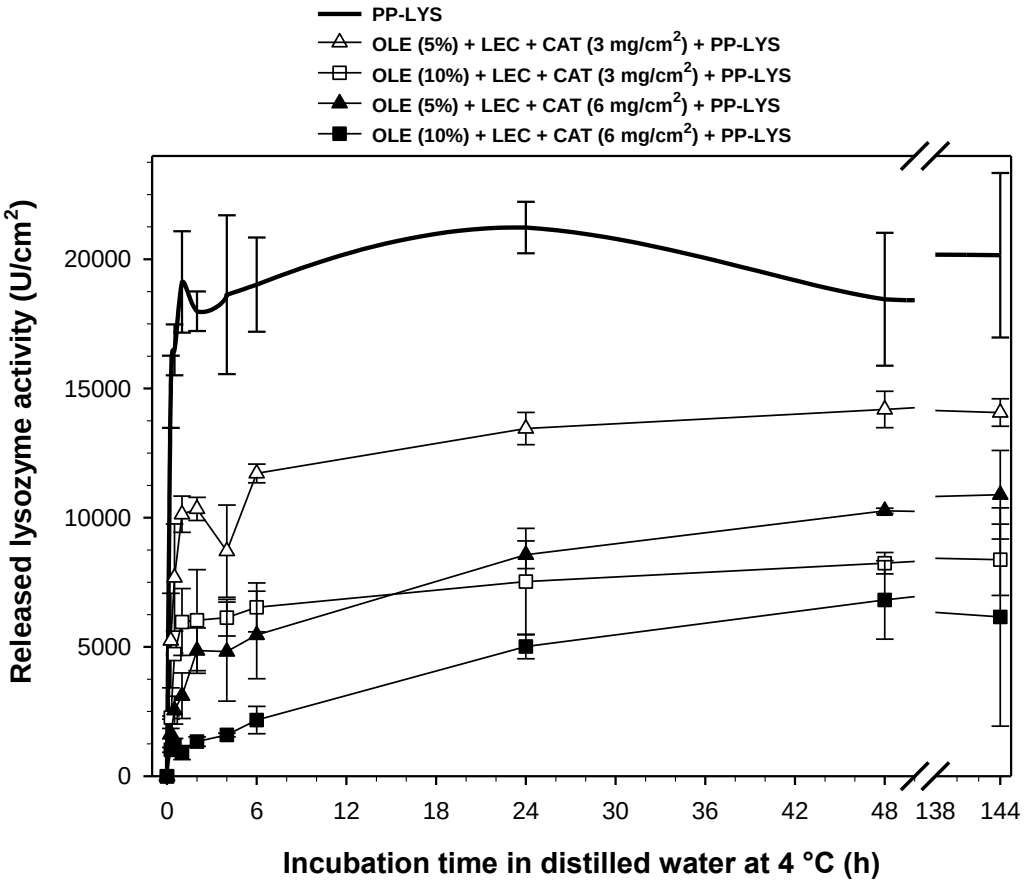


Figure 6.20. Release profiles of PP-LYS from catechin plasticized zein–OLE blend films. (PP-LYS concentration: 0.7 mg/cm^2; lecithin concentrations: 10% (w/w) of zein; CAT: catechin, PP-LYS: partially purified lysozyme, OLE: oleic acid, LEC: lecithin).

Table 6.14. Total soluble lysozyme activity and some kinetic parameters determined from release curves of catechin plasticized zein–OLE blend films

Film composition[a]				Initial lysozyme release rate	Total released lysozyme activity
PP-LYS (mg/cm²)	**CAT (mg/cm²)**	**OLE (%)[b]**	**LEC (%)[b]**	**(U/cm²/h)**	**(U/cm²)**
0.7	-	-	-	38289[c] (0-0.5)[d]	21226±994a[e] (24)[f]
0.7	3.0	5	10	16493 (0-0.5)	15023±895b (72)
0.7	3.0	10	10	9370 (0-0.5)	9062±1563c (72)
0.7	6.0	5	10	5400 (0-0.5)	10892±1713c (144)
0.7	6.0	10	10	2726 (0-0.5)	8319±1168c (72)

[a] PP-LYS: lysozyme; CAT: catechin; OLE: oleic acid; LEC: lecithin

[b] concentrations of oleic acid and lecithin as % of zein (w/w).

[c] determined from the slope of the initial linear portion of release curves.

[d] time periods (h) of data used in best fitting curves. r^2 of curves were between 0.7876 – 0.9994.

[e] different letters in each column show significant difference at $P<0.05$

[f] time (h) at which the equilibrium was reached for lysozyme release.

It seemed that the barriers formed against lysozyme diffusion when oleic acid and catechin were used in film making reduced the initial lysozyme release rates effectively. However, the total released enzyme activity from films containing PP–lysozyme was too low to obtain proper antimicrobial activity in real food systems due to entrapment of the enzymes in film matrix. Therefore the release tests were conducted by using films with 1.4 mg/cm² PP–lysozyme to obtain effective optimum soluble activity even in the film compositions showed partial enzyme entrapment.

6.2.2.2. Partially Purified Lysozyme Release Profiles of Zein–Fatty Acid Blend Films

It has been reported in previous section that some phenolic compounds including catechin can be used effectively as natural plasticizer for zein films which has commercialization problems as self-standing films due to their brittleness and lack of flexibility. The interaction of hydroxyl groups of phenolic compounds with carbonyl groups of zein biopolymer creates a plasticizing effect and causes modifications in film morphology depending on the molecular properties of phenolic compounds (Alkan et al., 2011). The major morphological change caused by catechin in zein films is the reduced film porosity. Thus, effects of catechin induced morphological changes in lysozyme release profiles of zein films was determined before evaluating the lysozyme release profiles of developed blend films. Figure 6.21 shows that the lysozyme release from catechin plasticized zein films are much slower than those of control zein film. The initial lysozyme release rates of 6 mg/cm² catechin containing film was almost 3 fold lower than that of control films containing PP–lysozyme (Table 6.15). These results clearly showed the effect of catechin on lysozyme release profiles and supported the previous findings that reported the reduced lysozyme release rate from zein films by catechin induced porosity reduction in film matrix.

For the films containing PP–lysozyme, the initial release rate of enzyme from catechin plasticized zein–OLE blend films were lower than those of control and catechin plasticized zein films (Figure 6.21). In addition to that, the initial lysozyme release rate was decreased as the oleic acid concentration in film composition increased (Table 6.15). Results clearly showed that, for catechin plasticized blend film containing 20% oleic acid the initial lysozyme release rate was almost 9 and 3 fold lower than those of control and catechin plasticized zein films. On the other hand, no significant differences were determined among the total released enzyme activity of zein-fatty acid blend films and zein films plasticized with catechin ($P>0.05$).

Furthermore, to investigate the effects of oleic acid on the lysozyme release profiles, zein–OLE blend film was produced without the catechin incorporation with selected OLE concentration (10% (w/w) of zein). Figure 6.21 shows that the release profiles of lysozyme from unplasticized zein–OLE blend films are not much differ from that of control films. Moreover, the initial lysozyme release rate was slightly increased

in zein–oleic film containing PP-lysozyme. These results were in conflicted with the findings of Ouattara et al. (2000), who reported reduction in diffusion coefficient of acetic acid from chitosan film by addition of fatty acid lauric acid. Therefore, increasing the hydropobicity of the zein films by adding oleic acid could not be the major diffusion barrier against lysozyme release.

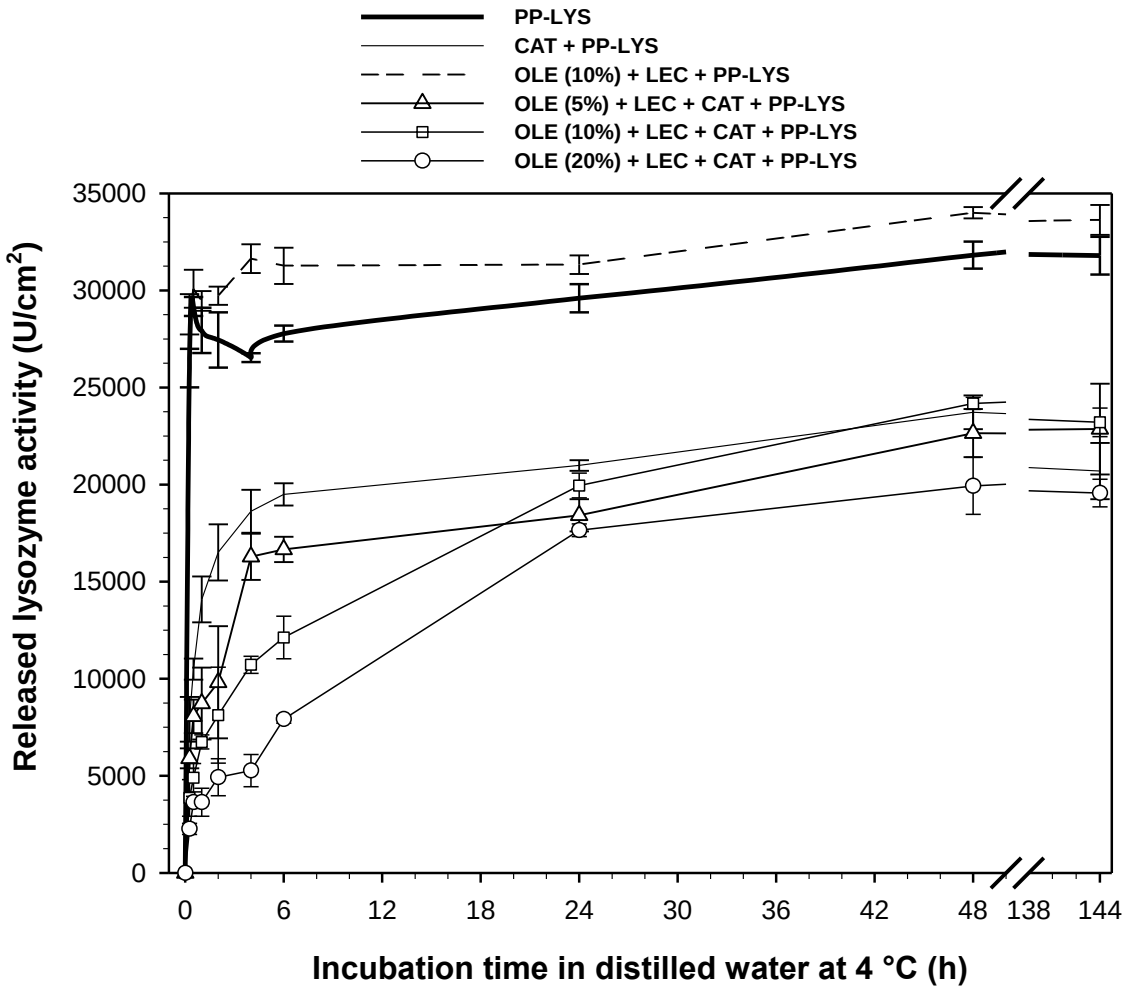


Figure 6.21. Release profiles of PP-LYS from catechin plasticized zein–OLE blend films. (PP-LYS concentration: 1.4 mg/cm^2; catechin concentrations: 6.0 mg/cm^2; lecithin concentrations: 10% (w/w) of zein; OLE: oleic acid, LEC: lecithin, CAT: catechin, PP-LYS: partially purified lysozyme).

