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Development and Characterization of Antimicrobial Food Coatings Based on Chitosan and Essential Oils

Shuang Chi
University of Tennessee - Knoxville

To the Graduate Council:

I am submitting herewith a thesis written by Shuang Chi entitled "Development and Characterization of Antimicrobial Food Coatings Based on Chitosan and Essential Oils." I have examined the final electronic copy of this thesis for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Master of Science, with a major in Food Science and Technology.

Svetlana Zivanovic, Major Professor

We have read this thesis and recommend its acceptance:

Frances Ann Draughon, Jochen Weiss

Accepted for the Council:
Carolyn R. Hodges

Vice Provost and Dean of the Graduate School

(Original signatures are on file with official student records.)

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Vice chancellor and
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Development and characterization of antimicrobial food coatings based on chitosan and essential oils

A Thesis

Presented for the

Masters of Science Degree

The University of Tennessee, Knoxville

SHUANG CHI

August, 2004

DEDICATION

This thesis is dedicated to my parents

Mr. Pizhen Chi

And

Mrs Wei Jiang

who have given me invaluable educational opportunities.

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Abstract

The objectives of this research were (1) to evaluate antimicrobial effects of essential oils incorporated in chitosan edible films against *Listeria monocytogenes* and *Escherichia coli* O157:H7, (2) to evaluate effectiveness of the films on a meat product, (3) to characterize physical properties of chitosan films enriched with essential oils, and (4) to determine whether the addition of oregano essential oil to bologna can influence consumer liking of the product.

Anise, basil, coriander, and oregano essential oils were tested for antimicrobial activity by paper disc diffusion test with concentration of 1 to 24 μl/disc. Chitosan films incorporated with those essential oils with concentration of 1 to 3 % (3 to 9 μl/disc) were tested for antimicrobial property by film disc diffusion test. Oregano essential oil – chitosan films were applied on thick bologna slices to determine antimicrobial efficacy on a real food product. Solvent extraction and GC/MS analysis were performed to identify and quantify the dominant compounds in oregano essential oil incorporated into the chitosan films before and after application on bologna. Physico–chemical properties of oregano essential oil enriched chitosan films were characterized for thickness, water vapor permeability (WVP), puncture strength, tensile strength, and elongation. The influence of additional oregano EO (45 ppm and 90 ppm) to bologna slices on consumer liking was tested with computerized hedonic scales and just about right scale.

Results showed that oregano essential oil exhibited the strongest antimicrobial activity towards *L. monocytogenes* and *E. coli* O157:H7 compared to other tested essential oils (oregano >> coriander > basil > anise). Although both tested pathogens were affected, *Listeria monocytogenes* appeared to be more sensitive to oregano essential oil than *Escherichia coli* O157:H7. Application of pure chitosan films reduced pathogen counts on meat product (bologna) from 1 to 3 logs and chitosan films enriched with 1 and 2 % oregano essential oil were sufficient for 4 logs reduction of *L. monocytogenes* and *E. coli* O157:H7 on bologna slices stored at 10 °C for 5 days. Results of physico–chemical tests showed that addition of oregano essential oil to chitosan films resulted in increased thickness, higher elasticity, reduced puncture and tensile strength, and lower WVP.

Application of the films on bologna resulted in increased thickness, higher WVP, higher elasticity, and reduced puncture and tensile strength of the films. Bologna slices covered with chitosan films with 1 % oregano essential oil and stored 5 days at 10 ^{o}C absorbed 60 ppm of the EO. Addition of 45 ppm oregano EO did not affect the consuming liking of the product compared to control.

Keywords: chitosan, edible film, essential oil, anti-microbial properties

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List of Symbols

g	gram
h	hour
kg	kilogram
L	liter
m	meter
cm	centimeter
mm	millimeter
mg	milligram
mL	milliliter
µL	microliter
kDa	kilo-Dalton
psi	pond per square inch
d	day
rpm	revolutions per minute
CFU	colony forming units
N	Newton
ppm	parts per million

Abbreviations

EO	essential oil
OEO	oregano essential oil
DA	degree of acetylation
DDA	degree of deacetylation
FFS	film forming solution
MW	molecular weight
GC	gas chromatography
MS	mass-spectrometry
HPLC	high performance liquid chromatography
SPME	solid-phase microextraction
WVP	water vapor permeability
GP	gas permeability
ATCC	American type culture collection
TS	tensile strength
NIST	National Institute of Standards and Technology

Part 1

Introduction

Microbial pathogens remain the serious problem causing foodborne illness in the United States. Recent outbreaks associated with Escherichia coli O157:H7, Listeria monocytogenes, and Salmonella sp. have raised attention and requests for safe, nonspecific, and highly effective antimicrobial agents that are applicable in broad variety of food products. Food industry has invested significant resources to find ways to inhibit bacterial growth, extend product shelf life, and ensure food safety. To achieve this goal, naturally occurring substances with antibacterial activity are becoming more desirable to the food industry as well to the consumers. The most common natural compounds investigated for their antimicrobial efficacy include bacteriocins, such as nisin, natamycin, and pediocin, enzyme lysozyme, protein lactoferrin, polysaccharide chitosan, and essential oils and their constituents – phenols (eugenol, carvacrol, thymol), terpenes (carvone, camphor, pinene), and acids (cinnamic rosemarinic, carnosic, ursolic).

Antimicrobial effects of chitosan have already been utilized in medicine, pharmacy, dentistry, and agriculture. Free amino groups on chitosan molecules are protonated at pH bellow 6, what enables interaction of the biopolymer with negatively charged molecules on microbial surface. The interactions result in cell wall rupture and membrane permeation and lead to microbicidal effect. Furthermore, chitosan is a good film-forming, thickening, and gelling agent what provides additional advantages for its use. Chitosan has been commercialized as a dietary supplement for weight loss due to its lipid-binding properties and as flavor, color and drug carrier. Being recognized as an edible material, chitosan gained interest for application as an antimicrobial coating for fresh and processed foods.

Essential oils have been used for centuries as food additives, fragrance, but also in medical therapy. Their antimicrobial effects have recently been re-evaluated and confirmed. Several mechanisms of antimicrobial activity of essential oils have been proposed. They include (a) the interference of active compounds of essential oils with the phospholipids bilayer of the cell membrane causing increased permeability and loss of cellular constituents; (b) interruption of various enzyme systems, including those involved in the production of cellular energy and synthesis of structural components; and (3) damaging of genetic material. However, the highly volatile nature of these

compounds usually limits their applications in foods and cause reduction of antimicrobial effects over time.

Combination of two naturally occurring antimicrobial components, chitosan and essential oils, may provide a unique system with enhanced antimicrobial properties. Incorporation of essential oils into the chitosan films could reduce losses of active components due to evaporation and establish possibilities for prolonged antimicrobial action and improved safety of foods.

The objectives of this research were (1) to evaluate antimicrobial effects of essential oils incorporated in chitosan edible films against *L. monocytogenes* and *E. coli* O157:H7, (2) to evaluate effectiveness of the antimicrobial films on a meat product, (3) to characterize physical properties of chitosan films enriched with essential oils, and (4) to determine whether the addition of oregano essential oil on bologna can influence consumer liking of the product.

Part 2

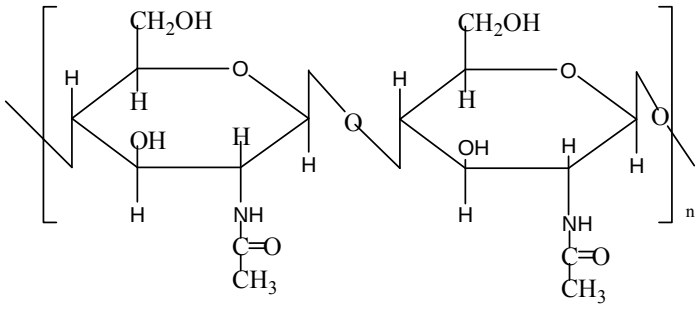
Literature Review

Chitin and Chitosan

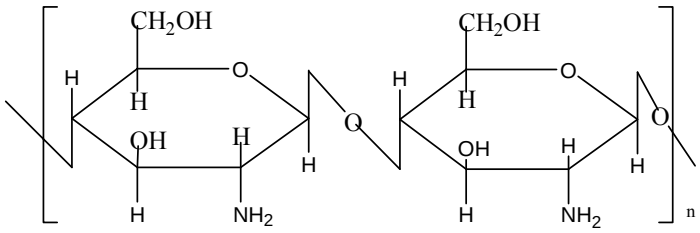
Chitin is the major structural component of the exoskeleton of invertebrates, cuticles of insects, and the cell walls of fungi. It is considered to be the second most abundant biopolymer on earth after cellulose. Chitin is a linear, highly crystalline polysaccharide composed of acetylglucosamine units linked by β-1,4 glycosidic bonds (Figure 1A). Chitosan is a polycationic polysaccharide obtained by deacetylation of chitin (Figure 1B).The degree of deacetylation (DDA) in chitin usually ranges from 5 to 15 % and in chitosan from 70% to 95%. High degree of acetylation (DA) and crystallinity of chitin molecules make them insoluble in common solvents. Reduced acetylation level in chitosan results in free amino groups, which become easily protonated in acidic environment. This makes chitosan soluble in aqueous acidic solutions and enables expression of its bioactivity. However, physico-chemical properties and biological activity of chitosan are strongly affected by manufacturing processes.

Since chitosan is produced by deacetylation of chitin, the chitosan's properties, such as purity, viscosity, deacetylation, molecular weight, and crystallinity can have wide differences based on the manufacturing process. For example, source of chitin may result in different crystallinity of chitin itself, but also in bioactivity of produced chitosan. Chitin from squid pens exists in β-form which has more open structure (parallel chain alignment) compared to α-chitin (antiparallel chain alignment) found in crustacean exoskeletons (Shepherd and others 1997). Parallel arrangement of chitin molecules results in much weaker intermolecular hydrogen bonding of the main chains and more extensive swelling and higher reactivity during deacetylation of β-chitin compared to crustacean α-chitin (Tolaimate and others 2000).

Generally, deacetylation of chitin is conducted by alkali treatments. However, concentration of alkali, temperature, and time of the process affect the distribution of remaining acetylated groups. The most common processes involve 40 – 50 % NaOH treatments at 100 – 160 °C for 0.5 - 5 hours and result in the heterogeneous N-deacetylation with blocks of aceylated monomers (Kurita 2001). On the other hand, lower sodium hydroxide concentrations (~ 10 %) at room temperature for prolonged periods



A



B

Figure 1 - Structure of chitin, chitosan. A – Chitin, B – Chitosan.

result in randomly distributed acetyl residues (Aiba 1991). Application of mild deacetylation treatments is preferred when high molecular weight of chitosan is required, since the hot concentrated alkali solutions can split glycosidic bonds (Tolaimate and others 2003).

Antimicrobial activity

Depending on the substrate in which it is applied, chitosan may have different mechanisms of antimicrobial action. For example, when applied for plant protection for treatment of growing or stored crops, chitosan activates plant's defensive responses. Concentrations as low as 18 mg/l have been shown to elicit synthesis of phytoalexins and stimulate accumulation of phenolics and lignin by host plants (Allan and Hadwiger 1979, Reddy and others 1999). Similarly, chitosan-treated wheat seeds have improved seed germination and increased resistance against plant-pathogenic fungi (Reddy and others 1999).

In contrast, when applied in systems such as processed food or microbiological media, chitosan apparently directly affects microbial cells by one of two (or both) proposed mechanisms. In one mechanism, the polycationic positively charged chitosan interacts with negatively charged components at the cell surface, e.g., anionic cell wall polysaccharides, proteins, or phospholiopids in the cytoplasmic membrane (Helander and others 2002, Young and Kauss 1983). Depending on the type and location of the interaction, this may have various effects. Binding of chitosan with the cell wall anionic macromolecules apparently forms an impervious layer around the cell, which can prevent transport of nutrients to the cell (Ralston and others 1964). Interaction with cell membrane constituents may alter permeability resulting in leakage of intracellular electrolytes, glucose, enzymes, and other proteinaceous cytoplasmic material (Fang and thers 1994, Leuba and Stossel 1986, Tsai and Su 1999, Young and Kauss 1983). The second proposed mechanism involves penetration of chitosan molecules into the nucleus of eukaryotes which interferes with DNA transcription and mRNA and protein synthesis (Hadwiger and others 1986). In order to penetrate into the nucleus, chitosan molecules have to be of low molecular weight. It has been shown that the heptamer exhibited

maximum antifungal activity, but saccharides with three or less units were inactive (Hirano and Nagaro 1989, Kenedra and Hadwiger 1984).

Regardless of the mode of action, effectiveness of chitosan depends on the concentration and characteristics of the molecule itself, on environmental conditions, and the type of microorganism. It appears that chitosan has stronger bactericidal effect against gram-positive than against gram-negative bacteria (Dagry and others 2000, No and others 2002a). This is probably due to difference in the net electronegativity of the cell wall between gram-positive and gram-negative bacteria. Gram-positive cell wall structural polysaccharides are typically acidic carrying negative charges. Additional surface electronegativity originates from phosphoryl groups of teichoic and teichuronic acids. In gram-negative species, however, the outer lipopolysaccharide membrane suppresses the net charges by providing the partial hydrophobicity and by reducing the exposure of unsubstituted acidic groups.

