INNOVATIVE FOOD APPLICATIONS OF NOVEL MULTIFUNCTIONAL ACTIVE EDIBLE GEL FILLINGS AND COATINGS

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Dedicated to my grandmother Güler and my grandfather Ahmet Samim

ABSTRACT

INNOVATIVE FOOD APPLICATIONS OF NOVEL MULTIFUNCTIONAL ACTIVE EDIBLE GEL FILLINGS AND COATINGS

This thesis organized as two chapters aimed to develop and test multifunctional novel gel filling and coating. In Chapter 1, the thesis focuses on developing antimicrobial and antibrowning gelatin based gel filling to produce safe caramel apples. For this purpose, water holding capacity and mechanical stability of gelatin (GEL) gels were improved by the incorporation of soy proteins (SP) and inulin (IN), respectively. The water activity (a_w) of gels was also reduced to < 0.9 by the addition of sucrose (SUC). The apples were cored and the void cores were filled with GEL-SP-IN-SUC blend gel solution containing synergetic antimicrobials, nisin and lysozyme, and antibrowning agent ascorbic acid (each at 1% in gel). Candy sticks were then placed into gel-filled void cores, and apples were kept at 4ºC for 20 h for gelation of filling before caramel dip-coating. The caramel apples with antimicrobial GEL-SP-IN-SUC filling showed 2.4 – 2.9 D lower *Listeria* count than traditional caramel apples during 7-day cold storage. The active blend gel-filling also prevented discoloration of apple core. In Chapter 2, the thesis focuses on controlling postharvest sprouting and microbial risks associated with shallot bulbs by active chitosan based coating loaded with eugenol (EUG). The sustained release of EUG was achieved by applying ultrasonic homogenization (US) to prepare a composite of chitosan (CHI) with chickpea proteins (CP) (CHI:CP ratio of films = 2). The CHI-CP-EUG coating successfully reduced the sprouting and inhibited the inoculated *E. coli* and *L. innocua* in shallot bulbs by 2.3 log and 1.7 log, respectively.

ÖZET

YENİLİKÇİ YENİLEBİLİR ÇOK FONKSİYONLU JEL DOLGULAR VE KAPLAMALARIN İNOVATİF GIDA UYGULAMALARI

Bu tez iki bölümden oluşmakta olup temel amacı çok fonksiyonlu yenilikçi jel dolgu ve kaplama materyallerinin geliştirilmesi ve gıdalarda test edilmesidir. Bölüm 1'de tezin spesifik amacı antimikrobiyal ve enzimatik esmerleşme karşıtı etki gösteren jelatin (GEL) temelli dolgu materyalleri üretmek ve bunları güvenli karamel elma şekeri üretiminde kullanmaktır. Bu amaçla jelatin jellerinin su tutma kapasitesi ve mekaniksel stabilitesi sırasıyla soya proteini (SP) ve inulin (IN) katılarak artırılmıştır. Ayrıca jellerin su aktivitesi (a_w) sükroz (SUC) ilavesiyle 0.9 değerinin altına getirilmiştir. Uygulama sırasında elmaların eşelek kısmı çıkartılmış ve sinerjetik etki gösteren lizozim-nisin karışımı ve enzimatik esmerleşme karşıtı askorbik asit (her bir ajan jel içerisinde %1) ilave edilmiş GEL-SP-IN-SUC karışım jel çözeltisi ile doldurulmuştur. Ardından jel dolgu içerisine elma şekeri çubukları yerleştirilmiş ve jeller 4°C'de 20 saat katılaşma için bekletildikten sonra daldırılarak karamelle kaplanmıştır. Bu innovatif uygulamayla üretilmiş kontamine edilmiş karamel elma şekerleri geleneksel yöntemle üretilmiş kontamine edilmiş olanlara göre bir hafta soğukta depolama sırasında 2.4 – 2.9 log daha düşük *Listeria* sayımı göstermişlerdir. Ayrıca jel dolgu elma eşelek yuvasının kararmasını başarıyla engellemiştir. Bölüm 2'de tezin spesifik amacı arpacık soğanında hasat sonrası oluşan filizlenmenin ve mikrobiyal risklerin eugenol (EUG) içeren aktif kitosan (CHI) temelli kompozit kaplamalarla önlenmesidir. Kompozit hazırlamada kitosanın nohut proteiniyle (CP) karışımı (CHI:CP ratio of films = 2) ve ultrasonik homojenizasyon (US) kullanılarak kontrollü EUG salınımı yapan filmler üretilmiştir. Elde edilen CHI-CP-EUG kaplamalar arpacık soğanlarda filizlenmeyi geciktirmiş ve inoküle edilmiş *E. coli* ve *L. innocua* bakterilerini sırasıyla 2.3 ve 1.7 log inhibe etmiştir.

LIST OF ABBREVIATIONS

a*	redness
a_w	water activity
A	absorbance
AA	L-ascorbic acid
AFM	atomic force microscopy
AGF_{apple}	cored inoculated apple filled with GEL/SP/IN/SUC gel with LYS+NIS+AA
ANOVA	analysis of variance
ATCC	American Type Culture Collection
AUC	area under the curve
b*	yellowness
BHI	Brain Heart Infusion
BI	browning index
BSA	bovine serum albumin
C*	chroma
CDC	Centers for Disease Control and Prevention
cfu	colony forming unit
CHI	chitosan
CIE	Commission Internationale de I'Eclairage
CIPC	chlorpropham
CP	chickpea protein concentrate
CPE_{bulb}	non-sonicated CHI-CP-EUG coating coated bulbs
Ctr_{apple}	control cored inoculated apple
Ctr_{bulb}	uncoated bulbs
D	decimal
D[3,2]	surface-weighted mean particle diameter or Sauter mean
D[4,3]	volume-weighted mean particle diameter or De Brouckere mean
db	dry basis
DC	dry matter content
DMSO	dimethyl sulfoxide

DNA	deoxyribonucleic acid
DP	degree of polymerization
E. coli	*Escherichia coli*
EO	essential oil
EUG	eugenol
FAO	Food and Agriculture Organization of the United Nations
FDA	U.S. Food and Drug Administration
FFS	film forming solution
FTIR	Fourier transform infrared spectroscpy
FW	fresh weight
G	GEL/SP/IN/SUC blend gel
GEL	gelatin
GF_{apple}	cored inoculated apple filled with GEL/SP/IN/SUC gel
GMIA	Gelatin Manufacturers Institute of America
GRAS	generally recognized as safe
h*	hue
HLB	hydrophilic-hydrophobic balance
HMG-CoA	3-hydroxy-3-methylglutaryl coenzyme A
IAEA	International Atomic Energy Agency
IEP	isoelectric precipitation
IN	inulin
IU	international unit
k′	first-order rate constant
L*	lightness
L.	standard abbreviation used for “Linnaeus”
L. innocua	*Listeria innocua*
Lb. plantarum	*Lactobacillus plantarum*
LDPE	low density polyethylene
LPS	lipopolysaccharide
LYS	lysozyme
MA	malic acid
MANOVA	multivariate analysis of variance
MC	moisture content

MIC	minimum inhibitory concentration
MFU	McFarland unit
MRS	De Man, Rogosa and Sharpe
MW	molecular weight
N	Newton
NADPH	nicotinamide adenine dinucleotide phosphate
NBPSDHU	North Bay Parry Sound District Health Unit
NIS	nisin
NRRL	Northern Regional Research Laboratory
OPP	oriented polypropylene
P	p-value or probability value
Pa	Pascal
PF	peak force or penetration force
PG	polygalacturonase
pI	isoelectric point
pK_a	acid dissociation constant
PME	pectin methylesterase
PPC	protein-polysaccharide complex
PPCO	protein-polysaccharide conjugate
PPO	polyphenol oxidase
R_a	arithmetic mean roughness
R_q	root-mean-square roughness
RH	relative humidity
RNA	ribonucleic acid
ROS	reactive oxygen species
rpm	revolutions per minute
RSHM	Refik Saydam Hıfzısıhha Merkezi
SC_{apple}	standard inoculated caramel apple
SC_{bulb}	sonicated CHI coating coated bulbs
SCE_{bulb}	sonicated CHI-EUG coating coated bulbs
SCP_{bulb}	sonicated CHI-CP coating coated bulbs
$SCPE_{bulb}$	sonicated CHI-CP-EUG coating coated bulbs
SD	standard deviation

SDS-PAGE	sodium dodecyl sulfate-polyacrylamide gel electrophoresis
SEM	scanning electron microscopy
SP	soy protein isolate
sp.	species (singular)
spp.	species (plural)
subsp.	subspecies
SUC	sucrose
SW	swelling degree
T	transmittance
$t_{1/2}$	half-life
TF	toughness
TPA	texture profile analysis
TS	tensile strength
TSS	total soluble solid
U	unit
UNECE	United Nations Economic Commission for Europe
US	ultrasound
UV	ultraviolet
v/v	volume/volume
W	Watt
wb	wet basis
WBC	water binding capacity
WHO	World Health Organization
WS	water solubility
WVP	water vapour permeability
WVTR	water vapour transmission rate
w/v	weight/volume
w/w	weight/weight
YM	Young's modulus
ΔE*	colour change
ζ	zeta
2D	two dimensional
3D	three dimensional

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CHAPTER 1

AN INNOVATIVE DESIGN AND APPLICATION OF NATURAL ANTIMICROBIAL GELATIN BASED FILLING TO CONTROL RISK OF LISTERIOSIS FROM CARAMEL APPLES

1.1. Introduction

The prevention of food contamination by *Listeria monocytogenes*, and application of hurdles to prevent listerial growth in risky food are critically important since this bacterium may cause deadly infections in pregnant women, old people, and the immunosuppressed people (Vázquez-Boland et al. 2001; Álvarez-Ordóñez et al. 2015). The prepacked caramel apple was designated as a *Listeria* risk-food after it had caused dead of 7 people and hospitalization of 34 from listeriosis in 12 states of United States of America (Angelo et al. 2017; CDC 2015). This infection occurred unexpectedly since apples are too acidic, and the caramel coating applied at a high temperature (71 – 88^{o}C) is too dry ($a_w < 0.80$) to support *Listeria* growth (Bouvier 2015; Glass et al. 2015; Salazar et al. 2016; Ward, Bedale, and Glass 2022). However, the research by (Glass et al. 2015) clearly showed how *Listeria* developed in caramel apples during storage. These workers showed that stick insertion into apples at the stem-end that contains the potential *Listeria* contaminant causes leakage of apple juice from the core region to the interface between the apple surface and caramel coating. Then, during cold storage the released apple juice neutralized by the basic caramel becomes a suitable medium for the growth of *Listeria monocytogenes* contaminated at apple peel, calyx or stem regions. The findings of (Salazar et al. 2016) also supported that *Listeria* contaminated at the stem-end of apples is highly protected, and it might survive and grow in caramel apples.

Although the mechanism of listerial development in caramel apples has been characterized sufficiently, efforts to develop technological solutions to this problem are very limited. For example, Gustafson and Ryser (2017) tried thermal inactivation of

Listeria monocytogenes by increasing temperature of caramel dipping solutions for current industrial practice from 82ºC to 99ºC. However, it was reported that the high temperature caramel coating process cannot ensure *Listeria* elimination at the stem-end of apples, and it causes undesirable cooking of apple surface as well as formation of a too thin caramel coating (Gustafson and Ryser 2017; Salazar et al. 2016). Murray et al. (2018) achieved the inactivation of *Listeria monocytogenes* on surface and scar tissue of apples by applying the combination of UV-C light, ozone, and hydrogen peroxide via forced air circulation. Moreover, Carstens et al. (2018) evaluated the efficiency of impregnating different antimicrobials such as ascorbic acid, nisin, potassium sorbate and sodium benzoate into wooden sticks inserted into caramel apples. However, none of these methods prevent the leakage of apple juice at the interface between caramel coating and apple surface. Moreover, apples processed by the traditional method still contain risks associated with *Listeria* contaminated at protected sites like stem-end and calyx.

Different edible antimicrobial materials could be applied as a fruit coating to suppress the respiration rate and inactivate spoilage and pathogenic bacteria in whole or minimally processed sliced fruits (Park 1994; Rojas-Graü et al. 2009). In contrast, the application of edible materials capable of producing hydrogels as an antimicrobial and antioxidant gel filling for fruit preservation is scarce. However, different basic research studies have been conducted to develop gel-based delivery systems incorporated with natural active compounds suitable for biomedical, pharmaceutical and food applications. For example; Campia et al. (2017) showed that aerogels of galactomannan extracted from leguminous plant sources could be promising for biomedicinal and food packaging purposes to deliver antibiotics, bacteriocins, and enzymes (such as polymyxin B, nisin, lysozyme, protease and lipase). Abhari et al. (2017) developed starch hydrogels cross-linked by trisodium citrate for controlled release of caffeine. Moritaka and Naito (2002) investigated the flavor release properties of agar and gelatin gel. Aloe vera gel has also been tested as a fruit coating to prolong shelf-life and quality of grapes and nectarine during cold storage (Ahmed, Singh, and Khan 2009; Serrano et al. 2006). A hydrogel could be employed not only as a high capacity reservoir to deliver active compounds (antimicrobials and antioxidants) onto food surface, but also it could be used as an absorbent pad to bind drip-loss fluids from food (Batista et al. 2019).

In the current study, a smart preservation system was formed by an innovative application of antimicrobial and antioxidant gelatin (GEL) based blend gel as a

multifunctional filling material for caramel apples. The developed composite gels formed by mixing GEL with soy proteins (SP), inulin (IN) and sucrose (SUC) were incorporated with generally recognized as safe (GRAS) antimicrobials, nisin (an antimicrobial peptide) and lysozyme (an antimicrobial enzyme), that synergetic action against *Listeria* frequently exploited in different systems (Gill and Holley 2000a, 2000b; Monticello 1990; Morsy et al. 2018; Proctor and Cunningham 1993; Sozbilen and Yemenicioğlu 2020; Takahashi et al. 2012). The gel pH was lowered and browning of cored apple was prevented by addition of the antioxidant ascorbic acid. This work is the first study in the literature that applied a tailor-made antimicrobial gel-filling in an innovative way to prevent risk of listeriosis from a minimally processed fruit product as caramel apple.

1.2. Background Information

1.2.1. Food Gels

A gel can be defined as a cross-linked three-dimensional (3D) network entrapping a very high amount of continuous fluid phase (liquid or gas) giving the structure viscoelastic property between a liquid-like and a solid-like rheological behaviour (Harris, 1990; McClements 2016; Nishinari 2021). Gels can be classified in different ways according to their cross-linking type, reversibility, source, structure and medium. Gelation occurs via cross-linking of polymer chains. According to the cross-linkage, chemical gels can be formed from cross-linking through covalent bonds and physical gels can be formed from cross-linking through non-covalent bonds (Foegeding and Davis 2011; McClements 2016). In chemical gels, high bond energy leads to gelation being irreversible, whereas gelation is reversible in physical gels due to the weak bond energy. Gels are also categorized as hydrogels, organogels and aerogels/xerogels depending on their swelling medium; water, organic solvent and air, respectively. For example; food gels are a type of hydrogel. According to the structural organization, food gels can be divided into 2 groups (Foegeding 2006; McClements 2016): (1) Particulate gels consist of a collection of large particles of aggregated biopolymer molecules. These gels are optically opaque because large particles can scatter light strongly. (2) Filamentous gels

consist of thin filaments of aggregated biopolymer molecules. These gels are optically transparent because the width of thin filaments is too small to scatter light enough. Yogurt, cheese, tofu, cooked egg white, jams, jellies, surimi, marshmallows, etc. are traditional food products that are gels. Namely, polysaccharides, proteins and lipids are capable of producing gels of different strengths and textures for various applications.

Polysaccharide gels are achieved at a low polymer concentration (Harris 1990). Some polysaccharides can show thermal-induced gelation due to the promotion of the conformational changes in their molecular structure (such as coil-helix transition upon cooling in gellan and double helices formation in carrageenan) followed by aggregation and network formation via junction zones (Kuhn, Picone, and da Cunha 2012). According to the gelling behaviour, thermal-induced polysaccharide gels can be divided into 2 groups: heat-set gels and cold-set gels. Heat-set gels comprise polysaccharide solutions that form gels under heating (starch, cellulose, curdlan, etc.), whereas cold-set gels comprise hot polysaccharide solutions that form gels during cooling (agar, carrageenan, gellan, etc.). On the other hand, some electrically charged polysaccharides such as alginate and low-methyl ester pectin can show ion-induced gelation because the addition of salts functioning as cross-linkers promotes the formation of ionic cross-linking via bridges of cations between two carboxylate groups from two neighboring chains in close contact, known as “egg-box model” gelation (Fraeye et al. 2010). Gels result from the electrostatic interactions between negatively charged carboxylate groups in guluronate blocks in polysaccharide molecules and positively charged cations leading to the formation of ionically cross-linked gel network.

Protein gels are achieved at a high polymer concentration (Harris 1990). Protein gelation needs a driving force to unfold the native protein structure followed by a protein aggregation with depletion of electrostatic repulsion and then junction zones formation between molecules. It can be formed physically-induced (via heat or pressure), chemically-induced (via acidification, ionic strength or cold gelation) and enzymatically-induced (Kuhn, Picone, and da Cunha 2012). Heat-set gelation is a one-step heating process involving denaturation, unfolding and aggregation until gelation. Acidification (through acid addition or fermentation) can change pH toward pI of protein resulting in hydrophobic interactions, aggregation and gel formation. Increased ionic strength reduces or neutralizes electrostatic repulsion between proteins, thereby strengthening protein-protein interactions and forming a gel. Cold-set gelation is a two-step process leading to

more intense protein-protein interactions. If protein denaturation is performed in low protein concentration than critical gelation concentration at pH far from pI and at low ionic strength, soluble protein aggregates are formed. Then, gelation is induced by adding salts or changing pH toward pI resulting in insoluble protein aggregation and network formation. Limited enzymatic hydrolysis of proteins can enhance aggregation and gelation by exposing hydrophobic residues. Gelation of fibrous proteins such as collagen and gelatin can be easily promoted by cross-linking with enzymes such as transglutaminase, protease, and rennet; in contrast, the compact structure of globular proteins such as casein and whey proteins hinders the accessibility of enzymes to the targeted active site on aminoacid residues.

As a type of organogel, oleogels are 3D systems obtained by solidifying liquid oil using oleogelator such as surfactant (Perța-Crișan et al. 2022). Some replacement studies have been conducted based on the usage as healthier alternatives to saturated fats, trans-fats, shortening and margarine (Hwang, Singh, and Lee 2016; Palla et al. 2017; Silva et al. 2018; Silva et al. 2021).

1.2.2. Gelatin – Soy Protein Blend Gel

Gelatin is a fibrous protein obtained from collagen by acid hydrolysis (Type A gelatin) or alkaline hydrolysis (Type B gelatin). Different processes cause differences in isoelectric points: Type A gelatin with pI ≈ 7.0 – 9.0 and Type B gelatin with pI ≈ 4.7 – 5.4 (Hartel, von Elbe, and Hofberger 2018; Kavur and Yemenicioğlu 2020). An important structural feature of gelatin is that it is a mixture of α-chains (free), β-chains (two α-chains covalently cross-linked), and γ-chains (three α-chains covalently cross-linked) (Haug and Draget 2011). The content of the chain components gives it the unique rheological properties of gelatin, such as high viscosity and high gel strength (Johnston-Banks 1990; Chen et al. 2017; Liu et al. 2021). Gel strength is represented by bloom number. Bloom number is a measure of maximum force (g) needed to compress a 12.7 mm diameter probe 4 mm into 6.67% gelatin gel prepared at 60°C and cooled to 10°C for 17 h (GMIA 2019). Gelatins with high bloom number form firm, elastic and strong gels. Gelatin exhibits cold-set thermoreversible gelation owing to the role of intermolecular hydrogen bonds. At elevated temperatures (T > 40°C), gelatin chains are in disordered random coil

conformation in solution, but upon cooling coil-helix transition occurs and ordered helix formation results in the generation of junction zones and gel network. The incorporation of sugar improves its setting time, melting point and gel strength (Johnston-Banks 1990).

Soy proteins are one of the most widely used commercial plant proteins. As globular proteins, soy proteins are successfully used in many food formulations due to their abundance, low cost, desirable texture, excellent processability and good techno-functional properties such as solubility, emulsification, gelation and hydration (water absorption, water retention and swelling) (Kinsella 1979). It is least soluble at isoelectric point pI ≈ 4.5 (Kavur and Yemenicioğlu 2020) but most soluble at pH 1.5 – 2.5 and pH 7.0 – 12.0 (Lusas and Rhee 1995). Soy proteins absorb water and tend to retain it. Thus, the unique functional property of this protein is high water binding capacity due to the large amounts of charged polar aminoacids which can bind considerably more water than uncharged polar aminoacids (Wolf, Cowan and Wolff 1971; Chou and Morr 1976). pH, temperature and abundance of polar aminoacids significantly affect its water retention capacity. For example; water retention of soy proteins is high at pH 6.0 – 8.0 and at 40 – 70^{o}C (Wang and Zayas 1991). Ma et al. (2022) also found that soy protein isolate (7.5 g/g) had the highest water holding capacity among pulse protein isolates extracted from faba bean, pea and lentil.

Mixed gels can be formed by which only one of two polymers actively forms gel network by entrapping the other or both polymers are incorporated into the gel network (Brownsey and Morris 1988). Mixing ratio enables the production of tailor-made mixed gelatin/globular protein systems. In this study, gelatin/globular protein mixture was produced at conditions (pH, temperature and concentration) where gelatin actively forms a gel and no electrostatic interaction among each other can be observed.

1.2.3. Lysozyme

Lysozyme is an antimicrobial enzyme that shows strong antimicrobial activity against gram-positive bacteria by hydrolyzing β-glycosidic bonds between C-1 on N-acetylmuramic acid and C-4 on N-acetylglucosamine in the peptidoglycan cell wall (Losso, Nakai, and Charter 2000; Johnson and Larson 2005). This disruption on the mechanical strength of bacterial cell wall results in cell death by lysis (Düring et al. 1999).

Gram-negative bacteria become more susceptible to lysozyme after the disruption of protective lipopolysaccharide (LPS) layer on the outer membrane by EDTA, organic acids, lactoferrin, nisin and modified lysozyme (Wu et al. 2019). There are also some studies demonstrating its antifungal activity against some fungal pathogens (Utkhede and Bogdanoff 2003; Wang et al. 2005; Wang, Ye, and Rao 2012). Lysozyme is naturally present in a wide variety of organisms such as animals (egg white, mammalian organs, tissues and fluids such as milk, saliva, serum and tears), plants (papaya, turnip, leguminous lysozyme isolated from mung bean and cranberry bean) and microorganisms (Fleming 1922; Alderton, Ward, and Fevold 1945; Howard and Glazer 1967; Tsugita et al. 1963; Bernier et al. 1971; Wang et al. 2005; Wang, Ye, and Rao 2012).

A hydrophobic core with hydrophilic aminoacid side chains opening toward the surface maintains lysozyme stability. Due to its disulfide bonds, lysozyme is highly heat stable even in acidic conditions (Proctor and Cunningham 1988). Its isoelectric point is pI ≈ 11.0 (Losso, Nakai, and Charter 2000; Kavur and Yemenicioğlu 2020). Its activity is high at pH within 4.5 – 6.5 range (Sozbilen and Yemenicioğlu 2021), but low stability and activity are observed in highly acidic and alkaline conditions (Proctor and Cunningham 1988). Sugars increase the heat stability of lysozyme by enhancing protein-protein interactions (Uedaira and Uedaira 1980). Egg white lysozyme was regarded as GRAS by FDA in 2000 (GRAS Notice No. GRN 64) and also lysozyme produced by *Trichoderma reesei* was recently approved as GRAS by FDA in 2020 (GRAS Notice No. GRN 853). According to European Commission Regulation (EC) No 2066/2001 of 22 October 2001, it can be used in winemaking up to 500 mg/L to control malolactic fermentation.

1.2.4. Nisin

Nisin is an antimicrobial peptide usually produced by fermentation of some *Lactococcus lactis* subsp. *lactis* strains in a whey or milk-based medium (Thomas, Clarkson, and Delves-Broughton 2000). It shows strong antimicrobial activity against gram-positive bacteria by its pore-forming activity throughout the target cell membrane. Positively charged nisin binds to cell membrane containing anionic lipids with its carboxyl (C)-terminal part via electrostatic interactions and forms pores by the

penetration of its more hydrophobic amino (N)-terminal part into the lipid phase of the membrane (Breukink and de Kruijff 1999, 2006; Zhang 2019). Pore formation disrupts the proton motive force resulting in cell death (Zhang 2019). Gram-negative bacteria become more susceptible to nisin after disrupting protective LPS layer on the outer membrane by EDTA, organic acids, lactoferrin and lysozyme (Harris, Fleming, and Klaenhammer 1992). Moreover, it can inhibit the growth of bacterial spores such as *Clostridium botulinum* (Scott and Taylor 1982), *Clostridium difficile* (Lay et al. 2016) and *Bacillus coagulans* (Campbell and Sniff 1959). There are also scarce studies demonstrating its antifungal activity against some fungal pathogens (Paster et al. 1999; Lay et al. 2008; Thanjavur et al. 2022).

The thio-ether bridged rings give nisin a rigid screw-like structure and hydrophobic residues are located at oppositely with hydrophilic residues (Breukink and de Kruijff 1999). This ring structure provides the protection from thermal and enzymatic degradations (Khan and Oh 2016). Nisin stability depends on its solubility. For example; it is highly heat stable even at 2.5 pH, but insoluble at neutral and alkaline pH values (Hurst 1981). Its isoelectric point is pI ≈ 9.0 (Zhang 2019; Kavur and Yemenicioğlu 2020). Nisin was regarded as GRAS by FDA in 2000 (GRAS Notice No. GRN 65). According to European Commission Regulation (EC) No 1333/2008 of 16 December 2008, it can be used in ripened and processed cheese and cheese products up to 12.5 mg/kg, in pasteurized liquid egg up to 6.25 mg/L, in some desserts (semolina and tapioca puddings and similar products) up to 3 mg/L and in mascarpone cheese and clotted cream up to 10 mg/kg.

1.3. Materials and Methods

1.3.1. Materials

Type B gelatin (from bovine skin) was kindly provided by Seljel (Sel Sanayi, Balıkesir, Turkey) (bloom strength: 220, ≥ 70% protein, viscosity: 2.5 mPa.s at the concentration of 6.67%). Commercial soy protein isolate (Dunasoy 90 at 90% w/w protein) obtained from non-GMO defatted and dehulled soybeans was kindly provided

by Euroduna Food Ingredients GmbH (Barmstedt, Germany). Sucrose (MW: 342.30 g/mol) was purchased from Panreac Quimica SLU (Barcelona, Spain). The commercial inulin (from chicory root) was obtained from Beneo-Orafti HSI (Oreye, Belgium) (minimum 90% inulin, DP ≥ 10). Lyophilized powder of lysozyme from chicken egg white (L6876) (Product information: ≥ 90% protein, activity: ≥ 40000 U/mg) and nisin from *Lactococcus lactis* (N5764) (Product information: 2.5%, activity: ≥ 10^6 IU/g) were purchased from Sigma-Aldrich (St. Louis, MO, USA). L-ascorbic acid was purchased from (Merck, Darmstadt, Germany). *Listeria innocua* NRRL-B 33314 (ATCC 1915) was from the culture collection of the microbiology laboratory of the Department of Food Engineering at Izmir Institute of Technology. Whole fresh Starking Delicious apples (average weight: 169.0 ± 3.7 g) and commercial caramel sauce (Kenton, Istanbul, Turkey) were purchased from a local retail supermarket.

1.3.2. Methods

1.3.2.1. Production of Gelatin Based Blend Gels

The gel solution was prepared by blending and dissolving gelatin (GEL), inulin (IN) and sucrose (SUC) at concentrations of 14, 31 and 31% (w/v) in warm water (≈ 50ºC) by stirring at 500 rpm, respectively. The soy protein isolate (SP) was then added into the blend at the concentration of 1.4% (w/v), and the blend was homogenized at 10000 rpm for 2 min using a homogenizer (Heidolph Instruments, Silent Crusher M, Schwabach, Germany). In this formulation, SUC was used to control the development of *Listeria* and other bacteria in the gel by reducing its water activity (a_w) < 0.9, while SP and IN were employed mainly to increase water holding capacity and mechanical stability of GEL based gel, respectively. The concentrations of each component were optimized with detailed preliminaries that targeted optimization of specified gel properties. The blend GEL/SP/IN/SUC gel solution was heated in a water bath at 85ºC for 45 min to induce gel formation of proteins. After cooling, nisin (NIS), lysozyme (LYS) and ascorbic acid (AA) were added and then dissolved (each at 1% w/v alone or in combination) in warm gel solutions by stirring. The heated gel solutions were then poured into molds (12-

well cell culture plate having well depth of 17 mm and well diameter of 22 mm) and incubated for 20 h at 4ºC to achieve complete gelation. The cooled gels were removed gently from molds and used in different characterization studies. During application for caramel apples, the heated gels were directly used as a filling material.

1.3.2.2. Mechanical Properties of Gels

Texture profile analysis (TPA) of gels was characterized using a TA-XT plus texture analyzer (Stable Micro Systems Ltd., Godalming, UK) equipped with a round compression plate (plate diameter: 75 mm, crosshead speed: 0.5 mm/, cell load: 50 kg). Test conditions used by Fiszman and Damásio (2000) were applied with slight modifications based on our gel properties. Cylindrical gel samples (22 mm in diameter × 15 mm in height) at 4ºC were compressed up to 50% deformation. From force – time curve (see Figure. B1 in Appx. B), hardness (N), springiness or elasticity (mm), resilience (dimensionless), cohesiveness (dimensionless) and gumminess (N) were determined according to (Bourne 2002). Experiments of each gel were replicated twice with five repetitions.

1.3.2.3. Physicochemical Properties of Gel Forming Solutions and Gels

Total soluble solid (TSS) content of gel forming solutions was determined with a digital refractometer (Atago 3830, PAL-3, Tokyo, Japan). The equipment was calibrated with distilled water. Measurements were replicated twice with three repetitions.

The turbidities of gel forming solutions were determined by measuring their transmittance (T%) at 640 nm using a spectrophotometer (Shimadzu UV-Vis, Model 2450, Tokyo, Japan). Measurements were replicated twice with three repetitions.

Water activity (a_w) of gels was measured directly by placing 7.5 g gel in a bench-top a_w-meter (HygroLab, Rotronic AG, Bassersdorf, Switzerland).

pH of gels was determined using a digital pH-meter (inoLab, Terminal, Level 3, WTW GmbH, Weilheim, Germany) as described in Brewer (2012). The samples were

prepared by homogenizing 1 g of gel with 9 mL of distilled water. Measurements were replicated twice with three repetitions.

Water binding capacity (WBC) of gels was determined according to a gravimetric method of Witono et al. (2014) at 4°C. Gels were accurately weighed after annealing for 20 h at 4°C (M_1). Each gel was immersed in 100 mL of distilled water shaking at 80 rpm at 4°C. After 72 hours (at which water equilibrium uptake) gels were taken out, excess water on the gel surface was slightly wipped off using tissue paper and reweighed for final weight (M_2). WBC was calculated as g water/g gel using the following equation (Eq. 1.1). Measurements were replicated twice with three repetitions.

$$WBC = (M_2 - M_1) / M_1 \quad \text{(Eq. 1.1)}$$

The colour of gels was determined with a digital portable colorimeter (chromometer type, Konica Minolta, CR-400, Tokyo, Japan associated with illuminant D65, standard observer 2° and illumination area of 8 mm diameter) standardized with a white plate (Y = 93.80, X = 0.3159, y = 0.3322). For standardization, a white background was placed under the gels and the CIELab colour scale was used; L* (0, dark; 100, light), a* (-a, greenness; +a, redness; 0, neutral) and b* (-b, blueness; +b, yellowness; 0, neutral). Experiments of each gel were replicated twice with five repetitions.

1.3.2.4. Antimicrobial Activity of Gels against *L. innocua* in Broth Media

The antimicrobial tests were conducted by *L. innocua* growth and adapted at 4°C. Stock culture of *L. innocua* was maintained in Nutrient Broth (Merck, Darmstadt, Germany) supplemented with 20% sterile glycerol in a 1:1 ratio (v/v) at -20°C prior to the analysis. *L. innocua* was prepared by transferring one loop of frozen culture to Nutrient Broth and incubating culture at 37°C for 24 h. One-mL aliquot from the active culture was transferred into fresh Nutrient Broth, and it was incubated at 4°C for 24 h to achieve the adaptation of its growth at 4°C. Discs of gels (≈ 10 g, diameter: 6.6 cm, thickness: 0.5 cm) were placed into flasks containing 45 mL Nutrient Broth and 5 mL bacterial culture (10^7 cfu/mL). The flasks were sealed and placed into a shaker working at 80 rpm and 4°C. The bacteria were counted at day 0, 1, 4, 7 and 14. For this purpose,

1 mL aliquot from each flask was serially diluted and spread-plated onto Oxford Listeria Selective Agar (Merck, Darmstadt, Germany) enriched with Oxford Listeria Selective Supplement (Merck, Darmstadt, Germany). The plates were incubated at 37ºC for 24 h, the colonies formed were counted and expressed as log cfu/mL. Experiments of each gel were replicated twice with two repetitions.

1.3.2.5. LYS and NIS Release Profiles of Gels

To determine their soluble/bound LYS and NIS contents, gels were subjected to release tests in distilled water at 4ºC. Discs of gels (diameter: 22 mm, thickness: 5mm) were placed into beakers containing distilled water ten times their weight (25 mL) and shaken at 80 rpm. The release curves were formed by plotting the calculated released activities [Unit (U) and International Unit (IU) per g of gel for LYS and NIS, respectively] versus time. The release tests continued until reaching equilibrium for release of LYS or NIS. All calculations were corrected by considering the activity removed from the aqueous media during sampling. The recoveries (%) of LYS and NIS were calculated considering the ratio of total activities (U or IU) released and total activities added into gels. Experiments of each gel were replicated twice with three repetitions.

1.3.2.5.1. Monitoring of LYS Activity

LYS activity was determined by the spectrophotometric method given by Arcan and Yemenicioğlu (2013) using *Micrococcus lysodeikticus* ATCC No. 4698 (Sigma-Aldrich, St. Louis, MO, USA) as a substrate. For this purpose, the reaction mixture was prepared by mixing 0.1 mL release test solution and 2.4 mL *Micrococcus lysodeikticus* cell suspension (substrate absorbance was adjusted to 0.6 – 0.7 at a concentration of 0.26 mg/mL) kept at 30ºC. The reduction in absorbance at 660 nm was monitored for 150 sec using a spectrophotometer (Shimadzu UV-Vis, Model 2450, Tokyo, Japan). The slope of the initial linear portion of absorbance versus time curve was used for the calculation of enzyme activity. One Unit of LYS activity (U) corresponds to an absorbance decrease of

0.001 per minute. The amount of released LYS was expressed as mg LYS/g gel. The release profiles were formed by plotting recovery of LYS (%) versus time (h).

1.3.2.5.2. Monitoring of NIS Activity

NIS activity was determined by the classical zone inhibition assay against *Lactobacillus plantarum* NRRL-B4496 (used as nisin bioindicator) as described by Teerakarn et al. (2002). Stock culture of *Lb. plantarum* was maintained in MRS Broth (Merck, Darmstadt, Germany) supplemented with 20% sterile glycerol in a 1:1 ratio (v/v) at -20ºC prior to the analysis. *Lb. plantarum* was prepared by transferring one loop of frozen culture to MRS Broth and incubating culture at 30ºC for 24 h. The turbidity of bacteria suspension was adjusted to 0.5 MFU (McFarland Unit) by transferring of active culture into fresh 0.1% peptone water. Then, 1 mL diluted culture was seeded into 100 mL MRS test agar freshly prepared by adding 0.75% of bacto grade agar (Becton, Dickinson and Company, USA) and 20 mL/L of 50% Tween 20 (Sigma-Aldrich, St. Louis, MO, USA) into MRS Broth. After the test agar poured into the Petri dishes solidified, 3 test wells per plate were opened on the agar surface using a sterile 6 mm diameter cork-borer and wells were filled with 50 µL release test solution. Then, Petri dishes were incubated in an anaerobic jar with BD GasPack (Becton, Dickinson and Company, USA) at 37ºC for 16 – 18 h. The diameter of each well and clear zone were measured using a digital caliper (Mitutoyo IP67, Japan) because the diameter of the inhibition zone equals the difference between the diameters of clear zone and well. The serial dilutions of NIS (500, 250, 125, 62.5, 31.25, 15.62, 7.81, 3.90, 1.95, 0.97 IU/mL) in sterile 0.05 M citrate-phosphate buffer (pH 4.0) were used to prepare a standard curve (see Figure. A1 in Appx. A) plotting by the logarithm of NIS concentration versus the diameter of inhibition zone. One International Unit of NIS activity (IU) equals to the activity of 1 µg of the standard preparation. The amount of released NIS was expressed as mg NIS/g gel. The release profiles were formed by plotting recovery of NIS (%) versus time (h).

1.3.2.6. Application of Developed Antimicrobial Gels to Produce Safe Caramel Apples

Briefly, apples washed extensively with tap water were cored using a stainless steel apple coring knife (Figure. 1.1A). This eliminates stem-ends and calyx that provide a protective site for internalization of pathogenic contaminants. The stem-end side of apples were then wrapped with a plastic stretch film to prevent leakage of gel filling (Figure. 1.1B), and apples were then placed onto a flat surface keeping their stem-ends at the bottom. Gel solution was then poured inside the void cores from the blossom-end of each apple (Figure. 1.1C). After that, sticks were immersed into gel fillings along the vertical axis of apple core and apples were incubated at 4°C for 20 h for solidification of antimicrobial gel filling (Figure. 1.1D). At the end of incubation period, the stretch film was removed and apples were dip-coated with commercial caramel solution at ≈ 45°C (Figure. 1.1E).

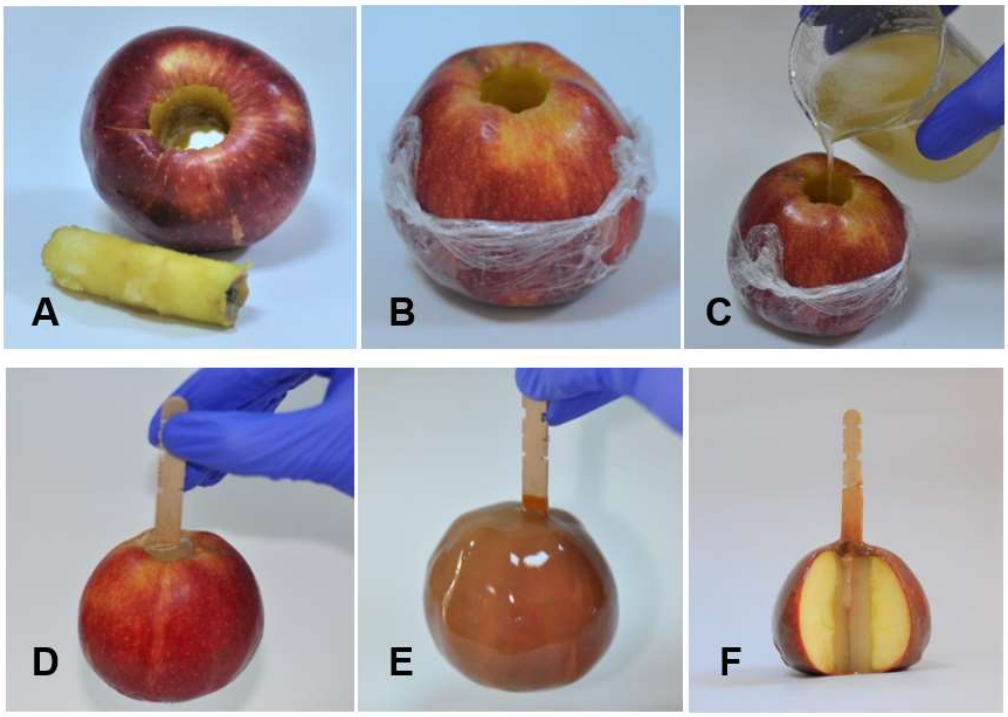

Figure 1.1. Representative images of the novel method used in caramel apple production (A: coring of apple, B: wrapping of stem-end with a stretch film to prevent leakage of the gel filling, C: pouring of gel forming solution from blossom-end, D: solidification of gel filling following stick immersion, E: caramel coating of apple, F: cross-sectional view of obtained caramel apple).

1.3.2.7. Effect of Gel Filling on *Listeria* Counts of Inoculated Caramel Apples

L. innocua used in the inoculation tests was prepared by transferring one loop of frozen culture to Nutrient Broth and incubating culture at 37°C for 24 h. One-mL aliquot from the active culture was transferred into fresh Nutrient Broth, and it was incubated at 4°C for 24 h to achieve the adaptation of its growth at 4°C. The initial number of the culture was ≈ 10^8 cfu/mL. The inner surfaces of cylindrical holes opened in apples by coring were inoculated with 0.5 mL of inoculum by spreading with a sterile plastic rod (this represents contamination of inner surface of core during processing). The inoculated apples were kept under aseptic conditions in safety cabinet for 15 min for absorption of the inoculum. The inoculated cored apples were then processed into caramel apple as described at section 1.3.2.6. Two different gels, GEL/SP/IN/SUC and GEL/SP/IN/SUC with LYS+NIS+AA, were filled into cored apples to obtain control gel-filled cored apples (GF_{apple}) and active gel-filled cored apples (AGF_{apple}), respectively. Some inoculated cored apples without gel fillings and caramel coating were wrapped with stretch films to obtain control apples (Ctr_{apple}). Standard caramel apples (SC_{apple}) were also prepared by inserting sticks into apples through stem-end, pipetting 0.5 mL of inoculums between inserted stick amd stem-end tissues of apples, and then coating apples with the caramel as described above. All apple samples were prepared in duplicate and stored at 4°C for 7 days for microbiological analysis. Samples were enumerated for *Listeria* at day 0, 1, 3 and 7. For sampling, all caramel apples were first halved, and then the solidified gel at their center was removed and discarded. The inoculated inner surface of apple cores was then carefully excised with a sterile knife, and a 20 g sample put into a sterile stomacher bag (BagFilter, Interscience, France) was homogenized for 210 s with 180 mL sterile 0.1% peptone water in a stomacher (BagMixer 400, Interscience, France). The serial decimal dilutions prepared from this homogenate were spread-plated onto Oxford Listeria Selective Agar (Merck, Darmstadt, Germany) enriched with Oxford Listeria Selective Supplement (Merck, Darmstadt, Germany). The plates were incubated at 37°C for 24 h, the colonies formed were counted and expressed as log cfu/g apple. Experiments of each caramel apple were replicated twice with three repetitions.