Table 6.15. Total soluble lysozyme activity and some kinetic parameters determined from release curves of catechin plasticized zein–OLE blend films

Film composition[a]				Initial lysozyme release rate	Total released lysozyme activity
PP-LYS (mg/cm²)	**CAT (mg/cm²)**	**OLE (%)[b]**	**LEC (%)[b]**	**(U/cm²/h)**	**(U/cm²)**
1.4	-	-	-	67472[c] (0-0.5)[d]	32501±723a[e] (72)[f]
1.4	-	10	10	71152 (0-0.5)	34002±294a (48)
1.4	6.0	-	-	23124 (0-0.5)	23725±867bc (48)
1.4	6.0	5	10	17623 (0-0.5)	22861±2337bc (72)
1.4	6.0	10	10	10944 (0-0.5)	25011±1718b (72)
1.4	6.0	20	10	7632 (0-0.5)	20972±886c (72)

[a] PP-LYS: lysozyme; CAT: catechin; OLE: oleic acid; LEC: lecithin

[b] concentrations of oleic acid and lecithin as % of zein (w/w)

[c] determined from the slope of the initial linear portion of release curves

[d] time periods (h) of data used in best fitting curves. r^2 of curves were between 0.7392 – 0.9763

[e] different letters in each column show significant difference at $P<0.05$

[f] time (h) at which the equilibrium was reached for lysozyme release

The use of fatty acids with different aliphatic chain length was also tested to evaluate possibility of creating further modifications in morphology and lysozyme release profiles of blend structures (Figure 6.22). The formation of blend film by lauric acid (LAU) instead of oleic acid did not considerably change the release profiles of lysozyme, but the initial lysozyme release rate of zein–LAU films was almost 1.5 fold higher than those of zein–OLE blend film (Table 6.16). On the other hand, similar

initial lysozyme release rates were obtained by incorporating oleic acid ($C_{18:1}$) and linoleic acid ($C_{18:2}$; LINO) that have same aliphatic chain length (Figure 6.23). So the addition of lauric acid (C_{12}) which has shorter aliphatic chain length was less effective on decreasing the initial lysozyme release rate. These results clearly showed the lysozyme release rates from zein-fatty acid blend films reduced as the length of the aliphatic chain of fatty acid increased.

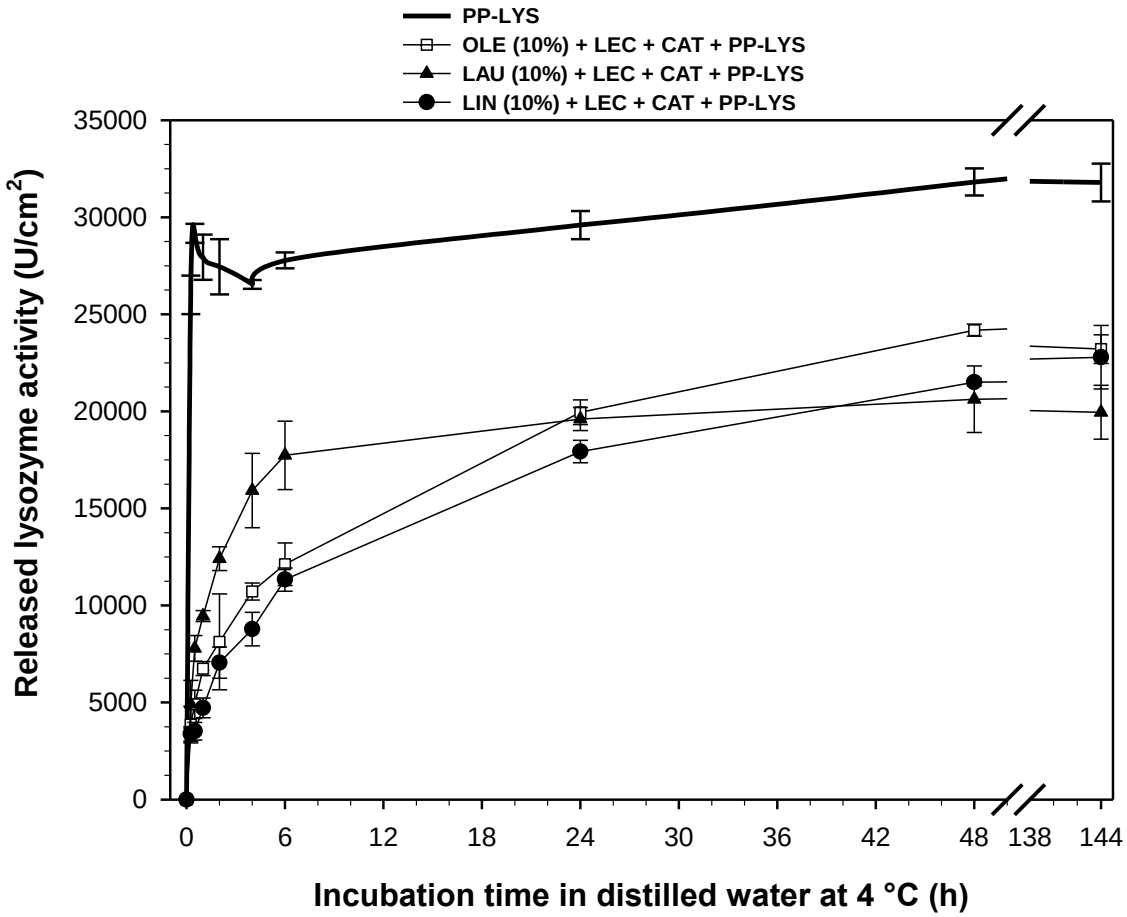


Figure 6.22. Release profiles of PP-lysozyme from catechin plasticized zein–oleic acid blend films. (PP-lysozyme concentration: 1.4 mg/cm^2; catechin concentrations: 6.0 mg/cm^2; fatty acid and lecithin concentrations: 10% (w/w) of zein; OLE: oleic acid, LAU: lauric acid; LIN: linoleic acid; LEC: lecithin, CAT: catechin, PP-LYS: partially purified lysozyme).

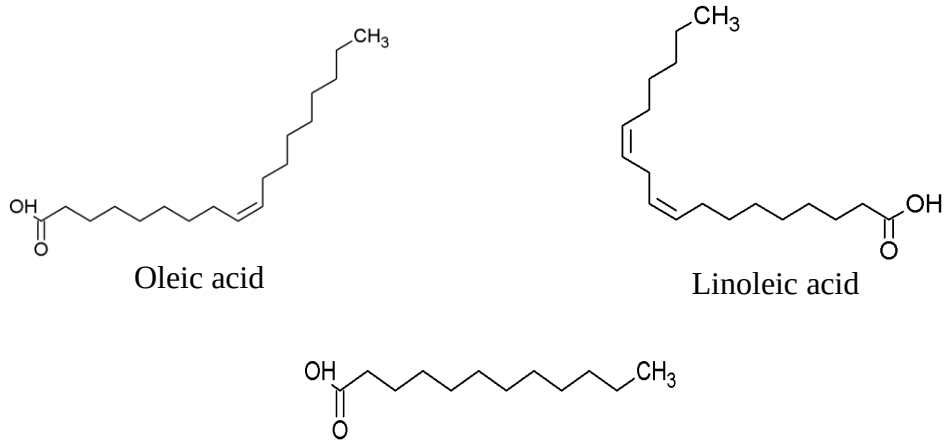


Figure 6.23. Chemical structures of fatty acids used in blend film composition

Table 6.16. Total soluble lysozyme activity and some kinetic parameters determined from release curves of catechin plasticized zein–fatty acid blend films

Film composition[a]				**Initial lysozyme release rate**	**Total released lysozyme activity**
PP-LYS (mg/cm²)	**CAT (mg/cm²)**	**FA (%)**[b]	**LEC (%)**[b]	**(U/cm²/h)**	**(U/cm²)**
1.4	-	-	-	67472[c] (0-0.5)[d]	32501±723a[e] (72)[f]
1.4	6.0	10(OLE)[f]	10	10944 (0-0.5)	25011±1718b (72)
1.4	6.0	10(LAU)	10	16387 (0-0.5)	20941±1301c (72)
1.4	6.0	10(LIN)	10	8346 (0-0.5)	22789±1632bc (72)

[a] PP-LYS: lysozyme; CAT: catechin; FA: fatty acid; LEC: lecithin

[b] concentrations of fatty acids and lecithin as % of zein (w/w)

[c] determined from the slope of the initial linear portion of release curves

[d] time periods (h) of data used in best fitting curves. r^2 of curves were between 0.7392 – 0.9763

[e] different letters in each column show significant difference at $P<0.05$

[f] time (h) at which the equilibrium was reached for lysozyme release

[f] OLE: oleic acid, LAU: lauric acid, LIN: linoleic acid

6.2.2.3. Commercial Lysozyme Release Profiles of Zein–Fatty Acid Blend Films

C–lysozyme content of the zein films was set to 0.7 mg/cm² because it showed much higher in vitro activity (71875 U/mg powder) than that of partially purified one (12548 U/mg powder). Figure 6.24 shows that the lysozyme release from catechin plasticized zein films are much slower than those of control zein film. The initial lysozyme release rates of 6 mg/cm² catechin containing film was almost 2 fold lower than that of control films containing C–lysozyme. So it can be concluded that, catechin induced morphological changes were also very effective on C-lysozyme release from zein films. Moreover, due to the interaction between lysozyme and catechin partial enzyme entrapment was observed for C-lysozyme since the total released lysozyme activity at the equilibrium reached to 73 and 82 % that of catechin plasticized control films (Table 6.17). In addition to that, much slower initial release rate of lysozyme was also obtained than those of control and catechin plasticized zein films by developing zein–OLE blend films (Fig. 6.24). Moreover, similar to blend films containing PP-lysozyme, the initial lysozyme release rate of zein–OLE blend films containing C–lysozyme decreased when the oleic acid concentration increased from 5% to 10%. However, increasing the oleic acid concentration to 20% did not affect the initial lysozyme release rate further (Table 6.17). In addition to that, the addition of oleic acid did not cause significant amount of enzyme activity trapping in films containing C–lysozyme (P>0.05). These results also showed that zein–OLE blend films were more effective on PP–lysozyme than C–lysozyme to slow down the initial release rates of enzymes. The differences between the effects of oleic acid on PP– and C–lysozyme could be explained by the presence of impurities in enzyme powder. The possible presence of proteins beside enzyme in PP-lysozyme powder (Jin, Davidson, Zivanovic, & Zhong, 2009), could also affect the release profiles of lysozyme from zein films (Gucbilmez et al., 2007).