Antimicrobial activity of chitosan apparently increases with degree of deacetylation (Roberts 1992). Thus, chitosan with DA of 25 % caused reduction of the optical density in *E. coli* suspensions within 2 hours of application, while more deacetylated chitosan exhibited prolonged activity over 16 hours (Liu and others 2001). Conflicting results have been reported regarding the correlation of molecular weight and antimicrobial activity of chitosan. Liu and others (2001) found that antimicrobial activity increased with the chitosan molecular weight from 5.0×10^3 to 9.16×10^4 and decreased when MW further increased to 1.08×10^6. However, chitosan with molecular weight of 4.0×10^5 displayed a much weaker antibacterial effect as compared to larger molecules of chitosan glutamate and chitosan lactate, both with MW in the range of 1.0 to 1.5×10^6 (Sudarshan and others 1992). Joen and others (2001) found that chitosan molecular weight of at least 10 kDa is required for effective inhibition of gram-positive and gram-negative bacteria. Similarly, No and others (2002a,b) found that chitosans with molecular weight from 28 kDa to 1671 kDa had much greater antibacterial properties than chitosan oligomers with molecular weights from 1 kDa to 22 kDa. Furthermore, Zivanovic and others (2004) confirmed that chitosan polysaccharide expressed stronger bactericidal

effects towards *Listeria monocytogenes* and *Salmonella Typhymurium* strains compared to chitosan oligosaccharide.

Chitin and chitosan in films and coatings

As edible films and coatings gained great interest to extend shelf life and improve the quality of food product, chitin and chitosan have been successfully used as food packaging materials due to their film-forming properties. Thus, chitosan coatings can be used to modify the internal atmosphere, decrease the transpiration losses, and delay the ripening of fruits (El Ghaouth and others 1991, 1992). Furthermore, the chitosan coatings have showed antibacterial activity (Shahidi and others 1999). Coma and others (2002) found that chitosan film completely inhibited *L. monocytogenes* for at least 8 days. However, they observed a decrease in bactericidal effect with time, which they explained to be the result of a decreased availability of amino-groups of chitosan. Similarly, it was observed that the use of chitosan as edible coating on pizza (0.079 g/100 g pizza) delayed growth of *Alternaria* sp, *Penicillium* sp, and *Cladosporium* sp. but showed a little effect on *Aspergillus* sp (Rodriguez and others 2003).

Chitosan films have been evaluated as carriers of bacterial inhibitors, such as organic acids and cinnamaldehide, to preserve meat products (Ouattara and others 2000). The authors found that chitosan-based antimicrobial films significantly reduced the growth of Enterobacteriaceae at the surface of pastrami and resulted in the complete inhibition of growth on bologna during the 21-day storage. In contrast, the films had no or little effect on the lactic acid bacteria count on either of the products.

Essential Oils

Essential oils are complex mixtures of numerous compounds from various parts of the plants. Some of the main groups of found in essential oils include alcohols, aldehydes, esters, ethers, ketones, phenols and terpenes (Orav 2001). Each of the group consists of numerous compounds. For example, terpenes include monoterpenes,

diterpenes, sesquiterpenes, sesquiterpene lactones, etc, which are an important class of volatile constituents and may have bioactive properties (Orav 2001).

Essential oils have been used for flavoring foods and beverages for hundreds of years. Due to their pleasant fragrance, many essential oils have been used in cosmetic products and perfumes. They had also been used as pain releasing agents in traditional Chinese medications (Yip and Tse 2004).

Antimicrobial activities of essential oils have been recognized for many years and recently have been extensively researched (Daferera and others 2003, Ela and others 1996, Elgayyar and others 2001, Reddy and others 1998, Tassou and others 2000). However, the most of the studies have focused on the activity *in vitro*, and only very few authors have documented their antimicrobial activity on food products (Ouattara and others 2000a,b, Skandamis and Nychas 2001). Oregano, basil and coriander oils have been shown to be strongly active against *L. monocytogenes, S. aureus, E. coli, Salmonella Typhimurium, Y. enterocolitica, P. aeruginosa, A. niger, G. candidum* and *Rhodothorula*, while anise, although not particularly inhibitory to bacteria, strongly inhibits molds (Conner and Buchat 1984, Ela and others 1996, Elgayyar and others 2001). Application of essential oils against foodborne pathogens is a promising technology as a supplement to conventional preservation methods. Application of 0.8 % oregano essential oil on meat surface resulted in an initial 2 - 3 log reduction of the majority of bacterial population and death of *L. monocytogenes* after 10 days at 5°C (Tsigarida and others 2000). Similarly, 0.5 % oregano essential oil effectively delayed microbial growth on meat stored at 5 °C in air and modified atmosphere (Skandamis and Nychas, 2001). Examining survival of *E. coli* in eggplant salad, Skandamis and Nychas (2000) found that 0.7 % oregano essential oil caused significant reduction during storage but the death rate was depended on pH and temperature. Paster and others (1995) showed that thyme and oregano essential oils could be used as alternative preservatives for stored grains since both oils successfully inhibited natural microflora of wheat grains.

Generally, phenolics and terpenoids are the major contributors to antimicrobial effects of essential oils. Essential oils that contained a high percentage of monoterpenes, eugenol, cinnamaldehyde, thymol and carvacrol had been reported to have strong

antibacterial activities (Lis-Balchin and Deans 1997). The antimicrobial activity of essential oils is considered to be by one (or more) of the proposed mechanisms. The proposed mechanisms include (1) interference with the phospholipids bilayer of the cell membrane, causing increased permeability and loss of cellular constituents (Lambert and others 2001); (2) interference with activity of variety of enzyme systems, including those involved in the production of cellular energy and synthesis of structural components; and/or (3) damaging of genetic material (Kim and others 1995).

Beside for their antimicrobial activity, essential oils and various plant extracts have been widely evaluated to be used as natural antioxidants. Ruberto and Baratta (2000) tested about 100 pure components of essential oils for their antioxidant effectiveness and found that phenols possess the highest antioxidant activity. The function was due to the presence of hydroxyl groups in their molecules (Shahidi and others 1992). Sokmen and others (2004) found that the methanol extracts obtained from herbal parts showed better antioxidative effect than that of butylated hydroxytoluene.

The concentration and ratio of the active compounds in essential oils depend on the plant variety, origin, time of harvest, and conditions of processing and storage (Deans and Ritchie 1987). For example, oregano (*Origanum vulgare*) harvested from the northern parts of Greece was rich in thymol (30.3 – 42.8 %) and had low carvacrol levels (1.7 – 2.5 %), whereas those from the southern parts were rich in carvacrol (57.4 – 69.6 %) but had only 0.2 – 4.1 % thymol (Kokkini and others 1997). The *Origanum majorana* L. (Lamiaceae) grown in the southwestern part of the Mediterranean region, was characterized by rich oil yield with high carvacrol content, but *O. majorana* grown in the western part of Turkey was poor in oil and contained only trace amounts of carvaerol (Tabanca 2004).

Oregano, characteristic spice of the Mediterranean cuisine, is obtained by drying leaves and flowers of *Origanum vulgare* subsp. hirtum plants. The extract of the herb has been traditionally used to improve the sensory characteristics and extend the shelf-life of foods. Oregano essential oil has been found to have antioxidative, antimicrobial, and antiviral properties and carvacrol and thymol have been recognized as the principle constituents and responsible compounds for its functional properties (Lambert and others

2001, Sokmen and others 2004). Oregano essential oil has the ability to inhibit the growth of gram-positive, gram-negative bacteria, fungi and molds (Elgayyar and others 2001, Lambert and others 2001, Hammer and others 1999, Sokmen and others 2004). Tsigarida and others (2000) reported that addition of 0.8 % of oregano essential oil on meat resulted in reduction of the bacterial population for 2 - 3 logs. Lactic acid bacteria and *L. monocytogenes* showed the most apparent decrease but the gram-negative pseudomonads were apparently resistant to the oil. Helander and others (1998) found that carvacrol and thymol decreased the intracellular ATP content of *E. coli* cells while the extracellular ATP simultaneously increased, which indicated a disruptive action of the compounds on the plasma membrane. Studies showed that oregano essential oil can also reduce lipid oxidation and be applied on meat or other lipid containing products to help extend the shelf-life (Botsoglou and others 2002, Lagouri and others 1993, Milos and others 2000).

The essential oil of anise is normally obtained by steam distillation of the crushed anise seed. The major constituent in anise essential oil is anethole (90 %) which significantly effects fungal development and subsequent mycotoxin production in wheat grains (Soliman and Badeaa 2002).

Essential oil of basil (distilled from *Ocimum basilicum L*) contains methyl chavicol and linalool as the principal constituents. Basil essential oil has been reported to antimicrobial against a variety of gram-positive, gram-negative bacteria and yeasts and molds (Wan and others 1998). Soliman and Badeaa (2002) reported that the methyl chavicol fraction in basil oil was about 50% of the oil and acted as the major component of the oil's antifungal activity. The oil has been extensively used in flavors, for confectionery, baked goods, sauces, and in spiced meats, sausages, etc.

Coriander essential oil is commercially produced by distillation from coriander leaves and/or seeds. The active components of the oil are α-pinene, borneol, linalool, cineole, and linalool (Delaquis and others 2002, Wichtl 1994). Delaquis and others (2002) reported that the minimum inhibitory concentrations of coriander oil toward *E. coli* O157:H7 and *L. monocytogenes* were 0.23 and 0.47 % (v/v), respectively. Wangensteen and others (2004) found that coriander leaves showed stronger antioxidant activity than the seeds and the ethyl acetate extract contributed to the strongest activity

regardless on the part of the plant from which was extracted. They also reported that the total phenolic content in the coriander extracts have positive correlations with the antioxidant activity.

Biodegradable Packaging

The purposes of food packaging are to protect foods from outside contamination during distribution, transmission, and storage, to maintain the correct moisture, oxygen or carbon dioxide content in a product or maintain a desired atmosphere in the headspace around a product. Materials, especially those used in the food and agriculture industries, have rapidly developed in last few years. The availability in large quantities at low cost and favorable functional characteristics enabled the broad application of petrochemical based plastics such as polyolefins, polyesters, polyamides, and etc. However, their non-biodegradable characteristics lead to environmental pollution and packaging material has been the target of environmental and consumer activist groups as being a major contributor to the solid waste stream. Biodegradable packaging materials received a great attention because of their functionality and environmental - friendly attributes. Among them, edible coatings and films show special advantages in increasing the shelf-life of product.

Edible coatings and films

Edible packaging and coatings must be free of toxic compounds and should have a high biochemical, physico-chemical and microbiological stability, before, during and after application (Risch 2000). They also should have good sensory qualities and good barrier and mechanical properties.

The main components of edible packaging that provide good film–forming properties are polysaccharides, proteins, and lipids. They are not only biodegradable products from various food sources, but can also serve as carriers for certain additives, such as antioxidants, preservatives, flavors, etc. (Day 1998). Edible coatings could also

provide a barrier against visible and/or UV light which can modify the food characteristics via oxidation of lipids and pigments (Risch 2000).

Polysaccharide and protein based films have good mechanical properties and present excellent barriers for gases, aromas, and lipids, but are highly permeable to moisture (Krochta and Mulder–Johnson 1997). Wu and others (2001) described that starch-alginate-based edible films had the ability to limit moisture loss and lipid oxidation of precooked beef patties but the abilities differed with the composition of films. Films made from high amylose starch showed lower water vapor permeability (WVP) and gas permeability (GP) than regular corn starch films while addition of oil decreased WVP of starch-based films (Garcia and others 2000).

Protective coatings based on zein are commercially available for use on confectionery items, shelled nuts, and pharmaceutical tablets. As the other protein films, zein films have high water vapor permeability. Weller and others (1998) tested zein and zein/lipid films and found that the addition of plasticizer and lipid to film matrix lowered the WVP and increased the elasticity of the films.

Studies showed that, contrary to hydrophilic polysaccharide and protein based films, hydrophobic lipid based films have poor mechanical properties but high moisture resistance (Yang and Paulson 2000). Therefore, significant efforts have been directed towards development of edible films by incorporation of both hydrophilic and hydrophobic molecules into the film-forming matrix to improve film's physico-chemical properties. Yang and Paulson (2000) showed that addition of lipids to gellan films significantly improved the water vapor permeability, but lower the mechanical properties and caused the films to become opaque. Garcia and others (2000) found that the addition of sunflower oil to starch-based film decreased the water vapor permeability and lowered the crystalline-amorphous ratio compared to films without additives. Chick and Hernandez (2002) reported that the increase of wax content in lactic acid-casein based edible films could significantly decrease water vapor permeability. Sebti and others (2002) introduced stearic acid in cellulose films what resulted in decreased water vapor transmission rate, increased contact angle, decreased tensile strength, and lowered air permeability of the films. Similarly, Ozdemir and Floros (2003) reported that increasing

the amount of beeswax resulted in decreased potassium sorbate diffusivity in whey protein films.