1.3.2.8. Effect of Gel Filling on Browning of Caramel Apples

The colour measurements were conducted using a digital portable colorimeter (Chromometer type, Konica Minolta, CR-400, Tokyo, Japan associated with illuminant D65, standard observer 2^{o} and illumination area of 8 mm diameter) standardized with a white plate (Y = 93.80, X = 0.3159, y = 0.3322) at the inner parts of halved caramel apples, after solidified gels inside their cores were removed. Results were expressed as CIELab scale using L* (0, dark; 100, light), a* (-a, greenness; +a, redness; 0, neutral) and b* (-b, blueness; +b, yellowness; 0, neutral). The measured L*, a* and b* values were also used for calculation of browning index (BI) at day 0, 1, 3 and 7 days of cold storage in the following equation (Eq. 1.2) expressed as Palou et al. (1999). Experiments of each sample were replicated twice with five repetitions.

$$BI = \frac{100(X - 0.31)}{0.172} \qquad \text{Eq. (1.2)}$$

where $X = \frac{a^* + 1.75L^*}{5.645L^* + a^* - 3.012b^*}$

1.3.2.9. Adhesion Strength of Gel Filling against Sticks Placed for Handling of Caramel Apples

Tension test was performed to determine the adhesion strength of solidified gel filling within cores of caramel apples against handling sticks placed within gel before complete solidification. This test reflected the work required to pull and liberate the stick from solidified gel in the core of caramel apple. The following texture parameters were determined by TA.XT plus texture analyzer (Stable Micro systems Ltd., Godalming, UK) equipped with tensile grips (crosshead speed: 0.5 mm/s, cell load: 50 kg) using stress – strain curve: tensile strength (TS) value is determined by finding maximum stress needed to liberate stick from gel-filling, elongation at break (EB) value is determined from percent elongation before stick liberated from gel-filling, Young's modulus (YM) value is calculated from slope of the linear region of stress – strain curve. The area under the curve (AUC) value determined from the area of stress – strain curve gives the required

energy before stick liberated from gel-filling. Peak force (PF) value is the maximum force to liberate stick from gel-filling. Measurements were done with three repetitions.

1.3.2.10. Statistical Analysis

One-way analysis of variance (ANOVA) was used to process the data of gel samples while two-way ANOVA was performed to evaluate the storage period analysis of caramel apple samples using IBM SPSS Statistics for Windows, version 23.0 (IBM Crop., USA). Statistical differences among means were compared with Duncan's multiple range test at a significance level of $P < 0.05$.

1.4. Results and Discussion

1.4.1. Preliminary Experiments for Gel Preparation

1.4.1.1. Determination of Gel Formula

Preliminary experiments were performed to optimize the gel-filling formula. Gel formulation was determined considering potentially the most influential responses that are worthy of being studied. The concentrations of components [gelatin (8, 10, 12, 14, 16% based on gel forming solution, w/v), soy protein (0, 10, 20, 30, 40% based on gelatin, w/w), inulin and/or sucrose (each at 28, 31, 34% based on gel forming solution, w/v)] were tested and the formula was optimized with detailed preliminaries that targeted optimization of certain specific gel properties. Gel composition was determined by considering the following information: (1) To measure the mechanical properties with a 50 kg load cell, the critical minimum concentration of gelatin gel, which avoided the disruption of gel integrity during the test, was found to be 8% in the preliminaries. So, the gelatin employed as a gelling agent was changed between 8 and 16% to obtain sufficient strength in the GEL. (2) The soy protein employed to increase water holding capacity was tested at different concentrations. The selected concentration was the limit

at which insoluble aggregates began to be observed within GEL. (3) The sucrose was employed to reduce the water activity of GEL below the critical value of 0.92 which is the minimum water activity demand by *Listeria monocytogenes*. The inulin was employed mainly to increase the mechanical stability of GEL and also to reduce water activity. Therefore, sucrose and inulin concentrations were changed between 28 and 34% to determine the minimum concentrations necessary to drop water activity to the target level.

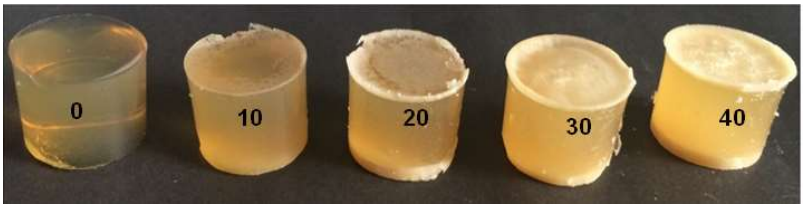

Figure 1.2. Representative image of gelatin gels containing different concentrations of soy protein.

The mechanical properties of gelatin gel series were presented in Table 1.1. Gel strength significantly increased with increasing gelatin concentration ($P < 0.05$) probably due to more intense intermolecular contacts and stronger protein-protein interactions (Zayas 1997; Porayanee, Katemake, and Duangmal 2015). Similar findings were reported by Porayanee et al. (2015) and Lau et al. (2000). In an active gel-filling design, 14% gelatin concentration was sufficient to gain an intermediate hardness because blending with other gel components would give additional hardness by decreasing the amount of liquid phase in the system. However, its low melting temperature (28 – 32^{o}C) and high tearability may limit its usage as gelatin gel alone in the current study. To avoid these drawbacks, soy protein was adopted to blend with gelatin. Soy protein has high hydrophilic character due to the high presence of aspartic and glutamic acid contents. Using soy proteins, textural properties and water binding capacity of gelatin gels were regulated. However, there was a segregative phase separation at high protein concentration (see Figure 1.2). Thus, soy protein concentration was optimized at 10% based on gelatin (w/w) or 1.4% based on gel forming solution (w/v), so that there was no phase separation (see Table 1.2).

Table 1.1. Texture profile analysis of gel series prepared with different gelatin concentrations[a,b].

Gelatin concentration (%)	Hardness (N)	Cohesiveness	Gumminess (N)	Springiness (mm)	Chewiness (N.mm)	Resilience
8	10.19 ± 1.84^{c}	0.65 ± 0.11^{bc}	6.70 ± 1.38^{d}	0.56 ± 0.07^{b}	3.74 ± 0.92^{d}	1.31 ± 0.11^{a}
10	28.90 ± 0.38^{b}	0.21 ± 0.07^{d}	6.01 ± 2.13^{d}	0.61 ± 0.14^{b}	3.70 ± 1.53^{d}	0.96 ± 0.05^{bc}
12	31.35 ± 2.71^{b}	0.44 ± 0.10^{cd}	13.73 ± 2.71^{c}	0.74 ± 0.07^{a}	10.23 ± 2.28^{c}	1.06 ± 0.10^{b}
14	31.31 ± 2.84^{b}	0.84 ± 0.33^{ab}	25.70 ± 7.35^{b}	0.84 ± 0.07^{a}	21.77 ± 7.00^{b}	0.97 ± 0.13^{bc}
16	37.58 ± 1.70^{a}	0.97 ± 0.23^{a}	36.38 ± 8.23^{a}	0.85 ± 0.05^{a}	30.61 ± 5.81^{a}	0.89 ± 0.11^{c}

[a] Different lower letter superscripts in the same column indicate significant differences ($P < 0.05$)
[b] Values are represented as mean ± SD (n = 5).

Table 1.2. Composition of gels prepared with different concentrations of inulin and/or sucrose.

Gel samples	Gelatin* (%)	Soy* (%)	Inulin* (%)	Sucrose* (%)
GEL	14	0	0	0
GEL/SP	14	1.4	0	0
GEL/IN	14	0	28 31 34	0
GEL/SP/IN	14	1.4	28 31 34	0
GEL/IN/SUC	14	0	28 31 34	28 31 34
GEL/SP/IN/SUC	14	1.4	28 31 34	28 31 34

* w/v of gel forming solution.

Table 1.3. Water activity values of gel-fillings prepared with 28% inulin and/or sucrose.

Gel samples[a]	a_w
GEL	0.983
GEL/SP	0.966
GEL/IN	0.979
GEL/SP/IN	0.983
GEL/IN/SUC	0.918
GEL/SP/IN/SUC	0.917

Table 1.4. Water activity values of gel-fillings prepared with 34% inulin and/or sucrose.

Gel samples[a]	a_w
GEL	0.973
GEL/SP	0.954
GEL/IN	0.931
GEL/SP/IN	0.933
GEL/IN/SUC	0.872
GEL/SP/IN/SUC	0.891

Water activity (a_w) of developed gel-filling must be lowered below 0.92 which is a critical growth limit of *Listeria monocytogenes* (Nolan, Chamblin, and Troller 1992) in the current work. The detected a_w values of gel-fillings were shown in Tables 1.3 – 1.4. Without any humectants, a_w values of GEL and GEL/SP gels were found to be really high enough and suitable for microbial growth, especially pathogens. *Clostridium botulinum* could even grow before *Listeria monocytogenes* in gels formed from these formulas. Given that IN alone was not enough to reduce a_w, equal amounts of IN and SUC were used in the gel formula. While a_w was high even by adding 28% IN and 28% SUC, fortunately adding 34% IN and 34% SUC reduced the a_w of GEL/SP/IN/SUC gel. However, this high sugar concentration made the gel to become very sticky. Thus, 31% IN and 31% SUC concentrations were chosen as average levels.

1.4.2. Development of GEL Based Filling with Desired Mechanical Properties

The stick used in candied apple products is a characteristic and essential component since it enables practical handling during consumption (Glass et al. 2015). Thus, the solidified form of developed blend GEL based filling should gain the desired mechanical properties (high firmness, gumminess, cohesiveness and resilience) essential

to hold and to support the stick placed at the void apple core strongly (see Figure 1.1F). A filling containing only GEL alone forms brittle gels with low adhesion to stick and it developed deep cracks and detachments during handling and storage (see Figure C1 in Appx. C). Thus, to improve the mechanical properties of developed gel, GEL matrix was supported by IN a prebiotic oligosaccharide that has been known for its ability to act synergistically with different hydrocolloids to improve their gel strength (Roberfroid 2005; BeMiller 2018; Delgado and Bañón 2018). Texture profile analysis results given at Table 1.5 clearly showed the significant improvements in hardness, cohesiveness, gumminess, chewiness and resilience of GEL gels by addition of IN ($P < 0.05$). On the other hand, SP and SUC showed significant improvements only in cohesiveness and gumminess/chewiness of GEL gels, respectively ($P < 0.05$). The combination of SUC with IN in GEL gels also gave significantly more cohesive and gummy gels than control GEL and GEL/IN gels. These results clearly showed that the improved mechanical characteristics of GEL gel originated from IN and SUC that improve gelation, networking and viscoelastic behaviour of GEL gels.

1.4.3. Performance of Developed GEL Based Filling with Stick Placed at Cores of Caramel Apples

It was a desired phenomenon to adhere the stick and to hold the caramel apple straight in the current study. The results in Table 1.6 showed the effects of different gel components on mechanical performance determined during the pulling of sticks from cores of caramel apples containing solidified gels by using the texture analyzer. TS, EB and YM values observed during pulling of sticks from solidified GEL, GEL/SP and GEL/SP/IN gel-fillings within caramel apples were not significantly different ($P > 0.05$). However, caramel apples with GEL/SP/IN gel-filling showed 1.8 and 2.0 fold higher AUC and PF values than those of GEL filled control. Moreover, caramel apples with GEL/SP/IN/SUC gel-filling exhibited a very high adhesion to sticks and showed almost 1.9, 2.8, 1.7, 3.2, 2.2 fold higher TS, EB, YM, AUC and PF values than control caramel apples filled with GEL filling. Furthermore, the gel-filling with SP and IN showed no cracking during handling and cold storage in contrast to GEL filled control (see Figure C1 in Appx. C).

Table 1.5. Development of mechanical properties of GEL gel to obtain a suitable caramel apple filling[b,c].

Gel samples[a]	Hardness (N)	Cohesiveness	Gumminess (N)	Springiness (mm)	Chewiness (N × mm)	Resilience
GEL	41.38 ± 6.06[b]	0.55 ± 0.13[d]	22.44 ± 5.82[d]	0.87 ± 0.07[c]	19.63 ± 5.46[d]	0.62 ± 0.06[b]
GEL/SP	43.70 ± 6.25[b]	0.62 ± 0.08[c]	26.80 ± 4.47[d]	0.91 ± 0.04[abc]	24.31 ± 4.34[d]	0.65 ± 0.05[b]
GEL/IN	51.93 ± 8.20[a]	0.77 ± 0.02[b]	39.92 ± 7.03[c]	0.91 ± 0.04[abc]	36.35 ± 7.62[c]	0.72 ± 0.04[a]
GEL/SP/IN	54.43 ± 5.14[a]	0.79 ± 0.02[ab]	42.89 ± 4.25[bc]	0.89 ± 0.06[bc]	38.25 ± 5.68[bc]	0.70 ± 0.03[a]
GEL/IN/SUC	58.00 ± 5.51[a]	0.84 ± 0.01[a]	48.77 ± 5.03[a]	0.95 ± 0.02[a]	46.30 ± 5.05[a]	0.72 ± 0.02[a]
GEL/SP/IN/SUC	56.70 ± 6.14[a]	0.84 ± 0.01[a]	47.64 ± 5.30[ab]	0.94 ± 0.01[ab]	44.67 ± 5.37[ab]	0.71 ± 0.03[a]

[a] GEL: gelatin, SP: soy protein, IN: inulin, SUC: sucrose.
[b] Different lower letter superscripts in the same column indicate significant differences ($P < 0.05$).
[c] Values are represented as mean ± SD (n = 10).

Table 1.6. Adhesion strength of solidified gel-filling against sticks placed at apple cores (Note: sticks were placed at cores before solidification of gels)[b,c].

Gel samples[a]	TS (MPa)	EB (%)	YM (MPa)	AUC (MPa)	PF (N)
GEL	1.72 ± 0.40[b]	2.91 ± 0.38[b]	0.69 ± 0.04[b]	6.67 ± 0.51[c]	29.81 ± 2.13[b]
GEL/SP	1.74 ± 0.16[b]	3.72 ± 1.02[b]	0.82 ± 0.04[b]	9.43 ± 1.09[bc]	34.84 ± 3.17[b]
GEL/SP/IN	2.53 ± 0.74[ab]	4.74 ± 2.05[b]	0.91 ± 0.03[ab]	12.15 ± 5.30[b]	59.24 ± 2.56[a]
GEL/SP/IN/SUC	3.25 ± 0.11[a]	8.08 ± 2.48[a]	1.15 ± 0.31[a]	21.21 ± 0.55[a]	64.99 ± 2.34[a]

[a] GEL: gelatin, SP: soy protein, IN: inulin, SUC: sucrose.
[b] Different lower letter superscripts in the same column indicate significant differences ($P < 0.05$).
[c] Values are represented as mean ± SD (n = 3).

These results clearly showed the suitability of mechanical properties for GEL/SP/IN/SUC as a gel-filling to tightly hold the sticks placed at apple cores before gel solidification.

The adhesion strength of gels on various materials such as biomaterials, wood, plastic, glass and metal has been previously studied, but there were limited studies on foods in the literature. However, there are some reports to determine peeling strength for meats from sausage casings (Nishino, Tanaka, and Yokoyama 1990a; 1990b; Nishino, Tanaka, and Yokoyama 1991; Yokoyama 1966), given that keeping the quality and integrity of sausage products is related to the degree of adhesion of meat to the casing surface. For example; Yokoyama (1966) designed an adhesion strength tester to determine the peeling strength between casing as an adhesive and fish sausage and kamaboko as a model substrate system. Moreover, Nishino et al. (1991) investigated the degree of meat adhesion to different kinds of plastic casings on fish sausages.

1.4.4. Physicochemical Properties of GEL Based Gel

Some physicochemical properties of developed gels were shown in Table 1.7. The gel development studies were focused not only to obtain desired mechanical and textural properties, but also to minimize a_w of gels and to increase their water binding capacity that is essential to prevent leakage of fruit juice from cored apples during storage. The addition of IN increased the total soluble solids (TSS) of GEL solutions to almost 40% while addition of IN with SUC further increased the TSS by close to 60%. Thus, the aw of GEL/SP/IN/SUC dropped below a_w of 0.92 which is a critical limit for growing of *Listeria monocytogenes* (Snyder 2018; Wagner and McLauchlin 2008). SP did not cause an increase in TSS of gel solutions since it formed a colloidal dispersion. However, colloidal dispersion of SP turned clear GEL solutions into highly turbid; thus, this caused dramatic drops in gel transmittance (T) values. IN alone caused less reduction in T than SP alone, but the combination of IN with SUC caused a dramatic reduction in T similar to that caused by SP. It seems that the presence of SUC turned soluble IN into a colloidal form due to the increased competition of both carbohydrates for binding with water molecules (Cui et al. 2013). Thus, GEL gels with SP, IN and SUC were highly turbid with T values lower than 1%. However, visual appearance is not a critical parameter in the current work since developed gel-fillings will serve as a filler at the cores of apples.

Table 1.7. Total soluble solid content (%), water activity, pH, transmittance (%) and water binding capacity (g water/g gel) of GEL based gels[b,c].

Gel samples[a]	TSS (%)	a_w	pH	T (%)	WBC[d] (g water/g gel)
GEL	15.8 ± 0.40[c]	0.96 ± 0.005[a]	5.42 ± 0.20[b]	92.0 ± 1.75[a]	0.35 ± 0.07[b]
GEL/SP	16.2 ± 0.32[c]	0.97 ± 0.01[a]	5.64 ± 0.20[ab]	1.8 ± 0.12[cd]	0.60 ± 0.11[a]
GEL/IN	40.9 ± 1.01[b]	0.93 ± 0.005[b]	5.50 ± 0.16[ab]	31.0 ± 2.15[b]	0.38 ± 0.07[b]
GEL/SP/IN	40.2 ± 1.37[b]	0.94 ± 0.008[b]	5.77 ± 0.12[a]	2.20 ± 0.30[c]	0.61 ± 0.10[a]
GEL/IN/SUC	59.7 ± 2.15[a]	0.90 ± 0.01[c]	5.39 ± 0.20[b]	0.64 ± 0.05[d]	0.36 ± 0.08[b]
GEL/SP/IN/SUC	59.1 ± 0.90[a]	0.89 ± 0.006[c]	5.61 ± 0.09[ab]	0.60 ± 0.06[d]	0.55 ± 0.06[a]

[a] GEL: gelatin, SP: soy protein, IN: inulin, SUC: sucrose.
[b] Different lower letter superscripts in the same column indicate significant differences ($P < 0.05$).
[c] Values are represented as mean ± SD (n = 6).
[d] Data referred to 72 h after water immersion of gels.

The SP was added to GEL solutions to improve their water binding capacity (WBC). The results of WBC tests showed that the addition of SP increased the WBC of different gels (GEL/SP, GEL/SP/IN and GEL/SP/IN/SUC) 1.6 – 1.7 fold, while IN and SUC had no considerable effect on WBC of gels. These results were inconsistent with those of Zhang and Zhao (2013) who reported that the combination of gelatin with soybean protein isolate (cross-linked with enzyme transglutaminase) gave a hydrocolloid blend with higher water holding capacity. Finally, it is important to note that the pH of gels showed a limited variation between pH 5.4 and 5.8. The isoelectric points (pIs) of GEL type B and SP are ≈ 4.8 and ≈ 4.5, respectively (Lusas and Rhee 1995; Hartel, von Elbe, and Hofberger 2018). Thus, it is clear that both GEL and SP are mainly negatively charged with the gels.

1.4.5. LYS and NIS Release Profiles of GEL Based Gel

The release profiles of major antimicrobial compounds, LYS and NIS, from developed GEL/SP/IN/SUC (G) blend gels with or without AA were presented in Table 1.8 and Table 1.9, respectively and also graphed in Figure 1.3. The release tests were conducted with G gels with LYS or NIS alone (not with a combination of LYS+NIS) to prevent interaction of LYS with NIS determination method that depends on a zone inhibition test (see section 1.3.2.5.2).

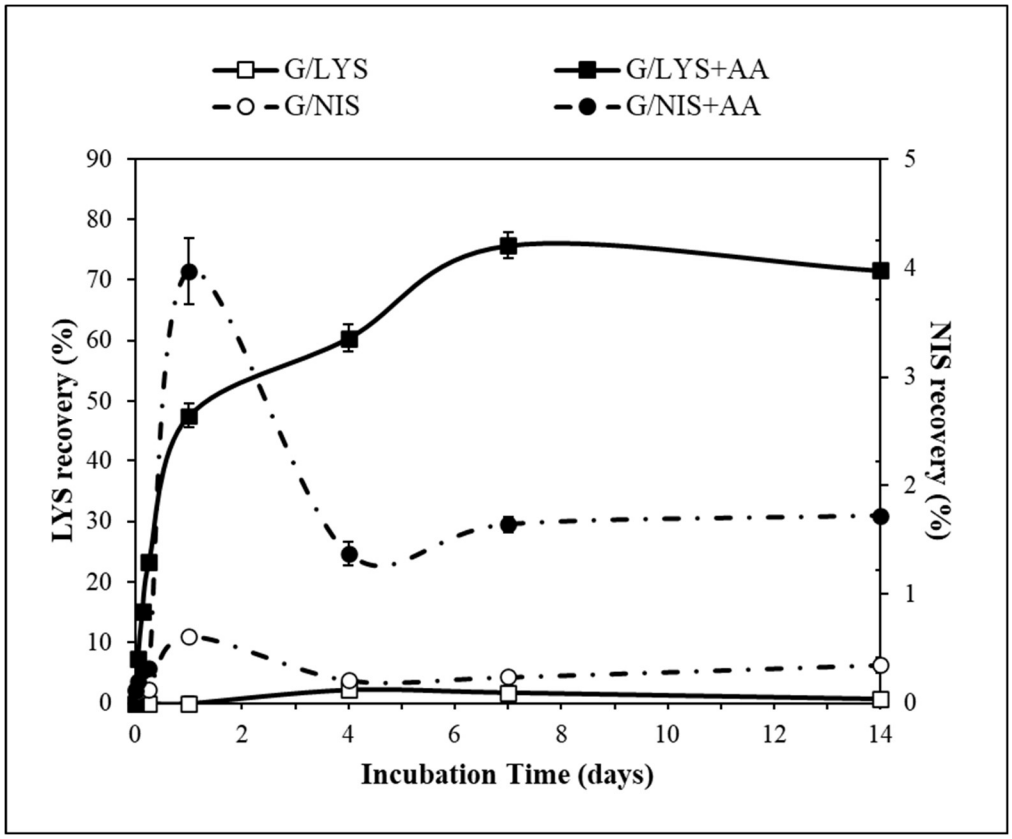

Figure 1.3. LYS (primary y-axis) and NIS (secondary y-axis) release profiles of GEL/SP/IN/SUC gels (G) with or without ascorbic acid (AA) during incubation in distilled water at 4°C.

Table 1.8. LYS release profiles of GEL/SP/IN/SUC gels (G) with or without ascorbic acid (AA) during incubation in distilled water at 4°C[b,c].

Parameter	Gel samples[a]	Incubation Times						
		1 h	***3 h***	***6 h***	***Day 1***	***Day 4***	***Day 7***	***Day 14***
Released Amount (mg LYS/g gel)	G/LYS	-	-	-	-	$0.22 \pm 0.08^{a,B}$	$0.17 \pm 0.08^{a,B}$	$0.07 \pm 0.02^{b,B}$
	G/LYS+AA	0.74 ± 0.05^{g}	1.51 ± 0.10^{f}	2.34 ± 0.05^{e}	4.75 ± 0.22^{d}	$6.04 \pm 0.25^{c,A}$	$7.57 \pm 0.23^{a,A}$	$7.16 \pm 0.53^{b,A}$
Released Activity (U/g gel)	G/LYS	-	-	-	-	$847 \pm 346^{a,B}$	$677 \pm 324^{a,B}$	$282 \pm 92^{b,B}$
	G/LYS+AA	2856 ± 213^{g}	5842 ± 423^{f}	9057 ± 192^{e}	18428 ± 865^{d}	$23425 \pm 985^{c,A}$	$29349 \pm 898^{a,A}$	$27752 \pm 2060^{b,A}$
Recovery* (%)	G/LYS	-	-	-	-	$2.18 \pm 0.89^{a,B}$	$1.75 \pm 0.84^{a,B}$	$0.73 \pm 0.24^{b,B}$
	G/LYS+AA	7.37 ± 0.55^{g}	15.07 ± 1.09^{f}	23.36 ± 0.50^{e}	47.53 ± 2.23^{d}	$60.42 \pm 2.54^{c,A}$	$75.70 \pm 2.32^{a,A}$	$71.58 \pm 5.31^{b,A}$

[a] G/LYS: GEL/SP/IN/SUC gel with lysozyme, G/LYS+AA: GEL/SP/IN/SUC gel with lysozyme and ascorbic acid.
[b] Different lower and capital letter superscripts in the same row and column indicate significant differences ($P < 0.05$), respectively.
[c] Values are represented as mean ± SD (n = 6).
* Recovery (%) = (Released amount of LYS/Incorporated amount of LYS)*100

Table 1.9. NIS release profiles of GEL/SP/IN/SUC gels (G) with or without ascorbic acid (AA) during incubation in distilled water at 4°C[b,c].

Parameter	Gel samples[a]	Incubation Times							
		0 h	***1 h***	***3 h***	***6 h***	***Day 1***	***Day 4***	***Day 7***	***Day 14***
Released Amount (mg NIS/g gel)	G/NIS	$0.013^{e,A}$	$0.010^{f,B}$	$0.013^{e,B}$	$0.012^{ef,B}$	$0.061^{a,B}$	$0.021^{d,B}$	$0.024^{c,B}$	$0.035^{b,B}$
	G/NIS+AA	$0.011^{e,B}$	$0.020^{de,A}$	$0.030^{de,A}$	$0.032^{d,A}$	$0.397^{a,A}$	$0.137^{c,A}$	$0.164^{b,A}$	$0.172^{b,A}$
Released Activity (IU/g gel)	G/NIS	$12.69 \pm 1.40^{e,A}$	$9.57 \pm 0.56^{f,B}$	$12.93 \pm 0.35^{e,B}$	$11.54 \pm 1.14^{ef,B}$	$60.73 \pm 4.94^{a,B}$	$20.65 \pm 2.26^{d,B}$	$24.27 \pm 3.42^{c,B}$	$34.71 \pm 7.28^{b,B}$
	G/NIS+AA	$11.46 \pm 1.35^{e,B}$	$20.36 \pm 1.09^{de,A}$	$29.61 \pm 1.85^{de,A}$	$31.95 \pm 3.37^{d,A}$	$397.36 \pm 49.80^{a,A}$	$136.56 \pm 11.07^{c,A}$	$164.31 \pm 7.37^{b,A}$	$171.53 \pm 57.44^{b,A}$
Recovery* (%)	G/NIS	$0.13 \pm 0.01^{e,A}$	$0.10 \pm 0.005^{f,B}$	$0.13 \pm 0.003^{e,B}$	$0.12 \pm 0.01^{ef,B}$	$0.61 \pm 0.05^{a,B}$	$0.21 \pm 0.02^{d,B}$	$0.24 \pm 0.03^{c,B}$	$0.35 \pm 0.07^{b,B}$
	G/NIS+AA	$0.11 \pm 0.01^{e,B}$	$0.20 \pm 0.01^{de,A}$	$0.30 \pm 0.01^{de,A}$	$0.32 \pm 0.03^{d,A}$	$3.97 \pm 0.50^{a,A}$	$1.37 \pm 0.11^{c,A}$	$1.64 \pm 0.07^{b,A}$	$1.72 \pm 0.57^{b,A}$

[a] G/NIS: GEL/SP/IN/SUC gel with nisin, G/NIS+AA: GEL/SP/IN/SUC gel with nisin and ascorbic acid.
[b] Different lower and capital letter superscripts in the same row and column indicate significant differences ($P < 0.05$), respectively.
[c] Values are represented as mean ± SD (n = 18).
* Recovery (%) = (Released amount of NIS/Incorporated amount of NIS)*100

The very low minimum recoveries of LYS (≈ 2% at day 4) and NIS (≈ 0.6% at day 1) from G/LYS and G/NIS gels clearly showed that the G gel binds the protein based antimicrobial compounds effectively. This result was expected since LYS (pI: 11.4) and NIS (pI: 9) (Losso et al. 2000; Silvetti et al. 2017; Zhang, 2019) are positively charged while major proteins of gel matrix, GEL and SP, are negatively charged at the G gel pH of 5.6. On the other hand, the maximum recoveries of LYS and NIS from G/LYS+AA and G/NIS+AA gels were almost 76% and 4%, respectively. The addition of AA into G gel reduced the gel pH from 5.6 to 4.1, a pH below the pI values of GEL and SP. Thus, it appeared that the reduced negative charges but increased positive charges of G gel matrix caused the liberation of a significant portion of positively charged bound LYS from the gels. In contrast, only a slight portion of bound NIS was liberated from the G gels by the pH change caused by AA. This finding suggested that the binding of NIS by the G gel matrix occurs by some other bonds and/or interactions (such as hydrophobic interactions and/or hydrogen bonds).

1.4.6. Antimicrobial Activity of GEL Based Gel against *L. innocua* in Broth Media

The antimicrobial effects of GEL/SP/IN/SUC (G) blend gel with different active agents (LYS, NIS, AA) alone or in combination on *L. innocua* was studied in broth media incubated at 4ºC. The results were demonstrated in Table 1.10 and graphed in Figure 1.4. There were no significant differences among initial *L. innocua* counts of broth cultures containing different gels ($P > 0.05$). The control culture lacking G gel and culture with G gel showed no significant change in *Listeria* counts during 1 week of incubation ($P > 0.05$). The G/AA gel was not found to be effective on *L. innocua* and caused only a very limited drop (-0.3 log) in initial bacterial counts within 1 week. However, dramatic drops were observed in *L. innocua* counts of cultures with G/NIS (-1.6 log), G/LYS+NIS (-1.9 log), or G/LYS+NIS+AA (-2.8 log) after 1 day of incubation ($P < 0.05$). The *L. innocua* counts of cultures incubated 1 day with G/NIS and G/LYS+NIS were not significantly different from each other, while culture incubated with G/LYS+NIS+AA for 1 day had significantly lower *L. innocua* count than cultures with other gels ($P < 0.05$). The G/LYS was less effective than these three potent gels and it took 4 days for this gel to cause a

significant drop (-1 log) in initial *L. innocua* counts of culture. The respective log reductions in initial *Listeria* counts of cultures with different active gels after 1 and 2 weeks of cold storage showed that the ranking of antimicrobial potency of gels from lowest to highest is as follows: G/LYS (-0.9 and -1.8 log) < G/NIS (-3.4 and -5.1 log) < G/LYS+NIS (-4.4 and -6.3 log) < G/LYS+NIS+AA (-5.2 and > -6.6 log). These results clearly proved the effectiveness of NIS alone on *Listeria*, but the most potent gels were obtained by combinational use of LYS+NIS or LYS+NIS+AA. It is important to note that according to the release test results, the highest potency of G gels with LYS+NIS+AA originated from the solubilization of LYS from these gels due to the pH drop created by AA (see section 1.4.5). However, the synergy between LYS and NIS against *Listeria* has also recently been demonstrated by our research group both for their soluble and bound forms incorporated into chitosan films (Sozbilen and Yemenicioğlu 2020).

Table 1.10. Antimicrobial activity of GEL based gels against *L. innocua* in broth media incubated at 4°C.

Gel samples[a]	***L. innocua* (Log cfu/mL)**[b,c]				
	Day 0	***Day 1***	***Day 4***	***Day 7***	***Day 14***
Control	$7.6 \pm 0.04^{a,A}$	$7.6 \pm 0.12^{a,A}$	$7.6 \pm 0.08^{a,A}$	$7.6 \pm 0.04^{a,A}$	$7.5 \pm 0.06^{a,A}$
G	$7.5 \pm 0.13^{a,A}$	$7.4 \pm 0.31^{a,A}$	$6.8 \pm 0.73^{a,BC}$	$6.8 \pm 0.64^{a,BC}$	$7.2 \pm 0.90^{a,A}$
G/LYS	$7.6 \pm 0.08^{a,A}$	$7.4 \pm 0.16^{a,A}$	$6.6 \pm 0.26^{b,C}$	$6.7 \pm 0.36^{b,C}$	$5.8 \pm 0.21^{c,B}$
G/NIS	$7.6 \pm 0.05^{a,A}$	$6.0 \pm 0.40^{b,B}$	$4.2 \pm 0.50^{c,D}$	$4.2 \pm 0.16^{c,D}$	$2.5 \pm 0.58^{d,C}$
G/AA	$7.6 \pm 0.05^{a,A}$	$7.6 \pm 0.07^{a,A}$	$7.4 \pm 0.04^{b,AB}$	$7.3 \pm 0.08^{b,BA}$	$7.0 \pm 0.14^{c,A}$
G/LYS+NIS	$7.6 \pm 0.02^{a,A}$	$5.7 \pm 0.11^{b,B}$	$3.3 \pm 0.75^{c,E}$	$3.2 \pm 0.15^{c,E}$	$1.3 \pm 0.02^{d,D}$
G/LYS+NIS+AA	$7.6 \pm 0.05^{a,A}$	$4.8 \pm 0.33^{b,C}$	$3.7 \pm 0.40^{c,DE}$	$2.4 \pm 0.35^{d,F}$	< 1

[a] Control: control culture (no gel), G: GEL/SP/IN/SUC gel, G/LYS: GEL/SP/IN/SUC gel with lysozyme, G/NIS: GEL/SP/IN/SUC gel with nisin, G/AA: GEL/SP/IN/SUC gel with ascorbic acid, G/LYS+NIS: GEL/SP/IN/SUC gel with lysozyme and nisin, G/LYS+NIS+AA: GEL/SP/IN/SUC gel with lysozyme, nisin, and ascorbic acid.
[b] Different lower and capital letter superscripts in the same row and column indicate significant differences ($P < 0.05$), respectively.
[c] Values are represented as mean ± SD (n = 4).

The synergy between NIS and LYS has also been demonstrated by different workers against *Listeria monocytogenes*, *Brochothrix thermosphacta, Staphylococcus aureus, Carnobacterium* sp. and food spoilage lactobacilli (Anderson et al. 1991; Chung and Hancock 2000; Gill and Holley 2000a, 2000b; Monticello 1990; Nattress et al. 2001; Proctor and Cunningham 1993; Takahashi et al. 2012). It was reported that the decomposition of peptidoglycan layer of bacterial cell walls by LYS enables easy access and then subsequent binding of NIS to the sensitive cell membranes which otherwise exist

buried beneath the cell surface (Dawson et al. 1996; Monticello 1990). Thus, combinational use of LYS and NIS has been suggested not only to increase the effectiveness of bacterial inactivation but also to prevent bacterial resistance problems (Pereda et al. 2017; Sudagidan and Yemenicioğlu 2012).

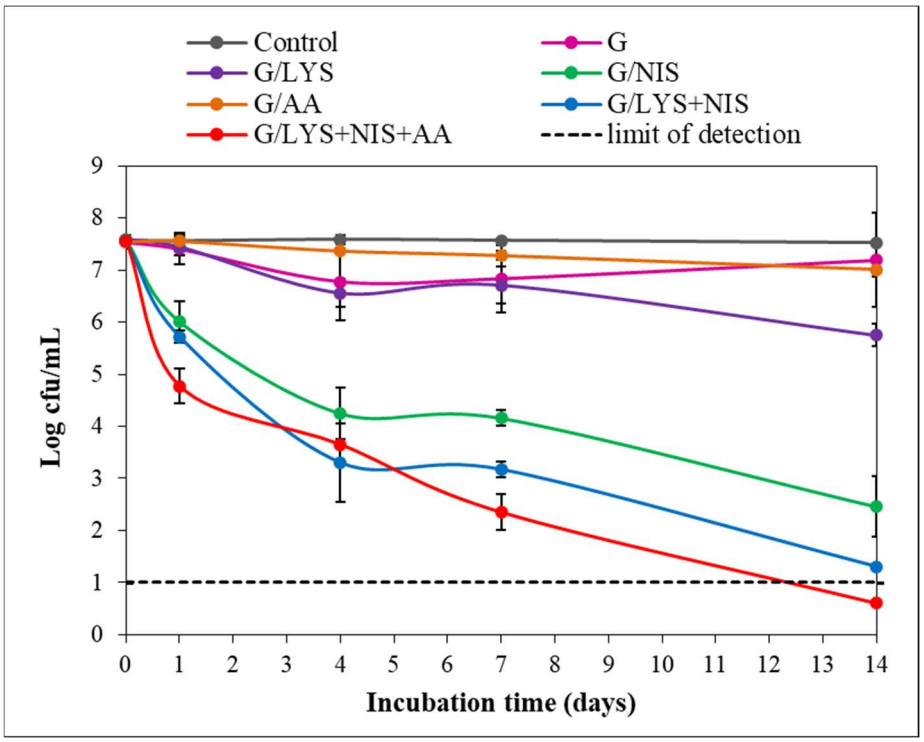

Figure 1.4. Antimicrobial activity of GEL based gels against *L. innocua* in broth media incubated at 4°C.

1.4.7. Effect of GEL Based Filling on *Listeria* Counts of Inoculated Caramel Apples

The results showing antimicrobial effect of developed G (GEL/SP/IN/SUC) gel with LYS+NIS+AA when it was filled into cores of *Listeria* inoculated caramel apples during 1 week of cold storage was presented in Table 1.11 and graphed in Figure 1.5. There were no significant differences among the initial (0^{th} day) *Listeria* counts of Ctr_{apple}, SC_{apple} and GF_{apple} while AGF_{apple} which contains the developed antimicrobial gel had 2 to 2.5 log lower initial *Listeria* counts than the controls and other caramel apples. No significant changes occurred in *Listeria* count of Ctr_{apple} within 3 days, but extended cold storage for 7 days caused a significant increase in *Listeria* count of this control sample (*P*

< 0.05). The *Listeria* counts of SC_{apple} and GF_{apple} samples at the end of 3 days were significantly higher than those of their respective initial counts on 0^{th} day ($P < 0.05$). The significant increase in *Listeria* load of SC_{apple} also continued between 3^{rd} and 7^{th} days while GF_{apple} did not show a further significant increase in its *Listeria* load between 3^{rd} and 7^{th} days. In contrast, the *Listeria* counts of AGF_{apple} on 3^{rd} and 7^{th} days were not significantly different than that at 0^{th} day. This result showed that the developed antimicrobial gel filling caused a significant reduction in initial *Listeria* load of caramel apples and prevented the significant development of *Listeria* in these samples during 7 days of cold storage.

Table 1.11. Effect of GEL based antimicrobial gel-filling on *L. innocua* counts of inoculated caramel apples stored at 4°C.

Samples[a]	***L. innocua* (Log cfu/g)**[b,c]			
	Day 0[d]	***Day 1***	***Day 3***	***Day 7***
Ctr_{apple}	$5.3 \pm 0.98^{b,A}$	$5.8 \pm 0.27^{b,A}$	$5.6 \pm 0.28^{b,B}$	$6.8 \pm 0.12^{a,A}$
SC_{apple}	$5.8 \pm 0.07^{c,A}$	$5.7 \pm 0.17^{c,A}$	$6.0 \pm 0.34^{b,A}$	$6.9 \pm 0.55^{a,A}$
GF_{apple}	$5.8 \pm 0.24^{c,A}$	$6.1 \pm 0.20^{bc,A}$	$6.4 \pm 0.35^{ab,A}$	$6.5 \pm 0.28^{a,A}$
AGF_{apple}	$3.3 \pm 0.26^{ab,B}$	$2.9 \pm 0.89^{b,B}$	$3.6 \pm 0.44^{ab,C}$	$4.0 \pm 0.69^{a,B}$

[a] Ctr_{apple}: control cored inoculated apple, SC_{apple}: standard inoculated caramel apple, GF_{apple}: cored inoculated apple filled with GEL/SP/IN/SUC gel, AGF_{apple}: cored inoculated apple filled with GEL/SP/IN/SUC gel with LYS+NIS+AA (each at 1% concentration).
[b] Different lower and capital letter superscripts in the same row and column indicate significant differences ($P < 0.05$), respectively.
[c] Values are represented as mean ± SD (n = 4).
[d] *Listeria* load of apples immediately after caramel coating.

In the literature, the LYS+NIS combination has been tested against *Listeria* spp. in different risky food such as sausages (Monticello 1990), hotdog (V A Proctor and Cunningham 1993), ham or bologna (Gill and Holley 2000a, 2000b; Mangalassary et al. 2008), minced meat (Morsy et al. 2018) and seafood products such as smoked salmon (Datta et al. 2008), minced tuna and salmon roe (Takahashi et al. 2012). Recently synergetic mixture of LYS and NIS incorporated into chitosan films has been successfully used to inactivate *Listeria* on seeds destined for edible sprout production (Sozbilen and Yemenicioğlu 2020). Moreover, considerable *Listeria* inactivation was achieved in milk using LYS and NIS in combination with mild heating below pasteurization temperatures as a biopreservation application (Sozbilen and Yemenicioğlu 2021). However, to our knowledge, the current work is the first report about the antilisterial potential of LYS+NIS combination in minimally processed fruits.

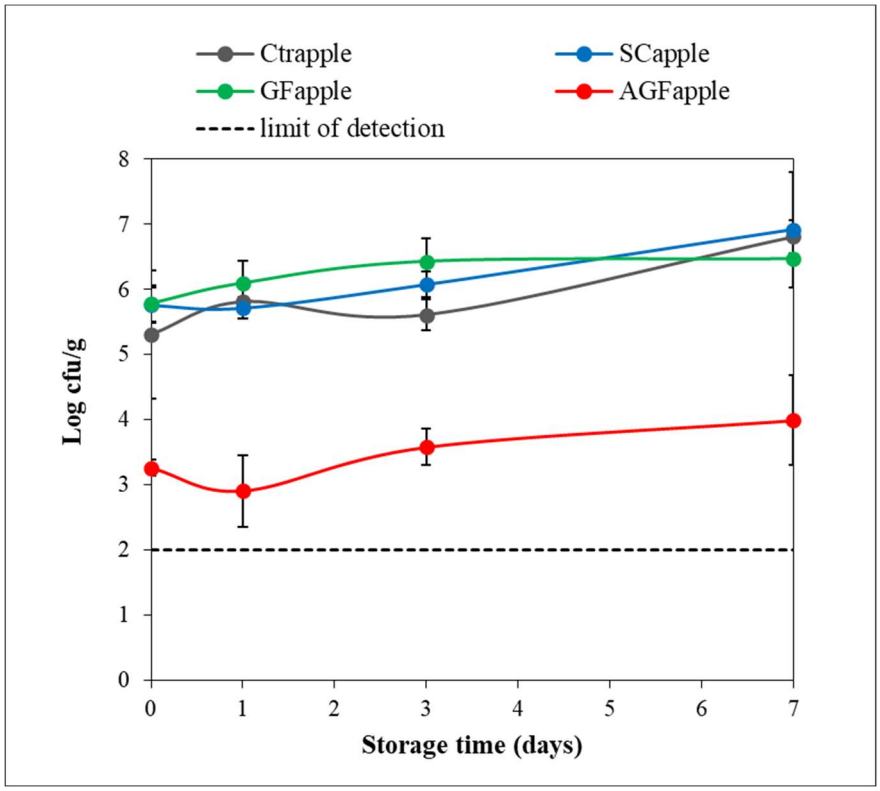

Figure 1.5. Effect of GEL based antimicrobial gel-filling on *L. innocua* counts of inoculated caramel apples stored at 4°C.

