DEVELOPMENT OF ACTIVE FILM FROM GIANT CATFISH SKIN GELATIN INCOPORATED WITH ANTIOXIDANT EXTRACT FROM SOME TROPICAL FRUIT SEEDS

SAMART SUI-UT

MASTER OF SCIENCE

PROGRAM IN FOOD TECHNOLOGY

MAE FAH LUANG UNIVERSITY

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THIS THESIS IS A PARTIAL FULFILLMENT OF
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EXAMINING COMMITTEE

..... CHAIRPERSON
(Asst. Prof. Dr. Romanee Sanguandeekul)

..... COMMITTEE
(Asst. Prof. Dr. Saroat Rawdkuen)

..... COMMITTEE
(Dr. Akkasit Jongjareonrak)

..... COMMITTEE
(Dr. Chuleeporn Thanomsilp)

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Author	Samart Sai-Ut
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Supervisory Committee	Asst. Prof. Dr. Saroat Rawdkuen Dr. Akkasit Jongjareonrak

ABSTRACT

This study was aimed to investigate the extraction of gelatin and development the active gelatin film from giant catfish skin incorporated with fruit seeds antioxidant extracts. Gelatin was extracted from giant catfish (*Pangasianodon gigas*) skin using distilled water at 45°C for 12 h. The re-extraction process was also investigated to obtain a high gelatin recovery. The first extraction was done by incubating the pretreated acid-treated fish skin at 45 °C for 12 h. The remnant was re-extracted at temperatures of 60–90 °C for 1–12 h. The gelatin yield of the first extraction was 10.14%, while the re-extraction at 90 °C provided higher recovery (19.5%). Low band intensity of α_1 and α_2 chains of gelatin was observed after re-extraction at high temperature for a longer time. The absorption bands of amide I (1,653 cm^{-1}) and II (1,542 cm^{-1}) from both extracted gelatins were similar. The obtained results suggested that the gelatin re-extraction process could be applied for other skin sources to obtain high gelatin recovery with the desired properties.

Solid–liquid extraction and response surface methodology (RSM) were used to optimise conditions for the extraction of phenolic compounds from longan (*Dimocarpus longan* Lour.),

lychee (*Litchi chinensis* Sonn.), passion fruit (*Passiflora edulis*), and rambutan (*Nephelium lappaceum*) seeds. The independent processing variables were ethanol concentration, temperature and time. Ethanol concentration and temperature significantly affected extraction yield, extractable phenolic content (EPC), and antioxidant activities including DPPH-, ABTS-radical scarvenging activity, and ferric reducing/antioxidant power (FRAP). According to the prediction value at optimum condition, longan seed had highest extraction yield, EPC, and antioxidant activities compared with lychee, passion fruit, and rambutan seed extracts. The optimal conditions for longan seed extraction based on combination responses were 53 % (v/v) ethanol, 58 °C, and 139 min. These optimum conditions yielded EPC of 5,804 mg gallic acid equivalents (GAE)/100 g dry sample with DPPH-, ABTS- radical scanvenging activity, and FRAP values of 2,442, 8,620, and 3,609 GAE/100 g dry samples, respectively. The experimental values were well agreed with those predicted values. Thus longan seed was used to extract antioxidant compound for development of active gelatin film.

Gelatin films incorporated with longan seeds (LS) extract or butylated hydroxytoluene (BHT) at different concentrations were developed and characterized. The properties including tensile strength (TS), elongation at break (EAB), protein solubility and FTIR spectra of the films were similar to the film made without LS or BHT. The ncrease in redness (a^*) and yellowness (b^*) values of film were observed with the increasing concentration of LS extract ($P < .05$). The results showed some interactions of phenolic compounds in LS extracts, especially when LS was added at higher concentration as evidenced by the higher glass transition temperature, decreased in EAB, and increased water solubility of the films. Films with LS extract or BHT incorporated showed preventive effect on lipid oxidation of soybean oil. The peroxide formation (PV), conjugated diene (CD), and thiobarbituric acid reactive substances (TBARS) values of soybean oil slowly changed compared with the control film.