As film-forming biopolymers have the ability to act as carriers of small molecules, various additives have been applied in the films and coatings. Incorporation of essential oils into the films and coatings may not only improve the mechanical characteristics of the films, but also enhance their antimicrobial properties. Combination of naturally occurring antimicrobial components, chitosan and essential oils, may provide a unique system with enhanced antimicrobial properties. Incorporation of essential oils into the chitosan films could reduce losses of active components due to evaporation and establish possibilities for prolonged antimicrobial action and improved safety of foods. Furthermore, the hydrophobic compounds of the oils may enhance barrier and mechanical properties of the films.

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Part 3

Antimicrobial activity of chitosan films enriched with essential oils

Antimicrobial activity of chitosan films enriched with essential oils

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This chapter is a lightly revised version of a paper by the same name submitted to the Journal of Food Science by Svetlana Zivanovic, Shuang Chi, and Frances Ann Draughon.

My primary contributions to this paper include (1) experiments with chitosan film forming, determination of antimicrobial properties of essential oils and films, (2) most of the data gathering and interpretation, (3) most of the writing.

Abstract

Antimicrobial and physico-chemical properties of chitosan films and chitosan films enriched with essential oils (EO) were determined in vitro and on processed meat. Antimicrobial effects of pure EO of anise, basil, coriander, and oregano, and of chitosan-essential oil films against *Listeria monocytogenes* and *Escherichia coli* O157:H7 were determined by an agar diffusion test. The antibacterial effects of the EO were similar when applied alone or incorporated in the films. The intensity of antimicrobial efficacy was in the order: oregano >> coriander > basil > anise. The chitosan films and chitosan-oregano EO films were applied on inoculated bologna samples and stored 5 d at 10 °C. Pure chitosan films reduced L. monocytogenes by 2 logs while the films with 1 and 2 % oregano EO decreased the number of both bacteria by about 4 logs. The films have potential to be used as active biodegradable films with strong antimicrobial effects.

Keywords: chitosan, edible films, essential oils, antimicrobial films

Introduction

Chitosan is a cationic polysaccharide obtained by deacetylation of chitin, which is the major constituent of the exoskeleton of crustaceans. Chitosan has gained significant attention and has been evaluated for numerous applications in the medical, food, agricultural, and chemical industries. Dissolved chitosan has been used to coat grain seeds to increase germination rate and provide resistance to plant pathogens (Freepons 1997), as antimicrobial additive (Helander and others 2001, Roller and Covill 1999; Sudarshan and others 1992, Zivanovic and others 2004), for binding and recovery of proteins and metals from food processing waste waters (Pinotti and others, 1997; Selmer-Olsen and others, 1996), and for preparation of wound healing sponges (Ueno and others 2001).

Due to its high molecular weight and solubility in acidic aqueous solutions, chitosan can form gels, films, and fibers (Ghanem and Skonberg 2002, Hirano and others 1999). However, antimicrobial and functional properties of chitosan's solutions and films depend on characteristics of chitosan molecule itself (degree of acetylation, molecular weight), other compounds in the system (type and concentration of the acid, presence of proteins, lipids, ions and other food ingredients), and environmental conditions (temperature and relative humidity). Chitosans with low degree of acetylation (< 15 %) and molecular weight of 28 kDa to 1671 kDa have shown the strongest antibacterial effects in aqueous solutions regardless of the type of acid used for solubilization (No and others 2002). However, type of acid used for film preparation significantly affects the film properties. Acetic and formic acid-based films have shown the highest tensile strengths followed by the films prepared with lactic, propionic, and citric acids (Begin and Van Calsteren 1999, Caner and others 1998). Begin and Van Calsteren (1999) found that the film thickness and flexibility increased with increased molecular weight of acid used as a solvent. On the other hand, Park and others (2002) determined that increased molecular weight of the chitosan molecules resulted in increased strength of the films but did not significantly affect water vapor permeability of the films. Since the water vapor and oxygen permeability are important parameters in evaluation of films as food packaging, various additives have been examined for their potential to alter permeability

of the chitosan films. Although addition of 0.25 and 0.5 % glycerol has increased permeability and decreased tensile strength of the films (Butler and others 1996), low relative humidity and presence of lauric acid in the films have significantly reduced permeability of water vapor and increased transmission rates of CO_2 and O_2 (Wong and others 1992).

Chitosan has intrinsic antimicrobial activity which is effectively expressed in aqueous systems (Papineau and others 1991, Sudarshan and others 1992, Wang 1992). Long positively charged chitosan molecules interact with negatively charged bacteria membranes causing disruption and death of the cell (Helander and others 2001, Young and Kauss 1983). However, antimicrobial properties may become negligible when chitosan is in a form of insoluble films (Ouattara and others 2000).

Many types of edible films have been evaluated for controlled release of antimicrobial agents in food products (Cagri and others 2002, Ozdemir and Floros 2003). Antimicrobial activity of essential oils (EO) were recognized long ago, but their application as natural antimicrobials has recently received increased attention in the food industry (Davidson 2001, Draughon 2004). Advantage of EO is not only in their plant origin but in synergistic effects of their compounds as evidenced in greater activity when applied as natural EO than by summary of the effects of the individual substances (Duke and Beckstrom-Stenberg 1994). Incorporation of EO in chitosan films may not only enhance film's antimicrobial properties but also reduce water vapor permeability and slow lipid oxidation of the product on which the film is applied (Botsoglou and others 2002, Yanishlieva and others 1999). Some of the main chemical compounds of EO include alcohols, aldehydes, esters, ethers, ketones, phenols, and terpenes. Although each type of EO consists of over a hundred compounds, usually just a few of them dominate. Thus, anethole makes over 90 % of anise EO (Soliman and Badeaa 2002), methyl chavicol and linalool are the principal constituents of basil EO, α-pinene, borneol, linalool and cineole dominate in coriander EO (Delaquis and others 2002), and carvocrol and thymol are the principal constituents of oregano EO (Lambert and others 2001). Generally, phenolics and terpenes are major contributors to antimicrobial effects of EO. A number of reports established a good correlation between strong antibacterial activity

and the presence of monoterpenes, eugenol, cinnamaldehyde, carvacrol, and thymol in EO (Kim and others 1995, Lis-Balchin and Deans 1997, Ouattava and others 1997, Suresh and others 1992). Numerous studies have shown that oils of basil, black cumin, clove, cinnamon, oregano, rosemary, and thyme have strong antimicrobial activity (Arora and Kaur 1999, Deans and Ritchie 1987, Delaquis and others 2002, Elgayyar and others 2001, Meena and Sethi 1994, Ozkan and others 2003, Tepe and others 2004). On the other hand, although anise EO has low effectiveness towards bacteria, it acts as a strong antimycotic agent (Elgayyar and others 2001).

The objectives of this research were (1) to determine antimicrobial effects of chitosan films and chitosan films enriched with essential oils against *L. monocytogenes* and *E. coli* O157:H7, (2) to determine antimicrobial effectiveness of the films on a meat product, and (3) to characterize physical properties of chitosan films enriched with essential oils.

Materials and Methods

Bacteria strains and culture maintenance. Cultures of *L. monocytogenes* (ATCC 7644) and *E. coli* O157:H7 (ATCC 43889) were obtained from the Food Safety Center collection, the University of Tennessee, Knoxville, TN. Test strains of bacteria were pre-cultured into trypticase soy broth (TSB) (Difco Laboratories, Detroit, MI) containing 0.6 % (w/v) yeast extract (TSBYE) (Difco) at 35 °C for 24 h. The cultures were kept refrigerated on tryptose soy agar slants during the experiment. PALCAM medium (Difco) and rhamnose sugar test were performed to determine the viability and purity of L. monocytogenes. Sorbital MacConkey agar medium and API 20ETM system were used to test the viability and purity of E. coli O157:H7 culture.

Antimicrobial effects of essential oils. Food grade essential oils (EO) of anise, basil, coriander and oregano were purchased from Essential Oil Company (Portland, OR). Antimicrobial activity of the oils was tested by the filter paper disc diffusion method (NCCLS 2000). Twenty-four-hour-old culture of L. monocytogenes or E. coli with 10^7 CFU/mL was spread on Mueller Hinton II Agar plates (0.1 mL/plate). One sterile 6 mm-

diameter filter paper disc was placed in the center of inoculated plate, and the required amount of EO was carefully pipetted onto it (drop-by-drop). The oils were tested in concentrations of 1, 2, 3, 4, 5, 6, 12, 18 and 24 µl per paper disc. All plates were incubated at 35 °C for 2 d, after which the inhibition zones were measured with a caliper and recorded in millimeters. All tests were performed in triplicates.

Film preparation. Medium molecular weight chitosan (450 kDa) was purchased from Aldrich Chemical Co. (Milwaukee, WI). Chitosan stock solution was prepared with 1.5 % w/w chitosan in 1.5 % v/v acetic acid. To achieve complete dispersion of chitosan, the solution was stirred overnight at room temperature, filtrated through miracloth (Calbiochem-Novabiochem Corp., San Diego, CA) to remove impurities, and sterilized at 121 °C for 15 min. The EOs were first mixed with Tween®20 (Aldrich Chemical Co. Milwaukee, WI) to help distribute and completely incorporate the oils in chitosan matrix and than added to the chitosan stock solution. The final film forming solution (FFS) consisted of 1 % chitosan, 1 % acetic acid, 0.5 % Tween®20, and 1, 2, 3 or 4 % EO. The FFS was homogenized under aseptic conditions at 21,600 rpm for 1 min (Polytron Kinamatica Inc. PV, Cincinnati, OH) and poured into 50-mm i.d. sterile petri dishes. All the films were prepared with 20 g of FFS per petri dish (one film) which ensured 10 mg chitosan/cm^2. Control films were prepared identically but without addition of EO. After drying under 5 psi vacuum at 30 °C, films were kept in sealed petri dishes at 4 °C until analysis.

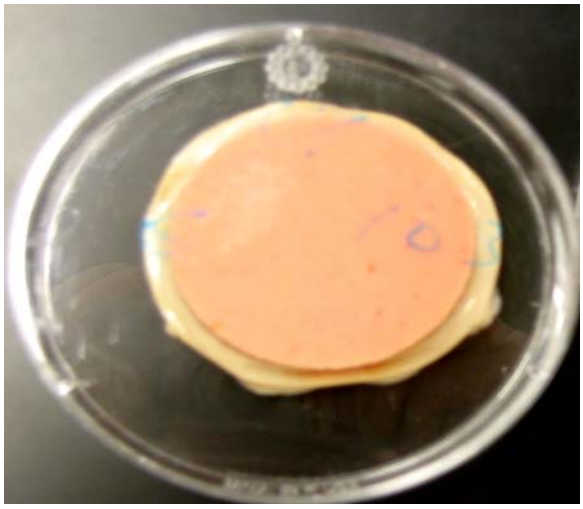
Antimicrobial effects of essential oil incorporated into chitosan films. The bacteria suspensions were prepared by following the same procedure as for the paper disc diffusion test. The inoculum was evenly spread on Mueller Hinton II Agar plates resulting in 10^6 CFU/plate. Uniform 6.6-mm-diameter discs were cut with a hole-puncher from the prepared chitosan films, and one film disc was placed in the center of the inoculated petri dish. Concentrations of the EO in the film forming solutions of 1, 2, 3, and 4 % corresponded to 3.1, 6.2, 9.3, and 12.3 µl EO per film disc, respectively. Chitosan films with no EO served as control. The plates were incubated at 35 °C and measurements were taken after 48 h. During the incubation, the film discs slightly swelled due to water absorption and resulted in enlarged diameter (6.9 mm). Therefore,

both inhibition zone and disc diameter were measured and width of inhibition ring was recorded. The tests were performed in duplicates.

Film application on processed meat. Extra thick bologna slices were purchased from a local grocery store. According to the processor, the composition (w/w) was: 29 % total fat, 5.4 % total carbohydrates, 10.7 % proteins, and 0.9 % sodium. Uniform bologna slices (5.25 mm thick and 48 mm diameter, 11 g weight) were placed in sterile petri dishes. The petri dishes were sealed with parafilm and stored at 4 °C until use but no longer than 2 d.

Bologna slices were inoculated by spreading of 0.1 mL 10^4 CFU/mL, 10^5 CFU/mL, 10^6 CFU/mL and 10^7 CFU/mL bacterial suspensions on one side of each slice. Films prepared with 1 or 2 % oregano EO were placed between two equally inoculated slices to make a 'sandwich' (Figure 1). Each 'sandwich' was placed in a 100 x 15-mm petri dish, The petri dish was sealed with para-film and stored at 10 °C for 5 d. Inoculated 'sandwich' samples without application of the films and 'sandwich' samples with chitosan films with no EO served as controls. After inoculation, the films were aseptically removed and two slices of bologna from the same 'sandwich' were homogenized with bacteria enrichment broth in 400 mL stomach bags for 1 min. Buffered Listeria enrichment broth with modified Listeria selective enrichment supplement (Oxoid limited, Hampshire, England) was used for *L. monocytogenes* and modified tryptone soy broth with novobiocin supplement (Oxoid) was used for *E. coli* O157:H7. Appropriate dilutions were surface-plated on PALCAM Medium Base Agar (Difco) and MacConkey Sorbitol Agar (Difco) to quantify *L. monocytogenes* and *E. coli* O157:H7, respectively.

