Preparation and Characterization of Antimicrobial Packaging Films from Cricket Chitosan Enriched with *Schisandra Chinensis* Extract

Vita Jarolimkova

MASTER´S THESIS

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Abstract

Chitosan films for food packaging applications were prepared from shrimp and cricket chitosan, enriched with *Schisandra chinensis* extract. Physicochemical and antibacterial properties of the films were studied and compared with references studying similar chitosan films. Chitosan is a biodegradable polymer, mostly harvested from shells of crustaceans, which has been studied for packaging applications, as it can improve shelf life of food products, especially when enriched with antibacterial and antioxidant compounds. The addition of these substances, such as herbal extracts, influences the physicochemical properties of the film, which are important parameters for characterization of packaging material, as it is necessary to assess its ability to fulfill the necessary packaging requirements. It was shown that insect chitosan film has superior or equivalent physicochemical properties and superior antimicrobial properties to shrimp chitosan. Addition of *S. chinensis* extract improved antibacterial and water vapor permeability barrier properties, however resistance to water solubility decreased. The originality of the work lies in the use of extract from *S. chinensis*, which has not been used in films before and which demonstrated antibacterial effect on Gram-positive bacteria, and in harvesting chitosan from crickets (*Gryllus bimaculatus*) as an alternative chitosan source, as all previous experiments with chitosan films have been done with crustacean chitosan. Moreover, during the extraction of insect chitosan, novel and effective decolorization method was discovered.

Executive Summary

Introduction

Renewable, biodegradable and active packaging is currently considered to be a very important part of the research and development in packaging industry. Considering the negative environmental impact of oil-derived plastics as well as food losses due to microbiological spoilage or oxidative quality deterioration, packaging produced and disposed of in a sustainable way which can also prolong food shelf-life is certainly desirable.

Therefore, innovative bioplastics derived from microbial, plant or animal sources have been explored as replacement for conventional plastics. Currently, the main deterrent to mass use of bioplastics is their high production cost and in many cases inferior mechanical and physicochemical properties. These challenges can however be overcome with further research leading to production optimization and to exploration of blends and coatings which could improve the ability of the polymers to fulfill necessary functions.

One of such biopolymers currently being explored as potential source for manufacturing bioplastics is chitosan, which is partially deacetylated form of chitin, one of the most abundant biopolymers on earth. It is currently harvested from crustacean exoskeletons, namely crabs and shrimps, giving thus added value to formerly a waste product. It is a very interesting and versatile material, as it can form films when solubilized in mild acids, homogenized and casted. It has also inherent antioxidant and antibacterial properties.

However, with aquaculture experiencing issues with sustainability, many researchers have started looking for alternative sources of chitin and chitosan. Insect-derived chitosan appears particularly promising, as it was shown to be very similar to crustacean chitosan, displayed antioxidant and antimicrobial properties and it can be harvested as a by-product of insect protein purification.

Chitosan packaging films have fairly good water vapor permeability and oil leakage resistance, oxygen barrier properties and high potential to have very favorable life cycle assessment and reduced price once production is optimized. Their current disadvantage is low resistance to water solubility and pH and difficulty in processing. In terms of active packaging potential, chitosan films have antibacterial and antioxidant properties. Antibacterial properties of chitosan and chitosan films appear to be dependent on many factors, such as molecular weight, deacetylation degree, pH, film preparation method, film properties, food matrix present and the characteristic of the microbial strain.

Antibacterial and antioxidant properties of chitosan films can be further improved by incorporation of natural extracts, many of which appear to be very compatible with chitosan matrix. These substances can have positive or negative impact on the physicochemical and mechanical properties of chitosan films, for which reason different herbal substances are currently being explored.

Two promising plants with antioxidant and antibacterial properties are *Schisandra chinensis* and *Bupleurum falcatum*. These extracts were shown to inhibit a wide spectrum of bacteria, moreover, *S. chinensis* leaves, which are currently discarded as a waste product, have superior antioxidant and antibacterial properties in comparison with the fruits of the plant.

Therefore, the aim of this study was to evaluate the possibility to purify chitosan from new insect with potential as a food protein source (*Gryllus bimaculatus*), assess its ability to form films and enrich those films with antibacterial and antioxidant extracts from the two above mentioned Korean herbs. As far as the author is aware, insect chitosan has never been used to cast films, and *S. chinensis* and *B. falcatum* extracts have never been used in active packaging films either.

Methodology

Reagents were of analytical grade or higher, crickets were obtained from an insect farm and plant material from herbal market and supermarket.

Methodology was based on relevant previous studies whenever available, and when necessary adjusted to fit the scale of chitosan production, which in turn influenced the maximum area of films that could be produced. All adjustments of methodology were explained in detail and taken into consideration during evaluation of results.

All experiments except for chitosan purification and antimicrobial compound extraction were done at least in triplicate with appropriate controls. Statistical analysis was performed by Excel, difference between means was assessed by ANOVA (one-way analysis of variance) with $P < 0.05$ being the threshold for significance.

Results and Discussion

Cricket chitin was successfully purified by a procedure consisting of previously described deproteinization and demineralization method, supplemented with newly discovered decolorization method using substance X. Cricket chitosan was then purified by a novel method optimized by Kyo-Sung Chae. The large scale purification showed that difficulties arise from the scale of the process, mainly due to difficult protein removal. Therefore, the deacetylation degree was relatively lower than the usual deacetylation degree of shrimp chitosan. Molecular weight was estimated from previous experiment conducted by Chae Kyo Sung, and expected to be lower than 7 kDa, which is much lower than commercial shrimp low molecular weight chitosan.

S. chinensis fruit ethanol extract and *B. falcatum* root ethanol and methanol extracts were prepared. The methanol extract was subsequently fractionated into hexane, ethyl acetate, ethyl ether and water fraction. However, the only fractions which were water-soluble and could therefore be used in the following experiments were the two ethanol extracts and *B. falcatum* root methanol extract water fraction. Total polyphenols of these three fractions

were measured. *S. chinensis* fruit ethanol extract contained the highest level of polyphenols, which was comparable to other studies. *B. falcatum* root extracts were found to be less rich in polyphenols, however no similar studies were conducted previously. The high level of polyphenols in fruits of *S. chinensis* was considered to be also indicative of the possible even higher level of polyphenols in leaves. As total polyphenols have been shown to correlate well with antioxidant activity, the results were considered to indicate high possibility that the higher polyphenol content is indicative of higher antioxidant activity, and as protection from oxidation is a desirable feature of active packaging, the two ethanol extracts with higher levels of polyphenols were selected for further testing.

S. chinensis fruit ethanol extract and *B. falcatum* root ethanol extracts were then standardized by dilution with distilled water to contain equal levels of polyphenols and their minimum inhibitory concentration was tested against two Gram-positive (*B. cereus* and *L. monocytogenes*) and two Gram-negative (*E. coli* and *V. parahaemolyticus*) strains by disc-diffusion method. *V. parahaemolyticus* failed to grow, which was likely caused by adverse incubation conditions. *E. coli* was not susceptible to any extract at any concentration tested (up to 0.750 mg total polyphenols/ml), however *L. monocytogenes* was inhibited by *S. chinensis* extract at 0.750 mg total polyphenols/ml and *B. cereus* by 0.500 mg total polyphenols/ml. While higher susceptibility of Gram-positive strains to *S. chinensis* extract was expected, literature review suggested that Gram-negative strains should have been inhibited, albeit at higher concentration. The lack of antibacterial activity of *B. falcatum* extract was surprising, as ethyl ether and ethyl acetate fractions of ethanol extract were reported in literature to possess very strong antibacterial activity against both Gram-positive and Gram-negative strains. It appears that the antimicrobial compounds were not extracted in sufficient quantity by ethanol extraction. Based on these results, *S. chinensis* fruit extract was considered to be a more suitable candidate for incorporation in chitosan films, also as *S. chinensis* leaves were shown previously to possess even stronger antimicrobial activity than fruits. It was decided that *S. chinensis* fruit extract would be incorporated in the films at 0.750 mg total polyphenols/ml film-forming solution, which was equivalent to approximately 4% (w/w) of total solids.

Subsequently, four types of films were casted: film prepared from cricket chitosan, film prepared from shrimp chitosan, film prepared from cricket chitosan enriched with *S. chinensis* extract and film prepared from shrimp chitosan enriched with *S. chinensis* extract. In all cases, peelable films were formed, which proved that cricket chitosan has film-forming properties. During the experiment, it was observed that cricket chitosan film-forming solutions had higher viscosity, possibly due to lower deacetylation degree, and that solutions containing *S. chinensis* extract had higher wetting properties, likely due to interaction with the chitosan hydroxyl and amino groups.

Shrimp chitosan film without extract did not exhibit any antibacterial activity, cricket chitosan film without extract inhibited *B. cereus* after prolonged incubation, but not other strains. This results is most likely caused by the ability of very low molecular weight chitosan to diffuse from the film, while higher molecular weight shrimp chitosan is tightly bound within the film matrix. Films infused with *S. chinensis* extract inhibited both Gram-positive strains, with *L. monocytogenes* seeming sensitive only to *S. chinensis* extract and

B. cereus sensitive to the extract and also cricket chitosan. Compared to other antibacterial chitosan films prepared, *S. chinensis* extract at 4% concentration performs better than certain essential oil which have been used for film enrichment, but in some cases is inferior.

As for physicochemical properties, cricket and shrimp chitosan films were comparable in terms of thickness, density, moisture content, water vapor permeability, but differed in water solubility and swelling degree, possibly due to difference in deacetylation degree. Color was also different, possibly due to different color of the chitosan powder used to prepare the gels.

Addition of *S. chinensis* extract led to increase in thickness, density, water solubility, swelling degree, and moisture content. On the other hand, water vapor permeability was decreased. Such behavior was observed in studies with similar hydrophilic compounds as can be explained by the extract fitting within the matrix and binding to chitosan amino and hydroxyl bonds through hydrogen bonds, thus increasing thickness and density and preventing water from binding to these groups and decreasing moisture content. Less intermolecular hydrogen bonding also makes the gel more readily soluble in water and less resistant to swelling. The extract binding to chitosan polar groups also limits water solubility in the film and the high density decreases void space through which water could diffuse. Upon incorporation of the extract, color also changed into red-brown.

Conclusions

The present study showed that cricket chitosan can be purified and used to prepare thin films enriched with *S. chinensis* extract suitable for use as packaging polymers. Shrimp chitosan was also compatible with the extract. The prepared films enriched with *S. chinensis* extract manifested antibacterial activity against Gram-positive bacteria and pure cricket chitosan film also inhibited *B. cereus*. Concerning physicochemical properties, cricket and shrimp chitosan films were comparable in terms of thickness, density, moisture content, water vapor permeability, but cricket chitosan had lower water solubility and swelling degree, which means cricket chitosan gels might be more resistant when used for packaging of food with higher moisture content. Color was also different, possibly due to different color of the chitosan powder used to prepare the gels. Addition of *S. chinensis* extract led to increase in thickness, density, water solubility, swelling degree, and moisture content. On the other hand, water vapor permeability was decreased. Upon incorporation of the extract, color also changed into red-brown. *S. chinensis* extract therefore improved barrier properties against water vapor, however it also made the films less resistant to solubilization in water. The darkened color might possibly influence light absorbance properties, but that could not be assessed in the present study. Antioxidant activity of the films was not measured, but it can be expected to correlate with the total polyphenol content in the films enriched with extract.

Additionally, novel decolorization method of highly pigmented cricket chitin was discovered in the process of chitosan purification, which is relatively safe, economical and does not require harsh conditions. Moreover, behavior of the film-forming solution

suggested that rheological properties of chitosan solutions might be dependent strongly on deacetylation degree.

In summary, it was shown that cricket chitosan is equivalent or superior (in the case of water solubility as well as antibacterial activity against *B. cereus*) to shrimp chitosan as source for chitosan packaging films and that addition of *S. chinensis* fruit extract improves water vapor barrier properties and antimicrobial properties, however the films become less resistant to water.

List of Abbreviations

DDA	Degree of deacetylation/Deacetylation degree
FT-IR	Fourier-Transform Infrared Spectroscopy
Mw	Molecular weight
LMW	Low molecular weight
HMW	High molecular weight
MC	Moisture content
MIC	Minimum Inhibitory Concentration
N/A	Not available
SD	Swelling degree
WVP	Water vapor permeability
WS	Water solubility
WVTR	Water vapor transmission

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1 Introduction

1.1 Renewable and Biodegradable Food Packaging

In the global quest for sustainability, biodegradable packaging based on renewable resources is a very topical subject, as it has lower environmental impact. Bioplastics market is expected to grow by almost 37% between 2014 and 2020 (Persistence Market, 2014) and it has been suggested that in the near future, most oil-derived plastics, will be replaced by plastics derived from renewable sources (Chen, 2014), which will also be supported by consumer demand for more "natural" packaging (Nayak, 2014). The trend seems to be particularly pronounced for food packaging (Siracusa, Rocculi, Romani & Rosa, 2008). This appears to be a very likely scenario, as Carlsberg, one of the leading beer companies, has just recently announced the start of a project to develop fully bio-based and biodegradable bottle (2015a) and AIMPLAS, plastics research and development service provider, is developing novel bio-degradable packaging for fresh foods (2014). Already nowadays, certain foods are packed in biopolymers, such as fruits and vegetables and long shelf-life foods which do not require particularly high water and oxygen barrier properties (Peelman et al., 2013).

Bio-based and biodegradable are parameters which can, but do not have to, go hand in hand. While bio-based polymers originate from renewable biological sources, biodegradability is not linked to the origin of the monomers comprising the packaging polymer, but to the chemical structure (Chen, 2014), which determines whether microbial degradation is possible or not (Singh, Kaushik & Biswas, 2014). Compostability is particularly desirable, as it is not as energy-intensive as plastic recycling and it contributes to soil fertilization (Siracusa, Rocculi, Romani & Rosa, 2008).

There are two major concerns limiting the use of biodegradable bioplastics. One of the challenges of bioplastics development are inferior mechanical, barrier and physicochemical properties compared to oil-derived plastics, which can be improved by additional coatings, blending biopolymers with other materials such as nanoparticles or blending together different biopolymers, and by physical or chemical modification of polymers (Peelman et al., 2013). The other one is the production cost. Bioplastics are more expensive to manufacture than conventional plastics (Peelman et al., 2013; Siracusa, Rocculi, Romani & Rosa, 2008). Therefore, research has been focused on overcoming these challenges in order to make biodegradable bioplastics more appealing as an alternative to oil-based plastics (Kumar, 2011).

1.2 Active Food Packaging for Microbial Growth and Oxidation Control

Another current trend in the packaging industry is active packaging, which is packaging that can interact with its environment or contents, and particularly packaging prolonging shelf-life (Chen, 2014; Dobrucka, 2013) due to reduced oxidation and microbial spoilage (Nayak, 2014).

Such properties are highly desirable and useful, as up to 40% of the food produced worldwide is wasted due to microbial spoilage or loss of quality. Even though food losses occur at different stages in the supply chain in developed versus developing countries, inability of packaging and storage conditions to prevent microbial growth and nutrient oxidation is an issue common to both (Gustavsson, Cederberg, Sonesson, Van Otterdijk & Meybeck, 2011).

Moreover, control of foodborne diseases caused by bacteria is still challenging. The incidence of many infections does not show a particularly decreasing trend and is often above target levels to which governments are attempting to bring the number of incidents per year. For example *Vibrio* infections in the US increased in 2013 compared to previous years, and *Listeria* infection rates remained unchanged (Crim et al., 2014). In South Korea, total incidence of foodborne diseases, and particularly outbreaks concerning for example *Bacillus cereus* and *Escherichia coli*, increased in the period of 2006 – 2010 compared to 2001 – 2005 (Chon, Lee, Lee, Bang & Seo, 2014). Foodborne diseases linked to fresh produce in particular are continuously persisting in both US and EU, and do not seem to be diminishing (Callejón, Rodríguez-Naranjo, Ubeda, Hornedo-Ortega, Garcia-Parrilla & Troncoso, 2015). Foodborne pathogens pose an alarming threat in all developing economies – South East Asia, Central and South America, and Africa (Akhtar, Sarker & Hossain, 2014). Elderly and children are especially vulnerable groups with higher death rates (Crim et al., 2014), which is of major concern as the population ages. It is also expected that foodborne diseases will become more prevalent with increasing global warming due to environmental and behavioral factors (Schuster-Wallace, Dickin & Metcalfe, 2014). Also, lipid and protein oxidation has been linked to quality deterioration (Domiszewski, Plust & Wasilewska, 2013; Muela, Monge, Sañudo, Campo & Beltrán, 2015; Scheidegger, Radici, Vergara-Roig, Bosio & Pesce, 2013; Tabee, 2008; Zhang, Xiao & Ahn, 2013).

1.3 Potential of Chitosan Films as Renewable, Biodegradable and Active Packaging with Antimicrobial and Antioxidant Properties

1.3.1 Basic Characterization of Chitin, Chitosan and Chitooligosaccharides

Chitin, poly (β-(1→4)-N-acetyl-D-glucosamine (Figure 1), is a polysaccharide commonly found in the arthropod exoskeletons, such as shells of crabs and shrimps (which are a waste product of aquaculture) or insects, in fungal cell walls and in *Saccharomyces cerevisiae* (Nayak, 2014; Rinaudo, 2006). It is one of the most abundant natural polymer after cellulose, and the most common form is α-chitin, which is present in crabs, shrimps and insects as well as fungi and yeasts (Rinaudo, 2006). For commercial applications, chitin is obtained from crustacean shells, of which it comprises 15 – 20% (Mathur & Narang, 1990). It is purified from exoskeletons by deproteinization via alkaline treatment, demineralization by acids, and decolorization, however the procedure needs to be adapted to each source due to differences in composition (Rinaudo, 2006).

Chitosan (Figure 1) can be obtained by partial deacetylation (at least 50%) of chitin, commonly through treatment with concentrated sodium hydroxide at high temperature (Mathur & Narang, 1990; Nayak, 2014; Rinaudo, 2006). Commercial chitosan produced from crab and shrimp shells has deacetylation degree (DDA) equal to 70 – 90% (Mathur & Narang, 1990) and molecular weight (Mw) ranging from 50 to 2000 kDa, the high variability in Mw being caused by low control of chitosan purification process during which partial depolymerization occurs (Rege, Garmise & Block, 2003). Chitosan is mostly insoluble at neutral and basic pH (Nayak, 2014), however it can be solubilized in weak acids and the solubility is dependent on many factors regarding the chitosan and the solvent (Rinaudo, 2006). Chitosan has been shown to have antioxidant (Yen, Yang & Mau, 2008) and antibacterial (Giner et al., 2012) properties. Chitooligosaccharides, which are very short chitosan chains with low molecular weight and low degree of polymerization, were also shown to exhibit antioxidant (Yang, Shu, Shao, Xu & Gu, 2006) and antibacterial properties (Choi, Kim, Yoo, Oh, Choi & Kim, 2001).

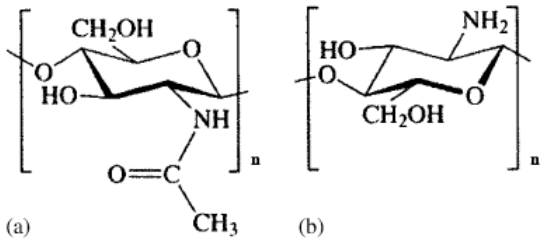

Figure 1 Chitin (a) and chitosan (b) structure (Rinaudo, 2006)

1.3.2 Insects as an Alternative Source of Chitosan

Until the present day, the vast majority of research has been concentrated on crustacean chitosan and its applications. Possibly the main reason for that is the abundance of shrimp and crab shells as waste products of aquaculture, and the content of chitin in these shells (30%) (Kerton, Liu, Murphy & Hawboldt, 2014) The potential of insects as an alternative source of chitosan has only been gathering attention recently and remains a rather little explored topic. Yet the studies available suggest that insect chitosan is very suitable for the same applications as chitosan purified from shrimps and crabs. Harvesting chitosan from insects could thus become an interesting alternative to relying on aquaculture, which has in its current state created negative environmental impact (such as destruction of mangroves and marshes leading to ecosystem destabilization, use of antibiotics with possible leakage in surrounding wild ecosystems, and improper sediment disposal) and experienced decline in production due to poor planning and management (Paez-Osuna, 2001; Páez-Osuna, 2001).

Insect chitin and chitosan seem to be in general very similar to shrimp chitosan. Chitin occurs in the alpha form (Kaya, Baran, Erdoğan, Menteş, Özüsağlam & Çakmak, 2014; Liu et al., 2012) and appears to have comparable physicochemical properties (Liu et al., 2012). Chitosan prepared from housefly larvae was comparable to food-grade crustacean chitosan (MiAo & Wu, 2011).

One difference however appears to be generally lower molecular weight of insect chitosan compared to shrimp chitosan. Insect chitosan seems to have low molecular weight ranging from 2.6 kDa for Colorado potato beetle (Kaya, Baran, Erdoğan, Menteş, Özüsağlam & Çakmak, 2014) to 501 kDa of blowfly chitosan (Song, Yu, Zhang, Yang & Zhang, 2013). It was also shown that very short chitooligosaccharides (polymerization degree of 4.5) can be prepared by further treatment of cicada chitosan (Wu, Pan, Wang & Wu, 2013).