1.4.8. Effect of GEL Based Filling on Browning Index and Colour of Caramel Apples

Colour change inevitably occurs during the storage of some minimally processed products due to tissue damage. In the current work, the apple coring process provoked excessively damaged tissues. The polyphenol oxidase (PPO), an oxidoreductase group enzyme that catalyzes the oxidation of polyphenols, is responsible for the browning of apples. The control of discoloration in the core regions of apples is a highly challenging issue since PPO locates mainly around these tissues (Bhowmik and Dris 2004). L*, a* and b* values of core regions for controls and caramel apples were noted in Tables 1.12 – 1.14. The decrease in L* value and increases in a* and b* values are related to the occurrence of browning reactions in fresh-cut apples during storage (Perez-Gago, Serra, and Río 2006; Piagentini et al. 2012; Song et al. 2013). For example; Piagentini et al. (2012) reported that decreases in L* and h* (hue) and increases in a*, b* and C* (chroma) represented browning in fresh-cut apples. These trends were normally observed in the

core section of all apple samples during storage. However, colour findings indicated that the decreasing trend for L* and also the increasing trend for a* and b* of AGF_{apple} were significantly lower than Ctr_{apple} and GF_{apple} ($P < 0.05$). Thus, colour of AGF_{apple} slightly changed compared to Ctr_{apple} and GF_{apple} during storage for 7 days at 4°C due to the antibrowning effect of AA.

The browning index (BI) of core regions for controls and caramel apples were presented in Table 1.15. There were no significant differences among BI of apples used for Ctr_{apple}, GF_{apple} and AGF_{apple} immediately after coring. The BI of all apples increased significantly during the time past between coring and caramel coating ($P < 0.05$), but the increases in BI of Ctr_{apple} and GF_{apple} (3.4 and 3.0 fold, respectively) on 0^{th} day of cold storage were considerably higher than that of AGF_{apple} (1.9 fold) containing ascorbic acid (AA) within its gel-filling. As expected, the BI of Ctr_{apple} lacking any gel-filling within its core increased most rapidly and reached significantly higher levels than those of GF_{apple} and AGF_{apple} within 3 days ($P < 0.05$). The BI of GF_{apple} reached a peak point on 1^{st} day and it declined slightly with further storage. It is clear that the control gel-filling lacking AA is also slightly beneficial to suppress browning, possibly by limiting contact of PPO with air oxygen. It is important to note that the BI of AGF_{apple} did not change significantly during cold storage ($P > 0.05$). The photos of Ctr_{apple}, GF_{apple} and AGF_{apple} samples after 1 week of cold storage clearly showed the beneficial effect of using active gel-filling with AA (Figure 1.6). The cores of Ctr_{apple} and GF_{apple} turned dark brown (Figure 1.6A) and brown (Figure 1.6B), respectively. In contrast, AGF_{apple} maintained its light yellowish colour (Figure 1.6C). These results clearly showed the effective inhibition of browning by AA that is not only a reducing agent that turns enzyme-oxidized quinones back to diphenols, but also inactivates enzyme PPO by showing competitive inhibition (Mishra, Gautam, and Sharma 2012). The gel-fillings with AA have not been applied previously to prevent enzymatic browning of cored apples. However, different reports exist related to use of 1% AA in edible coatings (those from whey protein-beeswax composite, alginate, pectin and gellan gum) to prevent enzymatic browning in fresh-cut apples (Moreira et al. 2015; Perez-Gago, Serra, and Río 2006).

Table 1.12. Effect of active gel-filling on L* value of caramel apples stored at 4°C.

Samples[a]	**L*[b,c]**				
	After coring[d]	***Day 0***[e]	***Day 1***	***Day 3***	***Day 7***
Ctr_{apple}	$70.18 \pm 4.99^{a,A}$	$47.42 \pm 2.44^{b,C}$	$45.59 \pm 3.43^{b,B}$	$42.60 \pm 1.85^{c,C}$	$42.16 \pm 1.92^{c,C}$
GF_{apple}	$69.77 \pm 4.33^{a,A}$	$51.02 \pm 3.33^{b,B}$	$40.04 \pm 2.93^{c,C}$	$45.59 \pm 2.71^{c,B}$	$44.95 \pm 2.05^{c,B}$
AGF_{apple}	$69.32 \pm 4.74^{a,A}$	$57.09 \pm 3.30^{b,A}$	$53.40 \pm 3.00^{c,A}$	$55.51 \pm 1.33^{bc,A}$	$54.15 \pm 2.14^{bc,A}$

[a] Ctr_{apple}: control cored apple, GF_{apple}: cored apple filled with GEL/SP/IN/SUC gel, AGF_{apple}: cored apple filled with GEL/SP/IN/SUC gel with LYS+NIS+AA (each at 1% concentration).
[b] Different lower and capital letter superscripts in the same row and column indicate significant differences ($P < 0.05$), respectively.
[c] Values are represented as mean ± SD (n = 10).
[d,e] Colour of apples immediately after coring of apples and caramel coating, respectively.

Table 1.13. Effect of active gel-filling on a* value of caramel apples stored at 4°C.

Samples[a]	**a*[b,c]**				
	After coring[d]	***Day 0***[e]	***Day 1***	***Day 3***	***Day 7***
Ctr_{apple}	$0.24 \pm 1.52^{d,A}$	$4.28 \pm 2.07^{c,B}$	$13.33 \pm 1.30^{b,A}$	$14.54 \pm 1.48^{ab,A}$	$14.97 \pm 0.77^{a,A}$
GF_{apple}	$0.44 \pm 1.46^{c,A}$	$9.26 \pm 1.47^{b,A}$	$12.36 \pm 1.52^{a,A}$	$13.50 \pm 2.50^{a,A}$	$13.28 \pm 1.56^{a,B}$
AGF_{apple}	$0.28 \pm 1.40^{b,A}$	$3.68 \pm 2.40^{a,B}$	$3.83 \pm 1.40^{a,B}$	$3.64 \pm 1.53^{a,B}$	$2.28 \pm 2.22^{a,C}$

[a] Ctr_{apple}: control cored apple, GF_{apple}: cored apple filled with GEL/SP/IN/SUC gel, AGF_{apple}: cored apple filled with GEL/SP/IN/SUC gel with LYS+NIS+AA (each at 1% concentration).
[b] Different lower and capital letter superscripts in the same row and column indicate significant differences ($P < 0.05$), respectively.
[c] Values are represented as mean ± SD (n = 10).
[d,e] Colour of apples immediately after coring of apples and caramel coating, respectively.

Table 1.14. Effect of active gel-filling on b* value of caramel apples stored at 4°C.

Samples[a]	**b*[b,c]**				
	After coring[d]	***Day 0***[e]	***Day 1***	***Day 3***	***Day 7***
Ctr_{apple}	$24.08 \pm 5.30^{c,A}$	$37.62 \pm 2.73^{b,C}$	$38.80 \pm 2.32^{ab,A}$	$39.57 \pm 1.13^{a,A}$	$36.54 \pm 2.06^{b,A}$
GF_{apple}	$24.87 \pm 4.89^{b,A}$	$36.92 \pm 1.09^{b,B}$	$34.96 \pm 3.23^{a,B}$	$37.47 \pm 3.30^{a,A}$	$35.41 \pm 2.05^{a,A}$
AGF_{apple}	$23.97 \pm 3.98^{c,A}$	$31.64 \pm 3.21^{b,A}$	$27.74 \pm 4.86^{b,C}$	$29.46 \pm 2.00^{ab,B}$	$27.68 \pm 2.71^{b,B}$

[a] Ctr_{apple}: control cored apple, GF_{apple}: cored apple filled with GEL/SP/IN/SUC gel, AGF_{apple}: cored apple filled with GEL/SP/IN/SUC gel with LYS+NIS+AA (each at 1% concentration).
[b] Different lower and capital letter superscripts in the same row and column indicate significant differences ($P < 0.05$), respectively.
[c] Values are represented as mean ± SD (n = 10).
[d,e] Colour of apples immediately after coring of apples and caramel coating, respectively.

Table 1.15. Effect of active gel-filling on browning of caramel apples stored at 4°C.

Samples[a]	**Browning index*[b,c]**				
	After coring[d]	***Day 0***[e]	***Day 1***	***Day 3***	***Day 7***
Ctr_{apple}	$41.75 \pm 14.31^{d,A}$	$140.49 \pm 18.48^{c,A}$	$174.35 \pm 26.02^{b,A}$	$202.74 \pm 22.65^{a,A}$	$181.35 \pm 14.07^{b,A}$
GF_{apple}	$42.97 \pm 13.62^{d,A}$	$129.01 \pm 15.27^{c,A}$	$185.33 \pm 43.22^{a,A}$	$165.91 \pm 30.58^{ab,B}$	$154.85 \pm 22.95^{b,B}$
AGF_{apple}	$42.11 \pm 11.98^{b,A}$	$81.79 \pm 15.38^{a,B}$	$76.02 \pm 18.36^{a,B}$	$76.77 \pm 9.58^{a,C}$	$71.16 \pm 9.16^{a,C}$

[a] Ctr_{apple}: control cored apple, GF_{apple}: cored apple filled with GEL/SP/IN/SUC gel, AGF_{apple}: cored apple filled with GEL/SP/IN/SUC gel with LYS+NIS+AA (each at 1% concentration).
[b] Different lower and capital letter superscripts in the same row and column indicate significant differences ($P < 0.05$), respectively.
[c] Values are represented as mean ± SD (n = 10).
[d,e] Colour of apples immediately after coring of apples and caramel coating, respectively.

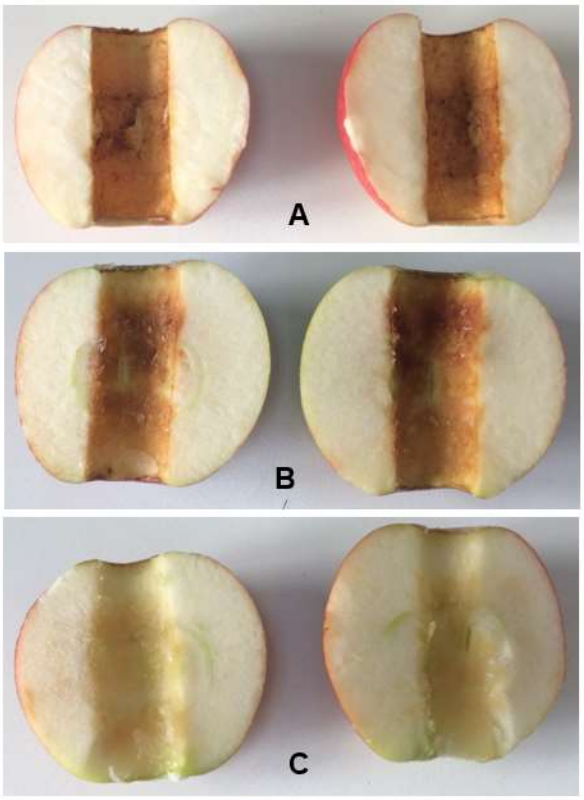

Figure 1.6. Cross-section photos of different cored apples after 1 week cold storage at 4ºC [A: Ctr_{apple}: control cored apple, B: GF_{apple}: cored apple filled with GEL/SP/IN/SUC gel, C: AGF_{apple}: cored apple filled with GEL/SP/IN/SUC gel with LYS+NIS+AA (each at 1% concentration)] (Note: gels were removed from core just before photographing).

1.5. Conclusion

The current thesis clearly showed possibility of changing classical candied apple processing method and adapting active gel-filling in their production with an innovative design. GEL based gel supported with SP, IN and SUC forms hard, mechanically stable, adhesive gel when it is filled within cored apples. This gel-filling strongly holds and supports the stick placed within the core of caramel apple before gel solidification and prevents leakage of apple juice. The active compounds (LYS, NIS and AA) added into gel-filling successfully inhibit *Listeria* and enzymatic browning in caramel apples. This work is the first study that employs an active gel filling to increase safety and quality of a minimally processed fruit product. Further studies are needed to improve in industrial applicability of developed safe caramel apple production technique against *Listeria*.

CHAPTER 2

AN INNOVATIVE DESIGN AND APPLICATION OF NOVEL CHITOSAN – CHICKPEA PROTEIN EDIBLE COMPOSITE COATING TO CONTROL SPROUTING AND POTENTIAL PATHOGENIC CONTAMINANTS IN SHALLOT BULBS (*Allium cepa* L. Aggregatum group)

2.1. Introduction

The onion varieties (*Allium* spp.) are widely cultivated and traded as popular fresh-market crops worldwide. Onion is one of the most consumed ingredients required for year-round operations. Freshly harvested onion bulbs are metabolically active and tend to grow during storage resulting in sprouting. Sprouted bulbs are undesirable, unsaleable, and unsuitable for processing due to the changes in sugar composition profile, bulb weight and shape. For example; reducing sugar content controls the degree of the non-enzymatic browning reaction during heating in dried and fried onion products (Krähmer et al. 2021) and also, the respiration and water loss result in weight loss and shrinkage. Moreover, there are some problems with the sorting and grading of sprouted ones. Although onion bulbs have one of the highest storage potentials even under ambient conditions, commercial onion processing generates a 5 – 50% loss in the storage of marketable onions, 85% of which is the reason for by sprouting and rooting (Comin 1961). Therefore, control of sprouting gets into focus for postharvest management to remain economically competitive. Unfortunately, when bulbs are harvested, there are limited opportunities to retain bulb quality. Therefore, there is a need to develop postharvest applications based on a deep knowledge of plant physiology. The physiological process of freshly harvested onion bulbs can be divided into 3 periods: rest, dormancy and sprouting. Many studies have revealed the main roles of several plant hormones in regulating physiological stages. For example; gibberellin, cytokinin and

auxin are growth promoters, whereas abscisic acid is an inhibitor. Immediately after harvest, the activity of inhibitors is high while the activity of growth promoters is very low during the rest period, so bulbs do not show any growth activity. Dormancy is a physiological state during which sprouting does not begin even under optimal sprouting conditions. Although no external morphological changes can be detected in dormant bulbs, dormancy release is a complicated mechanism involving various biochemical and physiological changes. Hormonal regulation, carbohydrate metabolism, reactive oxygen species (ROS) produced by NADPH oxidase and antioxidant metabolism have been reported to control dormancy (Foreman et al. 2003; Bailly, El-Maarouf-Bouteau, and Corbineau 2008; Liu et al. 2017). When dormancy releases, internal buds start to grow in bulbs. Since sprouting is a critical issue in the conservation of bulbs, different strategies have been used to extend dormancy and minimize sprouting such as low temperature storage, application of chemical sprout suppressants (chlorpropham, maleic hydrazide, ethylene, etc.) and irradiation. However, such treatments are not fully environmental-friendly. For example; long-term low temperature storage is a major economic cost in terms of carbon footprinting and irradiation is not allowed in certified organic systems. Actually, sprout control chemicals provide a practical and less expensive approach. However, legislation increasingly limits the use of many of these chemicals. For instance, The European Commission (2019) officially banned sprout inhibitor chlorpropham (CIPC) in 2020 (EU 2019/989 of 17 June) and bans on common synthetic suppressants have become widespread. There is thus considerable interest in natural alternatives including essential oils (EOs) or new strategies such as edible films and coatings. Certain oils of herbs and spices, forest residues such as bark extracts, essential oils (caraway, coriander, clove, orange, mint oils, etc.) and volatile terpenes (carvone, cineole, eugenol, limonene, menthol, etc.) which are the main components of EOs have been shown to be potent sprout suppression activity (Reynolds 1987; Vaughn and Spencer 1991; Vokou, Vareltzidou, and Katinalds 1993; Coleman, Lonergan, and Silk 2001; Kleinkopf, Oberg, and Olsen 2003; Darabi et al. 2011; Afify et al. 2012; Gómez-Castillo et al. 2013; Santos et al. 2020; Boivin et al. 2021; Belay et al. 2022; Frazier, Olsen, and Kleinkopf 2004; Teper-Bamnolker et al. 2010). Nowadays, there are some EOs containing natural sprout suppressive products in the market such as mint oil based Biox-M, clove oil based Biox-C and caraway oil based Talent. Due to their heterogeneous chemical structures, there is still limited information on EOs' action mechanisms during dormancy period. However,

the possible mode of action of monoterpenes has been shown on the molecular level to suppress sprouting by affecting the synthesis and activity of plant hormones (Gumbo, Magwaza, and Ngobese 2021). For example; cineole and menthol (Clegg et al. 1980) and S-carvone (Oosterhaven, Hartmans, and Huizing 1993) postpone sprouting by inhibiting the activity of HMG-CoA reductase. HMG-CoA reductase is a rate-limiting enzyme for the mevalonate pathway in the cytoplasm (Goldstein and Brown 1990), a crucial pathway for the synthesis of plant hormones, so it has a vital role in cell growth and development (see B5 in Appx. B). For instance; Suttle, Olson, and Lulai (2016) also reported that 1,8-cineole inhibited sprout growth by reducing the bioactive gibberellin content in potato tubers. It is previously suggested that volatility is important for EO efficacy (Vaughn and Spencer 1991).

Apart from sprouting, onion bulbs are prone to microbial contamination through contact with soil, irrigation water and food processing surfaces. The low acidity and high soluble solid content of onions present a suitable environment for microbial growth. Especially, whole fresh onions have frequently been linked to *Salmonella* spp. outbreaks in the USA (CDC, 2020, 2021). Additionally, some foodborne outbreaks and recalls associated with the consumption of whole and processed onions have also been reported. For example; room stored freshly diced onions used as a garnish for burgers were found to be linked to *Escherichia coli* O157:H7 outbreak at a restaurant in Canada in 2008 (NBPSDHU 2009). The findings of Lieberman et al. (2015) also supported that diced onions should be kept in refrigeration conditions. There have been food recalls linked to fresh-cut onions alone or in vegetable mixtures (CDC 2016; FDA, 2022) following the isolation of *Listeria monocytogenes* from product and environmental samples. After harvest, whole onions are often stored at refrigeration conditions, but they are commonly shipped and retailed at ambient conditions. Although the onion skin is discarded before consumption, some studies on fresh produce informed how bacteria from the outer surface readily transferred into inner edible flesh during processing such as peeling, cutting and slicing (Vadlamudi et al. 2012; Penteado, de Castro, and Rezende 2014; Jung et al. 2017). Scollon et al. (2016) clearly showed the potential cross-contamination of *Listeria* from inoculated to sterile onions during sequential slicing. For minimal processing, onions are peeled, washed with chlorinated water and conveyed to a slicer or dicer. Scollon et al. (2016) explained the differences between two processes given that diced onions are rewashed, but sliced onions are packed directly without further washing

to keep the integrity of slices. This policy can be another critic point if there is cross-contamination. Although the mechanism of bacterial transmission into edible flesh has been characterized sufficiently, efforts to develop technological solutions to this problem are very limited. For example; Coskun et al. (2021) tried the thermal inactivation of *Salmonella* spp. by far-infrared radiation. However, it was reported that lower log reductions were obtained on the outer skin of white onions than yellow ones due to the increased thermal resistance of pathogens at a low water activity of skin. Chang et al. (2018) achieved the inactivation of fungal spores using corona discharge air plasma (CDAP), but active particles generated by CDAP were not found to be effective for the inhibition of fungal mycelium. Sharma et al. (2020) kept the onions in marketable quality for up to 3 months at ambient conditions using gamma irradiation at 120 Gy, but a low dose (0.02 – 0.09 kGy) has been recommended for sprout inhibition (WHO 1988; IAEA 1997). Puspita et al. (2022) informed that the nano-chitosan coated shallot bulbs remained marketable for 9 weeks at ambient conditions. Zhang et al. (2023) evaluated the efficiency of oregano and clove oil to control onion soft rot caused by *Pectobacterium carotovorum* subsp. *carotovorum*. Rajini (2021) first reported the inhibition effect of eugenol on black mold incidence of onion caused by *Aspergillus niger*. Generally, the antimicrobial action mechanism of EOs includes a decrease in permeability of cell membrane after penetrating the cell wall due to their hydrophobicity, an increase in membrane fluidity resulting in leakage of ions and intracellular contents, and finally cell death (Álvarez-Martínez et al. 2021; Angane et al. 2022). Certain terpenes, such as eugenol, which have a phenolic -OH group, can cross the bacterial cell membrane causing an alteration in the permeability (Gill and Holley 2006a). Also, free -OH group is able to inhibit the action of some bacterial enzymes such as histidine decarboxylase (Wendakoon and Sakaguchi 1995), protease and amylase (Thoroski, Blank, and Biliaderis 1989) by binding them via hydrogen bonds. It is important to note that eugenol is able to inhibit the activity of the membrane-bound ATPase in *Escherichia coli* and *Listeria monocytogenes* (Gill and Holley 2006b). Furthermore, intracellular reactive oxygen species generated by eugenol can induce oxidative damage and cell death by reducing the superoxide dismutase activity (Bai et al. 2022). On the other hand, eugenol shows antifungal activity by inhibiting ergosterol biosynthesis, which is the major sterol component of the phospholipid bilayer of fungal cell membrane regulating membrane permeability (Ahmad et al. 2010; de Oliveira Pereira et al. 2013).

A valuable benefit of using EOs is that they serve a dual purpose acting as antisprouting and antimicrobial (antibacterial and antifungal) agents. Moreover, it has been reported that some EOs in vapour form is more effective compared with those in liquid form (Evenari 1949; Moleyar and Narasimham 1986; Tyagi and Malik 2011; Rajini 2021). For example; EOs containing phenol, alcohol, ketone, ester and hydrocarbon functional groups show higher antimicrobial activity in the vapour phase; in contrast, the inhibition effect of EOs containing aldehyde functional groups comes from diffusion (Inouye et al. 2006). Inouye et al. (2003) explained the possible reason by the fact that vapour form allows free attachment of EOs to the organism while the lipophilic EOs in an aqueous solution tends to be formed micelles, which suppress the attachment. Given that these compounds leave behind little or no residue due to high volatility (Frazier, Olsen, and Kleinkopf 2004), there are some limitations in usage as free form. They can be characterized by low chemical and thermal stability, high sensitivity and volatility; thus, their activities show variability. They evaporate rapidly in free form, so repeated applications are necessary for long-term control. Lastly, one of the main drawbacks is also high cost of EOs (Boivin et al. 2021). One of the promising strategies to overcome these problems is to encapsulate, protect and carry of EOs in a compatible emulsion matrix to prepare active edible films. This strategy could solve these problems by providing a sustained release of the volatiles into produce over a targeted time to keep food quality and safety, thus using a limited dose as possible.

In the current study, a novel composite edible coating has been designed and investigated to control postharvest sprouting and microbial risks associated with shallot bulbs. To fabricate an active edible film, linear rod-like chitosan and globular chickpea proteins were used as biopolymer building blocks for complex formation via ultrasound at pH 5.0, a condition close to the isoelectric point of protein favoring associative phase separation (where individual biopolymer charges were opposite). For this purpose, a chitosan/chickpea protein binary complex particle emerged from electrostatic interaction and eugenol was loaded with improved stability generated by ultrasound treatment. The developed emulsion composite film was formed by complex coacervation of chitosan (CHI) and chickpea proteins (CP) for encapsulating natural inhibitor, eugenol (EUG). This work is the first study investigating the efficacy of a tailor-made film as a coating for inhibiting sprouting and pathogenic contaminants in bulbous vegetables.

2.2. Background Information

2.2.1. Emulsion-Based Edible Films and Coatings

Edible films and coatings can be defined as a continuous protective and/or functional matrix produced mainly from food-grade biopolymers such as proteins, polysaccharides and/or lipids in various forms (mono-component, blend and composite or monolayer/multilayer) and applied as a thin layer onto food surface for acting as a barrier to mass transfer (moisture, light, aroma and gases, etc.) or as a carrier of active agents (Baldwin and Hagenmaier 2012; Han 2014; Bertuzzi and Slavutsky 2017; Yemenicioğlu 2022). Films are formed as stand-alone sheets and then used as a food wrap while coatings are directly formed on foods. There are 2 common processes for film preparation (Guilbert and Gontard 2005): (1) wet process (casting) based on the phase transition of a film forming solution from polymer-in-solvent to solvent-in-polymer; (2) dry process (thermoforming) based on thermoplastic properties of biopolymers such as glass transition and gelatinization when processed into compression molding, extrusion or heat pressing. On the other hand, dipping, spraying and brushing are some of the basic methods for coating preparation.

Edible biopolymer films can be classified according to structural materials: polysaccharide-based films (prepared from plant-based materials such as starch, pectin, cellulose, guar gum, locust bean gum, marine-based materials such as alginate, agar, carrageenan, chitosan and microbial-based materials such as xanthan, gellan, pullulan), protein-based films (prepared from plant-based sources such as zein, soy, wheat, legumes and animal-based sources such as gelatin, collagen, milk proteins, whey, casein, egg proteins, myofibrillar proteins) and lipid-based films (prepared from plant-based sources such as wax, resin and animal-based sources such as beeswax, shellac, tallow) (Galus and Kadzińska 2015; Yemenicioğlu et al. 2020). These environmentally friendly films should satisfy a number of specific functional requirements such as structural integrity, colour and appearance, mechanical handling, gas exchange control, moisture barrier and nontoxicity (Sengupta and Han 2014). When mono-component materials could not meet these different functional requirements, blends and composites appear to be a promising strategy to design multicomponent films and coatings having improved characteristics.

Blends are formed by mixing at least two different materials with blending into each other. Composites are formed by combining at least two different materials without dissolving or blending into each other. Emulsion-based films, bilayer films and nanostructured films are some types of composite films. Of them, emulsion composite films are formed by dispersing and entrapping hydrophobic lipidic components in a hydrophilic matrix. In general, films made from polysaccharides and proteins show good mechanical and oxygen barrier properties, but have poor water resistance, light and moisture barrier properties. Conversely, films made from lipids show excellent water resistance and moisture barrier properties, but have poor strength. Therefore, it is reasonable to incorporate hydrophobic compounds into hydrophilic matrix to overcome these drawbacks.

Emulsion films and coatings are prepared by high emulsification of lipidic compounds (dispersed phase) into hydrocolloid based film forming solution (continuous phase). According to Yemenicioğlu (2022), such a system should form a good emulsion supported by suitable emulsifying agents; otherwise, stuck small oil droplets merge together to form larger ones (flocculation and then coalescence) and loss rapidly on the film surface by evaporation. For this purpose, polysaccharide-protein complexes have remarkable emulsifying properties thanks to polysaccharides' self-associating and proteins' amphiphilicity (Xie et al. 2023). For example; pea protein-gum arabic and whey protein-dextrin complexes stabilized oil-in-water emulsions (Zha et al. 2019; Pan et al. 2020). Shih and Daigle (2000) found that xanthan gum improved emulsifying activity of rice proteins. Proteins and polysaccharides can link together by covalent bonds generating Maillard-type conjugates and by non-covalent interactions such as electrostatic interactions, hyrophobic interactions, hydrogen bonding, Van der Waals, etc. generating coacervates and precipitates (McClements 2006; Patino and Pilosof 2011; Zhang et al. 2021) (see A and B in Figure 2.1). Such combine particles have been extensively used for emulsion stabilization, encapsulation and controlled release of active agents in food and pharmaceutical applications. Due to their amphiphilic nature, proteins rapidly adsorb at oil-water interface acting as surface active agents and support the complex to position on the interface while hydrophilic groups of polysaccharides extend into aqueous phase creating repulsive forces against droplet aggregation (Dickinson 2008; Warnakulasuriya and Nickerson 2018). On the other hand, the applied homogenization technique is important while preparing film forming emulsions because size distribution of oil droplets

is another determining factor both for the stability of film forming solutions and the physicochemical properties of self-standing films. High shear devices such as high pressure homogenizers, ultrasonicators and microfluidizers presents emulsions with fine droplets and ideal film homogeneity. Ultrasonication is gaining interest in food industry because it can produce small lipid droplets with a narrow size distribution.

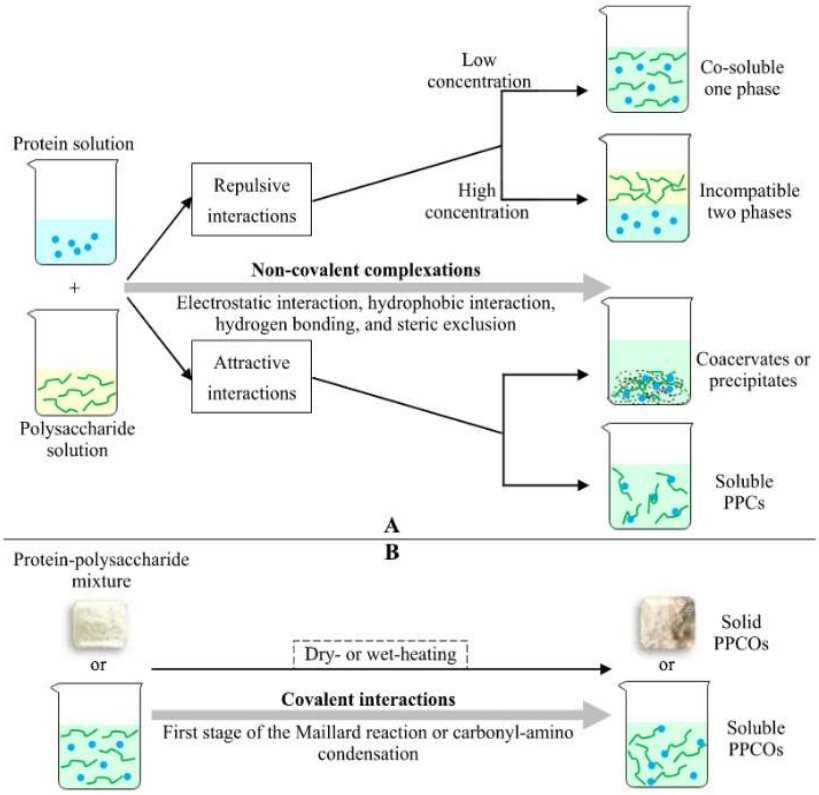

Figure 2.1. Possible scenarios to form PPCs (protein-polysaccharide complexes) and PPCOs (protein-polysaccharide conjugates) (Source: Zhang et al. 2021; copyright © 2021 with permission from Elsevier).

2.2.2. Chitosan – Chickpea Protein Composites

When oppositely charged proteins and polysaccharides are mixed in solution, they tend to behave in 3 different possible ways (Tolstoguzov 2006): (1) segregative phase separation which they repel each other resulting in two phases, one rich in polysaccharide and the other one rich in protein; (2) cosolubility which they mix well resulting in stable solution; (3) associative phase separation which they attract each other resulting in a polyelectrolyte complex. Proteins are negatively and positively charged respectively at

higher and lower pH values than pI. Thus, they can be electrostatically bonded to positively charged polysaccharides such as starch, dextran, chitosan ($pH < pK_a$) and negatively charged polysaccharides such as alginate, carrageenan, pectin, xanthan gum, gum arabic ($pH > pK_a$). In this study, we focus on using positively charged chitosan and negatively charged chickpea protein as biopolymer building blocks for complex formation assisted by ultrasound at pH 5.0, a condition close to pI of protein favoring associative phase separation (where each biopolymer charges were opposite).

Chitosan is a linear polysaccharide produced by alkaline deacetylation of chitin. It is positively charged below physiological pH ($pK_a \approx 6.5$) due to the protonation of its lateral amino groups under acidic condition. Reactive amino ($-NH_2$) and hydroxyl (-OH) groups of chitosan present many advantages, such as forming electrostatic complexes, antimicrobial activity, cross-linking, antioxidant activity, modification, chelating ability and biocompatibility (Fortunati 2016; Feng and Wang 2022). For example; antimicrobial activity of chitosan comes from its ability to bind negatively charged bacterial cell surfaces (LPS outer membrane on gram negatives and teichoic acids in gram positives due to the presence of phosphate groups) with its protonated amino groups ($-NH_3^+$) and thus disrupting the stability of cell structure (Ke et al. 2021). It has been known that gram-negative bacteria can be more susceptible to chitosan than gram-positive bacteria due to their more phosphorylated groups on LPS. Also, the thick peptidoglycan cell wall of gram-positive makes it difficult for chitosan to bind to the plasma membrane. Furthermore, chitosan is capable of chelating ions and nutrients required for microbial survival. Molecular weight and deacetylation degree have a considerable impact on its inherent antimicrobial activity (Yemenicioğlu 2022). For instance; low molecular weight chitosan and chitooligosaccharides can easily penetrate through bacterial or fungal cell walls leading to inhibition of DNA/RNA or protein synthesis (Ke et al. 2021). Chitosan has been previously used to fabricate complex coacervate with some proteins such as whey proteins (Lee and Hong 2009), casein and bovine serum albumin (Kurukji, Norton, and Spyropoulos 2016), pea protein isolate (Zhang et al. 2020), soy protein isolate (Huang et al. 2012; Dong et al. 2021), canola protein isolate (Chang et al. 2016) and rice protein hydrolysate (Xie et al. 2023). However, there were not found any studies generating the chitosan-chickpea protein complex.

Plant proteins are considered sustainable food proteins because they are safe and affordable. Given that their solubility is less than animal proteins, complexation strategies

can be used to improve their performance in food systems (Warnakulasuriya and Nickerson 2018). As a globular protein, chickpea protein shows some important functional properties such as high water and oil binding capacity, emulsification and gelation properties (Grasso et al. 2022; Yemenicioğlu 2022). Therefore, it potentially acts as a natural food emulsifier. Moreover, owing to its bland flavour, it can be easily mixed with other ingredients without overpowering the flavour.

2.2.3. Eugenol

Eugenol is a hydrophobic monoterpene mainly obtained from clove essential oil. It exhibits several antioxidant, antimicrobial, antibiofilm, and anti-inflammatory activities (Ulanowska and Olas 2021). It is listed by FDA as GRAS by FDA (21CFR184.1257). However, in practice, the utilization of eugenol as free form is still limited due to its low chemical and thermal stability, high sensitivity to oxidation, and high volatility resulting in high concentration and high cost. Encapsulation technique involving edible biopolymer complex can be a promising solution to optimize its potency within the required minimum concentration and keep its bioactivity and stability so that it can be used in the food industry.

Protein-polysaccharide complexes are powerful tools to achieve encapsulation, emulsion stabilization and controlled/sustained release of active agents under various conditions. Complex coacervation based encapsulation obtain in the following steps: emulsification of oil in polysaccharide-protein mixture, pH optimization to complex coacervation point to coat the emulsified lipid droplets by coacervate layer and production of fully coated lipid droplets (Eratte et al. 2018). This method presents some advantages, such as high loading capacity when required, high stability, targeted delivery, sustained release, limited evaporation loss, protection of entrapped core material by a shell, flavour masking, etc. Different research studies have been priorly conducted in the literature to encapsulate some hydrophobic bioactive such as orange essential oil, ergosterol, lycopene, curcumin and polyunsaturated fatty acids by use of chitosan-protein coacervates (Rojas-Moreno et al. 2018; Rudke et al. 2019; Lv et al. 2020; Okagu, Jin, and Udenigwe 2021; Chebotarev et al. 2022). For example; Chebotarev et al. (2022)

successfully fabricated milk protein-chitosan supramolecular complexes to encapsulate essential lipids such as omega-3 and omega-6.

2.3. Materials and Methods

2.3.1. Materials

Chitosan (448869, powder) having low molecular weight (50 – 190 × 10^3 Da) and 75 – 85% deacetylation, and GRAS EOs [eugenol (E51791), citral (W230316) and limonene (183164)] were purchased from Sigma-Aldrich (St. Louis, MO, USA). Glycerol (Merck, Darmstadt, Germany) was used as a plasticizer and polyoxyethylene sorbitan monooleate under the commercial name Tween 80 (Merck, Darmstadt, Germany) having hydrophilic/lipophilic (HLB) of 15.0 and a density of 1.07 g/cm^3 was used as a non-ionic surfactant in oil-in-water system. Chickpea protein concentrate (at ≈ 70% w/w protein and ≈ 20% w/w carbohydrate) was extracted from dry chickpeas (Koçbaşı variety) by classical isoelectric precipitation method described in section 2.3.2.1. As non-pathogenic surrogates for *Listeria monocytogenes* and *Escherichia coli* O157:H7, *Listeria innocua* NRRL-B 33314 (ATCC 1915) and *Escherichia coli* RSHM 4024 (ATCC 25922) were respectively selected from the culture collection of the microbiology laboratory of the Department of Food Engineering at Izmir Institute of Technology. Whole fresh shallots (average weight: 5.0 ± 1.0 g), with dry outer skin and no visible green tissue, were purchased in a commercial ripening stage from a local retail supermarket.

2.3.2. Methods

2.3.2.1. Protein Extraction from Chickpeas

Protein concentrate was obtained using isoelectric precipitation (IEP) method after alkaline extraction according to Aydemir et al. (2014) with a slight modification. For this purpose, 50 g of dry chickpea was rehydrated overnight in 500 mL of deionized

water at 4ºC. The mixture was then homogenized in a Waring blender equipped with a stainless steel jar for 2 min at high speed. For protein isolation, the pH of the homogenate was adjusted to 9.5 with 1 M NaOH and stirred using magnetic stirrer for 45 min at ambient temperature. The slurry was thinned by filtering with a synthetic cheesecloth. Fine slurry was clarified by centrifugation for 15 min at 9600 × g at 4ºC (Sigma 6K15 centrifuge, Rotor No. 12500, Osterode am Harz, Germany). The supernatant was collected and then precipitated at 4.5 pH with 1 M acetic acid. The precipitated proteins were collected by centrifugation and resuspended in distilled water. The pH of the suspension was again adjusted to 9.5 with 1 M NaOH. The suspension was clarified by centrifugation for 15 min at 9600 × g at 4ºC. The supernatant was again collected and once more precipitated at 4.5 pH with 1 M acetic acid. The precipitated proteins were collected by centrifugation and resuspended in distilled water adjusted to 7.0 pH with 1 M NaOH and lyophilized (Labconco, FreeZone, 6 L, Kansas City, MO, USA). Chickpea protein concentrate (≈ having 70% protein content detected by the Kjeldahl method and ≈ 20% carbohydrate content detected by the phenol-sulphuric acid method), contained mainly globulins, was stored at -18ºC.

2.3.2.2. Preparation of Composite Films

1.5% (w/w) chitosan was dissolved in 0.5% (v/v) glacial acetic acid solution by stirring at 300 rpm for 20 h. After overnight agitation, 0% and 50% chickpea protein concentrate (based on CHI, w/w) was added and homogenized at 12000 rpm for 3 min using a homogenizer (Heidolph Instruments, Silent Crusher M, Schwabach, Germany). To functionalize chickpea protein, additional high-power sonication or ultrasound (US) was performed at ambient temperature using an ultrasonic processor (Vibra-Cell VC505, Sonics & Materials Inc., Newtown, CT, USA) with an ultrasonic probe of 13 mm diameter and an ultrasonic converter for producing sonic waves at an operating frequency of 20 kHz. Exposure time was adjusted with a preliminary study that targeted successful protein solubility. The 150 mL glass beakers were used as a sonoreactor for 100 mL film solution and the tip of probe was submerged to a depth of approximately 3 cm. The solution was sonicated at 75% amplitude for 0 and 10 min under 500 W sonication power. The sonicated samples were cooled to room temperature. The solution was filtered using

a cheesecloth to remove insoluble residues. Then, glycerol (chitosan/glycerol: 1:1, w/w) and Tween 80 at 0.1% (w/w) were added into the solution by stirring at 300 rpm for 30 min. Eugenol at a concentration of 0 and 0.64% (w/w) was then added into the film forming solution followed by stirring at 800 rpm for 15 min. The dispersion was again homogenized at 15000 rpm for 5 min to form the emulsion. Moreover, to produce the emulsion with higher stability in sonicated samples, the dispersion was further sonicated for 2 min more (Ultrasonic warming for 2 min was measured and the temperature did not exceed 50°C. Since vapour pressure of eugenol (< 1 mmHg at ≈ 50°C) is much lower than atmospheric pressure, eugenol cannot easily evaporate (see Figure B7 in Appx. B)). The classical casting method was used to obtain self-standing films for characterization studies. For this purpose, a constant amount (20 g) of film forming solution was poured into sterile petri dishes (8.5 cm in diameter), dried at 45°C for 20 h and peeled from the casting plates prior to analyses. The concentrations of each component were optimized with detailed preliminaries that targeted optimization of specified film properties.

2.3.2.3. Microplate Turbidimetric Growth Inhibition Assay

The antibacterial activity of three GRAS EOs was tested against *L. innocua* and *E. coli* strains using a microplate turbidimetric growth inhibition assay applied by Teixeira et al. (2013) with some modifications. Stock cultures of *L. innocua* and *E. coli* were respectively maintained in Brain Heart Infusion (BHI) Broth (Merck, Darmstadt, Germany) and Nutrient Broth supplemented with 20% sterile glycerol in a 1:1 ratio (v/v) at -20°C prior to the analysis. To prepare the inocula, one loop (10 µL) of stock cultures was individually transferred into 9 mL BHI Broth and incubated at 37°C for 24 h. The initial number of inocula was 10^8 cfu/mL and serially diluted to 10^6 cfu/mL. Then, 20 µL of each bacterial suspension and 20 µL of different concentrations of EOs in 1% dimethyl sulfoxide (DMSO/C_2H_6OS) (Merck, Darmstadt, Germany) were pipetted into sterile 96-well plates (polystyrene, flat bottom, Isolab, Wertheim, Germany) containing 160 µL BHI Broth. The final volume of each well was 200 µL, the final concentration of bacterial suspensions was 10^5 cfu/mL, the final concentration of EOs was in the range of 400 – 12800 ppm (µL/mL) and the final concentration of DMSO was 1% (v/v). In this experiment, positive control (growth control) contained inoculated broth without any EO,

while negative control (sterility control) contained non-inoculated broth (see Figure 2.2). The plate was incubated at 37ºC for 24 h. To assess the antimicrobial activities, growth curves were obtained by measuring the turbidity of each well at 600 nm every 30 min up to 24 h on a microplate reader (Varioskan Flash Spectroscopy, Thermo, Finland). Any concentration of EOs in which the bacteria failed to exhibit any growth (no turbidity) was considered as minimum inhibitory concentration (MIC). Measurements were done with two repetitions.

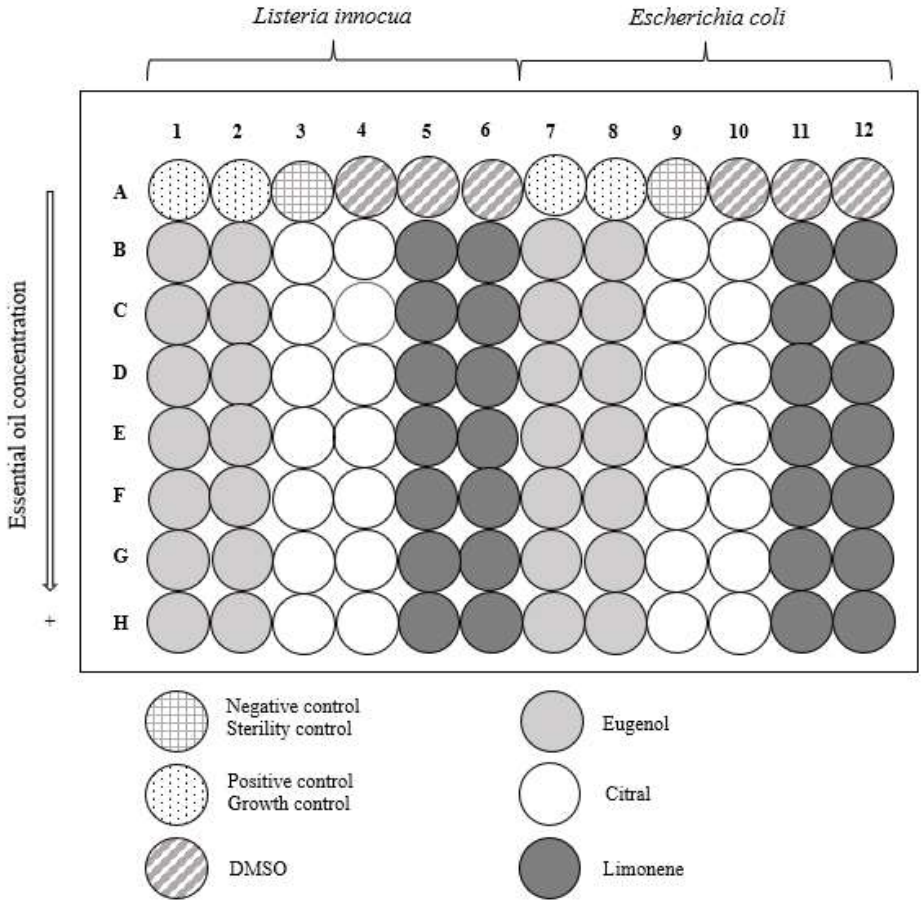

Figure 2.2. Experimental design for determining minimum inhibitory concentration (MIC) of three different essential oils: eugenol, citral, limonene.