Films recovered from the 'sandwich' samples inoculated with 10^6 CFU/mL were flooded with sterile peptone water (pH = 6.8) and left at the room temperature for 5 min with occasional stirring. The plate counts of bacteria in the wash peptone water were performed on selective media (PALCAM and MacConkey Sorbitol agar). Tests were done in triplicates.



“Sandwich”

Figure 1. The “sandwich” prepared with two inoculated bologna slices and chitosan film placed between them.

Physical characterization of chitosan films. Film thickness was assessed on 24 films per treatment with average measurements at 5 points for each film (microcaliper; Mitutoyo, Japan). Puncture strength was measured on 8 films per treatment with the TA.XTplus Texture Analyzer (Texture Technologies Corp., Scarsdale, NY/Stable Micro Systems, Godalming, Surrey, UK). The instrument was equipped with TA-108S fixture and 2 mm-diameter needle probe (TA-52) moving with a test speed of 1 mm/s. Tensile strength (TS) was determined on bone-shaped film specimens by TA.XTplus Texture Analyzer in tensile mode (test speed 1 mm/s). The TS was calculated as TS [N/mm^2] = F/(w∗d) where 'F' was force at break [N], 'w' was width of the film at the point of break [mm], and 'd' was thickness of the film [mm]. Elongation (%E) was determined as the maximum extension of the film before the break. Water vapor permeability (WVP) of the films was determined as a weight difference of Fisher/Payne permeability cups (Fisher Scientific, Pittsburgh, PA) filled with 5.0 g d.i. water and sealed with the films, before and after 24-h of incubation at 25 °C and 50 % RH.

Results and Discussions

Antimicrobial activity of essential oils. All essential oils used in this study (anise, basil, coriander, and oregano) expressed antimicrobial activity against *L. monocytogenes* and *E. coli* 157:H7 (Figure 2). As expected, antimicrobial activity was stronger at the higher concentrations of EOs. Oregano oil showed the strongest inhibition towards both pathogens. The growth of L. monocytogenes was completely inhibited (clear plates) with 12 or more μL of oregano EO. Even the lowest tested amount of oregano EO (1 μL) caused inhibition zone of 21.5 mm and 17.1 mm for *L. monocytogenes* and *E. coli* O157:H7, respectively. The inhibition zone caused by 24 μL coriander oil was 31.1 mm and 34.6 mm for *L. monocytogenes* and *E. coli* O157:H7, respectively. Basil EO expressed only slight inhibitory effects, e.g., 24 μL of the oil resulted in 23.3 mm and 13.2 mm zones for *L. monocytogenes* and *E. coli* O157:H7, respectively. Anise EO showed the weakest antibacterial effects compared to other tested essential oils. No inhibition of *L. monocytogenes* was observed with less than 4 μL anise EO or with less than 12 μL for *E. coli* O157:H7.

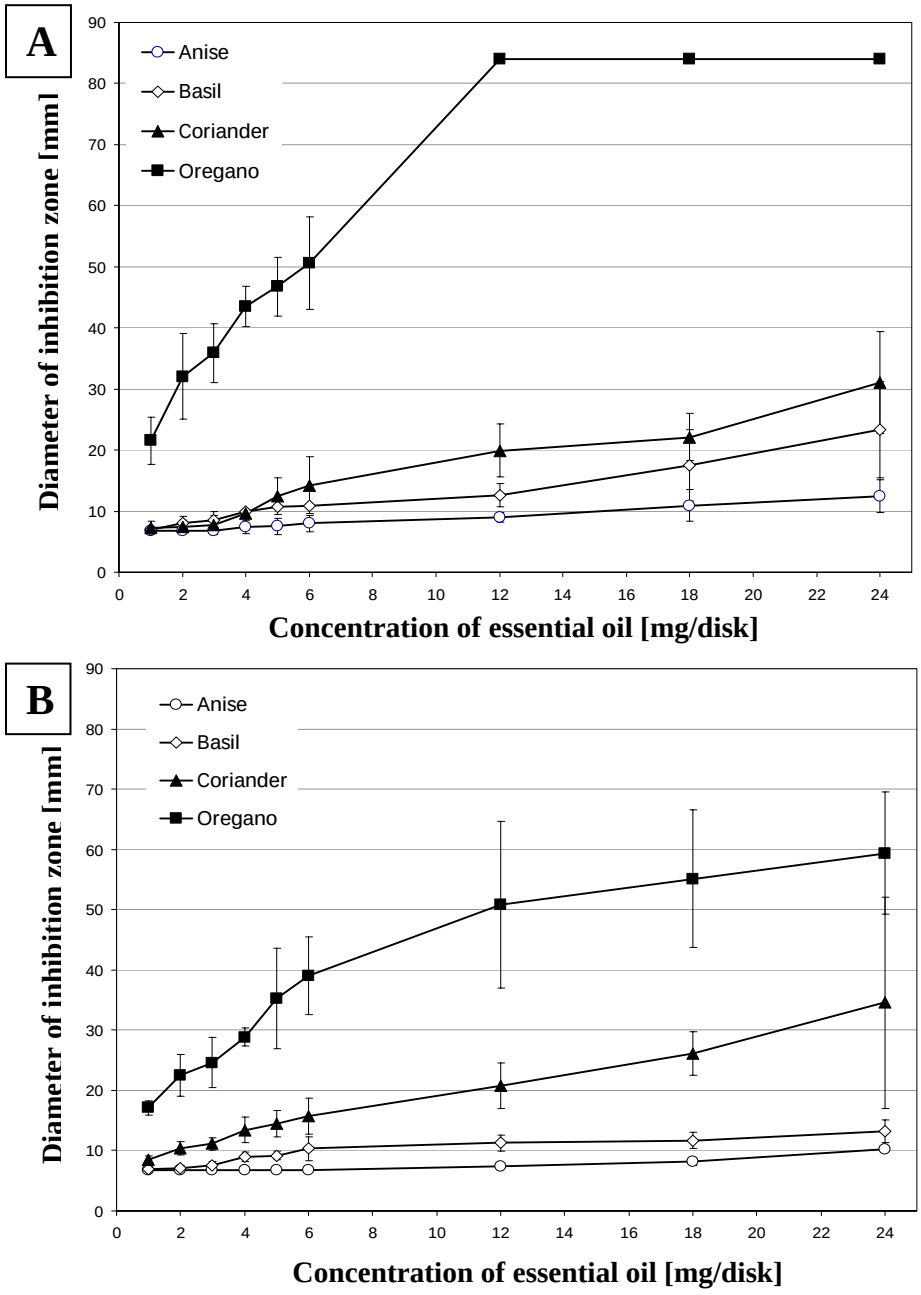


Figure 2. Effects of essential oils of anise, basil, coriander, and oregano on (A) *L. monocytogenes* and (B) *E. coli* O157:H7 determined by agar diffusion test.

Plates were inoculated with 10^6 CFU/plate and incubated 48 h at 35 °C.

The paper disk diameter was 6 mm, and the inside diameter of petri dish was 84 mm.

These results are similar to those of Elgayyar and others (2001). In their report, oil of oregano completely inhibited the growth of *E. coli* and strongly inhibited *L. monocytogenes* and basil EO had strong inhibitory effects towards *E. coli* but only mild effects against *L. monocytogenes*. On the other hand, in that study, *L. monocytogenes* was moderately and *E. coli* just weakly susceptible to coriander EO while anise EO had very little effect on either pathogens. Similarly, Deans and Ritchie (1987) found that 10 µL pure essential oil of anise, basil, and coriander added in wells of the iso-sensitest agar did not show inhibitory effects against *E. coli* after a 48-h incubation at 25 °C. Although most researchers have recognized oregano EO as one of the oils with the strongest bacteriostatic and bactericidal properties towards *E. coli* and *L. monocytogenes* (Burt and Reinders 2003; Friedman and others 2002), that option may not always be true (Ouattara and others 1997). The major compounds of oregano EO are carvacrol and thyme. However, the concentration and ratio of the active compounds depend on the plant variety, origin, time of harvest, and conditions of processing and storage. For example, the oregano variety *Origanum vulgare* harvested from the northern parts of Greece was rich in thymol (30.3 to 42.8 %) and had low carvacrol levels (1.7 to 2.5 %), whereas that from the southern parts was rich in carvacrol (57.4 to 69.6 %) but had only 0.2 to 4.1 % thymol (Kokkini and others 1997). In the experiment of Ouattara and others (1997) the oregano EO that showed very low antimicrobial activity towards meat spoilage bacteria had only 5.19 % carvacrol and 0.37 % thymol and these low levels were probably the reason for the lack of activity.

Our results showed that oregano EO was more effective against gram-positive *L. monocytogenes* than against gram-negative *E. coli* O157:H7 (Figure 2). The results were similar to those reported elsewhere (Demetzos and others, 2001; Nakatani, 1994). The proposed mechanism of antimicrobial activity of phenolic compounds of EOs is in their attack on the phospholipid cell membrane, which causes increased permeability and leakage of cytoplasm (Kim and others 1995), or in their interaction with enzymes located on the cell wall (Farag and others 1989, Wendakoon and Sakaguchi 1995). Thus, the resistance of Gram-negative bacteria to the essential oils lies in the protective role of their cell wall lipopolysaccharides.

Antimicrobial effects of chitosan films. The film discs, placed on the inoculated agar evenly spread with 0.1 mL of bacteria suspension, absorbed water and bent upward. An incense in inoculation volume of bacterial suspension to 0.5 mL resulted in swollen films that stayed in full contact with the inoculated agar but their diameter enlarged from 6.6 mm to 6.9 mm. Therefore, inhibition zone and disc diameter were measured and the width of inhibition ring is reported (Figure 3).

Pure chitosan films, with no EO, served as a control to determine potential antimicrobial effects of chitosan films per se, but we did not observe any inhibition of the tested bacteria by the control films (Figure 3). There are two possible reasons for these results. First, the inoculum in this experiment was 10^6 CFU per petri dish while others have used much lower inoculum (< 10^2 CFU/petri dish) for similar experiments (Coma and others 2002). Thus, the high number of bacteria may exceed the inhibition activity of chitosan. Another explanation may be in the fact that chitosan has to be dissolved in the order to act as an antimicrobial. In this case, chitosan molecules were tightly bound within the film what prevented expression of the antimicrobial action.

The inhibition effects of oregano EO incorporated into the chitosan films were lower than that of pure EO. Similar to the results with the pure EO, oregano EO incorporated into the chitosan films, regardless of the concentration applied, exhibited the strongest inhibition of tested pathogens compared to other EO. Oil of anise, basil and coriander had weak inhibition of both bacteria and were excluded from further study. The inhibition of *L. monocytogenes* and *E. coli* O157:H7 caused by oregano films on Mueller Hinton II Agar plates is shown in Figure 3. The possible reason for the decrease in activity of the EO incorporated in the chitosan films compared to activity of pure EO may be due to potential partial loss of highly volatile compounds of the EO during film preparation. The other reason may be due to slower/controlled release of active compounds from the chitosan film than from cellulose filter paper. Similar to the effects of the pure oil, *L. monocytogenes* appeared to be more sensitive to oregano films than *E. coli* O157:H7.

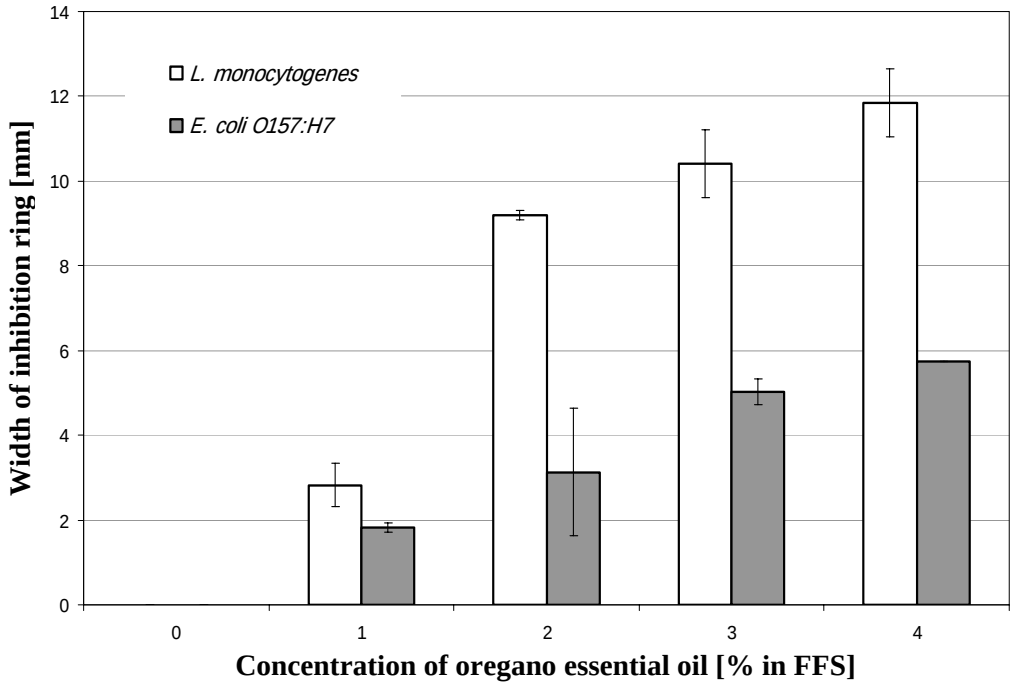


Figure 3. Inhibition of *L. monocytogenes* and *E. coli* O157:H7 with chitosan films enriched with oregano essential oil.

