รายงานวิจัยฉบับสมบูรณ์

โครงการ แอคทิฟฟิล์มจากไคโตซานที่มีสมบัติยังยั้งจุลินทรีย์ เพื่อใช้เป็นบรรจุภัณฑ์อาหาร

โดย

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สัญญาเลขที่ RMU 5080063

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แอคทิฟฟิล์มจากไคโตซานที่มีสมบัติยังยั้งจุลินทรีย์เพื่อใช้เป็นบรรจุภัณฑ์อาหาร

บทคัดย่อ

งานวิจัยนี้มีวัตถุประสงค์เพื่อพัฒนาแอคทิฟฟิล์มจากไคโตซานที่มีสมบัติยังยั้งจุลินทรีย์และต้านการเกิดปฏิกิริยาออกซิเดชันโดยการเติมสารสกัดชาเขียว เพื่อใช้เป็นบรรจุภัณฑ์อาหาร โดยแบ่งงานวิจัยเป็น 3 ขั้นตอน ขั้นแรกเป็นการศึกษาภาวะที่เหมาะสมในการเตรียมฟิล์มไคโตซานและปรับปรุงคุณสมบัติทางกายภาพของฟิล์มโดยใช้พลาสติไซเซอร์ ฟิล์มไคโตซานเตรียมได้โดยใช้ไคโตซาน 2 % (w/v) ในสารละลายกรดอะซิติกเข้มข้น 1 % (w/v) อย่างไรก็ตามฟิล์มที่ได้มีความยืดหยุ่นต่ำ การปรับปรุงสมบัติทางกลของฟิล์มไคโตซานทำโดยใช้กลีเซอรอลเป็นพลาสติไซเซอร์ ที่ความเข้มข้น 0, 10, 20, 30, 40 และ 50% โดยน้ำหนักของไคโตซาน ติดตามผลโดยการวัด ความหนา ค่า tensile strength, elongation at break, อัตราการซึมผ่านของไอน้ำ พบว่ากลีเซอรอล 30% เป็นความเข้มข้นที่เหมาะสมเนื่องจากให้ฟิล์มที่มีความยืดหยุ่นเพิ่มขึ้น ในขณะที่ tensile strength และคุณภาพด้านอื่นๆ ของฟิล์มอยู่ในเกณฑ์ที่เหมาะสม

ขั้นที่สองเป็นพัฒนาแอคทีฟฟิล์มให้มีสมบัติยับยั้งการเจริญของจุลินทรีย์และต้านการเกิดออกซิเดชันโดยการเติมสารสกัดชาเขียว ศึกษาผลของการเติมสารสกัดชาเขียวที่มีความเข้มข้น 0, 2, 5, 10 และ 20% (w/v) ต่อค่า tensile strength , elongation at break, ความหนาแน่น ค่าสี ค่าการซึมผ่านของไอน้ำ (water vapor permeability, WVP) ปริมาณฟีนอลิกทั้งหมด (total phenolic content, TPC), ปฏิกิริยาการจับอนุมูล 2,2-diphenyl-1-picrylhydrazyl (DPPH) และ การยับยั้งการเจริญของจุลินทรีย์ 4 ชนิด ได้แก่ *Staphylococcus aureus* TISTR 118, *Salmonella* Enteritidis DMST 17368, *Escherichia coli* TISTR 780 และ *Pseudomonas fluorescens* TISTR 358 ด้วยวิธี agar diffusion รวมทั้งวิเคราะห์การเกิดปฏิกิริยาระหว่างไคโตซานและสารประกอบฟีนอลิก โดยใช้ Fourier Transform Infrared (FTIR) spectrometry ผลการทดลองพบว่าเมื่อเพิ่มความเข้มข้นของสารสกัดชาเขียว ค่า tensile strength, elongation at break ความหนาแน่น, ค่าสีแดง (a) ค่าสีเหลือง (b) TPC และเปอร์เซนต์การจับอนุมูล DPPH เพิ่มขึ้น ส่วนค่า WVP และค่าความสว่าง (L) มีค่าลดลง นอกจากนี้ยังพบว่าการเติมสารสกัดชาเขียวช่วยเพิ่มประสิทธิภาพการยับยั้งจุลินทรีย์โดยพบบริเวณการยับยั้ง (inhibition zone) และมีการยับยั้งการเจริญของจุลินทรีย์บริเวณใต้แผ่นฟิล์ม ส่วนฟิล์มไคโตซานที่ไม่เติมสารสกัดชาเขียว ไม่พบ inhibition zone แต่มีการยับยั้งการเจริญของจุลินทรีย์บริเวณใต้แผ่นฟิล์มเท่านั้น อย่างไรก็ตามการเพิ่มความเข้มข้นของสารสกัดชาเขียวไม่มีผลต่อประสิทธิภาพการยั้บยั้งจุลินทรีย์ เมื่อพิจารณาผลการวิเคราะห์ด้วย FTIR พบว่า มีการเกิดพันธะไฮโดรเจนระหว่าง amine group ของไคโตซานและ hydroxyl group ของสารประกอบฟีนอลิกจากชาเขียว ส่งผลให้สมบัติทางกลของฟิล์มดีขึ้น และค่า WVP ลดลง เมื่อความเข้มข้นของสารสกัดชาเขียวเพิ่มขึ้น และการเพิ่มของสารประกอบฟีนอลิก ส่งผลให้ประสิทธิภาพการต้านการเกิดออกซิเดชันและการยับยั้งการเจริญของจุลินทรีย์เพิ่มขึ้น

ในขั้นสุดท้ายเป็นการศึกษาการประยุกต์ใช้ฟิล์มไคโตซานที่พัฒนาได้ในการยืดอายุการเก็บรักษาผลิตภัณฑ์ไส้กรอกหมู ทำโดยห่อผลิตภัณฑ์ไส้กรอกด้วยฟิล์มที่เติมสารสกัดชาเขียว

เปรียบเทียบกับตัวอย่างที่ห่อด้วยฟิล์มไคโตซานที่ไม่เติมสารสกัดชาเขียว (C-film) และตัวอย่างควบคุม (Control) ซึ่งไม่มีการห่อด้วยฟิล์มไคโตซาน ติดตามการเปลี่ยนแปลงคุณภาพของผลิตภัณฑ์ทางกายภาพ ได้แก่ ค่าสี และเนื้อสัมผัส (แรงตัดขาด), ทางเคมี ได้แก่ ค่า pH และค่ากรดไทโอบาร์บิทูริก (Thiobarbituric acid value, TBA), ทางจุลินทรีย์ได้แก่ ปริมาณแบคทีเรียทั้งหมด ยีสต์ รา และแบคทีเรียแลกติก และการทดสอบทางประสาทสัมผัส โดยพิจารณา กลิ่นผิดปรกติ, สี, การเกิดเมือก และ การยอมรับโดยรวม ของผลิตภัณฑ์ไส้กรอก พบว่าตัวอย่างที่ห่อด้วย มีการเปลี่ยนแปลงของค่าแรงตัดขาด, pH ค่าสี และค่า TBA น้อยกว่าตัวอย่างที่ห่อด้วย และตัวอย่างควบคุม ตามลำดับ และพบว่าตัวอย่างที่ห่อด้วย มีการเจริญของแบคทีเรียทั้งหมด ยีสต์ รา และแบคทีเรียแลกติก ช้ากว่าตัวอย่างอื่น ๆ ส่งผลให้ ค่า pH มีการเปลี่ยนแปลงน้อยกว่าเมื่อเทียบกับตัวอย่างอื่นๆ สอดคล้องกับผลการทดสอบทางประสาทสัมผัสซึ่งพบว่า เมื่อระยะเวลาการเก็บเพิ่มขึ้น ผลิตภัณฑ์ไส้กรอกที่ห่อด้วย มีการเปลี่ยนของสี, กลิ่น และการยอมรับโดยรวม ช้ากว่าตัวอย่างอื่น และพบว่าตัวอย่างควบคุมมีอายุการเก็บรักษาน้อยกว่า 12 วัน ส่วนตัวอย่างที่ห่อด้วย มีอายุการเก็บรักษาน้อยกว่า 20 วัน ในขณะที่ตัวอย่างที่ห่อด้วย มีคุณภาพทางประสาทสัมผัสเป็นที่ยอมรับตลอดระยะเวลาการเก็บรักษาได้นาน 20 วัน ที่อุณหภูมิ 4 $^{\circ}$C ดังนั้นจึงสามารถสรุปได้ว่าฟิล์มไคโตซานที่เติมสารสกัดชาเขียวช่วยลดการเกิดปฏิกิริยาออกซิเดชัน และชะลอการเจริญของจุลินทรีย์ จึงสามารถรักษาคุณภาพและช่วยยืดอายุการเก็บรักษาของผลิตภัณฑ์ไส้กรอกหมูได้นานขึ้น

Keywords: บรรจุภัณฑ์แอคทีฟ; ไคโตซานฟิล์ม; โพลิฟีนอลจากชาเขียว; การยืดอายุการเก็บอาหาร; สมบัติการต้านการเจริญของจุลินทรีย์; สมบัติการต้านอนุมูลอิสระ

Active Chitosan-Based Film with Antimicrobial Property for Food Packaging Application

ABSTRACT

This research was aimed to develop an active film from chitosan film incorporated with green tea extract to enhance antioxidant and antimicrobial properties in order to be used for food shelf life extension. The experiments were divided into 3 parts. Firstly, chitosan-based film preparation and modification were determined. The results suggested that the optimum chitosan films could be prepared from 2% chitosan in 1% acetic acid. However, the chitosan film was brittle and had low flexibility. Mechanical property of chitosan film was modified by adding different concentrations of glycerol, as a plasticizer, including 0, 10, 20, 30, 40 and 50% (w/w of chitosan). The effect of plasticizer concentration on the mechanical properties of the chitosan film was determined by measuring their tensile strength, elongation at break, thickness, surface colors and water vapor transmission rate. The results showed that 30% glycerol was the optimum concentration to improve flexibility, while maintaining tensile strength and other physical properties of the film.

Secondly, chitosan-based film was incorporated with green tea extract (GT) in order to improve film's antioxidant and antimicrobial properties. The optimum concentration of green tea extract was then determined by adding 0, 2, 5, 10 and 20% (w/v) of green tea in film-forming solution, and tensile strength, elongation at break, water vapor permeability (WVP), density, total phenolic compounds (TPC), radicals scavenging 2,2-diphenyl-1-picrylhydrazyl (DPPH) of the film were determined. Fourier Transform Infrared (FTIR) spectrometry was carried out to observe the potential modifications of the chitosan films when incorporated with GTE. The ability to inhibit *Staphylococcus aureus* TISTR 118, *Salmonella enteritidis* DMST 17368, *Escherichia coli* TISTR 780 and *Pseudomonas fluorescens* TISTR 358 was conducted using agar diffusion method. It was found that WVP and lightness (L) decreased, while density, TPC, DPPH scavenging activity, redness (a), and yellowness (b) increased with increasing green tea concentration. The results also showed that films containing green tea extract had inhibition zone and could inhibit bacterial growth underneath film, while chitosan-alone film had no inhibition zone. The results suggested that incorporation of GT into chitosan films improved mechanical and water vapor barrier properties and enhanced polyphenolic content,

antioxidant activity and antimicrobial property of the films. Changes in the FTIR spectra of the chitosan films were observed when GTE was incorporated, suggesting some interactions occurred between amine group of chitosan and hydroxyl group of green tea polyphenols.

Lastly, the chitosan film containing green tea extract (CGT-film) was used as an active film for shelf life extension of pork sausage. Qualities of pork sausages wrapped with CGT-film were compared with those wrapped with chitosan-alone film without green tea incorporation (C-film) and those without chitosan film wrapping (Control). Changes in the physical qualities including color values and texture, the chemical qualities including pH and thiobarbituric value (TBA), the microbiological qualities including total plate count, yeasts/moulds, and lactic acid bacteria were determined throughout the storage. The sensory qualities including odor, color, slime formation, and overall acceptance were also evaluated using Quantitative Descriptive Analysis. The results showed that samples wrapped with CGT-film had higher cutting force, lightness and yellowness values, but lower TBA and microbial growth than those wrapped with C-film and control. Based on microbiological analysis and sensory evaluation, control samples and those wrapped with C-film had shelf life of less than 12 and 20 days, respectively. Samples wrapped with CGT-film had better qualities than other samples and had shelf life of up to 20 days at 4 $^{\circ}$C. Incorporation of GT into chitosan film could enhance the antioxidant and antiomicrobial properties of the film. CGT-film reduced the lipid oxidation and inhibited microbial growth and, consequently, could maintain qualities and extended shelf life of the pork sausage.

Keywords: Active packaging; Chitosan film; Green tea polyphenols; Food shlelife extension; Antimicrobial property; Antioxidant property

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

ทุนเพิ่มขีดความสามารถด้านการวิจัยของอาจารย์รุ่นกลาง

โครงการ แอคทิฟฟิล์มจากไคโตซานที่มีสมบัติยังยั้งจุลินทรีย์เพื่อใช้เป็นบรรจุภัณฑ์อาหาร

ชื่อหัวหน้าโครงการ รศ.ดร. อุบลรัตน์ สิริภัทราวรรณ

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EXECUTIVE SUMMARY

JUSTIFICATION

Due to the major concerns of food quality and safety in the food industry, antimicrobial and antioxidant packaging is a main focus in the new generation of active packaging. Not only a big effort to extend the shelf life and enhance food quality, reducing packaging waste has also been a major concern and has encouraged the exploration of new bio-based packaging materials, such as edible and biodegradable films from renewable resource. Hence, bio-based active packaging is gaining interest from researchers and industry due to its potential to provide quality and safety benefits as well as non-polluting property. The development of plastics or packaging films based on biopolymers has attracted attention due to their environmentally friendly nature and their potential use in the food and packaging industry. As one of the candidates for such biopolymers, chitosan, a polymer of 1,4 linked b-D-glucosamine and N-acetyl glucosamine units, is abundant, renewable, and biodegradable, making it attractive materials for bioplastics.

Chitosan is prepared by deacetylation of chitin, has been proved to be nontoxic, biodegradable, bifuncitonal, biocompatible and thus becomes a promising biodegradable polymer for active food packaging. Chitosan is a functional biopolymer, having intrinsic antimicrobial and antioxidant properties and consequently has high potential to be used as a biodegradable active packaging. Although, chitosan has antioxidant property, there are some limitations in being a practical antioxidant. Moreover, many reports indicate that only organisms in direct contact with the active sites of chitosan are inhibited since chitosan is in a solid form and incapable to diffuse through the food component. Because of the characteristic inhibitory mechanism and the specific activity of chitosan against specific target microorganisms, the film has a limited antimicrobial spectrum. Incorporation of antioxidant and/or antimicrobial agents into chitosan film can widen this limited function spectrum.

Incorporation of antioxidants/antimicrobials into packaging materials has become popular since oxidation and microbial contamination are major problems affecting the food quality. Active ingredients can be incorporated into biodegradable films and coatings. They may carry antioxidants, antimicrobial agents, colorants, flavors, and/or fortified nutrients. These functions are promising and will receive more attention in the near future. Currently, the use of natural ingredients instead of synthetic chemicals is a worldwide trend affecting most consumer products. Due to this consumer preference, more research and development will focus on the use of natural antioxidant and/or antimicrobial agents.

Green tea (*Camellia sinensis*) is a good source of polyphenolic compounds. Green tea catechins, including (–)-epicatechin (EC), (–)-epigallocatechin (EGC), (–)-epicatechin gallate(ECG), (–)-epigallocatechin gallate (EGCG), (–)-catechin (C), (–)-gallocatechin gallate (GCG), (–)-catechin gallate (CG), and (–)-gallocatechin (GC), are proved to exhibit antimicrobial activity against some bacteria and have good antioxidant activity. The beneficial effects of the phenolic compounds are thought to result from their ability to scavenge reactive oxygen and nitrogen species (Chan et al., 2007; Rohn, Rawel & Kroll 2004). Therefore, this research was aimed to improve antioxidant and antimicrobial efficacy of biodegradable chitosan-based film by incorporating with green tea extract, as a natural active agent, and to determine the feasibility of using the developed chitosan-based film as an active packaging for food shelf life extension. The inherent biodegradability is also a strong advantage of this film.

OBJECTIVE

The overall objective of this research was to develop a bio-based active food packaging from chitosan film incorporated with green tea for food shelf life extension. The specific objectives included:

1. To determine the optimum condition for preparing chitosan-based film and the optimum centration of plasticizer to improve the physical properties of the chitosan-based film

2. To improve antioxidant and antiomicrobial properties of the chitosan film by incorporating with natural green tea extract and to study the effect of green tea incorporation on the chemical, physical and microbiological properties of the film

3. To determine the feasibility of the developed chitosan film incorporated with green tea as an active packaging for food shelf life extension

METHODOLOGY

This research was separated into 3 main parts including

(1) Chitosan-based film preparation and modification. This part was to determine the optimum condition for preparing chitosan-based film and the optimum concentration of plasticizer to improve mechanical properties of the chitosan film. A series of preliminary experiments were conducted in order to determine the optimum type and concentration of acidic solvent and plasticizer used for preparing the chitosan-based films. The effect of plasticizer concentration on the mechanical properties of the chitosan film plasticized using glycerol, at different concentration was determined by measuring their tensile strength, elongation at break, thickness, surface colors and water vapor transmission rate.

(2) Chitosan film incorporated with green tea extract to improve antioxidant and antimicrobial properties. The antioxidant and antimicrobial properties of chitosan film by incorporation with natural green tea extract (GT) at various concentrations and to study the effect of GT incorporation on the tensile strength, elongation at break, density, surface colors, opacity, water vapor permeability, total phenolic content and antioxidant activity of the film. Fourier Transform Infrared spectrometry was carried out to observe the structural interactions of chitosan films incorporated with GT polyphenols. Antimicrobial activity assay of chitosan films against *Staphylococcus aureus* TISTR 118, *Salmonella enteritidis* DMST 17368, *Escherichia coli* TISTR 780 and *Pseudomonas fluorescens* TISTR 358 was carried out using agar diffusion.

(3) Shelf life extension of food product using chitosan film incorporated with GT. The chitosan film containing green tea extract (CGT-film) was used as an active film for shelf life extension of pork sausage. The results were compared with those wrapping with chitosan-alone film without green tea incorporation (C-film) and those without chitosan wrapping (Control). Changes in the physical qualities including color values and texture, the chemical qualities including pH and thiobarbituric value (TBA), the microbiological qualities including total plate count, yeasts/moulds, and lactic acid bacteria were determined throughout the storage. The sensory qualities including odor, color, slime formation, and overall acceptability were evaluated using Quantitative Descriptive Analysis.

RESULTS

The optimum chitosan-based films could be prepared from 2% chitosan in 1% acetic acid. However, the obtained chitosan film was brittle and had low flexibility. The mechanical properties of chitosan film was then modified by adding glycerol, as a plasticizer, at different concentrations including 0, 10, 20, 30, 40, and 50 % w/w of chitosan. The results showed that an increase in the amount of the plasticizer caused an increase in elongation at break (extensibility), a decrease in tensile strength and an increase in water vapor transmission rate of the film. The elongation of the film increased with increasing plasticizer concentration, but at high concentration there were decreases in both tensile strength and elongation. The changes in mechanical properties of the film could be attributed to an alteration of intermolecular and intramolecular interaction occurring in the films. The results suggested that 30% glycerol was the optimum concentration to improve flexibility, while maintaining tensile strength and other physical properties of the film.