2.3.2.4. Physicochemical Properties of Film Forming Solutions

pH of film forming solutions was directly measured by immersing probe of a digital pH-meter (inoLab, Terminal, Level 3, WTW GmbH, Weilheim, Germany) into the solutions. Measurements were replicated twice with three repetitions.

Soluble protein content of film forming solutions was determined by Bradford assay (Bradford 1976) using bovine serum albumin (BSA) (Sigma-Aldrich, St. Louis, MO, USA) as protein standard. For Bradford protein reagent preparation, 100 mg Coomassie Brilliant Blue G-250 was dissolved in 50 mL 95% ethanol and then 100 mL 85% ortho-phosphoric acid was added. The solution was diluted to a final volume of 1 L with distilled water. After filtering, the dye was ready for use. 50 µL of protein solution was added into 2.5 mL of Bradford solution and incubated in the dark for 1 hour. Absorbance was read at 595 nm. A standard curve of BSA (0.05, 0.1, 0.2, 0.4, 0.6, 0.8, 1 mg/mL) was used to calculate protein content in samples (see Figure A2 in Appx. A). Results were expressed as g soluble protein/100 g FFS. Experiments were replicated twice with three repetitions.

The light transmittance of film forming solutions was determined by measuring their transmittance (T%) at 600 nm and ambient temperature using a spectrophotometer (Shimadzu UV-Vis, Model 2450, Tokyo, Japan). Measurements were replicated twice with three repetitions.

2.3.2.5. Physical Stability of Film Forming Solutions

About 2.5 mL of film forming solution was put into a plastic cuvette and size distribution was directly measured by dynamic light scattering method on a particle size analyzer (NanoPlus 3, Particulate Systems, Micromeritics, GA, USA) with a scattering angle of 165º at ambient temperature. The results of droplet size expressed as volume-weighted mean particle diameter (D[4,3]) and surface-weighted mean particle diameter (D[3,2]) were given as µm. Experiments of each solution were replicated twice with three repetitions.

Zeta (ζ-) potential value of film forming solutions, contained individual CHI and CP and their complexes were determined using a zeta potential analyzer (NanoPlus 3, Particulate Systems, Micromeritics, GA, USA) at ambient temperature. Solutions were 10-fold diluted with 0.01 M acetate buffer adjusted to 5.0 pH and directly injected into the capillary cell of the analyzer. Experiments of each solution were replicated twice with three repetitions.

2.3.2.6. Hydrodynamic Properties of Films

Moisture content of films was determined by a gravimetric method. Test films were cut into pieces of 15 × 7.5 mm and weighed before (W_1) and after (W_2) drying in a vacuum oven at 70ºC and 20 mmHg for 24 h. Moisture content (MC) was expressed in percent, both wet basis (wb) and dry basis (db), and also dry matter content (DC) was expressed in percent from the following equations (Eqs. 2.1 – 2.3). Experiments of each film were replicated twice with five repetitions.

$$MC_{wb}\ (\%) = [(W_1 - W_2) / W_1] \times 100 \qquad \text{Eq. (2.1)}$$

$$MC_{db}\ (\%) = [(W_1 - W_2) / W_2] \times 100 \qquad \text{Eq. (2.2)}$$

$$DC\ (\%) = 100 - MC_{wb} \qquad \text{Eq. (2.3)}$$

Film solubility in water was determined by a gravimetric method proposed by Ferreira et al. (2016). Test films were cut into pieces of 15 mm × 7.5 mm, dried in a vacuum oven at 70ºC and 20 mmHg for 24 h and weighed for initial dry weight (W_1). Then, films were immersed in 10 mL of distilled water during 24 h under orbital shaking with a shaking rate of 80 rpm at ambient temperature. Afterwards, they were redried and reweighed for final weight (W_2). Water solubility (WS) was expressed in percent from the following equation (Eq. 2.4). Experiments of each film were replicated twice with five repetitions.

$$WS\ (\%) = [(W_1 - W_2) / W_1] \times 100 \qquad \text{Eq. (2.4)}$$

To evaluate the ability of the film to absorb water, swelling test was carried out using a gravimetric method proposed by Ferreira et al. (2016). Test films were cut into pieces of 15 mm × 7.5 mm, dried in a vacuum oven at 70ºC and 20 mmHg for 24 h to become completely dehumidified and weighed for initial dry weight (W_1). Then, films were immersed in 10 mL of distilled water for 24 h under orbital shaking with a shaking rate of 80 rpm at ambient temperature. Films were taken out, excess water on the film surface was slightly wiped off using tissue paper and the swelled films were reweighed for final weight (W_2). Swelling degree (SW) was expressed in percent from the following equation (Eq. 2.5). Experiments of each film were replicated twice with five repetitions.

$$SW\ (\%) = [(W_2 - W_1) / W_1] \times 100 \qquad \text{Eq. (2.5)}$$

2.3.2.7. Optical Properties of Films

The ultraviolet and visible light barrier properties of the films were determined by measuring the light transmittance (%) at selected wavelengths (200 – 800 nm) using a spectrophotometer (Shimadzu UV-Vis, Model 2450, Japan) according to Fang et al. (2002). Mean thickness values were 0.10 ± 0.05 mm for the films. Experiments of each film were replicated twice with three repetitions.

Film opacity was determined by using absorbance values of films at 600 nm (Abs_{600nm}) with a spectrophotometer (Shimadzu UV-Vis, Model 2450, Japan). Rectangular pieces of films (10 × 30 mm) were placed in a spectrophotometer test cell and the empty cell was used as a reference. Film thickness was measured using a digital micrometer (Palmer, Comecta, Barcelona, Spain) with a sensitivity of 0.001 mm. Opacity was calculated using the following equation (Eq. 2.6) given by Han and Floros (1997). Experiments of each film were replicated twice with three repetitions.

$$\text{Opacity (A/mm)} = Abs_{600}/\text{film thickness (mm)} \qquad \text{Eq. (2.6)}$$

The colour of films was determined with a digital portable colorimeter (chromometer type, Konica Minolta, CR-400, Tokyo, Japan associated with illuminant D65, standard observer 2° and illumination area of 8 mm diameter) standardized with a white plate (Y = 93.80, X = 0.3159, y = 0.3322). Film specimens were placed on a white plate and the CIELab scale was used; L* (0, dark; 100, light), a* (-a, greenness; +a, redness; 0, neutral) and b* (-b, blueness; +b, yellowness; 0, neutral). The change of colour was evaluated by comparing total colour differences between films. The colour difference (ΔE*) was calculated using the following equation (Eq. 2.7) given by (Gennadios et al. 1996). Values were expressed as the mean of five measurements on different areas of each film. Experiments of each film were replicated twice with three repetitions.

$$\Delta E^* = \sqrt{\left(L^*_{standard} - L^*_{sample}\right)^2 + \left(a^*_{standard} - a^*_{sample}\right)^2 + \left(b^*_{standard} - b^*_{sample}\right)^2} \qquad \text{Eq. (2.7)}$$

where they are differentials between the colour parameter of the samples and the colour parameter of the white standard (L* = 97.54, a* = -5.02, b* = 7.05) used as the film background.

2.3.2.8. Barrier Properties of Films

Permeability was determined by the gravimetric method standardized by ASTM Standard Method E96-95 (1995) using Payne permeability cups (Elcometer SPRL, Hermalle/s Argenteau, Belgium). There are two versions of this technique: (1) the desiccant method and (2) the water method. In the current work, the desiccant method was used as described in Çavdaroğlu et al. (2023). Test cups (anodized aluminum test cups having a 10 cm^2 opening) were filled with 3 g dried silica beads as a desiccant to produce 0% RH below the film. A film (diameter: 6 cm), oriented with the shiny side facing up, was mounted to the top of the cup and then sealed with three clamps after putting the gasket ring fixed with a thin layer of silicone vacuum grease as a sealant. After taking initial weights, the cups were incubated in an environmental chamber at 50% RH and 25°C for 2 days. Test cups were reweighted periodically for 48 h. The change in the weight of the cup was plotted against time and the slope of each line was calculated by linear regression ($R^2 \geq 0.99$). Water vapour transmission rate (WVTR), permeance and water vapour permeability (WVP) were calculated from the following equations (Eqs. 2.8 – 2.10). Experiments of each film were replicated twice with two repetitions.

$$WVTR\ \left(\frac{g}{m^2.day}\right) = \frac{\Delta w}{t \times Area} \qquad \text{Eq. (2.8)}$$

$$Permeance\ \left(\frac{g}{m^2.day.kPa}\right) = \frac{WVTR}{\Delta P} \qquad \text{Eq. (2.9)}$$

$$WVP\ \left(\frac{g.mm}{m^2.day.kPa}\right) = permeance \times thickness = \frac{\Delta w \times e}{t \times Area \times \Delta P} \qquad \text{Eq. (2.10)}$$

where Δw/t is the flux calculated as the slope obtained by linear regression of mass gain (g) of the film versus time (s), Area is test area (m^2) determined by cup diameter, e is film thickness (mm) measured using a digital micrometer (Palmer, Comecta, Barcelona, Spain) with a sensitivity of 0.001 mm, ΔP is vapour pressure difference calculated with $\Delta P = S\ (R_1 - R_2)$ where S is the saturation vapour pressure at test temperature (3.169 kPa at 25°C), R_1 is RH of test chamber (50%) and R_2 is RH within test cup (0%).

2.3.2.9. Mechanical Properties of Films

Mechanical properties of films were evaluated by measuring tensile strength (TS), elongation at break (EB), Young's modulus (YM) and toughness (TF) in accordance with ASTM Standard Method D882-02 (2002). For this purpose, films were cut into 8 mm wide and 80 mm length strips. Tensile properties were determined by TA.XT plus texture analyzer (Stable Micro Systems Ltd., Godalming, UK) equipped with tensile grips (crosshead speed: 50 mm/min, initial grip distance: 50 mm, cell load: 5 kg) using stress-strain curve (see Figure B2 in Appx. B). Film thickness was measured using a digital micrometer (Palmer, Comecta, Barcelona, Spain) with a sensitivity of 0.001 mm. Experiments of each film were replicated twice with five repetitions.

2.3.2.10. EUG Release Profiles of Films

To determine their soluble/bound EUG contents, films were subjected to release tests in model solutions and air. The release tests continued until reaching equilibrium for the release of EUG. A standard curve of EUG (0.0078, 0.0156, 0.03125, 0.0625, 0.125, 0.25 mg/mL) was used to calculate released/retained EUG content from/in films (see Figure A3 in Appx. A).

2.3.2.10.1. Release Test Performed in Model Solutions

To determine their soluble eugenol content, films were subjected to release tests in two ethanolic food simulants based on the European Commission Regulation (EU) No 10/2011 of 14 January 2011 on plastic materials and articles intended to come into contact with food: a hydrophilic food simulant A (ethanol 10% v/v) and a hydrophobic food simulant D1 (ethanol 50% v/v) incubated both at 4°C and 25°C. For this purpose, film samples were cut into 3 cm × 6 cm and immersed in 50 mL of the corresponding model solutions in flasks. Then, each film formulation – food simulant system was kept under stirring at 80 rpm at 4°C and 25°C throughout the assay. Eugenol released from films was

monitored at different time intervals and the absorbance was determined spectrophotometrically three times by using 0.1 mL of the take aliquot from the release test solution in a single measurement according to the Folin-Ciocalteu method of Singleton and Rossi (1965). The release profile over time was determined by the absorbance measurements using the standard curve (see A3 in Appx. A). The amount of released eugenol was expressed as mg EUG/cm^2 film. The release curves were formed by plotting released eugenol content (%) versus time (h). Released eugenol content (%) was calculated considering the ratio of the amount found (mg/cm^2 film) to the amount added into films (mg/cm^2 film). Experiments of each film were replicated twice with three repetitions.

2.3.2.10.2. Release Test Performed in Air

To determine their bound eugenol content, films were subjected to release tests in the air at 25ºC. For this purpose, discs of films (8.5 cm in diameter) were exposed to air for 4 weeks of room storage. One-quarter of a film (14.186 cm^2) were peeled off from the plate and then cut into small pieces with a scissor. Then, film pieces were homogenized at 18000 rpm for 3 min in 5 mL 100% ethanol and then centrifuged at 10000 × g for 15 min. Eugenol retained in films was monitored at different time intervals and the absorbance was determined spectrophotometrically three times by using 0.1 mL of the take aliquot from the supernatant in a single measurement according to the Folin-Ciocalteu method of Singleton and Rossi (1965). A standard curve of eugenol (0.0078, 0.0156, 0.03125, 0.0625, 0.125, 0.25 mg/mL) was used to calculate retained eugenol content in films (see Figure A3 in Appx. A). The retention profile over time was determined by the absorbance measurements using the standard curve. The amount of retained eugenol was expressed as mg EUG/cm^2 film. The retention curves were formed by plotting retained eugenol content (%) versus time (h). Retained eugenol content (%) was calculated considering the ratio of amount found (mg/cm^2 film) to amount added into films (mg/cm^2 film). Rate constant and half-life values were also calculated from the semi-logarithmic retained EUG concentration-time plot. Experiments of each film were replicated twice with three repetitions.

2.3.2.11. Surface Investigation of Films

The surface and cross-section morphologies of the freeze-dried films were examined using a scanning electron microscope (SEM) (FEI Quanta 250 FEG, FEI Company, USA) at different magnifications 2500× and 5000× without any sputter coating application. Before cross-sectional photographed in the SEM, film strips were fractured in liquid nitrogen. Thickness was determined from image analysis of SEM micrographs showing cross-sectional views of the films.

Surface roughness of the freeze-dried films was examined using an atomic force microscope (AFM) (Digital Instruments, MMSPM Nanoscope IV, Bruker, USA). Film samples with an area of 2 μm × 2 μm were scanned at a rate of 1 Hz using tapping mode in the air at ambient temperature using the NCHV model tip (Bruker). The captured images (3 for each sample) were analyzed by Nanoscope Analysis software v.1.7 (Bruker, Germany). The AFM images also provided quantitative data such as roughness. The surface roughness (R_a = arithmetic mean roughness, R_q = root-mean-square roughness) was measured to obtain information about the surface topology in detail.

2.3.2.12. Antimicrobial Activity of Inoculated Films

The antimicrobial activity of films was tested on inoculated films according to Boyacı et al. (2019). Films were exposed to UV-light under a laminar flow hood for 15 min. Then, they were cut into 3 cm × 6 cm pieces using a template and a sterile lancet at aseptic conditions and placed into sterile Petri dishes. Two pieces of films were used for each film type. *L. innocua* or *E. coli* used in the inoculation tests were prepared by transferring one loop of frozen culture to Nutrient Broth and incubating culture at 37ºC for 24 h. The cultures were then diluted with 0.1% peptone water to obtain their inoculums with 10^7 cfu/mL. One side of each film surface was inoculated with 225 μL of *L. innocua* (10^7 cfu/mL) or *E. coli* (10^7 cfu/mL) cultures which were spread using a sterile glass Drigalski spatula. The films were kept under a laminar flow hood for 20 min for inoculum absorption from the film surface. The Petri dishes containing the inoculated films were stored at ambient temperature. The microbial load of the films was determined on the

freshly-prepared samples (day 0) and after 1 and 5 days of incubated samples at 25ºC with a detection limit set at 2 log cfu/g. For this purpose, inoculated films (≈ 0.2 g) were put into a sterile Erlenmeyer flask using sterile tweezers and diluted 20-fold with 0.1% peptone water under constant shaking at 160 rpm for 15 min at ambient temperature. The serial decimal dilutions were prepared using peptone water and they were spread-plated onto Oxford Listeria Selective Agar (Merck, Darmstadt, Germany) enriched with Oxford Listeria Selective Supplement (Merck, Darmstadt, Germany) and Violet Red Bile Agar (Merck, Darmstadt, Germany) for enumeration of *L. innocua* and *E. coli*, respectively. The plates were incubated at 37ºC for 24 h for enumeration and the colonies were counted. Microbial counts were expressed as log cfu/g of each film. Experiments of each film were replicated twice with six repetitions.

2.3.2.13. Effect of Antimicrobial Coating on *L. innocua* and *E. coli* Counts of Inoculated Bulbs

Experimental work was carried out in September. Prior to coating, fresh shallot bulbs with no wounds or decays were selected, surface-sterilized with 1% sodium hypochlorite (NaClO) solution for 15 min and rinsed. After that, they were dried overnight in a fume hood and kept at ambient temperature. Stock cultures of *L. innocua* and *E. coli* were maintained in Nutrient Broth supplemented with 30% sterile glycerol in a 1:1 ratio (v/v) at -20ºC prior to the analysis. To prepare the inocula, 150 µL of stock cultures were individually transferred into 135 mL 1% peptone water and incubated at 37ºC for 24 h. The initial number of inocula was 10^8 cfu/mL. A high initial bacterial concentration was set in this experiment to be able to count the reduction caused by antimicrobial agents, which have strong antibacterial effects. Bulbs were immersed into the culture individually and stirred with a sterile plastic rod for 15 min to distribute the inoculum evenly. The inoculated bulbs were placed into sterile Petri dishes and dried for 24 h in a biosafety cabinet. Coating solutions with or without eugenol were prepared as described previously in section 2.3.2.2. Bulbs were dipped into flasks containing the film solutions (5 bulbs immersed in 50 mL FFSs), and the contents were stirred with a sterile plastic rod for 3 min to distribute the solution evenly. Five uncoated and coated bulbs

were placed in sterile Petri dishes. Afterward, they were dried overnight in a fume hood kept at ambient temperature. All bulb samples were prepared in duplicate.

Microbiological tests were carried out on the freshly-prepared samples (day 0, coating after 15 min) and after 1 and 5 days of incubated samples at 25ºC with a detection limit set at 2 log cfu/g. For bacterial count evaluation at day 0, five whole bulbs were diluted 10-fold with 1% peptone water and stirred vigorously in an Erlenmeyer flask at 320 rpm for 30 min. For bacterial counts evaluation on days 1 and 5, the outermost papery skin layer of bulbs was carefully excised with a sterile knife, and a 0.5 g excised skin sample was homogenized in 10 mL sterile 1% peptone water at 6000 rpm for 5 min using a high speed dispenser (Ultra Turrax tube dispenser, IKA Werke GmbH & Co. KG, Staufen, Germany). The serial decimal dilutions prepared from this homogenate were spread-plated onto Oxford Listeria Selective Agar (Merck, Darmstadt, Germany) enriched with Oxford Listeria Selective Supplement (Merck, Darmstadt, Germany) and Violet Red Bile Agar (Merck, Darmstadt, Germany) for enumeration of *L. innocua* and *E. coli*, respectively. The plates were incubated at 37ºC for 24 h and the colonies were counted. Microbiological counts were expressed as log cfu/g bulb. Experiments of each bulb were replicated twice with three repetitions.

2.3.2.14. Physicochemical Properties of Coated Bulbs

Experimental work was carried out in March. Coating solutions with or without eugenol were prepared as described previously in section 2.3.2.2. Bulbs were dipped into flasks containing the film solutions (5 bulbs immersed in 50 mL FFSs), and the contents were stirred with a plastic rod for 3 min to distribute the solution evenly. Five uncoated and coated bulbs were placed on styrofoam plates. Afterward, they were dried overnight in a fume hood kept at ambient temperature. All bulb samples were prepared in duplicate. All analyses were carried out periodically on bulbs during 28 days of room storage.

Total soluble solid (TSS) content of bulbs was determined using a digital refractometer (Atago 3830, PAL-3, Tokyo, Japan) according to Roldán-Marín et al. (2009), after the crushed juice had been manually extracted by squeezing a skinned bulb wrapped in cheesecloth. The equipment was calibrated with distilled water. Measurements were replicated twice with five repetitions.

pH of bulbs was determined using a digital pH-meter (inoLab, Terminal, Level 3, WTW GmbH, Weilheim, Germany) according to Roldán-Marín et al. (2009) after blending a skinned bulb in 30 mL distilled water with a homogenizer (Heidolph Instruments, Schwabach, Germany). Measurements were replicated twice with five repetitions.

Titratable acidity of bulbs was determined using the colorimetric titration method in accordance with AOAC Official Method 942.15 (2000). After blending a skinned bulb in 30 mL distilled water with a homogenizer (Heidolph Instruments, Schwabach, Germany), titration was performed with standard 0.1 N NaOH solution using a few drops of 1% phenolphthalein solution as an indicator. Results were expressed as percent malic acid (MA) or g MA/100 g fresh weight (FW). Measurements were replicated twice with five repetitions.

The weights of individual bulbs stored in summer at ambient conditions (≈ 25ºC and ≈ 45% RH) were recorded on each sampling day. Weight loss was determined from the change in the weight compared with that at the beginning of the storage and expressed as a percentage loss. Measurements were replicated twice with ten repetitions.

2.3.2.15. Textural Properties of Coated Bulbs

Bulb texture was measured by a penetration test performed on both polar and equatorial regions of onions using a TA.XTplus Texture Analyzer (Stable Micro Systems Ltd., Godalming, England) equipped with a needle probe attachment (crosshead speed: 0.5 mm/s, cell load: 5 kg). Test conditions used by Maw et al. (1996) were applied with slight modifications. A whole bulb was positioned in the center of the platform and the probe penetrated into the polar region of the bulb to 50% from the surface. Then, bulbs were halved vertically, each half was positioned in the center of the platform and the probe penetrated into the equatorial section of the bulb to 75% from the surface. The following texture parameters were determined using force – time curve: penetration force (PF) is the maximum force required to insert the neddle into bulb to a depth that causes irreversible crushing, and area under the curve (AUC) value determined from the area of force – time curve gives the required work.

2.3.2.16. Sprout Assessment of Coated Bulbs

The incidence of external sprouting of room stored bulbs was detected visually and recorded. A bulb was considered as sprouted when the sprout leaves had emerged from the neck (Miedema 1994), and sprouted ones were counted on each storage day. In a similar way to the method of Temkin-Gorodeiski et al. (1972), after bulbs were cut in half (vertically from top to bottom), the presence of green coloration was also determined visually using a scale of 1 to 4, which represents 1: white, 2: yellow, 3: yellowish green and 4: green (see Figure 2.3). Internal photographs were also taken at each bulb to record the sprouting status.

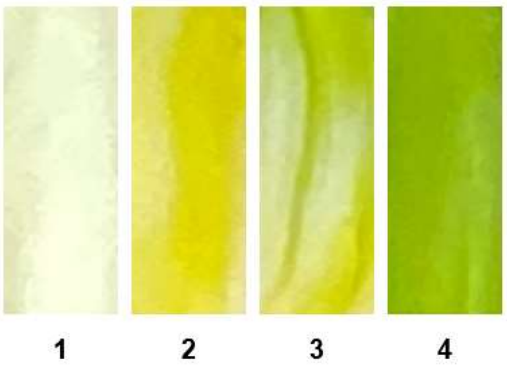

Figure 2.3. Green colour intensity scale generated from sprouts.

2.3.2.17. Sensory Evaluation of Coated Bulbs

Sensory evaluation was carried out in the sensory room at Food Engineering Department by the paired comparison test using trained panellists pre-selected from the postgraduate students of the Izmir Institute of Technology, Izmir, Turkey. Ten adults aged between 25 and 35 years took part in this evaluation. During the test, panellists judged 2 stimuli in 2 different pairs. After bulbs had been washed, two different film solutions, CHI-CP and CHI-CP-EUG, were respectively coated on bulbs to obtain control (SCP_{bulb}) and treatment ($SCPE_{bulb}$) groups, and then coated bulbs were air-dried for 24 h in a fume hood. Uncoated bulbs (Ctr_{bulb}) represented the conventional onion. After 0, 1, 7, 14, 21, 28 days of ambient storage, bulbs were coded with random three-digit numbers and

served to the panellists on trays. Fresh air was obtained between each assessment. The panellists were presented with two evaluation cards (see Figures B3 and B4 in Appx. B). In sensory evaluation, the attributes measured were as follows: general appearance and odour. The rating levels considered two response categories “yes” or “no” for present and absent reactions to the difference in sight and smell between two different bulbs.

2.3.2.18. Statistical Analysis

One-way analysis of variance (ANOVA) was used to process the data of the characterization of film and FFS samples while two-way ANOVA was performed to evaluate the storage period analysis of film and bulb samples using IBM SPSS Statistics for Windows, version 23.0 (IBM Crop., USA). The effects of two process variables (sonication time vs. component concentration) on film properties were also determined using two-way ANOVA on film properties. Statistical differences among means were compared with Duncan’s multiple range test at a significance level of $P < 0.05$.

2.4. Results and Discussion

2.4.1. Preliminary Experiments for Coating Preparation

2.4.1.1. Selection of Phenolic Compound

Antimicrobial efficacy of three selected EOs were compared given that eugenol (21CFR184.1257), citral and limonene (21CFR182.60) are considered as GRAS by FDA. Their minimum inhibitory concentrations were determined against *L. innocua* and *E. coli*. EO showing potentially the most influential antimicrobial response was selected as a result of the microplate turbidimetric growth inhibition assay. Lag time and the percentage of bacterial growth inhibition were summarized in Tables 2.1 – 2.2.

Table 2.1. Effect of EOs on *L. innocua* inhibition[a].

Samples	EO concentration (ppm)	Lag (min)	Inhibition (%)
Control	0	257.55 ± 28.42	0
Control + DMSO	0	267.11 ± 41.34	0
Eugenol	400	243.62 ± 17.10	4.91
	800	448.46 ± 80.85	37.50
	1600	-	100
	3200	-	100
Citral	400	219.42 ± 27.13	14.11
	800	374.23 ± 60.87	23.31
	1600	485.08 ± 61.27	62.50
	3200	-	100
	4800	-	100
Limonene	6400	351.83 ± 19.66	4.17
	7200	252.63 ± 18.71	29.17
	8400	375.14 ± 28.79	29.17
	12800	221.38 ± 16.09	62.50

Table 2.2. Effect of EOs on *E. coli* inhibition[a].

Samples	EO concentration (ppm)	Lag (min)	Inhibition (%)
Control	0	40.77 ± 0.76	0
Control + DMSO	0	46.50 ± 2.86	0
Eugenol	800	7.15 ± 10.10	80
	1600	-	100
	3200	-	100
Citral	800	16.59 ± 4.36	58.57
	1600	28.96 ± 2.06	58.57
	3200	187.42 ± 60.67	38.57
	4800	163.87 ± 46.85	50.00
	6400	333.57 ± 79.90	55.71
	7200	316.48 ± 160.83	67.14
	8400	377.06 ± 71.34	51.43
	12800	425.58 ± 2.23	44.29
Limonene	1600	48.73 ± 3.56	4.29
	3200	23.89 ± 12.63	35.71
	6400	11.43 ± 4.00	42.86
	7200	14.73 ± 12.86	41.43
	8400	-	62.86
	12800	-	70.00

EOs were prepared in 1% DMSO solution, after it had been confirmed that DMSO did not affect the growth of the tested bacteria. Similarly, Wadhwani et al. (2009) found that there was no adverse effect on bacterial growth at 1% and 2% concentrations of DMSO, but at above 3% concentration of DMSO some tested bacteria showed a significant decrease in growth. Minimum inhibitory concentration (MIC) is defined as the

lowest antimicrobial agent concentration that produces in-vitro inhibition of microbial growth (Rankin 2005). When MICs of selected EOs were compared, some differences were observed between MICs of eugenol, citral and limonene. As seen, *L. innocua* was highly sensitive to eugenol and citral, whereas *E. coli* were highly resistant to citral and limonene. The efficacy of EOs against *L. innocua* was in the following order: eugenol > citral > limonene, while the efficacy order of EOs against *E. coli* was: eugenol > limonene > citral. Higher concentrations of citral resulted in a longer lag time for *E. coli*. Lag time extension means that antimicrobial concentration is high enough to allow bacteria to show stress response, but still low enough that it does not fully inhibit bacterial growth (Štumpf et al. 2020). Moreover, lag time extension can also cause bacteria to develop antimicrobial resistance. Our results showed 100% growth inhibition of both *L. innocua* and *E. coli* when only 1600 ppm eugenol was present in the medium. So, eugenol was selected due to showing strong antimicrobial activity with the lowest MIC value among tested GRAS EOs against target microorganisms. Its concentration range in film formulation was adjusted depending on the outcome of the preliminary work.

2.4.1.2. Determination of Coating Formula

The job of coating is to find the perfect match between the different combinations of ingredients, the formulation used to produce the coating and the uniformity of the coating. Preliminary experiments were performed to optimize the coating formula. Film forming solutions were adjusted to maintain a consistent mass of solids on the casting plate for each formula. Film formulation was determined considering potentially the most influential responses that are worthy of being studied.

The concentrations of components [chitosan (1.5% based on the film forming solution, w/w), chickpea protein (0, 10, 30, 50% based on CHI, w/w), eugenol (0, 0.16, 0.32, 0.64% based on the film forming solution, w/w)] were tested and the formula was optimized with detailed preliminaries that targeted optimization of certain specific film properties.

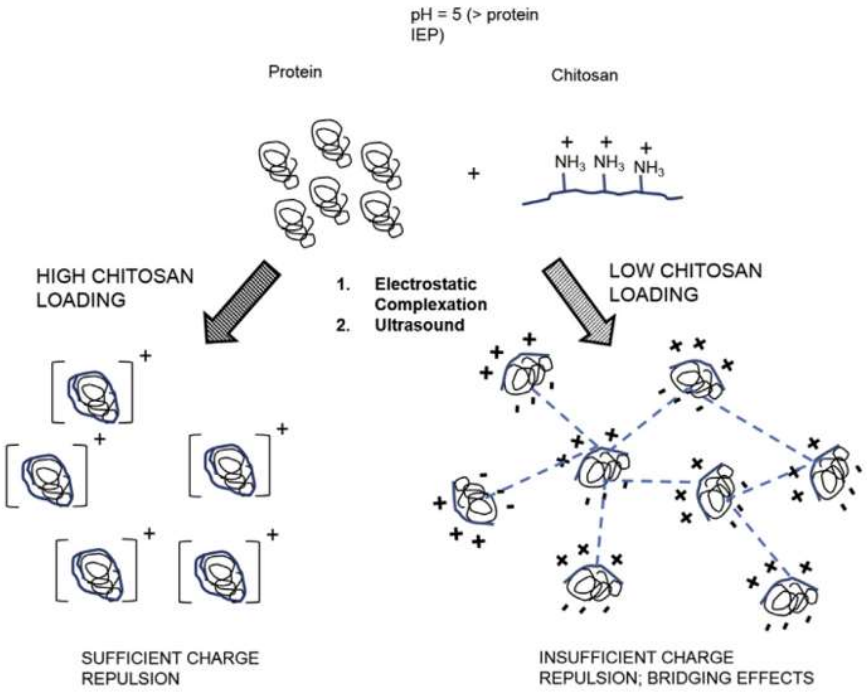

Figure 2.4. Types of complexes that can be produced by the complexation of chitosan and protein: coacervate or droplets (left-side) (liquid-liquid phase separation) and solid precipitate or particles (right-side) (liquid-solid phase separation) (Source: Kurukji, Norton, and Spyropoulos 2016).

Film composition was determined by considering the following information: (1) Chitosan (CHI) was used at 1.5% (w/w) concentration because below 1.5%, physical chain entanglements were not sufficient and more junctions needed to be formed to induce gelation and enhance mechanical strength (Montembault et al. 2005). (2) Chickpea proteins (CP) were added at a level 0, 10, 30 and 50% of chitosan by weight because excess protein resulting in low chitosan can cause insufficient charge repulsion between the individual complexes due to not fully electrostatic coverage (see Figure 2.4). As a surface active plant protein, the reason for using chickpea protein is its high oil binding capacity, emulsifying activity and stability (Aydemir and Yemenicioğlu 2013). (3) Eugenol (EUG) was used at 0, 1, 2 and 4 times the detected MIC value because EUG would be encapsulated to obtain sustained release, not in free form. It is known that practical applications are required to be higher than the theoretical value obtained under laboratory conditions. (4) 1:1 (w/w) ratio of chitosan/glycerol was used since the films would be too brittle without using glycerol, and it would be impossible to perform their analysis. Also, Jackson (1987) mentioned that a concentration of at least 50% of glycerol could be used to obtain good consistency in a gel-like membrane. (5) Tween 80 was

selected as a surfactant. It is supported by the research work of Ahmad et al. (2019) where the use of Tween 80 could minimize the globule size more effectively due to its low molecular weight compared to polymeric surfactants. However, high surfactant concentration can suppress the antibacterial action of EO by firmly encapsulating it within the surfactant micelles (Ghazy et al. 2021). Moreover, free -OH groups of Tween 80 can prevent the interaction between EO and bacteria by interacting with phenolic compounds (El-Sayed et al. 2017). Thus, Tween 80 at a level of 0.1% (w/w) was added into film forming solution to assist EO dissolution according to a study by Casariego et al. (2008).

2.4.1.3. Determination of Exposure Time of Ultrasonic Treatment

Using ultrasonic treatment with the 20 kHz probe at a power output of 500 W, the ultrasonic intensity was 63 W/cm^2 in the current work. Yuan et al. (2021) optimized the ultrasonic power to form chitosan-brown alga composite films and they reported a remarkable increase in TS and E when sonication power was over 500 W. Yan et al. (2021) obtained less soy protein solubility when subjected to ultrasonic power of 600 W compared to 400 W. Conversely, Cao et al. (2021) gained high quinoa protein solubility when ultrasonic power was both 300 and 400 W. Thus, the power of the ultrasonicator would be sufficient to achieve the desired effects in this study.

The purpose of determining mechanical properties is to obtain the strength, ductility and flexibility of a material when subjected to a load. Mechanical properties are important factors for the durability of packaging films. Films and coatings must have suitable mechanical properties to withstand external forces during the transportation, handling, storage, marketing and use of film-packed foods (Das et al. 2022). Therefore, sufficient mechanical strength is needed to ensure film integrity. For these reasons, the exposure time of sonication was adjusted according to the tensile properties of CHI-CP composite films. According to a study by Ahmadi et al. (2011), the mechanical properties of methylcellulose film improved after 5 min sonication while increasing exposure time up to 30 min caused a decrease in TS and EB values. Researchers concluded that an increase in the size and number of cavitation bubbles probably caused space between molecules and reduced film strength. Thus, the duration of sonication was set to 0-5-10 min at ambient temperature in the preliminary work.

Table 2.3. Tensile properties of non-sonicated and sonicated chitosan films containing different concentrations of chickpea protein[b,c].

Parameters[b,c]	Film samples[a]	Duration (min)		
		0	***5***	***10***
TS (MPa)	CHI-CP0	$3.78 \pm 0.21^{a,B}$	$2.57 \pm 0.21^{b,B}$	$1.63 \pm 0.30^{c,C}$
	CHI-CP10	$3.69 \pm 0.32^{a,B}$	$2.30 \pm 0.34^{c,B}$	$3.02 \pm 0.28^{b,B}$
	CHI-CP30	$2.65 \pm 0.59^{a,C}$	$2.59 \pm 0.34^{a,B}$	$2.82 \pm 0.44^{a,B}$
	CHI-CP50	$5.18 \pm 0.14^{a,A}$	$3.95 \pm 0.32^{c,A}$	$4.63 \pm 0.10^{b,A}$
EB (%)	CHI-CP0	$33.90 \pm 1.66^{b,C}$	$50.28 \pm 3.63^{a,C}$	$52.62 \pm 3.88^{a,C}$
	CHI-CP10	$36.18 \pm 2.30^{b,C}$	$52.24 \pm 2.91^{a,C}$	$49.19 \pm 4.36^{a,C}$
	CHI-CP30	$65.30 \pm 5.36^{a,A}$	$62.80 \pm 1.87^{a,B}$	$67.40 \pm 4.11^{a,B}$
	CHI-CP50	$57.70 \pm 1.81^{b,B}$	$75.06 \pm 4.83^{a,A}$	$74.12 \pm 5.26^{a,A}$
YM (MPa)	CHI-CP0	$0.08 \pm 0.003^{a,A}$	$0.03 \pm 0.003^{b,A}$	$0.02 \pm 0.002^{c,C}$
	CHI-CP10	$0.07 \pm 0.002^{a,B}$	$0.03 \pm 0.002^{c,A}$	$0.05 \pm 0.006^{b,A}$
	CHI-CP30	$0.02 \pm 0.005^{a,D}$	$0.03 \pm 0.003^{a,A}$	$0.03 \pm 0.004^{a,B}$
	CHI-CP50	$0.06 \pm 0.004^{a,C}$	$0.03 \pm 0.002^{c,A}$	$0.04 \pm 0.005^{b,A}$
TF (MPa)	CHI-CP0	$57.83 \pm 5.27^{a,B}$	$53.82 \pm 5.18^{a,C}$	$36.75 \pm 8.27^{b,C}$
	CHI-CP10	$60.71 \pm 8.32^{a,B}$	$53.61 \pm 9.23^{a,C}$	$67.64 \pm 7.74^{a,B}$
	CHI-CP30	$67.00 \pm 15.34^{a,B}$	$70.35 \pm 8.72^{a,B}$	$79.25 \pm 15.55^{a,B}$
	CHI-CP50	$128.86 \pm 5.25^{b,A}$	$115.23 \pm 13.47^{b,A}$	$145.86 \pm 7.17^{a,A}$

[a] CHI: chitosan, CP: chickpea protein, 0-10-30-50: CP level.
[b] Different lower and capital letter superscripts in the same row and column indicate significant differences ($P < 0.05$), respectively.
[c] Values are represented as mean ± SD (n = 5).

Improved tensile strength is a desirable property. TS is the maximum stress required by the film to be resistant to the applied stress, and a high TS means the film is more resistant to breaking. EB is determined from the percentage increase in length the film achieved before the break and represents how stretchable a film is. YB is calculated from the slope of the linear region of the stress – strain curve, and a high YB means film is more rigid/stiff. T is calculated from the area under the stress – strain curve and indicates how much energy a film can absorb until breaking. The tensile properties of non-sonicated and sonicated CHI-CP films were shown in Table 2.3. There was no statistically significant change in the tensile properties of CHI-CP30. However, after sonication, TS and YM values of other films decreased slightly while EB values significantly increased. The highest tensile results were obtained in CHI-CP50 films after sonication. CHI-CP50 scored the highest EB values after both 5 and 10 min sonication time. According to Oyeoka et al. (2021), EB must be proportional to the ability of the film to withstand tensile stress, even though the packaging applications favour high EB values. At that point, interpreting the TF values of films makes sense. Given that a material must be tough enough to withstand both high stresses and strains, it must be both strong and ductile to be tough. For example; if two materials have the same strength, the

one with the higher ductility is desirable because high ductility helps to prevent brittle fracture. Since toughness is a representative property of the combination of strength and ductility, the duration of ultrasound was chosen according to this property. Especially, 10 min sonicated CHI-CP50 was found to be the most ductile film with the highest TF value. Therefore, sonication time was selected as 10 min.

2.4.1.4. Determination of Chickpea Protein Concentration

Ultrasounds are acoustic waves (mechanical pressure waves) with a frequency at the same level or higher than the threshold level of human audio detection, 20 kHz (human beings can detect sound in the range between 20 Hz – 20 kHz). It can be divided into 2 categories according to frequency (O'Sullivan et al. 2017): (1) ultrasound with high frequency (> 100 kHz) and low intensity (< 1 W/cm^2) is known as low power sonication or simply, diagnostic ultrasound; (2) ultrasound with low frequency (20 – 100 kHz) and high power intensity (> 1 W/cm^2) is known as high power sonication or simply, power ultrasound. Diagnostic ultrasound is commonly used to analyze physicochemical properties of food whereas power ultrasound can be used for processing, preservation and extraction since providing physical or chemical modification of food. Sonication presents a multi-force interaction including thermal effects, mechanical shear, agitation, vibration, pressure, cavitation and sound influx, etc. Commercial ultrasound systems can be available in both contact and non-contact types (Charoux et al. 2017). In contact types, transducers directly transfer ultrasonic waves to product using a liquid as a coupling medium whereas in non-contact types, transducers transfer ultrasonic waves to the product using air as a coupling medium. The impact of ultrasound on liquid systems mainly results from the cavitation of liquids. For example; in the food protein industry, ultrasound can be used to modify the functional properties of proteins due to causing some changes in the molecular structure. The cavitation effect of ultrasound disrupts globular protein structure, thus causing partial unfolding (it means a conformational change) (Yang et al. 2022; Su and Cavaco-Paulo 2021). According to the FTIR and SDS-PAGE results of Cao et al. (2021), quinoa protein solubilization was found to increase in α-helix, β-turn and surface hydrophobicity. After sonication, FTIR results of Zhao et al. (2022) showed a fall in β-turn and a raise in α-helix, β-sheet and random coil content of

the secondary structure of walnut protein isolate. Moreover, the results of CD spectroscopy conducted by Hu et al. (2013) pointed out lower α-helix, random coil and higher β-sheet content of sonicated soy protein isolate with 200 W power whereas higher α-helix, random coil and lower β-sheet content of sonicated soy protein isolate with 600 W power. Different studies have been performed to improve the functional properties of various proteins in literature. For example; O'Sullivan et al. (2016) found that high power sonication increased the solubility and emulsifying properties of wheat and soybean proteins while low power sonication was found to be insufficient to destroy the covalent bonds. Sheng et al. (2018) obtained the highest foaming ability and protein solubility in egg white after power sonication (20 kHz, 360 W for 10 min). Shen et al. (2017) stated that power sonication decreased particle size and increased surface hydrophobicity, free sulphydryl groups, solubility, emulsion activity index and emulsion stability index. Hu et al. (2013) demonstrated that due to the results of partial unfolding and reduced intermolecular interactions, power sonication improved the water holding capacity, gel strength and gel firmness of soybean protein isolate leading to denser and more uniform gel networks. Jambrak et al. (2008) also reported that power sonication (20 kHz, 600 W for 15 min) increased the solubility of whey proteins. Rahman et al. (2022) achieved an increase in surface hydrophobicity, emulsification and foaming properties of soy protein using power sonication. However, Gharibzahedi and Smith (2020) mentioned some limitations. Researchers reported that non-optimal conditions such as high power or long sonication times could lead to reactive free radicals formed through the decomposition of water molecules. Also, there is a temperature rise due to the heat generated during this process. Considering the benefits and drawbacks highlighted above, CP concentration was determined at which the highest protein solubility would be achieved.