Plates were inoculated with 10^6 CFU/plate and incubated 48 h at 35 °C. The initial disk diameter was 6.6 mm, and the inside diameter of petri dish was 84 mm.

Inhibition of pathogen growth on bologna by oregano chitosan films. Pure chitosan films placed between two inoculated slices of bologna reduced number of *L. monocytogenes* and *E. coli* O157:H7 compared to the control with no films applied (Table 1). Thus, chitosan films reduced the number of *L. monocytogenes* by 1 to 3 logs depending on the initial bacterial number. However, *E. coli* O157:H7 was less susceptible to the pure films. For example, when inoculated with the 2 x 10^6 CFU/'sandwich', the number of *E. coli* O157:H7 cells was reduced to 2.51 x 10^5, while the number for *L monocytogenes*, decreased to 5.01 x 10^3 CFU/'sandwich'. Apparently, chitosan films absorbed water from the meat product and became effective "on contact". Furthermore, a small amount of residual acetic acid in the films (used for film preparation) may have provided additional antimicrobial effect. Incorporation of oregano EO into the films significantly lowered the number of surviving cells (Table 1). Higher concentration of EO in the films resulted in stronger antibacterial effect. Chitosan films with 2 % oregano EO reduced the number of both pathogens to less than 10^2 CFU/'sandwich' when the inoculum was 2 x 10^3 or 2 x 10^4 CFU/'sandwich'. When inoculum was as high as 2 x 10^6 CFU/'sandwich', the surviving number of *L. monocytogenes* was 1.25 x 10^2, and of E. coli O157:H7 it was 2.51 x 10^2 CFU/'sandwich'. The results suggested that addition of oregano EO into the chitosan film improved the film's antimicrobial properties and strongly reduced pathogens on meat products.

After incubation of 'sandwich' samples at 10 °C for 5 d, chitosan films with and without oregano EO were removed and analyzed for surviving bacteria (Table 2). More viable *E. coli* O157:H7 remained on chitosan films (3.16 x 10^4 CFU/film) than did *L. monocytogenes* (3.98 x 10^2 CFU/film). Population of *E. coli* O157:H7 on oregano films, both with 1 % and 2 % EO, was less than 10 colonies per film while the count of *L. monocytogenes* on 1 % oregano films was 3.16 x 10 and less than 10 colonies on 2 % oregano films.

Table 1: Bacterial effects of chitosan films enriched with oregano essential oil towards *L. monocytogenes* and *E. coli* O157:H7 inoculated on bologna discs and stored 5 d at 10 °C.

Film	Inoculum (CFU/'sandwich')	Survived bacteria	
		L. monocytogenes (CFU/'sandwich')	*E. coli* O157:H7 (CFU/'sandwich')
Control (no film)	2×10^3	3.16×10^2	3.98×10^2
	2×10^4	3.98×10^4	2.51×10^3
	2×10^5	1.26×10^5	1.99×10^4
	2×10^6	2.51×10^6	3.16×10^5
Chitosan film	2×10^3	2.51×10^2	5.01×10^2
	2×10^4	3.98×10^2	1.99×10^2
	2×10^5	1.00×10^3	3.98×10^4
	2×10^6	5.01×10^3	2.51×10^5
1% Oregano Chitosan film	2×10^3	$<1.00 \times 10^2$	1.26×10^2
	2×10^4	1.00×10^2	1.58×10^2
	2×10^5	1.58×10^2	$<1.00 \times 10^2$
	2×10^6	6.31×10^2	3.16×10^2
2% Oregano Chitosan film	2×10^3	1.00×10^2	$<1.00 \times 10^2$
	2×10^4	$<1.00 \times 10^2$	1.00×10^2
	2×10^5	1.25×10^2	1.58×10^2
	2×10^6	1.25×10^2	2.51×10^2

Table 2: Population of bacteria on films removed from bologna sandwich (inoculated with 2 x 10^6 CFU/ 'sandwich') after 5 d storage at 10 °C.

Film	Average population on the film[a] (CFU/film)	
	L. monocytogenes	***E. coli* O157:H7**
Pure chitosan	1.50 x 10^2	4.00 x 10^4
1% Oregano-chitosan	< 30	< 10
2% Oregano-chitosan	< 10	< 10

[a] One film was applied between two inoculated slices of bologna (1 film per 'sandwich')

Physical properties of chitosan films with and without essential oils. Chitosan films made from film-forming solution with no EO were transparent and colorless. Incorporation of the emulsifier and EO resulted in thicker and opaque films. Pure chitosan films with 10 mg chitosan /cm^2 were 89 μm thick whereas addition of 2 % oregano EO in film-forming solution resulted in more than a 3-fold increase in film thickness (Figure 4). The films easily absorbed water and after application on bologna during 5 d at 10 °C, thickness further increased. However, the enlargement was the highest in the films with addition of emulsifier only and the lowest with addition of EO. These results were expected because EO, although being complex mixtures, are highly hydrophobic, and the increase in hydrophobicity of the film matrix should reduce water absorption. Similarly, water vapor permeability decreased with increased fraction of the hydrophobic compound (Figure 5). This activity offers the possibility not only to control the antimicrobial efficiency of the films but also to improve the barrier properties of chitosan films by EO.

The chitosan films prepared with only acetic acid had puncture strength of 310.7 N/mm, tensile strength of 105.7 N/mm^2, and elongation at break of 5 % (Figures 5-7). These results are comparable with those of Begin and Van Calsteren (1999) who determined stress at break to be about 55 MPa and elongation at break of 5 % for the films prepared with 2 % acetic acid. Addition of the oregano EO to our films not only increased the thickness but significantly reduced the strength of the films. However, after application of films on the meat product for 5 d, the strength of the films further decreased resulting in slightly rubbery and elastic texture. Resulting films did not have tendency to tear apart but were easy to handle. The strength of the chitosan films depends on the components of the film-forming solution. Type of acid has a significant effect on the strength and elasticity of the films. For example, films made with hydrochloric and acetic acid are firm and brittle, but those with lactic acid and citric acid are more flexible. Begin and Van Calsteren (1999) found that increased molecular weight of the counter ion resulted in thicker and more elastic but less strong (tough) films. These qualities explain our results in decreased puncture force and tensile strength and increased elongation percentage when the EOs were introduced into the films.

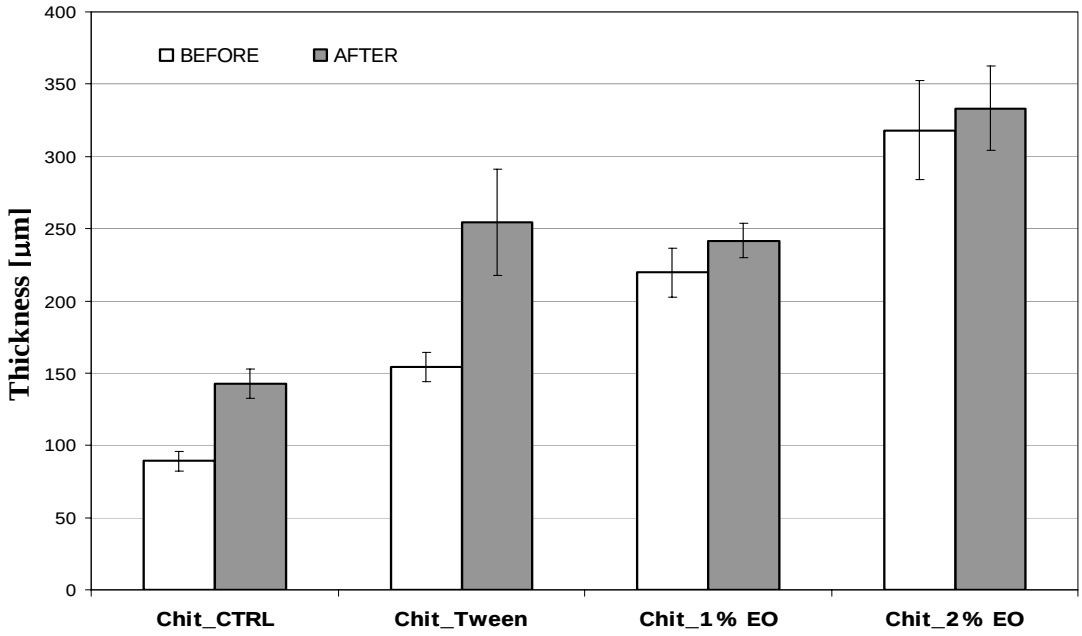


Figure 4. Thickness of pure chitosan films (Chit_CTRL), chitosan films with surfactant (Chit_Tween), and with 1 % (Chit_1% EO) and 2 % (Chit_2% EO) oregano essential oil before and after application on bologna.

Error bars represent standard deviation (n=24).

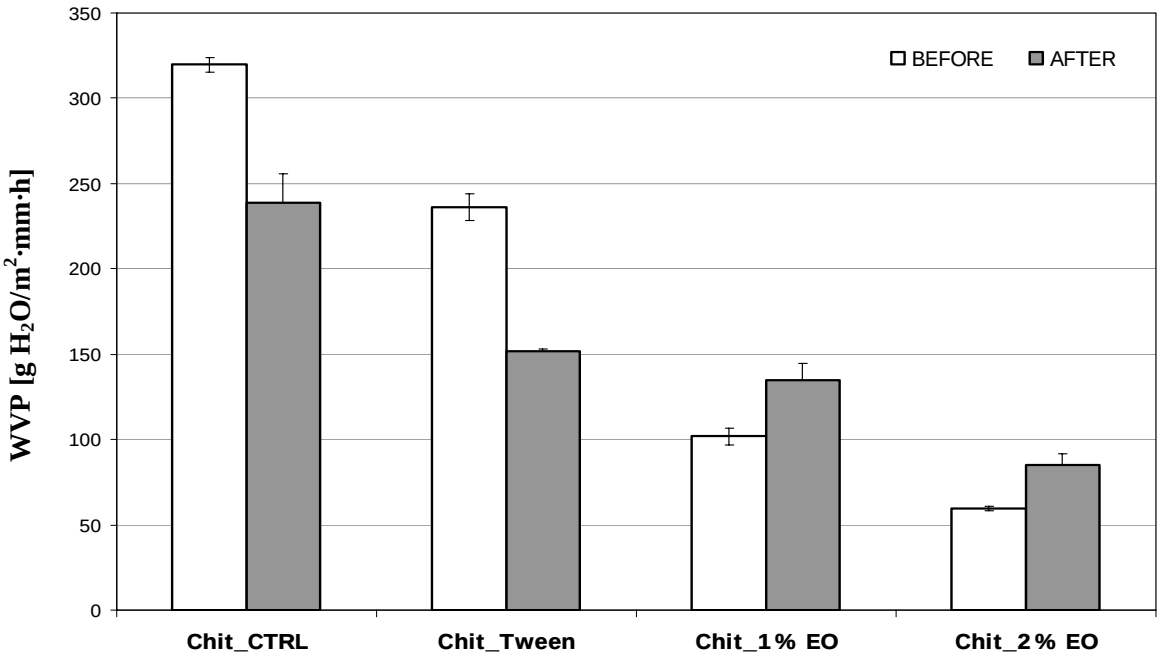


Figure 5. Water vapor permeability (WVP) of pure chitosan films (Chit_CTRL), chitosan films with surfactant (Chit_Tween), and with 1 % (Chit_1% EO) and 2 % (Chit_2% EO) oregano essential oil before and after application on bologna.

Error bars represent standard deviation (n=3).