Keywords: Active film / Antioxidant / Extraction / Giant catfish skin gelatin / Tropical fruit seed

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CHAPTER 1

INTRODUCTION

1.1 Introduction

Nowadays, packaging research is receiving a considerable attention due to the development of eco-friendly materials made from natural polymers, often from wastes agricultural-by-product, livestock raising or fishing. Such polymers may be protein, lipid, or polysaccharide based, and may be incorporated with compounds such us plant extracts and essential oils with specific antioxidant and/or antimicrobial activities. Fish gelatins as filmogenic agents has been already reported in the scientific literature. The studies dealing with fish gelatin films are mainly focused on the evaluation and improvement of the film forming ability and to a lesser extent on the incorporation of functional compounds with antioxidant activity such as polyphenol-rich plant extracts, butylated hydroxytoluene (BHT), and α-tocopherol. The use of antioxidant agents from natural sources such us plant extracts instead of synthetic antioxidants is of growing interest in westernized countries, since synthetic antioxidants are suspected of causing some safety concerns and have been restricted in their use as food additives. On the one hand, those phytochemicals may improve the stability of foods because of their properties as antioxidants, antimicrobials and/or enzyme inhibitors. By the way, research supports that the consumption of such phytochemicals may provide benefits against cancer, coronary heart disease, diabetes, high blood pressure, inflammation, microbial, viral and parasitic infections, ulcers, etc.

Gelatin, obtained by hydrolysis from collagen, is a protein with a wide range of functionalities and applications, including the film forming ability. However, the functional properties, thermostability and film forming ability of gelatin are highly dependent on its characteristics, which are related to the species origin (mammalian, cold water fish, warm water fish), wastes origin (bones, skin), animal age (affecting the collagen crosslinking), severity of the

extraction procedure (pH, temperature), etc. Currently, increasing the extraction temperature in order to increase the yield of gelatin extraction, even in detriment of quality, is a common practise in the gelatin extraction procedures. This practise is especially applied in gelatins obtained from mammalian sources such as pig skins or cow hides, which are the most available gelatins nowadays, but it may be also applied to fish gelatin extraction. For this reason, specific studies are needed for each type of gelatin. Giant catfish skin gelatin has been previously characterized and it has shown reasonable good physical properties and thermostability as compared to other fish species. However, its film forming ability and delivery bioactive compound to the film has not been reported yet.

There are considerably higher ratios of waste and by-products arising from fruit-processing industry and derived products have experienced growing worldwide popularity. It would be beneficial, in improving the complete utilization of the seeds, if they could be used as a source of natural food additives and ingredients. Fruits and vegetables contain antioxidant nutrients, in addition to vitamin C or E and carotenoids, that significantly contribute to their total antioxidant capacity. Among phytochemicals, special attention has been paid to polyphenols. Phenolic substances such as flavonoids are an example: they are the most common compounds in fruits and vegetables and have strong antioxidant capacity. The previous studies, longan seeds contained high levels of gallic acid, corilagin and ellagic acid and five compounds were identified from litchi seed, namely gallic acid, procyanidin B2, (-)-gallocatechin, (-)-epicatechin, and (-)-epicatechin-3-gallate. Longan and lychee seeds presented relatively high antioxidant activity has been reported, which could be ascribed to its phenolic content of 6,300 mg GAE/100 g seeds and 239 mg GAE/100g seed (dry matter), respectively. The ethanolic extract of the rind of rambutan has a high free radical-scavenging activity (762-822 mg/g GAE) (Palanisamy, Cheng, Masilamani, Subramaniam, Ling & Radhakrishnan, 2008). Passion fruit has low levels of phenolic compounds but very strong antioxidant capacity. These fruits extract could be a good choice to be incorporated into edible films with antioxidant properties.

Edible films may prevent the determination of food, mainly as a result of their ability to act as barriers to water, preventing dehydration, and to oxygen and light, reducing lipid oxidation. Antioxidant releasing packaging is a kind of food preservation system, in which an antioxidant or a mixture of antioxidants is incorporated into the package, contributing to the shelf-life extension

of food. To our knowledge, little research has been done on fish gelatin active films with antioxidant properties. In addition, giant catfish gelatin used as biomaterial would solve the problem of waste disposal and also the size of these fish provides the higher gelatin yield.