The effects of incorporation of green tea extract at different concentrations including 0, 2, 5, 10, and 20% w/v of green tea in the chitosan film-forming solution were determined. Incorporation of GT improved mechanical properties, water vapor barrier property, total phenolic compounds, DPPH scavenging activity, and antimicrobial properties of the resulting films. The DPPH scavenging activity of the films significantly increased with increasing GT concentration. As the GT increased in the film formulation, so did the expected antioxidant character of the active film. The lower WVP of the films incorporated with GT may be because, according to the FTIR analysis, the hydrogen and covalent interactions between chitosan network and polyphenolic compounds limit the availability of hydrogen groups to form hydrophilic bonding with water, subsequently lead to a decrease in the affinity of chitosan film toward water.

Chitosan films without incorporation of GT (control films) showed antimicrobial

The antimicrobial and antioxidant efficacy of the developed chitosan film incorporated with green tea extract for shelf life extension of pork sausages were investigated. The physical, chemical, microbiological and sensory qualities of pork sausages wrapped with CGT-film were compared with those wrapped with C-film and control samples without chitosan film wrapping. It was found that samples wrapped with CGT-film showed lower changes in color, pH, TBA, texture, microbial growth and sensory quality than those wrapped with C-film and control, respectively. The results suggested that incorporation of GT into chitosan film could enhance the antioxidative and antimicrobial effects of the film, and thus maintained the qualities and prolonged the shelf life of pork sausages.

The antimicrobial activity of green tea polyphenols is attributed to green tea polyphenols are capable of bactericidal activity, by inhibition of DNA and RNA synthesis of bacterial cells (*E. coli, S. aureus, S.*Typhimurium), inhibition of cytoplasmic membrane function of bacteria, resulting in leakage of intramembranous materials, and interfering with energy metabolisms of bacteria. Successful inhibition of lipid oxidation and microbial growth in the refrigerated pork sausage was possible with chitosan film and chitosan film incorporated with GT since they kept the sensory characteristics within an acceptable condition throughout the storage period. Based on the sensory quality and microbiological quality with regard to the TISI's Standard for Hotdog sausages-pork sausage (Thai Industrial Standards Institute, Ministry of Industry, Thailand), control pork sausage samples and those wrapped with chitosan-alone film had shelf life of less than 12 days and less than 20 days, respectively. Chitosan incorporated with GT could

extend shelf life of the samples for up to 20 days at 4 ºC, without any significant loss of odor, color, and overall acceptability or significant microbial growth.

RESEARCH CONTENT

ทุนเพิ่มขีดความสามารถด้านการวิจัยของอาจารย์รุ่นกลาง

โครงการ แอคทิฟฟิล์มจากไคโตซานที่มีสมบัติยังยั้งจุลินทรีย์เพื่อใช้เป็นบรรจุภัณฑ์อาหาร

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1. INTRODUCTION

Consumer concerns of food quality and safety lead to the development of active packaging, which is an innovative packaging performing some functions in the preservation of the food other than providing a barrier property. Active compounds and ingredients can be incorporated into packaging materials to provide several functions that do not exist in conventional packaging systems. Active packaging may carry antioxidants, antimicrobial agents and/or nutrients. Moreover, due to the health concerns of the consumers and environmental problems, current research in active packaging has focused on the use of natural preservatives in biodegradable packaging materials (Suppakul, Sonneveld & Bigger, 2003; Yingyuad et al., 2006).

Chitosan, a linear polysaccharide of randomly distributed β-(1-4)-linked D-glucosamine and N-acetyl-D-glucosamine, is a deacetylated product of chitin and is considered a biobased environmentally friendly material (Caner and Cansiz, 2007; Fernandez-Saiz, Lagaron and Ocio, 2009). Chitosan has potential to be used as alternative resources for active food packaging. Chitosan has intrinsic antioxidant and antimicrobial properties, which are affected by its molecular weight and concentration. Chitosan has been used as antimicrobial films and coatings due to its effectiveness of inhibiting the growth of not only Gram-positive and Gram-negative bacteria but also yeasts and moulds. Chitosan antimicrobial activity comes from its positive charges that would interfere with the negatively charged residues of macromolecules on the microbial cell surface, causing the membrane leakage (Caner and Cansiz, 2008; Fan et al., 2009; Dutta et al., 2009; Hernández-Muñoz et al., 2008).

Incorporation of antioxidants/antimicrobials into packaging materials has become popular since oxidation and microbial contamination are major problems affecting the food quality. Currently, the most frequently used antioxidants in active packaging are butylated hydroxyanisol (BHA) and butylated hydroxytoluene (BHT). Although these synthetic antioxidants can effectively be used in active food packaging because of high stability, low cost and efficiency, there are significant concerns related to their

toxicological aspects. Moreover, use of synthetic antioxidants is under strict regulation due to the potential health risk caused by such compounds. Therefore, extensive research has been conducted to employ some natural antioxidants such as phenolic compounds as alternatives to synthetic antioxidants (Chan, Lim and Chew, 2007; Jongjareonrak et al., 2008). Current research concerning use of natural antioxidant in edible films includes the addition of vitamin E into chitosan-based film (Park and Zhao, 2004) incorporation of tetrahydrocurcuminoids into chitosan film (Portes et al., 2009) and use of antioxidant borage extract in gelatin film (Gómez-Estaca et al., 2009).

Green tea (*Camellia sinensis*), nonfermented products, is a good source of polyphenolic compounds having strong antioxidant property (Chan et al., 2007). The important polyphonolic compounds in tea leaves include catechin, theaflavins and thearubigenes. (Gramzaa et al., 2006). Green tea catechins, including (–)-epicatechin (EC), (–)-epigallocatechin (EGC), (–)-epicatechin gallate(ECG), (–)-epigallocatechin gallate (EGCG), (–)-catechin (C), (–)-gallocatechin gallate (GCG), (–)-catechin gallate (CG), and (–)-gallocatechin (GC), are proved to exhibit antimicrobial activity against some bacteria and have good antioxidant activity. The beneficial effects of the phenolic compounds are thought to result from their ability to scavenge reactive oxygen and nitrogen species (Chan et al., 2007; Rohn, Rawel and Kroll 2004). Green tea has been reported to delay the onset of lipid oxidation in various foods including marine oil (Wanasundara and Shahidi, 1998), soybean oil and corn oil (Yanishlieva and Marinova, 2001) and dry-fermented sausage (Bozkurt, 2006).

As mentioned earlier, biodegradable active packaging is the main focus of current food packaging research and developments. Chitosan is a functional biopolymer, having intrinsic antimicrobial and antioxidant properties and consequently has high potential to be used as a biodegradable active packaging. Although, chitosan has antioxidant properties, there are some limitations in being a practical antioxidant. The possibility to improve antioxidant and antimicrobial properties of the chitosan film is to incorporate with active agents. As a good source of polyphenols, GTE may be used as active agent and incorporated into film. Accordingly, the aim of this research was to develop an environmentally friendly active film from chitosan incorporated with aqueous green tea extract as a natural active agent and to determine if the developed film would have potential to be used as an active packaging for food shelf life extension.

2. OBJECTIVES

The overall objective of this research was to develop a bio-based active food packaging from chitosan film incorporated with green tea for food shelf life extension. The specific objectives included:

2.1 To determine the optimum condition for preparing chitosan-based film and the optimum centration of plasticizer to improve the physical properties of the chitosan film

2.2 To improve antioxidant and antiomicrobial properties of the chitosan film by incorporating with natural green tea extract and to study the effect of green tea incorporation on the chemical, physical and microbiological properties of the film

2.3 To determine the feasibility of the developed chitosan film incorporated with green tea as an active packaging for food shelf life extension

3. LITERATURE REVIEW

3.1 Bio-based and/ or edible films

With the increasing population and stress on limited resources and the environment, uses of renewable resources to produce edible and biodegradable films that can improve product quality and/or reduce waste disposal problems are being explored (Krochta, 2002). There has been an increasing research interest in edible and biodegradable packaging films during the last decade, possibly due to their numerous advantages over synthetic packaging films, especially reducing the environmental problems associated with packaging (Kim et al., 2006; Srinivasa et al., 2007).

Biodegradable films for food packaging applications must be shown safe for such use (Krochta, 2002). The challenge of biodegradable films for food packaging is that the film can provide specific function safely and effectively for the time needed. Edible films are one category of biodegradable films. Edible films could be their uses in multilayer food packaging materials together with non edible films. In such case, the edible films are usually used as the internal layers in direct contact with food materials. The advantages of edible films over other conventional polymeric packaging material are that the films can be consumed with the packaged products and/or can contribute to the reduction of environmental pollution, and the films could be functioned as carriers for antimicrobial and antioxidant agents or flavorings, colorings, sweeteners (Kim et al., 2006).

Production of edible films makes less waste and pollution, however, their permeability and mechanical properties are generally poorer than synthetic films. Materials being used to form edible films include proteins, polysaccharides, lipids (waxes), and their composites (Conca and Yang, 1993).

3.1.1 Type of bio-based and/or edible films

Materials available for forming films and film coatings fall generally into the categories of proteins, polysaccharides, lipids, and resins. Plasticizers are often added to reduce film or coating brittleness. Other constituents can include antioxidants and antimicrobials to enhance the film or coating effectiveness.

Proteins: Proteins cover a broad range of polymeric compounds that provide structure or biological activity in plants or animals. Proteins are distinguished from

polysaccharides because they are based on approximately 20 amino acid monomers, rather than just a few or even one monomer, such as glucose in the case of cellulose and starch.

Protein film-forming materials include collagen, gelatin, fish myofibrillar protein, keratin, egg white protein, casein, and whey protein. Protein film-forming materials derived from plant sources include corn zein, wheat gluten, soy protein, peanut protein, and cottonseed protein (Krochta, 2002).

Lipids: Edible lipids include beeswax, candelilla wax, carnauba wax, triglycerides, acetylated monoglycerides, fatty acids, fatty alcohols, and sucrose fatty acid esters. Edible resins include shellac and terpene resin. Because lipid and resin materials are not polymers, they do not generally form cohesive stand-alone films. However, along with often providing desirable gloss, they can be used to coat on food or drug surface to provide a moisture barrier or to provide the moisture-barrier component of a composite film. Composite films can consist of a lipid layer supported by a protein or polysaccharide layer, or lipid material dispersed in a protein or polysaccharide matrix (Krochta, 2002).

Polysaccharides: Polysaccharide film-forming materials include starch and starch derivatives, cellulose derivatives, alginate, carrageenan, chitosan, pectinate, and various gums. Proteins can be combined with polysaccharides to modify film mechanical properties (Arvanitoyannis and Biliaderis, 1998).

3.1.2 Functions of bio-based and/or edible films

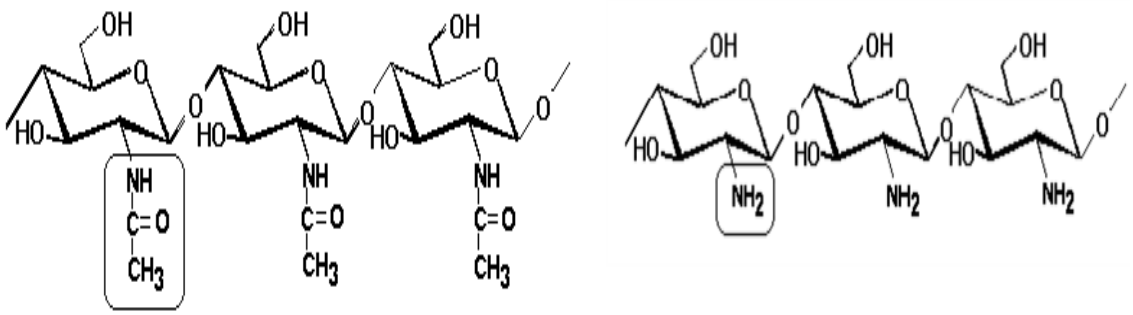
Most commonly, edible films and coatings are intended to function as a barrier to moisture, oxygen, flavor, aroma, and/or oil, thus improving food quality and shelf life. An edible film or coating may also provide some mechanical protection for a food, reducing bruising and breakage and thus improving food integrity. When an edible film or coating provides a moisture, flavor, aroma, or oil barrier between food components of different water activity, flavor, aroma, and/or oil content in a heterogeneous food, the quality and shelf life of the food are increased. Rather, they are intended to work with conventional packaging to improve product quality and shelf life. However, the amount of conventional protective packaging may be reduced (source reduction); and the remaining, simpler package may be more recyclable.

The protective function of edible films and coatings may be enhanced with addition of antioxidants or antimicrobials to the film or coating. Depending on the nature of the food, an edible coating may also carry flavors, nutrients, etc., to enhance the quality of the food. Finally, an edible coating can provide additional important sensory attributes to foods, including gloss, color, and non-greasy, non-sticky, or non-color-bleeding surface (Kim et al., 2006; Srinivasa et al., 2007; Siripatrawan and Harte, 2010).

3.2 Chitosan-based film

Chitosan is a deacetylated product of chitin (Figure 3.1) obtained from the cuticle of the marine crustaceans. Chitosan, a linear polysaccharide of randomly distributed β-(1-4)-linked D-glucosamine and N-acetyl-D-glucosamine, is considered a biobased environmentally friendly material (Caner and Cansiz, 2007; Fernandez-Saiz, Lagaron and Ocio, 2009). Chitosan has potential to be used as alternative resources for active food packaging. Chitosan has intrinsic antioxidant and antimicrobial properties, which are affected by its molecular weight and concentration. Chitosan has been used as antimicrobial films and coatings due to its effectiveness of inhibiting the growth of not only Gram-positive and Gram-negative bacteria but also yeasts and moulds. Chitosan antimicrobial activity comes from its positive charges that would interfere with the negatively charged residues of macromolecules on the microbial cell surface, causing the membrane leakage (Caner and Cansiz, 2008; Fan et al., 2009; Dutta et al., 2009; Hernández-Muñoz et al., 2008).

Chitosan film is a new generation of bio-based environmentally friendly materials and its potential use as alternative resources to be used in packaging applications, has been extensively studied (Buonocore et al., 2005; Li et al., 2006; Rhim et al., 2007). Durango et al. (2005) stated that coating minimally processed carrots with chitosan could inhibit mesophilic aerobes, psychrotrophic bacteria, yeasts and mold. Chitosan has also received attention as a potential antimicrobial packaging for meat products because they have been found to inhibit the growth of many pathogens in various meat products (Coma et al., 2002). However, films made only from chitosan lack water resistance and have poor mechanical properties. A deficiency of chitosan is due to its inherent water sensitivity and relatively low flexibility and strength (Rhim et al., 2007).



Chitin **Chitosan**

Figure 3.1 Chitin and chitosan (Nishiyama, 1991)

3.3 Modification of bio-based film properties using plasticizers

A plasticizer may be defined as a compound, when added to another material and under given conditions, modifies certain physical and mechanical properties of material. The addition of plasticizer to films produces films, which are less likely to break and more flexible and stronger. The reduction of the intermolecular bonds between the polymer chains, and thus the overall cohesion, facilitated elongation of the films and reduced its glass transition temperature. This is manifested by a reduction in the barrier properties to gases, vapors, and film solutes (Banker, 1966).

Generally, two types of plasticizers were distinguished. First, Internal plasticization is a result of modifications to the chemical structure of the polymer, for example, by copolymerization or selected hydrogenation or transesterification in the case of edible fats or similar. Second, external plasticization is obtained by adding an agent which modifies the structure and energy within the three-dimensional arrangement of the film polymer (Banker, 1966). Plasticizing agents are essential generally to overcome the brittleness of the chitosan films. Srinivasa *et al*. (2007) reported that chitosan blended with sorbitol gave better tensile strength than those with glycerol, polyethylene glycol and fatty acid.

Relatively small molecular weight hydrophilic plasticizers are often added to improve the physical properties of the films. The result of plasticizer addition is a lowering of the film glass transition temperature and an improvement in film flexibility

(lowering of film elastic modulus). Unfortunately, plasticizers generally also decrease the film's ability to act as a barrier to moisture, oxygen, aroma and oils. Plasticizers acceptable and generally used for bio-based films include glycerol, propylene glycol, sorbitol, sucrose, polyethylene glycol, fatty acids and monoglycerides.

Several studies on plasticization of chitosan films revealed that poly (ethylene glycol) (PEG) could improve the elastic properties of chitosan (Caner et al., 1998). Butler et al. (1996) found the water barrier and mechanical properties of plasticized chitosan films with glycerol changed during storage. Hosokawa et al. (1990) used glycerol to plasticize chitosan/cellulose composites, whereas Arvanitoyannis et al. (1998) used sorbitol and sucrose to plasticize chitosan/polyvinyl alcohol (PVA) blends. They stated that the elongation of the bio-based films increased with increasing plasticizer contents, but at high plasticizer contents there were decreases in both tensile strength and modulus. The film aging and the amount and type of plasticizer are important issues in the application of chitosan as biodegradable or edible films.

3.4 Active packaging from chitosan-based film

Active packaging technologies involve interactions between food, packaging material, and internal gaseous atmosphere to extend the shelf life of foods while maintaining their quality and safety. The packaging may be termed active when it performs specific desired role in food preservation other than providing gas or moisture barrier to external conditions. Active compounds and ingredients can be incorporated into packaging films and coatings to provide several functions that do not exist in conventional packaging systems. Active packaging may carry antimicrobial agents, antioxidants, colorants, flavors, fortified nutrients, and/or spices (Ahvenainen, 2003).

Chitosan films and coatings showed some antimicrobial effect for food application. However, many reports indicate that only organisms in direct contact with the active sites of chitosan are inhibited since chitosan is in a solid form and incapable to diffuse through the food component (Coma et al., 2002; Pranoto et al., 2005). Many reports have shown incorporating antimicrobial agents into chitosan film improved antimicrobial efficacy of chitosan, as diffused antimicrobial would add to nonmigrated antimicrobial potency of chitosan (Pranoto et al., 2005).

Consumer concerns of food quality and safety lead to the development of active packaging, which is an innovative packaging performing some functions in the preservation of the food other than providing a barrier property. Active compounds and ingredients can be incorporated into packaging materials to provide several functions that do not exist in conventional packaging systems. Active packaging may carry antioxidants, antimicrobial agents and/or nutrients (Ouattara et al., 2000; Sorrentino et al., 2007). Moreover, due to the health concerns of the consumers and environmental problems, current research in active packaging has focused on the use of natural preservatives in biodegradable packaging materials (Suppakul, Sonneveld and Bigger, 2003; Yingyuad et al., 2006).

Due to the consumer preference, more research and development has focused on the use of natural antimicrobials. Bactericidal agents, such as lysozyme, that destroy peptidoglycan in cell walls can effectively eliminate most gram-positive pathogens. However, it is difficult to inhibit the growth of gram-negative bacteria by cell wall destruction. For example, to simultaneously inhibit *Listeria monocytogenes* and *E. coli* O157:H7 using a single antimicrobial film/coating, the antimicrobial film must carry a mixture of antimicrobial agents (e.g., lysozyme and EDTA). Studies on gram-negative inhibition are required to develop films and coatings with a wider antimicrobial spectrum.

3.5 Active agents incorporated into films

There are many antimicrobial agents that exist and are widely used. Various antimicrobial agents may be incorporated in the packaging system, which are chemical antimicrobials, antioxidants, biotechnology products, antimicrobial polymers and natural antimicrobials.