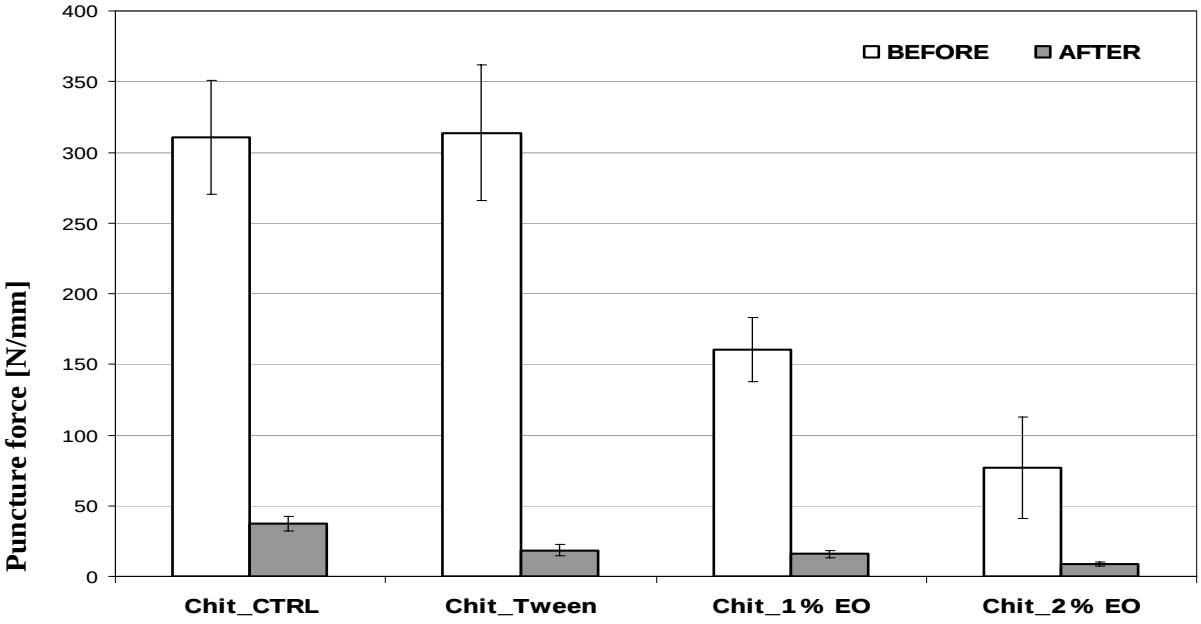


Figure 6. Puncture force of pure chitosan films (Chit_CTRL), chitosan films with surfactant (Chit_Tween), and with 1 % (Chit_1% EO) and 2 % (Chit_2% EO) oregano essential oil before and after application on bologna.

Error bars represent standard deviation (n=8).

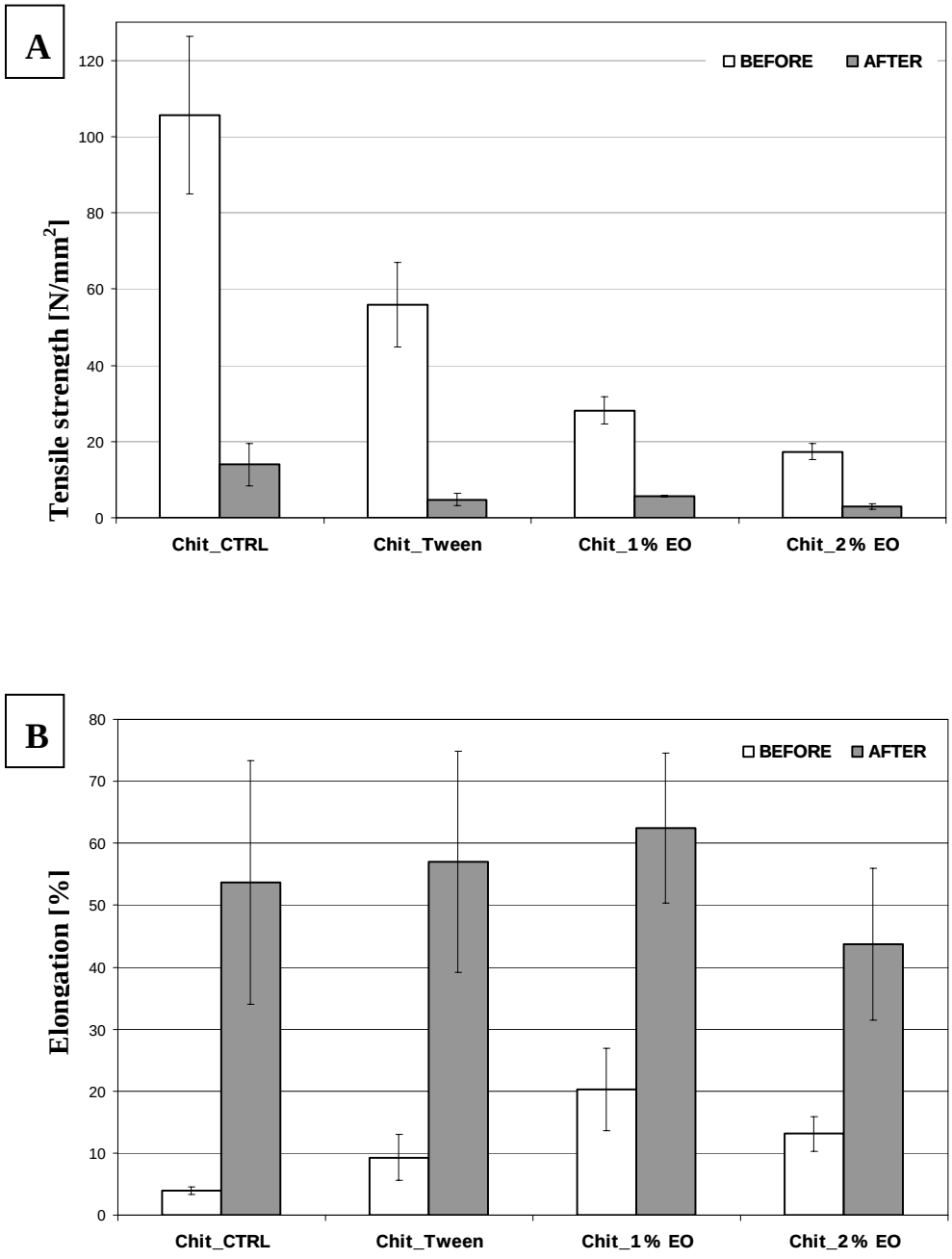


Figure 7. Tensile strength (A) and elongation (B) of pure chitosan films (Chit-_CTRL), chitosan films with surfactant (Chit_Tween), and with 1 % (Chit_1% EO) and 2 % (Chit_2% EO) oregano essential oil before and after application on bologna.

Error bars represent standard deviation (n=8).

Future research will focus on quantification of active compounds in essential oils and determination of potential loses during chitosan film preparation. Full characterization of stability, diffusion of the active compounds from the chitosan film into the product, and resulting antimicrobial effects will assist in optimizing the application of the active films on meat products.

Conclusions

Oregano essential oil exhibited the strongest antimicrobial activity towards L. monocytogenes and E. coli O157:H7 compared to other tested essential oils (oregano EO >> coriander EO > basil EO > anis EO). Although both pathogens were affected, L. monocytogenes appeared to be more sensitive to oregano EO than E. coli O157:H7. Application of pure chitosan films reduced pathogen counts on a meat product (bologna) from 1 to 3 logs, and chitosan films enriched with 1 and 2 % oregano EO were sufficient for 4 logs reduction of L. monocytogenes and E. coli O157:H7 on bologna slices stored at 10 °C for 5 d. Chitosan-EO films prepared with all natural ingredients have the potential to be used as an active packaging material for controlled release of active compounds in prevention and control of food pathogens on processed meat.

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Part 4

Incorporation of oregano essential oil in chitosan films for application on processed meat

Abstract

Distribution of active compounds of oregano essential oil (OEO) was determined during preparation and application of chitosan-OEO films on bologna samples. Oregano EO was extracted with hexane from film-forming solution and films before and after application on bologna. Essential oil compounds were separated and analyzed by GCMS. The results indicated that the concentration of volatile components of essential oil sharply decreased during film processing, *e.g.* from 757.7 ppm thymol in film-forming solution to 2.1 ppm in dried films. No thymol was detected in the films after application on bologna for 5 days at 4 °C due to diffusion of the compound into bologna. Results suggested that the essential oil compounds were firmly bound to the chitosan film matrix but the moisture and high lipid content of the meat product helped the release and their diffusion from the polysaccharide film into the product. Sensory evaluation of bologna with addition of OEO suggested that 45 ppm oregano oil would be acceptable by consumers. Results support the potential use of chitosan-OEO films as an antimicrobial packaging material for processed meat.

Keywords: chitosan, oregano essential oil, thymol, film, consumer acceptance

Introduction

Although essential oils (EOs) have been used as food flavoring agents for centuries, their application as antimicrobial and antioxidative additives has recently attracted attention of both researchers and the food industry. Essential oils are complex mixtures of numerous compounds and can be extracted from various parts of the plants. Some of the main chemical groups found in essential oils include alcohols, aldehydes, esters, ethers, ketones, phenols and terpenes. Principal compounds of EOs are dictated by the botanical origin of the oil. Thus, linalool dominates in the oils of cilantro and coriander (Delaquis and others 2002), trans-cinnamaldehyde in cinnamon (Lens-Lisbonne and others, 1987), α-pinen and 1,8-cineole in rosemary (Pintore and others 2002), and carvacrol and thymol in oregano and thyme (Kokkini and others 1997, Russo and others 1998). However, composition of EO from a particular species may vary between geographical regions and harvesting time (Kokkini and others 1997).

Oregano is characteristic spice of the Mediterranean cuisine obtained by drying leaves and flowers of *Origanum* sp. The herb and its essential oil have been traditionally used to improve sensory characteristics and extend the shelf-life of foods. It has been shown that oregano EO can reduce lipid oxidation in meat or other lipid containing products and, thus, extend the shelf-life of the product (Botsoglou and others 2003). Oregano essential oil has the ability to inhibit the growth of bacteria, fungi and molds (Elgayyar and others 2001, Lambert and others 2001). Our results from Chapter 3 confirmed that not only pure oregano essential oil has strong antimicrobial property, but the chitosan film incorporated with oregano EO can cause significant reduction of food-borne pathogens, such as *Listeria monocytogenes* and *Escherichia coli* O157:H7.

Many researchers have studied the antimicrobial components in oregano oil. Lambert and others (2001) reported that cavracrol and thymol are the principle constituents and responsible for its functional properties and Ultee and others (2002) found that the phenolic hydroxyl group of cavracrol was essential for its action against food-borne pathogens. Baydar and others (2004) tested oregano oil from different species and found the major constituent of the oils was cavracrol while Ettayebi and others (2000) and Karrpmar and Aktug (1987) found thymol as the dominant antimicrobial

compound of oregano EO. Because of the variation of the EO due to different geographical regions and harvesting seasons, as well as plant species, commercially available oregano EOs have considerable variations in carvacrol and thymol content. It was found that carvacrol concentration may very from trace amounts to 80 %, thymol from trace to 64 %, γ-terpinene from 2 to 52 %, and p-cymene from trace to 52 % (Burt 2004). Content of p-cymene and γ-terpinene in oregano EO appears to be significant since *p*-cymene (1-methyl-4-(1-methylethyl)-benzene) and γ-terpinene (1-methyl-4-(1-methylethyl)-1,4-cyclohexadiene) are the precursors of carvacrol (2-methyl-5-(1-methylethyl)phenol) and thymol (5-methyl-2-(1-methylethyl)phenol), respectively. Although, the ratio and absolute concentration of carvacrol and thymol can differ significantly, Kokkini and others (1997) found that the sum of the amounts of these four compounds present in Greek oregano plants were almost equal in specimens derived from different geographical regions. This indicates that the four compounds are biologically and functionally closely associated.

Antimicrobial properties of oregano EO have been widely evaluated but the necessary concentration for microbial reduction has not been firmly established. Minimal inhibitory concentration (MIC) of oregano EO differs depending on the bacterial species. Thus, MIC for *L. monocytogenes* was found to be 0.2 μl/ml (Smith-Palmer and others 1998), for *E. coli* 0.5 to 1.2 μl/ml (Prudent and others 1995, Hammer and others 1999, Burt and Reinders, 2003), *S. typhimurium* 1.2 μl/ml (Hammer and others 1999), and for *Staph. aureus* 0.5 to 1.2 μl/ml (Prudent and others 1995, Hammer and others 1999). However, the effective levels of EO in food products are usually higher than those established in laboratory media. Furthermore, certain oils appeared to be better antibacterials than others for applications in particular food products. Eugenol, coriander, clove, oregano and thyme oils were found to be effective at levels of 5 to 20 μl/g for inhibition of *L. monocytogenes* and autochthonous spoilage flora in meat products while mustard, cilantro, mint and sage oils were less effective or ineffective (Burt 2004). A high fat content seems to be responsible for significant reduction of the antimicrobial action of EOs in meat. For example, mint and cilantro EOs were not effective in pâté with 30 – 45 % fat and when applied with canola oil on ham (Tassou and others 1995, Gill and others

2002). Interestingly, incorporation of cilantro EO in a gelatine gel improved the antibacterial activity against *L. monocytogenes* in ham (Gill and others 2002). Similarly, encapsulated rosemary oil was much more effective than standard rosemary EO against *L. monocytogenes* in pork liver sausages (Pandit and Shelef 1994).