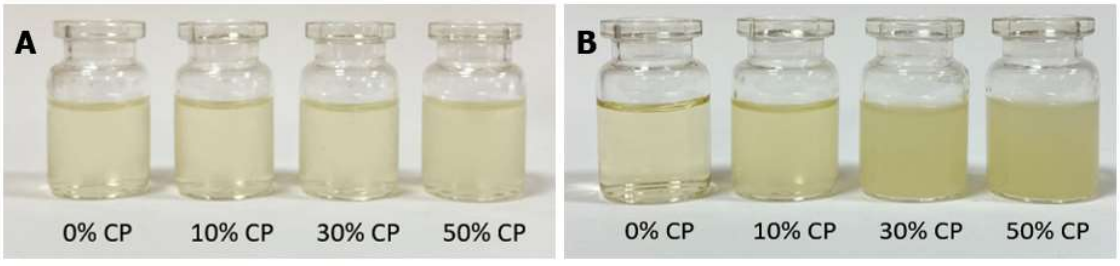

Figure 2.5. Photos non-sonicated (A) and sonicated (B) chitosan FFSs containing different concentrations of chickpea protein.

Table 2.4. Soluble protein (g protein/100 g film solution), pH and transmittance (%) of non-sonicated and sonicated chitosan FFSs containing different concentrations of chickpea protein[b,c].

Duration of sonication (min)	FFS samples[a]	Soluble protein (g protein / 100 g)	pH	T (%)
0	CHI-CP0	0.008 ± 0.003^{e}	5.05 ± 0.03^{a}	91.07 ± 3.48^{a}
	CHI-CP10	0.079 ± 0.02^{d}	5.07 ± 0.01^{a}	76.66 ± 5.48^{b}
	CHI-CP30	0.094 ± 0.02^{d}	5.09 ± 0.02^{a}	59.29 ± 5.90^{c}
	CHI-CP50	0.129 ± 0.02^{c}	5.12 ± 0.01^{a}	6.24 ± 0.25^{de}
10	CHI-CP0	0.021 ± 0.008^{e}	5.06 ± 0.10^{a}	89.91 ± 4.27^{a}
	CHI-CP10	0.093 ± 0.04^{d}	5.05 ± 0.14^{a}	17.61 ± 0.07^{d}
	CHI-CP30	0.180 ± 0.02^{b}	5.09 ± 0.13^{a}	0.89 ± 0.19^{e}
	CHI-CP50	0.243 ± 0.02^{a}	5.13 ± 0.09^{a}	0.34 ± 0.04^{e}

[a] CHI: chitosan, CP: chickpea protein, 0-10-30-50: CP level.
[b] Different lower letter superscripts in the same column indicate significant differences ($P < 0.05$).
[c] Values are represented as mean ± SD (n = 6 for soluble protein and n = 4 for pH and transmittance).

Some physicochemical properties of film solutions were displayed in Table 2.4. Sonication significantly affected the protein solubility and transmittance values of film solutions. Solubility of proteins is associated with the surface hydrophobic (protein-protein) and hydrophilic (protein-water) interactions, so high solubility makes it easy for the adsorption of protein to the interface. As expected, soluble protein content significantly increased as increasing protein concentration ($P < 0.05$). However, it should be emphasized that sonication remarkably promoted the solubility of CP. Soluble protein content in both CHI-CP30 and CHI-CP50 film solutions increased ≈ 2.0 fold after sonication. Similar results were obtained by Wang et al. (2020) using high intensity ultrasound under 300 W for up to 20 min. Researchers were able to improve solubility, foaming, emulsifying and gelation properties of chickpea protein isolate as a result of increased free sulphydryl content, surface hydrophobicity and decreased particle size. Sonication probably caused the unfolding of protein structure by cleaving non-covalent interactions such as electrostatic interactions, hydrophobic interactions and hydrogen bonding and so that hydrophilic parts of amino acids from inside are opened toward the water and form stronger interactions between protein and water molecules. Good solubility usually gives better emulsion and foaming properties. So, sonicated CHI-CP30 and sonicated CHI-CP50 samples seem suited for the current work. A point worth noting is that the increased solubility can not be due to the hydrolysis of peptide bonds of CP protein molecules because ultrasound alone can not be enough to break peptide bonds and hydrolyze the proteins (Noor et al. 2021). This result was likely due to the conformational change and the formation of soluble protein aggregates from insoluble protein aggregates.

As CP protein concentration increased, the transmittance of non-sonicated film solutions decreased significantly ($P < 0.05$). Since more molecules were in the film solution, more light was blocked. Also, colloidal dispersion of CP tuned clear CHI solution to highly turbid and this caused dramatic drops of transmittance values after sonication. It seems that sonication generated a more turbid solution for high protein levels. For instance, sonicated CHI films with CP30 and CP50 were highly turbid with T values lower than 1%. Moreover, sample at 50% CP on B in Figure 2.5 was visually more turbid. Although transparent films are desirable in the food industry because of allowing consumers to see the products in the package clearly, opaque films can also be important for protecting light sensitive food products. pH of film solutions was between 5.0 – 5.1 and did not change significantly upon ultrasonication ($P > 0.05$).

Since the soluble protein content of both CHI-CP30 and CHI-CP50 samples increased in the same trend, a further experiment was conducted. Stefanović et al. (2014) concluded that power sonication improved the antimicrobial activity of egg white proteins. Thus, the antimicrobial performance of the films was compared with each other. The results of the film surface inoculation test were displayed in Tables 2.5 – 2.6. Sonication and CP level did not have any significant effect on *E. coli* count during the incubation time. The *E. coli* counts of all films were less than 2.3 and 2 log cfu/g at time 24 h and after 5 days, respectively. The *E. coli* inoculated on the control film (CHI-CP0) showed > 2.4 D reduction after 24 h and > 2.7 D reduction after 5 days. This indicates the inherent antimicrobial potential of chitosan. The *L. innocua* counts of all films were less than 2 log cfu/g after 5 days of incubation; thus, it could be concluded that *L. innocua* showed greater resistance to CHI-CP films than *E. coli*. Similarly, a previous study of Sozbilen and Yemenicioğlu (2020) showed that the antimicrobial activity of CHI was influenced not only by its molecular properties but also by the type of bacteria tested. Since significant differences were observed in *L. innocua* counts of samples at time 0 and day 1 ($P < 0.05$), it could be said that sonication and CP level had a significant effect on *L. innocua* count during the incubation time. Sonication resulted in an additional minimum 0.9 D reduction at time 0. Although complete inactivation (for a detection limit of 2 log cfu/g) was observed for all films at the end of the incubation period, *L. innocua* counts of sonicated CHI-CP0 and CHI-CP50 films already dropped below 2.3 log cfu/g at time 24 h. When sonicated, the decimal reduction on CHI-CP50 was higher than that of CHI-CP0 for all incubation times. So, the lowest *L. innocua* count on sonicated CHI-

CP50 film is a desired property in the current work. Considering eugenol would be further added to the film composition, it is suitable to select a high CP level also to take advantage of its emulsification capacity.

Table 2.5. Anti-*Escherichia coli* activity of non-sonicated and sonicated chitosan films containing different concentrations of chickpea protein[b,c].

Duration of sonication (min)	Film samples[a]	*E. coli* (Log cfu/g film)[b,c,d]		
		Day 0	*Day 1*	*Day 5*
0	CHI-CP0	$4.71 \pm 0.81^{a,A}$	$< 2.3^{a,B}$	$< 2.0^{a,C}$
	CHI-CP10	$4.63 \pm 0.69^{a,A}$	$< 2.3^{a,B}$	$< 2.0^{a,C}$
	CHI-CP30	$5.06 \pm 0.96^{a,A}$	$< 2.3^{a,B}$	$< 2.0^{a,C}$
	CHI-CP50	$4.59 \pm 0.65^{a,A}$	$< 2.3^{a,B}$	$< 2.0^{a,C}$
10	CHI-CP0	$4.65 \pm 0.80^{a,A}$	$< 2.3^{a,B}$	$< 2.0^{a,C}$
	CHI-CP10	$4.95 \pm 0.43^{a,A}$	$< 2.3^{a,B}$	$< 2.0^{a,C}$
	CHI-CP30	$4.67 \pm 0.95^{a,A}$	$< 2.3^{a,B}$	$< 2.0^{a,C}$
	CHI-CP50	$5.28 \pm 0.56^{a,A}$	$< 2.3^{a,B}$	$< 2.0^{a,C}$

[a] CHI: chitosan, CP: chickpea protein, 0-10-30-50: CP level.
[b] Different lower and capital letter superscripts in the same column and row indicate significant differences ($P < 0.05$), respectively.
[c] Values are represented as mean ± SD (n = 6).
[d] Initial *E. coli* count was 8.5 log cfu/mL.

Table 2.6. Anti-*Listeria innocua* activity of non-sonicated and sonicated chitosan films containing different concentrations of chickpea protein[b,c].

Duration of sonication (min)	Film samples[a]	*L. innocua* (Log cfu/g film)[b,c,d]		
		Day 0	*Day 1*	*Day 5*
0	CHI-CP0	$6.62 \pm 0.24^{a,A}$	$3.93 \pm 0.17^{c,B}$	$< 2.0^{a,C}$
	CHI-CP10	$6.15 \pm 0.25^{b,A}$	$4.24 \pm 0.70^{bc,B}$	$< 2.0^{a,C}$
	CHI-CP30	$6.61 \pm 0.18^{a,A}$	$4.28 \pm 0.46^{bc,B}$	$< 2.0^{a,C}$
	CHI-CP50	$6.23 \pm 0.24^{ab,A}$	$4.57 \pm 0.45^{abc,B}$	$< 2.0^{a,C}$
10	CHI-CP0	$5.68 \pm 0.08^{c,A}$	$< 2.3^{d,B}$	$< 2.0^{a,C}$
	CHI-CP10	$5.23 \pm 0.19^{d,A}$	$4.45 \pm 0.78^{bc,B}$	$< 2.0^{a,C}$
	CHI-CP30	$5.24 \pm 0.52^{d,A}$	$4.06 \pm 0.08^{c,B}$	$< 2.0^{a,C}$
	CHI-CP50	$4.77 \pm 0.34^{e,A}$	$< 2.3^{d,B}$	$< 2.0^{a,C}$

[a] CHI: chitosan, CP: chickpea protein, 0-10-30-50: CP level.
[b] Different lower and capital letter superscripts in the same column and row indicate significant differences ($P < 0.05$), respectively.
[c] Values are represented as mean ± SD (n = 6).
[d] Initial *L. innocua* count was 8.7 log cfu/mL.

A 50% level of CP based on the CHI weight means a 0.75% level of CP based on the weight of the film forming solution. It is an appropriate level for the current study, given that the surplus protein concentration may adversely affect the stability of the emulsion system. It was previously suggested that more than 1% protein concentration

promotes the free form of protein in the system and could result in unstable emulsions (Sharma et al. 2022).

2.4.1.5. Determination of Eugenol Concentration

Many studies have reported that emulsions with essential oils as the single oil phase are unstable (Ghaderi et al. 2017; Jang et al. 2019; Zhao et al. 2020). It is also known that Ostwald ripening and oiling-off phenomena are difficult to be prevented using a single emulsifier or surfactant (Jang et al. 2019; Zhao et al. 2020). Therefore, emulsion coatings were prepared by two stages of stabilization methods in the current study. EUG oil droplets were stabilized by Tween 80 as a surfactant and by an interfacial layer formed by the interaction of CHI and CP.

Different research studies on emulsion systems have indicated that the emulsification of eugenol did not affect its antimicrobial activity (Hu et al. 2016; Shao et al. 2018). For example; in a previous study by Shao et al. (2018) eugenol-chitosan nanoemulsions (eugenol is a core material while chitosan is its carrier) showed a lower MIC value than free eugenol. Recent studies have reported that emulsion encapsulation could protect EUG from environmental stressors and enhance its antibacterial activity (H. Chen, Zhang, and Zhong 2015; Li et al. 2015). For instance; strong anionic compounds such as chitosan or gum arabic may interact with the surface proteins on the bacterial cell membrane increasing membrane permeability and disrupting the bacterial wall integrity, thereby improving the antimicrobial activity of essential oils (Hu et al. 2016; Shao et al. 2018). Therefore, the system used in the emulsion coating preparation in the current study would not adversely affect the antimicrobial activity of eugenol. The antimicrobial performance of EUG containing films was shown in Tables 2.7 – 2.8. Whether sonicated or not, increased EUG level up to 0.64% dropped both *E. coli* and *L. innocua* counts below 2 log cfu/g at time 0.

Table 2.7. Anti-*Escherichia coli* activity of non-sonicated and sonicated films containing different concentrations of eugenol[b,c].

Duration of sonication (min)	Film samples[a]	*E. coli* (Log cfu/g film)[b,c,d]		
		Day 0	***Day 1***	***Day 5***
0	CHI	$7.51 \pm 0.11^{ab,A}$	$< 2.0^{b,B}$	$< 2.0^{b,B}$
	CHI-CP50	$7.70 \pm 0.12^{a,A}$	$< 2.0^{b,B}$	$< 2.0^{b,B}$
	CHI-EUG0.16	$6.74 \pm 0.19^{de,A}$	$< 2.0^{b,B}$	$< 2.0^{b,B}$
	CHI-EUG0.32	$5.18 \pm 0.42^{g,A}$	$< 2.0^{b,B}$	$< 2.0^{b,B}$
	CHI-EUG0.64	$< 2.0^{h,A}$	$< 2.0^{b,A}$	$< 2.0^{b,A}$
	CHI-CP50-EUG0.16	$7.19 \pm 0.11^{bc,A}$	$< 2.0^{b,B}$	$< 2.0^{b,B}$
	CHI-CP50-EUG0.32	$5.75 \pm 0.05^{f,A}$	$< 2.0^{b,B}$	$< 2.0^{b,B}$
	CHI-CP50-EUG0.64	$< 2.0^{h,A}$	$< 2.0^{b,A}$	$< 2.0^{b,A}$
10	CHI	$7.19 \pm 0.25^{bc,A}$	$< 2.0^{b,B}$	$< 2.0^{b,B}$
	CHI-CP50	$7.48 \pm 0.12^{ab,A}$	$< 2.0^{b,B}$	$< 2.0^{b,B}$
	CHI-EUG0.16	$6.93 \pm 0.38^{cd,A}$	$< 2.0^{b,B}$	$< 2.0^{b,B}$
	CHI-EUG0.32	$6.40 \pm 0.17^{e,A}$	$< 2.0^{b,B}$	$< 2.0^{b,B}$
	CHI-EUG0.64	$< 2.0^{h,A}$	$< 2.0^{b,A}$	$< 2.0^{b,A}$
	CHI-CP50-EUG0.16	$7.48 \pm 0.09^{ab,A}$	$< 2.0^{b,B}$	$< 2.0^{b,B}$
	CHI-CP50-EUG0.32	$5.74 \pm 0.18^{f,A}$	$< 2.0^{b,B}$	$< 2.0^{b,B}$
	CHI-CP50-EUG0.64	$< 2.0^{h,A}$	$< 2.0^{b,A}$	$< 2.0^{b,A}$

[a] CHI: chitosan, CP: chickpea protein, EUG: eugenol, 0-0.16-0.32-0.64: EUG level.
[b] Different lower and capital letter superscripts in the same column and row indicate significant differences ($P < 0.05$), respectively.
[c] Values are represented as mean ± SD (n = 6).
[d] Initial *E. coli* count was 8.2 log cfu/mL.

Table 2.8. Anti-*Listeria innocua* activity of non-sonicated and sonicated films containing different concentrations of eugenol[b,c].

Duration of sonication (min)	Film samples[a]	*L. innocua* (Log cfu/g film)[b,c,d]		
		Day 0	***Day 1***	***Day 5***
0	CHI	$6.20 \pm 0.09^{a,A}$	$2.63 \pm 0.28^{a,B}$	$< 2.0^{a,C}$
	CHI-CP50	$6.01 \pm 0.30^{ab,A}$	$2.62 \pm 0.30^{a,B}$	$< 2.0^{a,C}$
	CHI-EUG0.16	$5.91 \pm 0.25^{ab,A}$	$< 2.0^{b,B}$	$< 2.0^{a,B}$
	CHI-EUG0.32	$2.66 \pm 0.62^{e,A}$	$< 2.0^{b,B}$	$< 2.0^{a,B}$
	CHI-EUG0.64	$< 2.0^{f,A}$	$< 2.0^{b,A}$	$< 2.0^{a,A}$
	CHI-CP50-EUG0.16	$5.81 \pm 0.13^{ab,A}$	$< 2.0^{b,B}$	$< 2.0^{a,B}$
	CHI-CP50-EUG0.32	$2.67 \pm 0.23^{e,A}$	$< 2.0^{b,B}$	$< 2.0^{a,B}$
	CHI-CP50-EUG0.64	$< 2.0^{f,A}$	$< 2.0^{b,A}$	$< 2.0^{a,A}$
10	CHI	$6.12 \pm 0.06^{ab,A}$	$< 2.0^{b,B}$	$< 2.0^{a,B}$
	CHI-CP50	$5.75 \pm 0.20^{b,A}$	$< 2.0^{b,B}$	$< 2.0^{a,B}$
	CHI-EUG0.16	$6.09 \pm 0.15^{ab,A}$	$< 2.0^{b,B}$	$< 2.0^{a,B}$
	CHI-EUG0.32	$3.15 \pm 0.51^{d,A}$	$< 2.0^{b,B}$	$< 2.0^{a,B}$
	CHI-EUG0.64	$< 2.0^{f,A}$	$< 2.0^{b,A}$	$< 2.0^{a,A}$
	CHI-CP50-EUG0.16	$6.09 \pm 0.16^{ab,A}$	$< 2.0^{b,B}$	$< 2.0^{a,B}$
	CHI-CP50-EUG0.32	$4.05 \pm 0.26^{c,A}$	$< 2.0^{b,B}$	$< 2.0^{a,B}$
	CHI-CP50-EUG0.64	$< 2.0^{f,A}$	$< 2.0^{b,A}$	$< 2.0^{a,A}$

[a] CHI: chitosan, CP: chickpea protein, EUG: eugenol, 0-0.16-0.32-0.64: EUG level.
[b] Different lower and capital letter superscripts in the same column and row indicate significant differences ($P < 0.05$), respectively.
[c] Values are represented as mean ± SD (n = 6).
[d] Initial *L. innocua* count was 8.7 log cfu/mL.

Table 2.9. Some physicochemical properties of non-sonicated and sonicated FFSs containing different concentrations of eugenol[b,c].

Duration of sonication (min)	FFS samples[a]	Soluble protein (g protein / 100 g)	pH	T (%)
0	CHI	0.013 ± 0.008^{g}	5.07 ± 0.009^{d}	89.83 ± 3.73^{a}
	CHI-CP50	0.097 ± 0.005^{d}	5.13 ± 0.016^{bc}	6.24 ± 0.25^{b}
	CHI-EUG0.16	0.016 ± 0.006^{g}	5.07 ± 0.013^{d}	0.28 ± 0.06^{c}
	CHI-EUG0.32	0.015 ± 0.003^{g}	5.08 ± 0.004^{d}	0.06 ± 0.001^{c}
	CHI-EUG0.64	0.016 ± 0.004^{g}	5.09 ± 0.004^{cd}	0.03 ± 0.001^{c}
	CHI-CP50-EUG0.16	0.100 ± 0.015^{d}	5.15 ± 0.02^{ab}	0.32 ± 0.10^{c}
	CHI-CP50-EUG0.32	0.072 ± 0.015^{e}	5.16 ± 0.01^{a}	0.05 ± 0.003^{c}
	CHI-CP50-EUG0.64	0.067 ± 0.01^{ef}	5.17 ± 0.005^{a}	0.03 ± 0.003^{c}
10	CHI	0.015 ± 0.006^{g}	5.10 ± 0.001^{cd}	86.66 ± 0.24^{a}
	CHI-CP50	0.213 ± 0.018^{c}	5.15 ± 0.001^{ab}	0.30 ± 0.006^{c}
	CHI-EUG0.16	0.016 ± 0.005^{g}	5.10 ± 0.001^{cd}	0.33 ± 0.002^{c}
	CHI-EUG0.32	0.016 ± 0.01^{g}	5.10 ± 0.001^{cd}	0.05 ± 0.002^{c}
	CHI-EUG0.64	0.018 ± 0.008^{g}	5.10 ± 0.001^{cd}	0.03 ± 0.002^{c}
	CHI-CP50-EUG0.16	0.250 ± 0.01^{b}	5.17 ± 0.001^{a}	0.13 ± 0.001^{c}
	CHI-CP50-EUG0.32	0.250 ± 0.02^{b}	5.17 ± 0.001^{a}	0.05 ± 0.004^{c}
	CHI-CP50-EUG0.64	0.293 ± 0.003^{a}	5.17 ± 0.001^{a}	0.02 ± 0.002^{c}

[a] CHI: chitosan, CP: chickpea protein, EUG: eugenol, 0-0.16-0.32-0.64: EUG level.
[b] Different lower letter superscripts in the same column indicate significant differences ($P < 0.05$).
[c] Values are represented as mean ± SD (n = 6 for protein solubility and n = 4 for pH and transmittance).

After adding EUG, some physicochemical properties of film solutions were presented in Table 2.9. Sonication and EUG addition did not significantly affect the soluble protein content and pH of CHI film, but were effective on CHI-CP50 film. It is well known that the hydrophilicity/hydrophobicity balance of the particle surface could impact the protein solubility. Increased EUG levels significantly decreased the soluble protein content of non-sonicated CHI-CP50 film. This fact is most likely due to the hydrogen bonding between hydroxyl groups of EUG and peptide bonds of CP protein. So, the presence of less hydrogen bonding between protein and water lowered the solubility. On the contrary, high EUG levels significantly increased the soluble protein content of sonicated CHI-CP50. This fact is most probably due to the formation of complex coacervate by CHI and CP for encapsulating EUG. It is possible that CHI-CP complex coacervate was dominated by the electrostatic interactions between positively charged CHI with negatively charged CP and hydrophobic interactions among CHI, CP and EUG. The presence of less hydrophobic sites resulted in an increase in protein solubility. To strengthen these arguments, zeta potential or surface charge density should be determined. The addition of EUG turned clear CHI solution into highly turbid, and this caused dramatic drops in transmittance values (below 1%). It is important to note that the

pH of film solutions showed a limited variation between 5.1 – 5.2. The isoelectric point (pI) of CP is ≈ 4.5 (Ma et al. 2022). Due to its cationic polyelectrolyte nature, pK_a of primary amine of CHI is ≈ 6.5 (Aranaz et al. 2021; Nilsen-Nygaard et al. 2015). Thus, it is clear that CHI is positively charged (pH < pK_a) while CP is negatively charged (pH > pI) within the solution. Consequently, the experimental design and coding of the emulsion coatings used in the following sections were shown in Table 2.10.

Table 2.10. Experimental design of the current study.

Treatment	Chitosan* (%)	Chickpea* (%)	Eugenol* (%)	Coding
Non-sonicated	1.5	0	0	CHI
	1.5	0.75	0	CHI-CP
	1.5	0	0.64	CHI-EUG
	1.5	0.75	0.64	CHI-CP-EUG
Sonicated	1.5	0	0	CHI
	1.5	0.75	0	CHI-CP
	1.5	0	0.64	CHI-EUG
	1.5	0.75	0.64	CHI-CP-EUG

* w/w of film forming solution.

2.4.2. Hydrodynamic Properties of Films

Wet basis (wb) and dry basis (db) moisture content (MC), dry matter content (DC), water solubility (WS) and swelling degree (SW) of films were summarized in Table 2.11. Moisture content relates to an affinity of film components for water. The moisture content of films was in the following order: CHI > CHI-EUG > CHI-CP > CHI-CP-EUG whether sonicated or not. CHI films had the highest moisture content probably due to the high affinity of chitosan for water because CHI has plenty of amino groups ($-NH_2$) and hydroxyl groups (-OH) in its structure (Neto et al. 2005). For example; Rueda et al. (1999) found that the interaction of water with -OH groups is stronger than with $-NH_2$ groups present in chitosan. EUG addition significantly decreased the moisture content of CHI film ($P < 0.05$). Most probably, the formation of covalent bonds between CHI and EUG reduced the availability of -OH and $-NH_2$ groups of chitosan and limited chitosan-water interaction through hydrogen bonding.

Table 2.11. Moisture content (%, wb and db), dry matter content, water solubility (%) and swelling degree (%) of emulsion coatings[b,c].

Film samples[a]	MC (%, wb)	MC (%, db)	DC (%)	WS (%)	SW (%)
Non-sonicated CHI	34.00 ± 2.44^{a}	51.72 ± 5.94^{a}	66.00 ± 2.44^{d}	25.89 ± 2.65^{d}	50.02 ± 8.11^{bc}
Sonicated CHI	33.08 ± 2.70^{a}	49.67 ± 6.25^{a}	66.92 ± 2.70^{d}	27.61 ± 3.33^{d}	39.71 ± 5.55^{cd}
Non-sonicated CHI-CP	23.50 ± 2.55^{c}	30.85 ± 4.34^{c}	76.50 ± 2.55^{b}	30.94 ± 3.10^{c}	35.92 ± 11.39^{d}
Sonicated CHI-CP	22.07 ± 2.59^{c}	28.44 ± 4.21^{cd}	77.93 ± 2.59^{b}	28.34 ± 3.27^{cd}	36.90 ± 5.60^{d}
Non-sonicated CHI-EUG	27.05 ± 2.61^{b}	37.24 ± 5.00^{b}	72.95 ± 2.61^{c}	36.16 ± 2.98^{b}	29.17 ± 9.49^{d}
Sonicated CHI-EUG	26.66 ± 3.61^{b}	36.66 ± 7.04^{b}	73.34 ± 3.61^{c}	38.71 ± 2.19^{ab}	31.09 ± 5.50^{d}
Non-sonicated CHI-CP-EUG	18.43 ± 5.85^{d}	23.22 ± 9.57^{de}	81.57 ± 5.85^{a}	38.57 ± 4.31^{ab}	59.64 ± 13.30^{ab}
Sonicated CHI-CP-EUG	15.77 ± 4.08^{d}	18.98 ± 5.73^{e}	84.23 ± 4.08^{a}	40.82 ± 2.66^{a}	67.05 ± 11.57^{a}

[a] CHI: chitosan, CP: chickpea protein, EUG: eugenol.
[b] Different lower letter superscripts in the same column indicate significant differences ($P < 0.05$).
[c] Values are represented as mean ± SD (n = 10).

CP addition decreased the moisture content of CHI films more than EUG addition. The results suggest that CHI-CP had a more compact structure while CHI-EUG had a less compact structure. CHI-CP interactions were most probably produced by hydrogen bonding and electrostatic interactions between -COOH, -NH_2 and -OH groups of the aminoacids in CP and -OH and -NH_2 groups in CHI. CHI-CP-EUG film had the lowest moisture content in the presence of both CP and EUG limiting the availability of water on the structure. This could be explained by a competition between water and EUG for the same protein sites. It is possible that hydrogen bonds between water and CHI may be replaced by the bonds between CHI-CP and CP-EUG. On the contrary, the dry matter content of films was in the following order: CHI-CP-EUG > CHI-EUG > CHI-CP > CHI whether sonicated or not.

None of the films had any loss in integrity after 24 h of storage in water, which is an indicator of a highly stable structural network. Solubility may be crucial to determine the release of active compounds from the film when placed over the food surface (Gómez-Estaca et al. 2010), in addition to affecting the resistance of the film to water (hydrophobicity), especially in a humid environment (Bourtoom and Chinnan 2008; Peng, Wu, and Li 2013). Lower solubility generally indicates high water resistance. Pristine CHI film presented the lowest water solubility probably due to the strong interactions between chitosan and acetic acid. For example; high water resistance could be obtained due to the amide bond formation between citric acid and chitosan (Cui et al. 2011) The solubility in water of pristine CHI film was in the range of 25 – 28% whether sonicated or not. Similarly, Ferreira et al. (2016) found 30.5% water solubility of medium molecular weight chitosan film. However, sonicated CHI-CP-EUG film had the highest water solubility with a value of 40.8%. The ideal value of solubility is based on the desired film application. A low solubility is suitable for packaging foods with high water activity whereas a high solubility is desirable for packaging foods of immediate consumption or encapsulating additives (Herrera-Vázquez et al. 2022). That film system seems to be suitable for encapsulation and release of EUG in the current study.

Adding CP or EUG significantly decreased the swelling degree of CHI film due to their hydrophobic nature (e.g., CP globulins and hydrophobic compound EUG most probably increased CHI hydrophobicity). However, sonicated CHI-CP-EUG film presented the highest value showing a high affinity to water. Consequently, its application is foreseen in foods with low water content.

2.4.3. Optical Properties of Films

The pristine CHI films were visually colourless and transparent while the composite films were observed to be yellowish in colour, all films were flexible and easy to peel off from the moulds.

Table 2.12. Light transmission (%) and opacity (A/mm) of emulsion coatings[b,c].

Film samples[a]	Light transmittance (%)								Opacity (A/mm)
	Wavelength (nm)								
	200	***280***	***350***	***400***	***500***	***600***	***700***	***800***	
Non-sonicated CHI	0.12^{a}	3.7^{a}	15.9^{a}	53.7^{a}	78.8^{a}	83.7^{a}	85.0^{ab}	85.5^{ab}	0.68 ± 0.19^{de}
Sonicated CHI	0.10^{b}	0.9^{b}	6.6^{bc}	42.6^{b}	78.4^{a}	85.0^{a}	86.7^{a}	86.7^{a}	0.53 ± 0.14^{e}
Non-sonicated CHI-CP	0.08^{c}	0.1^{b}	9.1^{b}	35.5^{c}	57.7^{c}	63.8^{c}	65.1^{c}	64.6^{c}	1.48 ± 0.09^{c}
Sonicated CHI-CP	0.06^{d}	0.005^{b}	6.1^{bc}	28.9^{d}	50.5^{d}	57.5^{d}	60.0^{d}	60.1^{d}	1.83 ± 0.16^{b}
Non-sonicated CHI-EUG	0.09^{b}	0.002^{b}	6.9^{bc}	37.2^{c}	70.2^{b}	78.5^{b}	81.2^{b}	82.2^{b}	0.84 ± 0.05^{d}
Sonicated CHI-EUG	0.08^{c}	0.002^{b}	3.7^{cd}	29.5^{d}	67.9^{b}	78.0^{b}	81.0^{b}	82.0^{b}	0.81 ± 0.04^{d}
Non-sonicated CHI-CP-EUG	0.05^{d}	0.001^{b}	3.8^{cd}	21.9^{e}	44.1^{e}	51.1^{e}	52.9^{e}	52.6^{e}	2.17 ± 0.15^{a}
Sonicated CHI-CP-EUG	0.04^{e}	0.001^{b}	2.0^{c}	12.5^{f}	35.8^{f}	45.3^{f}	48.8^{e}	49.8^{e}	2.23 ± 0.44^{a}
LDPE*	13.1	67.5	79.9	83.4	85.6	86.9	87.8	83.6	3.05
OPP*	4.6	80.0	86.2	87.9	88.8	89.1	89.3	89.6	1.67

[a] CHI: chitosan, CP: chickpea protein, EUG: eugenol.
[b] Different lower letter superscripts in the same column indicate significant differences ($P < 0.05$).
[c] Values are represented as mean ± SD (n = 6).
* Some synthetic films; LDPE: low density polyethylene and OPP: oriented polypropylene. Data was obtained from Shiku et al. (2003).

The light transmittance at selected wavelengths from 200 to 800 nm compared to some commonly used synthetic films and the opacity of emulsion coatings were presented in Table 2.12. Very low transmission values were noted for all films at 200 nm and 280 nm in UV light. The results indicated that all films had good barrier properties against UV light, regardless of composition. Similar light barrier properties have also been observed in gelatin films (Mu et al. 2012), fish myofibrillar protein films (Shiku et al. 2003), whey protein films (Fang et al. 2002). However, some synthetic polymer films for household wrap can not prevent UV light passage. But, all developed films showed enhanced barrier characteristics in the visible range compared to synthetic polymer films. Especially, sonicated CHI-CP-EUG film effectively blocked most light in

the UV-visible range. So, light barrier properties could help the packaged food avoid discoloration and oxidative deterioration leading to short retail time.

Opacity is an expression of transparency of a film and it can reduce the light transition. A greater value of opacity indicates lower transparency. Different opacity values can be related to the internal structure of films (Villalobos et al. 2005). The results showed that adding CP or EUG significantly increased the opacity of CHI films. Increased opacity with the addition of protein was probably due to the intermolecular cross-linking between chitosan and protein. Protein molecules may closely interact with chitosan chains resulting in a more compact structure that could block light transmission. This is consistent with Azaza et al. (2022), Ma et al. (2013) and Prodpran et al. (2007), who observed high opacity in chitosan-protein blend films. EUG containing films were much more opaque than pristine CHI film because oil droplets dispersed in carbohydrate matrix prevent light transmission through the film. Actually, it is known that essential oils might block light transmission due to the light scattering resulting from the distribution of oil droplets (Villalobos et al. 2005; Fabra et al. 2009). Similar findings have been reported by Pereda et al. (2012) and Binsi et al. (2013) for EOs containing chitosan films.

The colour properties (L*, a* and b*) and colour difference (ΔE*) of the films were recorded in Table 2.13. The CIE (L*, a*, b*) system was used because it is more sensitive in measuring yellowness and dark colours than the Hunter (L, a, B) system (Hunter Associates Laboratory 2012). All films were observed to be slightly yellowish and greenish. Adding CP and/or EUG significantly reduced the lightness value of the pristine CHI films ($P < 0.05$).

ΔE* is a standard measurement for quantifying the difference between given colours and understanding how the human eye detects colour differences. When ΔE* is less than 1, colour difference is invisible to the human eyes, within 1-2, colour difference is visible through close observation, within 2-10 colour difference is visible at a glance, within 11-49 colour difference are more similar than opposite and within 50-100 colour difference is exactly opposite. In the current work, this parameter showed the difference between the colour of films and standard white plate. The increased values observed on ΔE* of EUG containing CHI films were probably related to the natural colour EUG used

for making the films. However, all films had a colour difference between 12 – 27, so films seem more similar in colour according to the rating scale.

Table 2.13. Lightness (L*), redness (a*), yellowness (b*) and colour difference (ΔE*) of emulsion coatings[b,c].

Film samples[a]	L*	a*	b*	ΔE*
Non-sonicated CHI	92.91 ± 0.24[a]	-8.05 ± 0.38[g]	22.29 ± 2.21[c]	16.21 ± 2.21[cd]
Sonicated CHI	92.60 ± 0.91[a]	-7.41 ± 0.27[f]	21.10 ± 2.69[c]	15.10 ± 2.78[d]
Non-sonicated CHI-CP	90.84 ± 0.31[b]	-6.41 ± 0.07[d]	17.69 ± 0.80[d]	12.65 ± 0.84[e]
Sonicated CHI-CP	90.26 ± 0.63[b]	-6.79 ± 0.25[e]	21.37 ± 0.76[c]	16.18 ± 0.81[cd]
Non-sonicated CHI-EUG	88.44 ± 0.58[d]	-5.75 ± 0.06[b]	26.10 ± 0.85[b]	21.13 ± 0.96[b]
Sonicated CHI-EUG	85.97 ± 0.62[e]	-5.48 ± 0.16[a]	31.72 ± 1.62[a]	27.25 ± 1.72[a]
Non-sonicated CHI-CP-EUG	89.32 ± 0.75[c]	-6.07 ± 0.08[c]	22.38 ± 1.48[c]	17.43 ± 1.62[c]
Sonicated CHI-CP-EUG	88.24 ± 1.14[d]	-5.91 ± 0.19[bc]	23.35 ± 4.37[c]	17.04 ± 0.58[cd]

[a] CHI: chitosan, CP: chickpea protein, EUG: eugenol.
[b] Different lower letter superscripts in the same column indicate significant differences ($P < 0.05$).
[c] Values are represented as mean ± SD (n = 6).

2.4.4. Barrier Properties of Films

Transfer parameters are critical because they are directly related to the ability of the film to control moisture transfer between the environment and food. Controlling moisture migration is important to maintain the quality of packaged respiring food products such as fresh produces since they have an ongoing metabolism (e.g., respiration and transpiration). The permeability properties of films were presented in Table 2.14. WVTR values of pristine CHI films were found to be respectively 264.0 and 270.6 g/m^2/day whether sonicated or not. Similar finding was reported by Wiles et al. (2000). CHI films showed poor WVTR because naturally high contents of -OH and -NH_2 groups most probably interacted with water molecules aiding the permeation of the water molecules through the film. CHI-CP composite films exhibited lower WVTR than CHI films. It is probably due to the high level of non-polar aminoacids in CP protein.

WVP of films was in the following order: non-sonicated CHI, CHI-EUG, CHI-CP-EUG > sonicated CHI, CHI-EUG > non-sonicated CHI-CP > sonicated CHI-CP > sonicated CHI-CP-EUG. As seen in Table 2.14, non-sonicated chitosan and its composite films had significantly higher WVP values than sonicated ones. Given inherent hydrophilicity, carbohydrate and protein based films have been repeatedly reported to display high WVP (Cheng and Cui 2021; Deng et al. 2022; Fang et al. 2018). Their

hydrophilic groups may interact with permeating water molecules triggering plasticization during permeation (Basiak, Lenart, and Debeaufort 2017). Therefore, different research studies have been conducted to solve this problem (to lower the WVP) either by changing structural conformation during film processing or by adding reinforcing materials such as lipids, plasticizers or cross-linking agents, etc. The moisture transfer is relevant to the hydrophilic/hydrophobic ratio of compounds in the film matrix. Alone EUG addition did not cause any change in WVP in this study. Maybe, EUG exhibited a non-homogenous distribution in chitosan matrix by increasing water vapour diffusion. Theoretically, adding a hydrophobic substance is expected to improve WVP of a film because insoluble particles can prevent water molecule migration taking the empty spaces of the porous film matrix. On the other hand, sonicated CHI-CP-EUG film had the lowest WVP value due to the coupling effect of sonication and EUG. This result probably came from the fact that a more tortuous path for water molecules to diffuse through the film can be created by making the particles smaller with sonication, thereby improving the barrier properties. Similar findings were observed for starch (Cheng et al. 2010; Liu et al. 2018) and soybean protein isolate based films (Wang et al. 2014).

Table 2.14. Water vapour transmission rate (g/m^2/day), permeance (g/m^2/daykPa) and water vapour permeability (gmm/m^2/daykPa) of emulsion coatings[b,c].

Film samples[a]	**WVTR (g/m^2/day)**	**Permeance (g/m^2/daykPa)**	**WVP (gmm/m^2/daykPa)**
Non-sonicated CHI	270.6 ± 5.3^{abc}	170.7 ± 3.3^{abc}	20.9 ± 1.2^{a}
Sonicated CHI	264.0 ± 3.3^{abc}	166.6 ± 2.1^{abc}	20.3 ± 1.6^{ab}
Non-sonicated CHI-CP	250.8 ± 13.9^{bc}	158.2 ± 8.7^{bc}	19.2 ± 1.4^{abc}
Sonicated CHI-CP	232.2 ± 13.7^{c}	146.5 ± 8.6^{c}	18.1 ± 1.8^{bc}
Non-sonicated CHI-EUG	289.8 ± 30.0^{ab}	182.8 ± 18.9^{ab}	21.8 ± 1.1^{a}
Sonicated CHI-EUG	279.0 ± 31.5^{abc}	176.0 ± 19.9^{abc}	20.1 ± 2.7^{ab}
Non-sonicated CHI-CP-EUG	298.8 ± 15.4^{a}	188.5 ± 9.7^{a}	21.5 ± 1.9^{a}
Sonicated CHI-CP-EUG	239.0 ± 27.8^{c}	150.8 ± 17.5^{c}	16.8 ± 1.0^{c}

[a] CHI: chitosan, CP: chickpea protein, EUG: eugenol.
[b] Different lower letter superscripts in the same column indicate significant differences ($P < 0.05$).
[c] Values are represented as mean ± SD (n = 4).

A point worth noting is that WVP results were in good agreement with measurements where CHI film showed the highest moisture content while CHI-CP-EUG films showed the lowest moisture content. It can be suggested that the combination of sonication and EUG addition promotes a better water vapor barrier property.

2.4.5. Mechanical Properties of Films

The mechanical properties of the films studied by the uniaxial tensile test were represented in Table 2.16. High TS is generally desired, but EB can be adjusted to the intended film application. According to Winding and Hiatt (1961), a soft and weak polymer can be characterized by a low TS and moderate EB, a hard and brittle polymer can be characterized by a moderate TS and low EB, a soft and tough polymer can be characterized by a moderate TS and high EB and a hard and tough polymer can be characterized by a high TS and high EB (Table 2.15). As seen, tensile strength and stretchability are important parameters for film characterization in terms of resistance to external stress and flexibility, respectively. Thus, we consider that an ideal film serving as a controlled release coating should be hard (high TS) and tough (high EB) to maintain its integrity.

Table 2.15. Characteristic of stress-strain curves as related to polymer properties (Source: Winding and Hiatt 1961).

Description of polymer	**Characteristics of stress-strain curve**			
	modulus	**yield stress**	**tensile strength**	**elongation**
Soft, weak	low	low	low	moderate
Soft, tough	low	low	yield stress	high
Hard, brittle	high	none	moderate	low
Hard, strong	high	high	high	moderate
Hard, tough	high	high	high	high

The mechanical properties of emulsified films can be affected by multiple factors such as homogenization techniques, nature of biopolymers, structure of biopolymers, nature and content of oil, droplet size and distribution (Hopkins et al. 2015; Xue et al. 2019). Tensile strength (TS) is the maximum stress that a film can sustain before breaking (Pereda, Amica, and Marcovich 2012). Compositing significantly increased TS of the pristine CHI film ($P < 0.05$) whether sonicated or not. Especially, sonication and protein blending remarkably increased TS values (see sonicated CHI-CP and sonicated CHI-CP-EUG films in Table 2.16). This high film strength is probably related to the formation of intermolecular chemical bonds between CHI and CP. For example; hydrogen bonds could be formed between chitosan and protein because -OH and -NH_2 groups in chitosan mostly

act as hydrogen bond donors and acceptors, respectively (Ogawa, Naito, and Nishiyama 2019). Chitosan -NH_2 groups were protonated to -NH_3^+ in the acetic acid solution whereas protein molecules were unfolded with sonication resulting in -OH^- groups being exposed to form hydrogen bonds with -NH_3^+ of chitosan. Moreover, electrostatic interaction (between ammonium -NH_3^+ groups of chitosan and carboxylate -COO^- groups of protein) and hydrophobic interaction between these macromolecules are established. Proteins can also interact through disulfide bonds when they are unfolded. So, increasing the internal stable network and cohesiveness increased TS values.

Table 2.16. Tensile strength, elongation at break, Young's modulus and toughness of emulsion coatings[b,c].