1.2 Objectives

1.2.1 To obtain gelatin with high recovery from farmed giant catfish skin and some characteristics.

1.2.2 To optimize the extraction of phenol compounds from tropical fruits seeds by using RSM

1.2.3 To study some properties of gelatin films incorporated with fruit seed extracts or synthetic antioxidant.

1.3 Scope of the Research

The research was divided into 3 major parts including gelatin extraction, phenolic antioxidants extraction, and active film formation. First, gelatin was extracted from farmed giant catfish skin (*Pangasianodon gigas*). The gelatin extraction process was performed by varying the cycle of extraction (1 and 2 times), temperatures (60-90°C) and extraction time (1-12 h). The gelatins obtained were determined the recovery and some biochemical characteristics.

Second, studied on the extraction of phenolic compounds from tropic fruits seeds (lychee, longan, passion fruit, and rambutan) of with some factors being investigated. The required solid-to-liquid ratio (1:5 – 1:40 w:v) for the extraction of the phenolic compounds from the seeds was determined. RSM was applied to optimize the extraction condition for obtaining the highest antioxidant compounds. Ethanol proportion (40-80%), temperature (40-80°C), and time (60-180 min) on the phenol extraction was studied. The extracts were determined extraction yield, extractable phenolic compound, DPPH radical-scavenging activity, ABTS radical-scavenging activity, and FRAP reducing power antioxidant activity.

Finally, gelatin based films incorporated with selected fruit extract was prepared. Determination of film properties in term of mechanical properties, physicochemical properties

and antioxidative activity during storage was monitored. In addition, application of prepared film for oil oxidation retardation was also measured in term of peroxide value, conjugated diene, and thiobarbituric acid reactive substances.

CHAPTER 2

LITERATURE REVIEW

2.1 Edible Films

2.1.1 Definition

Any type of material used for enrobing (i.e., coating or wrapping) various food to extend shelf life of the product that may be eaten together with food with or without further removal is considered an edible film. Edible films provide replacement and/or fortification of natural layers to prevent moisture losses, while selectively allowing for controlled exchange of important gases, such as oxygen, carbon dioxide, and ethylene, which are involved in respiration processes. A film can also provide surface sterility and prevent loss of other important components. Generally, its thickness is less than 0.3 mm.

2.1.2 Film Composition

Materials available for forming films fall generally into the categories of proteins, polysaccharides, lipids, and resins. A plasticizer must often be added to reduce film brittleness. A surface-active agent is also often necessary to aid film formation. Other constituents can include antioxidants and antimicrobials to enhance the film effectiveness. The U.S. Code of Federal Regulations provides the status of protein, polysaccharide, lipid, resin, plasticizer, emulsifier, preservative, and antioxidant materials related to acceptable use (Baldwin, 1999).

2.1.2.1 Polysaccharides

Polysaccharides are biologically produced (bio-based) materials that have a unique combination of functional properties and environmentally friendly features. Polysaccharides are also polymers and their long chain structures, as in man-made polymers, provide good mechanical properties for applications such as fibers, films, adhesives, melt processable plastics, thickeners, rheology modifiers, hydrogels, drug delivery agents, emulsifiers, etc. They are

renewable materials, produced from other biological compounds, and generally are non-toxic. These features make polysaccharide materials a natural fit for sustainable development. Commercially available products include starch, cellulose and its derivatives (such as cellulose acetate, carboxymethyl cellulose, and methyl cellulose), sodium alginate, xanthan gum, dextran, carrageenan, and hyaluronic acid. Research continues to bring us new materials with improved performance and lower costs. Proteins can be combined with polysaccharides to modify film mechanical properties (Gennadios, McHugh, Weller & Krochta, 1994).

2.1.2.2 Lipids

Edible lipids include beeswax, candelilla wax, carnauba wax, triglycerides (e.g., milk-fat fractions), 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 a 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 & De Mulder-Johnston, 1997).