The effects of blend film making on the initial release rate of lysozyme was also related the type of fatty acid used in film composition. Although the release profiles of lysozyme did not affected by the type of fatty acid used in film compositions (Figure 6.24), the initial lysozyme release rate was higher when lauric acid used in film instead of oleic acid and linoleic acid (Table 6.17). These results clearly showed that, similar to

the zein-fatty acid blend films containing PP-lysozyme, the effects of the fatty acid on C-lysozyme release profiles was also depended on the aliphatic chain of fatty acids.

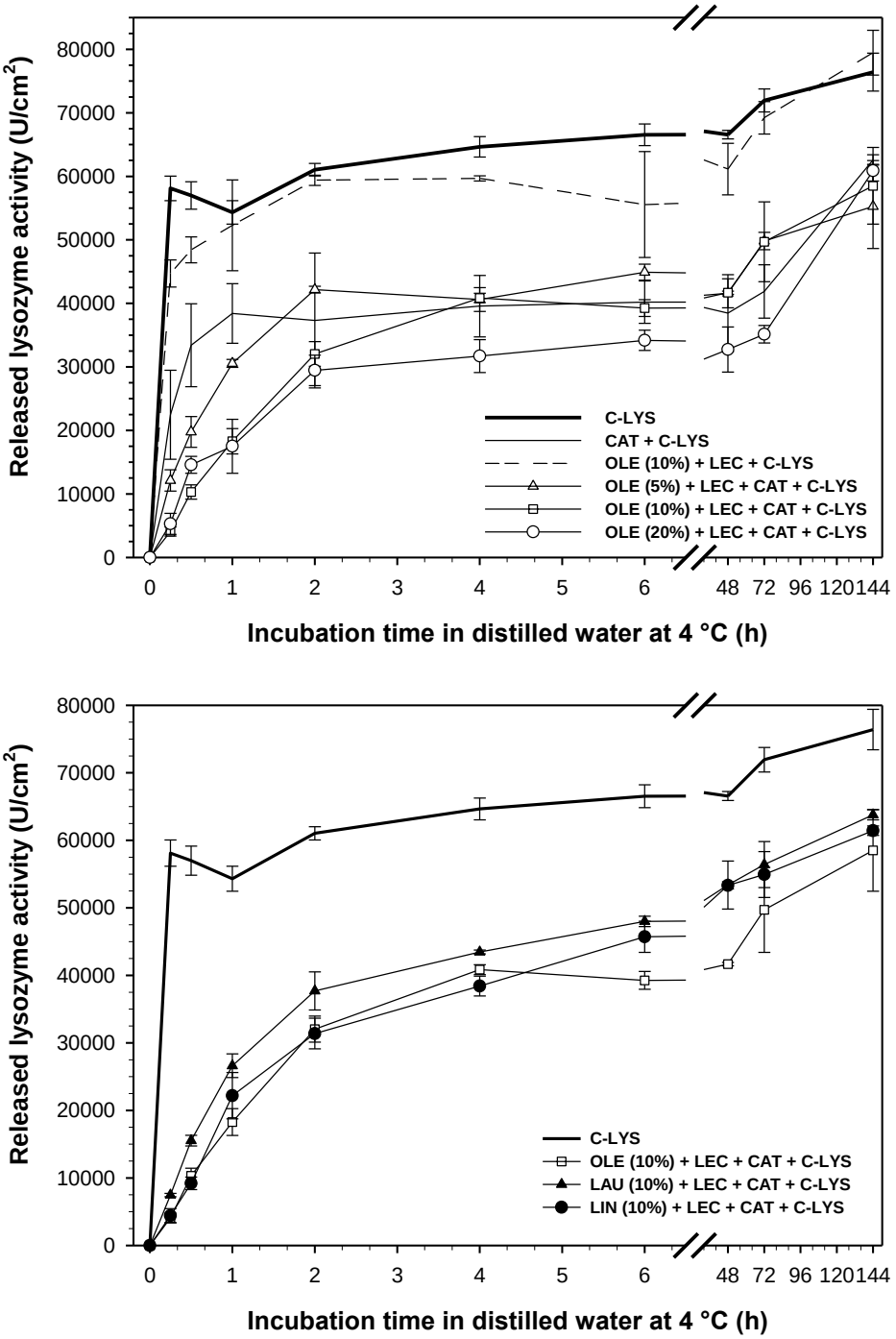


Figure 6.24. Release profiles of C-lysozyme from zein-fatty acid blend films (C-lysozyme concentration: 0.7 mg/cm^2; catechin concentration: 6.0 mg/cm^2; lecithin concentrations: 10% (w/w) of zein; CAT: catechin, C-LYS: C-lysozyme, OLE: oleic acid, LAU: lauric acid, LIN: linoleic acid, LEC: lecithin; C-LYS: commercial lysozyme).

Table 6.17. Total soluble lysozyme activity and some kinetic parameters determined from release curves of catechin plasticized zein–fatty acid blend films

Film composition[a]				Initial lysozyme release rate	Total released lysozyme activity
C-LYS (mg/cm²)	**CAT (mg/cm²)**	**FA (%)[b]**	**LEC (%)[b]**	**(U/cm²/h)**	**(U/cm²)**
0.7	-	-	-	137648[c] (0-0.5)[d]	76408±2987a[e] (144)[f]
0.7	-	10(OLE)[g]	10	113245 (0-0.5)	79468±3524a (144)
0.7	6.0	-	-	71418 (0-0.5)	62576±810b (144)
0.7	6.0	5(OLE)	10	41314 (0-0.5)	55255±6622b (144)
0.7	6.0	10(OLE)	10	19762 (0-0.5)	58513±6041b (144)
0.7	6.0	20(OLE)	10	19863 (0-0.5)	60868±1608b (144)
0.7	6.0	10(LAU)	10	30798 (0-0.5)	63786 ±749b (144)
0.7	6.0	10(LIN)	10	18249 (0-0.5)	61427 ±696b (144)

[a] C-LYS: commercial lysozyme; CAT: catechin; FA: fatty acid; LEC: lecithin

[b] Concentrations of oleic acid and lecithin as % of zein (w/w)

[c] Determined from the slope of the initial linear portion of release curves

[d] Time periods (h) of data used in best fitting curves. r^2 of curves were between 0.7392 – 0.9763

[e] Different letters in each column show significant difference at $P<0.05$

[f] Time (h) at which the maximum activity was reached for lysozyme release

[g] OLE: oleic acid, LAU: lauric acid, LIN: linoleic acid

6.2.2.4. SEM Analysis of Zein and Zein–Fatty Acid Blend Films

The SEM cross-sectional images of the developed films were obtained to understand the morphological changes in films derived by plasticization with catechin and formation of blend structures. As seen in Figure 6.25A, zein film containing PP–lysozyme had very porous structure. According to the findings of Wang et al. (2005), solvent evaporation was the driving force of the formation of highly porous structure; and this clearly explained the rapid released of lysozyme from zein films. As seen in Figure 6.25B, the incorporation of catechin into zein films reduced both the number and size of the pores and gave denser films. Catechin induced morphological changes were also observed on the surface (glass side) of the films (Figure 6.25C-D). Morphological changes observed in film structure clearly explained the reduced release rate of lysozyme in films containing catechin. On the other hand, the addition of fatty acids into zein films plasticized with catechin caused dramatic changes in film morphology and formed spherical capsules (microspheres) (Figure 6.26A-C). The existences of microspheres were also observed at the surface of the blend film as spherical pits (Figure 6.26D-F). The formation of micro sized zein spheres had been previously observed in zein solution after ethanol evaporation by Wang and Padua (2010, 2012). However, at high protein concentrations, as used in the present study, film structure was developed by coagulation of microspheres (Wang & Padua, 2010). In addition to concentration parameter, the amphiphilic characteristic of zein solution is also critical for the formation of microspheres (Wang, Su, Schulmerich, & Padua, 2013). Thus, in this study, an increase in the formation of microspheres with the addition of amphiphilic fatty acids and lecithin was expected. In fact, the morphological changes of film structure when zein mixed with OLE without use of lecithin was recently explained by Wang et al. (2008). According to these researchers the morphological changes in zein–OLE system occurred at three steps; (1) formation of large numbers of OLE coated zein spheres, (2) partial melting of the spheres by means of OLE, and (3) transformation of a sponge–like morphology by interconnection of spheres with channels and tunnels. The spheres observed by SEM of zein–fatty acid blend films in this work lacked the interconnections specified by Wang et al. (2008) (Figure 6.26A-C). Thus, it seemed that the lecithin emulsifier used in this work stabilized the fatty acid coating formed around zein spheres.