Chemical antimicrobial agents are the most common substances used in the industry. They include organic acids, fungicides, alcohols and antibiotics. Organic acids and their derivatives such as benzoic acids, parabens, sorbates, sorbic acid, propionic acid, acetic acid, lactic acid, medium-size fatty acids and their mixture possess strong antimicrobial activity and have been used as food preservatives, food contact substances and food contact material sanitizers. Benomyl and imazalil had been incorporated in plastic films and demonstrated antifungal activity. Ethanol has strong antibacterial and antifungal activity, however, it is not sufficient to prevent the growth of yeast. Ethanol may enhance some volatile flavor compounds but also causes a strong undesirable chemical

odor in most food products. Some antibiotics can be incorporated into animal feedstuffs for the purpose of disease treatment, disease prevention or growth enhancement as well as human disease curing. The use of antibiotics as package additives is not approved for the purpose of antimicrobial functions and is also controversial due to the development of resistant microorganisms. However, antibiotics may be incorporated for short-term use in medical devices and other non-food products. Antioxidants are effective antifungal agents due to the restrictive oxygen requirement of moulds. Food grade chemical antioxidants could be incorporated into packaging materials to create an anaerobic atmosphere inside packages, and eventually protect the food against aerobic spoilage (Smith *et al*., 1990).

Various bacteriocins that are produced by microorganisms also inhibit the growth of spoilage and pathogenic microorganisms. These fermentation products include nisin, lacticins, pediocin, diolococcin, and propionicins (Daeschul, 1989; Han, 2002). These biologically active peptides possess strong antimicrobial properties against various bacteria. Other non-peptide fermentation products such as reuterin also demonstrate antimicrobial activity. Besides the above food grade bacteriocins, other bacteriocins would be utilized for the development of antimicrobial packaging systems.

The interest in the development and application of natural antimicrobial agents as additives in packaging materials has increased markedly due to their potential safety advantages. Essential oils such as garlic oil and cinnamon oil are proved to be able to inhibit microbial growth although different results are observed depending on test conditions, microorganisms, and the source of the antimicrobial compounds. The study by Pranoto et al. (2005) indicated that the films containing antimicrobial agents including garlic oil, potassium sorbate and nisin could enhance antimicrobial activity.

Incorporation of antioxidants into packaging materials has become popular since oxidation is a major problem affecting the food quality. Currently, the most frequently used antioxidants in active packaging are butylated hydroxyanisol (BHA) and butylated hydroxytoluene (BHT). Although these synthetic antioxidants can effectively be used in active food packaging because of high stability, low cost and efficiency, there are significant concerns related to their toxicological aspects. Moreover, use of synthetic antioxidants is under strict regulation due to the potential health risk caused by such compounds. Therefore, extensive research has been conducted to employ some natural antioxidants such as phenolic compounds as alternatives to synthetic antioxidants (Chan, Lim and Chew, 2007; Jongjareonrak et al., 2008; Yen, Yang and Mau, 2008). Current

research concerning use of natural antioxidant in edible films includes the addition of vitamin E into chitosan-based film (Park and Zhao, 2004) incorporation of tetrahydrocurcuminoids into chitosan film (Portes et al., 2009) and use of antioxidant borage extract in gelatin film (Gómez-Estaca et al., 2009).

3.6 Green tea polyphenols

Green tea (*Camellia sinensis*), nonfermented products, is a good source of polyphenolic compounds having strong antioxidant property (Chan et al., 2007). The health benefits of green tea have received considerable attention in recent years, and there is general consensus that this is associated with its polyphenolic compounds, which belong to a subgroup of flavonoids. The important polyphonolic compounds in tea leaves include catechin, theaflavins and thearubigenes. (Gramzaa, Khokharb, Yokob, Gliszczynska-Swigloc, Hesa and Korczaka, 2006). Green tea catechins, including (–)-epicatechin (EC), (–)-epigallocatechin (EGC), (–)-epicatechin gallate (ECG), (–)-epigallocatechin gallate (EGCG), (–)-catechin (C), (–)-gallocatechin gallate (GCG), (–)-catechin gallate (CG), and (–)-gallocatechin (GC), are proved to exhibit antimicrobial activity against some bacteria and have good antioxidant activity. Major green tea polyphenols are shown in Figure 3.2. The beneficial effects of the phenolic compounds are thought to result from their ability to scavenge reactive oxygen and nitrogen species (Chan et al., 2007; Rohn, Rawel and Kroll 2004). Green tea has been reported to delay the onset of lipid oxidation in various foods including marine oil (Wanasundara and Shahidi, 1998), soybean oil and corn oil (Yanishlieva and Marinova, 2001) and dry-fermented sausage (Bozkurt, 2006).

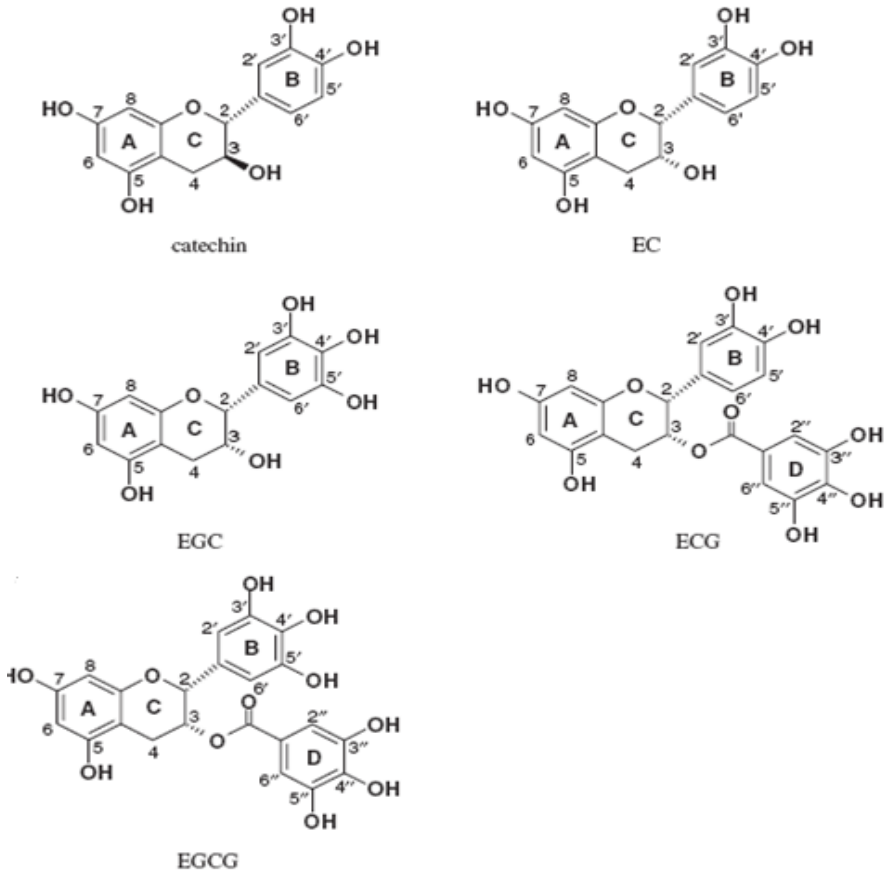


Figure 3.2 Molecular structures of green tea polyphenols (Pirker et al., 2008)

Many research has suggested that polyphenolic compounds are potential natural alternatives for synthetic antimicrobial and antioxidant agents. Green tea polyphenols are capable of bactericidal activity, by inhibition of DNA and RNA synthesis of bacterial cells (*E. coli, S. aureus, S.*Typhimurium) (Mori et al.,1987), inhibition of cytoplasmic membrane function of bacteria, resulting in leakage of intramembranous materials (Tsuchiya et alk., 1994), and interfering with energy metabolisms of bacteria (Haraguchi et al., 1998). Liu et al. (2004) demonstrated that among the four compounds polyphenol compounds, ECG, EGCG, EC, and caffeine, ECG and EGCG were the most active. Scanning electron microscopy (SEM) studies showed that ECG and EGCG altered bacterial cell morphology, which might have resulted from disturbed cell division. Wu et al. (2007) found that EC and caffeine in green tea could inhibit *S. aureus, Bacillus subtilis* and *E. coli*, and Hamilton-Miller (1995) who reported that polyphenolic compounds in green tea could inhibit *E. coli*, *Salmonella* Typhimurium*, Listeria monocytogenes*, *S. aureus* and *Campylobacter jejuni*.

4. MATERIALS & METHODS

This research was separated into 3 main parts including (1) Chitosan-based film preparation and modification. This part was to determine the optimum condition for preparing chitosan-based film and the optimum concentration of plasticizer to improve physical properties of the chitosan film, (2) Chitosan film incorporated with green tea (GT) to improve antioxidant and antimicrobial properties. This part was aimed to improve the antioxidant and antimicrobial properties of chitosan film by incorporation with natural green tea extract and to study the effect of green tea incorporation on the film properties. (3) Shelf life extension of food product using chitosan film incorporated with GT. This part was aimed to determine the efficacy of film incorporated with GT to maintain quality and extend shelf life of a food product.

4.1 Chitosan-based film preparation and modification

4.1.1 Chitosan-based film preparation

Commercial chitosan (Seafresh Industry Public Company Limited, Chumphon, Thailand) with degree of deacetylation of 95% was used to prepare chitosan-based film. Chitosan film forming solution was prepared according to the procedure of Yingyuad et al. (2006) with slight modification. A series of preliminary experiments were conducted in order to determine the optimum type and concentration of acidic solvent used for preparing the chitosan-based films. The preliminary results suggested that the optimum chitosan films could be prepared from 2% (w/v) chitosan in 1% (v/v) acetic acid.

A film-forming solution was prepared by dissolving specific amount of chitosan powder into 1% acetic acid solution. The solution was heated at 60 $^{\circ}$C in a water bath shaking incubator (SW 23, Julabo Labortechnik GmbH, Seelbach, Germany) at 100 oscillation/min for 30 min. The chitosan solution was filtered through a coarse sintered glass filter to remove undissolved impurities. The resulting solutions were homogenized for 2 min using a homogenizer (D-79282, Ystral GmbH, Ballrechten-Dottingen, Germany). The film-forming solutions were subsequently degassed to remove air bubbles using a sonicator (Ultrasonic Processor, Cole-Parmer, Vernon Hills, Illinois, USA). Portions of 90 $\pm$ 1 g from each film-forming solution were cast on a 12 x 28.5 cm ceramic plate and dried at ambient condition (27 $^{\circ}$C). The obtained films were conditioned in an environmental chamber at 25 $^{\circ}$C and 50 % relative humidity (RH) using saturated

4.1.2 Modification of chitosan-based film

The results in the previous section indicated that the chitosan film was brittle. Therefore, the mechanical properties of the films were improved by addition of plasticizer. In this experiment, glycerol was used as plasticizer. The optimum concentration of plasticizer was determined to improve the mechanical and barrier properties of the chitosan film. Different concentrations of glycerol (10, 20, 30, 40 and 50 %w/w of chitosan powder) were added into film-forming solution. The plasticized chitosan film was prepared and casted as previously described. The obtained films were conditioned in a humidity chamber at 50 % RH and 25 $^{\circ}$C for at least 48 h prior to testing. After conditioned, the films were examined for their physical properties. The effect of plasticizer at different concentrations on the physical properties of the films was determined by measuring their thickness, tensile strength, elongation at break, WVTR and color values.

4.1.3 Film thickness measurement

Film thickness was measured using a digital micrometer (Mitutoyo Absolute, Tester Sangyo Co., Ltd., Tokyo, Japan). Five replications were conducted for each sample treatment. Five measurements were taken at random positions around the film sample and the mean values were calculated.

4.1.4 Water vapor transmission rate

Water vapor transmission rate (WVTR) of the films was determined following ASTM standard test method (ASTM, 2003). Film samples, previously equilibrated at 50% RH for 48 h, were sealed to glass cups having 5 cm diameter containing silica gel. The film-covered cups were placed in an environmental chamber set at 25 $^{\circ}$C and 75% RH using saturated NaCl (Merck Company, Darmstadt, Germany). The cups were weighed periodically using an analytical balance (Sartorius A200S, Sartorius Mechatronics Co. Ltd., Bangkok, Thailand) until steady state was reached (±0.0001 g). The WVTR ($g.m^{-2}.day^{-1}$) of the films (Eq.1) was determined from the slope obtained from the regression analysis

of moisture weight gain (Δw) transferred through a film area (*A*) during a definite time (Δt), once the steady state was reached. At least six replications of each film treatment were tested for WVTR.

$$WVTR = \frac{\Delta w}{A\Delta t} \quad (1)$$

4.1.5 Mechanical properties

Mechanical properties including tensile strength (TS) and percentage of elongation at break (%E) were measured with an Instron Universal Testing Machine (Model 5655, Instron Corporation, Canton, MA, USA) following the ASTM Standard Test Method D 882-91 (ASTM, 2003). Each film strip (15 x 2.5 cm) was mounted between the grips of the instron and tested with an initial grip separation of 5 cm and crosshead speed of 1 mm/s. TS (Eq.2) was calculated by dividing the maximum load (F_{max}) by the initial cross-sectional area (ϕ) of the film sample expressed as MPa. Elongation at break (Eq.3) was calculated as the ratio of the film extension (Δl) at the point of sample rupture to the initial length (l_0) of a sample and expressed as a percentage. Measurements represent an average of at least nine samples.

$$TS = \frac{F_{\max}}{\Phi} \quad (2)$$

$$\%E = \frac{\Delta l}{l_0}100 \quad (3)$$

4.1.6 Color measurement

Hunter color (*L, a, b*) values were measured using a Minolta Chromameter (CR-300, Minolta Camera Co., Osaka, Japan). Films were cut into 15 x 2.5 cm and six readings at different positions on each film were measured. Five replications were conducted for each treatment and five film samples were used for each replication.

4.1.7 Statistical analysis

This experiment was conducted in six replications and the mean values were obtained from 3 measurements on separate samples. Completely randomized design (CRD) was used. The experimental data were subjected to one-way analysis of variance (ANOVA). The statistical significance of differences between mean values was

established at $p \leq 0.05$ with Duncan's New Multiple Range Test in the general statistical program.

4.2 Incorporation of chitosan film with green tea extract to improve antioxidant and antimicrobial properties of an active film from chitosan

4.2.1 Film preparation

Green tea leaves were purchased from a local hypermarket in Bangkok and ground using a blender (Toshiba MX-X10GMC, Toshiba Thailand Co., Ltd., Bangkok, Thailand). Green tea powders were kept in a linear low density polyethylene laminated aluminium pouch under vacuum condition using a vacuum packaging sealer (WEBOMATIC, Bochum, Germany) and stored at 8 $^{\circ}$C, until extraction. Green tea water extract solution was prepared by mixing ground tea powder in distilled water (1:5 w/w) controlled at 90 $^{\circ}$C in an Erlenmeyer flask and stirred in a water bath shaking incubator (SW 23, Julabo Labortechnik GmbH, Seelbach, Germany) at 100 oscillation/min for 10 min. The water extract of green tea was filtered through Whatman No.1 filter paper and was used for chitosan film-forming solution for further experiment.

Commercial chitosan (Seafresh Industry Public Company Limited, Chumphon, Thailand) with degree of deacetylation of 95% was used to prepare chitosan-based film. Chitosan film forming solution was prepared according to the procedure of Yingyuad et al. (2006) with slight modification. A series of preliminary experiments were conducted in order to determine the optimum type and concentration of acidic solvent and plasticizer used for preparing the chitosan-based films. The results suggested that the optimum chitosan films could be prepared from 2% chitosan in 1% acetic acid. The results also indicated that the mechanical properties of the films were improved by adding glycerol, as plasticizer, at the concentration of 30 % w/w of chitosan powder.

A film-forming solution was prepared by dissolving specific amount of chitosan powder into 1% acetic acid solution. Glycerol (Sigma Chemical Co., St. Louis, MO, USA), as plasticizer, was added to the film-forming solution at the constant concentration of 30 % w/w of chitosan powder. The solution was heated at 60 $^{\circ}$C in a water bath shaking incubator at 100 oscillation/min for 30 min. The chitosan solution was filtered through a

coarse sintered glass filter to remove undissolved impurities. The solution was cooled to room temperature and the GT solution was included to obtain the final concentrations of 0, 2, 5, 10, and 20 % (w/v) of green tea in the chitosan film-forming solution. The resulting solutions were homogenized for 2 min using a homogenizer (D-79282, Ystral GmbH, Ballrechten-Dottingen, Germany). The film-forming solutions were subsequently degassed to remove air bubbles using a sonicator (Ultrasonic Processor, Cole-Parmer, Vernon Hills, Illinois, USA). Portions of each film-forming solution were cast on a ceramic plate and dried at ambient condition. The obtained films were conditioned in an environmental chamber at 25 $^{\circ}$C and 50 % relative humidity (RH) for 48 h prior to testing. All property measurements were performed immediately after removing film specimens from the chamber to minimize moisture variances of these natural films.

4.2.2 Film thickness and density measurements

Film thickness was measured using a digital micrometer (Mitutoyo Absolute, Tester Sangyo Co., Ltd., Tokyo, Japan). Five replications were conducted for each sample treatment. Five measurements were taken at random positions around the film sample and the mean values were calculated. Film density was determined from the film weight and volume. The film volume was calculated from film the area and thickness.

4.2.3 Color and opacity measurement

Hunter color (*L, a, b*) values were measured using a Minolta Chromameter (CR-300, Minolta Camera Co., Osaka, Japan), as described in 4.1.6. Films were cut into 15 x 2.5 cm and six readings at different positions on each film were measured. Five replications were conducted for each treatment and five film samples were used for each replication.

Opacity was determined according to the method of Park et al. (2004) by measuring the film absorbance at 600 nm using a UV spectrophotometer (Thermo Scientific GENESYS 20, Thermo Fisher Scientific, Inc., Rochester, NY, USA). The films were cut into a rectangle piece and directly placed in a spectrophotometer test cell. An empty test cell was used as the reference. The opacity of the films was calculated by the following equation

$$T = \frac{Abs_{600}}{x} \quad (4)$$

where T is the transparency, Abs_{600} is the value of absorbance at 600 nm and x is the film thickness (mm). According to this equation, the high values of T indicate lower transparency and higher degree of opacity.

4.2.4 Water vapor permeability coefficient

Water vapor transmission rate (WVTR) of the films was determined following ASTM standard test method (ASTM, 2003), as described in 4.1.4. The WVTR of the films was then used to calculate the water vapor permeability coefficient (WVP) using Eq (5). At least six replications of each film treatment were tested for WVP.

$$WVP = WVTR\frac{x}{\Delta p} \quad (5)$$

where WVP is the permeability coefficient (g.mm. $m^{-2}.d^{-1}.kPa^{-1}$), x is the film thickness, Δp is the partial water vapor pressure gradient between the inner (p_1) and outer (p_2) surface of the film in the chamber.

4.2.5 Mechanical properties

Mechanical properties including tensile strength (TS) and percentage of elongation at break (%E) were measured with an Instron Universal Testing Machine (Model 5655, Instron Corporation, Canton, MA, USA) following the ASTM Standard Test Method D 882-91 (ASTM, 2003), as described in 4.1.5.