2.1.2.3 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. The amino acids are similar in containing an amino group (-NH,) and a carboxyl group (-COOH) attached to a central carbon atom. However, each amino acid has a different side group attached to the central carbon that lends unique character to that amino acid. The side group can be non-polar (hydrophobic), polar uncharged (hydrophilic), positively charged at pH 7, or negatively charged at pH 7 (Cheftel, Cuq, & Lorient, 1985). Most proteins contain 100-500 amino acid residues. Depending on the sequential order of the amino acids (primary structure of the protein), the protein will assume different structures along the polymer chain (secondary structure of the protein), based on van der Waals, hydrogen bonding, electrostatic, hydrophobic, and disulfide cross-link interactions among the amino acid units (Cheftel, et al., 1985). The tertiary protein structure reflects how the

secondary structures organize relative to each other, based on the same types of interactions, to form overall globular, fibrous or random protein structure. Finally, quaternary structure occurs when whole proteins interact with each other into associations to provide unique structure or biological activity. The secondary, tertiary, and quaternary structures of proteins can be modified by various physical and chemical agents, including heat, mechanical treatment, pressure, irradiation, lipid interfaces, acids and alkalis, and metal ions (Cheftel, et al., 1985). Such agents are often used in the formation of protein films and coatings to optimize protein configuration, protein interactions, and resulting film properties.

Protein film-forming materials derived from animal sources 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. Proteins that have successfully been formed into films and/or coatings are listed in Table 2.1 along with the solubility properties of the native proteins before formation into films or coatings.

2.1.2.4 Plasticizers

Protein films are often quite stiff and brittle due to extensive interactions between protein chains through hydrogen bonding, electrostatic forces, hydrophobic bonding, and/or disulfide cross-linking. Relatively small molecular weight hydrophilic plasticizers are often added, which mainly compete for hydrogen bonding and electrostatic interactions with the protein chains. The result of plasticizer addition is a reduction in protein chain-to-chain interactions, a lowering of the protein glass transition temperature (s), and an improvement in film flexibility (lowering of film elastic modulus). Also, film elongation (stretchiness or ductility) increases, and film strength decreases. 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 protein edible films include glycerol, propylene glycol, sorbitol, sucrose, polyethylene glycol, fatty acids, and monoglycerides. Water is also an important plasticizer for protein films. Thus, film moisture content, as affected by the surrounding environment's relative humidity (RH), has a large effect on film properties.

Table 2.1 Proteins Used for Edible and Biodegradable Films.

Protein	Protein Solvents			
	Water	**Acidic Water**	**Alkaline Water**	**Aqueous Ethanol**
Collagen		X		
Gelatin	X			
Fish myofibrillar protein		X	X	
Keratin				X
Egg white protein			X	
Casein	X			
Whey protein	X			
Corn zein				X
Sorghum kafirin				X
Wheat gluten		X	X	X
Rice bran protein		X	X	
Soy protein	X		X	
Peanut protein			X	
Cottonseed protein			X	

From Gennadios, A., McHugh, T. H., Weller, C. L., & Krochta, J. M. (1994). Edible Coatings and Films Based on Proteins. In J. M. Krochta, E. A. Baldwin, & M. O. Nisperos-Carriedo (Ed.), Edible Coatings and Films to Improve Food Quality (pp. 201-278). Lancaster, PA: Technomic Publishing Company.

The presence of hydrophilic plasticizers such as glycerol attracts additional moisture and additionally impacts film properties. Plasticizers that are commonly used with protein films and coatings are glycerol, propylene glycol triethylene glycol, sorbitol, sucrose, and polyethylene glycol.

2.1.2.5 Emulsifiers

Emulsifiers are surface-active compounds with both polar and non-polar character, which absorb at the water-lipid interface and reduce surface tension. To produce protein-lipid or polysaccharide-lipid composite films from aqueous solution, it is often necessary to add an emulsifier to allow dispersion of the lipid material in the solution. Also, for some food-coating applications, addition of a surface-active agent to a coating formulation may be necessary to achieve satisfactory surface wetting and spreading with the coating formulation and then adhesion of the dry coating. Some proteins are sufficiently surface-active that no emulsifier is necessary to form well-dispersed composite films or provide good surface wetting and adhesion.