Chitosan prepared from insects has antioxidant properties, as demonstrated by studies with housefly larvae, (Ai, Wang, Xia, Chen & Lei, 2012; Ai, Wang, Yang, Zhu & Lei, 2008), Colorado potato beetle adults and larvae, though adults had higher activity (Kaya, Baran, Erdoğan, Menteş, Özüsağlam & Çakmak, 2014), and blowfly (Song, Yu, Zhang, Yang & Zhang, 2013).

It has also been tested against microbial pathogens with positive results, as housefly larvae chitosan displayed antibacterial (Lai, Lei, Niu, Zhong & Jiang, 1998), antifungal (Ai, Wang, Xia, Chen & Lei, 2012) and antiviral (Ai, Wang, Xia, Chen & Lei, 2012) properties. Colorado potato beetle chitosan demonstrated antibacterial properties, with higher activity of adult beetle chitosan (Kaya, Baran, Erdoğan, Menteş, Özüsağlam & Çakmak, 2014). Chitooligosaccharides prepared from cicadas showed very good antibacterial properties as well (Wu, Pan, Wang & Wu, 2013).

Chitosan is usually purified from insects by similar methods that are used with crustaceans. Deproteinization is carried out with a strong base at high temperature (Ai, Wang, Yang, Zhu & Lei, 2008; Gu et al., 2010; Kaya, Erdogan, Mol & Baran, 2015; Liu et al., 2012; Song, Yu, Zhang, Yang & Zhang, 2013; Wu, Pan, Wang & Wu, 2013; Zhang, Haga, Sekiguchi & Hirano, 2000). Decolorization is performed with substances such as potassium permanganate (Ai, Wang, Yang, Zhu & Lei, 2008; Liu et al., 2012), mixture of chloroform, methanol and water (Kaya, Erdogan, Mol & Baran, 2015), mixture of hydrogen peroxide and hydrochloric acid (Majtán, Bíliková, Markovič, Gróf, Kogan & Šimúth, 2007), or sodium hypochlorite (Song, Yu, Zhang, Yang & Zhang, 2013). Demineralization is then done with acid (Ai, Wang, Yang, Zhu & Lei, 2008; Gu et al., 2010; Kaya, Erdogan, Mol & Baran, 2015; Liu et al., 2012; Majtán, Bíliková, Markovič, Gróf, Kogan & Šimúth, 2007; Song, Yu, Zhang, Yang & Zhang, 2013; Wu, Pan, Wang & Wu, 2013; Zhang, Haga, Sekiguchi & Hirano, 2000). Dacetylation then follows with sodium hydroxide at high temperature (Ai, Wang, Yang, Zhu & Lei, 2008; Gu et al., 2010; Song, Yu, Zhang, Yang & Zhang, 2013; Wu, Pan, Wang & Wu, 2013; Zhang, Haga, Sekiguchi & Hirano, 2000). The exact procedure, however, needs to be adjusted depending on the insect source, as the composition of insects differs (Barker, Fitzpatrick & Dierenfeld, 1998; MiAo & Wu, 2011; Mlcek, Borkovcova, Rop & Bednarova, 2014). The yields of chitin isolation from insects are lower than form crustaceans (Majtán, Bíliková, Markovič, Gróf, Kogan & Šimúth, 2007). It also appears that use of adult insects leads to higher yields in chitosan isolation as well as higher purity than larvae and it appears to have also superior properties (Kaya, Baran, Erdoğan, Menteş, Özüsağlam & Çakmak, 2014).

An extremely interesting point appears to be the possibility to obtain chitin-rich powder as a by-product of insect protein purification process developed to produce tofu-like product called C-Fu (Cadesky, 2015). This could significantly simplify the purification process, as most proteins are harvested for the product and removed from the meal, and it also improves sustainability of the process, as proteins present in the insects are not wasted. With this in consideration, while use of insects that are considered as pests is one way to approach the sustainability of insect chitosan production (Kaya, Baran, Erdoğan, Menteş, Özüsağlam & Çakmak, 2014), use of cricket, an insect with potential for commercial rearing for human consumption as it has good nutritional content and is already widely consumed in Asia (Barker, Fitzpatrick & Dierenfeld, 1998; Srinroch, Srisomsap, Chokchaichamnankit, Punyarit & Phiriyangkul, 2015) and coupling chitosan production with protein harvesting, is another sustainable approach.

1.3.3 Chitosan as a Source for Renewable and Biodegradable Plastic Films with Antimicrobial and Antioxidant Properties

1.3.3.1 Advantages and Disadvantages of Chitosan Films

Chitosan can be used to produce gels, films, fibers and sponges (Rinaudo, 2006) and it has been applied in medicine, agriculture, waste treatment, and food and cosmetic industry

(Rinaudo, 2006). (Chen, 2014) suggests that the term “bioplastics” is reserved for novel and innovative types of polymers derived from biological sources, which can, but do not have to be, biodegradable. Chitosan films can therefore be justly categorized as bioplastics.

There are several advantages of chitosan-based packaging polymers. They have been prepared from aquaculture by-products, as the main source of chitin and chitosan are shrimp and crab shells, adding value to waste material (Leceta, Guerrero, Cabezudo & de la Caba, 2013). They are biodegradable, with biodegradation requiring 90 – 150 days depending on the properties of the films and on the microorganisms present (Srinivasa & Tharanathan, 2007). In total life cycle assessment of chitosan films in comparison with life cycle of polypropylene, chitosan films had significantly lower impact in the categories of carcinogen generation and fossil fuel use (Leceta, Guerrero, Cabezudo & de la Caba, 2013). From the point of view of functionality, chitosan-based films have moderate resistance to water vapor permeability, superior barrier properties against oxygen transfer compared to low-density polyethylene and many bioplastics (though inferior compared to other kinds of plastics), and good barrier properties against oil and fat leakage (Kittur, Kumar & Tharanathan, 1998). Chitosan films also possess antioxidant properties (Altiok, Altiok & Tihminlioglu, 2010) and antimicrobial activity (Coma, Deschamps & Martial-Gros, 2003), which makes them good candidates for active packaging.

The main disadvantages of materials based on chitosan are low resistance to water (due to hydrophilic nature of chitosan which makes it water-soluble) and pH (as chitosan films are commonly soluble at acidic pH and very LMW films even at physiological pH) (Lim, Lee, Israelachvili, Jho & Hwang, 2015; Rinaudo, 2006) and the difficulty in processing them the same way as conventional plastics (Pelissari, Yamashita & Grossmann, 2011). Chitosan films are also significantly more expensive (at 15.5 USD/kg) than both oil-based plastics (estimated to cost around 2 USD/kg) and corn starch or cellulose-based plastics (0.8 to 1 USD/kg), which however can be a tolerable cost level for food packaging (Srinivasa & Tharanathan, 2007). As for their environmental impact, they perform worse than polypropylene in terms of land use, environmental charge, respiratory inorganics and minerals, however it should be considered that this is a result of not yet optimized larger-scale production process, and it is very likely that energy use as well as processing additives requirements will be lowered once production is adjusted (Leceta, Guerrero, Cabezudo & de la Caba, 2013). Mechanical properties are also inferior to those of conventional plastics (Kittur, Kumar & Tharanathan, 1998). Mechanical properties of chitosan films are affected by numerous factors, such as solvent used to dissolve the chitosan prior to film casting, deacetylation degree, pH (Kim, Son, Kim, Weller & Hanna, 2006), plasticizer type and content (Suyatma, Tighzert, Copinet & Coma, 2005), which implies that such factors must be carefully controlled during possible commercial film preparation in order to provide reasonable protection of the product.

1.3.3.2 Factors Influencing Antimicrobial Properties of Chitosan and Chitosan Films

A short overview of factors influencing antimicrobial activity of chitosan and chitosan films is provided in Table 1.

Many authors have observed stronger antimicrobial effect of Crustacean-derived chitosan films and edible coatings against Gram-positive than Gram-negative bacteria (Coma, Deschamps & Martial-Gros, 2003; Jridi et al., 2014; No, Young Park, Ho Lee & Meyers, 2002). Chitosan films have been shown to be highly effective against *Listeria monocytogenes*, including studies in food systems, such as chilled pork loins (Paparella, Serio, Di Pasquale, De Nicola & Chaves-Lopez, 2011). Other studies did not observe a significant difference in inhibition between the two types (Anaya, Cárdenas, Lavayen, García & O'Dwyer, 2013). And certain authors even observed higher effect against Gram-negative bacteria (Devlieghere, Vermeulen & Debevere, 2004). It has been shown, however, that method of film preparation influences antimicrobial activity of chitosan films (Anaya, Cárdenas, Lavayen, García & O'Dwyer, 2013). For example, water activity level (depending on drying) influences the number of active sites that can interact with the microorganisms (Arancibia, López-Caballero, Gómez-Guillén, Fernández-García, Fernández-Martín & Montero, 2015). Such film properties have been linked with the level of activity towards Gram-positive as compared to Gram-negative bacteria. Also, film-forming solution was shown to have higher antibacterial effect than resulting film, possibly due to lower availability of the functional groups in the dry film (Arancibia, López-Caballero, Gómez-Guillén, Fernández-García, Fernández-Martín & Montero, 2015).

For chitosan solutions (not necessarily intended for film preparation), similar discrepancy exists, suggesting that the form of solution or a film does not particularly influence the specificity of antimicrobial effect. In some cases stronger effect against Gram-negative than Gram-positive strains was observed (Younes, Sellimi, Rinaudo, Jellouli & Nasri, 2014), while fungi were inhibited more than both kinds of bacteria (Younes, Hajji, Frachet, Rinaudo, Jellouli & Nasri, 2014). Other studies however showed higher effect against Gram-positive than Gram-negative bacteria (Jeon, Park & Kim, 2001; Jung, Youn, Lee, No, Ha & Prinyawiwatkul, 2010; No, Young Park, Ho Lee & Meyers, 2002; Tao, Qian & Xie, 2011).

Correlation between molecular weight and activity was observed by some authors. Jeon, Park & Kim, 2001 concluded that minimum molecular weight that can inhibit microorganisms is 10 kDa, however Fernandes et al., 2008b demonstrated that chitooligosaccharides shorter than 2 kDa can inhibit bacterial growth and cicada chitooligosaccharides of very low molecular weight had antibacterial properties as well (Wu, Pan, Wang & Wu, 2013). LMW chitosan was shown to inhibit Gram-negative bacteria more effectively (Eaton, Fernandes, Pereira, Pintado & Xavier Malcata, 2008; Fernandes et al., 2008a; Kim, Min, Kim, Kimmel, Cooksey & Park, 2011; Liu et al., 2006; Younes, Hajji, Frachet, Rinaudo, Jellouli & Nasri, 2014; Younes, Sellimi, Rinaudo, Jellouli

& Nasri, 2014), while higher molecular weight correlated with higher inhibitory effect against Gram-positive bacteria and fungi (Chen, Chung, Woan Wang, Chen & Li, 2002; Fernandes et al., 2008a; Younes, Hajji, Frachet, Rinaudo, Jellouli & Nasri, 2014). However with this factor as well, there have been reports of the reverse. No, Young Park, Ho Lee & Meyers, 2002 documented higher activity against Gram-negative bacteria if higher molecular weight of chitosan was used, while Gram-positive bacteria were inhibited by a wide range of molecular weights and Chen, Chung, Woan Wang, Chen & Li, 2002 observed Gram-negative bacteria being inhibited by both low and high molecular weight chitosan. This could be explained by other properties of the chitosans tested being different (such as degree of deacetylation).

Table 1 Summary of factors influencing chitosan and chitosan films antimicrobial activity

Molecular Weight	Controversial
Degree of Deacetylation	Increased effect with increasing degree of deacetylation
pH	Must be below 6, highest effect between pH 3 and 4
Origin of chitosan	Fungal, crustacean and insect chitosan all have antimicrobial properties
Film properties	Little explored
Proteins in food matrix	Good activity if pH is below isoelectric point of the protein
Sodium chloride in food matrix	Controversial
Oil in food matrix	No effect
Bacterial strain	Individual differences possibly more important than belonging to Gram-positive or Gram-negative groups

Degree of deacetylation also seems to co-influence the antibacterial effect of chitosan, with higher degree of deacetylation leading to higher effect (Benhabiles, Salah, Lounici,

Drouiche, Goosen & Mameri, 2012; Chen, Chung, Woan Wang, Chen & Li, 2002; Younes, Sellimi, Rinaudo, Jellouli & Nasri, 2014).

Also pH influences the antibacterial activity of chitosan and it should be below 6 (isoelectric point of chitosan) for the amino groups to be protonated (Chung, Wang, Chen & Li, 2003; Helander, Nurmiaho-Lassila, Ahvenainen, Rhoades & Roller, 2001; Younes, Sellimi, Rinaudo, Jellouli & Nasri, 2014). While good antibacterial effect was observed around pH 4, it decreased at pH 6 (Devlieghere, Vermeulen & Debevere, 2004), and Chung, Wang, Chen & Li, 2003 observed the same trend, with highest inhibition at pH 3, which slowly decreased with increasing pH until pH 6, followed by a large decrease in activity at pH 7. The inhibitory effect at pH 1 and pH 2 was similar to that at pH 5 and pH 4, respectively. Similarly, it was reported that acid-soluble chitosan has higher antimicrobial effect than water-soluble chitosan (Jung, Youn, Lee, No, Ha & Prinyawiwatkul, 2010).

Similar trends have been observed with fungal chitosan, as antibacterial effect increased with higher deacetylation degree, lower molecular weight and lower pH (Tayel, Moussa, Opwis, Knittel, Schollmeyer & Nickisch-Hartfiel, 2010) and Gram-positive bacteria was found to be more susceptible to chitosan effect (Jeihanipour, Karimi & Taherzadeh, 2007; Tayel, Moussa, Opwis, Knittel, Schollmeyer & Nickisch-Hartfiel, 2010). However, Jeihanipour, Karimi & Taherzadeh, 2007 observed overall lower antimicrobial effect of fungal chitosan as compared to crustacean chitosan. Insect chitosan, on the other hand, exhibited very good antibacterial properties (Ai, Wang, Yang, Zhu & Lei, 2008; Kaya, Baran, Erdoğan, Menteş, Özüsağlam & Çakmak, 2014; Lai, Lei, Niu, Zhong & Jiang, 1998; Wu, Pan, Wang & Wu, 2013).

Some authors argue, that it is impossible to clearly conclude whether in general there is stronger effect against a particular group of bacteria (Arancibia, López-Caballero, Gómez-Guillén, Fernández-García, Fernández-Martín & Montero, 2015; Devlieghere, Vermeulen & Debevere, 2004), and the results might be dependent on the particular strain tested (Younes, Sellimi, Rinaudo, Jellouli & Nasri, 2014). They also question the influence of molecular weight and deacetylation degree (Arancibia, López-Caballero, Gómez-Guillén, Fernández-García, Fernández-Martín & Montero, 2015; Devlieghere, Vermeulen & Debevere, 2004). Jung, Youn, Lee, No, Ha & Prinyawiwatkul, 2010 observed that chitosan of different molecular weight and deacetylation degree inhibited completely or almost completely all bacteria tested and did not find a particular correlation between molecular weight and antibacterial effect, except for particular strains and particular deacetylation degree. Similarly, there was no relationship between deacetylation degree and antimicrobial activity. This experiment therefore also supports the opinion that the level of activity is a result of a particular combination of several factors – namely, microbial strain, molecular weight and deacetylation degree.

As for interaction with food matrix, gelatinized starch was shown to decrease the antimicrobial effect of chitosan, possibly due to protective effect or electrostatic interaction with the amino groups (Devlieghere, Vermeulen & Debevere, 2004).

Effect of proteins is dependent on the pH of the environment and the isoelectric point of the protein. If pH is lower than the isoelectric point, chitosan still exhibits good antimicrobial effect as amino groups of chitosan and side-chain groups of proteins are protonated, and their interactions are also restricted as a consequence (Devlieghere, Vermeulen & Debevere, 2004).

Addition of NaCl and increased ionic strength increases solubility of chitosan (Chung, Wang, Chen & Li, 2003; Devlieghere, Vermeulen & Debevere, 2004). However, its effect on antimicrobial activity has not been shown clearly yet. Devlieghere et al., 2004 described that it decreases its activity. Two phenomena occur; negatively charged chloride anions interact with positively charged chitosan amino groups, hence reducing the activity. At the same time, the sodium cations compete for binding at the bacterial cell wall with chitosan. Yet Chung, Wang, Chen & Li, 2003 observed increased activity of chitosan when NaCl was added to the solution.

Addition of oil had no effect on the activity (Devlieghere, Vermeulen & Debevere, 2004).

1.3.3.3 Mode of Action of Chitosan

Experiments suggested that the mode of action against Gram-negative bacteria of chitosan at low pH is binding to their outer membrane (containing negatively charged phospholipids) due to its positively charged amino groups, hereby reducing its barrier properties (Helander, Nurmiaho-Lassila, Ahvenainen, Rhoades & Roller, 2001; Li, Feng, Yang, Fu, Wang & Su, 2010; Tao, Qian & Xie, 2011). Moreover, the permeabilization of the membrane makes bacteria susceptible to other environmental stresses, leading to a synergic effect (Helander, Nurmiaho-Lassila, Ahvenainen, Rhoades & Roller, 2001). Observation of morphological changes in the cell wall of bacteria exposed to chitosan showed that morphology of Gram-negative bacteria is affected more severely, while few changes can be observed in Gram-positive bacteria, which can be related to the different thickness of peptidoglycan layer (Eaton, Fernandes, Pereira, Pintado & Xavier Malcata, 2008). In some cells, chitosan also led to release of intracellular DNA and RNA (Li, Feng, Yang, Fu, Wang & Su, 2010; Wang et al., 2012), however proteins were not released in the study of Wang et al., 2012 while Li, Feng, Yang, Fu, Wang & Su, 2010 observed release of proteins into the medium. The protoplasts also became more concentrated than in normal physiological state (Wang et al., 2012). Yet in other cells, the membrane thickened to prevent such release, leading also to blocking the possibility to absorb molecules form the environment (Li, Feng, Yang, Fu, Wang & Su, 2010; Wang et al., 2012). The interaction of chitosan with bacterial membranes is therefore believed to be very complex, with many processes happening simultaneously (Wang et al., 2012).

1.3.3.4 Use of Natural Extracts and Essential Oils for Improvement of Antioxidant and Antibacterial Properties of Chitosan Films

The antimicrobial effect can be further enhanced by addition of other agents such as plant essential oils (Georgantelis, Ambrosiadis, Katikou, Blekas & Georgakis, 2007; Valencia-Chamorro, Palou, del Río & Pérez-Gago, 2011; van den Broek, Knoop, Kappen & Boeriu, 2014) and extracts (Ferreira, Nunes, Castro, Ferreira & Coimbra, 2014; Sánchez-González, González-Martínez, Chiralt & Cháfer, 2010). Films with enhanced antioxidative properties prepared from chitosan and natural extracts and essential oils have also been studied (Ferreira, Nunes, Castro, Ferreira & Coimbra, 2014; Krkić et al., 2013; Wang, Tian, Feng, Fan, Pan & Zhou, 2014). The addition of extracts and essential oils, depending on their type and concentration, either does not significantly alter physiochemical properties of the film (Tripathi, Mehrotra & Dutta, 2008) or on the contrary influences the properties of the film such as mechanical resistance, water vapor permeability, opacity, solubility in water, antioxidant capacity; and therefore its subsequent application (Ferreira, Nunes, Castro, Ferreira & Coimbra, 2014; Sánchez-González, González-Martínez, Chiralt & Cháfer, 2010).

1.3.3.5 Potential of Schisadra chinensis and Bupleurum falcatum Extracts as Antixodiant and Antibacterial Additives in Chitosan Films

While many natural compounds are being explored as possible additives, *Schisandra chinensis* and *Bupleurum falcatum* have only been studied *in vitro* for now and have not been applied to packaging films as active substances despite their promising antibacterial properties.

S. chinensis fruit and leaf ethanol extracts were tested against *Staphylococcus aureus*, *Bacillus cereus*, *Listeria monocytogenes*, *Salmonella typhimurium*, and *Escherischia coli*. The leaf extract inhibited all bacteria at concentrations lower than 100 µg/ml and the fruit extract inhibited some of them at this concentration, and all of them at higher concentration (which was not explained clearly, but seems to be 1 or 10 mg/ml) (Mocan et al., 2014), though the study can be criticized in terms of lack of description regarding the extract concentration estimation and possible interference of the solvents used in the study. Antimicrobial effect was however supported by another study in which all antimicrobial solvents were removed prior to the experiment and where concentration was indicated clearly; 0.5% *S. chinensis* extract added to 10 ml broth with bacteria showed antimicrobial effect against *S. aureus* and *Aspergillus niger* (Sung, Kim, Kim & Nam, 2013). *S. chinensis* leaves and fruits were also rich in polyphenols, which provides them with good antioxidant properties. Similarly to the antibacterial activity, *S. chinensis* leaves contained seven times higher levels of total polyphenols and five times higher levels of total flavonoids, and these levels correlated with the antioxidant activity (Mocan et al., 2014). Correlation between antioxidant activity and total polyphenol and flavonoid content was also found in other studies with *S. chinensis* fruit (Wang, Chen, Zhang, Lan & Zhang, 2011). Particularly

leaves, which are a waste product of the *S. chinensis* processing industry, are a very promising and sustainable source of antibacterial and antioxidant compounds.