4.2.6 Total phenolic assay

For this purpose, 25 mg of each film sample were dissolved in 3 ml of distilled water. Total phenolic content (TPC) of the chitosan film samples was determined according to the Folin-Ciocalteu method as described by Singleton, Orthofer and Lamuela-Raventos (1999) with slight modifications. Briefly, 0.1 ml of film extract solution were mixed with 7 ml distilled water and 0.5 ml of Folin-Ciocalteau reagent (Merck Company, Darmstadt, Germany). The mixture was incubated for 8 min at room temperature before addition of 1.5 ml of sodium carbonate solution and 0.9 ml of distilled water. The mixture was stored in a dark chamber at room temperature for 2 h. The absorbance of the mixture was then measured at 765 nm using a spectrophotometer

(Thermo Scientific GENESYS 20, Thermo Fisher Scientific, Inc., Rochester, NY, USA). Gallic acid solutions (Fluka Chemical Company, St. Louis, MO, USA) in the specific concentration range were used to construct a calibration curve. The concentration of total phenolic compounds in the samples is expressed as gallic acid equivalents (GAE), which reflect the phenolic content as the amount of gallic acid in mg per gram dry weight of the sample, calculated by using an equation that was obtained from the standard graph (R^2 = 0.996), is given as:

$$Abs_{765} = 0.001 \text{ mg gallic acid} + 0.027 \qquad (6)$$

This estimation of total phenolic compounds in the samples was analyzed in five replications, and the results were averaged.

4.2.7 Determination of antioxidant activity

The antioxidant activity of the film samples was evaluated using DPPH (2,2-diphenyl-1-picrylhydrazyl) free radical scavenging assay according to Blois (1958) with slight modification. Briefly, 3 ml of film extract solution were mixed with 1 ml of 1 mM methanolic solution of DPPH (Fluka Chemical Company, St. Louis, MO, USA). The mixture was vortexed using a vortex-Genie 2 (Scientific Industries, Inc. Bohemia, New York, USA) and incubated in the dark at ambient temperature for 30 min. When the DPPH solution was mixed with the sample mixture acting as a hydrogen atom donor, a stable nonradical form of DPPH is obtained with simultaneous change of the violet color to pale yellow. The absorbance was then measured at 517 nm. The percentage of DPPH free radical quenching activity was determined using the following equation:

$$\text{DPPH scavenging effect (\%)} = \frac{Abs_{DPPH} - Abs_{extract}}{Abs_{DPPH}} 100 \qquad (7)$$

where Abs_{DPPH} is the absorbance value at 517 nm of the methanolic solution of DPPH and $Abs_{extract}$ is the absorbance value at 517 nm for the sample extracts. Each sample was assayed at least five times.

4.2.8 FTIR analysis

Fourier Transform Infrared (FTIR) spectrometry was carried out to observe the structural interactions of chitosan films incorporated with GT. The FTIR spectra of chitosan films were recorded from 4000-650 cm^{-1} at resolution of 1 cm^{-1} using a FTIR

spectrometer (PerkinElmer 1760X, PerkinElmer Life And Analytical Sciences, Inc., Waltham, Massachusetts USA).

4.2.9 Antimicrobial activity assay

Antimicrobial activity assay of chitosan films was carried out using agar diffusion, following the method of Pranoto, Rakshit and Salokhe (2005) with slight modification. For the antimicrobial assay, all tested microorganisms (*Staphylococcus aureus, Escherichia coli, Pseudomonas fluorescens* and *Salmonella* Enteritidis) were selected from food spoilage or pathogenic bacteria, which are commonly found in food products.

Staphylococcus aureus TISTR 118, *Escherichia coli* TISTR 780 and *Pseudomonas fluorescens* TISTR 358 were obtained from Thailand Institute of Scientific and Technological Research (TISTR), Thailand, and *Salmonella* Enteritidis DMST 17368 was obtained from Culture Collection for Medical Microorganism, Department of Medical Sciences, Thailand. Before used, the test strains were grown in nutrient broth (HiMedia Laboratories Pvt. Ltd., Bombay, India) to give a final density of 10^7 CFU/ml.

The films were cut into a disc form of 6 mm diameter. Film discs were placed on nutrient agar (Merck, Darmstadt, Germany) plates which had been previously seeded with inoculum containing indicator microorganisms. The plates were then incubated at 37 $^{\circ}$C for 24 h for *S. aureus*, *E. coli* and *S.* Enteritidis and at 30 $^{\circ}$C for 24 h for *P. fluorescens.* The diameter of inhibitory zone surrounding film discs and contact area of films with agar surface were measured. Inhibitory zone was calculated by subtracting overall clear zone by diameter of the film disc. If there was no clear zone surrounding the film disc, it was marked as no inhibitory zone. Contact area was also used to evaluate growth inhibition underneath the film discs in direct contact with target microorganisms on the agar.

4.2.10 Statistical analysis

A completely randomized design (CRD) was used as an experimental design, where film forming solutions containing different concentrations of GT were applied as treatments. Chitosan films without GT were used as control. The experimental data were subjected to one-way analysis of variance (ANOVA). The statistical significance of differences between mean values was established at $p \leq 0.05$ and the Duncan's New Multiple Range Test was applied for all statistical analyses.

4.3 Shelf life extension of pork sausage using chitosan film containing GT

4.3.1 Sample preparation

Pork sausages were obtained from a local supplier in Bangkok. Before use,

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4.3.2 Color measurement

CIELAB color (L* (lightness), a* (redness), b* (yellowness)) values were measured using a Minolta Chromameter (CR-300, Minolta Camera Co., Japan) with a 10 mm diameter aperture and a 10° standard observer. The values for *L**, *a**, and *b** were recorded to evaluate surface color changes of pork sausage during storage. All measurements and analyses were made directly on the surface of meat samples immediately after opening the packages.

4.3.3 Texture measurement

The texture (cutting force) was measured using a Texture analyzer (Lloyd Food Texture Analyzer, model TA 500, England) with a displacement speed of 2 mm. sec^{-1}. The force necessary to obtain a constant deformation of 30 mm was recorded in gram force.

4.3.4 pH measurements

Ten grams of pork sausage samples were homogenized in 90 ml distilled water at 1000 rpm using a stomacher (Seward stomacher, model 400, England). pH was

4.3.5 Lipid oxidation

TBA (2-thiobarbituric acid) measurement was determined following the method of Tokur et al. (2006). Ten grams of homogenized sample were added with 97.5 ml of distillated water and 2.5 ml of 4 N HCl. The mixture was heated with steam distillation. Five ml of distillate was added to 5 ml of thiobarbutiric reactive reagent containing 0.02 M TBA in 90 % glacial acetic acid and incubated for boiling water for 35 min. After cooling, the absorbance of the pink solution was measured at 538 nm using a spectrophotometer (Thermo Scientific GENESYS 20, Thermo Fisher Scientific, Inc., Rochester, NY, USA). The constant 7.8 was used to calculate the TBA number using Eq 8. The TBA value is expressed as milligrams of malonaldehyde per kg of sample.

$$\text{TBA value} = 7.8 \times \text{Abs}_{538} \quad (8)$$

4.3.6 Microbiological analysis

Twenty five grams of pork sausage were homogenized in 225 ml of 0.1% peptone buffer for 2 min using a Stomacher Lab Blender (Seward Laboratory, London, UK). Serial decimal dilutions were made with the same diluent. The samples were spread-plated on Plate Count Agar (PCA) (HiMedia Laboratories Pvt. Ltd., Bombay, India) and incubated at 37 $^{\circ}$C for 24 h for total viable count. For yeasts and moulds, the samples were spread-plated on Potato Dextrose Agar (PDA) (HiMedia Laboratories Pvt. Ltd., Bombay, India) and incubated at 25 $^{\circ}$C for 72 h. For lactic acid bacteria, the samples were pour-plated on Lactobacillus MRS Agar (MRS) (HiMedia Laboratories Pvt. Ltd., Bombay, India) and incubated at 37 $^{\circ}$C for 72 h, following the method of Bingol and Bostan (2007). The results were reported as colony forming unit (CFU/g).

4.3.7 Sensory evaluation

The sausages were brought to approximately 20 $^{\circ}$C before assessment. Quantitative Descriptive Analysis (Stone et al., 1974) was used to obtain four attributes to describe the samples. Odor attributes were assessed first, followed by appearance (color

4.3.8 Statistical analysis

This experiment was conducted in triplicate and the mean values were obtained from 2 measurements on the separate samples. Symmetric Factorial Experiment with Completelyly Randomized Design (CRD) was used for pH, color, texture and TBA measurements at each time interval over the storage period. Randomized Complete Block Design (Factorial in RCBD) was used for assessment of sensory evaluation. The statistical significance of differences between mean values was established at $p \leq 0.05$ with the Duncan's New Multiple Range Test in the general model of the SPSS statistical package (SPSS Inc. Version 10.0, Chicago, Illinois).

5. RESULTS AND DISCUSSION

5.1 Chitosan-based film and film modification

5.1.1 Mechanical property

The effect of plasticizer concentration on the mechanical properties of the chitosan film plasticized using glycerol, at different concentration was determined by measuring their thickness, tensile strength and elongation at break. The results showed that an increase in the amount of the plasticizers resulted in a significant ($p \leq 0.05$) increase in thickness (Figure 5.1), a decrease in mechanical resistance (decrease in tensile strength) and an increase in extensibility (increase in elongation at break). Tensile strength (Figure 5.2) decreased from 37.49 $\pm$ 3.27 to 11.52 $\pm$ 1.29 MPa when glycerol concentration increased from 0 to 50 % w/w. There was a decreasing trend when adding glycerol from 0-30% but was not statistically different.

The elongation at break (Figure 5.3) increased from 14.63 $\pm$ 2.56 to 63.51 $\pm$ 7.11 % with an increase in plasticizer concentration from 0-40%. However, when adding 50 % plasticizer, the elongation at break decreased to 46.53 $\pm$ 6.68, probably because high concentration of plasticizer may interrupt intermolecular and intramolecular interaction of the chitosan polymer. This result is in agreement with those of Hosokawa et al. (1990) and Arvanitoyannis et al. (1998) who found that the elongation of the bio-based films increased with increasing plasticizer concentration, but at high concentration there were decreases in both tensile strength and modulus. Changes in mechanical properties as affected by hydrophilic plasticizers were observed for various hydrocolloid-based films (Gontard *et al*., 1993; Butler *et al*., 1996; Caner *et al*., 1998; Srinivasa *et al*., 2007).

Generally, tensile strength and elongation at break are inversely correlated. The latter showed an increasing trend with the addition of plasticizers. Glycerol are low molecular weight hydrophilic molecules that could fit into chitosan chains and established hydrogen bonding with reactive groups of chitosan. By reducing internal hydrogen bonding between polymer chains, the density of intermolecular interaction in material decrease and the free volume between polymer chains increase (Cuq *et al*., 1997). The changes in mechanical properties of the film could be attributed to a decrease in density and an alteration of intermolecular and intramolecular interaction occurring in the films.

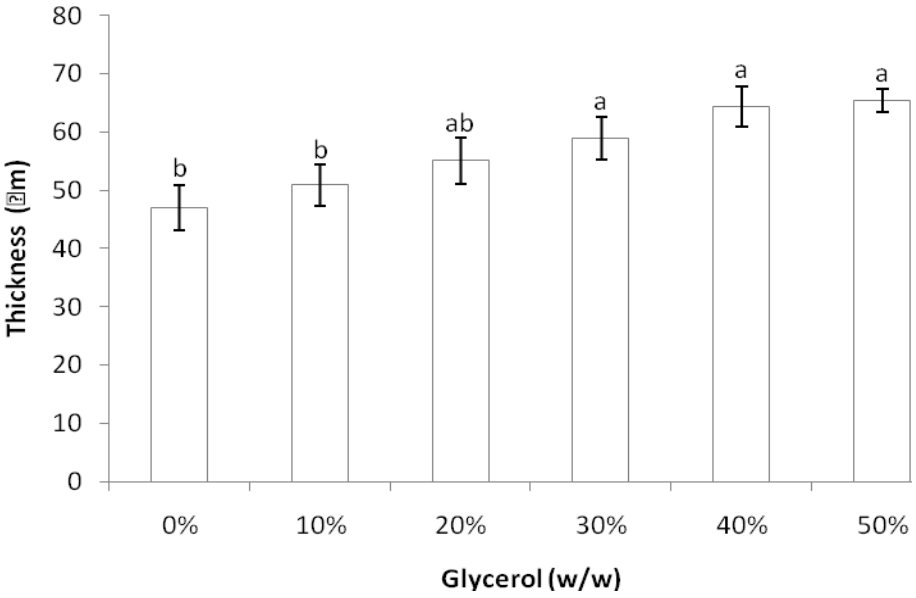


Figure 5.1 Thickness of chitosan films plasticized using different concentration of glycerol. Values are given as mean ± standard deviation. Different superscript letters in the same column indicate significantly different ($p \leq 0.05$) when analyzed using Duncan's New Multiple Range Test.

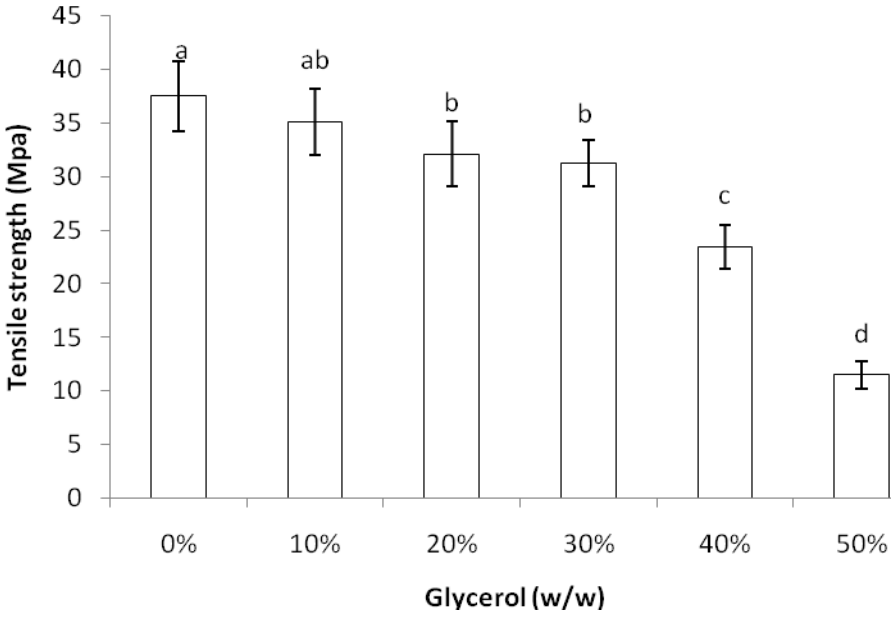


Figure 5.2 Tensile strength of chitosan films plasticized using different concentration of glycerol. Values are given as mean ± standard deviation. Different superscript letters in the same column indicate significantly different ($p \leq 0.05$) when analyzed using Duncan's New Multiple Range Test.

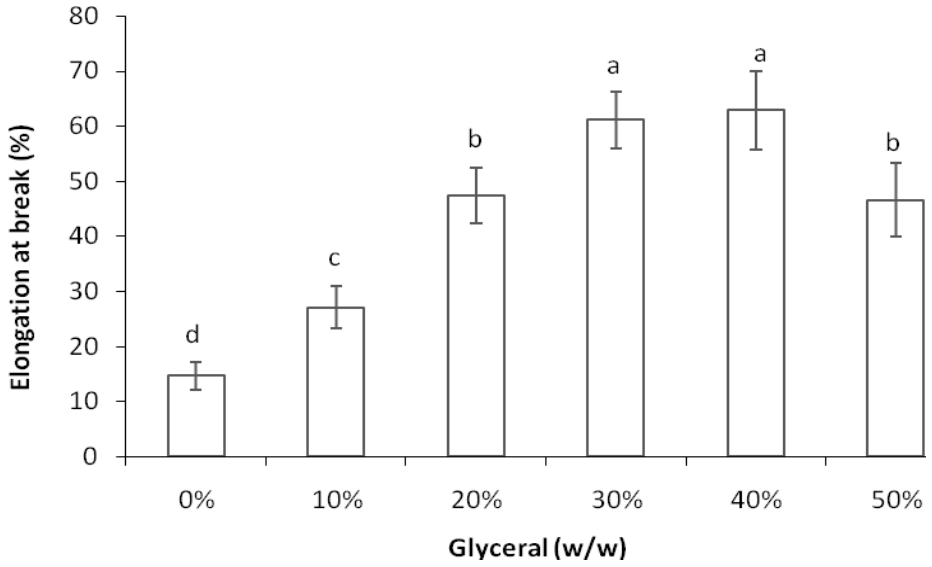


Figure 5.3 Elongation at break of chitosan films of chitosan films plasticized using different concentration of glycerol. Values are given as mean ± standard deviation. Different superscript letters in the same column indicate significantly different ($p \leq 0.05$) when analyzed using Duncan's New Multiple Range Test.

5.1.2 Water vapor transmission rate

Water vapor transmission rate of the chitosan films prepared using different concentration of plasticizer was examined. The WVTR increased significantly ($p \leq 0.05$) with concentration of plasticizer (Figure 5.4). The WVTR increased from 5.37 ± 0.43 to 7.64 ± 0.23 $g \cdot m^{-2} \cdot h^{-1}$, when the concentration of glycerol increased from 10 to 50 %w/w. Water vapor transmission rate increased with plasticizer concentration and this could be related to hydrophilicity of plasticizer molecules. In addition, this change could be explained by structural modifications of the chitosan network. The incorporation of plasticizer could modify the molecular arrangement of the chitosan network, resulting in an increase in free volume. Film network becomes less dense and, consequently, more permeable (Ashley, 1985). Incorporation of hydrophilic plasticizers has been reported to increase the water vapor permeability of hydrocolloid-based films (Gontard et al., 1993; McHugh et al., 1994; Srinivasa et al. 2007; Talja et al., 2007).

Relatively small molecular weight hydrophilic plasticizers are often added to improve the physical properties of the films. The result of plasticizer addition is a

lowering of the film glass transition temperature and an improvement in film flexibility (lowering of film elastic modulus). Unfortunately, plasticizers generally also decrease the film's ability to act as a barrier to moisture and oxygen (Caner et al., 1998).