2.1.2.6 Other Film Additives

As mentioned, edible films have the potential to be effective carriers and providers of antioxidants, antimicrobials, nutrients, flavors, and colors to enhance food safety, nutrition, and quality.

1. Antimicrobials

The main cause of spoilage for many food products is surface microbial growth. Reduction of water activity (*Aw*) and protection with moisture-proof packaging are common methods used to prevent spoilage in food products. Nevertheless, a reorganization of water inside the package, due to temperature changes, can induce condensation of moisture on the food surface, increasing the possibility for microbial growth. Growth of yeasts, molds, and bacteria during product storage and distribution can drastically reduce food quality and food safety.

Use of antimicrobials such as benzoic acid, sodium benzoate, sorbic acid, potassium sorbate, and/or propionic acid represents an additional means of food preservation. Edible coatings have been studied as antimicrobial carriers because of their effectiveness in retaining additives on food surfaces. Within the last decade, several groups have evaluated diffusivity of sorbic acid and potassium sorbate in model systems (Torres & Karel, 1985).

Early research studies applied antimicrobials and fungicides by dipping food in additive solution. This method was initially effective in reducing the total number of viable microorganisms, but on storage, preservative diffused into the food, allowing surface spoilage to occur. Application of fungicides in wax-based emulsions or water suspension has been studied on citrus fruit. The primary factor responsible for their effectiveness is dependent on method of

application. The emulsified method was less effective of the two treatments, possibly due to encapsulation of the additive in the lipid phase (Ecker & Kolbezen, 1977).

2. Antioxidants

Antioxidants increase stability of food components, especially lipids, and maintain nutritional value and color by preventing oxidative rancidity, degradation and discoloration. Acid or phenolic compounds act as antioxidants. Acid compounds, such as citric and ascorbic acid, are metal chelating agents. Phenolic compounds, such as butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT), tertiary butylated hydroxyquinone (TBHQ), propyl gallate and tocopherols inhibit lipid oxidation. These antioxidants can be incorporated into edible films, thus being retained on the food surface where they are most effective, since oxidation is a surface-air phenomenon.

3. Nutrients

Along with increased market demands on nutritionally-fortified foods, edible films with high concentrations of nutraceuticals would provide alternative ways to fortify foods that otherwise cannot be fortified, such as fresh fruits, fresh vegetables and other minimally-processed foods. Food products could either be wrapped with nutritionally-fortified films. A study developed by Mei and Zhao (2003) demonstrated that water vapor permeability and tensile properties of milk protein-based edible films, with calcium caseinate and whey protein isolate, may be compromised when high concentrations of calcium and vitamin E are incorporated. Vitamin E incorporation increased elongation percentage at break, and reduced tensile strength of films, probably because of its hydrophobic nature, plasticizing effect or due to the heterogeneous structure of the film.

Some nanoscale phenomena have been utilized in nutraceutical and functional food formulation and manufacturing processes. New concepts, based on nanotechnology, are being explored to improve product functionality and delivery efficiency (Chen, Wu, Gu & Chen, 2007). This understanding could lead to a potentially novel type of active packaging for food products that enhance solubility, facilitate controlled release, improves bioavailability, and protect stability of micronutrients and bioactive compounds during processing, storage and distribution.

4. Flavors

Solute polarity is an important factor in the sorption process. Some authors have shown that aroma compounds are adsorbed more easily on the polymeric film if their polarities are similar. However, very few authors have studied the effect of this factor on transfer through hydrophilic edible packaging. Furthermore, aroma barrier properties of edible films have not been as thoroughly studied as water and oxygen transfer (Miller & Krochta, 1997). Transfer of three methyl ketones through methylcellulose-based films is more complex than that for low-density polyethylene plastic (LDPE). Their transfer rate increases with the chain length of the volatile compound, while their diffusion coefficient decreases. There is a relationship between permeability and sorption and between permeability and saturated vapor pressure. Mass transfer seems to depend mainly on the affinity between the volatile compound and the polymer. This relationship reveals the presence of physicochemical interactions that modify the structure of the polymer (plasticization).