Film samples[a]	**TS (MPa)**	**EB (%)**	**YM (MPa)**	**TF (MPa)**
Non-sonicated CHI	3.54 ± 0.38^{d}	92.12 ± 4.70^{a}	0.02 ± 0.001^{f}	129.33 ± 16.37^{e}
Sonicated CHI	4.24 ± 0.67^{d}	17.69 ± 2.07^{d}	0.21 ± 0.02^{a}	36.70 ± 8.19^{f}
Non-sonicated CHI-CP	6.62 ± 1.00^{c}	81.75 ± 5.92^{b}	0.04 ± 0.005^{de}	235.74 ± 41.62^{c}
Sonicated CHI-CP	11.50 ± 0.85^{a}	61.32 ± 3.03^{c}	0.15 ± 0.01^{b}	321.51 ± 29.06^{b}
Non-sonicated CHI-EUG	6.06 ± 1.08^{c}	83.54 ± 5.47^{b}	0.03 ± 0.01^{e}	190.19 ± 36.83^{d}
Sonicated CHI-EUG	5.77 ± 0.38^{c}	63.46 ± 2.80^{c}	0.05 ± 0.01^{d}	149.27 ± 11.88^{e}
Non-sonicated CHI-CP-EUG	10.05 ± 1.05^{b}	82.91 ± 7.86^{b}	0.07 ± 0.01^{c}	339.81 ± 52.56^{b}
Sonicated CHI-CP-EUG	11.80 ± 1.34^{a}	91.68 ± 3.77^{a}	0.07 ± 0.01^{c}	418.41 ± 50.17^{a}

[a] CHI: chitosan, CP: chickpea protein, EUG: eugenol.
[b] Different lower letter superscripts in the same column indicate significant differences ($P < 0.05$).
[c] Values are represented as mean ± SD (n = 10).

Elongation at break (EB) is the maximum change in film length before breaking (Pereda, Amica, and Marcovich 2012). Pristine CHI film had the highest EB resulting from the formation of interchain hydrogen bonds during CHI film formation. All composite films, except for sonicated CHI-CP-EUG, had lower EB values than the pristine CHI film. It might be due to the increase in intermolecular interactions and decrease in intermolecular distance. Similar flexibility reductions have also been demonstrated in chitosan-whey protein films (di Pierro et al. 2006; Ferreira et al. 2009) and in chitosan-gelatin films (BenBettaïeb et al. 2015). However, sonicated CHI-CP-EUG was able to show as much ductility as the pristine CHI film. This result is inconsistent with the literature suggesting that increased tensile strength usually results in decreased flexibility. Similar flexibility of sonicated CHI-CP-EUG to the pristine CHI film may be caused by the high content of non-polar amino acids in CP protein leading to an increase in the distance among polymer chains.

The effects EOs on the mechanical properties of film is very complex due to the specific interactions between oil components and polymer matrix (Bonilla et al. 2018). Film strength loss can be explained by the partial replacement of stronger polymer-polymer interaction by weaker polymer-oil interaction in the emulsified film network (Shojaee-Aliabadi et al. 2013). In fact, lipid addition can disrupt the polymer network in the film resulting in an increase in EB and a decrease in TS (Xue et al. 2019). Interestingly, the incorporation of EUG into produced films had the opposite effect. This strengthening effect is probably due to the cross-linking of some compounds in EUG with protein. Studies on emulsion films in the literature contain contradictory tensile results. However, a similar finding was reported by Ojagh et al. (2010) in chitosan based films containing different concentrations of cinnamon EO. Researchers concluded that oil produced a cross-linker effect within chitosan matrix leading to a decrease the free volume and the molecular mobility of chitosan. This gave a lubricant sheet-like structure (oil layers located among chitosan sheets). The cross-linking effects between chitosan and oil were also reported by Pereda et al. (2012) and Vargas et al. (2009) for chitosan-olive oil and chitosan-oleic acid emulsion films.

Elastic modulus or Young's modulus (YM) is a measure of resistance to elastic deformation. It is linked to film rigidity/stiffness. The greater the modulus, the stiffer the film. Non-sonicated films were found to be less rigid, and sonication made the films more rigid. Sonication probably leads to enhancing the cohesion forces of the polymer network.

Toughness (TF) is the ability of a film to absorb the energy during deformation up to fracture (Zhang and Zhang 2012). The greatest amount of energy before fracture could be dissipated by sonicated CHI-CP-EUG film.

Consequently, composite films were strong, but rigid and more brittle than the pristine CHI films. However, sonicated CHI-CP-EUG showed as much ductility as the pristine CHI film. Due to its remarkable TS, EB and TF values, sonicated CHI-CP-EUG film was ideal for self-integrity and mechanically resistant in the current work. Therefore, sonicated group of this film pointed out superior toughness in combination with high strength and ductility showing an example of synergistic composite properties.

2.4.6. Stability of Emulsion Based Film Forming Solutions

2.4.6.1. Particle Size Distribution

Particle size is a considerable parameter used to explain the functional properties (mechanical, morphologic, release, barrier, etc.) of films. The sizes of polymer aggregates were expressed as hydrodynamic diameters, an indicator of the diameter of an equivalent sphere with the same translational diffusion coefficient as the particle or molecule being measured. The volume-weighted mean or De Brouckere mean diameter (D[4,3], μm) and the surface-weighted mean or Sauter mean diameter (D[3,2], μm) of film forming solutions were given in Table 2.17. Non-sonicated solutions had significantly larger particle sizes than sonicated ones ($P < 0.05$). Especially, CP containing non-sonicated composite samples had larger particle size probably due to the chickpea protein aggregation. Unsurprisingly, sonication drastically reduced the particle size ($P < 0.05$). For example; more than 3.0 fold and 2.0 fold reductions were obtained in D[4,3] and D[3,2] values of samples. Likewise, high intensity ultrasound at a frequency of 20 kHz under 300 W with increasing duration reduced the particle size of chickpea protein aggregates in solution (Wang et al. 2020). According to the overall results of both hydrodynamic diameters, small-sized CHI-CP-EUG particles after sonication (2.7 fold reduction in D[4,3] and 2.5 fold reduction in D[3,2]) would improve the mechanical,

release and barrier properties of its film due to the increased protein adsorption around oil-water interface.

Table 2.17. Particle size values of film forming solutions[b,c].

Samples[a]	D[4,3] (µm)	D[3,2] (µm)
Non-sonicated CHI	26.0 ± 4.8^{c}	6.8 ± 3.7^{de}
Sonicated CHI	3.7 ± 0.3^{e}	3.3 ± 1.8^{e}
Non-sonicated CHI-CP	54.8 ± 5.4^{a}	47.3 ± 6.0^{a}
Sonicated CHI-CP	13.8 ± 8.2^{d}	13.2 ± 4.5^{c}
Non-sonicated CHI-EUG	37.0 ± 6.7^{b}	30.2 ± 8.0^{b}
Sonicated CHI-EUG	6.0 ± 0.6^{e}	5.3 ± 0.6^{de}
Non-sonicated CHI-CP-EUG	32.9 ± 5.0^{b}	27.7 ± 4.7^{b}
Sonicated CHI-CP-EUG	12.3 ± 1.9^{d}	11.0 ± 1.7^{cd}

[a] CHI: chitosan, CP: chickpea protein, EUG: eugenol.
[b] Different lower letter superscripts in the same column indicate significant differences ($P < 0.05$).
[c] Values are represented as mean ± SD (n = 6).

2.4.6.2. Zeta (ζ-) Potential

ζ-potential is useful to describe the electric charge interactions between particles in colloidal solution and to understand the net charge on the particle surface and stability of this dispersed system. The behaviour of two oppositely charged biopolymers in a solution depends on solution pH. ζ-potential values of dispersions in acetate buffer at pH 5.0 were displayed in Table 2.18. The ζ-potential of native CHI particles was found to be positively charged at pH 5.0. The amino groups of chitosan are protonated at a pH less than pK_a: 6.5 value rendering a positive surface charge to itself (chitosan – NH_2 + H_3O^+ ↔ chitosan – NH_3^+ + H_2O). Similarly, Costa et al. (2018) found the ζ-potential of chitosan solutions as +60 mV at pH 3.3 and +13 mV at pH 6.9. This reduction is related to the closeness of the solution pH to pK_a, which leads to self-aggregated chitosan stabilized by hydrogen bonds and hydrophobic interactions (Philippova and Korchagina 2012). Sonication significantly caused an increase in the ζ-potential of CHI. This was probably because more protonated amino groups ($-NH_3^+$) on disrupted chitosan polymer chains were exposed after sonication. Ho et al. (2016) reported that sonication shortened the chitosan polymer chains and increased the number of chains, but reduced the hydrophobicity due to the decreased number of acetyl units per unit chain (Ho et al. 2016). However, Costa et al. (2018) informed that sonication led to the exposure of hydrophobic

portions on chitosan particles due to the size reduction and increased surface area. Researchers suggested that these particles might bind more strongly to the oils, thereby enhancing the emulsion stabilization. Proteins have a cationic character at pH lower than pI due to the protonation of amino and carboxyl groups, while they showed an anionic character at pH higher than pI due to the deprotonation of these groups. The ζ-potential of native CP particles was found to be negatively charged at pH 5.0, which is higher than 4.5 pI, and sonication did not change significantly ($P > 0.05$). CHI-CP and CHI-CP-EUG complexes generated at 2:1 CHI:CP weight ratio had high positive ζ-potential values indicating dominant electrostatic repulsion between the particles in solution. It is important to note that the ζ-potential of these formed complexes was still positive (whether sonicated or not), which means that besides the electrostatic interactions between CHI and CP, other types of interactions (hydrogen bonding, hydrophobic interactions, etc.) made the complexes more tightly bound and these solutions had more net charges than individual solutions. However, sonication had no significant effect on the ζ-potential of complexes ($P > 0.05$). The ζ-potential values showed CHI-CP and CHI-CP-EUG particles were monodisperse.

Table 2.18. ζ-potential values of solutions[b,c].

Samples[a]	**ζ-potential (mV)**
Non-sonicated CHI	13.25 ± 3.68^{c}
Sonicated CHI	20.04 ± 7.46^{b}
Non-sonicated CP	-5.20 ± 0.30^{d}
Sonicated CP	-4.47 ± 0.50^{d}
Non-sonicated CHI-CP	32.40 ± 0.90^{a}
Sonicated CHI-CP	29.82 ± 0.45^{a}
Non-sonicated CHI-CP-EUG	32.76 ± 1.07^{a}
Sonicated CHI-CP-EUG	30.08 ± 0.47^{a}

[a] CHI: chitosan, CP: chickpea protein, EUG: eugenol.
[b] Different lower letter superscripts in the same column indicate significant differences ($P < 0.05$).
[c] Values are represented as mean ± SD (n = 6).

Table 2.19. Stability level depending on ζ-potential (Cano-Sarmiento et al. 2018).

ζ-potential magnitude (mV)	**Stability behaviour**
From 0 to ± 10	Highly unstable
From ± 10 to ± 20	Relatively stable
From ± 20 to ± 30	Moderately stable
More than ± 30	Highly stable

Theoretically, dispersions having high ζ-potential are electrostatically-stabilized while dispersions having low ζ-potential tend to coagulate leading to poor physical stability behaviour (see Table 2.19). For example; high magnitude (± 30 mV) usually leads to monodispersity, while low magnitude (± 5 mV) leads to aggregation, coagulation or flocculation. In the current work, high ζ-potential values of CHI-CP and CHI-CP-EUG solutions resulted in a stable system because of their stronger repulsive forces than attractive forces. It can be noted that CHI-CP and CHI-CP-EUG complexes were highly stable because of the interactions between CHI and CP and that the surface charge of the complexes was not changed after sonication even though sonication significantly decreased the particle size.

2.4.7. Eugenol Release Profiles of Films

EOs are usually sprayed on the surfaces of fruits and vegetables for postharvest control. In this case, their effects can fade away quickly due to the high volatility of EOs. This challenge could be overcome by designing sustained formulations to retain volatile active agents for longer. In the current study, chitosan-chickpea protein complex was tailored to give unique functionality for creating a controlled release system for eugenol. Therefore, sustained release of eugenol from chitosan-chickpea protein complex was characterized to determine whether this formulation would provide the desired depot release for antibacterial and antisprouting effects.

In the design of active films and coatings, the retained and released concentrations of the loaded agent are substantial due to the fact that these parameters will determine the duration and efficacy of film activity. The swelling-controlled release is based on the changes in the structure of the polymer matrix (Wang et al. 2022). Polymer gradually swells in contact with a penetrant (any of fluids such as liquid media, solvent, air, water vapour). Interaction with the penetrant allows to increase in the space between polymer molecules (polymer undergoes a transition from glassy to rubbery state) by lowering the glass transition temperature, and then the penetrant dissolves the active agent. Thus, the active agent releases out of the swollen matrix.

2.4.7.1. Release Tests Performed in Model Solutions

The released amounts of EUG from developed films in two simulants (A and D1) with different polarities at 4ºC and 25ºC were reported in Table 2.20 and Table 2.21, respectively. Since increasing ethanol ratio decreases the dipole moment, simulant D1 is more non-polar than simulant A. Low polarity of the simulant increased the amount of released EUG from sonicated CHI-CP-EUG film both at 4ºC and 25ºC due to the stronger affinity of free EUG to simulant D1. Therefore, maximum EUG release would be expected in less polar foodstuffs such as oil-in-water emulsions and alcoholic beverages while lower release would occur in more aqueous foods. This is consistent with the results of other researchers for EOs delivery, which increased when the ethanol ratio rose in the food simulant (Sánchez-González et al. 2011; Narayanan et al. 2013; Requena, Vargas, and Chiralt 2017; Talón et al. 2019). Requena, Vargas, and Chiralt (2017) demonstrated due to the promotion of EO solubility faster EO release in less polar simulants while slower EO release in more aqueous systems.

The release profiles of EUG loaded films were also shown on graphs in Figures 2.6 – 2.7. As seen in Figure 2.6, the increasing temperature increased the amount of released EUG in the presence of simulant A. According to Multivariate ANOVA Test (MANOVA), the temperature was found to be significantly effective on the EUG release of more than half of stored films in simulant A. This result supports that high temperature can trigger the release of EUG. However, as seen in Figure 2.7, temperature change did not affect the EUG release too much in simulant D1. According to MANOVA test, it was observed that temperature did not affect EUG release in simulant D1.

Table 2.20. Released EUG amount of films immersed in 10% ethanol (simulant A) at 4ºC and 25ºC[b,c].

Film samples[a]	**Released EUG (mg/cm² film) at 4ºC**					
	0.5 h	***1 h***	***3 h***	***6 h***	***24 h***	***48 h***
Non-sonicated CHI-EUG	$1.080 \pm 0.08^{b,B}$	$1.212 \pm 0.06^{a,B}$	$1.149 \pm 0.05^{ab,B}$	$1.130 \pm 0.06^{ab,C}$	$1.168 \pm 0.09^{ab,C}$	$1.162 \pm 0.05^{ab,C}$
Sonicated CHI-EUG	$0.913 \pm 0.15^{c,C}$	$1.162 \pm 0.07^{ab,B}$	$1.141 \pm 0.02^{ab,B}$	$1.114 \pm 0.02^{b,C}$	$1.214 \pm 0.02^{a,BC}$	$1.181 \pm 0.01^{ab,C}$
Non-sonicated CHI-CP-EUG	$1.039 \pm 0.11^{d,BC}$	$1.169 \pm 0.10^{bc,B}$	$1.148 \pm 0.05^{c,B}$	$1.195 \pm 0.05^{abc,B}$	$1.290 \pm 0.07^{a,B}$	$1.258 \pm 0.06^{ab,B}$
Sonicated CHI-CP-EUG	$1.361 \pm 0.06^{d,A}$	$1.498 \pm 0.03^{ab,A}$	$1.417 \pm 0.02^{c,A}$	$1.417 \pm 0.02^{c,A}$	$1.504 \pm 0.03^{a,A}$	$1.457 \pm 0.01^{bc,A}$
Film samples[a]	**Released EUG (%) at 4ºC**					
	0.5 h	***1 h***	***3 h***	***6 h***	***24 h***	***48 h***
Non-sonicated CHI-EUG	$47.97 \pm 3.53^{b,B}$	$53.87 \pm 2.91^{a,B}$	$51.09 \pm 2.17^{ab,B}$	$50.23 \pm 2.67^{ab,C}$	$51.91 \pm 4.17^{ab,C}$	$51.63 \pm 2.59^{ab,C}$
Sonicated CHI-EUG	$40.58 \pm 6.69^{c,C}$	$51.64 \pm 3.33^{ab,B}$	$50.68 \pm 0.97^{ab,B}$	$49.49 \pm 0.93^{b,C}$	$53.94 \pm 1.14^{a,BC}$	$52.47 \pm 0.33^{ab,C}$
Non-sonicated CHI-CP-EUG	$46.16 \pm 5.11^{d,BC}$	$51.96 \pm 4.39^{bc,B}$	$51.04 \pm 2.21^{c,B}$	$53.13 \pm 2.28^{abc,B}$	$57.32 \pm 3.33^{a,B}$	$55.94 \pm 3.06^{ab,B}$
Sonicated CHI-CP-EUG	$60.47 \pm 2.69^{d,A}$	$66.55 \pm 1.63^{ab,A}$	$62.95 \pm 1.23^{c,A}$	$62.98 \pm 1.01^{c,A}$	$66.82 \pm 1.43^{a,A}$	$64.77 \pm 0.49^{bc,A}$
Film samples[a]	**Released EUG (mg/cm² film) at 25ºC**					
	0.5 h	***1 h***	***3 h***	***6 h***	***24 h***	***48 h***
Non-sonicated CHI-EUG	$1.249 \pm 0.10^{a,B}$	$1.315 \pm 0.08^{a,A}$	$1.302 \pm 0.08^{a,A}$	$1.209 \pm 0.09^{a,B}$	$1.271 \pm 0.08^{a,B}$	$1.249 \pm 0.10^{a,B}$
Sonicated CHI-EUG	$1.232 \pm 0.05^{b,B}$	$1.310 \pm 0.10^{a,A}$	$1.274 \pm 0.02^{ab,A}$	$1.159 \pm 0.03^{c,B}$	$1.251 \pm 0.02^{ab,B}$	$1.214 \pm 0.04^{bc,B}$
Non-sonicated CHI-CP-EUG	$1.218 \pm 0.40^{a,B}$	$1.325 \pm 0.42^{a,A}$	$1.361 \pm 0.41^{a,A}$	$1.282 \pm 0.36^{a,AB}$	$1.323 \pm 0.37^{a,B}$	$1.298 \pm 0.36^{a,B}$
Sonicated CHI-CP-EUG	$1.546 \pm 0.10^{a,A}$	$1.596 \pm 0.10^{a,A}$	$1.591 \pm 0.10^{a,A}$	$1.514 \pm 0.07^{a,A}$	$1.610 \pm 0.13^{a,A}$	$1.569 \pm 0.08^{a,A}$
Film samples[a]	**Released EUG (%) at 25ºC**					
	0.5 h	***1 h***	***3 h***	***6 h***	***24 h***	***48 h***
Non-sonicated CHI-EUG	$55.48 \pm 4.76^{a,B}$	$58.42 \pm 3.93^{a,A}$	$57.87 \pm 3.85^{a,A}$	$53.73 \pm 4.03^{a,B}$	$56.47 \pm 3.80^{a,B}$	$55.50 \pm 4.46^{a,B}$
Sonicated CHI-EUG	$54.75 \pm 2.50^{b,B}$	$58.24 \pm 4.69^{a,A}$	$56.62 \pm 1.10^{ab,A}$	$51.51 \pm 1.59^{c,B}$	$55.59 \pm 1.21^{ab,B}$	$53.97 \pm 1.80^{bc,B}$
Non-sonicated CHI-CP-EUG	$54.13 \pm 18.10^{a,B}$	$58.88 \pm 19.08^{a,A}$	$60.50 \pm 18.43^{a,A}$	$57.00 \pm 16.37^{a,AB}$	$58.82 \pm 16.65^{a,B}$	$57.68 \pm 16.24^{a,B}$
Sonicated CHI-CP-EUG	$68.68 \pm 4.42^{a,A}$	$70.95 \pm 4.40^{a,A}$	$70.71 \pm 4.63^{a,A}$	$67.28 \pm 3.19^{a,A}$	$71.35 \pm 6.03^{a,A}$	$69.73 \pm 3.93^{a,A}$

[a] CHI: chitosan, CP: chickpea protein, EUG: eugenol.
[b] Different lower and capital letter superscripts in the same row and column indicate significant differences ($P < 0.05$), respectively.
[c] Values are presented as mean value ± SD (n = 6).

Table 2.21. Released EUG amount of films immersed in 50% ethanol (simulant D1) at 4ºC and 25ºC[b,c].

Film samples[a]	Released EUG (mg/cm² film) at 4ºC						
	0.5 h	***1 h***	***3 h***	***6 h***	***20 h***	***26 h***	***44 h***
Sonicated CHI-EUG	$1.046 \pm 0.10^{bc,B}$	$1.061 \pm 0.05^{bc,B}$	$1.102 \pm 0.04^{ab,C}$	$1.110 \pm 0.05^{ab,B}$	$1.146 \pm 0.05^{a,BC}$	$1.005 \pm 0.06^{c,D}$	$1.084 \pm 0.02^{ab,C}$
Non-sonicated CHI-EUG	$1.115 \pm 0.17^{ab,B}$	$1.114 \pm 0.07^{ab,B}$	$1.078 \pm 0.05^{b,C}$	$1.055 \pm 0.06^{b,B}$	$1.075 \pm 0.05^{b,C}$	$1.142 \pm 0.06^{ab,C}$	$1.225 \pm 0.09^{a,B}$
Sonicated CHI-CP-EUG	$1.312 \pm 0.16^{e,A}$	$1.370 \pm 0.09^{a,A}$	$1.383 \pm 0.11^{a,A}$	$1.362 \pm 0.13^{a,A}$	$1.401 \pm 0.16^{a,A}$	$1.422 \pm 0.08^{a,A}$	$1.371 \pm 0.03^{a,A}$
Non-sonicated CHI-CP-EUG	$0.780 \pm 0.10^{b,C}$	$1.178 \pm 0.13^{a,B}$	$1.240 \pm 0.13^{a,B}$	$1.182 \pm 0.12^{a,B}$	$1.223 \pm 0.10^{a,B}$	$1.267 \pm 0.10^{a,B}$	$1.313 \pm 0.13^{a,AB}$
Film samples[a]	**Released EUG (%) at 4ºC**						
	0.5 h	***1 h***	***3 h***	***6 h***	***20 h***	***26 h***	***44 h***
Non-sonicated CHI-EUG	$49.56 \pm 7.63^{a,B}$	$49.53 \pm 3.30^{a,B}$	$47.88 \pm 2.18^{a,C}$	$46.92 \pm 2.73^{a,B}$	$47.79 \pm 2.26^{a,C}$	$50.78 \pm 3.03^{a,C}$	$54.45 \pm 4.06^{a,B}$
Sonicated CHI-EUG	$46.49 \pm 4.60^{bc,B}$	$47.17 \pm 2.48^{bc,B}$	$48.97 \pm 1.89^{ab,C}$	$49.35 \pm 2.54^{ab,B}$	$50.93 \pm 2.40^{a,BC}$	$44.67 \pm 6.96^{c,D}$	$48.18 \pm 1.08^{ab,C}$
Non-sonicated CHI-CP-EUG	$34.68 \pm 4.50^{b,C}$	$52.36 \pm 5.98^{a,B}$	$55.10 \pm 5.79^{a,B}$	$52.53 \pm 5.49^{a,B}$	$54.34 \pm 4.73^{a,B}$	$56.29 \pm 4.77^{a,B}$	$58.35 \pm 6.20^{a,AB}$
Sonicated CHI-CP-EUG	$58.28 \pm 7.44^{a,A}$	$60.89 \pm 4.11^{a,A}$	$61.48 \pm 5.12^{a,A}$	$60.52 \pm 6.09^{a,A}$	$62.26 \pm 7.11^{a,A}$	$63.18 \pm 3.97^{a,A}$	$60.91 \pm 1.77^{a,A}$
Film samples[a]	**Released EUG (mg/cm² film) at 25ºC**						
	0.5 h	***1 h***	***3 h***	***6 h***	***20 h***	***26 h***	***44 h***
Non-sonicated CHI-EUG	$1.080 \pm 0.02^{c,B}$	$1.116 \pm 0.06^{bc,BC}$	$1.066 \pm 0.03^{cd,B}$	$1.026 \pm 0.03^{d,C}$	$1.087 \pm 0.04^{c,B}$	$1.140 \pm 0.03^{b,B}$	$1.244 \pm 0.04^{a,BC}$
Sonicated CHI-EUG	$1.069 \pm 0.07^{a,B}$	$1.034 \pm 0.05^{a,C}$	$1.087 \pm 0.06^{a,B}$	$1.117 \pm 0.03^{a,B}$	$1.109 \pm 0.08^{a,B}$	$1.097 \pm 0.05^{a,B}$	$1.127 \pm 0.03^{a,C}$
Non-sonicated CHI-CP-EUG	$1.129 \pm 0.08^{bc,B}$	$1.145 \pm 0.07^{bc,B}$	$1.111 \pm 0.07^{bc,B}$	$1.065 \pm 0.07^{c,BC}$	$1.113 \pm 0.05^{bc,B}$	$1.169 \pm 0.07^{b,B}$	$1.288 \pm 0.08^{a,B}$
Sonicated CHI-CP-EUG	$1.514 \pm 0.10^{a,A}$	$1.521 \pm 0.11^{a,A}$	$1.497 \pm 0.07^{a,A}$	$1.481 \pm 0.08^{a,A}$	$1.489 \pm 0.03^{a,A}$	$1.544 \pm 0.11^{a,A}$	$1.575 \pm 0.18^{a,A}$
Film samples[a]	**Released EUG (%) at 25ºC**						
	0.5 h	***1 h***	***3 h***	***6 h***	***20 h***	***26 h***	***44 h***
Non-sonicated CHI-EUG	$48.01 \pm 1.10^{c,B}$	$49.62 \pm 3.02^{bc,BC}$	$47.36 \pm 1.69^{cd,B}$	$45.59 \pm 1.56^{d,C}$	$48.30 \pm 1.77^{c,B}$	$50.65 \pm 1.34^{b,B}$	$55.32 \pm 1.95^{a,BC}$
Sonicated CHI-EUG	$47.52 \pm 3.43^{a,B}$	$45.95 \pm 2.52^{a,C}$	$48.30 \pm 2.98^{a,B}$	$49.66 \pm 1.45^{a,B}$	$49.29 \pm 3.59^{a,B}$	$48.73 \pm 2.65^{a,B}$	$50.06 \pm 1.31^{a,C}$
Non-sonicated CHI-CP-EUG	$50.19 \pm 3.62^{bc,B}$	$50.88 \pm 3.45^{bc,B}$	$49.39 \pm 3.15^{bc,B}$	$47.31 \pm 3.19^{c,BC}$	$49.48 \pm 2.52^{bc,B}$	$51.94 \pm 3.45^{b,B}$	$57.24 \pm 3.79^{a,B}$
Sonicated CHI-CP-EUG	$67.29 \pm 4.78^{a,A}$	$67.60 \pm 5.00^{a,A}$	$66.54 \pm 3.45^{a,A}$	$65.84 \pm 3.65^{a,A}$	$66.17 \pm 1.46^{a,A}$	$68.62 \pm 5.08^{a,A}$	$70.00 \pm 8.16^{a,A}$

[a] CHI: chitosan, CP: chickpea protein, EUG: eugenol.

[b] Different lower and capital letter superscripts in the same row and column indicate significant differences ($P < 0.05$), respectively.

[c] Values are presented as mean value ± SD (n = 6).

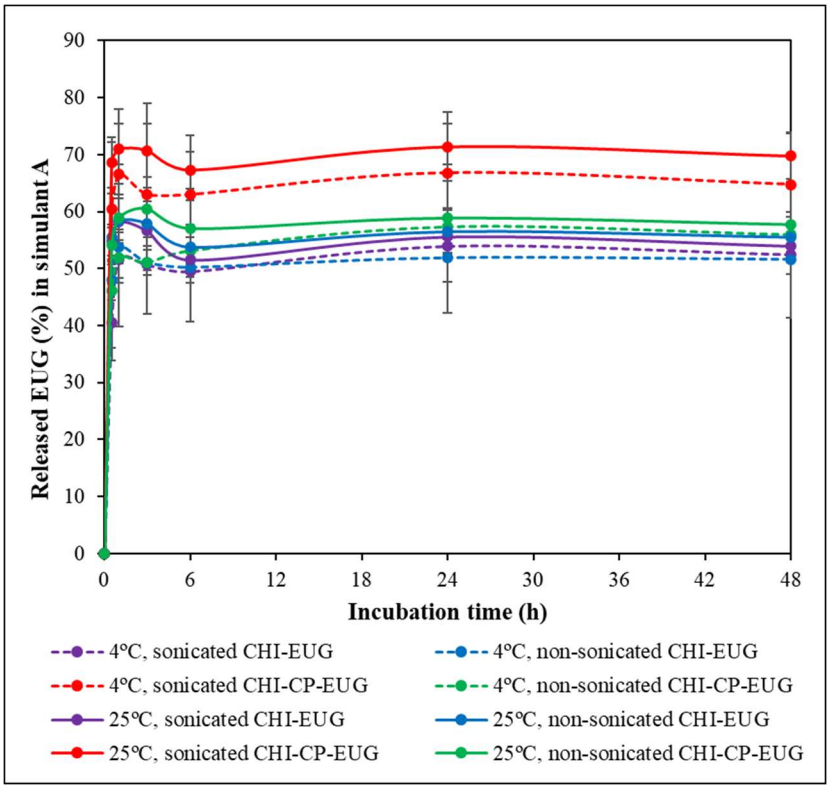

Figure 2.6. EUG release profiles of films during incubation in simulant A (10% ethanol) at 4°C and 25°C.

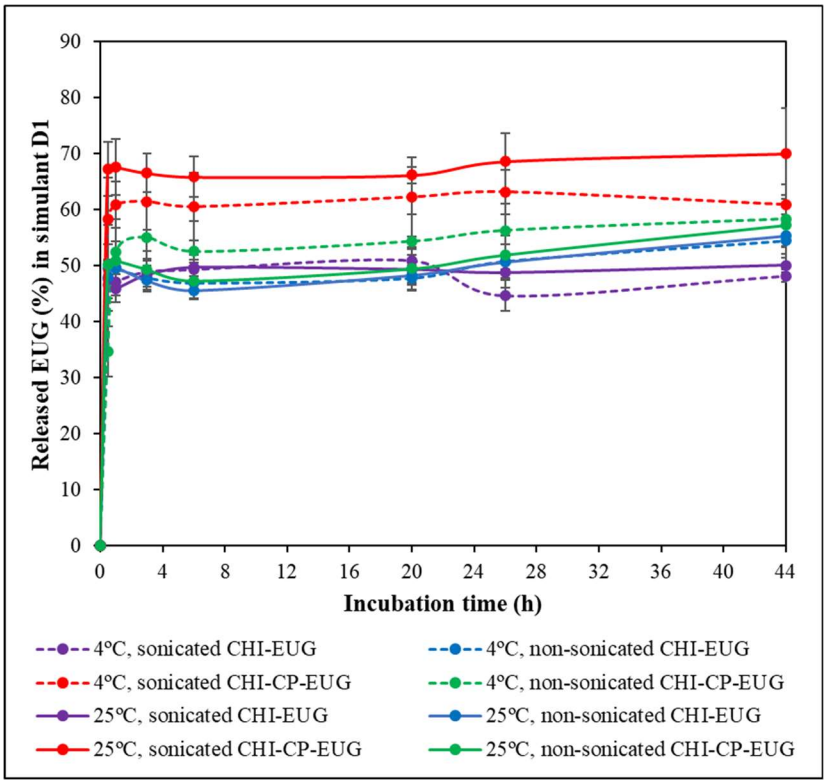

Figure 2.7. EUG release profiles of films during incubation in simulant D1 (50% ethanol) at 4°C and 25°C.

2.4.7.2. Release Tests Performed in Air

The release of EUG was analyzed in the air for 28 days of storage because onions are stored in ambient during the storage period until consumption. Based on the results in Figure 2.8, it was found that on the first day of storage, only about 3% of EUG was released from sonicated CHI-CP-EUG film while about 40% of EUG was quickly released from other films into the air (see Table 2.22 and subtract the percent values from 100). Rapid release of EUG during the first day was most likely due to the presence of free form of EUG. However, due to the sonication effect, EUG was firmly bonded to CHI-CP complex and released sustainably during storage of sonicated CHI-CP-EUG film. It is critical for effective postharvest control of onions in terms of inhibiting the sprouting and growth of inoculated microorganisms.

Although the release profiles showed that EUG release duration was about the same for all films suggesting that the presence of CP protein controls EUG release rate. To compare the kinetic parameters, a first-order graph was plotted after taking the common logarithm of the y-axis, since retained EUG (%) – time profile exhibited an exponential decrease on the arithmetic plot (see Figure 2.9). It is known that the trend of retained EUG profile can be transformed into a linear curve if plotted on a semi-logarithmic chart (the y-axis uses a logarithmic scale and the x-axis uses a linear scale) or after taking the common logarithm of values on the y-axis it would be linear on an arithmetic chart (both axes use linear scales). Only the initial linear portions (until the 7^{th} day) of the first-order plots were used to calculate kinetic parameters (see Figure 2.10).

Table 2.22. Retained EUG amount of films exposed to air at 25°C[b,c].

Film samples[a]	Retained EUG (mg/cm² film)						
	Day 1	***Day 2***	***Day 3***	***Day 7***	***Day 14***	***Day 21***	***Day 28***
Non-sonicated CHI-EUG	$1.37 \pm 0.05^{a,b}$	$1.00 \pm 0.05^{b,C}$	$0.67 \pm 0.05^{c,C}$	$0.22 \pm 0.02^{d,B}$	$0.06 \pm 0.006^{e,B}$	$0.028 \pm 0.002^{e,C}$	$0.025 \pm 0.002^{e,C}$
Sonicated CHI-EUG	$1.40 \pm 0.04^{a,b}$	$1.13 \pm 0.07^{b,B}$	$0.71 \pm 0.12^{c,BC}$	$0.23 \pm 0.01^{d,B}$	$0.05 \pm 0.006^{e,C}$	$0.030 \pm 0.008^{e,B}$	$0.027 \pm 0.002^{e,C}$
Non-sonicated CHI-CP-EUG	$1.39 \pm 0.20^{a,b}$	$1.13 \pm 0.08^{b,B}$	$0.77 \pm 0.05^{c,B}$	$0.23 \pm 0.04^{d,B}$	$0.06 \pm 0.005^{e,B}$	$0.031 \pm 0.009^{e,B}$	$0.030 \pm 0.008^{e,B}$
Sonicated CHI-CP-EUG	$2.17 \pm 0.12^{a,A}$	$1.87 \pm 0.06^{b,A}$	$1.41 \pm 0.03^{c,A}$	$0.76 \pm 0.16^{d,A}$	$0.08 \pm 0.006^{e,A}$	$0.037 \pm 0.001^{e,A}$	$0.034 \pm 0.001^{e,A}$
Film samples[a]	**Retained EUG (%)**						
	Day 1	***Day 2***	***Day 3***	***Day 7***	***Day 14***	***Day 21***	***Day 28***
Non-sonicated CHI-EUG	$61.00 \pm 2.56^{a,B}$	$44.54 \pm 2.30^{b,C}$	$29.57 \pm 2.21^{c,C}$	$10.03 \pm 0.95^{d,B}$	$2.53 \pm 0.26^{e,B}$	$1.27 \pm 0.07^{e,C}$	$1.10 \pm 0.09^{e,C}$
Sonicated CHI-EUG	$62.02 \pm 1.64^{a,B}$	$50.38 \pm 3.11^{b,B}$	$31.45 \pm 5.45^{c,BC}$	$10.39 \pm 0.45^{d,B}$	$2.04 \pm 0.27^{e,C}$	$1.35 \pm 0.03^{e,B}$	$1.18 \pm 0.08^{e,C}$
Non-sonicated CHI-CP-EUG	$61.59 \pm 8.87^{a,B}$	$50.05 \pm 3.73^{b,B}$	$34.20 \pm 2.17^{c,B}$	$10.39 \pm 1.61^{d,B}$	$2.41 \pm 0.25^{e,B}$	$1.37 \pm 0.03^{e,B}$	$1.35 \pm 0.04^{e,B}$
Sonicated CHI-CP-EUG	$96.65 \pm 5.58^{a,A}$	$82.91 \pm 2.57^{b,A}$	$62.59 \pm 1.21^{c,A}$	$33.73 \pm 7.29^{d,A}$	$3.34 \pm 0.29^{e,A}$	$1.65 \pm 0.05^{e,A}$	$1.51 \pm 0.07^{e,A}$

[a] CHI: chitosan, CP: chickpea protein, EUG: eugenol.
[b] Different lower and capital letter superscripts in the same row and column indicate significant differences ($P < 0.05$), respectively.
[c] Values are presented as mean value ± SD (n = 6).

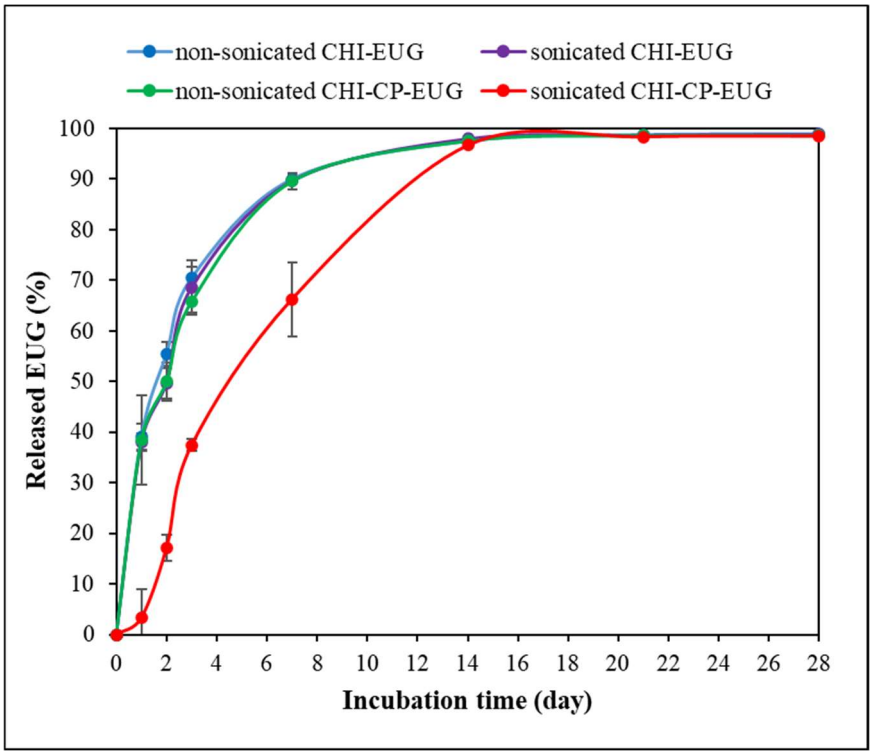

Figure 2.8. EUG release profiles of film during incubation in air at 25ºC.

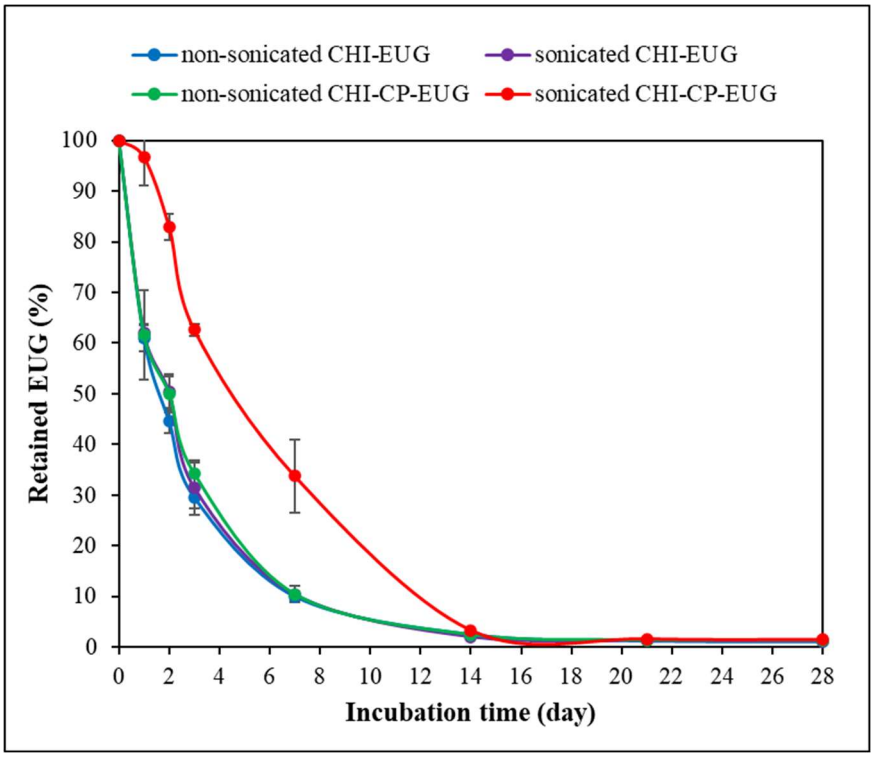

Figure 2.9. EUG retention profiles of films during incubation in air at 25ºC.

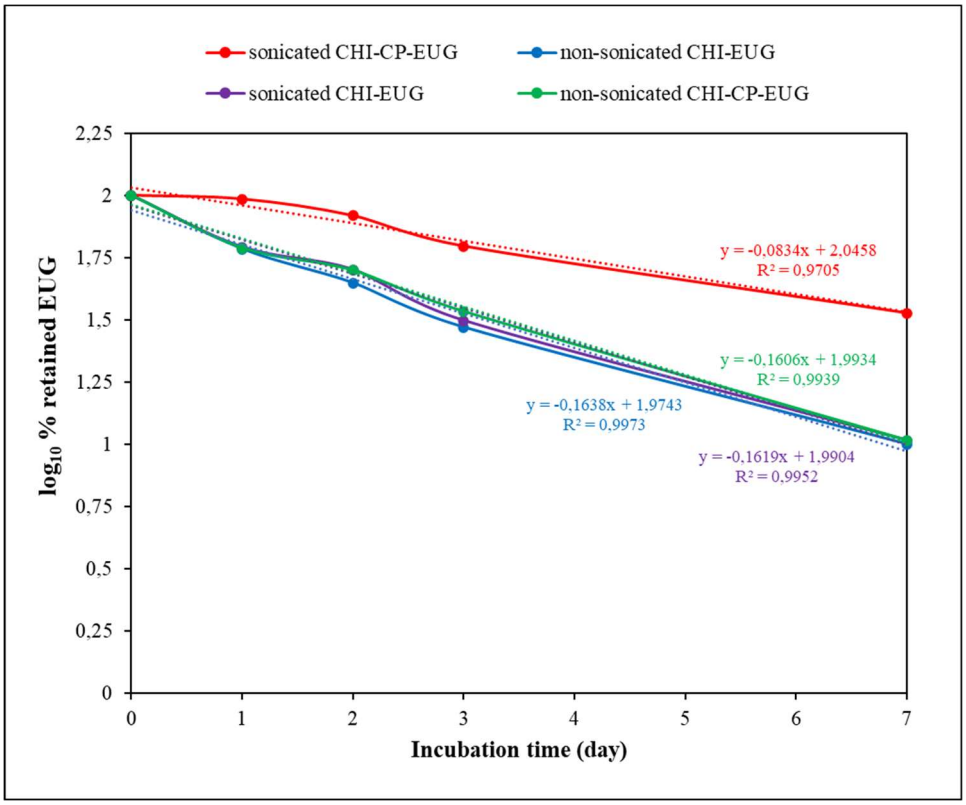

Figure 2.10. First-order plots for EUG-containing films.