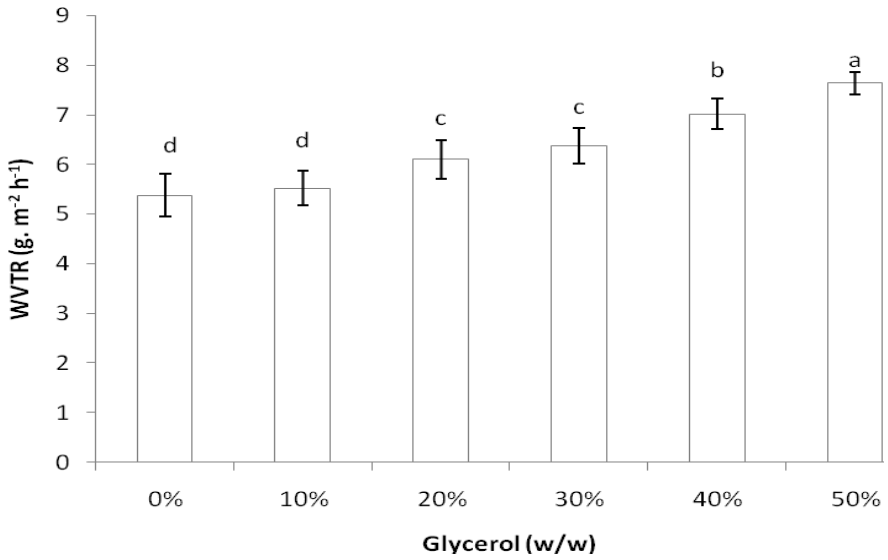
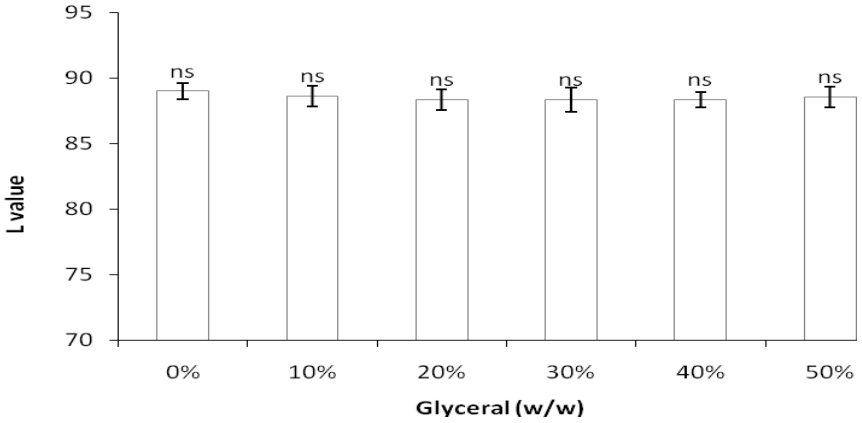


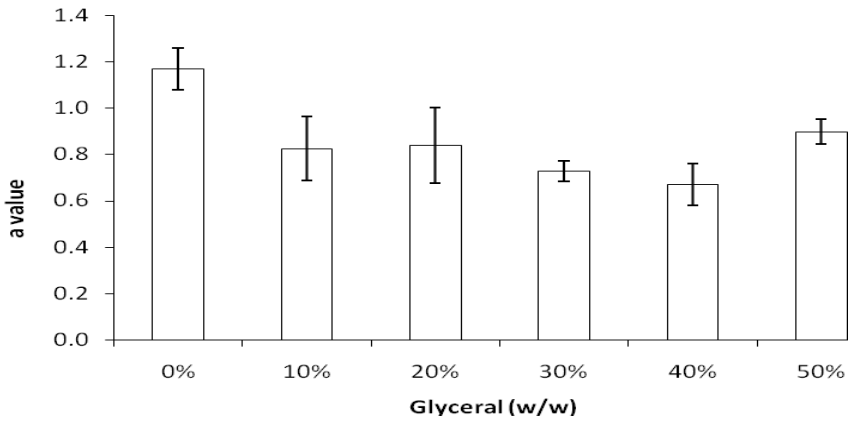
Figure 5.4 Water vapor transmission rate of chitosan films plasticized using different concentration of glycerol. Values are given as mean ± standard deviation. Different superscript letters in the same column indicate significantly different ($p \leq 0.05$) when analyzed using Duncan's New Multiple Range Test.

5.1.3 Surface color

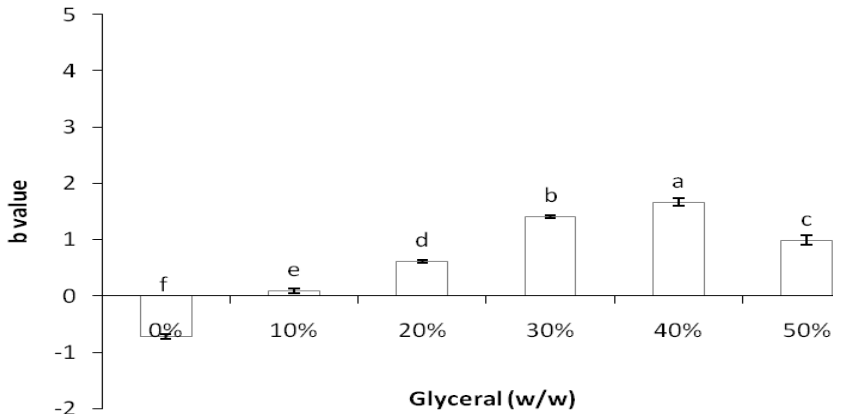
The effect of plasticizer type and concentration on film surface color is shown in Figure 5.5. Addition of plasticizer had no significant effect ($p > 0.05$) on the L (lightness/darkness) value (Figure 5a), but had a significant effect ($p \leq 0.05$) on a value (redness/greenness) and b value (yellowness/blueness). There was a decreasing trend of a value (indicator of the tendency towards greenness), when adding higher concentration of plasticizer (Figure 5b). b value increased significantly ($p \leq 0.05$) with addition of plasticizers (Figure 5.5 c). b value significantly increased (indicator of the tendency towards yellowness), when glycerol concentration increased from 10 to 40 %w/w (Figure 5.5 c). Cuq *et al*. (1996) also observed a relatively slight yellow color in glycerol-plasticized protein-based film. Labuza and Saltmarch (1981) reported that glycerol might participate in the browning reaction, but no specific mechanism was proposed.



(a)



(b)



(c)

Figure 5.5 Surface color including L-value (a), a-value (b) and b-value (c) of chitosan films plasticized using different concentration of glycerol. Values are given as mean ± standard deviation. Different superscript letters in the same column indicate significantly different ($p \leq 0.05$) and ns indicates no significant differences ($p > 0.05$), when analyzed using Duncan's New Multiple Range Test.

5.1.4 Conclusion

Addition of plasticizer could modify the molecular arrangement of the chitosan network, resulting in an increase in extensibility (increase in elongation at break), a decrease in mechanical resistance (decrease in tensile strength) and an increase in water vapor transmission rate. The changes in mechanical properties of the film could be attributed to a decrease in density and an alteration of intermolecular and intramolecular interaction occurring in the films. Based on physical properties, glycerol 30% w/w was the optimum concentration in that it gave the film with the optimum physical properties. The chitosan film had higher elongation at break (more flexible), while there was no significant change in tensile strength, when compared to the control film, and therefore was suitable for further experiment.

5.2 Incorporation of chitosan film with green tea extract to improve antioxidant and antimicrobial properties

5.2.1 Film color and opacity

The effects of GT concentration on film color and opacity are shown in Table 5.1. Adding GT into chitosan films significantly affected ($p \leq 0.05$) L (lightness/darkness), a (redness/greenness) and b (yellowness/blueness) values of the film surface. Films without GT were lighter (higher L value). L values of the films decreased from 87.50 ± 1.43 to 65.70 ± 0.97, but a increased from 1.11 ± 0.16 to 3.99 ± 0.28 (indicator of the tendency towards redness) and b values increased from 1.37 ± 0.1 to 40.02 ± 1.49 (indicator of the tendency towards yellowness), as the GT concentrations increased from 0 to 20 %.

The average film thickness of 62.08 ± 6.31 μm was obtained and used for calculating the opacity according to Eq 4. Chitosan films without GT were more transparent (lower opacity value) than those incorporated with GT (Table 5.1). The opacity of the film samples significantly increased ($p \leq 0.05$) with increasing GT concentration. An increase in film opacity as a consequence of the addition of antioxidant has also been reported in fish gelatin films containing borage extract (Gómez-Estaca et al., 2009).

Table 5.1 Color values and opacity index of chitosan films incorporated with GT

Film samples	*L*	*a*	*b*	T
Control	$87.50^{a} \pm 1.43$	$1.11^{c} \pm 0.16$	$1.37^{e} \pm 0.1$	$0.774^{e} \pm 0.023$
2% GT	$77.22^{b} \pm 0.24$	$2.83^{b} \pm 0.31$	$22.06^{d} \pm 1.3$	$1.685^{d} \pm 0.057$
5% GT	$74.93^{c} \pm 0.50$	$3.32^{ab} \pm 0.66$	$28.87^{c} \pm 0.83$	$2.229^{c} \pm 0.135$
10% GT	$72.61^{d} \pm 1.60$	$3.07^{ab} \pm 0.46$	$37.28^{b} \pm 1.89$	$2.732^{b} \pm 0.121$
20% GT	$65.70^{e} \pm 0.97$	$3.99^{a} \pm 0.28$	$40.02^{a} \pm 1.49$	$3.019^{a} \pm 0.124$

Values are given as mean $\pm$ standard deviation. Different superscript letters in the same column indicate significantly different ($p \leq 0.05$) when analyzed using Duncan's New Multiple Range Test.

5.2.2 Water vapor permeability coefficient and density

The water vapor permeability coefficient of a film is a constant value for permeation of the water vapor at a given temperature. The permeability of a film depends on its chemical structure and morphology, the nature of permeant and temperature of the environment. Water vapor permeability of the chitosan films incorporated with different concentrations of GT was examined at 25 $^{\circ}$C. The results showed that GT had a significant effect ($p \leq 0.05$) on WVP and density of the films as shown in Table 5.2. The permeability coefficient decreased from 0.256 $\pm$ 0.023 to 0.087 $\pm$ 0.012 g.mm. $m^{-2}.d^{-1}.$ kPa^{-1}, while the density increased from 1.21 $\pm$ 0.03 to 1.67 $\pm$ 0.03 $g.cm^{-3}$, as the concentration of GT increased from 0 to 20 %. Incorporation of GT into chitosan film caused the resulting films to become denser and less water vapor permeability.

There is a possibility that polyphenolic compounds may be able to fit into chitosan matrix and established interactions such as hydrogen or covalent bonding with reactive groups of chitosan (Rohn et al., 2004). Therefore, by increasing intermolecular

interactions within the chitosan polymer chain, the density of the chitosan-based film increased. The lower WVP of the films incorporated with GT may be because the hydrogen and covalent interactions between chitosan network and polyphenolic compounds limit the availability of hydrogen groups to form hydrophilic bonding with water, subsequently lead to a decrease in the affinity of chitosan film toward water. Spencer et al. (1988) reported similar effects on polyphenol-protein complexes and specified that polyphenols could form hydrogen and covalent bonds with the polar groups (e.g. amino and hydroxyl groups) of polypeptide surface resulting in a less hydrophilic layer of polyphenol-protein complexes. Curcio et al. (2009) also observed the formation of covalent bonds between gallic acid antioxidant and chitosan as verified by FTIR.

Similar finding was observed by Park et al. (2004) in a study on incorporation of mineral and vitamin into chitosan-based film. They found that by adding mineral or vitamin into the film matrix, interaction among adjacent molecules structures increased, resulting in a decrease in diffusivity of water vapor transmission rate through a film matrix and a decrease in hydrophilic tendency of the films. Gómez-Guillén, et al. (2007) also reported a decrease in availability of hydrogen groups due to cross-linking between tuna-fish gelatin and antioxidant extracts from murta leaves.

Table 5.2 Density and water vapor permeability coefficient of chitosan films incorporated with GT

Film samples	Density ($g.cm^{-3}$)	WVP ($g.mm.\ m^{-2}.d^{-1}.kPa^{-1}$)
Control	$1.21^{d} \pm 0.03$	$0.2562^{a} \pm 0.023$
2% GT	$1.25^{d} \pm 0.01$	$0.2250^{ab} \pm 0.037$
5% GT	$1.35^{c} \pm 003$	$0.2036^{b} \pm 0.027$
10% GT	$1.45^{b} \pm 0.04$	$0.1773^{b} \pm 0.019$
20% GT	$1.67^{a} \pm 0.03$	$0.0871^{c} \pm 0.012$

Values are given as mean $\pm$ standard deviation. Different superscript letters in the same column indicate significantly different ($p \leq 0.05$) when analyzed using Duncan's New Multiple Range Test.

5.2.3 Mechanical properties

The tensile strength is the measurement of maximum strength of a film to withstand against applied tensile stress and percent elongation represents the ability of a film to stretch (Park et al., 2004). The mechanical properties of chitosan films incorporated with different GT concentrations were statistically compared (Table 5.3). The results showed that GT significantly affected ($p \leq 0.05$) the mechanical resistance and extensibility of the chitosan-based films. Tensile strength and percent elongation at break did not significantly change when GT concentration increased from 0-5 %, but significantly increased from 25.13 ± 1.91 to 27.55 ± 3.46 MPa and from 54.76 ± 3.14 to 60.73 ± 3.37, respectively when GT concentration increased from 5 to 20 %. The effect of GT incorporation on the strengthening of the corresponding films may be explained by the similar assumption developed for WVP and density. The improvement in mechanical properties of the films incorporated with GT may be attributed to the interaction between chitosan matrix and polyphenolic compounds from GT.

Changes in mechanical properties as affected by polyphenolic compounds were also observed for other biopolymeric films, including vegetable tannins in sunflower protein isolate film (Orliac et al., 2002), murta leave extract in tuna-fish gelatin films (Gómez-Guillén et al., 2007) and antioxidant borage extract in fish gelatin films (Gómez-Estaca et al., 2009).

Table 5.3 Tensile strength and % Elongation of chitosan films incorporated with GT

Film samples	Tensile (MPa)	% Elongation
Control	$23.66^{b} \pm 2.63$	$54.62^{b} \pm 3.12$
2% GT	$25.00^{b} \pm 2.68$	$54.76^{b} \pm 3.14$
5% GT	$25.13^{b} \pm 1.91$	$58.14^{ab} \pm 4.24$
10% GT	$28.35^{a} \pm 3.51$	$60.39^{a} \pm 3.60$
20% GT	$27.55^{a} \pm 3.46$	$60.73^{a} \pm 3.37$

Values are given as mean ± standard deviation. Different superscript letters in the same column indicate significantly different ($p \leq 0.05$) when analyzed by Duncan's

5.2.4 Total phenolic content and antioxidant activity

Folin-Ciocalteu phenol reagent is used to obtain a crude estimate of the amount of phenolic groups present in the chitosan film. Phenolic compounds undergo a complex redox reaction with phosphotungstic and phosphomolybdic acids present in the Folin-Ciocalteu reactant. The color development is due to the transfer of electrons at basic pH to reduce the phosphomolybdic/phosphotungstic acid complexes (Curcio et al., 2009). The results showed that total phenolic content in the chitosan films significantly increased ($p \leq 0.05$) with increasing GT concentration (Figure 5.6).

DPPH scavenging assay was used to indicate antioxidant activity of the film. This assay is based on the ability of DPPH, a stable free radical, to be quenched and thereby decolorize in the presence of antioxidants resulting in a reduction in absorbance values (Enayat and Banerjee, 2009). In the DPPH test, the antioxidants reduce the DPPH radical to a yellow-colored compound, diphenylpicrylhydrazine, and the extent of the reaction depends on the hydrogen-donating ability of the antioxidants (Blois, 1958).

The results showed that DPPH scavenging activity of the films significantly increased ($p \leq 0.05$) with increasing GT concentration as shown in Figure 5.7. The chitosan films without GT extract showed some scavenging activity on DPPH. The scavenging mechanism of chitosan is related to the fact that free radical can react with the residual free amino (NH_2) groups to form stable macromolecule radicals, and the NH_2 groups can form ammonium (NH_3^+) groups by absorbing a hydrogen ion from the solution (Park, Je and Kim, 2004; Yen et al., 2008). In the films containing GT, the antioxidant activity increased in more than 15 folds in relation to the control samples. As the GT increased in the film formulation, so did the expected antioxidant character of the active film.

The results suggested that incorporation of GT into chitosan film improved mechanical and barrier properties and enhanced antioxidant activity of the film. Interactions between chitosan and polyphenolic compounds from GT may play a major role on the modification of film properties and will be discussed further based on the FTIR data obtained in the next section.

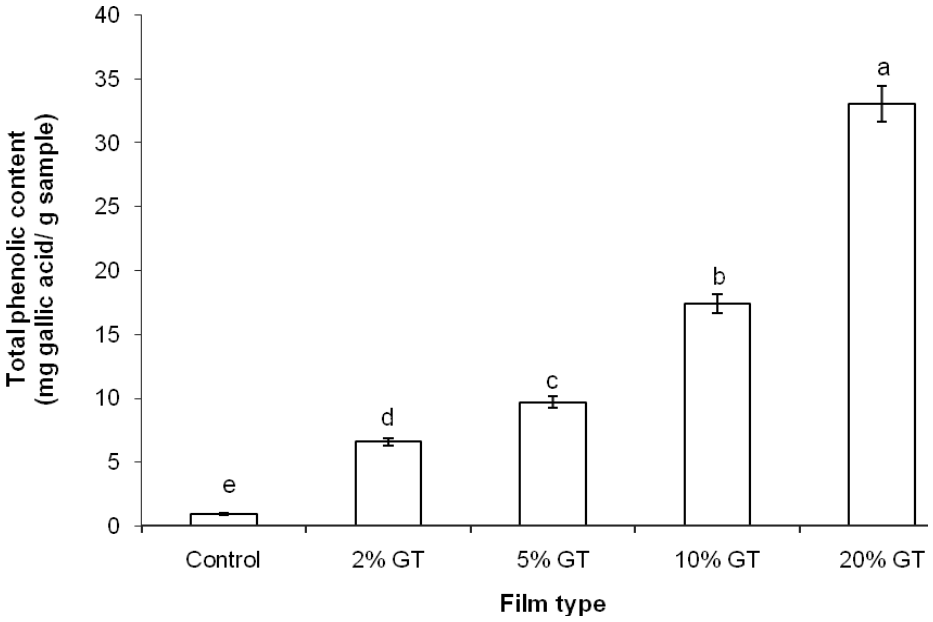


Figure 5.6 Total polyphenolic content of chitosan films incorporated with GT. Values are given as mean ± standard deviation. Different letters indicate significantly different ($p \leq 0.05$) when analyzed using Duncan's New Multiple Range Test.

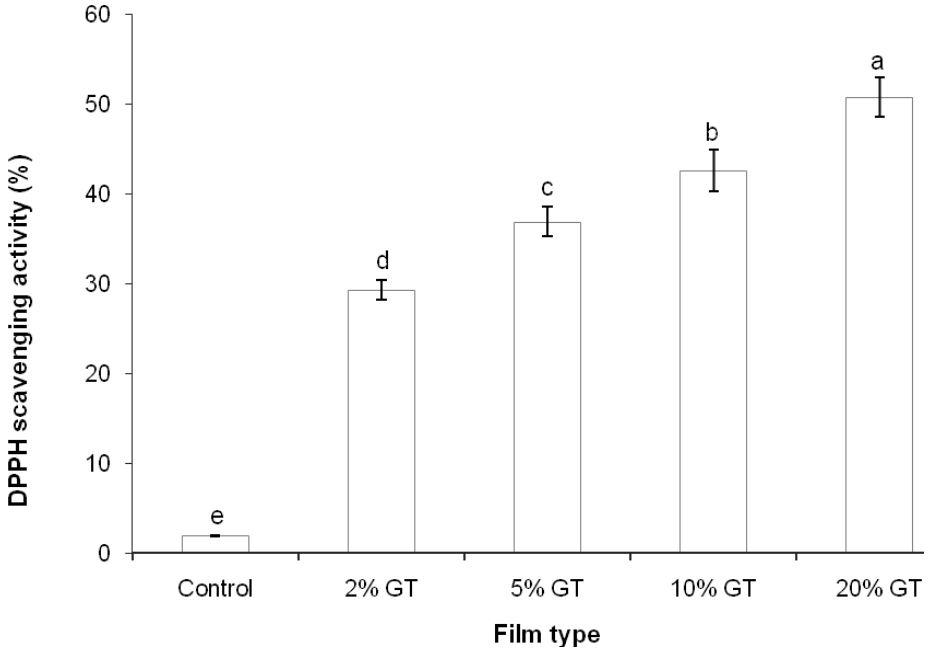


Figure 5.7 DPPH Scavenging of chitosan films incorporated with GT. Values are given as mean ± standard deviation. Different letters indicate significantly different ($p \leq 0.05$) when analyzed using Duncan's New Multiple Range Test.