Different essential oils obtained from parts of *Bupleurum* plants showed varying levels of antimicrobial activity (Akın, Saraçoğlu, Demirci, Başer & Küçüködük, 2012; Saraçoğlu, Akın, Demirci & Başer, 2012). *B. kaoi* Liu ethanol extract manifested god antioxidant properties related to its polyphenol content (Wang, Liu, Tseng & Yu, 2005). *B. falcatum* ethyl ether and ethyl acetate fractions of methanol extracts inhibited *B. cereus, B. subtilis*, *S.aureus*, *E. coli*, *Pseudomonas aeruginosa* at minimum inhibitory concentration (MIC) ranging from 0.10 to 0.25 mg/ml (Lee, Choi & Yun, 2014).

Therefore, the extracts of these plants appear to be highly effective against a range of Gram-positive and Gram-negative bacteria, making them suitable candidates for incorporation in active packaging based on chitosan films.

2 Objectives

It has been shown that biodegradable and active packaging development is an important need and trend in the packaging industry. As chitosan films, enriched with natural extracts, demonstrated potential to serve as such packaging polymers, the main objectives of the present research are:

- To prepare chitosan-based packaging films from cricket and shrimp chitosan with and without Korean herbal extracts (*Schisandra chinensis* and *Bupleurum falcatum*) so as to improve their antibacterial and possibly antioxidant properties;
- To evaluate their antimicrobial activity against Gram-positive and Gram-negative bacteria relevant to food hygiene;
- To assess their basic physicochemical properties which are relevant to the performance of food packaging.

Related to these, secondary objectives are:

- To purify chitosan from crickets (*Gryllus bimaculatus*) and test its similarity to shrimp chitosan;
- To extract antimicrobial and antioxidant compounds from *S. chinensis* and *B. falcatum*, determine their polyphenol content and antimicrobial activity.

3 Delimitations

The main delimitations of the study were linked to the time frame and available instruments. First, the films prepared constituted only of chitosan dissolved in mild acid with glycerol added as plasticizer, even though blending with other biopolymers can improve its properties, the time frame did not allow to explore complicated mixtures, moreover, it was essential to prepare a rather simple system so that the properties of novel chitosan could be assessed more clearly.

Second, due to the time-consuming nature of chitosan purification optimization and low production yields, there was rather high variability in quality of chitosan samples prepared from crickets, and despite the chitosan produced not being of the best quality, it was considered sufficient to test whether it was able to form films and exhibit antibacterial properties. To confirm its basic resemblance to commercial shrimp chitosan, FTIR analysis was performed.

Third, as *S. chinensis* leaves could not be obtained in time for the study due to seasonality of the material, *S. chinensis* fruit was used in their place to verify the antioxidant and antibacterial activity reported previously and to estimate the extract compatibility with chitosan matrix.

Fourth, total solids were only estimated for *S. chinensis* extract as *B. falcatum* extract was not used for the film-casting experiment.

Fifth, due to the scale and nature of total polyphenol content, minimum inhibitory concentration testing, and film casting, which would be necessary for all plant extracts and their fractions prepared, preliminary selection criteria were set to be their solubility in suitable solvent systems (necessary for total polyphenol testing as well as film casting) and total polyphenol content (which is an indicator of antioxidant and possibly antimicrobial activity). Therefore, only two extracts soluble in water with the highest total polyphenol content were selected for minimum inhibitory concentration testing. Subsequently, only the most effective extract was incorporated in the chitosan films.

Sixth, the methodology for assessing the films was chosen and adjusted, when necessary, to the area of films which could be casted, which was limited by the scale of cricket chitosan production. It was understood that these decisions might have had an impact on the reliability of the results, and therefore, all measurements were carefully examined and compared to relevant literature whenever such studies were available.

Seventh, regarding the novel nature of the work presented – as database search did not yield any results about films made from insect chitosan, or applications of the chosen extracts in active packaging, at times there was a lack of literature concerning the topic. In such cases, discussion was based on the context of results and theoretical knowledge about general properties of the substances studied.

4 Materials and Methods

4.1 Materials

Freeze dried adult crickets (*Gryllus bimaculatus*) were obtained from Hwasung farm (South Korea), dried organic Korean *S. chinensis* fruit was purchased from Natural Food company (South Korea) and dried *B. falcatum* root (imported from China) from Gyeongdong herbal and medicinal market (Seoul, South Korea). Cricket chitosan as well as herbal extracts used in relevant experiments were then prepared according to methods described below (powderized samples illustrated in Figure 2).

Figure 2 Cricket powder (left), *S. chinensis* powder (center), *B. falcatum* powder (right)

All chemicals were of analytical or higher grade, unless specified otherwise. Sodium hydroxide, sodium hypochlorite (8% solution), oxalic acid (99.0%), ethanol (99.9%), ethyl acetate, ethyl ether, gallic acid, sodium chloride, magnesium nitrate hexahydrate (> 98.0%), silicagel, and substance X were obtained from Samchun Pure Chemicals, Co., Ltd. (South Korea). Methanol, hexane and ethyl acetate were obtained from Daejung Chemicals and Metal Co., Ltd. (South Korea). Soybean oil was obtained from Sajo (South Korea). Folin-Ciocalteau's reagent was obtained from Sigma-Aldrich, Co. (Switzerland), low molecular weight (LMW) shrimp chitosan with deacetylation degree (DDA) of 75% - 85% was obtained from Sigma-Aldrich, Co. (China), and sodium carbonate from Sigma-Aldrich, Co. (Japan). Nutrient Broth and Ringers solution were obtained from Oxoid Ltd. (England). Difco™ Nutrient agar was obtained from Becton, Dickinson and Company (France). Sterile petri dishes were obtained from SPL Life Sciences (South Korea). Acetic acid (> 99.7%) and glycerol (> 99%) were purchased from Junsei Chemical CO., Ltd. (Japan). Paraffin was obtained from Kanto Chemical Co., Inc. (Japan), and glass beads from Glastechnique Mfg. (Germany).

Glass bottles used for the study had height of 9 cm, diameter of the bottom 3 cm, outer diameter and inner diameter of the opening 2.3 cm and 1.7 cm, respectively (3 mm wall thickness).

Bacteria (*E. coli*, *B. cereus*, *V. parahaemolyticus*, *L. monocytogenes*) were common strains used in the Department of Food and Nutrition and more detailed identification information was unavailable at the time.

All sterilization for microbiological work was carried out at 121 °C for 15 – 20 min (LTS Korea Corp., South Korea). Distilled water was prepared by Human Power I^+ (Human Corporation, South Korea).

Substance X and concentration/conditions A - E were used in place of real values in order to maintain the confidentiality of the project required by Hanyang University.

4.2 Methods

4.2.1 Cricket Chitosan Purification

4.2.1.1 Preparatory Chitin Purification - Optimization of Decolorization Step

Methodology for chitin purification was partially adapted from (Song, Yu, Zhang, Yang & Zhang, 2013), consisting of protein removal by sodium hydroxide, decoloration by sodium hypochlorite and demineralization by oxalic acid, with eventual replacement of the decolorization step by novel method in order to achieve lighter color comparable to commercial chitin and chitosan.

In all cases, freeze-dried crickets were ground to fine powder using a kitchen grinder (SMX-H3500GN, SI Shinil, South Korea) and sieved through a kitchen sieve to remove parts of legs that were not powderized. 5 chitin samples were then consecutively prepared from the powder, as the production of chitin had to be gradually optimized, particularly in terms of decolorization.

Sample 1:

For deproteinization, 10 g of powder was mixed with 100 ml 1M sodium hydroxide, incubated at 95 °C with shaking at 150 - 180 rpm for 6 h (SJ-850SL, Sejong Scientific Co., South Korea) and subsequently filtered through 200 µm mesh sieve and washed with distilled water until neutral pH of distilled water was reached. For decolorization, the sample was then mixed with 100 ml 0.5% sodium hypochlorite solution, incubated for 3 h at room temperature with moderate stirring, and subsequently filtered through 200 µm mesh sieve and washed with distilled water until pH of distilled water was reached. For demineralization, it was then mixed with 1 g/100 ml oxalic acid, incubated for 3 h at room temperature with stirring, filtered through 200 µm mesh sieve and washed with distilled water until pH of distilled water was reached. The whole sequence was repeated once more. Afterwards, two more steps of deproteinization with sodium hydroxide was performed, the sample was filtered through 200 µm mesh sieve and washed with distilled water until pH of distilled water was reached. It was then dried overnight at 50 – 60 °C (Han Baek Scientific Co., South Korea). Color was recorded after every round of deproteinization.

Sample 2:

In this case, 10 g of powder were submitted to four consecutive rounds of deproteinization and two consecutive rounds of demineralization (in same conditions as sample 1), the decolorization step was omitted. The washed sample was then dried for 8 h at 50 – 60 °C.

Sample 3:

For sample 3, 10 g of powder were submitted to deproteinization followed by demineralization, the decolorization step was omitted (same reaction conditions as

sample 1). This sequence was performed twice, followed by two more rounds of deproteinization. The total number of deproteinization steps was four, and the number of demineralization steps was 2.

Sample 4:

In this case, 10 g of powder were submitted to deproteinization performed in identical conditions as sample 1, with a single modification in washing the powder through 100 µm mesh sieve, followed by novel decolorization method, in which 2 g (wet weight) each of deproteinized and washed sample was mixed with 20 mL of 3 different concentrations of substance X. The samples were then incubated at room temperature for 60 h. Color of the solution was recorded after 3 h, 24 h and 60 h of incubation. The samples were not submitted to demineralization step.

Sample 5:

To prepare this sample, 10 g of powder were submitted to deproteinization step as sample 1: 0.28 g each of sample (dry weight) were mixed with 20 ml of two different concentration of substance X, and incubated at different reaction conditions with moderate stirring for 4 h and 20 minutes. Color was recorded after 5, 20, 35, 50, 90, 125, 200, 245 and 260 minutes. Both samples were washed with distilled water through 100 µm mesh sieve, demineralized by procedure described previously and dried for 48 h at 50 – 60 °C. The samples were then weighted.

4.2.1.2 Preparation of Chitin to Be Used in Subsequent Deacetylation

To prepare higher amount of chitin for deacetylation (sample 6), 40 g, divided into two separate batches of 20 g of powder, were deproteinized by mixing with 200 ml 1M sodium hydroxide and incubated at 95 °C with shaking for 6 h. The sample was subsequently filtered through 100 µm mesh sieve and washed with distilled water until neutral pH of distilled water was reached. Decolorization was performed by the novel method: the samples were mixed with 200 mL substance X solution, incubated for 5 h and 40 minutes at particular conditions with stirring, and subsequently filtered through 100 µm mesh sieve and washed with distilled water until pH of distilled water was reached. Demineralization was performed by mixing with 200 mL 1 g/100 ml oxalic acid, incubated for 3 h at room temperature with stirring, filtered through 100 µm mesh sieve and washed with distilled water until pH of distilled water was reached. It was then dried overnight at 50 – 60 °C. The dry weight of the obtained chitin was recorded.

4.2.1.3 Chitin deacetylation for Chitosan Production

Deacetylation was performed according to Song, Yu, Zhang, Yang & Zhang, 2013 and 0.43 g of chitin Sample 6 were mixed with 100 ml of 67 g/100 ml sodium hydroxide and incubated at 90 °C with shaking at 140 rpm for 3 h. The sample was then washed with

distilled water through 100 µm mesh sieve, transferred into fresh 100 ml of sodium hydroxide (same concentration), and incubated at identical conditions. This procedure was repeated once more, which meant that total incubation time was 9 h. The sample was then dried overnight at 50 – 60 °C and dry weight was recorded. The yield (% weight chitosan/weight *Gryllus bimaculatus* powder) of the chitosan preparation process was calculated.

4.2.1.4 Preparation of Chitosan for Film Casting

In order to prepare sufficient amount of chitosan for film casting, 240 g of cricket powder were deproteinized, decolorized and demineralized as described for sample 6, however deacetylation was adapted from Chae, 2015 (unpublished data) in order to increase yield.

4.2.1.5 Chitosan Analysis by FT-IR

Chitosan sample was crushed into fine powder in ceramic mortar and analyzed by FT-IR (Nicolet iS50 FT-IR and Nicolet iS50 ATR, Thermo Fisher Scientific Inc., USA). Absorbance was recorded between 400 and 4000 cm^{-1} with resolution of 4 cm^{-1}. Analysis was done by OMNIC Series 9.2.41 (Thermo Electron Scientific Instruments Corp., USA) Degree of deacetylation (DDA) was calculated from absorbance (A) ratios based on the following equations (Song, Yu, Zhang, Yang & Zhang, 2013; Van de Velde & Kiekens, 2004):

$$DDA = 97.67 - [26.486(\frac{A_{1655}}{A_{3450}})]$$

$$DDA = 100 - [31.918(\frac{A_{1320}}{A_{1420}}) - 12.20]$$

4.2.2 Extraction of Antibacterial and Antioxidant Compounds from *S. chinensis* Fruit and *B. falcatum* Root

Plant material was stored at room temperature in the dark prior to experiment. The *S. chinensis* fruit and *B. falcatum* root were ground to coarse powder by a kitchen grinder (MFP-1, Samsung, South Korea) with one speed setting at ambient temperature.

Ethanol Extract of *S. chinensis* fruit:

The ethanol *S. chinensis* extract was prepared by a method adapted from (Mocan et al., 2014). 160 g of *S. chinensis* fruit powder was mixed with 70% ethanol in 1:10 ratio (w/V) and extracted at 60 °C for 30 min using setting to “high level” of sonication (Power Sonic 505, Daihan Scientific Co., Ltd., South Korea). The extract was allowed to cool down to room temperature by keeping it at ambient conditions and subsequently filtered through filter paper No. 2 (Advantec, Hyundai Micro Co., Ltd., South Korea) and No. 1 (Whatman

International Ltd., UK) to remove impurities. The extract was concentrated to one tenth of its original volume in vacuo (VS-21SMTi, Konetic, South Korea) and filtered again through filter paper No. 1 (Whatman International Ltd., UK) and subsequently through 0.45 µm nylon filter (Bonna-Agela Technologies Inc., South Korea). The extract was stored in a sealed plastic container in the dark at room temperature.

Ethanol Extract of *B. falcatum* root:

The ethanol extraction of *B. falcatum* was modified from originally methanol extraction method (Lee, Choi & Yun, 2014). First, 200 g of *B. falcatum* root powder were mixed with 70% ethanol in 1:5 ratio (w/V) and slightly shaken (Baby Shaker™-II, BS-02, Toylab, Inc., South Korea) at room temperature for 88 hours in the dark. The extract was subsequently filtered through filter paper No. 2 (Advantec, Hyundai Micro Co., Ltd., South Korea) and No. 1 (Whatman International Ltd., UK). The extract was concentrated to one tenth of its original volume in vacuo (VS-21SMTi, Konetic, South Korea) and filtered again through filter paper No. 1 (Whatman International Ltd., UK) and subsequently through 0.45 µm nylon filter (Bonna-Agela Technologies Inc., South Korea). The extract was stored in a sealed plastic container in the dark at room temperature.

Methanol Extract of *B. falcatum* root:

The methanol extract of *B. falcatum* was prepared according to a method adapted from (Lee, Choi & Yun, 2014). First, 212 g of powder were mixed with methanol in 1:5 ratio (w/V) and slightly shaken at room temperature for 90 hours in the dark. The extract was subsequently filtered through filter paper No. 2 (Advantec, Hyundai Micro Co., Ltd., South Korea) and No. 1 (Whatman International Ltd., UK). The methanol extract was partitioned with 0.5 L hexane and both methanol and hexane fractions were concentrated in vacuo (HS-2001N, Hahn Shin Science Co., South Korea) until all solvent was evaporated and one third of the concentrated methanol extract was removed and stored in the dark at room temperature for future reference. The remaining two thirds of methanol extract were resuspended in distilled water and subsequently partitioned first with 0.5 L ethyl ether and then 0.5 L ethyl acetate. These ethyl ether and ethyl acetate fractions were also concentrated in vacuo (HS-2001N, Hahn Shin Scientific Co., South Korea) until all solvent was evaporated. The water fraction was concentrated to four fifths of the original volume and filtered through filter paper No. 1 (Whatman International Ltd., UK). The extracts were stored in a sealed plastic container in the dark at room temperature.

4.2.3 Solubility Testing of Methanol Extract and Its Fractions with Low Water Solubility

To test solubility, 3 µl of hexane extract, or 3 mg of methanol extract, ethyl acetate fraction, and ethyl ether fraction were mixed with 1 ml 70% ethanol and solution was visually inspected for occurrence of suspension. The solution was further diluted to 35% by adding 1 ml of distilled water and occurrence of suspension was inspected again.

Same quantities of fractions were also mixed with 1 ml of soybean oil and occurrence of suspension was checked. Afterwards, 1 ml of distilled water was added to the solution and mixed and both layers were checked for suspension.

4.2.4 Total Polyphenol Content of *S. chinensis* Fruit and *B. falcatum* Root Extracts

Total polyphenols were determined by Folin Ciocalteau's method adapted from Pantelidis, Vasilakakis, Manganaris & Diamantidis, 2007 in the three water-soluble samples: ethanol extract of *S. chinensis* fruit, ethanol extract of *B. falcatum* root and water fraction of *B. falcatum* root methanol extract. The ethanol extracts were diluted 1:100 and 1:1000 with distilled water, while the water fraction of methanol extract was diluted 1:10 with distilled water. Then, 0.1 ml of diluted extract was mixed with 0.5 ml of freshly prepared 1:10 diluted Folin-Ciocalteau's reagent and with 0.4 ml 7.5% (w/v) sodium carbonate. The samples were incubated in water bath for 5 min at 50 °C and absorbance was measured at 760 nm (Biomate 3S, UV-Visible Spectrophotometer). The polyphenol concentration was calculated using a standard curve (R^2 = 0.999) obtained from gallic acid standard (concentrations 0.01, 0.02, 0.03, 0.04, 0.05, 0.06, 0.07, and 0.08 mg/ml and submitted to identical reaction conditions.) All measurements were done in triplicate and results were indicated as mg gallic acid equivalents (GAE) per ml of concentrated extract and per g of plant material.

4.2.5 Minimum Inhibitory Concentration (MIC) Testing of *S. chinensis* and *B. falcatum* Root Ethanol Extracts

Bacterial inoculum was prepared by mixing 1 ml of bacterial stock solution in 9 ml of nutrient broth prepared as per manufacturer's instructions. All samples were incubated at 68 rpm and 35 °C overnight with mild shaking (Jisico, South Korea). The MIC assays were based on widely used methodology for agar diffusion testing (Hosseini, Razavi & Mousavi, 2009; Leceta, Guerrero, Ibarburu, Dueñas & De la Caba, 2013; Lee, Choi & Yun, 2014; Mocan et al., 2014; Pelissari, Grossmann, Yamashita & Pineda, 2009; Peng & Li, 2014). The overnight cultures were diluted with Ringers Solution to reach 10^7 – 10^8 cells/ml in inoculum (standardized by colony forming unit count and McFarland 0.5 standard; Absorbance at 625 nm = 0.08). 0.1 ml of inoculum was spread on sterile nutrient agar plates to obtain uniform lawn culture, with the exception of *V. parahaemolyticus*, which was spread on sterile nutrient agar plates containing 1% sodium chloride. The inoculum was left to dry shortly and 3 sterile paper discs (d = 12 mm) impregnated with extracts (20 – 25 µl per disc; concentrations 0.05, 0.1, 0.25, 0.5 and 0.75 mg polyphenols/ml) were placed on each plate. The plates were incubated at 35 °C (Han Baek Scientific Co., South Korea) for 24 and 72 h for *B. cereus*/*E. coli* and *L. monocytogenes*/*V. parahaemolyticus*, respectively. Plates without any discs and plates with paper discs impregnated with sterile distilled water were used as controls. The

inhibition zone was measured as the difference between the diameter of the inhibition zone and diameter of the disc with 1 mm precision and expressed in mm (Mocan et al., 2014), and also as the difference between total inhibition zone area and total disc area with 1 mm precision and indicated in mm^2 (Hosseini, Razavi & Mousavi, 2009). Growth under discs was inspected visually by lifting discs with sterile forceps. All assays were done in triplicate.

4.2.6 Determination of Total Solids in *S. chinensis* Extract

2 ml of *S. chinensis* fruit ethanol extract were put in 50 ml beaker (initial weight of empty beaker was recorded with 0.1 mg precision (BT 224 S, Sartorius, Germany)) and water and possible remaining ethanol were evaporated at 55 ± 3 °C (Han Baek Scientific Co., South Korea) until constant weight was reached. Concentration of the extract was determined as the difference of weight between the weight after evaporation and weight of the empty beaker, divided by 2 ml. This concentration (g/ml) was then used to calculate concentration of diluted extract in MIC tests and in chitosan films.

4.2.7 Shrimp and Cricket Chitosan with or without *S. chinensis* Extract Film Casting

Films were casted based on methodology adapted from similar studies published previously (Ferreira, Nunes, Castro, Ferreira & Coimbra, 2014; Hosseini, Razavi & Mousavi, 2009; Wang, Tian, Feng, Fan, Pan & Zhou, 2014).