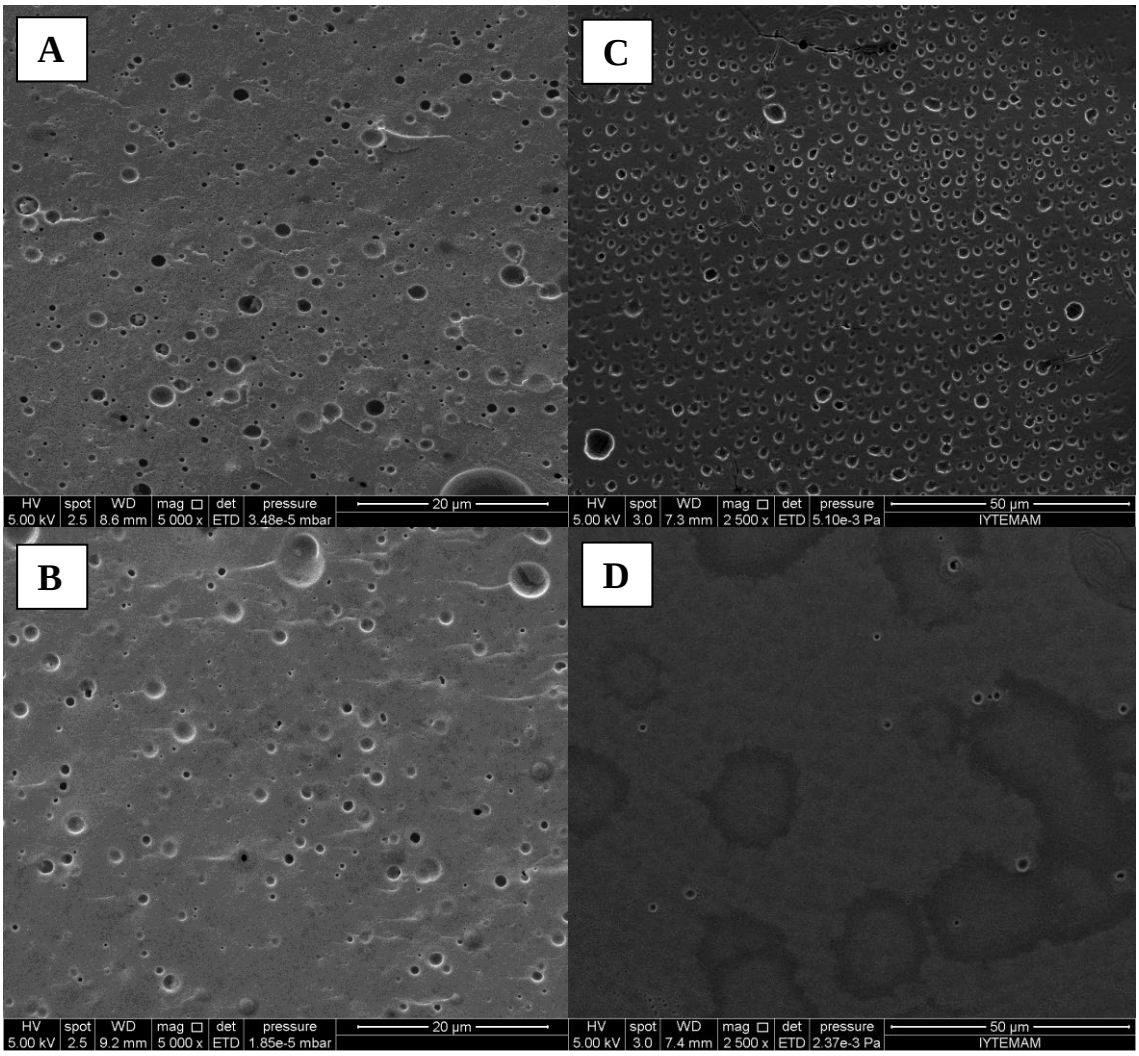


Figure 6.25. Cross-sectional and surface SEM images of the developed films: zein film containing PP-lysozyme (1.4 mg/cm^2)(A, C); zein film containing catechin (6 mg/cm^2) and PP-lysozyme (1.4 mg/cm^2) (B, D)

It appears that the repulsion formed by negative charges of lecithin at the fatty acid coating of formed zein spheres prevented the interaction and melting down of these spheres which formed the sponge–like structure described by Wang et al. (2008). Thus, it appeared that the reduced release rates of lysozyme in catechin containing zein–fatty acid blend films are in part due to the entrapment of lysozyme within spherical capsules which increased the barriers against enzyme diffusion. This model also explained the differences in the effectiveness of fatty acids on release rate of lysozyme. As indicated at Section 3.1.2, LAU has the smallest aliphatic chain length of all three fatty acid and gave higher initial lysozyme release rate. When LAU used in blend film compositions, an unstable coating layer around the zein microspheres with low

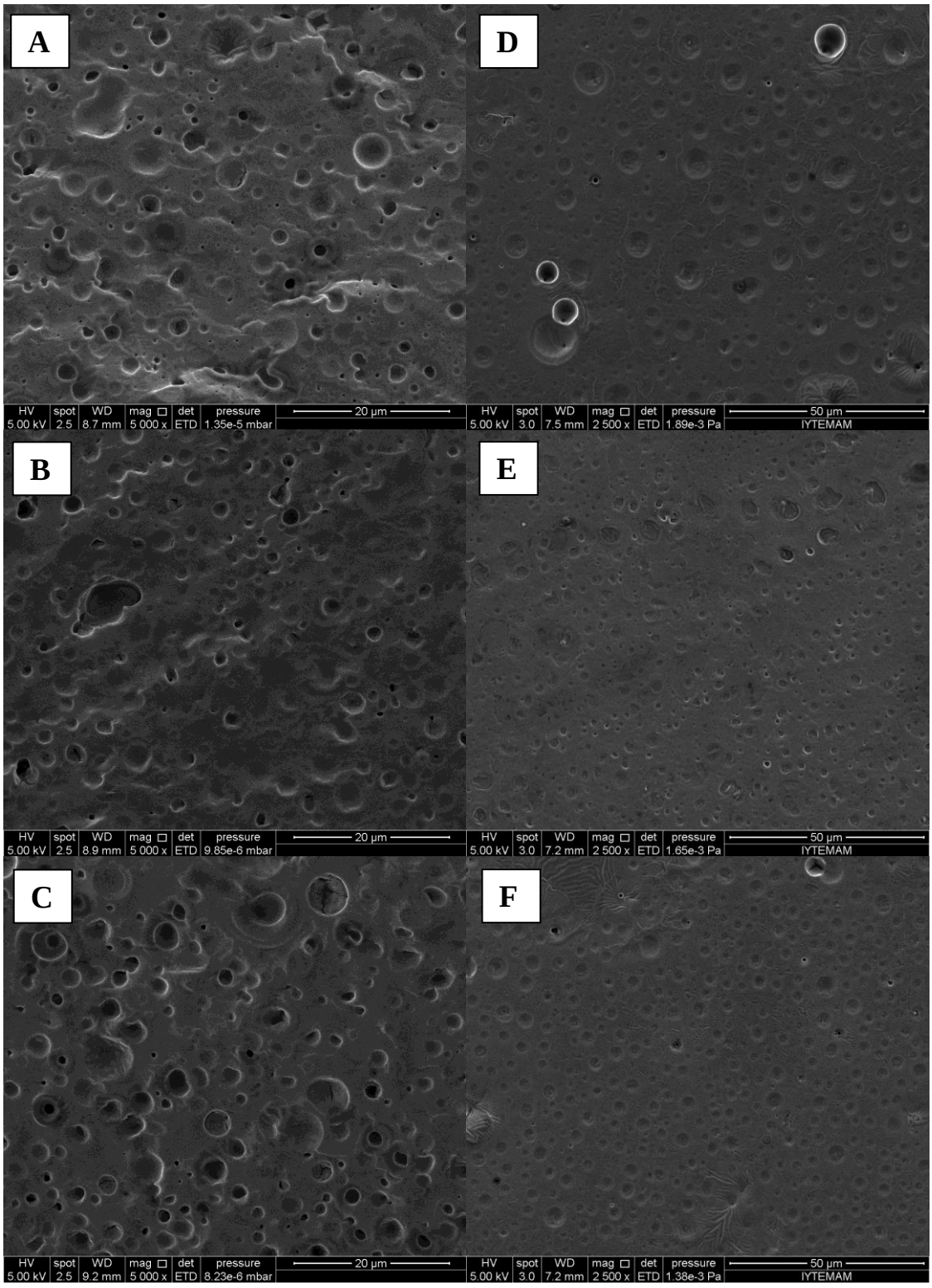


Figure 6.26. Cross-sectional and surface SEM images of the zein-fatty acid blend films: blend film containing OLE (10%), catechin (6 mg/cm^2) and PP-lysozyme (1.4 mg/cm^2) (A, D); blend film containing LAU (10%), catechin (6 mg/cm2) and PP-lysozyme (1.4 mg/cm^2) (B, E); blend film containing LIN (10%), catechin (6 mg/cm^2) and PP-lysozyme (1.4 mg/cm^2) (C, F)

hydrophobicity could be formed that might showed relatively low barrier properties against enzyme diffusion than that of other fatty acids. On the other hand, OLA and LIN could form more rigid and complex coating layer around the zein microspheres since they got a non-linear molecular shape (Fig. 1B and D) which might increase the interactions within the coating layers. Therefore it could be concluded that, the permeability of the coating layer could be affected by physical and chemical properties of the fatty acid type used in blend composition.

Morphological changes such as catechin induced porosity reduction and formation of zein microspheres derived by bled film making that effect the initial lysozyme release rate were also detected for zein and zein–fatty acid blend film containing C-lysozyme (Figure 6.27 and 6.28)