5.2.5 FTIR

FTIR spectroscopy was used as a tool to investigate the interaction between chitosan film and green tea polyphenols by measuring the absorbent during wavenumber range of 4000-650 cm^{-1} at resolution of 1 cm^{-1}. The FTIR spectra of control film and those incorporated with 2, 5, 10 and 20% GT are shown in Figure 5.8. Control film shows pattern with the absorption peaks at 3400 cm^{-1}, which indicates stretching of the O–H and N–H, and at 1030–1155 cm^{-1} for C–O bonds. Absorption peaks at 2900 cm^{-1} region and around 1450–1590 cm^{-1} and 1400 cm^{-1} correspond to C–H stretching, amine groups ($–NH_2$) and carboxyl groups ($–COO^-$), respectively. Special mention should be made to the peaks between 3500-3000 cm^{-1}, corresponding to stretching vibration of free hydroxyl and to asymmetric and symmetric stretching of the N-H bonds in amino group, respectively, are stronger in control film when compared to those incorporated with GT. In addition, two strong water bands at 1530 and 1400 cm^{-1}, associated to OH in-plane bending, are less discernible in the films incorporated with GT. These peaks became more flattened with increasing GT concentrations. Another important change takes place at 1700 and 1660 cm^{-1}. For film incorporated with GT, new peaks at 1700 cm^{-1}, ascribable to carbon-to-oxygen (C=O) stretching within the carboxylic group, and peak at 1660 cm^{-1}, correlated to carbon-to-carbon (C=C) stretching within the aromatic ring, which indicates functional group of phenolic compounds were detected and appeared to be more recognizable with increasing GT concentration.

An increase in the absorption bands at 1700 and 1660 cm^{-1} in the FTIR spectra was coincidental with the decrease in peaks at 3500-3000, 1530 and 1440 cm^{-1}. This observation supported the assumption that there could be a particular arrangement in the films due to the interactions of green tea polyphenolic compounds with hydroxyl and amino groups in chitosan matrix. These results were in good agreement with the study of physicochemical interaction between chitosan-catechin by Zhang and Kosaraju (2007) who found that amine functional groups of the chitosan decreased when incorporated with catechin. Similar findings were also observed by Curcio et al. (2009) in the formation of covalent bonds between gallic acid-chitosan and catechin-chitosan. From FTIR, it is evident that addition of GT, polyphenols could form hydrogen bonding and covalent bonding and thus occupied the functional group of chitosan matrix, subsequently lower the free hydrogen group which can form hydrophilic bonding with water. As a

result, improved mechanical and water barrier properties and enhanced antioxidant activity were observed in the chitosan films incorporated with GT.

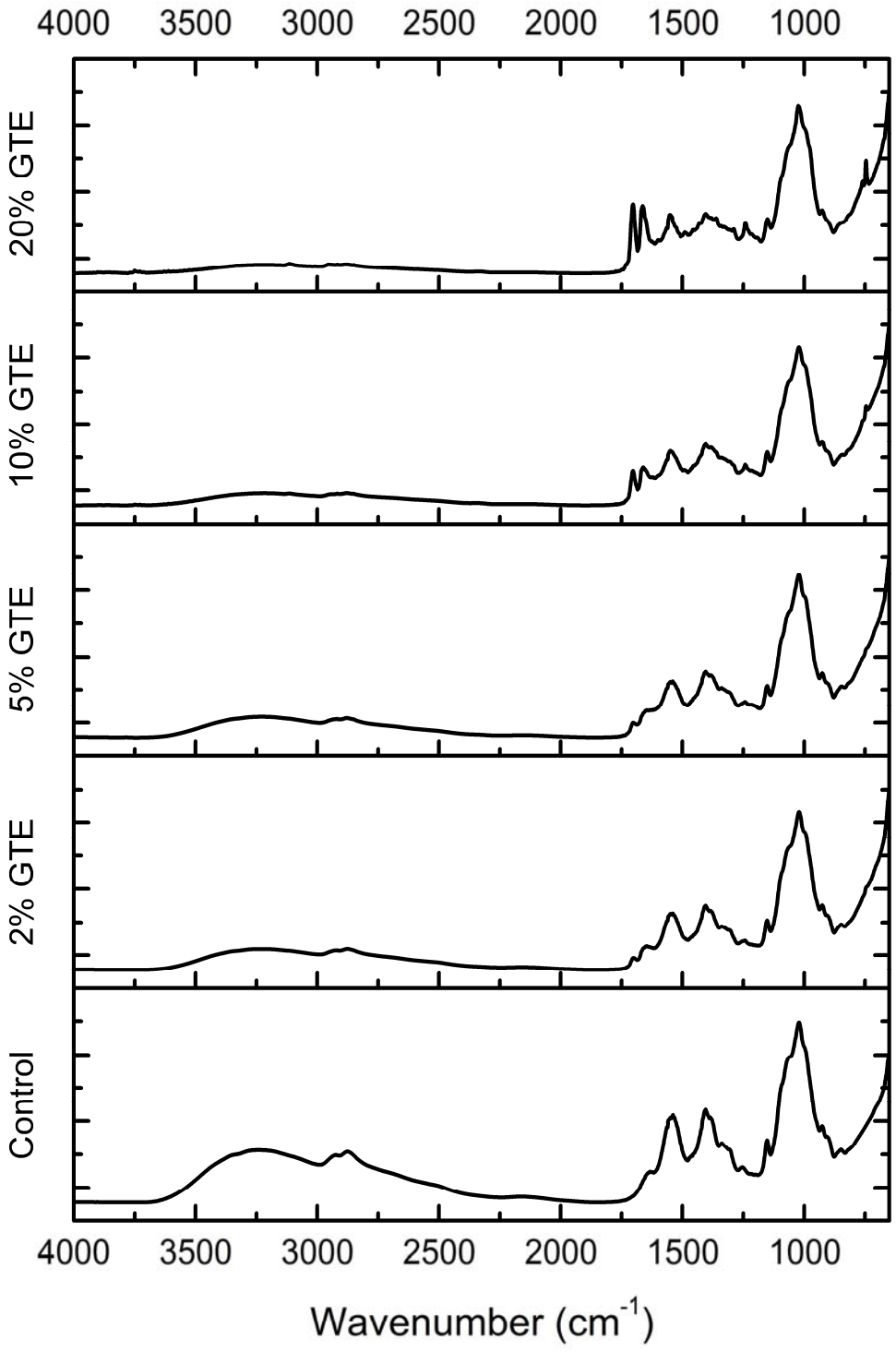


Figure 5.8 FTIR spectrum of chitosan films incorporated with GT.

5.2.6 Antimicrobial property of chitosan film containing GT

The antimicrobial property of chitosan film incorporated with GT was tested against pathogenic and/or spoilage microorganisms including Gram's positive (*Staphylococcus aureus*) and Gram's negative (*Escherichia coli* *Pseudomanas fluorescens* and *Salmonella* Enteritidis) bacteria. The indicator bacteria used in this experiment are common meat product contaminants. These bacteria are commonly known to cause food poisoning or food spoilage.

E. coli live in the intestines of humans and animals. Most varieties of *E. coli* are harmless or cause relatively brief diarrhea. However, a few particularly strains, such as *E. coli* O157:H7, can cause severe, bloody diarrhea and abdominal cramps. *E. coli* can be found in contaminated water or food, especially raw vegetables and undercooked meat products. Salmonella can cause diseases in humans and animals, and can be transmitted through the ingestion of contaminated food or water. Salmonella is a food safety concern with products from all animal sources. *S. aureus* can produce toxin which causes food poisoning. *S. aureus* food poisoning is often caused when a food handler contaminates food products that are served or stored at room- or refrigerator temperature. *P. fluorescens* is a food spoilage causing bacterium and can grow in a variety of foods. This spoilage is mostly due to the capability of producing heat-stable extracellular lipases, proteases and lecithinases that survive the thermal processing steps. There are several reports of spoilage of refrigerated foods with *P. fluorescens*, in particular of refrigerated meat products. The contamination of the meats results in changes in appearance and odor during prolonged storage (Delaquis and McCurday, 1990; Marshall et al., 1991).

The inhibitory activity was measured based on clear zone surrounding circular film discs. Measurement of clear zone diameter included diameter of film discs. Therefore, the values were always higher than the diameter of film discs (6 mm diameter) whenever clearing zone was present. Inhibition zone was then calculated by subtracting overall clear zone by diameter of the film disc. If there was no clear zone surrounding the film disc, it was marked as no inhibition zone, and the diameter was valued as zero. Moreover, contact area was used to evaluate growth inhibition underneath the film disc in direct contact with target microorganisms on the agar plate. The results of antimicrobial activity of chitosan films incorporated with GT against *S. aureus*, *E. coli*, *P. fluorescens* and *S.* Enteritidis are shown in Table 5.4.

From the results, chitosan films without incorporation of GT (control films) showed

The antimicrobial effect of chitosan occurred without migration of active agents

Chitosan film incorporated with GT showed antimicrobial activity, in terms of inhibition zone and limited growth underneath film discs, against all indicator bacteria. When GT was incorporated, there was diffusion of polyphenolic compounds through agar gel, resulting in a clearing zone on the bacterial growth. Therefore, Incorporation of GT improved antimicrobial efficacy of the chitosan film, as diffused antimicrobial agent would add to non-migrated antimicrobial efficiency of chitosan.

It is evident that film containing GT had better antimicrobial property than those of control film. This is to be expected because GT contains green tea polyphenols, which are capable of bactericidal activity, by inhibition of DNA and RNA synthesis of bacterial cells (*E. coli, S. aureus, S.*Typhimurium) (Mori et al., 1987), inhibition of cytoplasmic membrane function of bacteria, resulting in leakage of intramembranous materials (Tsuchiya et al., 1994), and interfering with energy metabolisms of bacteria (Haraguchi et al., 1998). These results are in good agreement with those of Wu et al. (2007) who found that epicatechin and caffeine in green tea could inhibit *S. aureus, Bacillus subtilis* and *E. coli*, and Hamilton-Miller (1995) who reported that polyphenolic compounds in green tea could inhibit *E. coli*, *Salmonella* Typhimurium*, Listeria monocytogenes*, *S. aureus* and *Campylobacter jejuni*.

Moreover, the results suggested that chitosan films incorporated with GT were more effective against Gram-positive bacteria (*S. aureus*) than the Gram-negative bacteria (*E. coli*, *P. fluorescens* and *S.* Enteritidis). This could be explained by the structural differences of the bacterial cell wall of Gram-positive and Gram-negative bacteria. Gram-negative bacteria, apart from the cell membrane, possess an additional outer layer membrane, which consists of phospholipids, proteins and lipopolysaccharides, and this membrane is impermeable to most molecules (Georgantelis et al., 2007).

The results also indicated that increasing GT level higher than 5 % did not significantly improve antimicrobial effect against all tested bacteria. The lack of increment on antimicrobial effect by increasing GT concentration was probably due to chemical interaction between amino group of chitosan and carboxyl group of GT (Chen, Yeh and Chiang, 1996), resulting in hindering the release of polyphenolic compounds from GT to inhibit microorganism surrounding film discs during agar diffusion assay. An interaction between functional groups of GT and chitosan was previously discussed in the FTIR analysis section.

It should be noted that a potent antimicrobial GT polyphenols may have a low rate of diffusion. From various published works, it has been suggested that besides the diffusion of the antimicrobial property of the GT polyphenols, there are many other variable factors. These include inoculum size, volume of agar, type of agar, size of paper discs, strain of certain bacterial species used, and incubation period.

Table 5.4 Antimicrobial activity of chitosan films containing GT against *S. Aureus, E. coli*, *P. fluorescens* and *S*. Enteritidis

Film samples	Inhibition zone*			
	S. aureus	*E.coli*	*P.fluorescens*	*S.*.Enteritidis
Control	0.00 (+)**	0.00 (+)	0.00 (+)	0.00 (+)
2% GT	1.00 (+)	0.67 (+)	0.00 (+)	0.00 (+)
5% GT	1.00 (+)	1.00 (+)	1.00 (+)	1.00 (+)
10% GT	1.00 (+)	1.00 (+)	1.00 (+)	1.00 (+)
20% GT	1.00 (+)	1.00 (+)	1.00 (+)	1.00(+)

*Inhibitory zone is clear zone surrounding film discs, measured diameter in mm, and

5.2.7 Conclusion

This study indicated that an active film from chitosan-based film could be achieved by incorporation with aqueous extract of green tea, as a natural antioxidant and antimicrobial agent. Incorporation of GT improved mechanical, water vapor barrier, antioxidant, and antimicrobial properties of the resulting films. These properties, as verified by FTIR analysis, could be attributed to the interactions between functional groups of chitosan and GT polyphenolic compounds. The chitosan films without GT showed some scavenging activity on DPPH. In the films containing GT, the antioxidant activity increased in more than 15 folds in relation to the control samples. As the GT increased in the film formulation, so did the expected antioxidant character of the active film. From antimicrobial testing against *S. aureus*, *E. coli*, *P. fluorescens* and *S.* Enteritidis using disc diffusion method, it was revealed that incorporation of GT into chitosan improved antimicrobial efficacy of the chitosan film. The chitosan film incorporated with GT shows potential to be used as an active packaging, in forms of antimicrobial wrapping film, coating or inner layer of multilayer packaging film for food products to inhibit microbial growth on the contact surface.

5.3 Shelf life extension of pork sausage using chitosan film containing GT

5.3.1 Color

Color values including L* coordinate (brightness indicator), a* value (indicator of the tendency towards the red for a* positive or green for a* negative) and b* value (indicator of the tendency towards yellow for b* positive or blue for b* negative) of control pork sausage samples and those wrapped with C-film and CGT-film during storage at $4^{\circ}C$ are shown in Figure 5.9 a, b, and c, respectively. All samples showed significant changes in the values of the L*, a* and b* values during storage.

L*, a* and b*values were affected ($p \leq 0.05$) by packaging conditions (control, C-film and CGT-film). Control sample showed significantly higher values of L* and b*values during storage than those wrapped with C-film and CGT-film, respectively. A significant increase in L* and b*values of control samples indicated discoloration. Changes found in color values of control samples were probably due to lipid oxidation and microbial spoilage. An accumulation of hydrogen peroxide produced by lactic acid bacteria during storage (Cayré, Garro, and Vignolo, 2005) may possibly be one of the main factors responsible for discoloration in control samples. The produced peroxide can react with nitric oxide hemochromogen or nitric oxide myoglobin and give oxidized porphyrin resulting in greening of the meat products (Jjaberg et al., 1970).

Inhibiting lipid oxidation and microbial growth would be beneficial in delaying product deterioration. Samples wrapped with C-film and CGT-film showed lower changes in color values probably due to the antioxidant and antimicrobial properties of the chitosan film. Chitosan has been reported to be able to inhibit the growth of lactic acid bacteria (Coma et al., 2002) which are predominant in cooked and cured meat (Samelis, Kakouri, and Rememtzis, 2000). The results also showed that samples wrapped with CGT-film has significantly lower changes ($p \leq 0.05$) in L* and b* values than those wrapped with C-film, probably due to the antioxidant and antimicrobial effect of polyphenolic compounds from green tea. The results suggested incorporation of GT into chitosan film could enhance the antioxidative and antimicrobial effects of the film and thus prolong the product shelf life while maintaining the qualities of the samples. The results are in good relation to the antioxidant property and antimicrobial assay of the film. As indicated previously, incorporation of GT into chitosan film increased the total phenolic compounds and DPPH scavenging activity of the film from 0.93 $\pm$ 0.07 to 33.03 $\pm$1.37 mg gallic acid/ g sample and from 1.94 $\pm$ 0.07 to 50.74 $\pm$ 2.2 %, respectively.

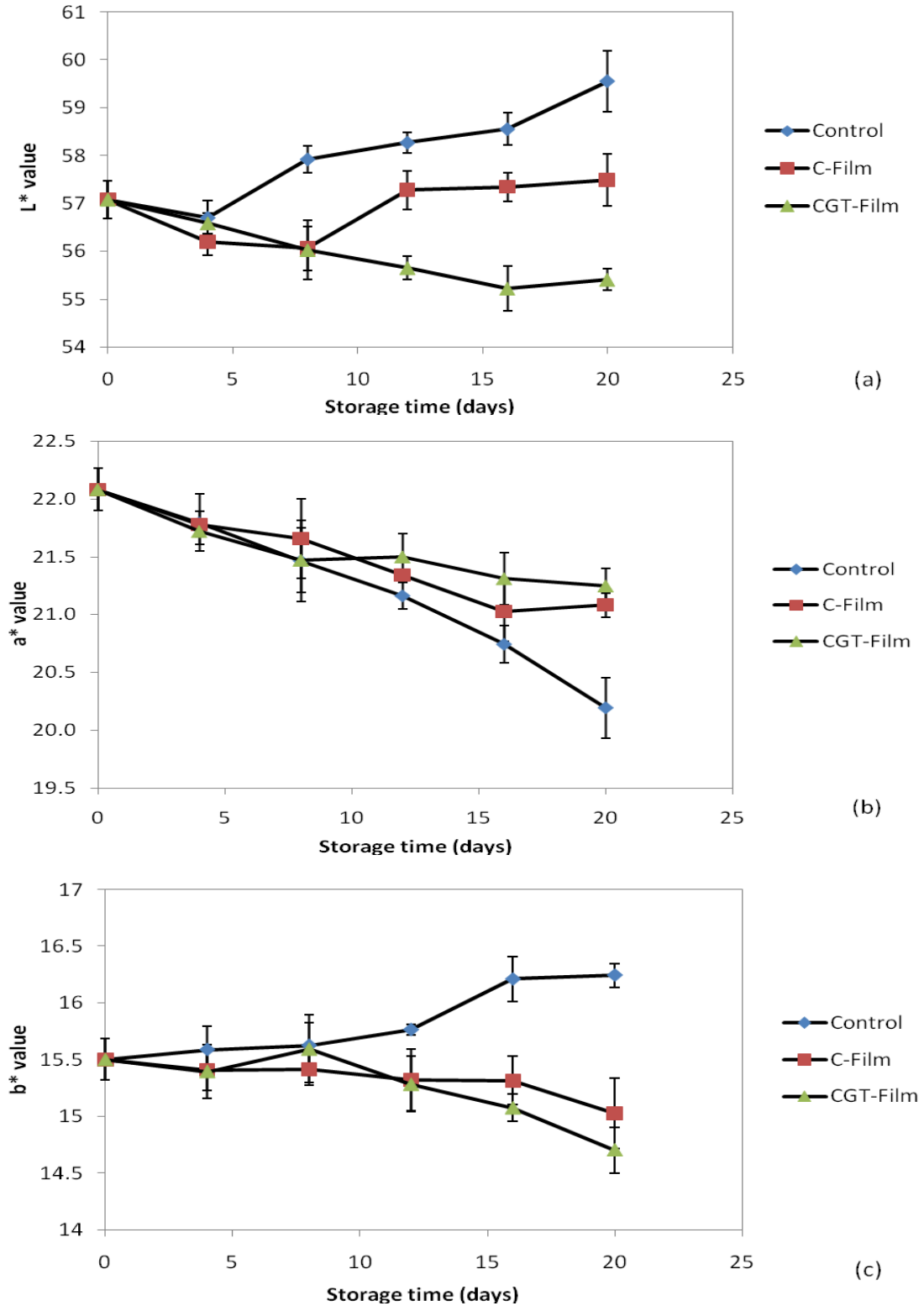


Figure 5.9 Color values of control pork sausage samples and those wrapped with C-film and CGT-film during storage at 4°C. Values are given as mean ± standard deviation. Different letters indicate significantly different ($p \leq 0.05$) when analyzed using Duncan's New Multiple Range Test.