Film-forming solution containing only shrimp chitosan was prepared by the following method:

1 g of chitosan powder was placed in 50 ml beaker and completely dissolved in 25 ml 2% (V/V) acetic acid at 50 °C and 200 rpm (HSD 120-03P, Misung Scientific Co., Ltd., South Korea), which required 65 min since placement of the beaker on the pre-heated stirring plate. The solution was covered with aluminum foil and occasionally carefully stirred with inoculation needle to accelerate dissolution. Afterwards, 25 ml of distilled water was added to the mixture and homogenized for 45 min at 50 °C, 200 rpm. Then, 0.53 g of glycerol (ratio of approximately 1:2 glycerol:chitosan, w:w) was added to the mixture and homogenized for additional 30 min at 50 °C, 200 rpm. The solution was then filtered through 6 layers of gauze to remove impurities and undissolved particles into a new 50 ml beaker placed on a hot plate pre-heated to 50 °C. The solution was subsequently degassed by sonication in water bath preheated to 50 °C for 10 min at “high” setting (Power Sonic 505, Daihan Scientific Co., Ltd., South Korea). Final concentration in the film-forming solution was therefore approximately 1% acetic acid (V/V), 2% chitosan (w/V), 1% glycerol (w/V).

Film-forming solution containing shrimp chitosan and *S. chinensis* fruit extract was prepared similarly, with only difference being replacing addition of 25 ml distilled water with the following: Instead of distilled water, 25 ml of *S. chinensis* fruit ethanol extract diluted with distilled water to contain 1 500 mg polyphenols/ml was added to the mixture. Final concentration in the film-forming solution was therefore approximately 750 mg polyphenols/ml (corresponding to approximately 4% w/V concentration of total solids).

Film-forming solution containing only *G. bimaculatus* chitosan was prepared by the following method:

0.7 g of chitosan powder was placed in 50 ml beaker and completely dissolved in 17.5 ml 2% (V/V) acetic acid at 50 °C and 200 rpm (HSD 120-03P, Misung Scientific Co., Ltd., South Korea), which required 45 min since placement of the beaker on the pre-heated stirring plate. The solution was covered with aluminum foil and occasionally carefully stirred with inoculation needle to accelerate dissolution. Afterwards, 17.5 ml of distilled water was added to the mixture and homogenized for 45 min at 50 °C, 200 rpm. Then, 0.37 g of glycerol (ratio of approximately 1:2 glycerol:chitosan, w:w) was added to the mixture and homogenized for additional 30 min at 50 °C, 200 rpm. The solution was then filtered through 6 layers of gauze to remove impurities and undissolved particles into a new 50 ml beaker placed on a hot plate pre-heated to 50 °C. The solution was subsequently degassed by sonication in water bath preheated to 50 °C for 10 min at "high" setting (Power Sonic 505, Daihan Scientific Co., Ltd., South Korea). Final concentration in the Film-forming solution was therefore approximately 1% acetic acid (V/V), 2% chitosan (w/V), 1% glycerol (w/V).

Film-forming solution containing *G. bimaculatus* chitosan and *S. chinensis* fruit extract was prepared similarly, with only difference being replacing addition of 17.5 ml distilled water with the following: Instead of distilled water, 17.5 ml of *S. chinensis* fruit ethanol extract diluted with distilled water to contain 1 500 mg polyphenols/ml was added to the mixture. Final concentration in the film-forming solution was therefore approximately 750 mg polyphenols/ml (corresponding to approximately 4% w/V concentration of total solids).

12 ml of film-forming solutions were then poured into Petri dishes with inner diameter of 8.5 cm (Area (A) = 56.7 cm^2) and spread to coat the dish evenly. Petri dishes were then placed in controlled temperature chamber (Han Baek Scientific Co., South Korea) in as horizontally balanced position as possible at 50 ± 1 °C for 11 – 14 h (until fully dried and possible to peel with forceps easily).

After drying, plates were allowed to cool down to ambient temperature (22 ± 2 °C) at ambient relative humidity (RH), peeled and stored in controlled humidity chamber at approximately 53 – 54% RH over saturated magnesium nitrate hexahydrate solution

(Wexler & Hasegawa, 1954) until following experiments (24 – 48 h for microbiological experiments, at least 7 days for physicochemical tests).

4.2.8 Properties of Shrimp and Cricket Chitosan Films with or without *S. chinensis* Extract

4.2.8.1 Evaluation of Antimicrobial Activity

Overnight culture of bacteria was prepared the same way as for MIC testing and diluted with Ringers Solution to obtain 1 000 CFU/ml. 0.1 ml was spread on sterile nutrient agar plates and incubated at 35 °C (Han Baek Scientific Co., South Korea) for 24 h (*B. cereus* and *E.* coli) or 48 h (*L. monocytogenes*). The plates were stored at 4 °C (FRB-4230N, Daewoo, South Korea) for 3 weeks prior to the experiment and regularly examined for contamination.

Bacterial inoculum was prepared by suspending 3 to 5 colonies of each strain in 10 ml of nutrient broth prepared as per manufacturer's instruction. All samples were incubated at 68 rpm and 35 °C (Jisico, South Korea) for 24 h (*B. cereus* and *E. coli*) or 48 h (*L. monocytogenes*). Agar diffusion test was based on former studies (Leceta, Guerrero, Ibarburu, Dueñas & De la Caba, 2013; Pelissari, Grossmann, Yamashita & Pineda, 2009; Wang et al., 2011). The overnight cultures were diluted with Ringers Solution to reach 10^7 – 10^8 cells/ml inoculum (standardized by colony forming unit count and OD_{600} measurement (Biomate 3S, Uv-Visible Spectrophotometer, Thermo Scientific, USA)). Then 0.1 ml of inoculum was spread on sterile nutrient agar plates to obtain uniform lawn culture. The inoculum was left to dry shortly and aseptically cut discs (d = 12 mm) from each of the four films produced were placed on the plates. Application of films was facilitated by placing the film circles on sterile filter paper or plastic discs first and then applying them on the plates with films facing towards the plate, being covered by the applicator disc. To improve adhesion, sterile Q-tips were used to gently press the applicator disc covering the film. Once films adhered to the surface, applicator discs were removed with sterile forceps. The plates were incubated at 35 °C (Han Baek Scientific Co., South Korea) for 10 days and inspected every 24 h for occurrence and size of inhibition zone. Plates without any discs and plates with paper discs impregnated with sterile distilled water were used as controls. The inhibition zone (IZ) was measured as the difference between total inhibition zone area and total disc area with 1 mm precision and indicated in mm^2 (Hosseini, Razavi & Mousavi, 2009; Ojagh, Rezaei, Razavi & Hosseini, 2010). Growth under discs was inspected visually by lifting discs with sterile forceps. All assays were done in triplicate.

4.2.8.2 Thickness, Density and Moisture Content (MC)

Thickness was measured with hand-held Digimatic Micrometer (MDC-25SX, Mitutoyo, Japan) with 0 – 25 mm range and 0.001 mm precision. Measurements were taken at 24 different positions and average value was reported.

Films conditioned to 53-54% RH were cut in squares with average side length (a) of 20.2 ± 0.3 mm. Density was calculated based on weight (M_1) (0.1 mg precision, BT 224 S, Sartorius, Germany), thickness (d) measurement in 5 locations (MDC-25SX, Mitutoyo, Japan), and the length of the sides with the following formula:

$$\rho = \frac{M_1}{da^2}$$

Moisture content (MC) was calculated from the weight of films cut in squares with average side length of 20.2 ± 0.3 mm after conditioning to 53% RH (M_1) compared to the weight after drying for 24 h at 105 ± 3 °C (M_2) (Han Baek Scientific Co., South Korea) and reported in % (Wang, Tian, Feng, Fan, Pan & Zhou, 2014):

$$MC = \frac{M_1 - M_2}{M_1} \times 100$$

The experiments to determine density and moisture content were done in triplicate.

4.2.8.3 Water Solubility (WS) and Swelling Degree (SD)

WS and SD were determined by methodology based on previous studies (Delville, Joly, Dole & Bliard, 2002; Peng & Li, 2014; Thakhiew, Devahastin & Soponronnarit, 2014; Wang, Tian, Feng, Fan, Pan & Zhou, 2014).

Squares of films conditioned to 53-54% RH with average side length of 20.2 ± 0.3 mm were weighted (M_1) (0.1 mg precision, BT 224 S, Sartorius, Germany), placed in 50 ml Falcon™ tubes, immersed in 20 ml distilled water and incubated for 24 h at 21 ± 1 °C in an orbital shaker set to highest speed (Baby Shaker™-II, BS-02, Toylab, Inc., South Korea). The films were then removed from the water, patted dry with filter paper, weighted (M_3) and dried at 70 ± 3 °C (Han Baek Scientific Co., South Korea) until constant weight was reached (M_4). The two parameters were then calculated by the following equations:

$$WS = \frac{(1 - MC)M_1 - M_4}{(1 - MC)M_1} \times 100$$

$$SD = \frac{M_3 - M_4}{M_4}$$

4.2.8.4 Water Vapor Permeability (WVP)

As it was possible to produce only a limited quantity and size of films, methodology was heavily adjusted to the available area of films, however the principle was based on Wang, Tian, Feng, Fan, Pan & Zhou, 2014.

Preparation of real specimens:

Films preconditioned to 53-54% RH were cut into circles and thickness was measured in three locations with hand-held Digimatic Micrometer (MDC-25SX, Mitutoyo, Japan) with 0 – 25 mm range and 0.001 mm precision. Glass bottles were filled with newly purchased silicagel until 1.5 to 2 cm below the opening (to enable mixing of the silicagel after each weighting) to provide 0% RH inside. The bottles were then sealed with circles cut from the films, which had total diameter of 23 mm (same diameter as the outer diameter of the bottle opening), out of which 17 mm was exposed to the water vapor). The films were attached to the bottles with melted paraffin. Upon solidification of the paraffin, adhesion of the films was inspected and all places of improper attachment were repaired. Three specimens were prepared for each film tested.

Preparation of dummy specimens:

One dummy specimen was prepared for shrimp chitosan film and for shrimp chitosan film containing *S. chinensis* extract. The specimens were prepared exactly the same way as real specimens, however silicagel was replaced by glass beads.

WVTR (Water Vapor Transmission Rate) and WVP Measurement

Initial weight was recorded (0.1 mg precision, BT 224 S, Sartorius, Germany) and specimens were placed inside a 75% RH chamber (maintained by saturated solution of sodium chloride) and incubated at 21 °C for 8 days. Every 12 h, weight was recorded and the silicagel was gently mixed without tampering with the films. The weight gain was plotted against time. There was no noticeable weight gain of the control dummy specimens. The weight gain of real specimens was linear over the duration of the experiment, and its slope yielded regression coefficients higher than 0.996. This slope, representing weight gain per unit of time, was then used to calculate WVTR and WVP:

$$WVTR = \frac{slope}{A}$$

$$WVP = \frac{WVTR \times d}{p \times (R_1 - R_2)}$$

Where A stands for film area in m^2, slope for weight gain per day in g, d for film thickness in mm, p for saturated vapor pressure at 21 °C (2.378 kPa as calculated by Antoine

equation through DDBST (2015b)), R_1 for RH inside the chamber (0.75) and R_2 for RH inside the glass bottles (0). WVP was then indicated in g mm m^{-2} day^{-1} kPa^{-1}.

4.2.8.5 Color measurement

Color was measured by portable colorimeter (Chroma Meter CR-400, Minolta Co., Ltd., Japan) by method described by Leceta, Guerrero, Ibarburu, Dueñas & De la Caba, 2013. White plate ($L^* = 96.06$, $a^* = 0.20$, $b^* = 2.02$) was used as standard and as background for measurement of the films. As explained by Leceta et al., the color scale was as follows: L^*: 0 – 100 (black – white), a^*: redness (+) or greenness (-), and b^*: yellowness (+) or blueness (-). 6 measurements were taken for each type of film and average values were recorded. C^*_{ab} (chrome/saturation), h^*_{ab} (hue) and ΔE^* (total color difference between samples placed on top of standard plate) were calculated based on equations provided by Leceta et al.:

$$C^* = \sqrt{a^{*2} + b^{*2}}$$

$$h^* = arctg\frac{b^*}{a^*}$$

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

h^*_{ab} values were then recalculated from Rad to °, and in the case of negative values in Rad, 180 ° were added (as arctg operates on $-\pi/2$ to $\pi/2$ interval, difference between $-a^*/+b^*$ and $+a^*/-b^*$ pairs must be corrected manually).

4.2.9 Statistical Analysis

All experiments except for chitosan purification and antimicrobial compound extraction were done at least in triplicate. Results were then reported as average of all measurements and standard deviation. Significant difference between means was evaluated through one-way analysis of variance (ANOVA), with $P < 0.05$ as threshold for statistical significance. Statistical analysis was performed with Microsoft Office Excel software package.

5 Results and Discussion

5.1 Cricket Chitosan Purification

5.1.1 Chitin Purification from *G. bimaculatus* – Optimization of Decolorization Process

Chitin from Sample 1 was prepared through twice repeated sequence of deprotenization, decolorization and demineralization, followed by additional deproteinization steps. The color was recorded after each round of deproteinization, as well as the color of the rinsed sodium hydroxide solution, and is presented in Figure 3.

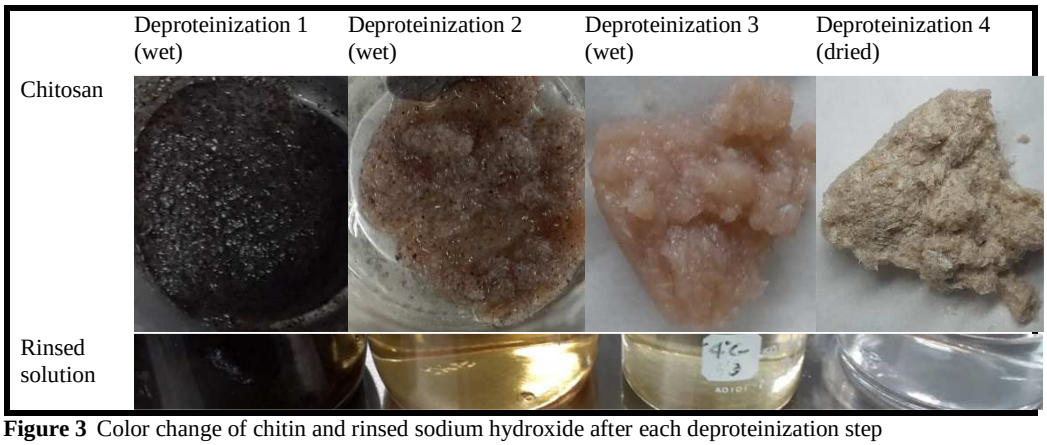

Figure 3 Color change of chitin and rinsed sodium hydroxide after each deproteinization step

It can be seen that the first three rounds of repeated deproteinization led to color removal, while the fourth one did not result in further bleaching. The color was only removed during the deproteinization step. No color removal was observed after the actual decolorization steps with sodium hypochlorite solution. This can be explained by the difference in insects used between the present study and reference, as the reference used *Chrysomya megalocephala* larvae (Song, Yu, Zhang, Yang & Zhang, 2013), and different insects and their life stages have different biochemical composition (Barker, Fitzpatrick & Dierenfeld, 1998; Mlcek, Borkovcova, Rop & Bednarova, 2014; Rockstein, 2012) and lower level of pigment. *Gryllus bimaculatus* has very dark cuticle, which is mainly due to melanin pigments that are bound to proteins as melanoproteins, which are difficult to solubilize. *Orthoptera* also contain tetrapyrrole pigments and carotenoids, which are also mostly bound to proteins (Rockstein, 2012). The fact that most melanins, which are responsible for the dark colour, as well as some other pigments are bound to proteins explains why color was removed during protein removal steps. However, after 3 repeated deproteinization steps, no more color was removed, the final sample was still rather dark and this process required harsh conditions and long time.

It was also observed that the rinsed solution of protein in strongly alkaline environment formed a gel.

To assess whether the sequence of deproteinization and demineralization steps has an impact on the decolorization, and whether not including the decolorization step has an impact on the color removal process, Samples 2 and 3 were prepared. Sample two underwent four consecutive rounds of deproteinization followed by two rounds of demineralization, while Sample 3 was submitted to two rounds of alternating deproteinization and demineralization steps, followed by two more deproteinization steps. The final color of the dried samples was recorded and compared with Sample 1, as shown in Figure 4.

Figure 4 Color of chitin prepared by different methods: Sample 1 prepared with a decolorization step by sodium hypochlorite, sample 2 prepared by 4 rounds of deproteinization followed by two rounds of demineralization, and sample 3 prepared by alternating rounds of deproteinization and demineralization.

It can be seen that the color of the three samples is similar shade of beige, with Sample 1 being slightly lighter than Sample 3; and Sample 2 being slightly darker than the other two samples. The difference is very small and does not suggest a significant influence of the sequence of the steps on the color removal process, or necessity to include the sodium hypochlorite decolorization step.

To produce chitin (and subsequently chitosan) with color comparable to commercially available shrimp chitosan, novel decolorization method was tested with samples 4 and 5. Substance X was expected to be suitable for decolorizing cricket chitin. To test its possible effect, Sample 4 was deproteinized and mixed with three different concentration of substance X. Figure 5 shows the color removal after 3 h, 24 h and 60 h of incubation.

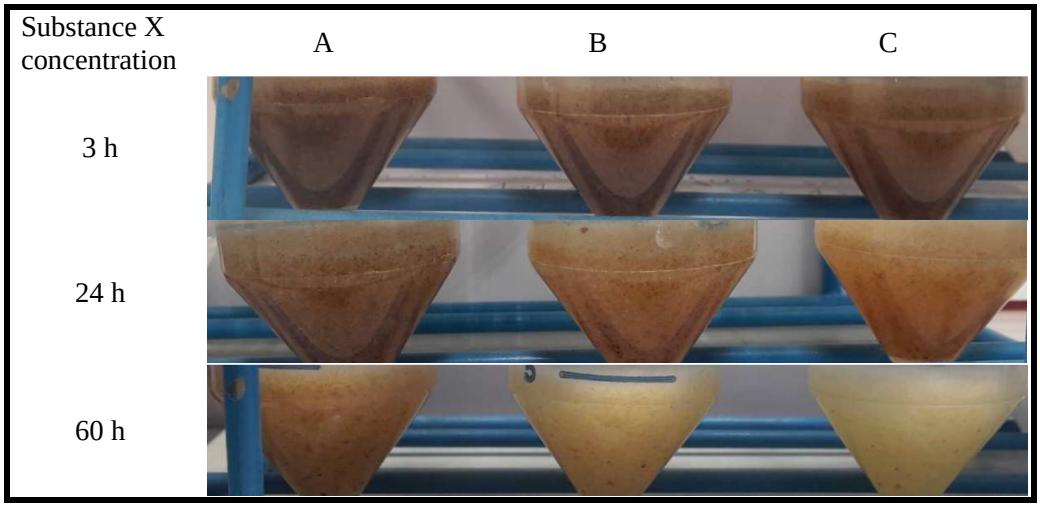

Figure 5 Color chance of chitin after incubation with different concentrations of substance X.

It can be seen that the bleaching effect was more effective at C concentration and with prolonged time. Fraction of sample 4 that was incubated for 60 h at a C concentration of substance X had color comparable to commercial shrimp chitosan.

Sample 5 was prepared to test whether change in reaction conditions would have impact on the speed of bleaching process. Therefore, 0.56 g deproteinized sample was obtained from 10.0 g of *Gryllus bimaculatus* powder and 0.28 g each were mixed with different substance X solutions and incubated at different conditions. The bleaching process is documented in Figure 6.