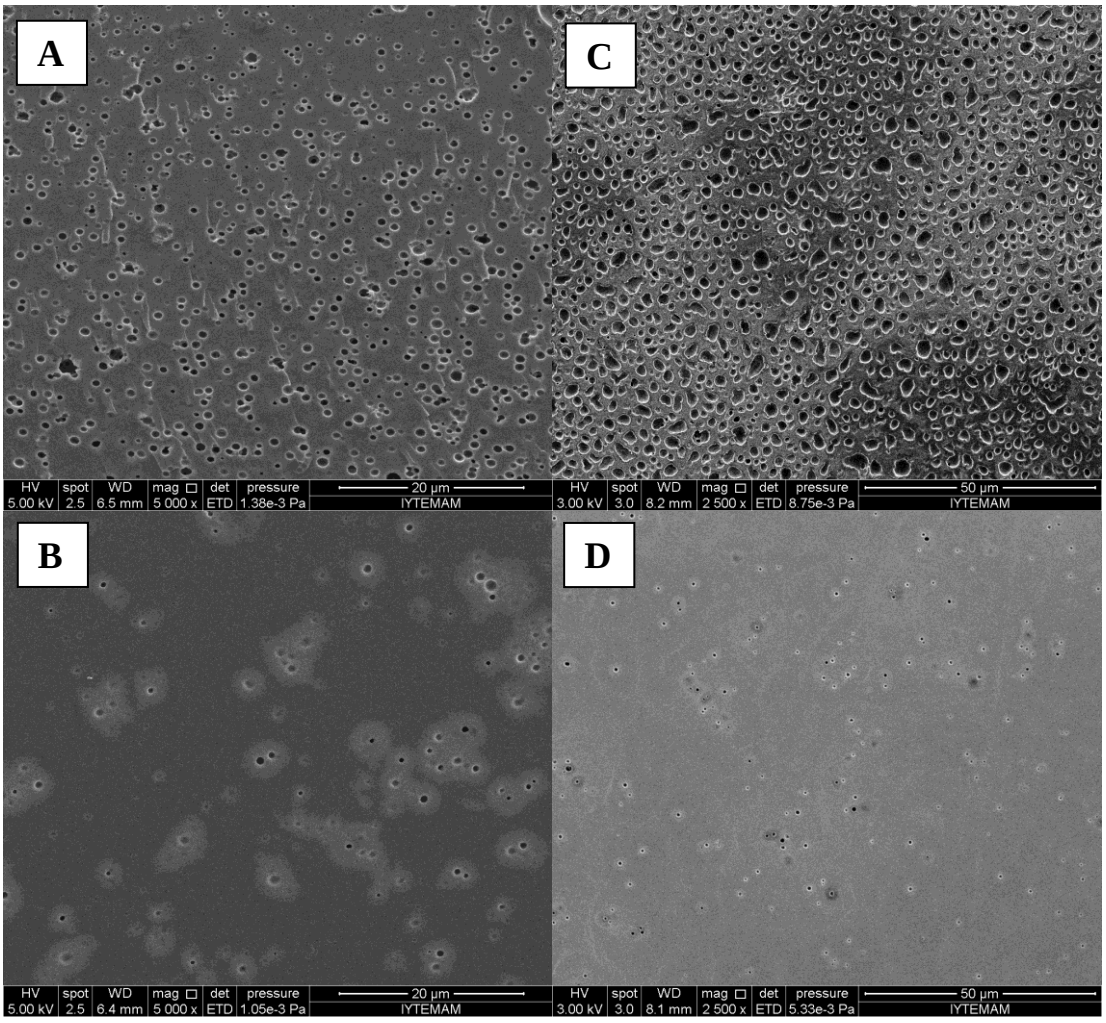


Figure 6.27. Cross-sectional and surface SEM images of the developed films: zein film containing C-lysozyme (0.7 mg/cm^2)(A, C); zein film containing catechin (6 mg/cm^2) and C-lysozyme (0.7 mg/cm^2) (B, D)

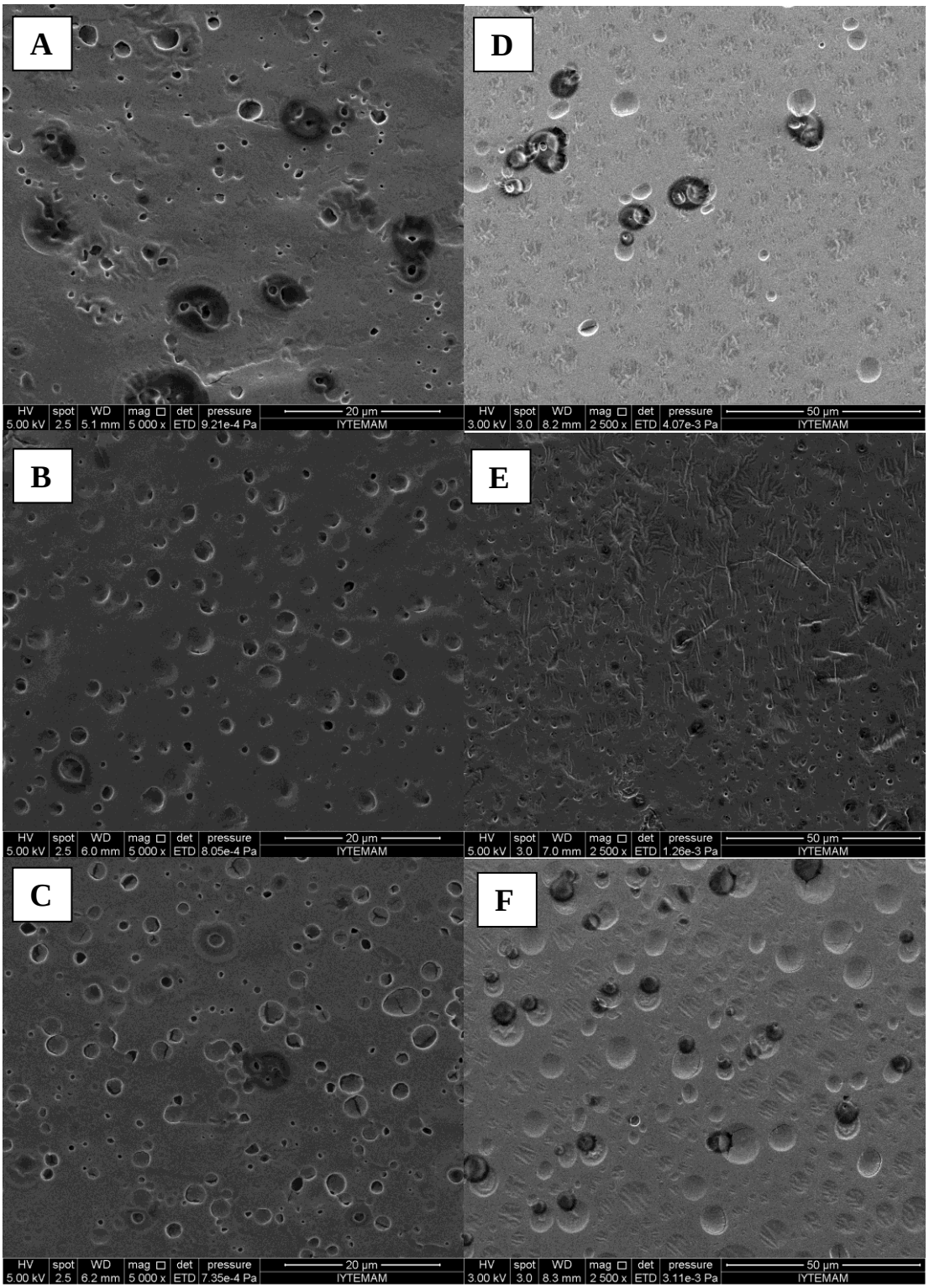


Figure 6.28. Cross-sectional and SEM images of the zein-fatty acid blend films: blend film containing OLE (10%), catechin (6 mg/cm^2) and C-lysozyme (0.7mg/cm^2) (A, D); blend film containing LAU (10%), catechin (6 mg/cm^2) and C-lysozyme (0.7mg/cm^2) (B, E); blend film containing LIN (10%), catechin (6 mg/cm^2) and C-lysozyme (0.7mg/cm^2) (C, F)

6.2.2.5. Antioxidant and Antimicrobial Properties of Zein–Fatty Acid Blend Films

The test of total flavonoid concentration in release mediums at the end of release experiments showed the soluble catechin contents of different films (Table 6.18). In catechin plasticized zein films, 59-81 % of the incorporated catechin was solubilized during the release tests since most of catechin existed free or weekly bound to protein in film structure. Therefore, developed films were performed high antioxidant potentials (61-91 μmol Trolox/cm²). The remaining catechin within the films was expected to be bound to the film matrix by H bonds. This hypothesis compares well with the previous findings of Alkan et al. (2011) who developed active zein films containing gallic acid and explained zein–gallic acid interaction with hydrogen bond formation between these components. The highest soluble catechin content as well as the highest antioxidant potential was measured for the zein–LIN blend films plasticized with catechin (P>0.05). The films lacking catechin also had antioxidant potential (1.1-5.4 μmol Trolox/cm²) since lysozyme and zein have antioxidant activities (You, Udenigwe, Aluko, & Wu, 2010; Zhang, Luo, & Wang, 2011b). However, compared to catechin containing films, the antioxidant potential of these films could be negligible (P>0.05). It is clear that the use of catechin is highly beneficial to improve antioxidant and bioactive status of food and reduce problems associated with brittleness during production, storage and handling of pre-cast films before food application.

The antimicrobial effects of the developed films were tested on *L. innocua* by using the classical disc diffusion method. The control zein film without lysozyme and catechin did not form any inhibition zones (Table 6.18). All other films containing lysozyme showed antimicrobial effect on *L. innocua* and formed clear zones around their discs. Although C–lysozyme has higher in vitro activity than that of PP–lysozyme, there were no significant differences among the antimicrobial performances of zein films containing only PP– or C–lysozyme (P> 0.05). The addition of catechin slightly decreased the antimicrobial potential of PP–lysozyme containing film, while the reduction in antimicrobial potential was found significant for the C–lysozyme containing film (P<0.05). In addition to that, catechin plasticized zein–OLE and zein–LIN blend films had lower antimicrobial potential than that of control films for both PP– and C–lysozyme containing ones. Especially antimicrobial potential of zein–OLE

blend film containing catechin were found significantly lower than that of control films (P<0.05). This result confirmed our release tests which showed trapping of lysozyme activity within the catechin plasticized zein and zein-fatty acid blend films. On the other hand, blend films containing LAU showed very high antimicrobial potential against *L.innocua.* This result showed parallelism with report of previous workers who tested antimicrobial potential of the zein films containing lauric acid against *Listeria monocytogenes* (Hoffman, Han, & Dawson, 2001).

Table 6.18. Total soluble catechin concentration, antioxidant and antimicrobial potential of zein and zein–fatty acid blend films

Film composition[a]				**Total released catechin**	**Antioxidant potential**	**Average zone area**
LYS (mg/cm^2)	**CAT (mg/cm^2)**	**FA (%)**[b]	**LEC (%)**[b]	**(mg/cm^2)**	**(µmol Trolox /cm^2)**	**(mm^2)**
-	-	-	-	-	-	-
PP-LYS						
1.4	-	-	-	-	5.4 ± 0.6c	65 ± 10a,AB[c]
1.4	6.0	-	-	3.99 ± 0.13ab	82.8 ± 5.8a	55 ± 26ab,ABC
1.4	6.0	10(OLE)[d]	10	3.84 ± 0.13b	73.1 ± 2.8b	35 ± 4b,C
1.4	6.0	10(LAU)	10	3.54 ± 0.03c	68.7 ± 4.5b	537 ± 52[e]
1.4	6.0	10(LIN)	10	4.29 ± 0.10a	90.7 ± 1.8a	34 ± 7b,C
C-LYS						
0.7	-	-	-	-	1.4 ± 0.1d	73 ± 26a,A
0.7	6.0	-	-	3.53 ± 0.06d	61.0 ± 1.2c	38 ± 7b,C
0.7	6.0	10(OLE)	10	3.76 ± 0.02c	65.5 ± 1.6c	43 ± 10b,BC
0.7	6.0	10(LAU)	10	4.26 ± 0.03b	74.6 ± 2.9b	506 ± 7[e]
0.7	6.0	10(LIN)	10	4.86 ± 0.07a	86.3 ± 4.2a	46 ± 7ab,ABC

[a] LYZ:lysozyme; CAT: catechin; FA: fatty acid; LEC: lecithin

[b] concentrations of fatty acids and lecithin as % of zein (w/w).