Table 2.23. Release kinetic parameters of EUG following exposure of films to air for 1 week at 25°C.

Film samples[a]	**slope**	**k´ (day^{-1})**	**$t_{1/2}$ (day)**
Non-sonicated CHI-EUG	-0.1638	0.38	1.8
Sonicated CHI-EUG	-0.1619	0.37	1.9
Non-sonicated CHI-CP-EUG	-0.1606	0.37	1.9
Sonicated CHI-CP-EUG	-0.0834	0.19	3.6

[a] CHI: chitosan, CP: chickpea protein, EUG: eugenol.

Release kinetic parameters were presented in Table 2.23. The rate constant (k´) represented the fraction of EUG that was released from the film during a given period of time. It was calculated from the slope (-k/2.303) of first-order plots. For example, k´ = 0.19 per day meant that approximately 19% of the amount remaining EUG in sonicated CHI-CP-EUG released into air (or volatilized) each day. According to the results of k´, EUG release rate in other films was found to be 2 times higher, so EUG will volatilize faster.

Half-life ($t_{1/2}$) was the time needed to release 50% of EUG in film. It was calculated from the rate constant. $t_{1/2} = 0.693 / k´$ where 0.693 was a constant derived

from the natural log (In) of the ratio of retained EUG at the beginning and end of one half-life, which definition was 2 (100%/50%) (In 2 = 0.693). The longer half-life means that the longer the EUG concentration of the film will remain above the minimum effective concentration. According to the results of $t_{1/2}$, the time required for 50% of EUG to be released was found to be 2 times longer in sonicated CHI-CP-EUG than other films. This is a desired result for the general scope of the current study. Similarly, Table 2.22 showed that 50% EUG retention was observed in stored non-sonicated CHI-EUG, sonicated CHI-EUG and non-sonicated CHI-CP-EUG films up till 2 days while that in stored sonicated CHI-CP-EUG film for up to 3 – 7 days. This finding clearly showed that the duration of action of encapsulated EUG in CHI-CP complex was the longest. For example; higher and long-term antisprouting and antibacterial effects are likely to be observed in bulbs coated with sonicated CHI-CP-EUG film because bulbs will be exposed to higher EUG concentrations for a more extended period. Consequently, the release studies demonstrated that EUG could be successfully encapsulated with coacervation complex of CHI-CP using sonication.

2.4.8. Surface Investigation of Films

Two powerful microscopy techniques for high resolution surface investigation are: scanning electron microscopy (SEM) and atomic force microscopy (AFM). The surface topography and roughness are highly influential characteristics on film performance.

2.4.8.1. SEM Visualization of Films

Figure 2.11 and Figure 2.12 respectively showed up-surface and cross-section images at 2500× or 5000× magnification. The surfaces of non-sonicated films were rough with some cracks. However, more smooth and continuous structures were observed after sonication. Obviously, sonication provided more uniform view compared with the non-sonicated films. This finding clearly showed that the film forming components became more compatible after being applied sonication. Addition of CP disrupted the smooth

structure and made the surface of pristine CHI film more rough due to protein aggregates. The small spherical particles appeared distributed over the film structure (see B in Figure 2.11). Sonication probably was able to promote adequately blending of CHI and CP, but still, there were found be some irregularities in sonicated CHI-CP film. Prodpran et al. (2007) suggested that the formation of non-covalent bonds between chitosan and protein strands may be caused roughness.

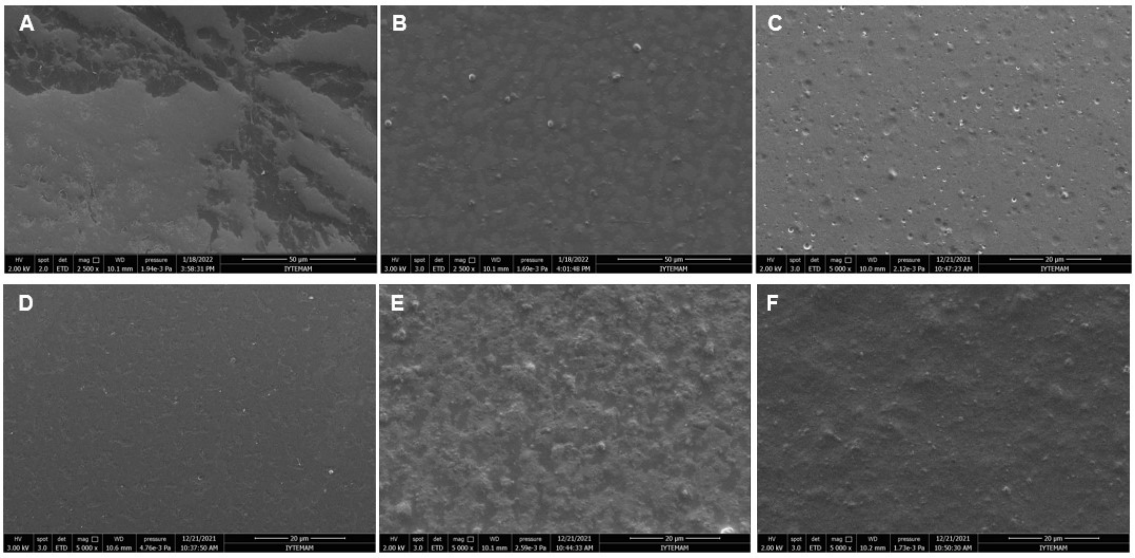

Figure 2.11. Surface images of films (A: non-sonicated CHI film; B: non-sonicated CHI-CP film; C: non-sonicated CHI-CP-EUG film; D: sonicated CHI film; E: sonicated CHI-CP film; F: sonicated CHI-CP-EUG film).

Oil droplets were visible in non-sonicated CHI-CP-EUG film (see C in Figure 2.11). Although EUG addition partially enhanced the smoothness of CHI-CP film, the cross-sectional view of non-sonicated CHI-CP-EUG film displayed some irregularities such as air bubbles or pores (see C in Figure 2.12). With the additional sonication, the incorporation of EUG into CHI-CP film remarkably enhanced the smoothness by filling the irregularities in the matrix (see F in Figures 2.11 and 2.12). It is important to note that sonication reduced air bubbles and gave denser CHI-CP-EUG film. Sonicated CHI-CP-EUG film showed more compact cross-section. This is probably due to the strong interaction and better compatibility thanks to sonication. A more compact and dense structure also helps to understand why this film showed an improvement in the moisture barrier properties and tensile strength.

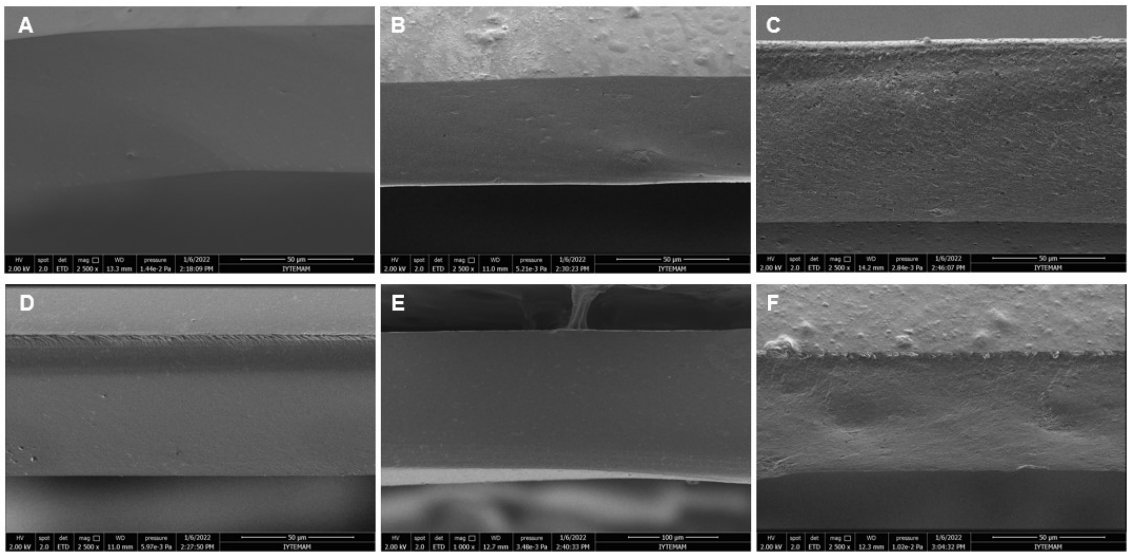

Figure 2.12. Cross-sectional images of films (A: non-sonicated CHI film; B: non-sonicated CHI-CP film; C: non-sonicated CHI-CP-EUG film; D: sonicated CHI film; E: sonicated CHI-CP film; F: sonicated CHI-CP-EUG film).

2.4.8.2. AFM Imaging of Films

The qualitative (topographic images) and quantitative (R_a and R_q) information on physical properties of films were determined by AFM. Both 2D and 3D topography of films were presented in Figure 2.13. All sonicated groups became pointed compared with the non-sonicated groups. This is consistent with Ruan et al. (2020), who reported that aggregated protein collapsed and formed uniform needle-like protrusions when utilized sonication to enhance protein solubility. Similar results on rice protein were also obtained by Ding et al. (2022). The cavitation and mechanical effects (mechanical agitation, microstreaming, shear force generation) of sonication may degrade large protein aggregates to form smaller particles (Chandrapala et al. 2012). Obviously, sonication led to a reduction in peaks and valleys. This finding was in accordance with the SEM tests and roughness results. As noted earlier, roughness of sonicated films were found to be lower than those of non-sonicated films. Most probably, sonication improved the ternary complex integration among CHI, CP and EUG in film matrix resulting in a more uniform and dense film.

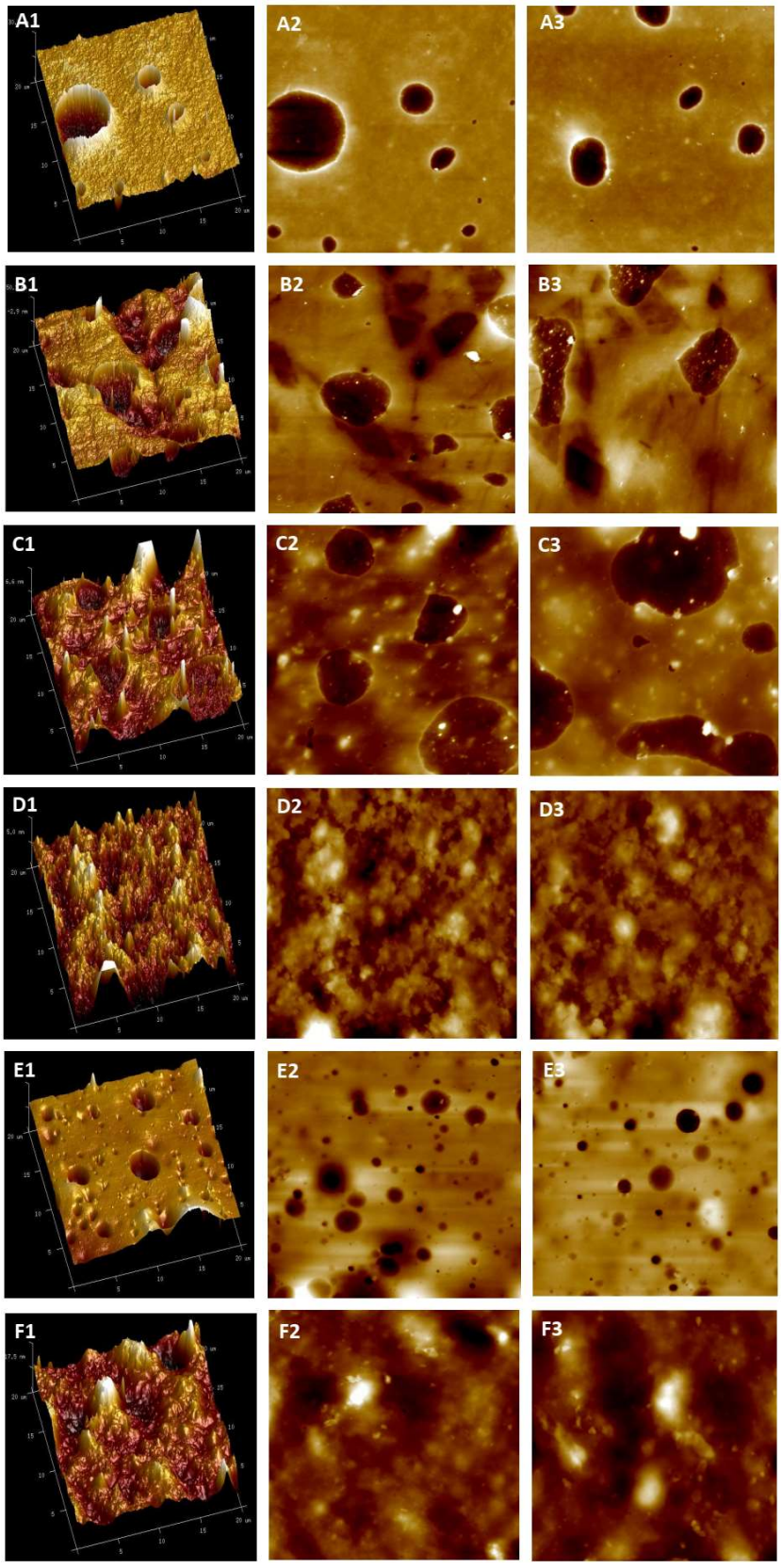

Figure 2.13. 2D (coded by 2 and 3 suffixes) and 3D (coded by 1 suffix) topographies of films (A: non-sonicated CHI film; B: non-sonicated CHI-CP film; C: non-sonicated CHI-CP-EUG film; D: sonicated CHI film; E: sonicated CHI-CP film; F: sonicated CHI-CP-EUG film).

2.4.8.3. Film Roughness and Thickness

Roughness is essential parameter in the current work because it likely had an effect on release performance and light scattering from film surface. Many studies reported that increased surface roughness on membranes/films increase flux due to the increase in surface area (Hirose, Ito, and Kamiyama 1996; Yao, Guo, and Zhang 2007). However, there are some contradictory results in the literature when the flux increases larger than the increase in surface area because the magnitude of increase in flux can be more or less than the increase in surface area depending on the geometry of the surface roughness (Goodyer and Bunge 2012).

Table 2.24. Roughness values and thickness of films[b,c].

Film samples[a]	**R_a (nm)**	**R_q (nm)**	**Thickness (µm)**
Non-sonicated CHI	29.57 ± 9.89^{a}	41.67 ± 12.26^{a}	91.55 ± 1.24^{a}
Sonicated CHI	13.63 ± 2.30^{b}	19.33 ± 2.38^{b}	59.49 ± 0.99^{e}
Non-sonicated CHI-CP	34.80 ± 4.69^{a}	45.63 ± 6.23^{a}	84.52 ± 0.70^{bc}
Sonicated CHI-CP	16.07 ± 4.14^{b}	22.53 ± 4.23^{b}	81.29 ± 0.98^{c}
Non-sonicated CHI-CP-EUG	4.86 ± 0.20^{c}	7.24 ± 1.38^{c}	88.24 ± 7.32^{ab}
Sonicated CHI-CP-EUG	3.26 ± 1.05^{c}	4.74 ± 1.39^{c}	74.09 ± 1.63^{d}

[a] CHI: chitosan, CP: chickpea protein, EUG: eugenol.
[b] Different lower letter superscripts in the same column indicate significant differences ($P < 0.05$).
[c] Values are represented as mean ± SD (n = 3).

Quantitative roughness measurements (R_a and R_q) were obtained from the AFM images were summarized in Table 2.24. Arithmetic mean roughness (R_a) is the mean height as calculated over the entire measured length/area. R_a values ranged from 3.26 to 34.80 nm. Root-mean-square of roughness (R_q) is square root of the distribution of surface height. R_q values ranged from 4.74 to 45.63 nm. Of all films, non-sonicated CHI and CHI-CP films had the greatest roughness while CHI-CP-EUG films had the least roughness whether sonicated or not. CP alone did not cause any significant change ($P > 0.05$) whereas CP and EUG coupling effect significantly decreased the roughness of the pristine CHI film ($P < 0.05$). A rougher can improve the availability of free functional groups on the surface (Rana and Matsuura 2010; Rastgar et al. 2017). As CHI, CP and EUG interacted with each other by chemical bonds, less functional groups were free on the film surface. Therefore, CHI-CP-EUG film offered a less rough surface compared with others. Roughness results were corroborated by SEM analysis. It can be presumed

that the films having higher roughness most probably have high surface area. It is worth noting that sonicated CHI-CP-EUG composite film presented more effective and prolonged release performance due to its smaller surface area. Also, this film scattered more light with a high opacity compared to other films due to its less roughness.

Thickness is also important in thin film technology. Food packaging applications generally consist of self-standing materials such as edible films, sheets, coatings, wraps, casings, mats and pouches(Janjarasskul and Krochta 2010). A thickness less than 254 μm represents a film, whereas higher than 254 μm thickness indicates a sheet (Janjarasskul and Krochta 2010). Thickness varied from 59.59 μm to 91.55 μm, so all samples met the criteria of being a thin film (see Table 2.24).

2.4.9. Selection of the Active Coating to be Used in Food Applications: Tests on Bulbs

The previous results showed that sonication should be used to achieve the desired film structure. Since sonicated (US treated) CHI-CP-EUG composite film had a superior mechanical, barrier and release performance showing synergistic composite film properties, it was tested on onion bulbs to control the risks of sprouting and microbial growth.

2.4.10. Physicochemical Properties of Stored Bulbs

The atmospheric composition, temperature and relative humidity of a store impact the length of storage of onions. Since high relative humidity induces root growth and high temperature induces sprouting, storage below freezing point of -1 to -2^{o}C is generally recommended (Gross, Wang, and Saltveit 2016). There are several technology options such as low temperature storage, high temperature storage, direct harvest storage and controlled atmosphere storage for bulk storage of onions. The storage conditions recommended by FAO (2003) for these systems are summarized in Figure B6 in Appx. B. In the current study, an active coating application was designed for storage conditions in grocery stores and at home after direct harvest storage. Thus, storage analyses were

done for 4 weeks at 25°C. According to market quality standards set by the United Nations Economic Commission for Europe (UNECE) bulbs must meet the minimum requirements of having at least one entire and intact skin, no bacterial or fungal disease (no rotting incidence), have no externally visible sprouts, etc. (UNECE 2019). Therefore, some physicochemical, textural and sprout assessment assays were done on stored bulbs.

Total soluble solid (TSS) content of coated and uncoated bulbs was given in Table 2.25. TSS is related to the storability or degree of postharvest conservation of bulbs, since it is positively correlated with sugar content. Uncoated Ctr_{bulb} and coated bulbs showed a level of TSS of 13 – 15% and there were no significant differences between bulbs during storage. Similar TSS values were obtained by Thivya et al. (2022). In literature, shallot bulbs have been reported to have higher dry matter content (15 – 18%) than onion bulbs (Rabinowitch 2021). Theoretically, the conversion of polysaccharides such as starch and pectins into soluble sugars causes an increase in TSS during storage. For example; Chope et al. (2006) demonstrated that rapid utilization of soluble sugars during sprouting decreased TSS content. However, there is no such a trend in the current work.

pH and titratable acidity values of uncoated Ctr_{bulb} and coated bulbs were shown in Table 2.26 and Table 2.27, respectively. The bulb juice was slightly acidic. The pH of bulbs was between 5.7 – 5.9, which was compatible with the results of Roberts and Kidd (2005). The acidity values of bulbs ranged from 0.16 to 0.30 g MA/100 g FW. Similarly, Thivya (2022) reported acidity levels within the interval 0.21 and 0.60% lactic acid (or 0.16 – 0.45% MA). Theoretically, pH and titratable acidity have an inverse relationship. However, both pH and titratable acidity presented a slightly decreasing tendency towards the end of the storage period in the current work. The reductions in pH and titratable acidity values during storage might be due to the degradation of organic acids. While sprouting starts, soluble sugars (sucrose, maltose, glucose, fructose, etc.) are decomposed into organic acids and transported for sprout growth. Undoubtedly, the respiration rate of stored bulbs for 28 days was higher than that of stored bulbs for 1 day.

Table 2.25. Total soluble solid content (%) of stored bulbs.

Samples	Storage time (days)				
	Day 1	***Day 7***	***Day 14***	***Day 21***	***Day 28***
Ctr_{bulb}	13.46 ± 1.64^{b}	14.08 ± 1.82^{ab}	13.57 ± 1.11^{b}	15.18 ± 1.43^{a}	15.33 ± 2.46^{a}
SC_{bulb}	13.15 ± 0.86^{c}	14.27 ± 1.47^{bc}	14.82 ± 0.76^{ab}	15.84 ± 0.92^{a}	13.59 ± 1.72^{c}
SCP_{bulb}	13.49 ± 1.78	14.37 ± 0.76	14.16 ± 1.11	14.58 ± 2.08	13.74 ± 1.34
$SCPE_{bulb}$	14.66 ± 1.80	14.01 ± 1.27	14.04 ± 1.14	15.48 ± 1.82	14.79 ± 1.91
CPE_{bulb}	14.76 ± 2.40	14.00 ± 1.14	14.25 ± 0.87	15.85 ± 1.42	14.31 ± 2.40

[a] Ctr_{bulb}: uncoated bulbs, SC_{bulb}: sonicated CHI coated bulbs, SCP_{bulb}: sonicated CHI-CP coated bulbs, $SCPE_{bulb}$: sonicated CHI-CP-EUG coated bulbs, CPE_{bulb}: non-sonicated CHI-CP-EUG coated bulbs.
[b] Different lower and capital letter superscripts in the same row and column indicate significant differences ($P < 0.05$), respectively.
[c] Values are presented as mean value ± SD (n = 10).

Table 2.26. pH values of stored bulbs.

Samples	Storage time (days)				
	Day 1	***Day 7***	***Day 14***	***Day 21***	***Day 28***
Ctr_{bulb}	$5.81 \pm 0.09^{a,C}$	$5.73 \pm 0.12^{abc,B}$	$5.76 \pm 0.09^{ab,A}$	$5.71 \pm 0.04^{bc,B}$	$5.66 \pm 0.09^{c,C}$
SC_{bulb}	$5.82 \pm 0.04^{a,BC}$	$5.75 \pm 0.05^{b,B}$	$5.82 \pm 0.04^{a,A}$	$5.74 \pm 0.01^{b,B}$	$5.75 \pm 0.05^{b,AB}$
SCP_{bulb}	$5.86 \pm 0.05^{a,ABC}$	$5.74 \pm 0.04^{c,B}$	$5.80 \pm 0.04^{b,A}$	$5.81 \pm 0.05^{b,A}$	$5.71 \pm 0.04^{c,BC}$
$SCPE_{bulb}$	$5.88 \pm 0.04^{a,A}$	$5.86 \pm 0.05^{ab,A}$	$5.83 \pm 0.06^{b,A}$	$5.84 \pm 0.08^{ab,A}$	$5.76 \pm 0.04^{c,A}$
CPE_{bulb}	$5.87 \pm 0.03^{a,AB}$	$5.83 \pm 0.05^{ab,A}$	$5.82 \pm 0.07^{ab,A}$	$5.81 \pm 0.07^{b,A}$	$5.72 \pm 0.03^{c,AB}$

[a] Ctr_{bulb}: uncoated bulbs, SC_{bulb}: sonicated CHI coated bulbs, SCP_{bulb}: sonicated CHI-CP coated bulbs, $SCPE_{bulb}$: sonicated CHI-CP-EUG coated bulbs, CPE_{bulb}: non-sonicated CHI-CP-EUG coated bulbs.
[b] Different lower and capital letter superscripts in the same row and column indicate significant differences ($P < 0.05$), respectively.
[c] Values are presented as mean value ± SD (n = 10).

Table 2.27. Titratable acidity (% MA or g MA/100 g FW) of stored bulbs.

Samples	**Storage time (days)**				
	Day 1	***Day 7***	***Day 14***	***Day 21***	***Day 28***
Ctr_{bulb}	$0.27 \pm 0.08^{a,A}$	$0.18 \pm 0.05^{bc,C}$	$0.16 \pm 0.05^{c,B}$	$0.22 \pm 0.04^{ab,A}$	$0.21 \pm 0.03^{bc,A}$
SC_{bulb}	$0.27 \pm 0.07^{a,A}$	$0.24 \pm 0.03^{ab,AB}$	$0.20 \pm 0.07^{c,AB}$	$0.23 \pm 0.03^{ab,A}$	$0.22 \pm 0.04^{c,A}$
SCP_{bulb}	$0.28 \pm 0.06^{a,A}$	$0.25 \pm 0.06^{ab,AB}$	$0.20 \pm 0.04^{b,AB}$	$0.25 \pm 0.05^{ab,A}$	$0.21 \pm 0.04^{b,A}$
$SCPE_{bulb}$	$0.28 \pm 0.09^{a,A}$	$0.21 \pm 0.02^{b,BC}$	$0.19 \pm 0.04^{b,AB}$	$0.24 \pm 0.06^{ab,A}$	$0.22 \pm 0.06^{b,A}$
CPE_{bulb}	$0.30 \pm 0.07^{a,A}$	$0.26 \pm 0.07^{a,A}$	$0.24 \pm 0.07^{a,A}$	$0.25 \pm 0.06^{a,A}$	$0.24 \pm 0.05^{a,A}$

[a] Ctr_{bulb}: uncoated bulbs, SC_{bulb}: sonicated CHI coated bulbs, SCP_{bulb}: sonicated CHI-CP coated bulbs, $SCPE_{bulb}$: sonicated CHI-CP-EUG coated bulbs, CPE_{bulb}: non-sonicated CHI-CP-EUG coated bulbs.
[b] Different lower and capital letter superscripts in the same row and column indicate significant differences ($P < 0.05$), respectively.
[c] Values are presented as mean value ± SD (n = 10).

Weight loss is a primary parameter representing the bulb's freshness and firmness because water loss continues over time due to the respiration and transpiration characteristics of fresh produce. According to general acceptance, onions remain marketable for up to 10% weight loss. Thamizharasi and Narasimham (1988) found that large proportion of water loss resulted from water vapor transmission through the skin rather than the neck and base. The driving force is the water vapor pressure difference between the inner bulb tissue and the surrounding air in the storage room. Physiological weight loss of coated bulbs based on water loss was detected in the current work. The coating was able to limit the weight loss until the end of storage. The weight lost ranged between 1 and 2% after 4 weeks of storage (results were not given) and it was below 10% which is the limit for marketability of onions.

2.4.11. Textural Properties of Stored Bulbs

The onion bulb consists of concentric layers emanating from the basal plate and culminating in the neck (Landahl and Terry 2022). Softening in shallot texture is normally expected during storage. Textural changes occur due to the convertion of insoluble polyuronide into more soluble forms. The softening mechanism is induced by the enzymatic role of pectolitic enzymes such as pectin methylesterases (PME) and polygalacturonases (PG). PMEs catalyze the hydrolysis of the pectin in the cell wall structure into pectate and methanol and this action softens the bulb tissue. Penetration force (PF) of polar and equatorial regions of bulbs were given in Tables 2.28 – 2.29 while area under the curve (AUC) results of polar and equatorial sections of bulbs were presented in Tables 2.30 – 2.31. PF and AUC represent hardness and work of penetration, respectively. The higher PF, the harder the sample and so it gives a large AUC. The hardness of Ctr_{bulb} significantly increased over time probably due to partial water loss of control bulbs leading to rigidity. The hardness of coated bulbs increased significantly on day 7 compared to day 1 probably due to the good surface drying of coatings leading to rigidity. However, hardness of coated bulbs slightly changed after the 7^{th} day. During storage, coated bulbs were found to be harder than control as a result of the hardening effect of coating on the bulb surface. It was found that PF at the polar direction was greater than that at the equatorial direction of bulbs. A similar finding was obtained by Eissa and Gamea (2003). This could be due to differences in tissue properties between fleshy layers at the equator and the dense compressed stem (base plate) at the polar.

Table 2.28. Penetration force of polar region of stored bulbs.

Samples	Storage time (days)				
	Day 1	***Day 7***	***Day 14***	***Day 21***	***Day 28***
Ctr_{bulb}	$4.42 \pm 1.18^{c,C}$	$6.95 \pm 2.22^{b,A}$	$7.38 \pm 2.68^{ab,A}$	$8.47 \pm 3.55^{a,A}$	$7.91 \pm 2.72^{ab,A}$
SC_{bulb}	$5.57 \pm 1.63^{b,AB}$	$7.78 \pm 2.50^{a,A}$	$8.79 \pm 3.03^{a,A}$	$9.06 \pm 3.95^{a,A}$	$8.85 \pm 4.05^{a,A}$
SCP_{bulb}	$5.87 \pm 1.52^{b,A}$	$7.57 \pm 2.23^{a,A}$	$7.99 \pm 2.96^{a,A}$	$8.40 \pm 3.63^{a,A}$	$8.09 \pm 3.02^{a,A}$
$SCPE_{bulb}$	$5.04 \pm 1.23^{b,BC}$	$7.60 \pm 2.87^{a,A}$	$7.87 \pm 2.97^{a,A}$	$8.77 \pm 3.33^{a,A}$	$8.80 \pm 4.08^{a,A}$
CPE_{bulb}	$4.85 \pm 1.41^{c,C}$	$8.55 \pm 3.14^{a,A}$	$9.06 \pm 3.12^{a,A}$	$8.11 \pm 3.89^{ab,A}$	$7.00 \pm 2.66^{b,A}$

[a] Ctr_{bulb}: uncoated bulbs, SC_{bulb}: sonicated CHI coated bulbs, SCP_{bulb}: sonicated CHI-CP coated bulbs, $SCPE_{bulb}$: sonicated CHI-CP-EUG coated bulbs, CPE_{bulb}: non-sonicated CHI-CP-EUG coated bulbs.
[b] Different lower and capital letter superscripts in the same row and column indicate significant differences ($P < 0.05$), respectively.
[c] Values are presented as mean value ± SD (n = 40).

Table 2.29. Penetration force of equatorial region of stored bulbs.

Samples	Storage time (days)				
	Day 1	***Day 7***	***Day 14***	***Day 21***	***Day 28***
Ctr_{bulb}	$0.82 \pm 0.58^{c,B}$	$6.68 \pm 1.01^{a,BC}$	$5.88 \pm 0.91^{b,C}$	$5.61 \pm 0.79^{b,B}$	$5.59 \pm 1.12^{b,B}$
SC_{bulb}	$1.78 \pm 1.45^{c,A}$	$7.40 \pm 1.12^{a,A}$	$6.65 \pm 1.40^{b,A}$	$6.74 \pm 0.78^{b,A}$	$6.62 \pm 1.31^{b,A}$
SCP_{bulb}	$1.83 \pm 1.16^{c,A}$	$7.10 \pm 0.86^{a,AB}$	$6.00 \pm 0.70^{b,BC}$	$6.75 \pm 1.24^{a,A}$	$6.03 \pm 0.85^{b,AB}$
$SCPE_{bulb}$	$1.64 \pm 0.75^{c,A}$	$6.77 \pm 0.85^{ab,BC}$	$6.41 \pm 1.17^{b,AB}$	$7.17 \pm 1.58^{a,A}$	$6.64 \pm 1.58^{ab,A}$
CPE_{bulb}	$1.82 \pm 1.15^{b,A}$	$6.35 \pm 0.78^{a,C}$	$6.50 \pm 0.72^{a,A}$	$6.06 \pm 1.41^{a,B}$	$6.24 \pm 1.30^{a,A}$

[a] Ctr_{bulb}: uncoated bulbs, SC_{bulb}: sonicated CHI coated bulbs, SCP_{bulb}: sonicated CHI-CP coated bulbs, $SCPE_{bulb}$: sonicated CHI-CP-EUG coated bulbs, CPE_{bulb}: non-sonicated CHI-CP-EUG coated bulbs.
[b] Different lower and capital letter superscripts in the same row and column indicate significant differences ($P < 0.05$), respectively.
[c] Values are presented as mean value ± SD (n = 40).

Table 2.30. AUC of polar region of stored bulbs.

Samples	Storage time (days)				
	Day 1	***Day 7***	***Day 14***	***Day 21***	***Day 28***
Ctr_{bulb}	$138.18 \pm 52.07^{d,C}$	$186.16 \pm 58.75^{c,C}$	$207.32 \pm 80.73^{bc,B}$	$245.38 \pm 91.11^{a,A}$	$223.09 \pm 63.48^{ab,A}$
SC_{bulb}	$176.94 \pm 60.27^{b,A}$	$237.53 \pm 74.20^{a,AB}$	$254.01 \pm 84.98^{a,A}$	$277.00 \pm 120.36^{a,A}$	$264.95 \pm 113.75^{a,A}$
SCP_{bulb}	$182.93 \pm 72.09^{b,A}$	$222.00 \pm 71.87^{ab,B}$	$227.01 \pm 82.69^{a,AB}$	$249.61 \pm 126.21^{a,A}$	$243.24 \pm 88.16^{a,A}$
$SCPE_{bulb}$	$167.25 \pm 56.11^{c,AB}$	$227.19 \pm 81.36^{b,AB}$	$237.51 \pm 88.57^{ab,AB}$	$267.47 \pm 107.38^{ab,A}$	$271.52 \pm 117.54^{a,A}$
CPE_{bulb}	$141.04 \pm 65.60^{b,BC}$	$260.83 \pm 96.15^{a,A}$	$264.72 \pm 96.04^{a,A}$	$263.07 \pm 125.92^{a,A}$	$230.90 \pm 80.82^{a,A}$

[a] Ctr_{bulb}: uncoated bulbs, SC_{bulb}: sonicated CHI coated bulbs, SCP_{bulb}: sonicated CHI-CP coated bulbs, $SCPE_{bulb}$: sonicated CHI-CP-EUG coated bulbs, CPE_{bulb}: non-sonicated CHI-CP-EUG coated bulbs.
[b] Different lower and capital letter superscripts in the same row and column indicate significant differences ($P < 0.05$), respectively.
[c] Values are presented as mean value ± SD (n = 40).

Table 2.31. AUC of equatorial region of stored bulbs.

Samples	Storage time (days)				
	Day 1	***Day 7***	***Day 14***	***Day 21***	***Day 28***
Ctr_{bulb}	$2.79 \pm 1.75^{c,B}$	$55.85 \pm 11.76^{a,B}$	$50.28 \pm 13.51^{b,B}$	$49.54 \pm 12.80^{b,C}$	$44.80 \pm 15.21^{b,B}$
SC_{bulb}	$8.24 \pm 10.44^{c,A}$	$69.51 \pm 15.84^{a,A}$	$58.36 \pm 14.62^{b,A}$	$67.02 \pm 16.02^{a,B}$	$58.32 \pm 16.29^{b,AB}$
SCP_{bulb}	$8.60 \pm 9.86^{c,A}$	$69.62 \pm 14.90^{a,A}$	$57.50 \pm 10.00^{b,A}$	$64.29 \pm 17.43^{a,B}$	$55.53 \pm 11.47^{b,AB}$
$SCPE_{bulb}$	$7.08 \pm 4.40^{d,A}$	$69.31 \pm 10.97^{b,A}$	$60.41 \pm 16.12^{c,A}$	$78.58 \pm 22.67^{a,A}$	$63.77 \pm 17.42^{bc,A}$
CPE_{bulb}	$8.72 \pm 6.75^{b,A}$	$64.68 \pm 15.11^{a,A}$	$61.70 \pm 9.81^{a,A}$	$61.04 \pm 17.45^{a,B}$	$59.21 \pm 15.83^{a,AB}$

[a] Ctr_{bulb}: uncoated bulbs, SC_{bulb}: sonicated CHI coated bulbs, SCP_{bulb}: sonicated CHI-CP coated bulbs, $SCPE_{bulb}$: sonicated CHI-CP-EUG coated bulbs, CPE_{bulb}: non-sonicated CHI-CP-EUG coated bulbs.
[b] Different lower and capital letter superscripts in the same row and column indicate significant differences ($P < 0.05$), respectively.
[c] Values are presented as mean value ± SD (n = 40).

2.4.12. Antimicrobial Effects of US Treated CHI-CP-EUG Composite Coating on Stored Bulbs

A preliminary experiment was conducted by Lieberman et al. (2015) to evaluate whether inoculated bacteria on the outermost papery onion skin internalized into the papery layers below. Researchers did not find any significant findings. Therefore, bacterial survival was evaluated after inoculation onto only the outer surface of whole onions in the current study. This test was designed to mimic the environmental contamination during distribution, retail and consumer home storage. The results of *L. innocua* and *E. coli* counts at time 0 and after 5 days of room storage for control (uncoated), stand-alone and composite films coated inoculated bulbs were presented in Table 2.32 and Table 2.33, respectively.

L. innocua counts of all coated samples were significantly lower than that of uncoated control during storage ($P < 0.05$). This result has clearly shown the effectiveness of CHI alone as an antimicrobial coating. At day 0, SC_{bulb} and SCP_{bulb} respectively showed 1.2 and 1.8 lower log *L. innocua* counts than the control. A significant increase was monitored in the *L. innocua* loads of the control and SCP_{bulb} during storage while a slight decrease was observed in the *L. innocua* load of SC_{bulb}. On the other hand, SCE_{bulb} and $SCPE_{bulb}$ coated with EUG containing emulsion films had 2 to 3.8 lower log initial (day 0) *L. innocua* counts than the control and other bulbs. Although the significant increase in *L. innocua* loads of SCE_{bulb} and $SCPE_{bulb}$ continued between days 1 and 5, the *L. innocua* counts of $SCPE_{bulb}$ were 0.7 and 0.6 lower log than SCE_{bulb} at days 1 and 5, respectively. $SCPE_{bulb}$ had 1.6 to 4.3 lower log *L. innocua* counts than the control and bulbs coated with EUG free films on day 1 while it had 1.2 to 3.8 on day 5.

There were no significant differences among the initial (day 0) *E. coli* counts of Ctr_{bulb} and SC_{bulb} ($P > 0.05$) while *E. coli* counts of SCP_{bulb}, SCE_{bulb} and $SCPE_{bulb}$ were significantly lower ($P < 0.05$). Especially, SCE_{bulb} and $SCPE_{bulb}$ coated with EUG containing emulsion films had 1.8 to 2.6 lower log initial (day 0) *E. coli* counts than the control and other bulbs. Although the changes in *E. coli* loads of Ctr_{bulb}, SC_{bulb} and SCP_{bulb} were not statistically significant during storage, *E. coli* counts of SCE_{bulb} and $SCPE_{bulb}$ were less than 2 log cfu/g after 1 day incubation. Although the significant increase in *L.*

innocua loads of SCE_{bulb} and $SCPE_{bulb}$ obtained on day 5, the *E. coli* counts of $SCPE_{bulb}$ were 0.7 lower log than SCE_{bulb} on day 5.

Table 2.32. Effect of US treated antimicrobial coatings on *L. innocua* counts of inoculated bulbs stored at ambient temperature.

Samples[a]	***L. innocua* (Log cfu/g)**[b,c,d]		
	Day 0[e]	***Day 1***	***Day 5***
Ctr_{bulb}	$5.82 \pm 0.41^{b,A}$	$7.10 \pm 0.07^{a,A}$	$7.02 \pm 0.30^{a,A}$
SC_{bulb}	$4.65 \pm 0.13^{a,B}$	$4.54 \pm 0.12^{ab,B}$	$4.47 \pm 0.07^{b,C}$
SCP_{bulb}	$4.07 \pm 0.20^{c,C}$	$4.47 \pm 0.05^{b,B}$	$4.99 \pm 0.19^{a,B}$
SCE_{bulb}	$< 2.0^{c,D}$	$3.55 \pm 0.10^{b,C}$	$3.83 \pm 0.18^{a,D}$
$SCPE_{bulb}$	$< 2.0^{c,D}$	$2.84 \pm 0.12^{b,D}$	$3.25 \pm 0.09^{a,E}$

[a] Ctr_{bulb}: uncoated bulbs, SC_{bulb}: sonicated CHI coated bulbs, SCP_{bulb}: sonicated CHI-CP coated bulbs, SCE_{bulb}: sonicated CHI-EUG coated bulbs, $SCPE_{bulb}$: sonicated CHI-CP-EUG coated bulbs.
[b] Different lower and capital letter superscripts in the same row and column indicate significant differences ($P < 0.05$), respectively.
[c] Values are presented as mean ± SD (n = 6).
[d] Initial *L. innocua* load is 8.7 log cfu/mL.
[e] *L. innocua* load of coated bulbs after 15 min.

Table 2.33. Effect of US treated antimicrobial coating on *E. coli* counts of inoculated bulbs stored at ambient temperature.

Samples[a]	***E. coli* (Log cfu/g)**[b,c,d]		
	Day 0[e]	***Day 1***	***Day 5***
Ctr_{bulb}	$4.57 \pm 0.30^{a,A}$	$5.06 \pm 0.36^{ab,A}$	$5.49 \pm 0.76^{a,A}$
SC_{bulb}	$4.40 \pm 0.20^{a,A}$	$4.25 \pm 1.13^{a,A}$	$4.12 \pm 0.80^{a,B}$
SCP_{bulb}	$3.81 \pm 1.00^{a,B}$	$4.21 \pm 1.49^{a,A}$	$4.97 \pm 0.50^{a,A}$
SCE_{bulb}	$< 2.0^{b,C}$	$< 2.0^{b,B}$	$3.37 \pm 0.48^{a,BC}$
$SCPE_{bulb}$	$< 2.0^{b,C}$	$< 2.0^{b,B}$	$2.71 \pm 0.65^{a,C}$

[a] Ctr_{bulb}: uncoated bulbs, SC_{bulb}: sonicated CHI coated bulbs, SCP_{bulb}: sonicated CHI-CP coated bulbs, SCE_{bulb}: sonicated CHI-EUG coated bulbs, $SCPE_{bulb}$: sonicated CHI-CP-EUG coated bulbs.
[b] Different lower and capital letter superscripts in the same row and column indicate significant differences ($P < 0.05$), respectively.
[c] Values are presented as mean ± SD (n = 6).
[d] Initial *E. coli* load is 8.7 log cfu/mL.
[e] *E. coli* load of coated bulbs after 15 min.

As a result, $SCPE_{bulb}$ coated with the developed antimicrobial emulsion film had the lowest *L. innocua* and *E. coli* counts among samples during storage. Since the release tests conducted in the air suggested that EUG was able to retain better in sonicated CHI-CP composite film, the enhanced antibacterial effect was most likely achieved due to the high amount of bonded EUG in the film matrix. The previously reported MIC value of EUG was 1600 ppm or 1.6 mg/mL for both *L. innocua* and *E. coli* in the current work. According to the EUG retention results in Table 2.22, only 1 day of contact time between sonicated CHI-CP-EUG film and suitable foodstuffs seemed to be enough to reach the

MIC of EUG against *L. innocua* and *E. coli*. However, overall results produced lower *E. coli* counts than *L. innocua* counts. This susceptibility difference was probably related to the strong electrostatic affinity of the positively charged particles of CHI-CP-EUG film towards the negatively charged outer membrane of *E. coli* thereby increasing the rate of particle attachment onto the cell surface. Antimicrobial test results were in good agreement with MIC measurements where *Listeria* was found to be more resistant to EUG than *E. coli*. EUG was more effective against *E. coli* probably due to the composition of the outer membrane of bacteria. Gram-negative bacteria have an outer membrane containing LPS, lipids and surface proteins or peripheral proteins. According to Gaysinsky et al. (2007), EUG can more easily interact with and solubilize into LPS, thereby delivering EUG to the cell because gram-negative bacteria such as *E. coli* have a thin peptidoglycan layer. The lack of an LPS layer in gram-positive bacteria such as *Listeria monocytogenes* decreases the affinity of EUG for the bacterial interface. Furthermore, polycationic CHI structure can easily interact with the predominantly anionic components such as LPS of gram-negative bacteria (Helander et al. 2001). Previous studies verified that the greater resistance of gram-positive bacteria might be due to their thick layer of peptidoglycan cell wall (Borges et al. 2013; Lopez-Romero et al. 2015).