For commercial application of essential oils in foods, the sensory impact of the oils must be evaluated. The potential problem is that threshold levels of EOs are usually at lower concentrations than those necessary for antimicrobial effects. However, foods commonly consumed with spices or seasonings would be less affected. Thus, the flavor of beef fillets treated with 0.8 % oregano EO was found to be acceptable after storage at 5 °C and cooking (Tsigarida and others 2000). The flavor, aroma, and color of minced beef with 1 % oregano EO improved during storage under modified atmosphere packaging and vacuum at 5 °C and EO was almost undetectable after cooking (Skandamis and Nychas 2001). The addition of thyme oil up to 0.9 % in a coating for cooked shrimps had no effects on the flavor or appearance but 1.8 % thyme oil in the coating significantly decreased the acceptability of the shrimps (Ouattara and others 2001, Ouattara and others 1997).

Because of the environmental problems and accumulation of large amounts of waste packaging materials, biodegradable films have been extensively researched in recent years. A good biodegradable film needs to be safe, biocompatible, and good gas and moisture barrier. Polysaccharide or protein based films have good mechanical properties. They are excellent gas, aroma, and lipid barriers but poor moisture barriers. On the other hand, lipid based films have rather poor mechanical properties but high moisture resistance (Yang and Paulson 2000). Wu and others (2001) found that starch-alginate-based edible films have the ability to limit moisture loss and lipid oxidation of precooked beef patties. Whey protein isolate based films containing sorbic and p-aminobenzoic acids clearly inhibited the growth of food-borne pathogens on meat products (Cagri and others 2002). Mchugh and Senesi (2000) reported that addition of lipids to apple-puree based edible films resulted in significant improvements in their water barrier properties. Incorporation of essential oils into biopolymer-based films can improve the mechanical characteristics of the films and enhance their antimicrobial

properties. Furthermore, addition of EO only to the film or coating would provide high concentrations of the active compounds on the surface of the product where microbial attack is expected, and reduce the total concentration of the EO within the product (when expressed on the total weight of the product).

Chitosan is a biopolymer composed glucosamine and *N*-acetylglucosamine units. It is a good coagulating agent and flocculant due to the high density of amino groups, which can interact with negatively charged substances (Goosen 1997). Dissolved chitosan is a good coating material, an antimicrobial additive (Helander and others 2001, Roller and Covill 1999, Sudarshan and others 1992, Zivanovic and others 2004), and a chelating agent that selectively binds proteins and metals (Jones and O'Melia 2000). Clinical trials have shown that chitosan does not cause any inflammatory or allergic reaction when applied either orally or by injection (Muzzarelli 1995).

Chitosan can easily form films from acidic aqueous solutions. However, the drawback of pure chitosan films, as of the other polysaccharide-based films, is in the high water vapor permeability. Our results in Part 3 showed the addition of oregano essential oil reduced the water vapor permeability and enhanced antimicrobial properties of the films. The chitosan-oregano EO films decreased the number of *L. monocytogenes* and *E. coli* O157:H7 for about 4 logs on bologna samples stored at 10 °C for 5 days.

The objective of this research were (1) to identify dominant compound in oregano essential oil; (2) to determine the concentration of the oil's dominant compound in film-forming solutions and films composed of chitosan and oregano EO; (3) to determine distribution of the EO compounds between the films and meat product during storage; and (4) to evaluate consumer liking of bologna with addition of oregano essential oil.

Materials and Methods

Film preparation. Medium molecular weight chitosan (450 kDa) was purchased from Aldrich Chemical Co. (Milwaukee, WI). Chitosan stock solution was prepared with 1.5 % w/w chitosan in 1.5 % v/v acetic acid. To achieve complete dispersion of chitosan, the solution was stirred overnight at room temperature, filtrated through miracloth (Calbiochem-Novabiochem Corp., San Diego, CA) to remove impurities, and sterilized at 121 °C for 15 min. The EO was first mixed with Tween®20 (Aldrich Chemical Co. Milwaukee, WI) to help distribute and completely incorporate the oil in chitosan matrix and than added to the chitosan stock solution. The final film forming solutions (FFS) consisted of 1 % chitosan, 1 % acetic acid, 0.5 % Tween®20, and 1 or 2 % EO. The final FFS were homogenized under aseptic conditions at 21,600 rpm for 1 min (Polytron Kinamatica Inc. PV, Cincinnati, OH) and poured into 50-mm i.d. sterile petri dishes. All the films were prepared with 20 g of FFS per petri dish (one film) which ensured 10 mg chitosan/cm^2. Control films were prepared identically but without addition of EO. After drying under 5 psi vacuum at 30 °C, fresh films were immediately analyzed with gas chromatography or used to prepare bologna 'sandwiches'.

Bologna manufacturing. To avoid interference of spices commonly used in commercial bologna, approximately 20 lb of bologna were prepared in the Department of Food Science and Technology. Beef and pork meat was purchased from a local grocery store. The meat (9.0 lb beef trim and 6.0 lb 50/50 pork trim) was ground through 3/16 inch grinder plate and homogenized with ice (3.0 lb), salt, sodium nitrite, non-fat dry milk, and phosphate. The mixture was stuffed into 3-inch fibrous casings and cooked in oven for about 2 hours until the internal temperature reached 69 °C (155 °F). The cooked bologna was cooled to room temperature and stored at 4 °C.

Film application on bologna. Uniform bologna slices (5.25 mm thick and 48 mm diameter, 11 g weight) were cut and one chitosan film was placed between two bologna slices to make a 'sandwich' as described in Part 3. Each 'sandwich' was placed in a petri-dish, sealed with parafilm, and stored at 4 °C. After 5 day-storage, the films and the bologna were separated and analyzed.

Extraction of essential oil from film forming solutions, films, and bologna. A twenty-gram of FFS was poured into glass centrifuge tube and mixed with 15 ml hexane (GC grade, Sigma-Aldrich, Allentown, PA). The mixture was shaken for 30 seconds and left 2 hours at room temperature. The hexane layer was separated and solvent was evaporated at room temperature under research grade nitrogen. Nonane (500 ppm, Sigma-Aldrich, Allentown, PA) was added as internal standard and volume was adjusted with hexane to 1 ml. For extraction of essential oil from the chitosan films, 10 ml hexane was mixed with a film (cut into small pieces) in 50 ml-flask and stirred for 1 hour at room temperature. The extract was decanted, and the residual film pieces were re-extracted with additional 5 ml hexane for 10 min with stirring. Preliminary experiments showed that the first 10 ml hexane extracts about 70 % of principle compound present in material and additional 5 ml extract about 25 % of the total amount. All 15 ml of hexane extract were combined in a glass centrifuge tube and solvent was evaporated under nitrogen (National Welders Supply Co., Knoxville, TN). The internal standard was added as described for FFS and the final volume was adjusted to 1 ml. The same procedure was performed with the films and bologna slices taken from the "sandwich". Minimum three replications for each sample were done throughout the experiment.

Instrumentation (GC/MS and columns). Essential oil composition was analyzed using a Shimadzu GCMS-QP5000 with SPB-1 fused silica column (30 m x 0.25 mm, 0.25 μm, Supelco, WI). The injector port was held at 250 °C. The carrier gas was helium (research grade, National Welder Supply Co., WC) at 1 ml/min constant flow. The oven temperature was set at 60 °C for 0.5 min, then ramped at 5 °C/min to 280 °C and held for 20 min (Diaz-Maroto and others, 2002). The injection volume was 1 μl and the analysis was conducted under splitless mode.

Sensory evaluation. To determine whether the addition of oregano EO to bologna can influence the consumer liking of the product, sensory evaluation was conducted using a 9-point hedonic scales and a just about right test. To mimic the release of essential oil from the films, a sunflower-oregano EO mixture was applied on bologna. Thick sliced bologna (Flavor of South) was purchased from a local store. According to the manufacturer (Bryan Foods, Cincinnati, OH), the composition was: 29 % total fat, 5.4

% total carbohydrates, 10.7 % protein, and 0.9 % sodium. Bologna slices were cut into 43-mm diameter discs and the mixture of food grade oregano essential oil and commercial sunflower oil were applied on one side of the disk. The concentrations of essential oil applied per bologna disc were 0 (control), 45 and 90 ppm. The samples were stored 5 days at 4°C prior to evaluation. All samples, labeled with 3-digit random numbers, were placed on small white paper plates and served immediately after being taken out of a 4 °C refrigerator. Presentation order was balanced to avoid psychological error and the panelists were asked to use low-sodium crackers to clean their palate and rinse with water between each product. Forty eight panelists evaluated the samples and data were analyzed by ANOVA ($p < 0.05$) using Fizz (Biosystems, Couternon, France). Penalty scores were evaluated as least-squares means of three groups scores derived from too much spice intensity, just about right, and too little spice intensity via SAS software.

Results and Discussions

Identification and quantification of oregano essential oil compounds in different matrices. GSMS analysis of oregano essential oil showed that thymol was the dominant compound with 59.57 % of total volatiles detected.

To evaluate the affect of emulsifier (Tween®20) on stability of EO dispersion within the chitosan film forming solution (FFS), EO was extracted from the solutions prepared with and without the emulsifier. The results of GC-MS analysis showed that 757.7 ± 99.7 ppm thymol was extracted from FFS prepared without Tween®20 and only 364.7 ± 39.9 ppm from the FFS with the emulsifier (Figure 1). Additionally, more peaks were detected when the emulsifier was not present in the FFS. The reason for the different levels of thymol detected in correlation with the presence of Tween®20 is in the interaction of the amphiphilic emulsifier's molecule with both chitosan (over hydrophilic part) and oregano essential oil's compounds (over hydrophobic part). These interactions stabilized the system and resulted in significantly lower levels of thymol extracted by the regular procedure. This clearly showed that incorporation of an emulsifier in polysaccharide-EO films, such as chitosan-oregano EO films, may slower losses of volatile compounds of the oil and help controlled release of active compounds during

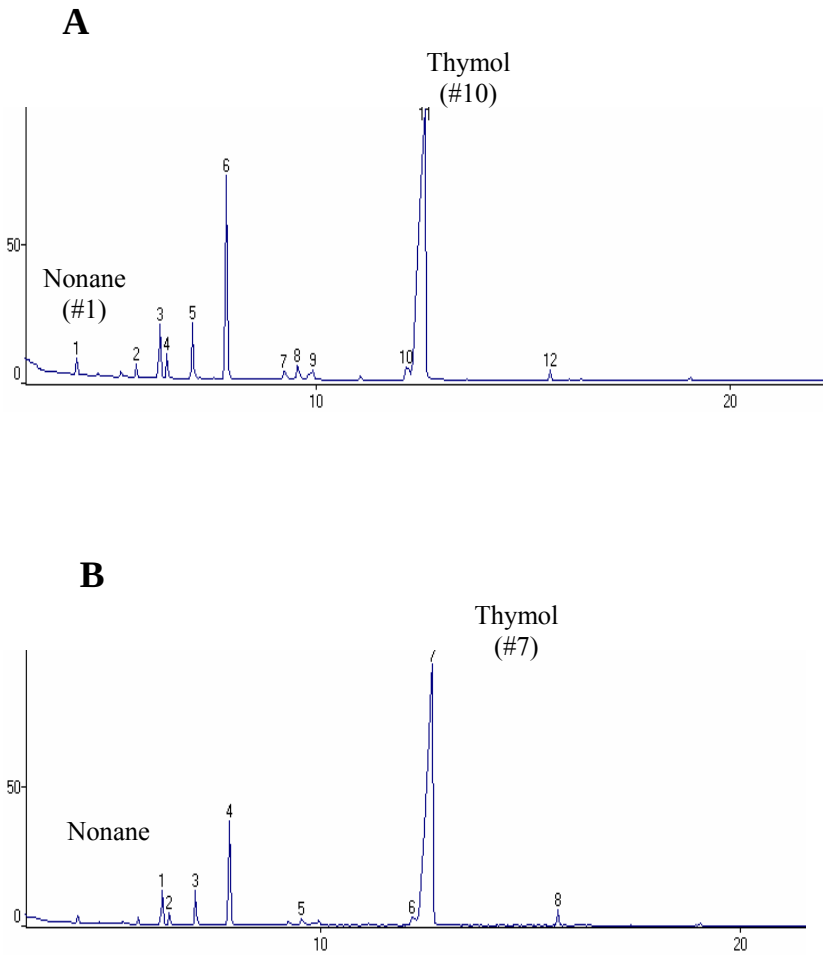


Figure 1. The chromatographs of chitosan–oregano EO film forming solutions (A) without and (B) with Tween®20.