[c] different letters in each column show significant difference at P<0.05. Small letters indicates the differences within each enzyme type. Capital letter indicates the differences within all results.

[d] OLE: oleic acid; LAU: lauric acid; LIN: linoleic acid

[e] not included in statistical analysis

6.2.2.6. Mechanical Properties of Zein and Zein–Faty Acid Blend Films

In order to analyze their mechanical properties tensile strength at break, elongation at break, and Young's modulus (YM) values of films were determined (Table 6.19). The control zein films lacking lysozyme and catechin showed very little elongation, but the tensile strength value of these films at the breaking point (17.67 MPa) were significantly higher than those of the catechin plasticized films (0.61-0.98 MPa) and blend films (0.45-1.23 MPa). The addition of lysozyme alone reduced the tensile strength and Young's modulus of zein films significantly (P<0.05), but it did not cause any significant change in film elongation (P>0.05). In contrast, the addition of catechin effectively plasticized the zein films which showed almost 20-54 fold higher elongation at break value than that of lysozyme containing control films. These finding is in line with that of reported in Section 6.1.1 that showed the plasticizing effect of phenolic compounds such as catechin, gallic acid, p-hydroxy benzoic acid, and ferulic acid on zein. On the other hand, PP–lysozyme containing films showed significantly lower elongation than C–lysozyme containing films since they had 2 fold protein amount in film matrix (P>0.05). However, no significant difference was observed in tensile strength at break and Young's modulus values of the zein-fatty acid blends films containing different type of lysozyme. It should also note that, the formation of blend films by using different fatty acid did not cause any significant changes in tensile strength, elongation and Young's modulus values (P<0.05). Although plasticizing effects of oleic acid and linoleic acid have been reported previously (Santosa & Padua, 1999), no plasticizing effect was observed for fatty acids in blend films at the studied concentration.

Table 6.19. Mechanical properties of zein and zein–fatty acid blend films

Film composition[a]				Tensile strength at break (MPa)	Elongation at break (%)	Young's modulus (MPa)	Film Thickness (µm)
LYZ (mg/cm^2)	**CAT (mg/cm^2)**	**FA (%)[b]**	**LEC (%)[b]**				
-	-	-	-	17.67 ± 0.93a[c]	4 ± 1a	775 ± 44a	113 ± 1
PP-LYS							
1.4	-	-	-	10.98 ± 0.96b	3 ± 1a	530 ± 32c	143 ± 1
1.4	6.0	-	-	0.98 ± 0.14c	61 ± 18b	55 ± 12d	167 ± 1
1.4	6.0	10(OLE)[d]	10	1.17 ± 0.26c	53 ± 9b	55 ± 19d	160 ± 2
1.4	6.0	10(LAU)	10	0.88 ± 0.10c	70 ± 10b	37 ± 9d	159 ± 2
1.4	6.0	10(LIN)	10	1.23 ± 0.10c	71 ± 15b	63 ± 9d	192 ± 5
C-LYS							
0.7	-	-	-	11.02 ± 0.67b	3 ± 1a	611 ± 22b	119 ± 3
0.7	6.0	-	-	0.61 ± 0.06c	161 ± 18c	28 ± 5d	160 ± 5
0.7	6.0	10(OLE)	10	0.52 ± 0.02c	164 ± 17c	23 ± 4d	162 ± 8
0.7	6.0	10(LAU)	10	0.45 ± 0.04c	166 ± 10c	22 ± 4d	184 ± 12
0.7	6.0	10(LIN)	10	0.49 ± 0.08c	131 ± 25c	22 ± 6d	173 ± 2

[a] LYZ:lysozyme; CAT: catechin; FA: fatty acid; LEC: lecithin

[b] concentrations of fatty acids and lecithin as % of zein (w/w).

[c] different letters in each column show significant difference at $P<0.05$

[d] OLE: oleic acid; LAU: lauric acid; LIN: linoleic acid

CHAPTER 7

CONCLUSIONS

In this work, the flexible zein based composite and blend films containing waxes or fatty acids were developed to control the release of phenolic compounds and lysozyme. The obtained controlled release properties were attributed to multiple factors including increased hydrophobicity of films, morphological changes in films formed by composite and blend film making methods, and the reduced pore sizes of films achieved by the plasticizer catechin. In zein–wax composite films, the development of highly hydrophobic and tourtous structure with aggregated wax particles formed a controlled release mechanism for the active agents. The effectiveness of the composite structure on the release profiles of active agent increased as the melting point of the wax used in films increased and as films got more tortuous with the aggregated wax particles. On the other hand, the morphological changes affecting the controlled release properties of zein–fatty acid blend films were related to the formation of extensive spherical zein capsules which entrapped and increased the diffusion barriers for active agents. In addition to that, both fatty acid concentration and carbon chain length were found to be effective on the release profiles of the active agents. The phenolic compounds incorporated into films acted not only as plastisizer but also they increased the antioxidant potential of films considerably.

The results of this work showed the possibility of producing flexible antimicrobial and/or antioxidant films with controlled release properties by using zein which is the major by-product of rapidly growing bioethanol industry. This work prepares a basis for the production of flexible active zein based self-standing films, coatings, or casings which can be employed for biopreservation of food.

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APPENDIX A

CALCULATION OF THE INITIAL LYSOZYME RELEASE RATE

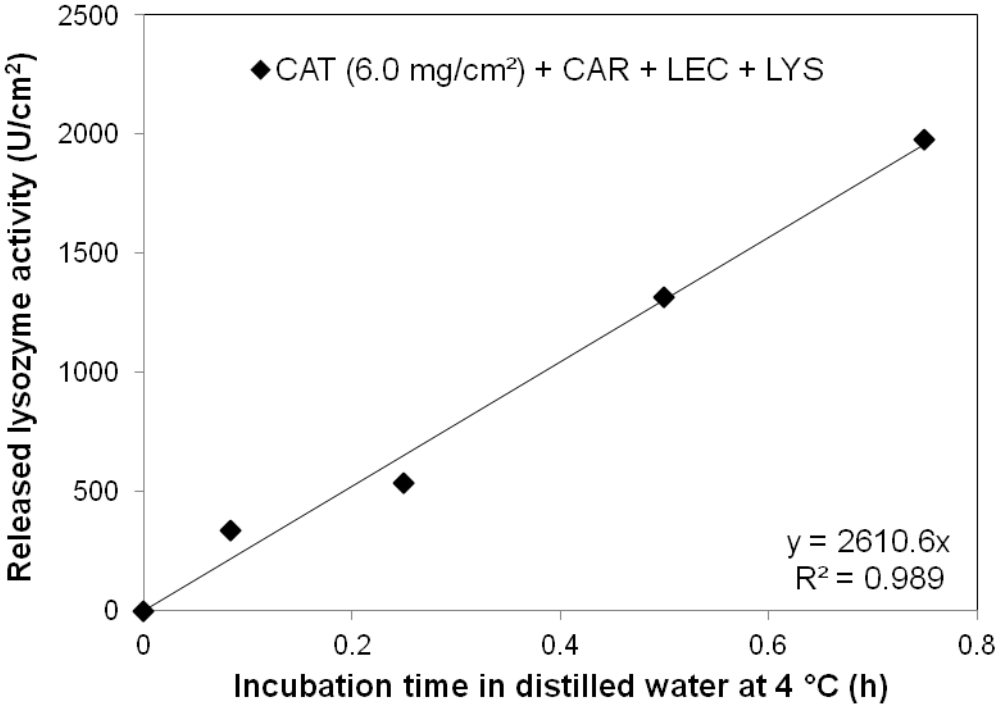


Figure A.1. The initial release rates of lysozyme were determined from the slope of the initial linear portion of release curve (The release rates were expressed as $U/cm^2/h$)