2.4.13. Antisprouting Effect of US Treated CHI-CP-EUG Composite Coating on Stored Bulbs

Apart from microbiological contamination and decay, sprout growth is another problem leading to the deterioration of stored bulbs. Evaluating the physicochemical quality parameters requires an in-depth understanding of bulb physiology. Physiological process of freshly harvested onion bulbs can be divided into 3 periods: rest, dormancy, regrowth (sprouting). Many studies have revealed the main roles of several plant hormones in regulation of physiological stages. For example; gibberellin, cytokinin and auxin are growth promoters/regulators whereas abscisic acid is an inhibitor. Ethylene can sometimes be a promoter or an inhibitor. Immediately after harvest, the activity of inhibitors is high while the activity of growth promoters is very low during rest period, so bulbs do not show any growth activity. Dormancy is a physiological state during which

sprouting does not begin even under optimal sprouting conditions. Although no external morphological changes can be detected in dormant bulbs, dormancy release is a complex process involving several physiological and biochemical changes. Carbohydrate metabolism, hormonal regulation, reactive oxygen species (ROS) produced by like NADPH oxidase (localized at the plasma membrane) and antioxidant metabolism have been reported to control of dormancy (Bailly et al. 2008; Foreman et al. 2003; Liu et al. 2018). For example; growth promoters accumulate gradually as time progresses in storage. ROS such as superoxide anions, hydrogen peroxide, hydroxyl radicals are generated inevitable by-products during metabolic reactions in plant tissue and they can be signaling molecules that associates with plant hormones for dormancy release and germination (Bailly, El-Maarouf-Bouteau, and Corbineau 2008; Leymarie et al. 2012; Ishibashi et al. 2015). Germination usually starts when the level of ROS reaches a certain level. ROS level is controlled by the concentration and activity of major plant ROS-scavenging antioxidant enzymes such as superoxide dismutase, ascorbate peroxidase, glutathione peroxidase and catalase. The metabolic activity in onion also depends on the source-to-sink transition; the source (scale leaves) is where the carbohydrates are produced and the sink (stem or base plate) is where they are consumed during sprouting (Sharma et al. 2016). When dormancy release, apical (terminal) buds start to grow with the transer of water and metabolites from scales to sink due to the increase in cell division rate. Sprouting is recorded when the sprout leaves extend beyond the neck of the bulb. If dormancy can be prolonged, bulb storage life would be extended.

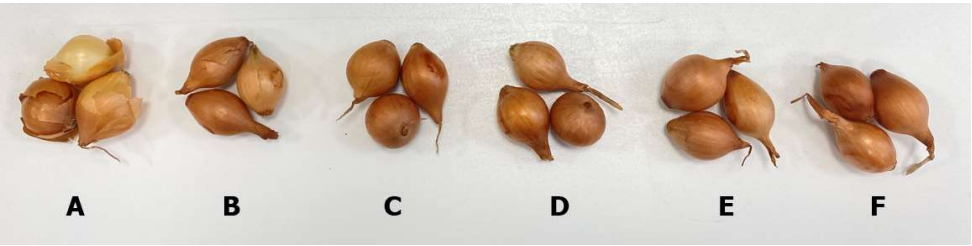

Figure 2.14. The views of the outer skins of uncoated and coated bulbs after 7 days of room storage (A: Ctr_{bulb}; B: SC_{bulb}; C: SCP_{bulb}; D: SCE_{bulb}; E: $SCPE_{bulb}$; F: CPE_{bulb}).

The views of the outer skins of uncoated and coated bulbs were given in Figure 2.14. Uncoated bulbs showed poor skin intactness over time due to the falling off dry scales. Coating application improved the intactness of the outer skin of bulbs by adhering

to the outermost scales after 7 days of room storage. This is an important finding because intact outer skin maintains an internal atmosphere within the bulb. Apeland (1969) informed that the respiration rate doubles and also water loss of bulbs increase when the outer skins of onions are removed. Bulbs with the skin removed also sprout more rapidly than those with intact skin (Tanaka, Yoshikawa, and Komochi 1985). Intact outer skins can be considered as a strong barrier to gas diffusion between the bulb interior and the external atmosphere resulting in low O_2 concentration and the corresponding decrease in the ratio of O_2:CO_2 within stored bulbs. Bulb sprouting delays with an elevation of internal CO_2 and a reduction of internal O_2. For example; Ladeinde and Hicks (1988) reported that paraffin waxing of root plate increased internal CO_2 and decreased internal O_2 in onion bulbs.

The number of sprouts growing out of bulbs in different batches from each day was noted in Table 2.34. Sprout growth started after 7 days in uncoated bulbs and after 14 days in coated bulbs. It should be emphasized that the number of sprouts in $SCPE_{bulb}$ was counted lesser than others during storage.

Table 2.34. Number of sprouts growing out of ten bulbs per week[b].

Samples[a]	**Storage time (days)**					
	0	***1***	***7***	***14***	***21***	***28***
Ctr_{bulb}	0/10	0/10	2/10	4/10	4/10	3/10
SC_{bulb}	0/10	0/10	0/10	4/10	4/10	4/10
SCP_{bulb}	0/10	0/10	0/10	3/10	3/10	2/10
$SCPE_{bulb}$	**0/10**	**0/10**	**0/10**	**1/10**	**1/10**	**1/10**
CPE_{bulb}	0/10	0/10	0/10	2/10	4/10	2/10

[a] Ctr_{bulb}: uncoated bulbs, SC_{bulb}: sonicated CHI coated bulbs, SCP_{bulb}: sonicated CHI-CP coated bulbs, $SCPE_{bulb}$: sonicated CHI-CP-EUG coated bulbs, CPE_{bulb}: non-sonicated CHI-CP-EUG coated bulbs.
[b] Different batches of bulbs were visually tested each day.

Greening is due to chlorophyll accumulation in chloroplasts. On exposure to light, green pigmentation occurs by the conversion of amyloplasts into chloroplasts and chlorophyll is accumulated. Poovaiah et al. (1972) found a positive relationship between chlorophyll development and high peroxidase activity in onion bulbs kept at ambient temperature because the catalytic activity of peroxidase on auxin and cytokinin shortens the dormancy period and stimulates sprouting. The greenness intensities of sprouted parts of stored bulbs were scored in Tables 2.35 – 2.39 to determine the sprouting status.

Table 2.35. The green colour intensity of sprouted parts of stored bulbs for 1 day[b].

Samples[a]	1	2	3	4	average
Ctr_{bulb}	-	7	3	-	2.3
SC_{bulb}	-	6	4	-	2.4
SCP_{bulb}	-	7	3	-	2.3
$SCPE_{bulb}$	-	9	1	-	2.1
CPE_{bulb}	-	8	2	-	2.2

[a] Ctr_{bulb}: uncoated bulbs, SC_{bulb}: sonicated CHI coated bulbs, SCP_{bulb}: sonicated CHI-CP coated bulbs, $SCPE_{bulb}$: sonicated CHI-CP-EUG coated bulbs, CPE_{bulb}: non-sonicated CHI-CP-EUG coated bulbs.
[b] Different batches of bulbs were visually tested each day.

Table 2.36. The green colour intensity of sprouted parts of stored bulbs for 7 days[b].

Samples[a]	1	2	3	4	average
Ctr_{bulb}	-	3	6	1	2.8
SC_{bulb}	-	3	6	1	2.8
SCP_{bulb}	-	2	7	1	2.9
$SCPE_{bulb}$	-	9	1	-	2.1
CPE_{bulb}	-	7	3	-	2.3

[a] Ctr_{bulb}: uncoated bulbs, SC_{bulb}: sonicated CHI coated bulbs, SCP_{bulb}: sonicated CHI-CP coated bulbs, $SCPE_{bulb}$: sonicated CHI-CP-EUG coated bulbs, CPE_{bulb}: non-sonicated CHI-CP-EUG coated bulbs.
[b] Different batches of bulbs were visually tested each day.

Table 2.37. The green colour intensity of sprouted parts of stored bulbs for 14 days[b].

Samples[a]	1	2	3	4	average
Ctr_{bulb}	-	-	5	5	3.5
SC_{bulb}	-	-	5	5	3.5
SCP_{bulb}	-	1	7	2	3.1
$SCPE_{bulb}$	-	5	5	-	2.5
CPE_{bulb}	-	3	5	2	2.9

[a] Ctr_{bulb}: uncoated bulbs, SC_{bulb}: sonicated CHI coated bulbs, SCP_{bulb}: sonicated CHI-CP coated bulbs, $SCPE_{bulb}$: sonicated CHI-CP-EUG coated bulbs, CPE_{bulb}: non-sonicated CHI-CP-EUG coated bulbs.
[b] Different batches of bulbs were visually tested each day.

Table 2.38. The green colour intensity of sprouted parts of stored bulbs for 21 days[b].

Samples[a]	1	2	3	4	average
Ctr_{bulb}	-	-	4	6	3.6
SC_{bulb}	-	-	3	7	3.7
SCP_{bulb}	-	2	3	5	3.3
$SCPE_{bulb}$	3	2	5	-	2.2
CPE_{bulb}	-	2	3	5	3.3

[a] Ctr_{bulb}: uncoated bulbs, SC_{bulb}: sonicated CHI coated bulbs, SCP_{bulb}: sonicated CHI-CP coated bulbs, $SCPE_{bulb}$: sonicated CHI-CP-EUG coated bulbs, CPE_{bulb}: non-sonicated CHI-CP-EUG coated bulbs.
[b] Different batches of bulbs were visually tested each day.

Table 2.39. The green colour intensity of sprouted parts of stored bulbs for 28 days[b].

Samples[a]	1	2	3	4	average
Ctr_{bulb}	-	-	4	6	3.6
SC_{bulb}	-	-	2	8	3.8
SCP_{bulb}	-	-	8	2	3.2
$SCPE_{bulb}$	1	4	4	1	2.5
CPE_{bulb}	-	-	5	5	3.5

[a] Ctr_{bulb}: uncoated bulbs, SC_{bulb}: sonicated CHI coated bulbs, SCP_{bulb}: sonicated CHI-CP coated bulbs, $SCPE_{bulb}$: sonicated CHI-CP-EUG coated bulbs, CPE_{bulb}: non-sonicated CHI-CP-EUG coated bulbs.
[b] Different batches of bulbs were visually tested each day.

In addition, the photographs of the bulbs were presented from Figures C4 – C8 in Appx. C. Although all bulbs had slight internal sprouts even on the first day of the experiment, the increasing trend in the greenness intensity of sprouts could be clearly observed in the following storage days. The greenness intensities of bulbs did not show much difference on days 1 and 7, but $SCPE_{bulb}$ differentiated from others from day 14 to the end of storage. Also, $SCPE_{bulb}$ was rated with the lowest average greenness intensity during the experiment. Taking sprout assessments into account, it can be suggested that active coating can retard sprout growth.

2.4.14. Sensory Evaluation of US Treated CHI-CP-EUG Composite Coating on Stored Bulbs

There are 2 techniques for sensory evaluation; quantitative measurements which electronic sensing device is only used or the nose is used with some device and parametric measurements in which the nose is used without any device (Brattoli et al. 2011). Also, parametric evaluation can be done by analytical tests which are used to detect differences or affective tests which are used to detect individual preferences. Due to its simplicity, the paired comparison was used as a sensory discrimination test (both parametric and analytical test). The proportions of "yes" and "no" responses obtained for each targeted attribute between $SCPE_{bulb}$ and control bulbs (SCP_{bulb} and Ctr_{bulb}) were presented in Figures 2.15 – 2.18. At day 0, there was a considerable difference in terms of appearance between $SCPE_{bulb}$ and uncoated Ctr_{bulb} whereas there was a partial difference between $SCPE_{bulb}$ and SCP_{bulb}. However, after day 0, the proportions of "no" responses for appearance difference tended to be higher than those of "yes" responses. At the end of the storage (on day 28), no differences in appearance were detected between bulbs.

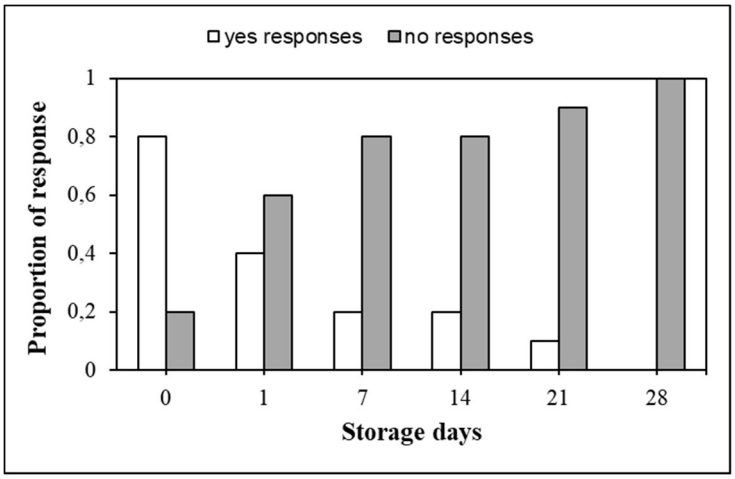

Figure 2.15. Appearance difference between $SCPE_{bulb}$ and Ctr_{bulb}.

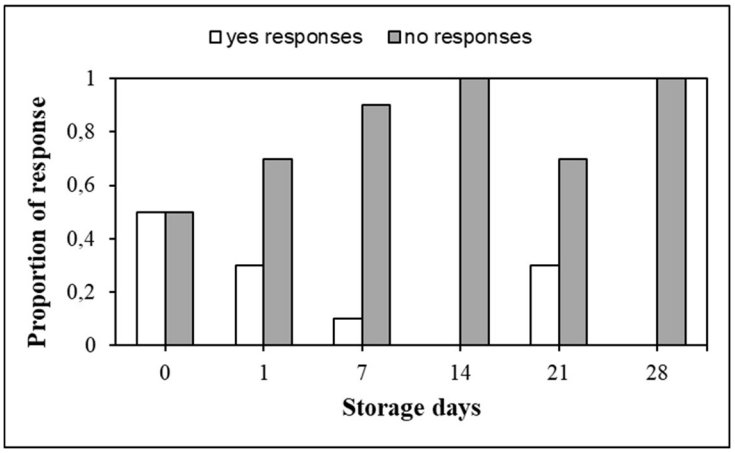

Figure 2.16. Appearance difference between $SCPE_{bulb}$ and SCP_{bulb}.

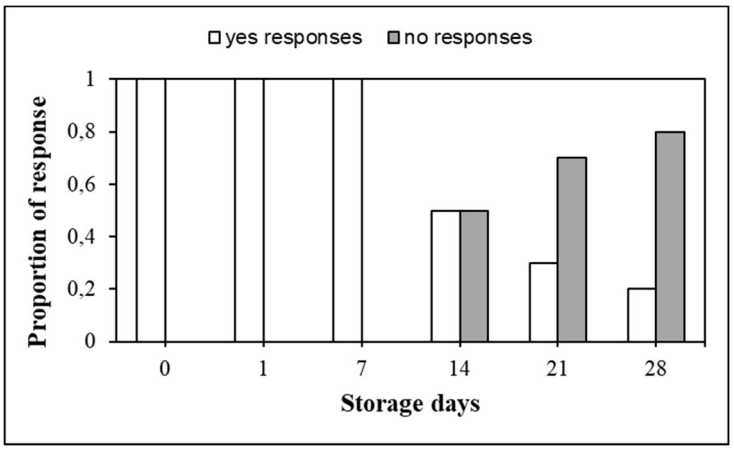

Figure 2.17. Odour difference between $SCPE_{bulb}$ and Ctr_{bulb}.

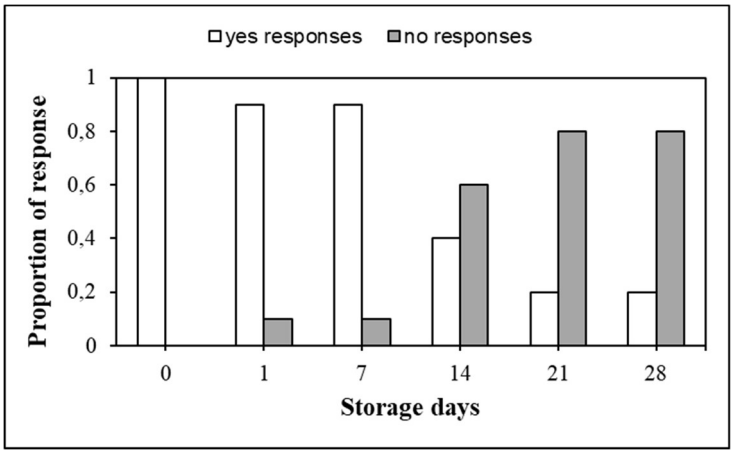

Figure 2.18. Odour difference between $SCPE_{bulb}$ and SCP_{bulb}.

Up to day 14, there was a considerable difference in terms of odour between $SCPE_{bulb}$ and SCP_{bulb} or Ctr_{bulb}. However, after day 14, the proportions of "no" responses for odour difference tended to be higher than those of "yes" responses. At the end of the storage (on day 28), little difference in odour could still be detected between bulbs.

2.5. Conclusion

The current thesis clearly showed the possibility of replacing synthetic antisprouting agents with an edible coating loaded with a naturally existing generally recognized as safe essential oil component. The developed chitosan-chickpea protein active film showed superior mechanical and moisture barrier properties and sustained eugenol release performance as well as antimicrobial and antisprouting activity than classical eugenol loaded chitosan film. Ultrasonication was the key process essential to obtain the chitosan-chickpea protein composite films since this process effectively increased protein solubility, encapsulated eugenol and homogeneity, and reduced particle size in film forming solution. To our knowledge, the chitosan-chickpea protein composite coating loaded with eugenol is the first natural active edible coating in the literature employed successfully to control both contamination and sprouting of shallot bulbs. There is a good potential to adopt developed coatings for alternative onion cultivars. Finally, the use of sustainable proteins of chickpea, a drought resistant pulse grown in semi-arid climates, as part of chitosan composite forms an alternative to chitosan composites obtained with animal proteins.

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APPENDICES

APPENDIX A. The Standard Curves Used for The Spectrophotometric Methods

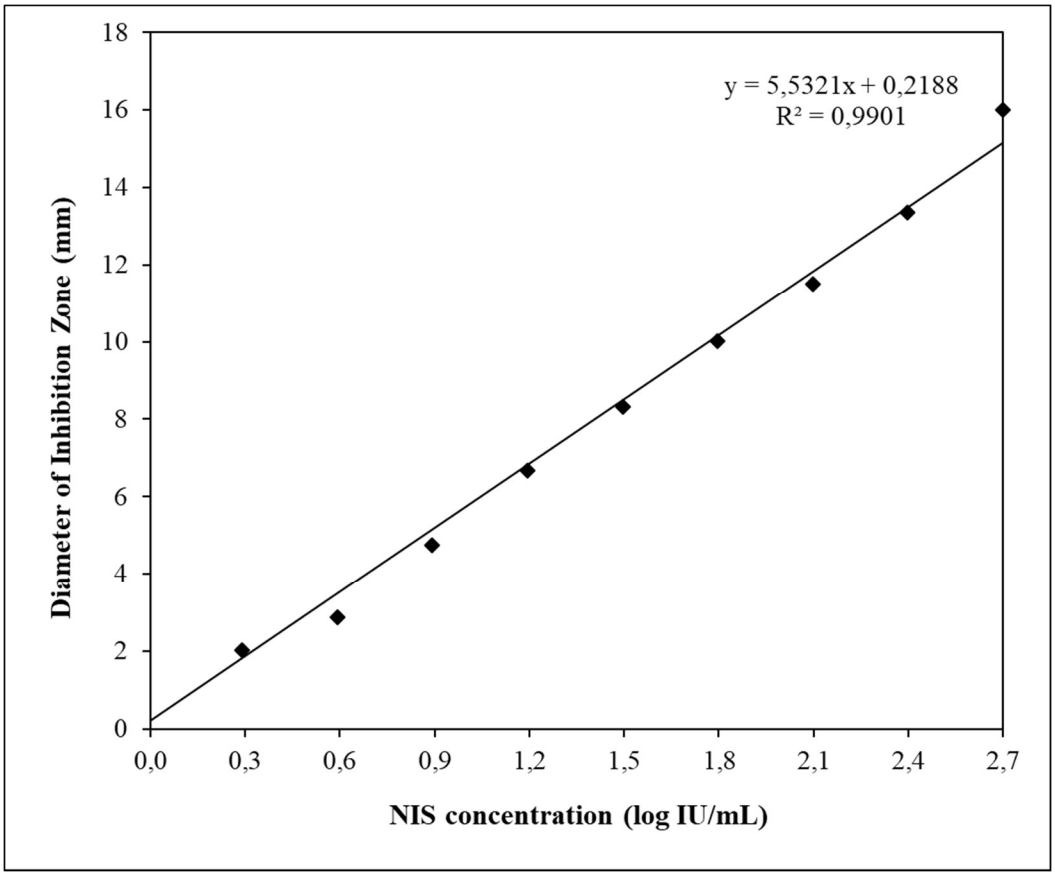

Figure A1. The standard curve prepared with nisin (NIS) for release tests. y = 5.532 x + 0.218 where y is diameter of inhibition zone in mm and x is the concentration of NIS in log IU/mL, respectively.

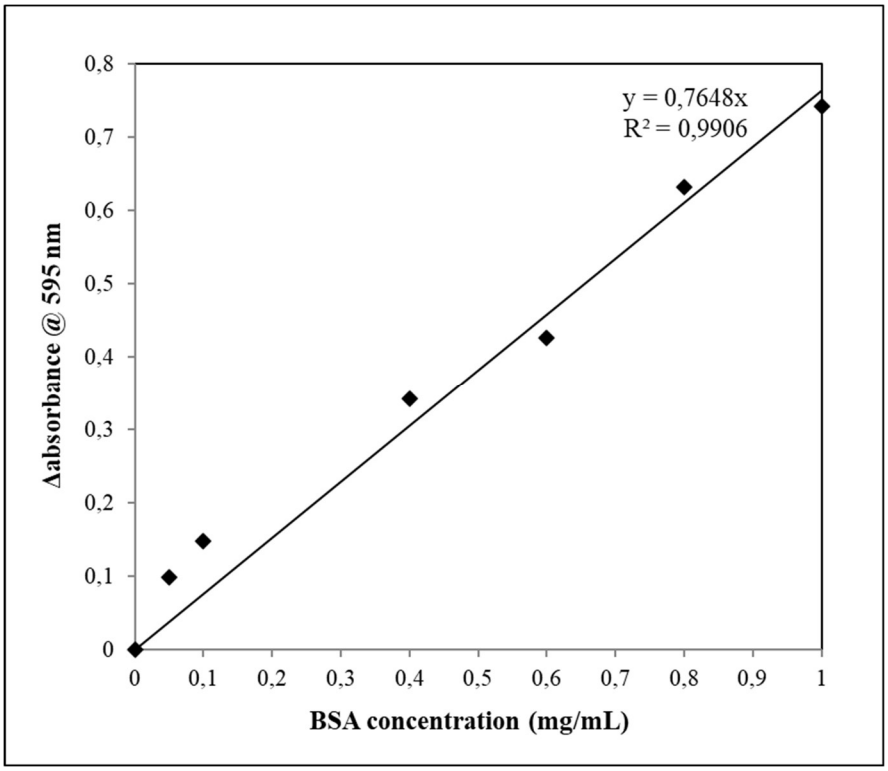

Figure A2. The standard curve prepared with bovine serum albumin (BSA) for soluble protein content measurement.

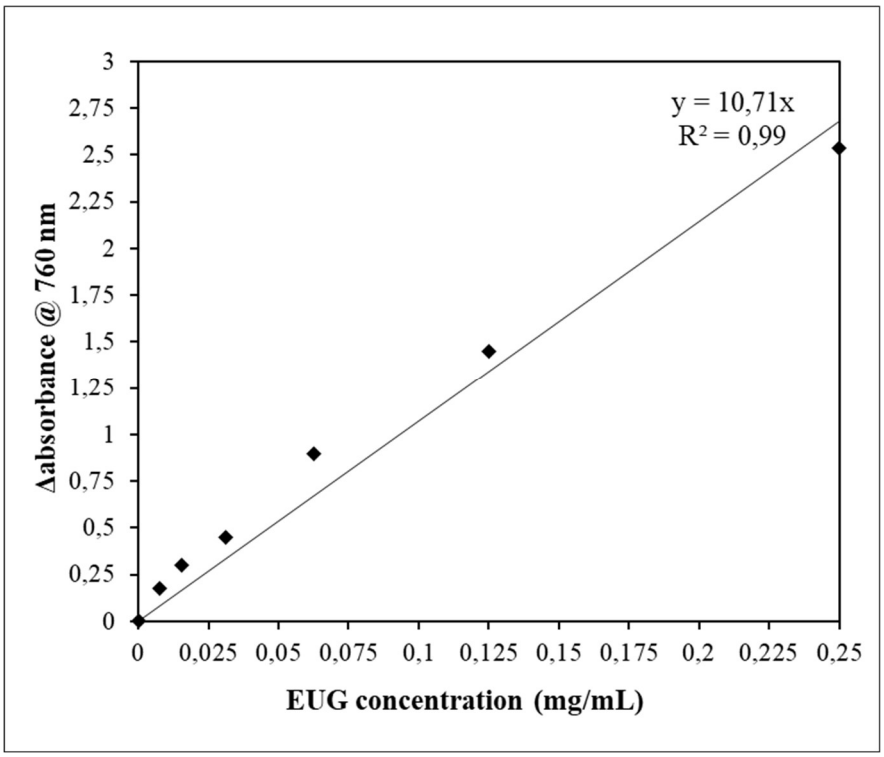

Figure A3. The standard curve prepared with eugenol (EUG) for release tests performed in model solutions and air at room temperature.

APPENDIX B. The Charts Used for the Analyses

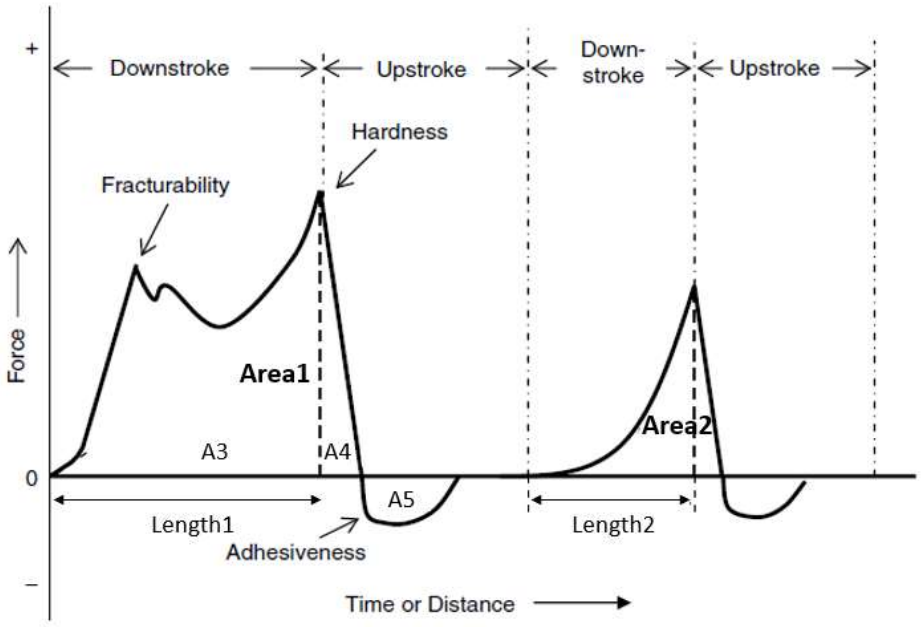

Figure B1. Typical force – time curve of TPA: maximum force of the 1st compression = hardness, force at the 1st peak = fracturability, cohesiveness = Area2/Area1, springiness = Length2/Length1, gumminess = hardness × cohesiveness, chewiness = gumminess × springiness, resilience = Area4/Area3, adhesiveness = Area5.

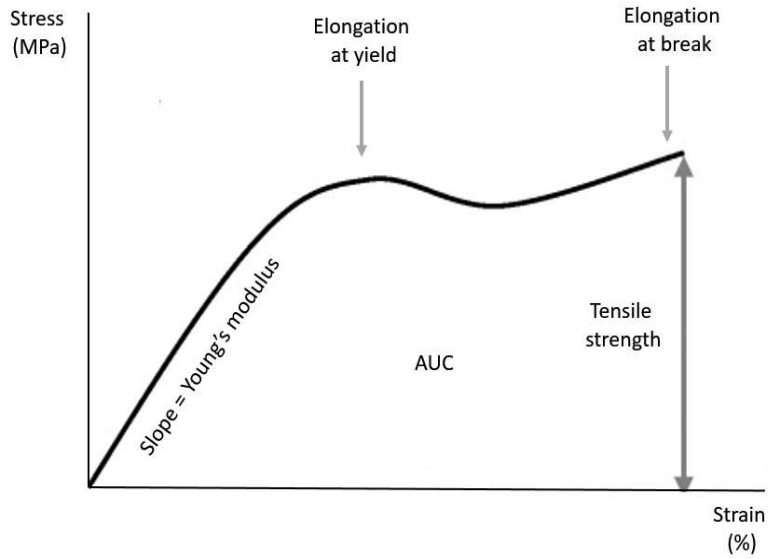

Figure B2. Typical stress – strain curve of tensile test.

Name:
Date:

You are presented with two coded sample. Please examine the appearance of samples in the order given. Can you detect a difference between the samples?

Yes No

Figure B3. Sample evaluation card for paired comparison test (simple difference test for general appearance).

Name:
Date:

You are presented with two coded sample. Please smell the samples in the order given. Can you detect a difference between the samples?

Yes No

Figure B4. Sample evaluation card for paired comparison test (simple difference test for odour).

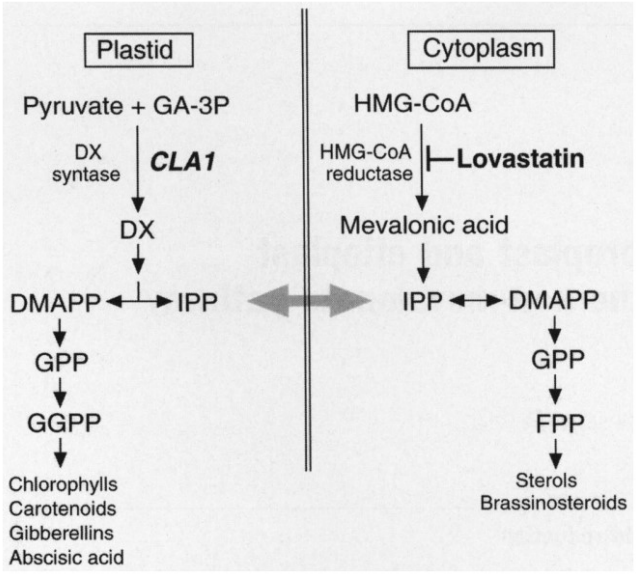

Figure B5. Two pathways for isoprenoid biosynthesis in higher plants (Source: Nagata et al. 2002; copyright © 2002 with permission from Springer Nature through Copyright Clearance Center).

Temperature (°C)	Relative humidity (%)	Length of storage
-3-0	70-75	6 months
-3	85-90	5-7 months
-2	75-85	300 days
(-2) - (-0.6)	75-80	6 months&
-1-0	70-80	6-8 months
-0.6	78-81	6-7 months
0	75-85	6 months
0	65-75	-
0	70-75	20-24 weeks*
0	70-75	-
0	65-70	1-2 months#
0	65-70	6-8 months†
0	-	230 days
0	70-75 or 90-95	up to 120 days
0	80-85	30-35 weeks§
1-2	80-85	30-35 weeks¥
1	87	-
1.1	70-75	16-20 weeks‡
4	-	170 days
8	-	120 days
12	-	about 90 days
20	-	25 days

*= With 16.3% loss (red onion); #= Bermuda cultivar; †= Globe cultivar; ‡= With 14.2% loss (red onion); &= Superba cultivar; §= Optimum storage conditions, 7% maximum water loss before becoming unsaleable; ¥= Probable practical storage conditions, 7-10 days shelf-life (approx.) at 20°C after storage, 7% maximum water loss before becoming unsaleable. Compiled from (Thompson, 1996; Thompson, 1982).

Figure B6. Recommended storage conditions for onion bulbs (Source: FAO, 2003).

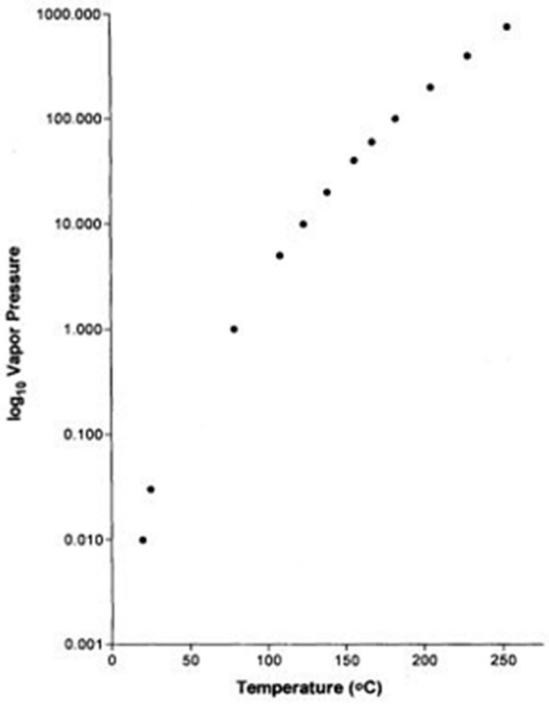

Figure B7. Vapour pressure of eugenol plotted on a logarithmic scale (Source: Yuwono et al. 2002; copyright © 2002 with permission from Elsevier through Copyright Clearance Center).

APPENDIX C. The Photographs of Fresh Produces

Figure C1. Images of GEL filled control apples showing some cracking during handling and cold storage.

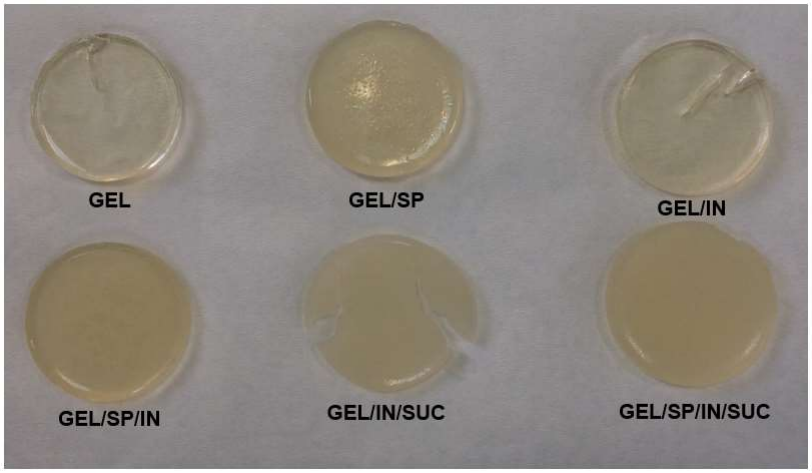

Figure C2. Gel-fillings water immersion at day 7.

Figure C3. Gel filled caramel apple production.

Figure C4. Cross-section photos of stored bulbs for 1 day at ambient temperature.

Figure C5. Cross-section photos of stored bulbs for 7 days at ambient temperature.

Figure C6. Cross-section photos of stored bulbs for 14 days at ambient temperature.

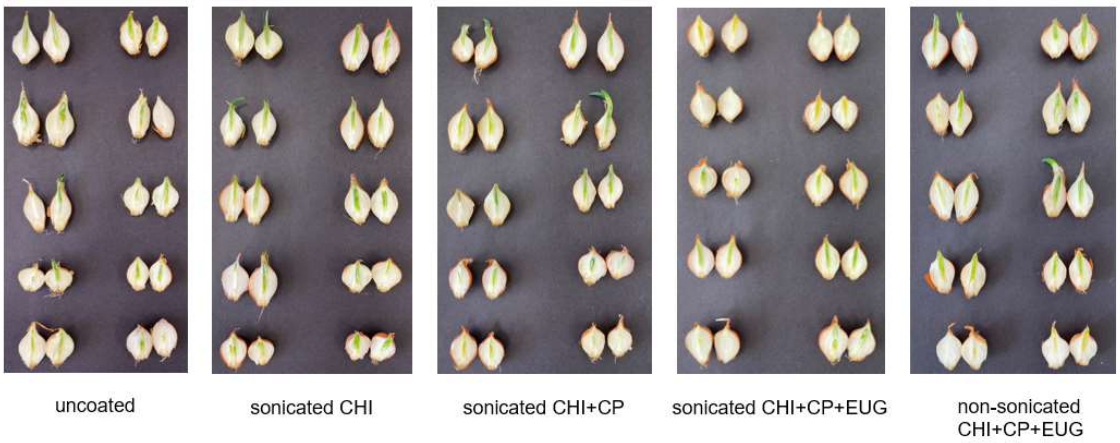

Figure C7. Cross-section photos of stored bulbs for 21 days at ambient temperature.

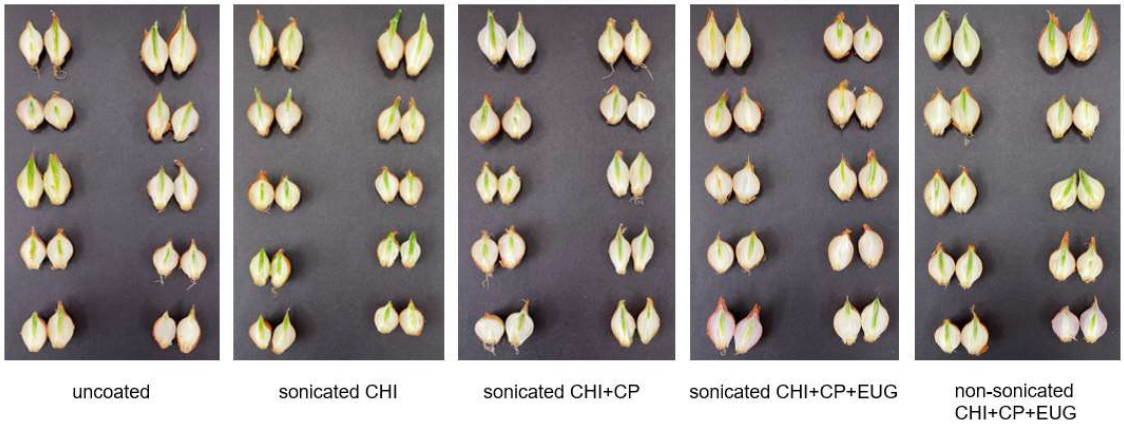

Figure C8. Cross-section photos of stored bulbs for 28 days at ambient temperature.

APPENDIX D. A Short Study Showing The Retarding Effect of Eugenol on Seed Germination and Bulb Sprouting

All seeds were stored in the dark at ambient temperature in the laboratory and protected from humidity before use. Broken and damaged seeds were eliminated by visual examination, intact and healthy seeds with uniform size were hand sorted. Prior to coating, seeds were washed with %1 sodium hypochlorite (NaClO) solution for 15 min with the seed weight (g) / solution volume (mL) ratio 1:5 (w/v) and rinsed. After that, they were dried in a fume hood at ambient temperature. Film solutions with or without EUG were prepared as described in section 2.3.2.2. Seeds were immersed into flasks containing the film solutions and the contents were stirred with a rod to distribute the solution evenly. The coated seeds were then dried in sterile Petri dishes kept at ambient temperature for 3 h under aseptic conditions. The germination study were designed according to the method given by Sozbilen and Yemenicioğlu (2020). Briefly, 20 portions of coated or uncoated seeds were placed onto moistened cotton placed into Petri dishes. The Petri dishes were then incubated in an environmental chamber at 22ºC for 5 days in the dark for the germination of seeds. Seeds were moistened daily with 20 mL water. A seed was considered germinated when its radicle was 2 mm long. The length of radicle was manually measured using a digital caliper on day 5.

Fyfield and Gregory (1989) informed that radicle length can be considered as a growth parameter for seedling growth. Thus, the performances of the prepared emulsion coatings were tested in a seed germination study. Unsurprisingly, radicle lengths of sonicated or non-sonicated CHI-CP-EUG coated seeds were significantly shorter than that of uncoated seeds (see Table D1). It is most probably due to the fact that film structure and eugenol adversely affected seedling growth. Similarly, Hu et al. (2017) concluded that eugenol treatment lowered the germination speed by reducing rice's sprouting rate and index. Its inhibitory effect had been enhanced with increasing eugenol concentration. In germination, α-amylase activity plays a vital role in hydrolyzing endosperm starch to soluble sugar forms as the principal energy for sprouting. Higher amylase activity contributes to faster germination process. Researchers found that eugenol postponed the synthesis of α-amylase, so low amylase activity delayed the germination speed.

Table D1. Radicle lengths (cm).

Samples[a]	Green Lentil	Mung bean
Ctr_{seed}	3.7 ± 1.0	3.5 ± 1.8
SC_{seed}	3.8 ± 2.6	2.9 ± 1.5
SCP_{seed}	2.6 ± 2.2	2.5 ± 1.6
$SCPE_{seed}$	0.7 ± 0.2	1.2 ± 0.7
CPE_{seed}	2.8 ± 1.7	1.8 ± 1.1

[a] Ctr_{seed}: uncoated seeds; SC_{bulb}: sonicated CHI coated seeds; SCP_{bulb}: sonicated CHI-CP coated seeds; $SCPE_{bulb}$: sonicated CHI-CP-EUG coated seeds; CPE_{bulb}: non-sonicated CHI-CP-EUG coated seeds.

The sprouting study was designed according to the applied in Chapter 2. Coating solutions with or without eugenol were prepared as described previously in section 2.3.2.2. Bulbs were dipped into flasks containing the film solutions (5 bulbs immersed in 50 mL FFSs), and the contents were stirred with a plastic rod for 3 min to distribute the solution evenly. Five uncoated and coated bulbs were placed on styrofoam plates. Afterward, they were dried overnight in a fume hood kept at ambient temperature. After 15 days, coated bulbs were halved and the sprouting status was visually tested. As seen in Figure D1, a considerable sprouting delay was observed in bulbs coated with sonicated CHI-CP-EUG coating. It has been thought that CHI-CP active coating loaded with EUG is able to find a successful application in root crops whose sprouting is undesirable.

Figure D1. Sprouting status of coated bulbs.

VITA

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EDUCATION

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Thesis: Innovative food applications of novel multifunctional active edible gel fillings and coatings.
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2010 – 2012 **M.Sc. in Food Engineering,** Ege University, İzmir, Turkey.
Thesis: Determination of the quality changes during storage of turkey rolls containing different proportions of PSE (pale, soft, exudative) like turkey meat.
Advisor: Prof. Dr. Meltem SERDAROĞLU

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