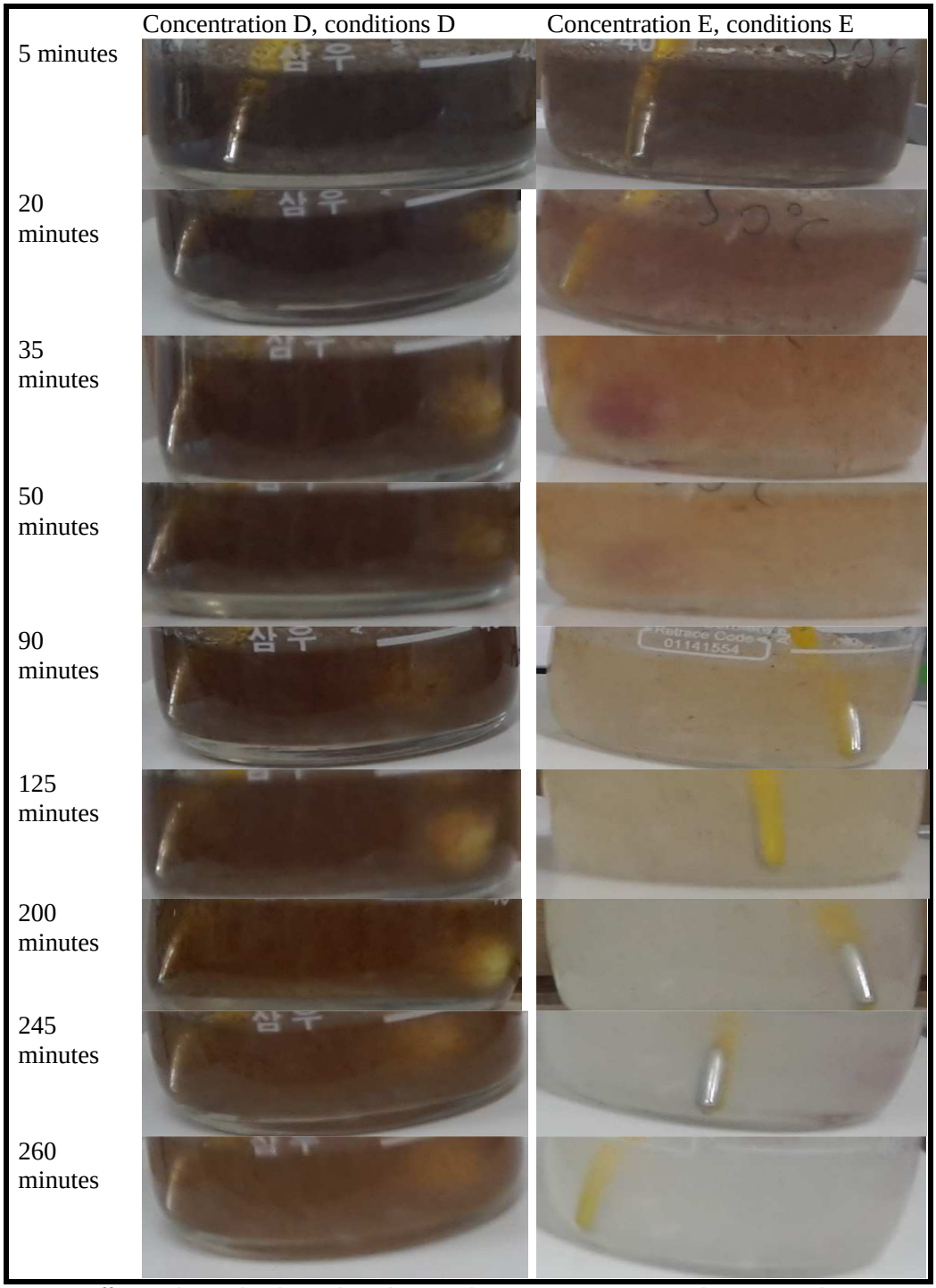

Figure 6 Effect on chitin color change depending on substance X concentration and reaction conditions.

It can be seen that the optimization of conditions and concentration, led to significant increase in the speed of decolorization and it was possible to obtain very white sample after 4 hours and 20 minutes of decolorization. Demineralization step was not performed.

Figure 7 shows comparison between samples 2, 3, 5 incubated with D concentration and E concentration.

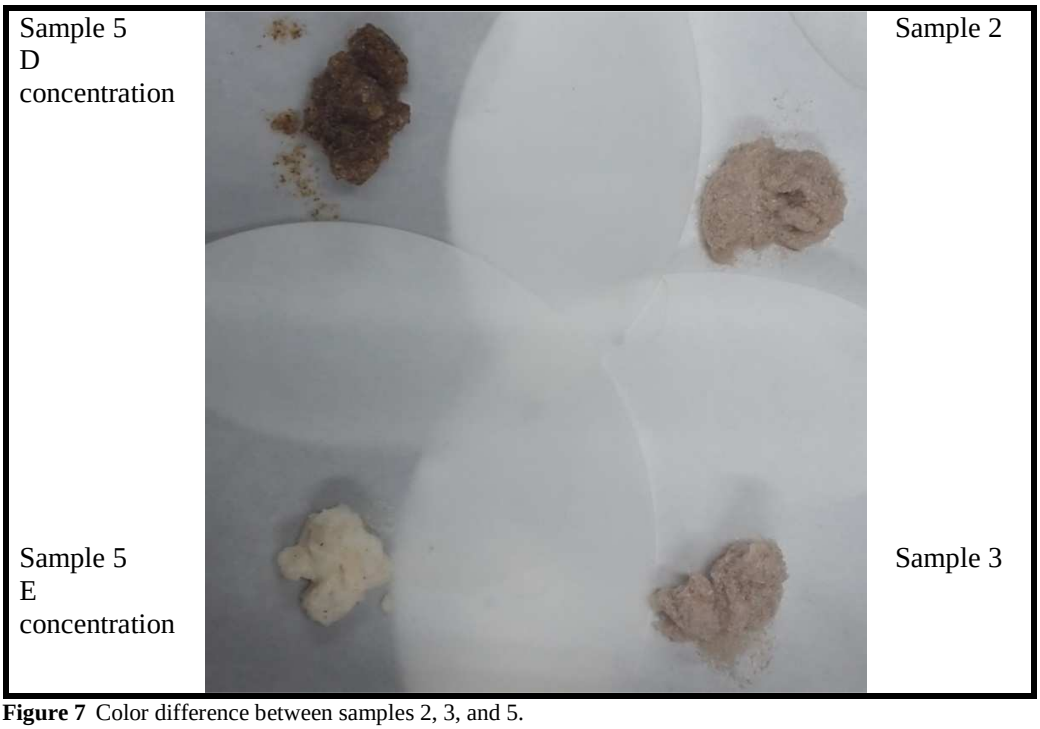

Figure 7 Color difference between samples 2, 3, and 5.

It can be seen that the chitin sample decolorized with E concentration has the lightest color of these four samples after only 4 hours and 20 minutes, which is much shorter time than the period that would be necessary to remove color with other concentrations, and than the time necessary to perform three consecutive deproteinization steps.

The lighter sample 5 (dry, E concentration) weighted 0.17 g, while the darker sample 5 (dry, D concentration) weighted 0.26 g. This could be an indicator that during decolorization, the pigments are also removed from the chitin sample. Compared to the decrease in weight during deproteinization (0.56 g of chitin obtained from 10.0 g of powder), the decrease in weight during the decolorization process was rather small. However, as the experiment was not performed in triplicate, it cannot be clearly determined whether the difference in weight between the two samples was not a result of handling. Further experiments would be necessary to determine what processes happen during decolorization to the pigments present in the sample.

Based on the results presented, it is clear that substance X is a very potent decolorizing agent that is able to efficiently decolorize the cricket chitin. The ratio of chitin to substance X solution was very generous, and it would therefore be worth exploring whether it would be possible to use smaller amount in order to decrease environmental impact as well as costs. Similarly, it might be possible to slightly change reaction conditions in order to fasten the reaction.

5.1.2 Chitin Deacetylation and Chitosan Preparation for Film Casting

Based on previous experiments, chitin Sample 6 was prepared by deproteinization (Song, Yu, Zhang, Yang & Zhang, 2013), followed by the novel decolorization method explained previously (which required about 50 minutes more than previously to achieve light color, which could be due to inefficient stirring, resulting in certain particles being in less contact with the solution) and by demineralization (Song, Yu, Zhang, Yang & Zhang, 2013). By this procedure, 0.86 g of chitin was obtained, but only half (0.43 g) was deacetylated (Song, Yu, Zhang, Yang & Zhang, 2013) into chitosan (0.031 g of chitosan was obtained from the 0.43 g of chitin). The yield was 0.2%, which was very unsuitable result for preparation of sufficient amount of chitosan for film casting. Therefore, the deacetylation procedure for large-scale chitosan production for film-casting was adjusted according to an optimized method, in which yield of at least 2% was expected (Chae, 2015) (unpublished data).

Difficulties were encountered during larger scale production of chitosan for film casting. Despite the chitin color becoming light exactly as observed with sample 6 after decolorization, the color became rather dark during deacetylation and the chitosan had gel-like appearance. The most likely explanation is possibly inefficient protein removal during the deproteinization step due to the scale of the process, considering that gel was formed during the deacetylation process in alkaline conditions, which was previously observed for *G. bimaculatus* proteins. Therefore, the sample was submitted to additional 3 hours of deacetylation. These issues suggest that large-scale production of cricket chitosan is very challenging, particularly due to the protein content. Therefore, it would be advisable to obtain chitin-rich byproduct of insect protein purification, as it would possibly make the removal of remaining proteins easier.

5.1.3 Chitosan Analysis by FT-IR

The spectra obtained for the chitosan used in film casting are presented in Figure 8. Previously, sample 6 was analyzed as well and showed high similarity to standard shrimp chitosan and degree of deacetylation equal to 84.19 % (Chae, 2015) (unpublished data). In the case of chitosan produced for film casting, it can be seen that the deacetylation degree is lower (68.79% compared to 75.72% of commercial shrimp chitosan, based on calculation with A_{1320} and A_{1420}, or 76.00% compared to 81.27% of commercial shrimp chitosan based on calculation with A_{1655} and A_{3450}), and the region between 1500 and 1700 cm^{-1} is similar to chitin, with signals typical for the secondary amide at 1558 and 1620 cm^{-1} and signals

typical for secondary amides of chitin visible also at 3107 and 1259 cm^{-1} (Van de Velde & Kiekens, 2004). One of typical chitosan bands at 1597 cm^{-1} is not present, however, signal at 1656 cm^{-1} is also typical for primary amines (Song, Yu, Zhang, Yang & Zhang, 2013) and indicates partial deacetylation. It appears that in the additional alkali treatment in prolonged deacetylation, remaining proteins were removed, as no signal appears at 1540 cm^{-1} (Majtán, Bíliková, Markovič, Gróf, Kogan & Šimúth, 2007). In conclusions, it appears that the cricket chitosan prepared has lower DDA than shrimp chitosan and is free of substantial protein contamination.

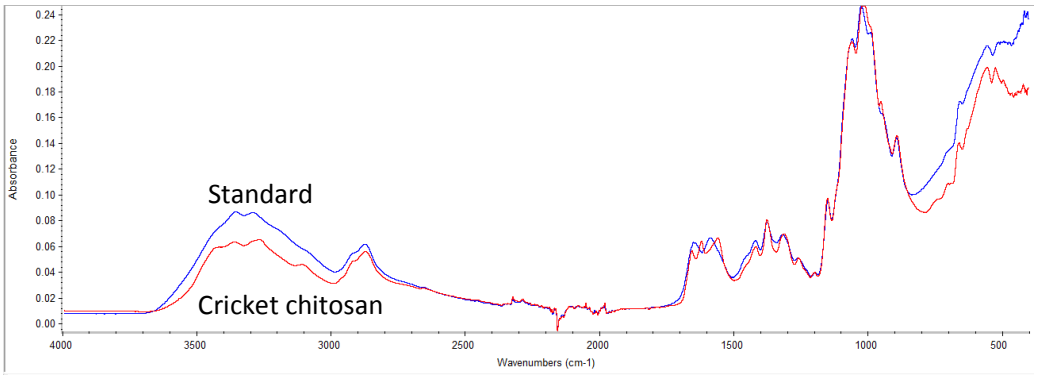

Figure 8 Comparison of spectra of standard shrimp chitosan and cricket chitosan

5.2 Extraction and Solubility of Antibacterial and Antioxidant Compounds from *S. chinensis* Fruit and *B. falcatum* Root

S. chinensis fruit and *B. falcatum* root extracts dissolved predominantly in distilled water, out of which majority of ethanol was evaporated, were obtained. The ten times concentrated *S. chinensis* fruit extract had dark red colour and a pleasant smell similar to Korean omija tea. *B. falcatum* root ethanol extract and all methanol extract fractions had brown color and smell reminiscent of oriental medicine. The methanol extract as well as the hexane, ethyl ether and ethyl acetate fractions, from which all solvent was evaporated, were very viscous.

Three extracts were already dissolved in water (ethanol extract of *S. chinensis* fruit, ethanol extract of *B. falcatum* root, and water fraction of *B. falcatum* root methanol extract) and therefore no solubility testing was performed. The results of solubility testing of the methanol extract and its fractions (except for the water fraction) are presented in Table 2.

Table 2 Solubility of selected extracts or their fraction of *B. falcatum* in water, ethanol and soybean oil.

Fraction	70% ethanol	35% ethanol	Soybean Oil	Water + Soybean Oil
Methanol	soluble	precipitate	precipitate	precipitate
Ethyl acetate	soluble	precipitate	precipitate	precipitate
Ethyl ether	precipitate	precipitate	precipitate	precipitate
Hexane	precipitate	precipitate	soluble	precipitate

Solubility in water is important in order to accurately perform comparative analysis of total polyphenols by Folin-Ciocalteau method. The occurrence of precipitate would affect the measured absorbance and decrease reliability of the results. Moreover, for preparation of homogenous chitosan films, water (or alternatively, oil) solubility is necessary, as films are commonly prepared from extracts soluble in water (Wang, Tian, Feng, Fan, Pan & Zhou, 2014) or from essential oils emulsified in the film-forming solution (Avila-Sosa et al., 2010). Moreover, for antimicrobial testing, it has been shown that most organic solvents commonly used for dissolving extracts non-soluble in water have antibacterial activity and might therefore distort the results (Wadhwani et al., 2009), rendering the results obtained for extracts dissolved in water incomparable to extracts dissolved in solvents such as methanol or DMSO (dimethyl sulfoxide). With regard to this fact, only the three extracts which were soluble in water without occurrence of precipitate – *S. chinensis* fruit ethanol extract, *B. falcatum* root ethanol extract (majority of ethanol evaporated) and *B. falcatum* root water fraction of methanol extract were chosen for subsequent experiments.

5.3 Total Polyphenol Content of *S. chinensis* Fruit and *B. Falcatum* Root Extracts and Implications for Antioxidant and Antimicrobial Activity

The results are presented in Table 3. For water fraction of *B. falcatum* methanol extract, the total polyphenols are calculated based on the measurement of 1:10 diluted extract, while for the ethanol extract, it is an average of measurements done with 1:100 and 1:1000 dilutions.

Table 3 Total polyphenol content of *S. chinensis* and *B. falcatum* extracts.

Sample	Total Polyphenol Content (mg GAE/g Plant Material)	Polyphenol concentration in the extract (mg GAE/ml)
S.chinensis fruit - ethanol extract	8.17 ± 0.23	8.17 ± 0.23
B. falcatum root - ethanol extract	3.22 ± 0.22	6.45 ± 0.43
B. falcatum root - water fraction of methanol extract	2.18 ± 0.01	0.77 ± 0.00

The total polyphenol content of *S. chinensis* fruit was determined to be 8.17 ± 0.23 mg GAE/g plant material, which is a result that is comparable to other researchers. (Mocan et al., 2014), whose methodology was used for the extraction, reported total polyphenols in the analyzed sample to be 9.20 ± 0.43 mg GAE/g. The difference could be explained by variation between the raw material in terms of for example harvest season, location or genetic factors (Bruni & Sacchetti, 2009) or by different length of storage of extracts prior to testing, as prolonged storage might be associated with decrease in polyphenol content (Tabart, Kevers, Sipel, Pincemail, Defraigne & Dommes, 2007).

Furthermore, the extraction method can also influence the amount of polyphenols extracted (Mocan et al., 2014). Wang, Chen, Zhang, Lan & Zhang, 2011, with a different extraction method as well as different source of *S. chinensis* plant material, measured total polyphenols in ethanol extract to be 26.79 ± 17.06 mg GAE/g. Kim & Park, 2010 reported 10.09 mg GAE/g. Despite this discrepancy in the result presented and other references, the measured total polyphenols seem to be a reasonable value, given the above mentioned factors influencing the levels of extracted bioactive molecules.

Based on literature review, compared to *S. chinensis* leaves, fruit is a less abundant source of polyphenols, (Mocan et al., 2014) reported approximately seven times higher levels of polyphenols in the leaf extract than in the fruit extract, while both extracts were prepared by the same method. This, together with the fact that while *S. chinensis* fruit is widely consumed in Asia while leaves are usually discarded, suggests that leaves would be a better candidate source of polyphenols, however, as leaves are not readily available outside of the harvest season, they could not have been obtained in time for the experiment.

B. falcatum root ethanol extract was less rich in polyphenols than *S. chinensis* fruit extract. The measured content was 3.22 ± 0.22 mg GAE/g plant material. No data for ethanol extract of this plant could be found for comparison. Ethanol extract of *Bupleurum kaoi* L., prepared by a different extraction method and fractionated with supercritical carbon dioxide, was shown to contain polyphenol levels ranging from 4.7 to 18.3 mg GAE/g plant material, depending on the fraction (Wang, Liu, Tseng & Yu, 2005). *B. falcatum* root water fraction of methanol extract was slightly less rich in polyphenols, containing

2.18 ± 0.01 mg GAE/g plant material. No reference could be found for total polyphenol content of *B. falcatum* root methanol extract or its fractions. The level of polyphenols present in the water fraction is possibly influenced by the extraction method (Mocan et al., 2014) and it is likely that part of the total extracted polyphenols may be present in one of the other fractions (Forsyth, 1952).

The total polyphenol content is an important indicator of antioxidant activity (Cai, Luo, Sun & Corke, 2004; Chew, Chan, Tan, Lim, Stanslas & Goh, 2011). Based on previous screening of 112 Chinese medicinal herbs, it was shown that in general, correlation coefficient between total polyphenol content and antioxidant activity was 0.967 for aqueous and 0.961 for methanolic extracts (Cai, Luo, Sun & Corke, 2004). It can therefore be assumed that extracts with higher polyphenol content would exhibit higher antioxidant activity. As it was not possible to conduct antioxidant activity testing in the frame of the project, this fact was used to select the two extracts with higher polyphenols for further testing as they are more likely to have higher antioxidant activity, which is a beneficial feature for active packaging polymers intended to prolong shelf-life.

Plant polyphenols were also shown to exhibit antimicrobial activity (Cowan, 1999; Karou, Dicko, Simpore & Traore, 2005; Moreno, Scheyer, Romano & Vojnov, 2006) and for example in rosemary extracts, there has been shown a link between polyphenol content and antimicrobial activity (Moreno, Scheyer, Romano & Vojnov, 2006). On the other hand, such a clear correlation was not observed in other studies (Chew, Chan, Tan, Lim, Stanslas & Goh, 2011), and therefore, total polyphenol content might not be a reliable indicator of antimicrobial activity, as this may be a result of synergic effect of many constituents of the extracts (Akın, Saraçoğlu, Demirci, Başer & Küçüködük, 2012).

5.4 Antibacterial Activity of *S. chinensis* Fruit and *B. falcatum* Root Ethanol Extracts against Selected Gram-positive and Gram-negative Bacteria

All *V. parahaemolyticus* petri dishes did not show any growth even after 72 hours, which was most likely caused by insufficient level of sodium chloride in the broth (Beuchat, 1973).

E. coli was not inhibited (no inhibition zone appeared) at any concentration tested of either *B. falcatum* root or *S. chinensis* fruit ethanol extract. There was no growth under the discs, however that was most probably a result of unfavorable growth conditions, as the control plates with paper discs impregnated with sterile distilled water only also did not exhibit any growth under the disc even without any infusion with antibacterial agents.

B. cereus was not inhibited (no inhibition zone appeared) by *B. falcatum* root ethanol extract at any concentration tested, however it was inhibited by *S. chinensis* fruit ethanol extract at 0.5 mg polyphenols/ml and 0.75 mg polyphenols/ml with inhibition zones of 41 ± 16 and 106 ± 22 mm^2, respectively. The MIC for *B. cereus* was

0.5 mg polyphenols/ml. There was no growth under the discs at all concentrations tested of both extracts, which, however, is linked to unfavorable growth conditions.

L. monocytogenes was inhibited by *S. chinensis* fruit ethanol extract at 0.75 mg polyphenols/ml with inhibition zone of 97 ± 11 mm^2. The *B. falcatum* root ethanol extract did not inhibit (no inhibition zone appeared) *L. monocytogenes* at any concentration tested. There was no growth under the discs at all concentrations tested of both extracts, which, however, is linked to unfavorable growth conditions. The results are summarized in Tables 4 and 5 and illustrated by Figure 9.