APPENDIX B

CATECHIN STANDARD FOR FOLIN–CHIOCALTEU METHOD

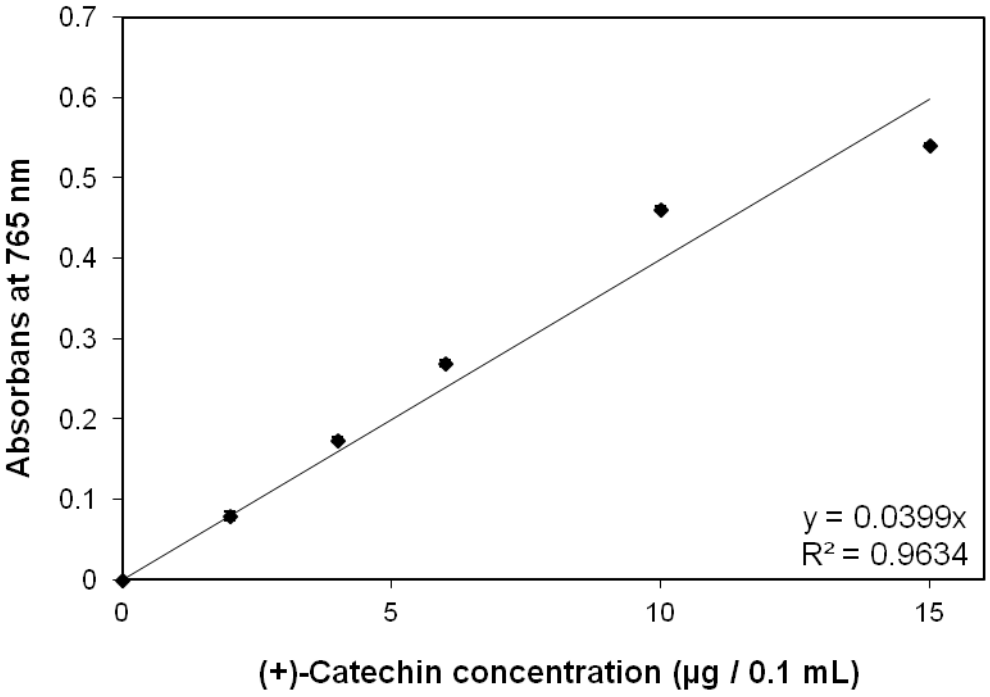


Figure B.1. Catechin standard for total phenolic content assay

APPENDIX C

GALLIC ACID STANDARD FOR FOLIN–CHIOCALTEU METHOD

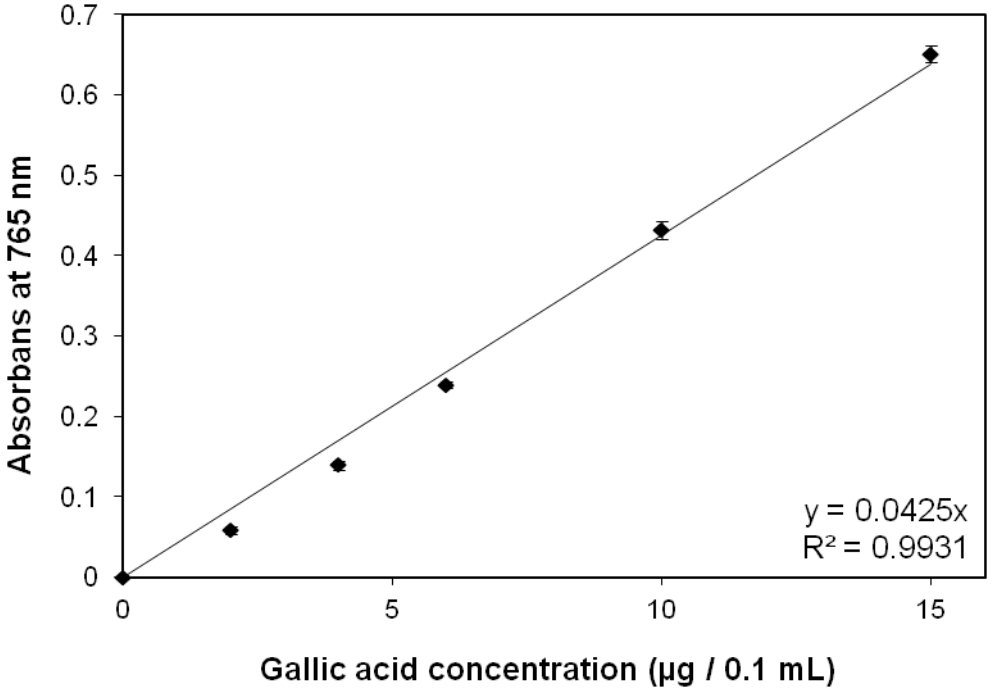


Figure C.1. Gallic acid standard for total phenolic content assay

APPENDIX D

CATECHIN STANDARD FOR ALUMINIUM CHLORIDE COLORIMETRIC METHOD

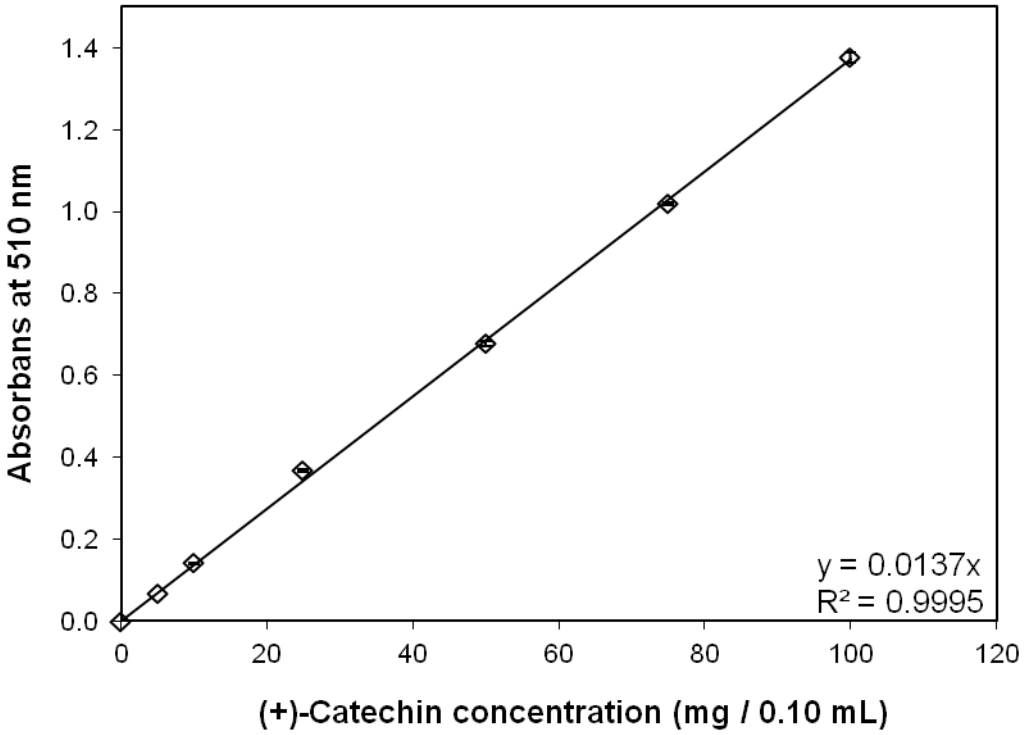


Figure D.1. Catechin standard for total flavonoid content assay

APPENDIX E

TROLOX STANDARD FOR ABTS RADİCAL DISCOLORATION ASSAY

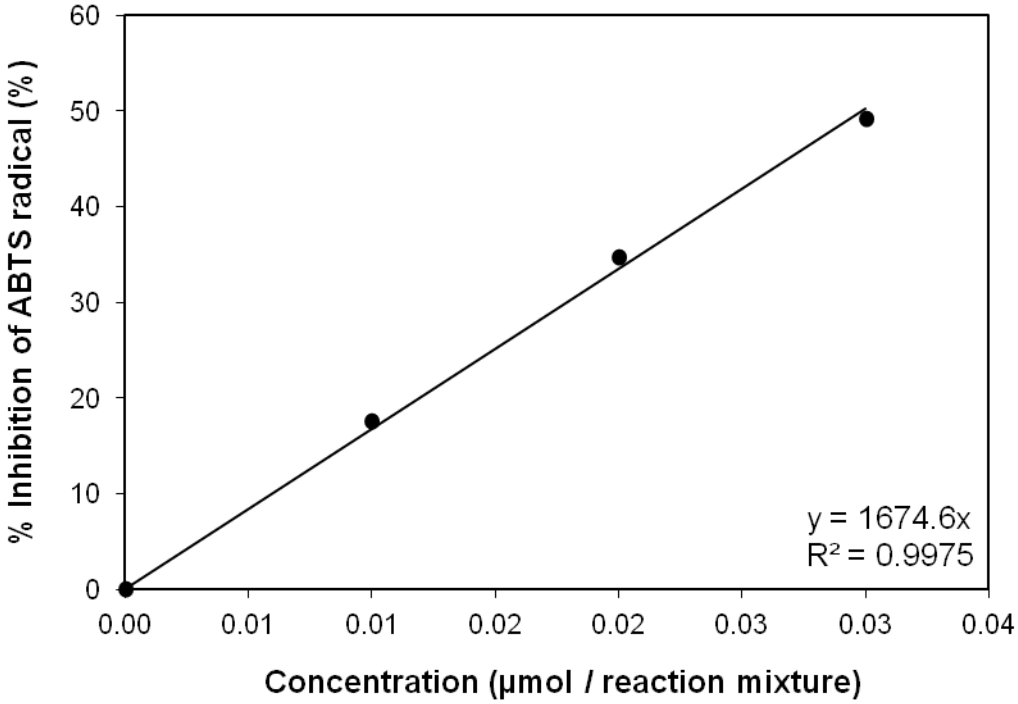


Figure E.1. Standard curve for Trolox

APPENDIX F

TROLOX STANDARD FOR AUC CALCULATION

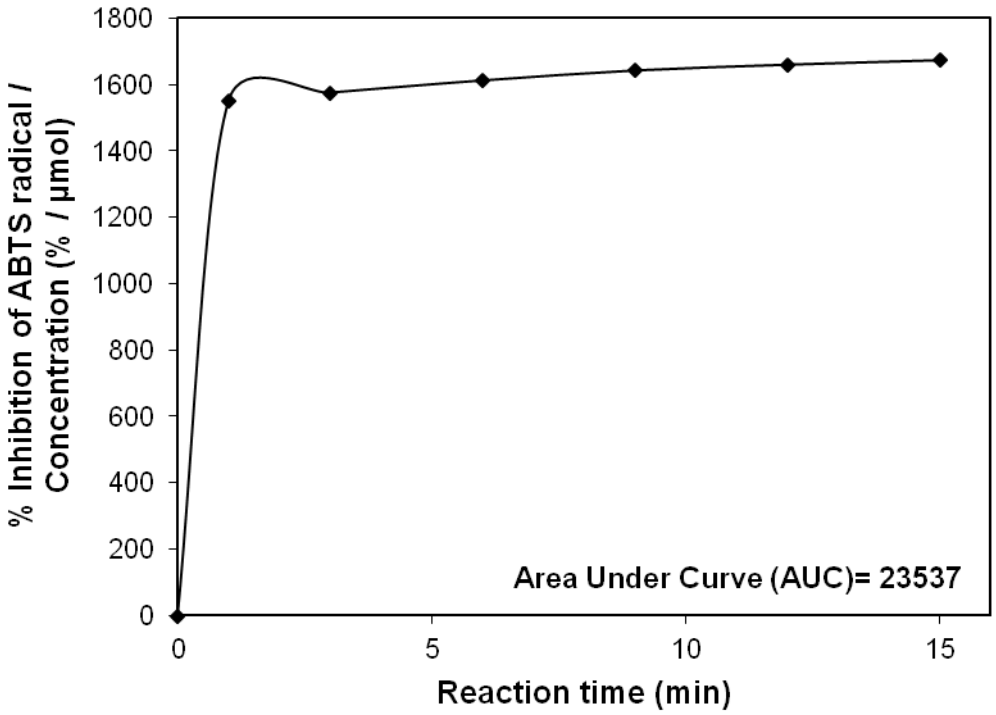
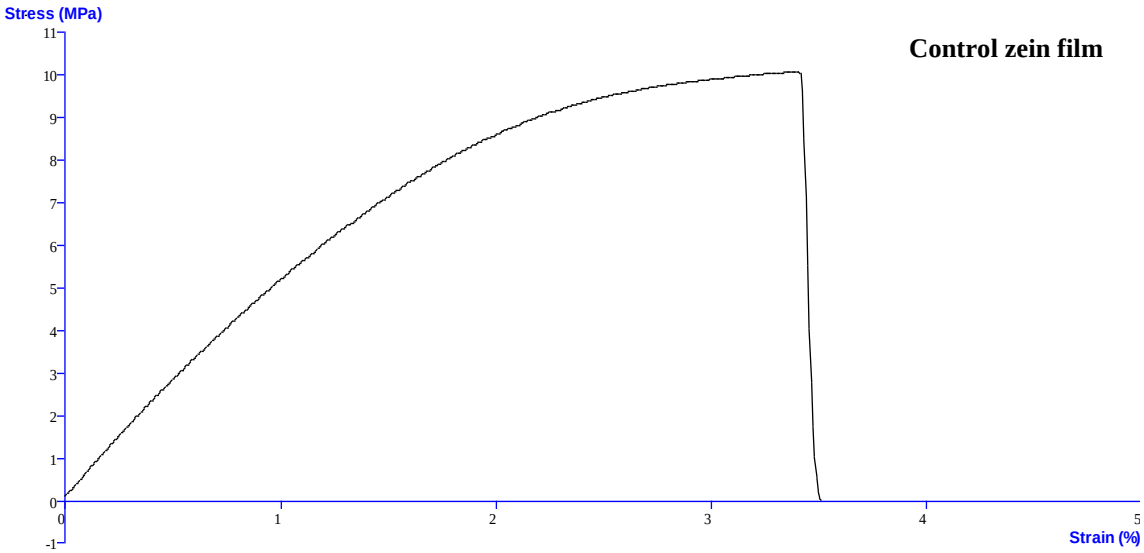