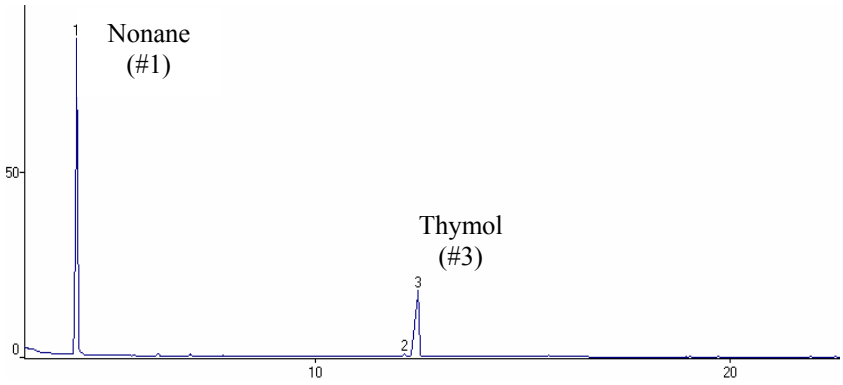
Peaks are shown by retention time (min) vs. total ion intensity.

extended time.

The film forming solution with addition of emulsifier and oregano essential oil was cast at 30 °C under the reduced pressure in the order to obtain chitosan-oregano EO films. The analysis of the resulting films showed significant decrease in detected thymol, only 2.1 ± 0.5 ppm, due to losses of volatile compounds during drying (Figure 2, A). However, after being applied on bologna slices for 5 days, no thymol was extracted from the films but several large peaks appeared with the similar retention times (Figure 2, B). The library search (NIST, Gaithersburg, MD) showed that those compounds had the similar molecular weights to thymol but had different structures. This may mean that degradation (oxidation, isomerization) of thymol occurred over time. Additionally, as seen in Part 3, after 5 days in direct contact with bologna, the films swelled and became rubbery. The presence of the emulsifier in the film matrix probably enhanced absorption of moisture and fats from bologna into the film. This, in turn, improved release of the EO compounds into the bologna as well as in the hexane during the extraction.

Preliminary analyses showed that commercial bologna contained significant amounts of thymol which could have interfered with our analysis. We, thus, prepared bologna with no spices (nor any other flavoring agents beside salt) and used it for determination of diffusion of thymol from the films. The prepared bologna had no detectable volatiles with retention times similar to thymol (Figure 3, A) but after 5 day-application of chitosan-oregano EO films, the thymol concentration on bologna reached 60 ± 13.3 ppm (Figure 3, B). The films used in this study were prepared from FFS with 1 % oregano EO. The same films were prepared for antimicrobial testing (Part 3) and showed 4 logs reduction of *L. monocytogenes* and *E. coli* under similar storage conditions. Compared with the results of Hammer and others (1999), Prudent and others (1995) and Smith-Palmer and others (1998), it appears that concentration of the active compounds expressed on the total product weight (60 ppm) was much lower than MIC of oregano EO (200 to 1200 ppm) for *L. monocytogenes* and *E. coli*. However, it is possible that the active compounds stayed at the surface of the bologna (in the close vicinity of the film) providing the

A



B

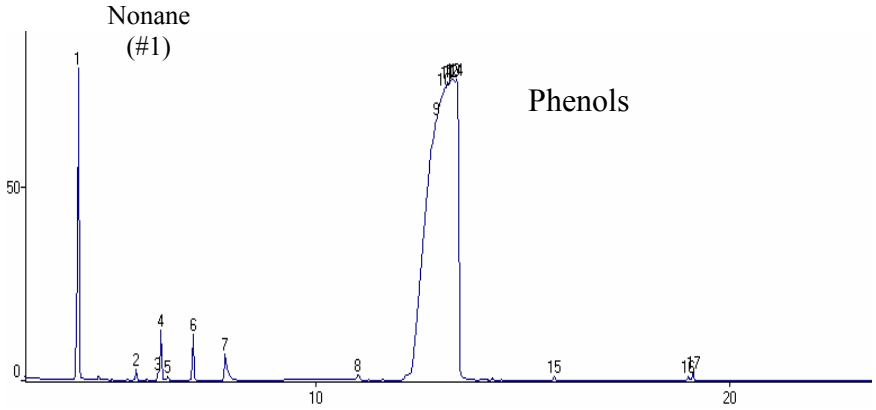


Figure 2. The chromatographs chitosan-oregano EO film samples (A) before and (B) after application on bologna.

Peaks are shown by retention time (min) vs. total ion intensity.

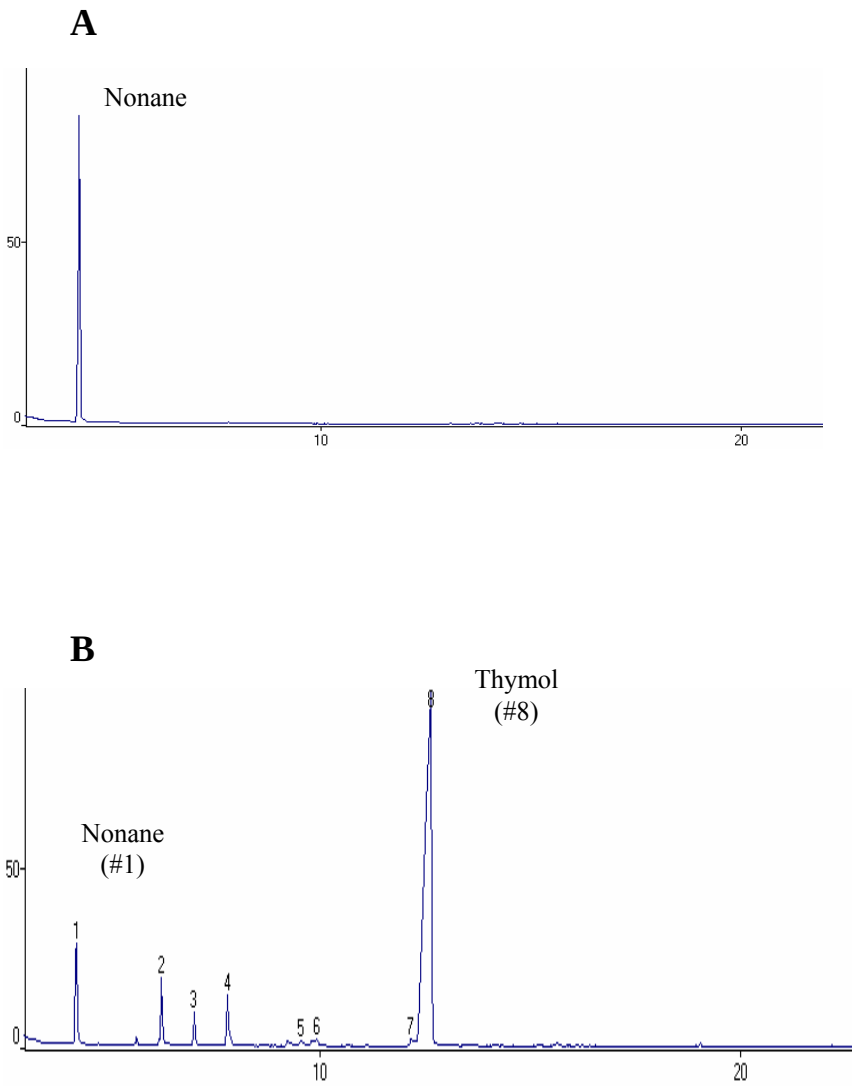


Figure 3. The chromatographs of bologna samples (A) before and (B) after the application of chitosan-oregano EO films.

Peaks are shown by retention time (min) vs. total ion intensity.

antimicrobial effect. These results provide support and justification for the application of oregano essential oil enriched chitosan films in food packaging.

Sensory evaluation. Results of analysis of variance showed that there were no significant differences ($p > 0.05$) among the three samples for hedonic scores for appearance, aroma, and texture. However, there were significant difference among three samples for overall liking, liking of flavor, and liking of the aftertaste (Table 1). The control (plain bologna) had the same liking level of overall, flavor, and after taste as that of the bologna sample with 45 ppm oregano EO. Both the control and the sample with 45 ppm oregano EO were liked more than the 90-ppm oregano sample based on those three attributes (Table 1). Panelists believed that bologna sample with 90 ppm oregano EO had too strong a flavor, and provided comments such as 'like eating straight oregano' and 'almost a burning sensation on the tongue'.

When calculating the frequency distributions of spice intensity, more than 25 % responses were shown on one side (too much intense or too little intense). Therefore, a penalty scores were computed to determine if the scores other than just right had an impact on the means for overall liking.

There was no significant difference between 'too little spice intensity' and 'just right' groups for control (plain bologna), which means that the evaluation of overall liking were alike for the two groups. The overall liking evaluation of group 'too little' and group 'too much spice intensity' were also alike. Panelists who thought the spice intensity was just right liked samples more than those considered there have too much spice intensity (Table 2).

The similar results were obtained with bologna samples with 45 and 90 ppm oregano EO. The evaluations of overall liking by groups that chose 'just right spice intense' and 'too little spice intense' were alike. The overall liking evaluation of group 'too little' and group 'too much spice intensity' were also alike. Panelists who thought the spice intensity was 'just right' liked sample more than those considered there have 'too much spice intensity' (Table 2).

Table 1: Means[a] for overall, flavor, and texture liking of three samples of bologna by 48 consumer panelists using the hedonic test[b.c]

Attribute	Control[d]	45 ppm oregano EO[e]	90 ppm oregano EO[f]
Overall	6.5 A	6.2 A	5.3 B
Flavor	6.4 A	6.1 A	5.1 B
Aftertaste	5.8 A	5.8 A	4.8 B

[a] Means within the same row sharing the same letter are not significantly different ($p > 0.05$).

[b] ANOVA for data analysis (Fizz, Biosystems, Couternon, France).

[c] 1 = dislike extremely, 5 = neither like nor dislike, 9 = like extremely.

[d] Bologna samples with 50 μl sunflower oil.

[e] Bologna samples with 50 μl mixture of oregano EO and sunflower oil (1:100).

[f] Bologna samples with 50 μl mixture of oregano EO and sunflower oil (1:50).

Table 2: Means for overall liking of bologna samples added with oregano essential oil evaluated by 48 panelists based on just about right test[a,b]

Least Square Mean	Too little spice intensity [c]	Just right	Too much spice intensity [d]
Control	6.1 AB (n = 17)	7.1 A (n = 24)	5.1 B (n = 7)
Oregano EO 45 ppm	6.6 AB (n = 7)	7.1 A (n = 22)	5.1 B (n = 19)
Oregano EO 90 ppm	6.0 AB (n = 9)	6.8 A (n = 11)	4.5 B (n = 28)

Least-squares means within the same **row** sharing the same letter are not significant different at the level of p = 0.05.

[a] Analyzed by SAS program.

[b] 1 = dislike extremely, 2 = dislike very much, 3 = dislike moderately, 4 = dislike slightly, 5 = neither like nor dislike, 6 = like slightly, 7 = like moderately, 8 = like very much, 9 = like extremely.

[c] Too little spice intensity group was those who indicated 'much too weak' and 'slightly too weak'.

[d] Too little spice intensity group was those who indicated 'much too intense' and 'slightly too intense'.

Conclusions

The GC-MS analysis showed that the chitosan film was a good matrix for incorporation of volatile compounds such those from oregano essential oil. Addition of the emulsifier Tween®20 further enhanced incorporation of oregano EO into the film and reduced the amount of compounds extracted with a common solvent (hexane). Application of chitosan-oregano EO films on bologna samples resulted in absorption of moisture and fat into the films and release of the active compound (thymol) into the product. The sensory evaluation suggested that 45 ppm oregano EO in bologna would be acceptable by consumers. The results support the use of chitosan-oregano EO films as packaging material for ready-to-eat meat products.

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Part 5

Conclusions

Oregano essential oil exhibited the strongest antimicrobial activity towards *L. monocytogenes* and *E. coli* O157:H7 compared to coriander, basil and anise essential oils (oregano EO >> coriander EO > basil EO > anis EO). Although both tested pathogens were affected, *Listeria monocytogenes* appeared to be more sensitive to active components from oregano EO than *Escherichia coli* O157:H7. Release of essential oils from the chitosan-EO films resulted in antimicrobial effects similar to those of the pure essential oils. Application of chitosan films (with no EO) on bologna slices for 5 days reduced *Listeria* and *E. coli* count from initial 5 x 10^4 cfu/g to 1.2 x 10^2 cfu/g and 6.3 x 10^3 cfu/g, respectively, while chitosan-EO films prepared from films forming solution (FFS) with 1 % oregano EO reduced count of both pathogens for 4 logs. Incorporation of oregano essential oil into chitosan film increased film thickness and elasticity, and reduced its resistance to puncture, tensile strength, and water vapor permeability. Chitosan film prepared from FFS with 1 % oregano EO released 60 ± 13.3 ppm thymol into bologna during 5 day-storage at 4 °C. Based on sensory evaluation conducted by the consumer panel, bologna with 45 ppm thymol would be accepted by consumers.

The overall conclusion of this research is that chitosan films enriched with oregano essential oil present an excellent system for controlled release of active compounds and can be successfully applied for prevention and control of food-borne pathogens on processed meat product.

Vita

This thesis was written towards fulfilling the requirements of the Masters of Science degree. Shuang chi received her Bachelor of Science in Medical Nutrition from the Qingdao University, Medical College. She is currently a Graduate Research Assistant at the University of Tennessee, Knoxville. In August 2004, she will be pursuing a doctoral degree in Food Science at University of Tennessee, Knoxville.