Table 4 Inhibition zones and minimum inhibitory concentrations of *S. chinensis* extract against selected bacteria

	Inhibition zone (mm^2)					**MIC**
SFE[a] concentration (mg polyphenols/ml)	0.05	0.1	0.25	0.5	0.75	
E. coli	-	-	-	-	-	N/A
V. parahaemolyticus	N/A	N/A	N/A	N/A	N/A	N/A
B. cereus	-	-	-	41 ± 16•	106 ± 22•	0.5
L. monocytogenes	-	-	-	-	97 ± 11	0.75

• Values followed by identical symbol are significantly (P < 0.05) different from each other.
[a]SFE = *S. chinensis* fruit extract

Table 5 Inhibition zones and minimum inhibitory concentrations of *S. chinensis* extract against selected bacteria

	Inhibition zone (mm)		**MIC**
SFE concentration (mg polyphenols/ml)	0.5	0.75	
E. coli	-	-	N/A
V. parahaemolyticus	N/A	N/A	N/A
B. cereus	2 ± 1	5 ± 1	0.5
L. monocytogenes	-	4	0.75

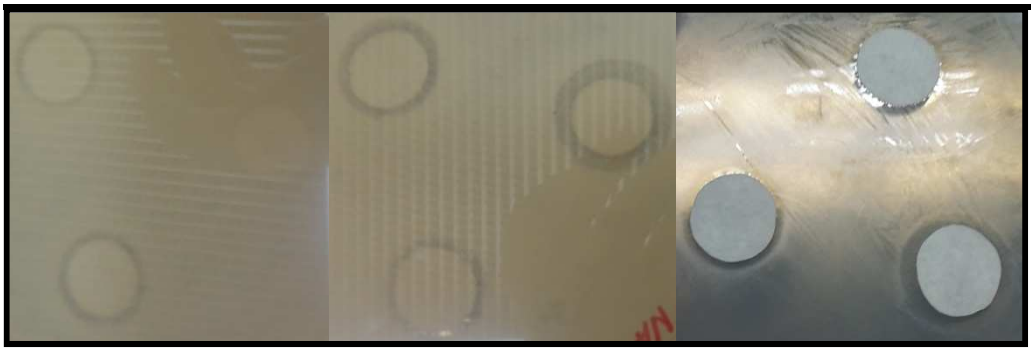

Figure 9 Left to right: *B. cereus* incubated with diluted *S. chinensis* extract containing 0.5 mg/ml polyphenols, B. cereus incubated with 0.75 mg/ml polyphenols, and *L. monocytogenes* incubated with 0.75 mg/ml polyphenols

It can be concluded that the compounds responsible for the antibacterial effect are specific to *S. chinensis*, as *B. falcatum* extract was standardized to contain equal levels of total polyphenols, yet *B. falcatum* extract did not exhibit any antibacterial properties at the same concentrations tested. It is not unusual that total polyphenol content does not necessarily correlate with antibacterial activity (Chew, Chan, Tan, Lim, Stanslas & Goh, 2011). Moreover, there are differences between the antibacterial activity depending on the polyphenol structure, for example, MIC against *B. cereus* and *L. monocytogenes* can range between 117 and 3 200, and between 67 and 3 200 µg polyphenols/ml, respectively (Taguri, Tanaka & Kouno, 2006).

While the sample of bacteria tested was narrow, consisting of only two Gram-positive (*B. cereus*, *L. monocytogenes*) and two Gram-negative (*E. coli*, *V. parahaemolyticus*) strains, and one of the Gram-negative bacteria did not exhibit any growth, the results seem to indicate that *S. chinensis* fruit ethanol extract is more potent against Gram-positive than Gram-negative bacteria, as both Gram-positive bacteria were inhibited, while the Gram-negative was not. Such trend had been observed with numerous plant extracts and seems to be related to the complicated structure of Gram-negative bacterial membrane (Shan, Cai, Brooks & Corke, 2007).

In comparison, Mocan et al., 2014 tested a range of concentrations of ethanol extracts of *S. chinensis* fruit and leaves up to 0.1 mg/ml to estimate MIC. No information was given whether the extract was dried before dilution or whether the original liquid extract was used, which makes direct quantitative comparison impossible, however, qualitatively, Gram-positive strains showed lower resistance than Gram-negative strains (bacteria tested were three Gram-positive and 2 Gram-negative, with common bacteria between this assay and Mocan et al. being *E. coli* and *L. monocytogenes*). In the study of Mocan et al., 1 mg/ml extract dissolved in DMSO created inhibition zones against all of the strains tested, however, during the MIC testing, the highest concentration (0.1 mg/ml) failed to inhibit *L. monocytogenes* as well as *E. coli*. In the present experiment, *L. monocytogenes* was inhibited while *E. coli* was not, suggesting that the MIC concentration for *E. coli* is higher than 0.75 mg polyphenols/ml, which corresponds to approximately 40 mg/ml concentration of total solids of the extract, which is much higher than the value given by Mocan et al. Other than possibly different way of expressing concentration of extracts, another difference between experiment of Mocan et al. and the present study is use of different solvents, which has influence on the results and can cause falsely positive results (Wadhwani et al., 2009). Mocan et al. used DMSO (9 parts to 1 part extract) of unknown concentration, while the present study was done with extract dissolved in water.

There are no data for antimicrobial effect of *B. falcatum* root ethanol extract. It was expected, based on assays done with ethyl ether and ethyl acetate fractions of methanol extract (Lee, Choi & Yun, 2014) that the plant compounds possess strong antibacterial properties against both Gram-positive and Gram-negative bacteria (MIC 0.1 mg/ml against *B. cereus*; 0.25 mg/ml against *E. coli*). There are several possible reasons why the ethanol extract failed to exhibit antimicrobial effect. First, it is possible that the active compounds

could only be extracted with methanol, as it has been shown previously that it is possible there are more compounds present in methanol than ethanol extracts of the same plant material, particularly within the group of bacterial inhibitors (Eloff, 1998). Alternatively, it is possible that the compounds were present in the ethanol extract, but they were less concentrated than they would be in the particular fractions, and therefore higher concentration of the ethanol extract would have been necessary to demonstrate ability to inhibit bacterial growth. The reason why ethanol extract was prepared and tested was the intended use of the extract as additive into food packaging material, which requires water or oil solubility and no toxicity, which limits the use of methanol extract as well as hexane, ethyl acetate and ethyl ether fractions, which are insoluble in both systems and which are toxic. Unfortunately, methanol extract and its fractions, which were expected to demonstrate very good antibacterial effect, could not be tested as they were not soluble in water, importance of which was discussed previously.

5.4.1 Solvent Interference

A concern that needs to be addressed when testing extracts is the solvent used to extract or solubilize the plant compounds. Ethanol is an antibacterial agent by itself and remaining ethanol in the extract after evaporation could possibly interfere with the results (Wadhwani et al., 2009). However, the two extracts, both extracted with 70% ethanol, were submitted to identical evaporation conditions. Both extracts were diluted to contain equal amount of polyphenols with distilled water, and *B. falcatum* extract, being less rich in polyphenols, was diluted less than *S. chinensis* extract. If ethanol was responsible for the inhibition of bacteria due to higher ethanol content in the less diluted extracts, it can be assumed that *B. falcatum* root ethanol extract would have seemingly exhibited antibacterial effect at 0.5 and 0.75 mg polyphenols/ml, which was not the case. It can therefore be concluded that the antibacterial effect of *S. chinensis* fruit extract is due to presence of antibacterial compounds.

5.4.2 Comparison with Other Plant Extracts

Evaluating the antimicrobial activity of the prepared *S. chinensis* fruit ethanol extract, as compared to other plant extracts, is challenging, as sometimes it is unclear from the methodology whether extracts were dried to solids, which were then resuspended in a solvent, or whether liquid extracts were considered to be the concentrated extract from which dilutions were made. Furthermore, rarely there are mentions of how influence of solvents on the assays was controlled. Finally, often *in vitro* assays are done in conditions which do not consider potential applications of the extracts, which are limited by the safety for ingestion by humans and animals and by compatibility with the matrix in which they are to be embedded.

What seems to be a major challenge for many screenings for antimicrobial substances from plants is finding those which could effectively inhibit Gram-negative bacteria, particularly

at lower concentrations (Chew, Chan, Tan, Lim, Stanslas & Goh, 2011; Mocan et al., 2014; Moreno, Scheyer, Romano & Vojnov, 2006) and those which would exhibit a wide effect against different bacteria. Most screenings showed that even within Gram-positive and Gram-negative groups, each specific strain reacts to the same dose of extract differently (Chew, Chan, Tan, Lim, Stanslas & Goh, 2011; Rauha et al., 2000).

While direct comparison is difficult due to varying ways of extract preparation and solvent systems used to resuspend or dilute extracts, *S. chinensis* fruit ethanol extract did not seem to be an extraordinarily strong antimicrobial agent. The MIC determined for Gram-positive bacteria ranged from 0.500 to 0.750 mg polyphenols/ml (corresponding to approximately 26 and 40 mg/ml concentration of total solids, respectively) and MIC for Gram-negative bacteria is expected to be even higher, if they can be inhibited by the extract at all. Yet, for example, *Rosmarinus officinalis* MIC against Gram-positive bacteria range between 2 and 15 µg/ml and against Gram-negative bacteria between 2 and 60 µg/ml (Moreno, Scheyer, Romano & Vojnov, 2006), which shows higher effectivity against bacteria than was exhibited by *S. chinensis* fruit ethanol extract. Many plant extracts inhibit bacteria in the range of hundreds of µg/ml (Taguri, Tanaka & Kouno, 2006).

A very interesting conclusion of (Mocan et al., 2014) was that *S. chinensis* leaves exhibited a stronger antibacterial effect than fruits. Leaves could not have been obtained for the present study due to seasonality, however as data for fruit extract are to a certain degree in accordance with those of Mocan et al., it can be expected that leaves would be an interesting source of antibacterial compounds. Not only are they are not utilized at all at present and are discarded during harvest, but they actually exhibit stronger effect than fruits. It is therefore a very good potential material, as it can be obtained cheaply and it does not compete with food production, unlike usage of *S. chinensis* fruits, which are sought after for various culinary applications, mainly for beverages (Lee & Kim, 2000; Mok, 2005).

5.5 Cricket and Shrimp Chitosan with or without *S. chinensis* extract Film Casting

Preliminary experiments (data not shown) indicated that 8 – 12 ml of film-forming solution containing 1:2 glycerol:chitosan (w/w) per Petri dish (8.5 cm diameter) resulted in films with reasonable plasticity and that drying for 11 – 15 h was sufficient to dry the films. Thicker films (made from 14, 16 and 25 ml of film-forming solution) had lower plasticity than thinner films and the same was observed for films dried for 24 h compared to those dried for up to 15 h. Samples of the films are illustrated in Figure 10.

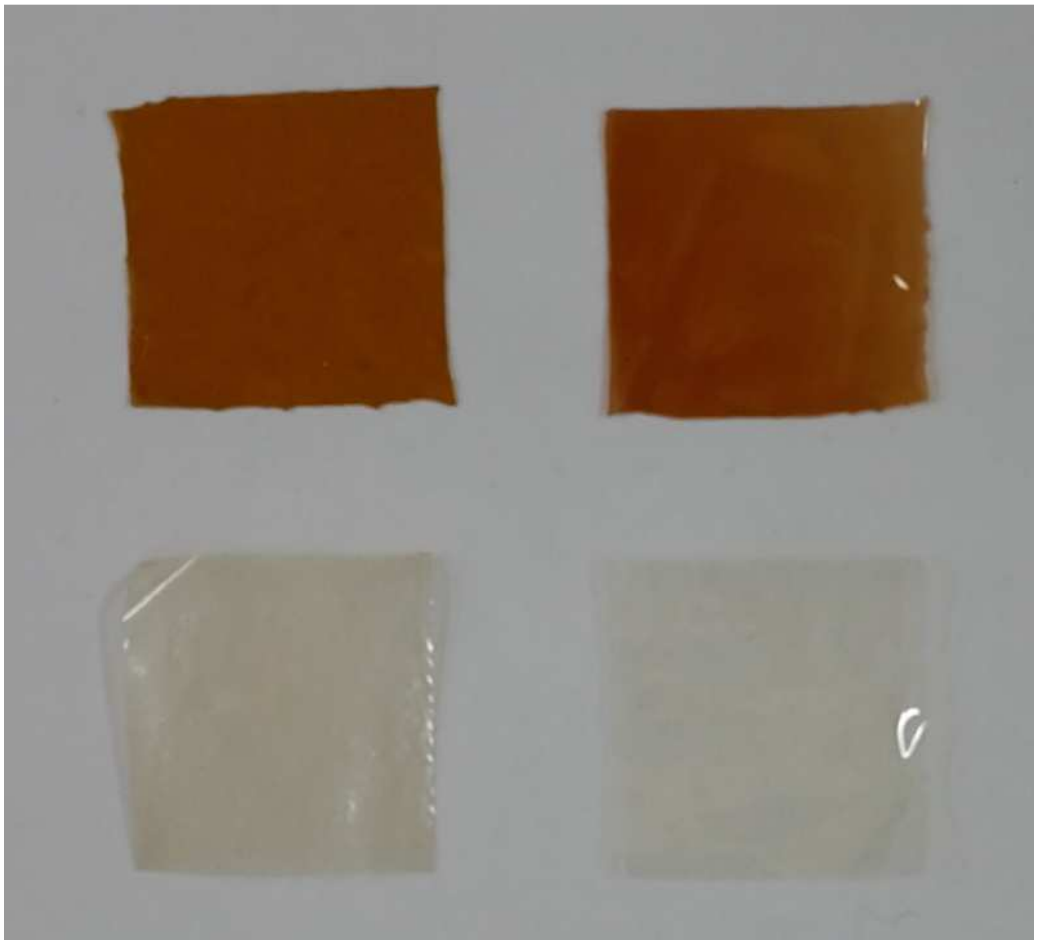

Figure 10 Top row, left: Cricket chitosan film containing *S. chinensis* extract, right: shrimp chitosan containing *S: chinensis* extract; bottom row, left: cricket chitosan film, right: shrimp chitosan film

During preparation of film-forming solutions, several differences between solutions made from shrimp and cricket chitosan were observed. The cricket chitosan dissolved faster than shrimp chitosan, most likely because of higher particle size of cricket chitosan, as the powder was dispersed more evenly in acetic acid solution than shrimp chitosan, which quickly formed clumps surrounded by gel layer, which slowed down dissolution. The fast dissolution in mild acetic acid also confirms that the substance prepared was indeed chitosan, as chitin does not seem to be easily soluble in such conditions (Pillai, Paul & Sharma, 2009).

The cricket chitosan film-forming solutions had also higher viscosity by subjectively perceived difference in filtration speed (viscosity was not measured scientifically). It was shown previously that chitosan solutions behave as shear-thinning liquids and at a given shear rate, their viscosity is dependent on Mw and DDA. Based on study with rheological

properties of chitosan solutions, viscosity increases with increased molecular weight and with decreased degree of deacetylation, however the limitation of the experiment was that chitosan with higher Mw had also lower DDA, therefore it was challenging to clearly show the link between viscosity and the two characteristics (Mucha, 1997). In this experiment, chitosan with lower Mw and lower DDA appeared to have higher viscosity, which possibly suggests that DDA influences viscosity more than Mw.

During film casting, there was a difference between film-forming solutions containing *S. chinensis* extract and film-forming solutions containing only chitosan (valid for both shrimp and cricket chitosan). While solutions containing extract easily coated the area of the Petri dish, solutions not containing extract required vigorous spreading in order to form a uniform layer, suggesting different wetting ability, with wetting being facilitated by the addition of *S. chinensis* fruit extract (Figure 11). In preliminary experiments (data not shown), the minimum volume that could form a uniform layer on the 8.5 cm petri dish of film-forming solution containing extract was 7 ml, while 12 ml was needed of solution not containing extract. Addition of *S. chinensis* extract therefore improves wetting, leading to the possibility to pour thinner films. Wetting, however, was not assessed by scientific methods. Experiments with diluted chitosan solutions in mild acids showed that surface tension is influenced mainly by inter-molecular hydrogen bonding (Qun & Ajun, 2006). As the surface tension of the solutions containing extract was apparently lower, it can be deduced that the presence of extract reduced the inter-molecular hydrogen bonding, possibly by interacting with the OH or NH_2 groups.

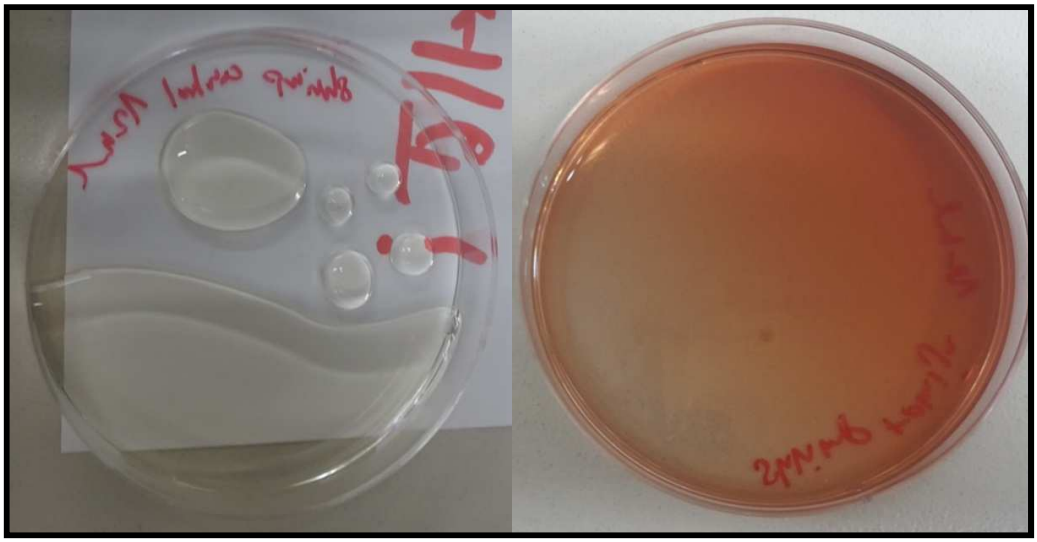

Figure 11 Left: shrimp chitosan film-foming solution; right: shrimp chitosan film-forming solution containing *S. chinensis* extract

The different behavior suggests that differences in physicochemical properties exist between different film-forming solutions based on the chitosan they contain and whether they contain *S. chinensis* extract or not and it would be interesting to assess these differences by appropriate methods. Also, prolonged ultrasonication has been used to reduce molecular weight of chitosan without affecting DDA in some cases (Baxter, Zivanovic & Weiss, 2005), therefore it would be interesting to measure molecular weight prior to sonication and after to confirm whether it has been affected by the degassing procedure or not.

5.6 Cricket and Shrimp Chitosan with or without *S. chinensis* Film Properties

5.6.1 Antimicrobial Activity of Chitosan Films with or without *S. chinensis* Extract

5.6.1.1 Film Application on Agar

Application of chitosan films on agar plates is challenging because of the difference in moisture content between the films and agar, which leads to shrinking, stretching and curling of the films. This behavior was observed in the current experiment, as well as by other researchers, and seems to be linked to the drying method (Leceta, Guerrero, Ibarburu, Dueñas & De la Caba, 2013), which influences moisture content of the films. Use of sterile plastic discs as applicators to ease adhesion of the films to agar is recommended, and showed better results than use of paper discs or use of forceps only (Figure 12).

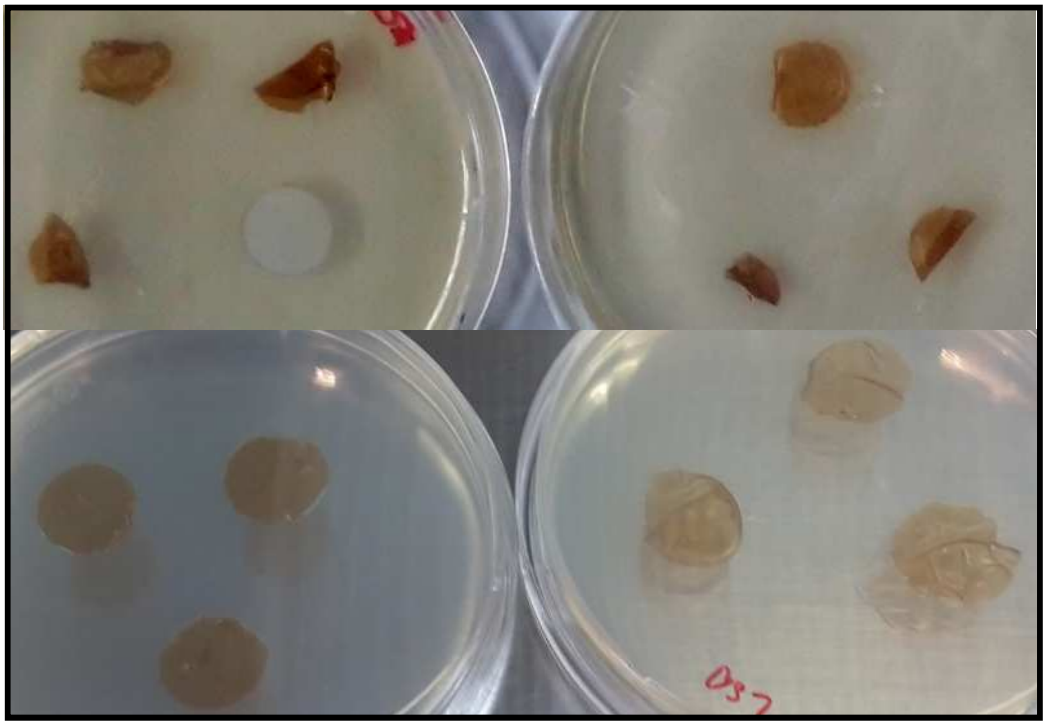

Figure 12 Above: use of forceps or paper discs, below: use of plastic applicators

5.6.1.2 Evaluation of Antimicrobial Activity through Agar Diffusion Test

Negative controls consisting of lawn cultures with sterile paper discs infused with sterile distilled water did not show any inhibitory zones, however there was no growth under the disc area. As distilled water does not have antibacterial properties, it was therefore assumed that no bacterial growth under the experimental films was due to adverse growth conditions caused by the adhesion of the disc to the agar surface and not by bacteriostatic properties.

E. coli was not inhibited by any of the films tested (shrimp chitosan film with or without *S. chinensis* extract, cricket chitosan film with or without *S. chinensis* extract) during the whole duration of the test (10 days).