5.3.2 Texture

Texture of control samples and those wrapped with C-film and CGT-film during storage at 4°C is shown in Figure 5.10. Cutting force of control samples was significantly lower than other samples throughout the storage. This is probably because the growth of spoilage microorganism in the sausage samples (Quintavalla and Vicini, 2002). Samples wrapped with C-film and CGT-film showed lower ($p \leq 0.05$) changes in cutting force than control samples due to antimicrobial property of chitosan to inhibit the growth of gram-positive and gram-negative bacteria, yeasts and mold (Shahidi, Arachchi, and Jeon, 1999). In addition, chitosan was reported to be able to inhibit the growth of lactic acid bacteria (Chen, Yeh, and Chiang, 1996; Coma et al., 2002) which are predominant in cooked and cured meat (Samelis, Kakouri, and Rememtzis, 2000). These results suggested the microbial growth in sausage samples could be minimized by using chitosan film. No significant ($p > 0.05$) differences between samples wrapped with C-film and CGT-film were observed throughout the storage period.

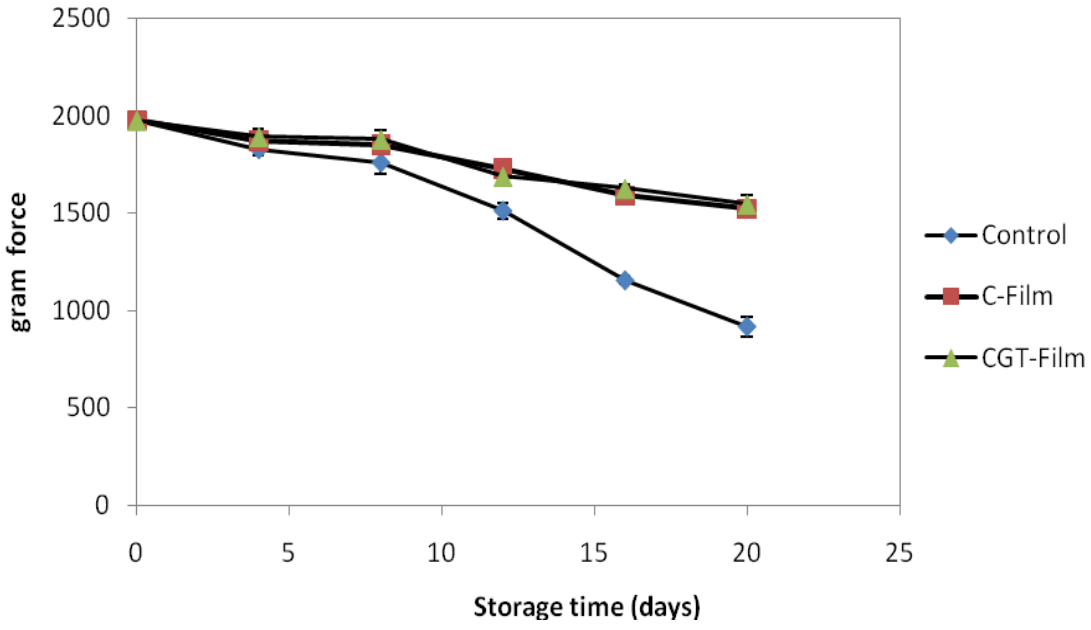


Figure 5.10 Texture (cutting force) of control pork sausage samples and those wrapped with C-film and CGT-film. Values are given as mean ± standard deviation. Different letters indicate significantly different ($p \leq 0.05$) when analyzed using Duncan's New Multiple Range Test.

5.3.3 pH

The results showed that pH of the samples from all packaging conditions decreased as the storage time increased (Table 5.5). The sausages without chitosan wrapping (control samples) showed the largest ($p \leq 0.05$) pH drop throughout the storage period, probably caused by the growth of lactic acid bacteria as well as other spoilage microflora which were predominant on cooked and cured meat products (Skandamis and Nychas, 2002). Cayré, Garro, and Vignolo (2005) indicated that *Pseudomonas,* lactic acid bacteria, and *Enterobacteriaceae* are spoilage microflora in refrigerated meat products. Lactic acid bacteria appeared to be the main causative agent of spoilage, which contribute to a decrease in pH of the sausages Ambrosiadis et al. (2004).

The similar results were obtained by Blixt and Borch (2002) who found a decrease in pH with an increase in the spoilage of packaged pork and beef. Samelis et al. (2000) also suggested a very good correlation between the growth of the spoilage microflora and the decrease in pH values of various cooked meat products stored at 4 ºC.

Samples wrapped with C-film and CGT-film showed lower ($p \leq 0.05$) pH changes during the storage than control samples, probably due to antimicrobial property of chitosan to inhibit the growth of gram-positive and gram-negative bacteria, yeasts and mold (Shahidi et al., 1999) In addition, chitosan was reported to be able to inhibit the growth of lactic acid bacteria (Chen, Yeh, and Chiang, 1996; Coma et al., 2002) which are predominant in cooked and cured meat (Samelis et al., 2000) and cause a pH drop in refrigerated meat products. These results suggested the microbial growth could be minimized by chitosan film.

Special mention should be made to the pH of samples wrapped with CGT-film, which decreased rapidly during the first four days of storage. This could be due to the acidic pH (~6.0-6.3) of green tea (Yoshida et al., 1999) incorporated into the film. However, pH of CGT-film wrapped samples did not significantly changed ($p > 0.05$) throughout the storage from 8-20 days. These results suggested that no significant growth of specific microflora that can cause a pH drop in the CGT-film wrapped samples.

Table 5.5 pH of control pork sausage samples and those wrapped with C-film and CGT-film during storage at 4°C.

Storage time (days)	Treatment	pH
0	Control	6.56^{a} ± 0.02
	C-Film	6.56^{a} ± 0.02
	CGT-Film	6.56^{a} ± 0.02
4	Control	6.54^{ab} ± 0.02
	C-Film	6.53^{ab} ± 0.02
	CGT-Film	6.47^{c} ± 0.01
8	Control	6.50^{bc} ± 0.01
	C-Film	6.48^{c} ± 0.00
	CGT-Film	6.40^{def} ± 0.01
12	Control	6.50^{bc} ± 0.01
	C-Film	6.48^{c} ± 0.01
	CGT-Film	6.39^{ef} ± 0.00
16	Control	6.41^{de} ± 0.01
	C-Film	6.50^{bc} ± 0.01
	CGT-Film	6.41^{de} ± 0.00
20	Control	6.36^{f} ± 0.00
	C-Film	6.44^{d} ± 0.01
	CGT-Film	6.39^{ef} ± 0.00

Values are given as mean ± standard deviation. Different superscript letters in the same column indicate significantly different ($p \leq 0.05$) when analyzed using Duncan's New Multiple Range Test.

5.3.4 Lipid oxidation

Lipid oxidation, with respect to TBA values of control sausage samples and those wrapped with C-film and CGT-film is shown in Figure 5.11. TBA values were affected ($p \leq 0.05$) by packaging conditions (control, C-film and CGT-film). During the storage, significantly higher ($p \leq 0.05$) TBA was evident in control samples than those wrapped with chitosan-alone film and chitosan incorporated with GT, respectively. This result suggested that lipid oxidation in sausage samples could also be minimized by the use of chitosan film probably due to antioxidant activity of chitosan (Park, Je and Kim, 2004) as well as its low oxygen permeability characteristic (Xu et al., 2005).

The antioxidant ability of C-film is thought to be due to chelation of free ion which is released from hemoproteins of meat during storage (Shahidi et al., 1999). As previously reported on the scavenging activity on DPPH of chitosan film in section 5.2.4, the scavenging mechanism of chitosan is related to the fact that free radical can react with the residual free amino (NH_2) groups to form stable macromolecule radicals, and the NH_2 groups can form ammonium (NH_3^+) groups by absorbing a hydrogen ion from the solution (Park, Je and Kim, 2004; Yen et al., 2008).

The effectiveness of chitosan on the oxidation reaction of meat product has also been reported by Soultos et al. (2008) and Shahidi et al. (1999). Incorporation of GT into chitosan film enhanced the antioxidant property of the film, as TBA values of CGT-film wrapped samples were lower than those wrapped with C-film. The results are in good agreement with the result of the antioxidant activity of chitosan film incorporated with GT. In the films containing 20% GT, the antioxidant activity increased in more than 15 folds in relation to the control samples. Tea extracts are powerful antioxidants, mainly owing to the presence of catechin, epicatechin, epigallocatechin, epigallocatechin gallate and epicatechin gallate (Salah et al., 1995). These compounds are effective free radical-scavengers and also effective by metal chelation (Salah et al., 1995; Shahidi et al., 1992). The reducing properties are generally associated with the presence of reductones, which have been shown to exert antioxidant action by breaking the free radical chain by donating a hydrogen atom. Reductones are also reported to react directly with peroxides and also with certain precursors of peroxides, thus preventing peroxide formation (Farhoosh et al., 2007).

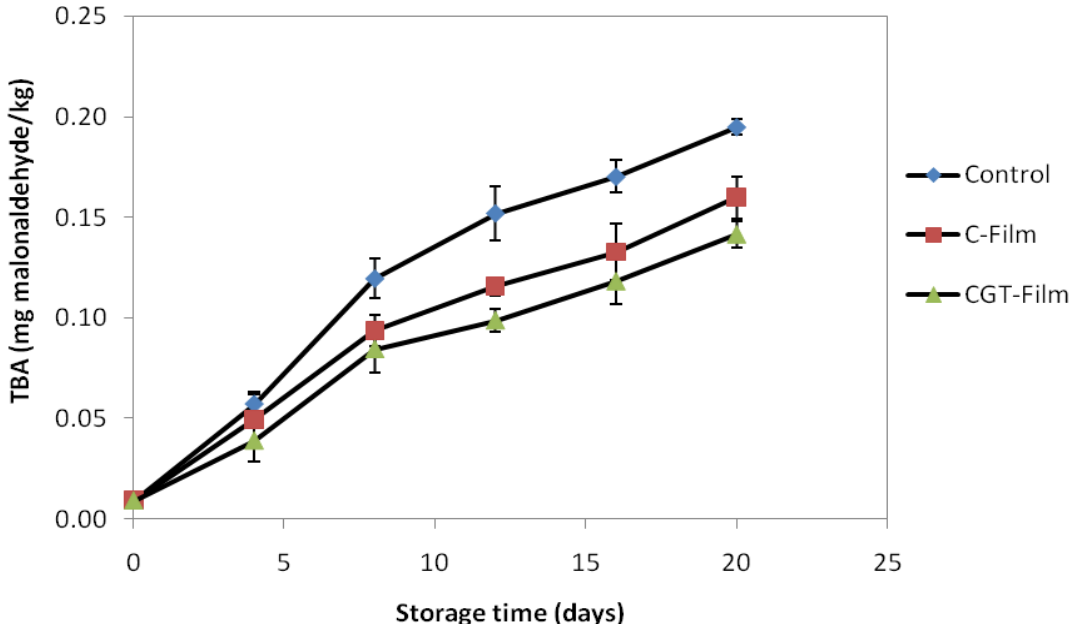


Figure 5.11 TBA values of control pork sausage samples and those wrapped with C-film and CGT-film during storage at 4°C. Values are given as mean ± standard deviation. Different letters indicate significantly different ($p \leq 0.05$) when analyzed using Duncan's New Multiple Range Test.

5.3.5 Microbiological analysis

Table 5.6 is shown total count, yeast/moulds, and lactic acid bacteria (CFU/g) of pork sausages during storage at 4°C. Microbial growth was affected by packaging condition (control, C-film, and CGT-film). Control sausage samples were found to have higher population of total aerobic count, yeast/moulds and lactic acid bacteria count. The mean value was 1.76×10^5, 3.78×10^3, and 3.35×10^2 CFU/g, respectively after 12 days of storage. Such a high population of these microorganisms in control samples affected the organoleptic properties. These microorganisms are considered as spoilage microflora and their presence in such high amounts is considered undesirable. For meat products, the growth of *Pseudomonas,* lactic acid bacteria, and *Enterobacteriaceae* are the major component of the spoilage microflora (Cayré, Garro, and Vignolo, 2005). Lactic acid bacteria were identified as the major spoilage population of sausages and other processed meats stored at refrigeration temperatures (Samelis et al., 2000). According to Ambrosiadis et al. (2004), lactic acid bacteria appeared to be the main causative agent of spoilage, which contribute to a decrease in pH of the sausages. Under anaerobic conditions Lactic acid bacteria may cause souring, slimy and/or greening, lactic acid,

ethanol and only small amounts of short-chain fatty acids causing off-odors. The population of yeasts and molds were also rather high, which may cause the slime formation on the sausage surface.

The microbial growth was more rapid in the control samples where the total viable count of 1.76×10^5 CFU/g was found after 12 days. At this point, the sample could be identified by off-odor and discoloration and would have been rejected by the consumers. According to the Thai Industrial Standards Institute (TISI) Standard No. 330 2547 for Hotdog sausages-pork sausage (Thai Industrial Standards Institute, Ministry of Industry, Thailand), the quality of pork sausage is unacceptable when the microbial count exceed 10^5 CFU/g. On the other hand, there was very low variability of total aerobic count, yeasts/moulds and lactic acid bacteria in the sausages wrapped with C-film and CGT-film. Low microorganisms in samples wrapped with C-film and CGT-film confirm the assumption that chitosan film could inhibit microbial growth and that incorporation of GT into chitosan film enhanced the antimicrobial property of the film. These results are in good agreement with the results of antimicrobial assay of chitosan-alone film and film incorporated with GT, which indicated that chitosan film incorporated with GT could inhibit *S. aureus, E. coli, P. fluorescens* and *S.* Enteritidis better than chitosan-alone film.

The antimicrobial effect of chitosan is thought to be related to electrostatic interaction between a positive charge on the NH_3^+ group of glucosamine monomer in chitosan molecules and negative charge of microbial cell membranes that lead to the leakage of intracellular constituents. Moreover, Xu et al. (2005) stated that chitosan film has low oxygen transmission rate and thus can create a significant anaerobic environment that prevents the growth of aerobic spoilage organisms, which generally are gram negative bacteria such as Pseudomonads or aerobic yeast and molds.

Incorporation of chitosan film with GT enhanced the antimicrobial property of the film, as total aerobic count, yeasts/moulds and lactic acid bacteria in the sausages wrapped with CGT-film were lower than those wrapped with C-film. The antimicrobial activity of green tea polyphenols is attributed to green tea polyphenols are capable of bactericidal activity, by inhibition of DNA and RNA synthesis of bacterial cells (*E. coli, S. aureus, S.*Typhimurium) (Mori et al.,1987), inhibition of cytoplasmic membrane function of bacteria, resulting in leakage of intramembranous materials (Tsuchiya et alk., 1994), and interfering with energy metabolisms of bacteria (Haraguchi et al., 1998). The results are also supported by those of Wu et al. (2007) who found that EC and caffeine in green tea

could inhibit *S. aureus, Bacillus subtilis* and *E. coli*, as well as Hamilton-Miller (1995) who reported that phenolic compounds in green tea could inhibit *E. coli*, *S.* Typhimurium*, L. monocytogenes*, *S. aureus* and *Campylobacter jejuni.* Lee et al. (2003) also found that green tea catechins are responsible for antibacterial properties against bacteria such as methicillin-, and ciprofloxacin-resistant Staphylococci, Enterococci, ciprofloxacin-resistant *Pseudomonas auruginosa* and *S. aureus*.

Table 5.6 Total count, yeast/moulds, and lactic acid bacteria (CFU/g) of pork sausages during storage at 4^{O}C

Storage time (days)	Samples	Total count	Yeast/moulds	Lactic acid bacteria
0	Control	<20	ND	ND
	C-Film	<20	ND	ND
	CGT-Film	<20	ND	ND
4	Control	3.57×10^{2}	ND	<20
	C-Film	<20	ND	ND
	CGT-Film	<20	ND	ND
8	Control	4.18×10^{2}	<20	<20
	C-Film	<20	<20	<20
	CGT-Film	<20	<20	ND
12	Control	1.76×10^{5}	3.78×10^{3}	3.35×10^{2}
	C-Film	<20	<20	<20
	CGT-Film	<20	<20	<20
16	Control	3.53×10^{5}	2.19×10^{4}	1.02×10^{3}
	C-Film	<20	6.93×10^{2}	<20
	CGT-Film	<20	<20	<20
20	Control	$>10^{9}$ *	$>10^{9}$ *	$>10^{9}$ *
	C-Film	4.45×10^{3}	1.11×10^{5}	<20
	CGT-Film	3.28×10^{2}	1.47×10^{4}	<20

ND= not detected

5.3.6 Sensory evaluation

Means of sensory attribute scores including off-odor, discoloration, slime and overall acceptance of control pork sausages samples and those wrapped with C-film and CGT-film, and stored at 4 $\pm$ 1 ºC are shown Figure 5.12-5.15, respectively. The sensory evaluation results appeared to be correlated to color index, texture, TBA values, and microbiological analyses. Due to high lipid oxidation and microbial growth, control samples showed spoilage as off-odor, slime formation and discoloration faster than those wrapped with C-film and CGT-film, respectively.

The odor quality was expressed in terms of off-odor scores, as shown in Figure 5.12, where the higher the scores, the lower the odor quality. The sensory evaluation results showed that off-odor scores increased with increasing storage period. Off-odor occurred in control samples faster than those wrapped with C-film and CGT-film, respectively. Off-odor was observed only on the control samples after 12 days of storage, which may be caused by the number of microorganism and the lipid oxidation during the storage since this scores were in relation to the number of microorganism and the TBA values. According to the microbiological results, the quality of pork sausage was unacceptable when the number of total aerobic bacteria was as high as 1.76×10^5 CFU/g which also exceeded the TISI Standard for Hotdog sausages-pork sausage (Thai Industrial Standards Institute, Ministry of Industry, Thailand). Samples wrapped with C-film and CGT-film had slightly off-odor after storage for 16 and 20 days, respectively, when the number of yeast were 1.11×10^5 and 1.47×10^4 CFU/g, and total aerobic counts were 4.45×10^3 and 3.28×10^2 CFU/g, respectively. These results suggested that using chitosan film could inhibit growth of microorganism and the antimicrobial property could be enhanced when the film was incorporated with GT.

For the appearance quality, discoloration scores are shown in Figure 5.13. The discoloration scores increased with increasing storage period. Control samples underwent discoloration after stored for 12 days, and the discoloration scores were significantly higher ($p \leq 0.05$) than those wrapped with C-film and CGT-film, respectively, throughout the storage. For samples wrapped with C-film and CGT-film, the discoloration scores were acceptable throughout the storage. The results were in good relation to the instrumental color measurement (Figure 5.9) which indicated that control sample had color changes higher than those wrapped with C-film and CGT-film. The color of control samples became grayish (an increase in lightness and a decrease in redness) as the

microbial growth and TBA values increased with an increase in storage period. The changes in color quality was caused by microbial growth and lipid oxidation during the storage since this scores were in good relation to number of microorganism and TBA values.

Color of samples wrapped with C-film and CGT-film slightly changed during the storage. Moreover, incorporation of GT into chitosan film improved the film properties as discoloration of samples wrapped with CGT-film was lower than those wrapped with C-film throughout the storage. Slime formation scores are shown in Figure 5.14. Slimy surface may be caused by yeasts and molds (Ambrosiadis et al., 2004). Slime formation of control samples was observed after storage for 16 days, where the number of total aerobic count, yeasts/moulds and lactic acid bacteria were as high as $1.76x10^5$, $3.78x10^3$ and $3.35x10^2$ CFU/g, respectively, while slime on samples wrapped with C-film was observed after 20 days, when the number of total aerobic, yeasts/moulds and lactic acid bacteria were $4.45x10^3$, $1.11x10^5$ and <20 CFU/g, respectively. Slime formation was not observed in CGT-film wrapped samples because of low number of total aerobic, yeasts/moulds and lactic acid bacteria.