Figure F.1. Trolox standard for AUC calculation

APPENDIX G

MECHANICAL TEST RESULTS OF CONTROL ZEIN FILMS AND ZEIN FILMS PLASTICIZED WITH PHENOLICS



Mechanical test result of control zein films

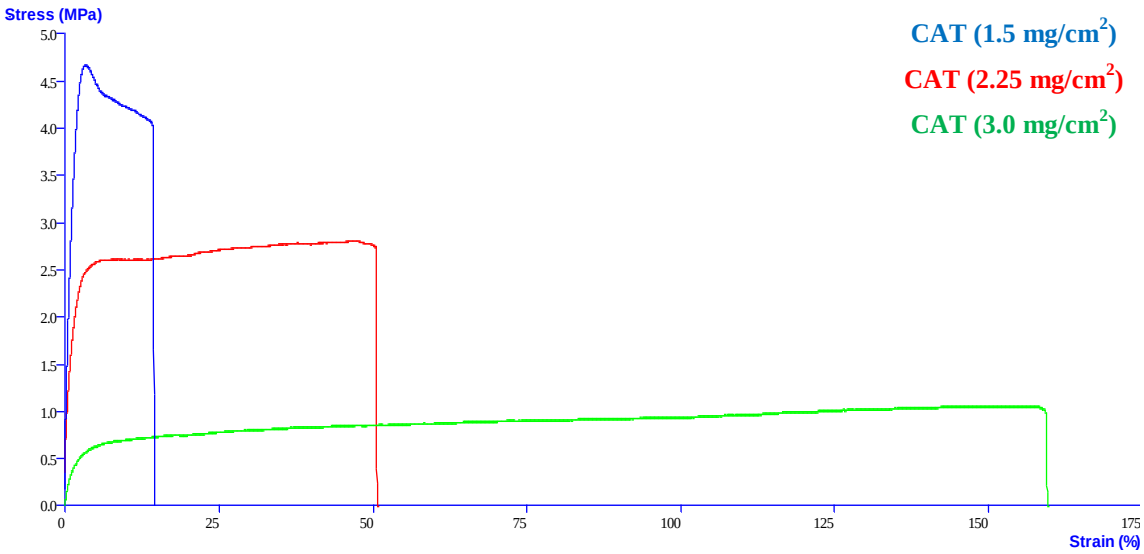


Figure G.2. Mechanical test result of catechin plasticized zein films

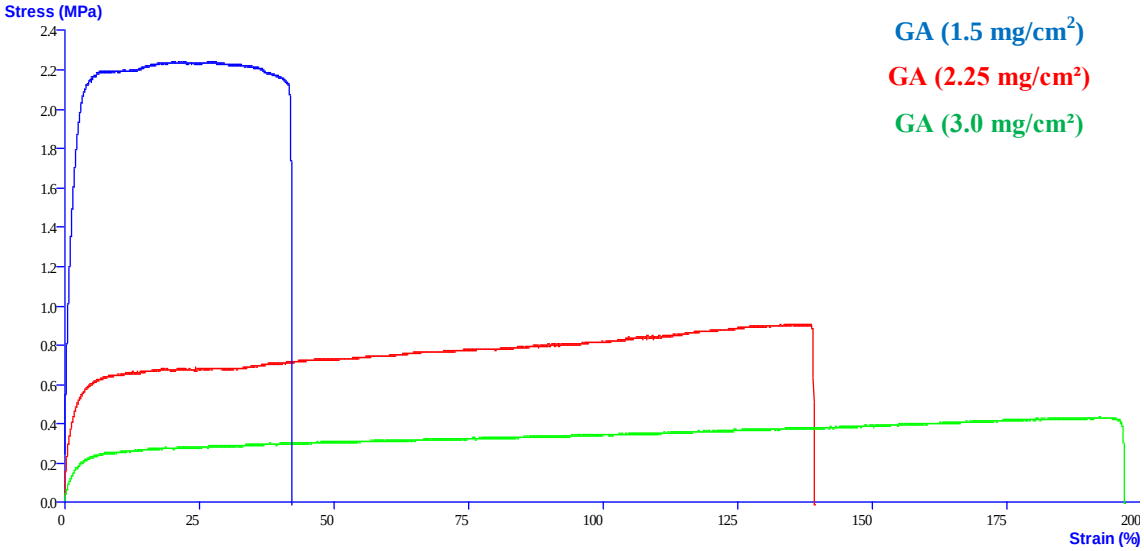


Figure G.3. Mechanical test result of gallic acid plasticized zein films

APPENDIX H

FTIR SPECTRUM OF ZEIN FILMS PLASTICIZED WITH CATECHIN OR GALLIC ACID

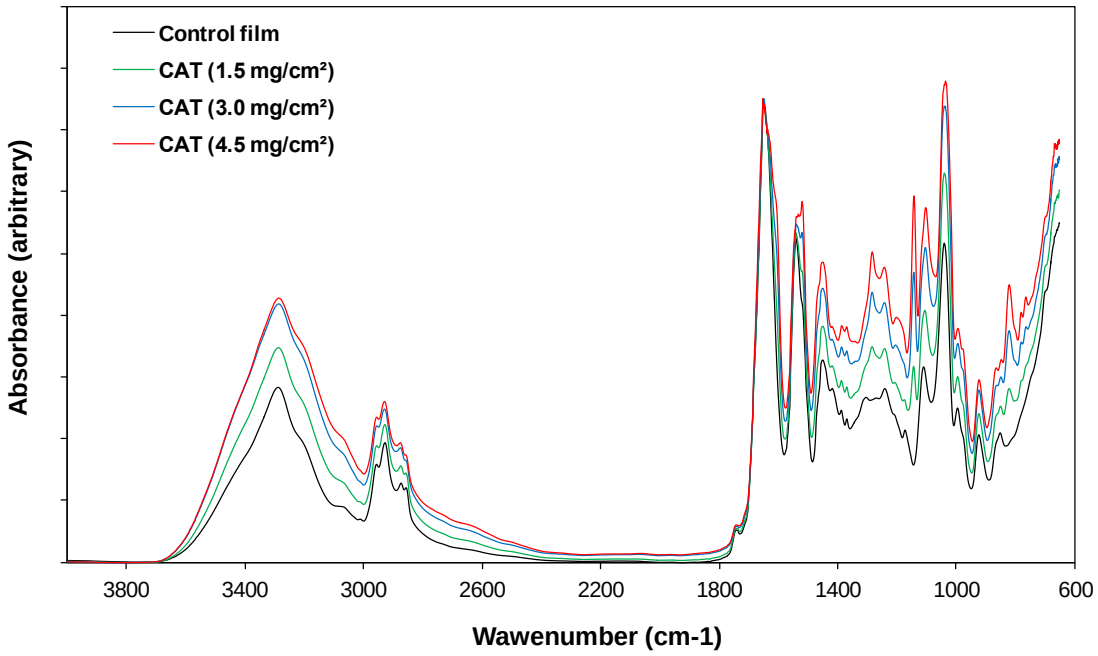


Figure H.1. FTIR spectrum of catechin plasticized zein films

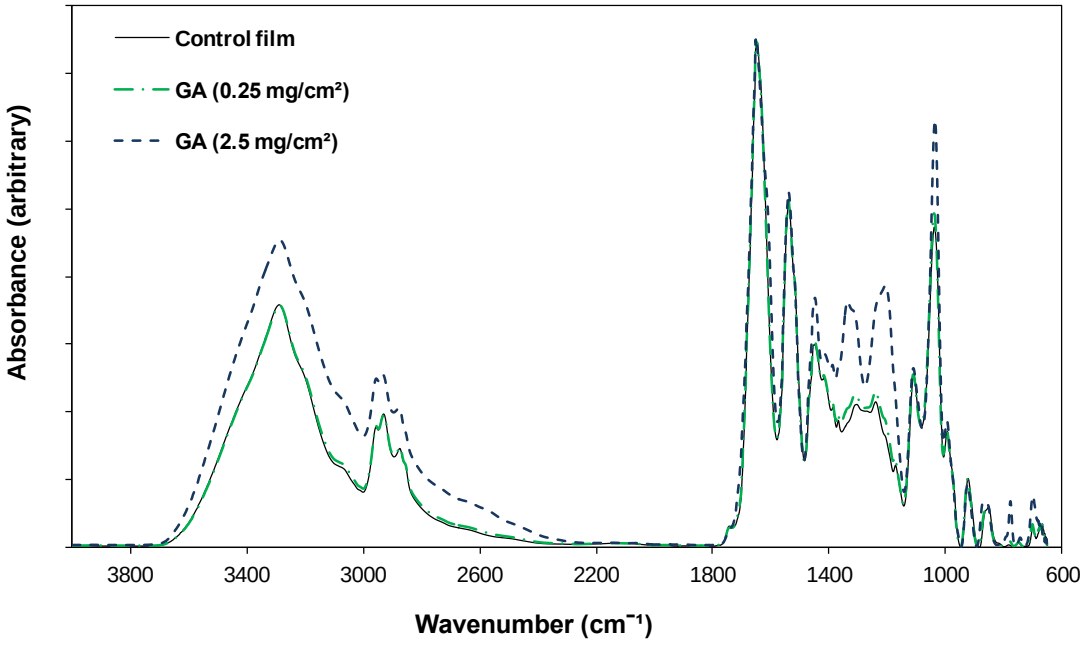


Figure H.2. FTIR spectrum of gallic acid plasticized zein films

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PUBLICATIONS

Arcan, I., & Yemenicioglu, A. (2013). Development of flexible zein-wax composite and zein-fatty acid blend films for controlled release of lysozyme. Food Research International, 51(1), 208-216.

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