L. monocytogenes was inhibited by both shrimp and cricket chitosan films containing *S. chinensis* fruit extract standardized to contain 0.75 mg polyphenols/ml film-forming solution (corresponding to approximately 4% (w/V) concentration of total solids). The size of the inhibition zones (IZ) is reported in Table 6 and illustrated by photographs (Figure 13), the size of the inhibition zones did not change throughout the duration of the assay. On the other hand, films not infused with the extract did not inhibit *L. monocytogenes* growth at all.

Table 6 Inhibition zones created against selected bacteria.

	Shrimp Chitosan IZ [mm^2]	Cricket Chitosan IZ [mm^2]	Shrimp Chitosan + *S. chinensis* IZ [mm^2]	Cricket Chitosan + *S. chinensis* IZ [mm^2]
E. coli	-	-	-	-
B. cereus	-	$280 \pm 69^{a\blacksquare}$	$169 \pm 44 / 82 \pm 43^{b\bullet\blacksquare\blacklozenge}$	$195 \pm 30^{c\bullet\star}$
L. monocytogenes	-	-	$591 \pm 72^{c\blacklozenge}$	$690 \pm 51^{c\star}$

[a] Inhibition zone formed after 6 days of incubation
[b] Size of inhibition zone decreased after 3 days of incubation
[c] Size of inhibition zone was constant during the whole duration of the test
$^{\bullet\blacksquare\blacklozenge\star}$ Values followed by identical symbol are significantly ($P < 0.05$) different

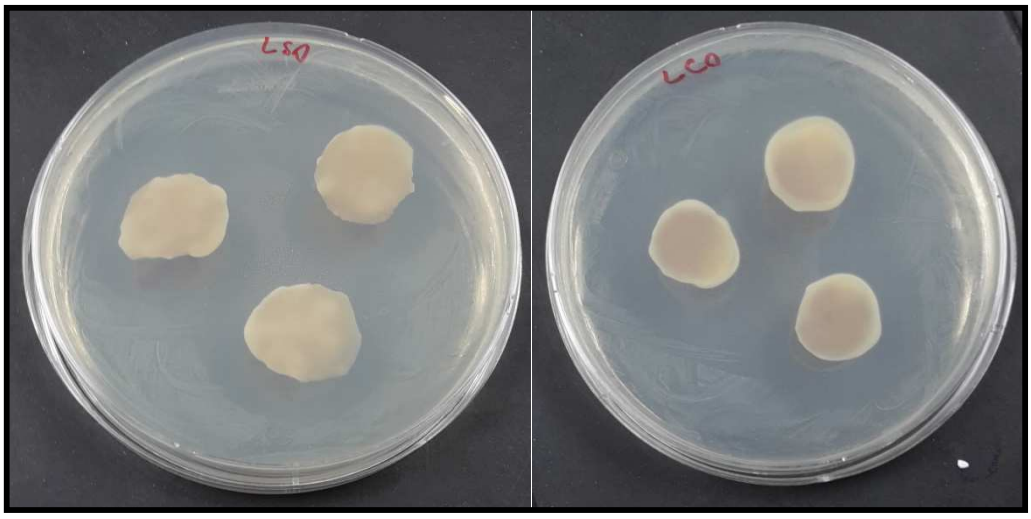

Figure 13 Left to right: *L. monocytogenes* incubated with shrimp chitosan film infused with *S. chinensis* fruit extract and with cricket chitosan film infused with *S. chinensis* fruit extract

B. cereus was inhibited by both shrimp and cricket chitosan films containing *S. chinensis* fruit extract standardized to contain 0.75 mg polyphenols/ml film-forming solution (corresponding to approximately 4% (w/V) concentration of total solids). The inhibition zones are reported in Table 6 and illustrated by photographs (Figure 14), the size of the inhibition zones did not change throughout the duration of the assay for cricket chitosan film containing extract, but it decreased in size for shrimp chitosan film containing the extract after 3 days, afterwards the size remained constant for the remaining period of the experiment. Film prepared only from cricket chitosan did not lead to formation of inhibition zones during the first 5 days of assay, however on the sixth day, clear inhibition zones formed (Figure 14) and maintained constant size throughout the remaining period of assay. No inhibition zones could be observed with films prepared only from shrimp chitosan.

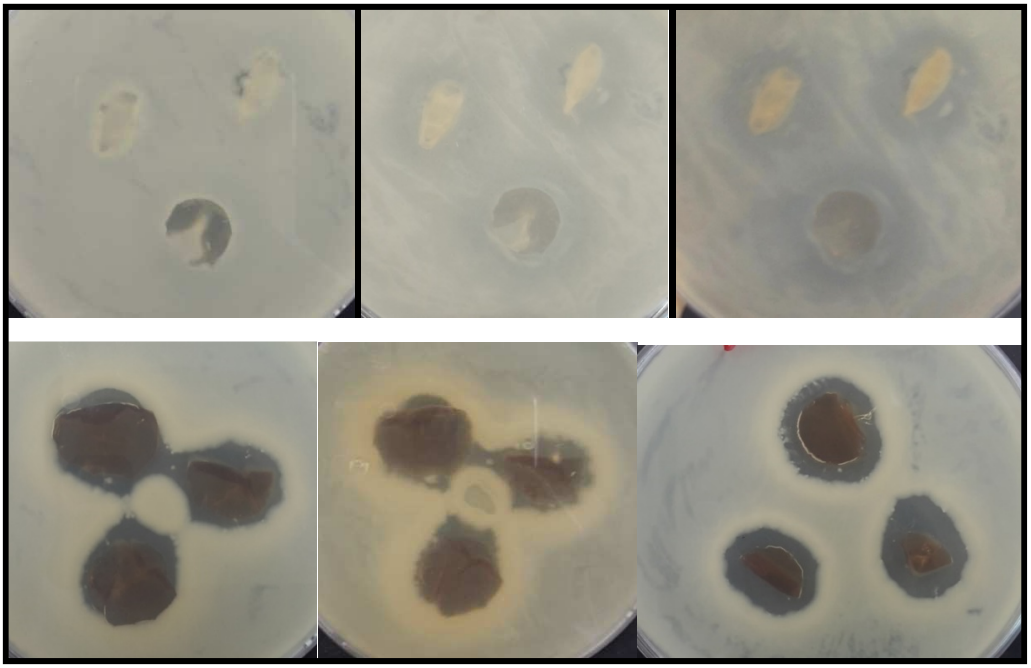

Figure 14 Above, left to right: *B. cereus* incubated with cricket chitosan discs after 1, 6 and 8 days. Below, left to right: *B. cereus* incubated with shrimp chitosan film infused with *S. chinensis* fruit extract (after 1 and 8 days) and with cricket chitosan film infused with *S. chinensis* fruit extract

5.6.1.3 Antimicrobial Properties of Cricket and Shrimp Chitosan Films

In the study of Leceta, Guerrero, Ibarburu, Dueñas & De la Caba, 2013, agar-diffusion tests performed both with films and film-forming solutions prepared from LMW and HMW commercial chitosan (DDA 75%) with added glycerol did not exhibit any antimicrobial effect, irrespective of the molecular weight and glycerol content. Assay with film incubation in bacterial medium also did not lead to decrease in the measured turbidity. On the contrary, experiment with film-forming solution incubated in bacterial culture led to decrease in absorbance, suggesting lethal effect towards the bacteria. These results suggest that due to molecular weight of chitosan, diffusion through agar is limited irrespective of whether the chitosan is dissolved or forms a film matrix. On the other hand, in bacterial culture, film-forming solution allowed for more efficient diffusion in the medium compared to chitosan forming a film. This supports the widely accepted fact that chitosan is antimicrobial substance, however the property can only be exhibited when chitosan molecules can interact with the bacterial membranes.

Slightly different observation was made by other researchers. Peng & Li, 2014 and Wang et al., 2011 observed antimicrobial effect against *E. coli* and *S. aureus* of pure commercial crustacean chitosan (DDA 85% - 90%) film-forming solutions in agar-diffusion test. This might have been possibly a result of difference in deacetylation degree of chitosan among

the studies, as higher deacetylation degree has been linked to higher antibacterial activity (Benhabiles, Salah, Lounici, Drouiche, Goosen & Mameri, 2012; Younes, Sellimi, Rinaudo, Jellouli & Nasri, 2014). However, once film was formed with 50 kDa chitosan, no antibacterial effect was observed, supporting the limited diffusion ability of LMW chitosan from film into agar (Wang et al., 2011).

Other researchers also did not observe any inhibition zones during study carried out with films prepared from low or medium molecular weight crustacean chitosan (DDA 75 - 85%) against any of the microorganisms tested including *L. monocytogenes* and *E. coli* (Altiok, Altiok & Tihminlioglu, 2010; Hosseini, Razavi & Mousavi, 2009; Ojagh, Rezaei, Razavi & Hosseini, 2010; Zivanovic, Chi & Draughon, 2005). Similarly, high molecular weight chitosan films (DDA 95%) did not inhibit *E. coli*, *L. monocytogenes*, *B. cereus* and other bacteria (Pranoto, Rakshit & Salokhe, 2005).

The result obtained is in agreement with the above mentioned studies, as none of the strains was inhibited by LMW shrimp chitosan films. The likely reason why cricket chitosan showed antibacterial effect against *B. cereus* and why this effect was only visible after prolonged incubation is therefore the lower molecular weight of cricket chitosan (at least partially in the chitooligosaccharide range) – approximately between 560 and 7457 Da (Chae, 2015) (unpublished data). The low molecular weight allows the substance to diffuse through the film, however at slower rates than antimicrobial compounds from *S. chinensis* extract, which are of much smaller size (Mocan et al., 2014). These findings are consistent with studies that observed higher activity of chitosan films against Gram-positive strains (Coma, Deschamps & Martial-Gros, 2003; Jridi et al., 2014; No, Young Park, Ho Lee & Meyers, 2002) and showed that actually in films, chitosan of very low molecular weight is more potent as it seems the molecules can easily diffuse from the films and be in contact with the bacterial membranes. No link between low molecular weight and higher potency against Gram-negative bacteria was found, however the study was limited to only one Gram-negative strain.

It is also not surprising that only *B. cereus* was inhibited while no effect was observed towards *L. monocytogenes* and *E. coli* as it has been shown that susceptibility to chitosan differs significantly among different bacterial strains (Wang et al., 2011) and it has been challenging to predict which bacteria in particular would be more susceptible. An interesting fact to be noted though is slightly lower deacetylation degree of the cricket chitosan (69 – 76%), which apparently did not interfere with its ability to inhibit growth of *B. cereus*. This observation further supports the general opinion that antibacterial activity of chitosan films depends on a complex combination of different factors, including molecular weight, deacetylation degree, pH, film moisture content, and the bacterial strain in question.

5.6.1.4 Antimicrobial Properties of Cricket and Shrimp Chitosan Films Infused with S. chinensis Fruit Extract

Incorporation of *S. chinensis* extract did not improve antimicrobial properties against *E. coli*, as was expected based on MIC results.

On the other hand, incorporation of the extract led to formation of inhibition zones on plates with both Gram-positive strains (*L. monocytogenes* and *B. cereus*). In the case of *L. monocytogenes*, there was no significant difference between the sizes of inhibition zones based on the type of chitosan used, which was in accordance with the failure of pure cricket/shrimp chitosan film to inhibit bacterial growth. It can be assumed that the inhibition zone was formed purely as a result of *S. chinensis* extract activity. The significant difference ($P < 0.05$) between sizes of inhibition zones formed by diluted *S. chinensis* extract (97 ± 12 mm^2) and sizes of inhibition zones formed by the films infused with the extract (591 ± 72 and 691 ± 51 mm^2 for shrimp chitosan and cricket chitosan films, respectively) is due to increased extract concentration per cm^3 of film as compared to the film-forming solution caused by evaporation of large volume of water from the film during drying.

In the case of *B. cereus*, initially, the size of inhibition zones was similar for both shrimp and cricket chitosan films containing the extract (no significant difference ($P < 0.05$) was observed). However, after 3 days of incubation, the size of inhibition zone created by the shrimp chitosan film decreased in size significantly ($P < 0.05$) compared to the constant size of inhibition zones caused by the cricket chitosan film. This can be explained by combined effect of *S. chinensis* extract and cricket chitosan. It is likely that initial growth was inhibited by diffusion of the extract, and after several days, the slow diffusion of cricket chitosan supported the effect. On the other hand, no chitosan diffusion occurred from shrimp chitosan films and therefore, bacteria could start growing nearer the disc than initially. Compared to the antimicrobial effect of *S. chinensis* extract standardized to contain 0.75 mg polyphenols/ml, there was significant ($P < 0.05$) difference in inhibition zone size only between cricket chitosan film infused with the extract (195 ± 30 mm^2) and the pure extract (106 ± 25 mm^2). There was no significant ($P > 0.05$) difference between the size of inhibition zones created by pure cricket chitosan film and cricket film containing extract. Considering that there was no significant difference between the activity of pure extract and extract added to shrimp chitosan film (169 ± 44 or 82 ± 43 mm^2), the increased area of inhibition zone in the case of cricket chitosan film containing extract can be attributed to the effect of cricket chitosan. This is also in line with the result obtained for pure cricket chitosan film, which inhibited *B. cereus*. This is different from the result obtained for *L. monocytogenes*, where both chitosan films (shrimp and cricket) infused with the extract created larger inhibition zones than the extract alone. The size of inhibition zones is surprising also considering the fact that *S. chinensis* MIC for *B. cereus* was determined to be 0.5 mg polyphenols/ml, and hence the film, containing higher concentration, should have led to occurrence of much larger inhibition zones, particularly

considering the effect of increased concentration on *L. monocytogenes*. Possible explanation for the occurrence of this effect could be higher number of CFU/ml than estimated by CFU plate counts which would lead to seemingly lower activity, local heterogeneities in the films tested, or most likely, the bad adhesion of the films to agar (Figure 13), which might have led to low amounts of antimicrobial compounds released into the agar.

5.6.1.5 Antimicrobial Effect of Cricket or Shrimp Chitosan Films Containing S. chinensis Extract in Comparison with Other Films Containing Plant Essential Oils

S. chinensis extract was used as an additive into chitosan films for the first time. Typically, plant essential oils rather than water-soluble extracts are used to enhance antibacterial properties of chitosan films. In the studies of Hosseini, Razavi & Mousavi, 2009 and Zivanovic, Chi & Draughon, 2005, *L. monocytogenes* was more susceptible to crustacean chitosan films incorporated with thyme, clove, cinnamon, or oregano oils than other strains tested. For *L. monocytogenes*, the inhibition zone observed in the present study had much larger area than the inhibition zones produced by all oils at 1% (oregano) or 1.5% (remaining oils) concentration (V/V) in film-forming solution, however the concentration of concentrated extract was 4% (w/V) in the film-forming solution. 1 - 4% oregano oil incorporated in chitosan film produced inhibition zones with diameter much smaller compared to *S. chinensis* fruit extract incorporated in cricket or shrimp chitosan films, even though preliminary experiment with pure oregano oil diluted to 1% concentration led to inhibition zones larger than those obtained with *S. chinensis*. It is therefore likely that oregano oil antimicrobial compounds are either sensitive and lost during film preparation, or they are not efficiently released into agar (Zivanovic, Chi & Draughon, 2005). On the other hand, garlic oil incorporated in chitosan film exhibited comparable activity to *S. chinensis* extract at 0.2 – 0.3% concentration (V/V) and created much larger inhibition zones at 0.4% (Pranoto, Rakshit & Salokhe, 2005). It can be therefore concluded that *S. chinensis* fruit extract has lower activity against *L. monocytogenes* than certain essential oils when incorporated in chitosan films, however it is more effective and has better diffusion ability than others. Moreover, it simplifies the preparation of films significantly as there is no need for addition of TWEEN, which is used to homogenize the dispersion of essential oils in chitosan solution (Zivanovic, Chi & Draughon, 2005), and it also increases wetting properties which are of interest during film casting.

1% lemon, thyme, cinnamon and oregano oils incorporated in chitosan films successfully inhibited *E. coli* (Altiok, Altiok & Tihminlioglu, 2010; Peng & Li, 2014; Zivanovic, Chi & Draughon, 2005), while *S. chinensis* extract at 4% (w/V) did not seem to have any effect against this bacterium. Cinnamon oil exhibited different effect in different studies, in some as low as 0.4% successfully inhibited growth of *E. coli* and other Gram-negative bacteria (Ojagh, Rezaei, Razavi & Hosseini, 2010), however other results showed that minimum amount necessary to be incorporated was 7.5% (Wang et al., 2011), which might be related

to difference in chitosan used for the study and different film preparation method. Clove oil dispersed in chitosan films inhibited *E. coli* at 5% concentration or higher (Wang et al., 2011). However, garlic oil incorporated in chitosan film did not inhibit *E. coli* at any concentration tested (0.1 - 0.4%) (Pranoto, Rakshit & Salokhe, 2005). These oils therefore perform much better than *S. chinensis* fruit extract against Gram-negative strains. There is however possibility that *S. chinensis* leaves would manifest good activity also against Gram-negative strains (Mocan et al., 2014).

B. cereus was inhibited by garlic oil from as low as 0.1% (V/V) more efficiently than by *S. chinensis* fruit extract (Pranoto, Rakshit & Salokhe, 2005).

In many studies, Gram-negative bacteria were on average more resistant than Gram-positive ones to films incorporated with plant essential oils (Hosseini, Razavi & Mousavi, 2009; Ojagh, Rezaei, Razavi & Hosseini, 2010; Pelissari, Grossmann, Yamashita & Pineda, 2009; Peng & Li, 2014; Pranoto, Rakshit & Salokhe, 2005; Wang et al., 2011; Zivanovic, Chi & Draughon, 2005), with difference occurring also among particular strains within the same Gram-positive or –negative group (Altiok, Altiok & Tihminlioglu, 2010; Hosseini, Razavi & Mousavi, 2009; Pelissari, Grossmann, Yamashita & Pineda, 2009; Pranoto, Rakshit & Salokhe, 2005). This is consistent with the effect observed in the present study, where Gram-negative strain was not susceptible while both Gram-positive strains were, with different inhibition zones.

5.6.1.6 Reliability of Agar Diffusion Method to Assess Antimicrobial Properties of Chitosan Films

Important consideration when assessing antimicrobial activity of films through agar diffusion method is the fact that substances which cannot diffuse well in agar medium will seemingly fail to exhibit antibacterial properties despite demonstrating antibacterial effect in other assays. An alternative method which overcomes such issue is incubation of films or film-forming solution (Leceta, Guerrero, Ibarburu, Dueñas & De la Caba, 2013) in bacterial culture with periodic recording of optical density (OD) and plating of appropriately diluted aliquots for CFU counts. The shortcoming of this alternative method is that the recommended film:culture ratio (Durango et al., 2006; Rhim, Hong, Park & Ng, 2006) requires large volume of film, which could not be prepared considering current scale of production and yield of cricket chitosan isolation. On the contrary, agar-diffusion tests require only very small area of films. Furthermore, considering the application of the prepared films as packaging polymers, agar diffusion assay seems to be the most reasonable *in vitro* approximation of the system. While assays performed with film-forming solutions in bacterial culture might be a better indication of the substances' absolute ability to inhibit bacterial growth due to unobstructed diffusion (Leceta, Guerrero, Ibarburu, Dueñas & De la Caba, 2013), ultimately, the polymer will be applied as a film and hence tests should be performed with the film rather than the solution. Similarly, considering the solubility of the chitosan films in water (as discussed in the following

chapters) and bacterial cultures being suspended in water-based broth, it can be argued that assays with film samples incubated in liquid bacterial cultures would not be an accurate representation of the intended function unless the polymer is used for beverage packaging.

5.6.2 Thickness, Density and Moisture Content (MC)

The average thickness, density and moisture content of the films is reported in Table 7.

Table 7 Comparison of thickness, density and moisture content of films prepared

	Thickness (µm)	Density (g/cm^3)	Moisture Content (%)
Shrimp	$53 \pm 8^{\bullet\blacksquare}$	$1.178 \pm 0.118^{\bullet}$	$35.67 \pm 1.30^{\blacksquare}$
Cricket	$61 \pm 6^{\bullet\blacklozenge}$	$0.994 \pm 0.061^{\blacksquare}$	$37.52 \pm 0.35^{\blacklozenge}$
Shrimp and *S. chinensis*	$111 \pm 16^{\blacksquare}$	$1.260 \pm 0.173^{\bullet}$	$22.49 \pm 0.36^{\bullet\blacksquare}$
Cricket and *S. chinensis*	$116 \pm 17^{\blacklozenge}$	$1.178 \pm 0.118^{\blacksquare}$	$20.56 \pm 0.13^{\bullet\blacklozenge}$

$^{\bullet\blacksquare\blacklozenge}$Values followed by identical symbol within the same column are significantly ($P < 0.05$) different.

It can be seen that the thickness and density of films significantly ($P < 0.05$) increased after addition of *S. chinensis* fruit extract, while moisture content significantly decreased. There was also significant ($P < 0.05$) difference in thickness between shrimp and cricket chitosan films, and in the moisture content between the shrimp and chitosan films containing *S. chinensis* extract, however these might be artifacts resulting from local heterogeneities in the films rather than due to a real difference, as there was no significant difference in the other two related parameters. This would be confirmed by a larger-scale study with higher number of casted films and more measurements, which could not be performed at this time due to limited scale of cricket chitosan production.