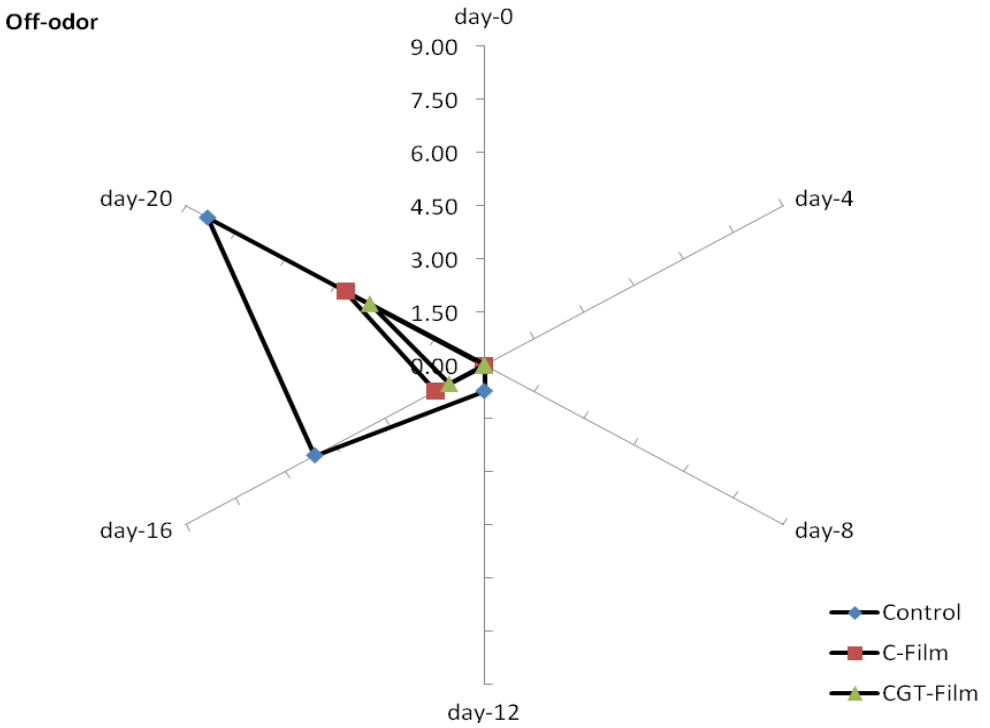


Figure 5.12 Off-odor scores of of control pork sausage samples and those wrapped with C-film and CGT-film during storage at $4^{\circ}C$

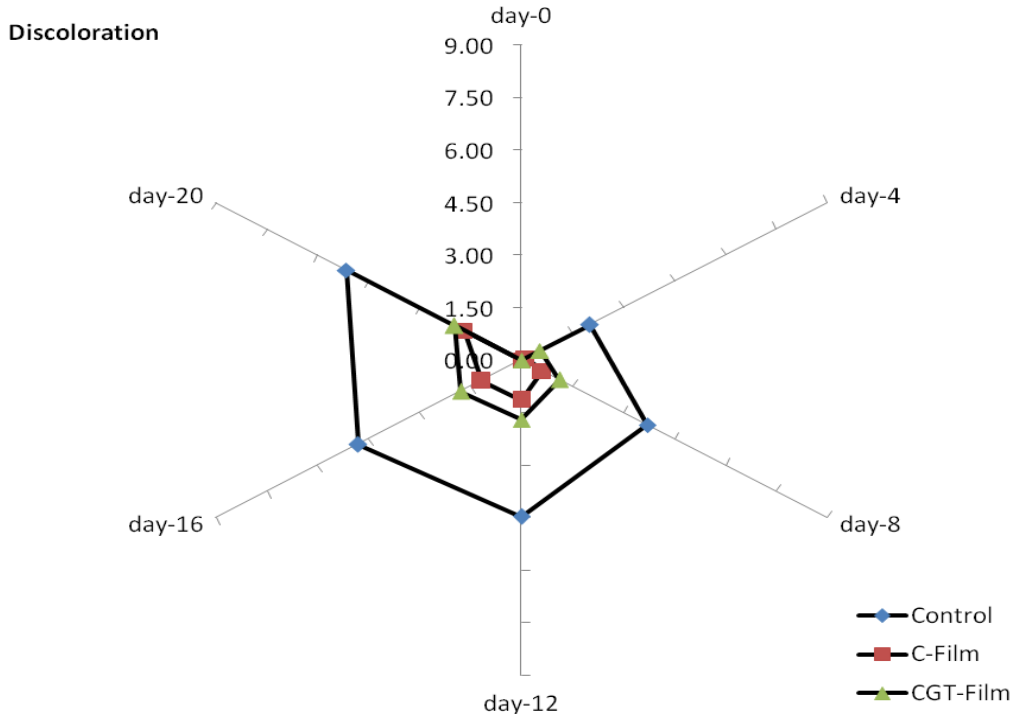


Figure 5.13 Discoloration scores of of control pork sausage samples and those wrapped with C-film and CGT-film during storage at 4^{O}C

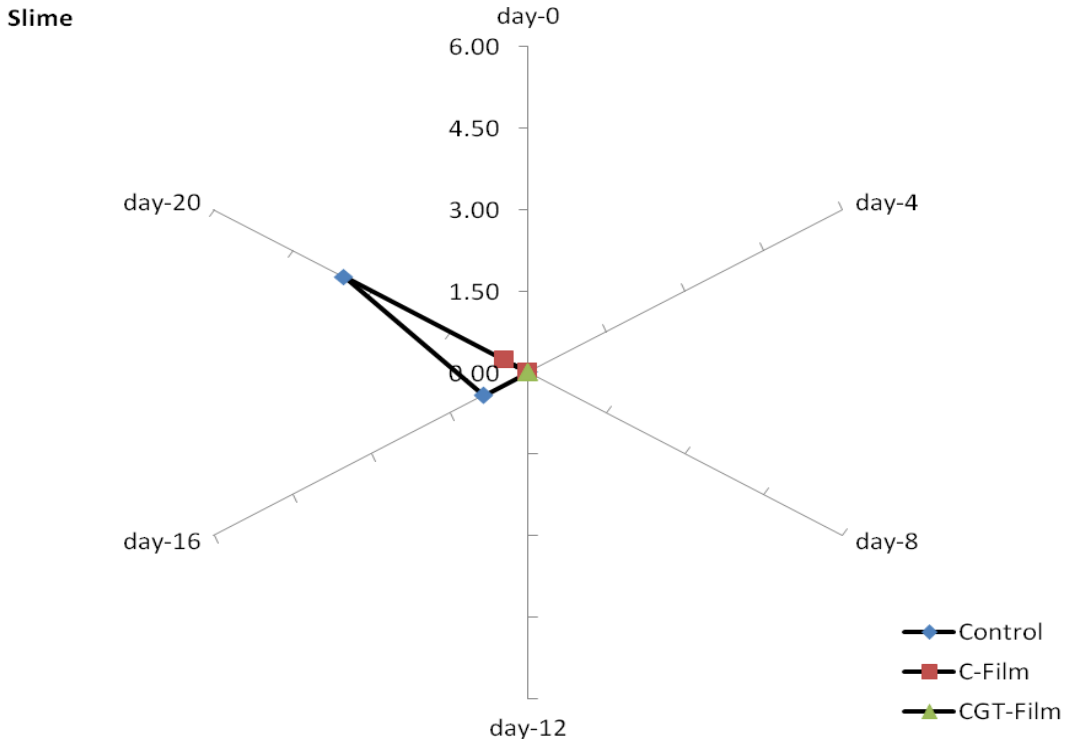


Figure 5.14 Slime formation scores of of control pork sausage samples and those wrapped with C-film and CGT-film during storage at 4^{O}C

The overall acceptance (Figure 5.15) of control samples was significantly lower than those wrapped with C-film and CGT-film throughout the storage. This was because the control samples had higher ($p \leq 0.05$) off-odor, discoloration and slime formation than those wrapped with C-film and CGT-film during storage, which affected the overall acceptability of the products. Based on sensory evaluation, control samples without chitosan wrapping and those wrapped with C-film reached unacceptable sensory scores after 12 and 20 days, respectively, while the samples wrapped with CGT-film could maintain acceptable sensory qualities throughout the storage period for 20 days.

Better sensory qualities of C-film and CGT-film wrapped samples confirm the assumption that chitosan film could inhibit microbial growth and that incorporation of GT into chitosan film enhanced the antimicrobial property of the film. Successful inhibition of lipid oxidation and microbial growth in the refrigerated pork sausage was possible with chitosan film and chitosan film incorporated with GT since they kept the sensory characteristics within an acceptable condition throughout the storage.

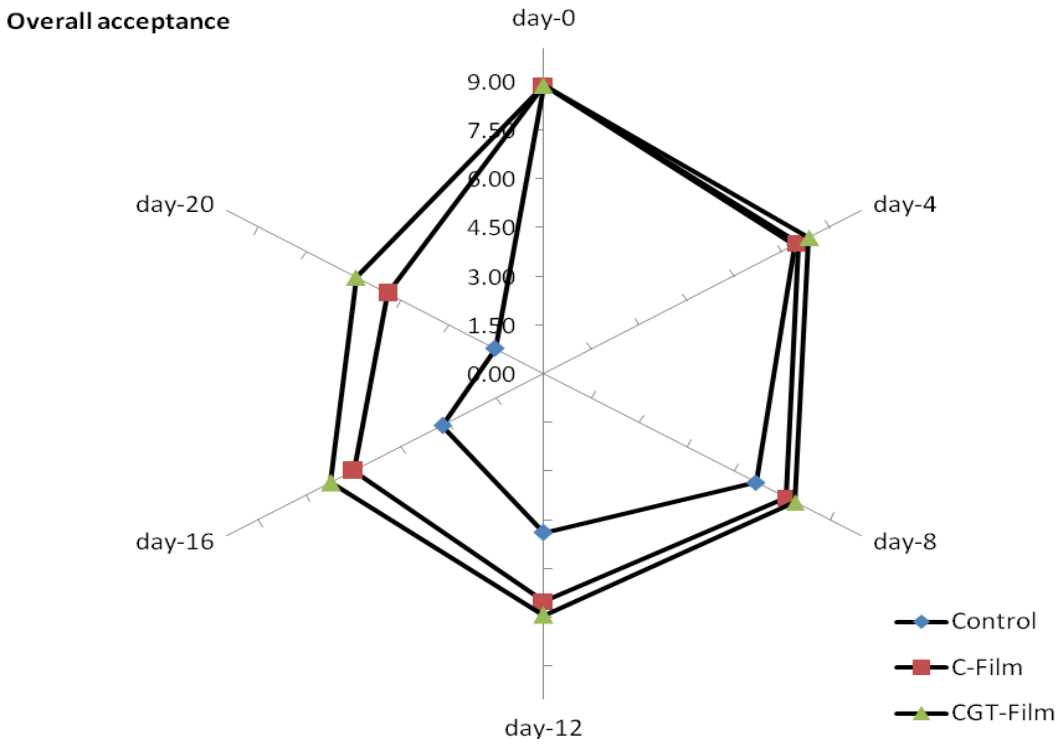


Figure 5.15 Overall acceptance scores of of control pork sausage samples and those wrapped with C-film and CGT-film during storage at 4°C

Based on the sensory evaluation and microbiological quality with regard to the TISI's Standard for Hotdog sausages-pork sausage (Thai Industrial Standards Institute, Ministry of Industry, Thailand), control pork sausage samples and those wrapped with chitosan-alone film had shelf life of less than 12 days and less than 20 days, respectively. Chitosan incorporated with GT could extend shelf life of the samples for up to 20 days at 4 ºC, without any significant loss of odor, color, and overall acceptability or significant microbial growth. Chitosan film incorporated with GT provided a type of active packaging to maintain quality and extend shelf life of the refrigerated pork sausage. GT could enhance the antioxidant and antimicrobial properties of the chitosan film.

5.3.7 Conclusion

Successful inhibition of lipid oxidation and microbial growth in the refrigerated pork sausage was possible with chitosan film and chitosan film incorporated with GT since they kept the sensory characteristics within an acceptable condition throughout the storage period. Chitosan incorporated with GT could extend shelf life of the samples for up to 20 days at 4 ºC, without any significant loss of odor, color, and overall acceptability or significant microbial growth, while control samples and those wrapped with chitosan-alone film had shelf life of less than 12 days and less than 20 days, respectively. Chitosan film incorporated with GT provided a type of active packaging to maintain quality and extend shelf life of the refrigerated pork sausage. GT could enhance the antioxidant and antimicrobial properties of the chitosan film.

6. CONCLUSION

The optimum chitosan-based films could be prepared from 2% chitosan in 1% acetic acid. However, the obtained chitosan film was brittle and had low flexibility. The mechanical properties of chitosan film was then modified by adding glycerol, as a plasticizer, at different concentrations including 0, 10, 20, 30, 40, and 50 % w/w of chitosan. The results showed that an increase in the amount of the plasticizer caused an increase in elongation at break (extensibility), a decrease in tensile strength and an increase in water vapor transmission rate of the film. The elongation of the film increased with increasing plasticizer concentration, but at high concentration there were decreases in both tensile strength and elongation. The changes in mechanical properties of the film could be attributed to an alteration of intermolecular and intramolecular interaction occurring in the films. The results suggested that 30% glycerol was the optimum concentration to improve flexibility, while maintaining tensile strength and other physical properties of the film.

The effects of incorporation of green tea extract at different concentrations including 0, 2, 5, 10, and 20% w/v of green tea in the chitosan film-forming solution were determined. Incorporation of GT improved mechanical properties, water vapor barrier property, total phenolic compounds, DPPH scavenging activity, and antimicrobial properties of the resulting films. The DPPH scavenging activity of the films significantly increased with increasing GT concentration. As the GT increased in the film formulation, so did the expected antioxidant character of the active film. The lower WVP of the films incorporated with GT may be because, according to the FTIR analysis, the hydrogen and covalent interactions between chitosan network and polyphenolic compounds limit the availability of hydrogen groups to form hydrophilic bonding with water, subsequently lead to a decrease in the affinity of chitosan film toward water.

Chitosan films without incorporation of GT (control films) showed antimicrobial effect on contact surface underneath film against Gram's positive (*Staphylococcus aureus*) and Gram's negative (*Escherichia coli Pseudomanas fluorescens* and *Salmonella* Enteritidis) bacteria, while there was no inhibition zone on the contact surface against all tested bacteria because chitosan is in a solid form and is incapable to diffuse through the adjacent agar media, and thus only organisms in direct contact with the active sites of

chitosan is inhibited. Chitosan could inhibit growth of bacteria due to the electrostatic interaction between NH_3^+ groups of chitosan and phosphoryl groups of phospholipid components of cell membranes, which caused the damage of structure, function and permeability of bacterial cell membranes, leading to the death of microbial cells. Chitosan film incorporated with GT showed antimicrobial activity, in terms of inhibition zone and limited growth underneath film discs, against all tested bacteria. When GT was incorporated, there was diffusion of polyphenolic compounds through agar gel, resulting in a clearing zone on the bacterial growth. Therefore, Incorporation of GT improved antimicrobial efficacy of the chitosan film, as diffused antimicrobial agent would add to non-migrated antimicrobial efficiency of chitosan. The chitosan film incorporated with GT shows potential to be used as an active packaging, in forms of antimicrobial wrapping film, coating or inner layer of multilayer packaging film for food products to inhibit microbial growth on the contact surface.

The antimicrobial and antioxidant efficacy of the developed chitosan film incorporated with green tea extract for shelf life extension of pork sausages were investigated. The physical, chemical, microbiological and sensory qualities of pork sausages wrapped with CGT-film were compared with those wrapped with C-film and control samples without chitosan film wrapping. It was found that samples wrapped with CGT-film showed lower changes in color, pH, TBA, texture, microbial growth and sensory quality than those wrapped with C-film and control, respectively. The results suggested that incorporation of GT into chitosan film could enhance the antioxidative and antimicrobial effects of the film, and thus maintained the qualities and prolonged the shelf life of pork sausages.

The antimicrobial activity of green tea polyphenols is attributed to green tea polyphenols are capable of bactericidal activity, by inhibition of DNA and RNA synthesis of bacterial cells (*E. coli, S. aureus, S.*Typhimurium), inhibition of cytoplasmic membrane function of bacteria, resulting in leakage of intramembranous materials, and interfering with energy metabolisms of bacteria. Successful inhibition of lipid oxidation and microbial growth in the refrigerated pork sausage was possible with chitosan film and chitosan film incorporated with GT since they kept the sensory characteristics within an acceptable condition throughout the storage period. Based on the sensory quality and microbiological quality with regard to the TISI's Standard for Hotdog sausages-pork sausage (Thai Industrial Standards Institute, Ministry of Industry, Thailand), control pork

sausage samples and those wrapped with chitosan-alone film had shelf life of less than 12 days and less than 20 days, respectively. Chitosan incorporated with GT could extend shelf life of the samples for up to 20 days at 4 ºC, without any significant loss of odor, color, and overall acceptability or significant microbial growth.

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OUTPUT

1. ผลงานตีพิมพ์ในวารสารวิชาการนานาชาติ

   1.1 บทความ “Physical properties and antioxidant activity of an active film from chitosan incorporated with green tea extract. Food Hydrocolloids, Volume 24, Issue 8, November-December 2010, Pages 770-775 Ubonrat Siripatrawan, Bruce R. Harte” ตีพิมพ์ในวารสาร Food Hydrocolloid (Impact factor = 2.511) (Manuscript แสดงไว้ใน Appendix I)

   1.2 อยู่ระหว่างเตรียมบทความเรื่อง “Chitosan film containing green tea extract for shelf life extension of pork sausage” คาดว่าจะนำส่งวารสาร Food Hydrocolloid (Impact factor = 2.511)

2. การนำผลงานวิจัยไปใช้ประโยชน์

   2.1 นำข้อมูลที่ได้จากงานวิจัยไปใช้ในการเรียนการสอน ในรายวิชา 22314533 Food Packaging ภาควิชาเทคโนโลยีทางอาหาร คณะวิทยาศาสตร์ จุฬาลงกรณ์มหาวิทยาลัย

   2.2 นำผลการวิจัยไปศึกษาต่อยอดเป็นโครงงานวิจัยสำหรับนิสิตปริญญาโทและปริญญาเอก

3. การเสนอผลงานในที่ประชุมวิชาการ

   3.1 นำเสนอผลงานเรื่อง “Effect of chitosan concentration and type of acid solutions on the physical properties of chitosan-based film” ในการประชุมทางวิชาการ The 5th International Packaging Congress and Exhibition จัดโดย Chamber of Chemical Engineers (CCE)/the Union of Chambers of Turkish Engineers and Architects (UCTEA) Izmir, Turkey November 20-27, 2007

   3.2 นำเสนอผลงานเรื่อง “Antimicrobial property of chitosan-based film” ในการประชุมทางวิชาการ The 58th Annual Meeting and Exhibition Industrial Microbiology and Biotechnology, จัดโดย The Society of Industrial Microbiology, San Diego, USA, August 10-14, 2008.