It has been previously reported that incorporation of certain bioactive compounds increases thickness (Hosseini, Razavi & Mousavi, 2009; Martins, Cerqueira & Vicente, 2012; Mei, Yuan, Guo, Wu, Li & Yu, 2013; Ojagh, Rezaei, Razavi & Hosseini, 2010; Park & Zhao, 2004; Peng & Li, 2014; Wang et al., 2011; Zivanovic, Chi & Draughon, 2005) and density (Ouattara, Simard, Piette, Begin & Holley, 2000; Siripatrawan & Harte, 2010; Wang, Tian, Feng, Fan, Pan & Zhou, 2014) of chitosan films. Sánchez-González, González-Martínez, Chiralt & Cháfer, 2010 however reported decrease in thickness after incorporation of tea tree oil in chitosan films and Moradi, Tajik, Razavi Rohani & Oromiehie, 2011 did not observe any significant difference in thickness after addition of extracts and essential oils. Park & Zhao, 2004 noted different effect on density based on which substance was incorporated. Moisture content was reported to decrease when extracts and essential oils are incorporated in the matrix (Martins, Cerqueira & Vicente, 2012; Ojagh, Rezaei, Razavi & Hosseini, 2010; Wang, Dong, Men, Tong & Zhou, 2013; Wang, Tian, Feng, Fan, Pan & Zhou, 2014). Alternatively, the change in moisture content (increasing or decreasing) was found to be dependent on the type of compound present and the way it affected the chitosan

matrix and its ability to bind water molecules (Hosseini, Razavi & Mousavi, 2009; Mei, Yuan, Guo, Wu, Li & Yu, 2013).

As for the mechanism in which thickness, density and moisture content are altered, it seems to be related to the looseness of the chitosan network, which is related to the void volume which can be occupied by water molecules (Wang et al., 2011), and by interactions between polyphenols and hydrophilic groups (Wang, Dong, Men, Tong & Zhou, 2013), which makes them less available to form hydrogen bonds with water molecules. It was shown that polyphenols interact moderately with chitosan and form non-covalent bonds (Kosaraju, D'Ath & Lawrence, 2006) and bind to the amino and hydroxyl groups (Wang, Dong, Men, Tong & Zhou, 2013). Incorporation of certain oils showed altered microstructure, which became looser, coupled with increased moisture content as water molecules filled the void volume of the film; or denser, coupled with lowered moisture content (Wang et al., 2011). Increase in density might also be a result of the extract fitting within the chitosan network (Wang, Tian, Feng, Fan, Pan & Zhou, 2014).

As both thickness and density were increased and moisture content decreased upon addition of *S. chinensis* extract (4%), it seems that the film matrix was altered and the extract fitted within the network, increasing the film density and blocking the hydrophilic groups from binding with water.

5.6.3 Water Solubility (WS) and Swelling Degree (SD)

The water solubility and swelling degree are reported in Table 8.

Table 8 Water solubility and Swelling degree of the films prepared

	WS (%)	SD
Shrimp	28.78 ± 10.74$^{■}$	7.13 ± 0.18$^{●♦}$
Cricket	13.12 ± 3.42$^{♦}$	1.15 ± 0.43$^{●⋆}$
Shrimp and *S. chinensis*	72.34 ± 1.61$^{●■}$	49.53 ± 6.48$^{■♦}$
Cricket and *S. chinensis*	56.80 ± 1.65$^{●♦}$	3.21 ± 0.38$^{■⋆}$

$^{●■♦⋆}$Values followed by identical symbol within the same column are significantly ($P < 0.05$) different.

Glycerol in general reduces water solubility in comparison with films that would be prepared without any additive (Leceta, Guerrero, Ibarburu, Dueñas & De la Caba, 2013). Addition of *S. chinensis* extract increased water solubility as well as swelling degree significantly ($P < 0.05$). Similar behavior was observed for thyme essential oil (Hosseini, Razavi & Mousavi, 2009; Peng & Li, 2014) (though the opposite behavior for thyme oil was observed in other studies, as discussed below), clove bud essential oil (Wang et al., 2011), tea polyphenols (Wang, Dong, Men, Tong & Zhou, 2013), and for grape pomace water extract, though not significant (Ferreira, Nunes, Castro, Ferreira & Coimbra, 2014). Swelling degree increased after addition of *Zataria multiflora* Boiss essential oil and grape seed extract (Moradi, Tajik, Razavi Rohani & Oromiehie, 2011). On the other hand,

incorporation of grape seed, clove, lemon essential oil, *Lycium barbarum* fruit extract, and cinnamon essential oil led to increased water solubility, which was explained by occurrence of covalent cross-linking bonds in the case of cinnamon essential oil and by the hydrophobicity of the other oils, and which might have been a result of different film preparation method in the case of *Lycium barbarum* (Ferreira, Nunes, Castro, Ferreira & Coimbra, 2014; Hosseini, Razavi & Mousavi, 2009; Ojagh, Rezaei, Razavi & Hosseini, 2010; Peng & Li, 2014; Wang et al., 2011; Wang, Tian, Feng, Fan, Pan & Zhou, 2014). Film swelling was also decreased with the addition of *Lycium barbarum* fruit extract (Wang, Tian, Feng, Fan, Pan & Zhou, 2014), lemon, thyme and cinnamon essential oils (Peng & Li, 2014), and galangal extract (Thakhiew, Devahastin & Soponronnarit, 2014). The reduced degree of swelling after incorporation of galangal extract was explained by increased cross-linking (Thakhiew, Devahastin & Soponronnarit, 2014). Addition of α-tocopherol did not cause any significant difference in water solubility (Martins, Cerqueira & Vicente, 2012). As discussed previously in section about moisture content, thickness and density, it seems likely that the bonds formed between *S. chinensis* extract and chitosan are of non-covalent nature, and it might be possible that as the compounds of the extract were bound through hydrogen bonds to chitosan amino and hydroxyl groups, the intermolecular forces among chitosan molecules were weakened. Hosseini, Razavi & Mousavi, 2009 also argues that the increased solubility after incorporation of thyme oil was due to disruption of the film network, and Wang et al., 2011 reported loose structure of the clove bud oil-containing film with increased solubility. Moreover, Moradi, Tajik, Razavi Rohani & Oromiehie, 2011 suggest that the interactions between chitosan molecules and polyphenols were responsible for increased swelling.

There was also a significant ($P < 0.05$) difference between films made from shrimp and cricket chitosan in both parameters, with comparison being done between both films not containing extract and both films containing extract, with one exception - the only pair which did not have significant difference was the water solubility for films not containing extract ($P > 0.05$). However, based on significant difference in the remaining three cases and large variation among the triplicate measurements, it can be argued that actually even in this case there might be significant difference, which was not manifested due to large variability between individual samples, possibly due to varying thickness. Cricket chitosan films exhibited lower water solubility as well as lower degree of swelling, and addition of *S. chinensis* extract had lower impact on swelling degree than in the case of shrimp chitosan. Possible explanation for the difference between shrimp and cricket chitosan is lower deacetylation degree and very low molecular weight, however no study concerning the use of insect-derived chitosan, or very low molecular weight/low deacetylation degree chitosan film could be found.

5.6.4 Water Vapor Permeability (WVP)

Low WVP is generally desirable for packaging polymers (Wang, Tian, Feng, Fan, Pan & Zhou, 2014). WVP of the chitosan films prepared in the present study is reported in Table 9.

Table 9 Water vapor permeability of prepared films

Specimen	WVP (g mm m^{-2} day^{-1} kPa^{-1})
Shrimp	5.282 ± 0.433
Cricket	6.279 ± 0.329•
Shrimp and *S. chinensis*	4.165 ± 0.486
Cricket and *S. chinensis*	4.522 ± 0.488•

•Values followed by identical symbol within the same column are significantly ($P < 0.05$) different.

The values obtained in this study were in general similar to those obtained by some researchers (Wang, Tian, Feng, Fan, Pan & Zhou, 2014), but different from others (Siripatrawan & Harte, 2010; Wang, Dong, Men, Tong & Zhou, 2013). It was suggested that method of film preparation, chitosan molecular weight and deacetylation degree, and amount of bioactive substances can be responsible for the variability among reported results (Wang, Dong, Men, Tong & Zhou, 2013), however glycerol content of films does not significantly alter WVP (Leceta, Guerrero, Ibarburu, Dueñas & De la Caba, 2013).

It can be seen that WVP of cricket chitosan films compared to shrimp chitosan films is not significantly ($P > 0.05$) different, for both pairs with and without *S. chinensis* extract. This is in agreement with the study of (Leceta, Guerrero, Ibarburu, Dueñas & De la Caba, 2013), who also observed only slight increase in WVP of films made form chitosan of lower molecular weight, possibly as a result of shorter chain length facilitating easier diffusion of water through the films. It is rather interesting that the difference between WVP of LMW shrimp chitosan and cricket chitosan of under 10 kDa is insignificantly small, which suggests that molecular weight plays a rather minor role in determining the WVP properties of chitosan films, or that there are rather large intervals of Mw which cause a particular increase in WVP.

In the case of shrimp chitosan, addition of *S. chinensis* extract caused a small decrease in WVP, though it was not significant ($P > 0.05$), however in the case of cricket chitosan, impregnation with the extract significantly ($P < 0.05$) reduced WVP. This observation was consistent with other studies. Incorporation of lemon, tea tree and oregano oil lowered WVP, possibly because of increased hydrophobicity of the films limiting water solubility (Peng & Li, 2014; Sánchez-González, González-Martínez, Chiralt & Cháfer, 2010; Zivanovic, Chi & Draughon, 2005). Addition of essential oils which loosened the film structure, such as clove essential oil, increased WVTR (Hosseini, Razavi & Mousavi, 2009; Wang et al., 2011), and same behavior was observed also for pine needle essential oil, which incurred holes in the matrix during film preparation (Mei, Yuan, Guo, Wu, Li & Yu, 2013), potassium sorbate, which caused loosening of the matrix (Pranoto, Rakshit & Salokhe, 2005), and nisin, a polypeptide which increased electrostatic repulsion with the chitosan molecules (Mei, Yuan, Guo, Wu, Li & Yu, 2013; Pranoto, Rakshit & Salokhe, 2005). Similarly, α-tocopherol and thyme oil can cause loosening of the film structure, hereby increasing permeability to water vapor (Hosseini, Razavi & Mousavi, 2009; Martins,

Cerqueira & Vicente, 2012), though in other studies, their hydrophobicity improved the barrier properties (Park & Zhao, 2004; Peng & Li, 2014), which could be explained by different film preparation method and different concentrations of its components. Incorporation of cinnamon oil, which did not disrupt the matrix and fitted within the chitosan molecules, did not lead to significant increase in WVTR (Hosseini, Razavi & Mousavi, 2009) or even lowered the vapor permeability (Ojagh, Rezaei, Razavi & Hosseini, 2010; Peng & Li, 2014); however in one study, it was found that while the structure of the film did not become loose, the stacked sheets were running in parallel, resulting in gaps permeable to water vapor (Wang et al., 2011). Incorporation of garlic oil did not significantly affect WVP (Pranoto, Rakshit & Salokhe, 2005). In the case of *Zataria multiflora* Boiss essential oil and grape seed extract, similar reduction in WVTR was observed, with the exception of when high concentrations of both substances were combined. Alterations in the matrix organization were debated to be responsible for the changes in WVTR (Moradi, Tajik, Razavi Rohani & Oromiehie, 2011). Most relevant to the present study, addition of *Lycium barbarum* fruit extract (Wang, Tian, Feng, Fan, Pan & Zhou, 2014), green tea extract (Siripatrawan & Harte, 2010) and tea polyphenols (Wang, Dong, Men, Tong & Zhou, 2013) to chitosan films significantly decreased WVP, and the reduction was greater with increasing extract or polyphenol concentration in the film. Therefore, reduction of WVP following incorporation of hydrophilic compounds such as polyphenols can be attributed to interaction between the amino and hydroxyl groups of chitosan molecules with components of the extract, which makes them less available to bind water and thus lowering water solubility in the film; or to increased density of the films which is indicative of reduced void space in the matrix, thus making diffusion of water molecules more difficult (Siripatrawan & Harte, 2010; Wang, Dong, Men, Tong & Zhou, 2013; Wang, Tian, Feng, Fan, Pan & Zhou, 2014). Addition of mineral salts, filling the void space within the matrix, also improved water vapor barrier properties (Park & Zhao, 2004). In summary, there seems to be two different principles in which WVP of chitosan films is decreased, one is increased hydrophobicity of the films upon incorporation of hydrophobic compounds, while the other one, which is valid for *S. chinensis* infused films, is lower permeability due to increased density limiting water diffusion and blocked amino and hydroxyl groups, which limits water solubility.

Considering that there was a significant increase in density of the shrimp chitosan films after *S. chinensis* extract incorporation, the results for cricket chitosan films and similar experiments discussed above, it is possible that the decrease in WVP not being significant for shrimp chitosan films after incorporation of *S. chinensis* extract is an artifact caused by the adjustments in methodology, namely the small area of films tested and the size and shape of bottles filled with silicagel.

As for packaging films, low permeability to water vapor is desirable (Wang, Tian, Feng, Fan, Pan & Zhou, 2014), the reduction of WVP upon incorporation of *S. chinensis* fruit extract is positive, as well as comparable WVP of cricket chitosan of very low molecular weight to that of LMW shrimp chitosan.

5.6.5 Color Measurement

The average values of L^*, a^*, and b^* measurements are reported in Table 10 and apparent color is documented in Figure 15. Comparison was made between the two films without *S. chinensis* (cricket-shrimp chitosan), and between films containing *S. chinensis* extract (cricket-shrimp chitosan). The difference between either of the films not containing extract and either of the films containing extract is significant ($P < 0.05$), as obviously the films without extract are pale yellow-green while films with extract are red-brown.

Table 10 Color measurement of the films prepared

	L^*	a^*	b^*
Shrimp chitosan	93.59 ± 0.50●	-0.86 ± 0.09■	6.84 ± 0.37♦
Cricket chitosan	89.22 ± 0.67●	-0.25 ± 0.04■	12.07 ± 0.87♦
Shrimp chitosan and *S. chinensis*	52.15 ± 6.74	22.31 ± 4.17	25.24 ± 1.30
Cricket chitosan and *S. chinensis*	48.23 ± 2.78	22.57 ± 1.48	26.42 ± 1.72

●■♦Values followed by identical symbol are significantly ($P < 0.05$) different, comparison was made between the two films without *S. chinensis* (cricket-shrimp chitosan), and between the two films containing *S. chinensis* extract (cricket-shrimp chitosan).

The C^*_{ab} (chrome), h^*_{ab} (hue), and ΔE^* (total color difference) values are reported in Table 11.

Table 11 The C^*_{ab}, h^*_{ab}, and ΔE^* values of films prepared

	C^*_{ab}	h^*_{ab} (Rad) / °	ΔE^*
Shrimp chitosan	6.89 ± 0.38●	-1.45 ± 0.01■ / 97.10 ± 0.34■	5.53 ± 0.51♦
Cricket chitosan	12.07 ± 0.87●	-1.55 ± 0.00■ / 91.16 ± 0.00■	12.17 ± 1.05♦
Shrimp chitosan and *S. chinensis*	33.93 ± 1.60	0.85 ± 0.12 / 48.90 ± 7.09	54.59 ± 6.33
Cricket chitosan and *S. chinensis*	34.82 ± 0.44	0.86 ± 0.06 / 49.49 ± 3.68	58.23 ± 2.22

●■♦Values followed by identical symbol are significantly ($P < 0.05$) different.

Based on both appearance and L^*, a^*, b^* measurement, the shrimp chitosan film is significantly ($P < 0.05$) whiter than cricket chitosan, most likely due to different color of the original chitosan powders, while there is no particular difference between the films containing *S. chinensis* extract. Both films containing *S. chinensis* extract are significantly ($P < 0.05$) darker than both chitosan films without extract. Both films containing extract are reddish brown, without significant difference ($P > 0.05$) between them, however both films not containing *S. chinensis* are very slightly yellow-green, with significant difference ($P < 0.05$) between them. The color of the films not containing extract has significant ($P < 0.05$) difference in saturation, with cricket chitosan film having more saturated color. There is no significant difference ($P > 0.05$) between the films containing extract in terms

of saturation. It can be therefore deduced that the amount of *S. chinensis* extract added had much higher influence on color than the chitosan, as while there was a significant difference in all parameters for pure chitosan films, the difference was erased once extract was added.

It can also be seen that the shrimp chitosan films appear more translucent and glossy, while cricket chitosan films appear more matte and opaque, however these qualities were not assessed scientifically.

Chitosan films differ in terms of L^*, a^*, b^*, C^*_{ab}, and h^*_{ab} depending on the chitosan properties and method of preparation. Different researchers obtained different values for the films prepared (Leceta, Guerrero, Ibarburu, Dueñas & De la Caba, 2013; Martins, Cerqueira & Vicente, 2012; Moradi, Tajik, Razavi Rohani & Oromiehie, 2011; Park & Zhao, 2004; Peng & Li, 2014; Siripatrawan & Harte, 2010; Wang, Tian, Feng, Fan, Pan & Zhou, 2014), which suggests that there is rather large variability even among chitosan obtained from crustaceans, and therefore the difference between shrimp and cricket chitosan films observed in this study might possibly be related not only to the source, but also to molecular weight and deacetylation degree. The values obtained in this study were rather similar to those of Peng & Li, 2014 and Leceta, Guerrero, Ibarburu, Dueñas & De la Caba, 2013. It was previously observed that addition of essential oils to chitosan films significantly influences the color and opacity of films (Peng & Li, 2014). Addition of *Lycium barbarum* (goji) fruit extract, *Zataria multiflora* Boiss essential oil combined with grape seed extract, and green tea extract to chitosan film showed exactly the same tendency as *S. chinensis* extract, with the color of the films becoming darker and more red and yellow (Moradi, Tajik, Razavi Rohani & Oromiehie, 2011; Siripatrawan & Harte, 2010; Wang, Tian, Feng, Fan, Pan & Zhou, 2014).

Figure 15 Top, left to right: shrimp chitosan film and cricket chitosan film; bottom, left to right: shrimp chitosan film containing *S. chinensis* extract, cricket chitosan film containing *S. chinensis* extract

6 Conclusions and Future Prospects

The present study showed that cricket chitosan can be purified and used to prepare thin films enriched with *S. chinensis* extract suitable for use as packaging polymers. Shrimp chitosan was also compatible with the extract. The prepared films enriched with *S. chinensis* extract manifested antibacterial activity against Gram-positive bacteria and pure cricket chitosan film also inhibited *B. cereus*. Concerning physicochemical properties, cricket and shrimp chitosan films were comparable in terms of thickness, density, moisture content, water vapor permeability, but cricket chitosan had lower water solubility and swelling degree, which means cricket chitosan films might be more resilient when used for packaging of food with higher moisture content. Color was also different, possibly due to different color of the chitosan powder used to prepare the gels. Addition of *S. chinensis* extract led to increase in thickness, density, water solubility, swelling degree, and moisture content. On the other hand, water vapor permeability was decreased. Upon incorporation of the extract, color also changed into red-brown. *S. chinensis* extract therefore improved barrier properties against water vapor, however it also made the films less resistant to solubilization in water. The darkened color might possibly influence light absorbance properties, but that could not be assessed in the present study. Antioxidant activity of the films was not measured, but it can be expected to correlate with the total polyphenol content in the films enriched with extract.

The main objectives of the work can therefore be considered as fulfilled, with the exception of *B. falcatum* extract not being incorporated in the films as the extracts prepared either failed to solubilize in suitable solvent or did not display antimicrobial properties. *S. chinensis* also failed to inhibit Gram-negative bacteria, despite the opposite being expected.

Additionally, novel decolorization method of highly pigmented cricket chitosan was discovered in the process of chitosan purification, which is relatively safe, economical and does not require harsh conditions. Moreover, behavior of the film-forming solution suggested that rheological properties of chitosan solutions might be dependent strongly on deacetylation degree.

In summary, it was shown that cricket chitosan is equivalent or superior (in the case of water solubility as well as antibacterial activity against *B. cereus*) to shrimp chitosan as source for chitosan packaging films and that addition of *S. chinensis* fruit extract improves water vapor barrier properties and antimicrobial properties, however the films become less resistant to water.

However, there are many questions left unanswered for further research. First of all, considering the tedious process of protein removal and the potential of insect protein to contribute to feeding the world's population, it would be very interesting to explore the possibility to couple chitosan purification to insect protein production and to obtain

protein-poor chitin-rich byproduct of such industry. Furthermore, the decolorization process can most likely be optimized in terms of ideal conditions and chitin:substance X ratio.

Second, while cricket chitosan appears to be very similar to shrimp chitosan and similar results were obtained concerning chitosan isolated from other insects, some differences between the two were observed in this study. Considering the lack of understanding how factors such as deacetylation degree and molecular weight exactly influence properties of chitosan and its film, it would be worthwhile to design s study which would explore the individual as well as combined effect of these parameters.

Third, it would be very interesting to repeat the experiment with *S. chinensis* leaf ethanol extract, which appears to be very promising in terms of antimicrobial activity, antioxidant properties and also sustainability.

Last, additional film properties should be tested, which are relevant for packaging, such as light absorbance, gas barrier properties, wettability and mechanical properties.

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