5. Colors

Food coloring (colouring) is any substance that is added to food or edible film to change its color. Food coloring is used both in commercial food production and in domestic cooking. Due to its safety and general availability, food coloring is also used in a variety of plastic film applications.

A growing number of natural food dyes are being commercially produced for edible film, partly due to consumer concerns surrounding synthetic dyes. Some examples include: caramel coloring, made from caramelized sugar, used in cola products and also in cosmetics, annatto, a reddish-orange dye made from the seed of the achiote. A green dye made from chlorella algae (chlorophyll). Cochineal, a red dye derived from the cochineal insect, *Dactylopius coccus*, betanin extracted from beets, turmeric (curcuminoids), saffron (carotenoids), paprika, elderberry juice, pandan (*Pandanus amaryllifolius*), a green food coloring, and butterfly pea (*Clitoria ternatea*), a blue food dye.

To ensure reproducibility, the colored components of these substances are often provided in highly purified form, and for increased stability and convenience, they can be formulated in suitable carrier materials (solid and liquids). Hexane, acetone and other solvents break down cell walls in the fruit and vegetables and allow for maximum extraction of the

coloring. Residues of these often remain in the finished surface film, but they do not need to be declared on the product. This is because they are part of a group of substances known as carry-over ingredients (Dangaran, Tomasula & Qi, 2010).

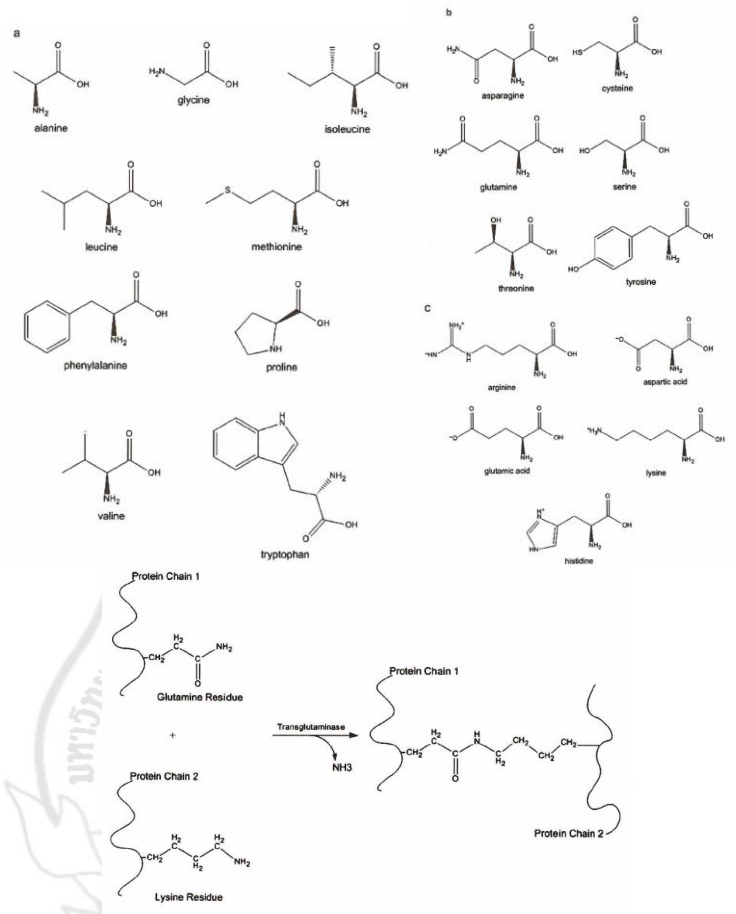
2.2 Protein-Based Edible Films

Research and development on films made from various agricultural proteins has been conducted over the past 20 years, but is of heightened interest, due to the demand for environmentally-friendly, renewable replacements for petroleum based polymeric materials and plastics. To address this demand, films have been made from renewable resources, such as casein, whey, soy, corn zein, collagen, wheat gluten, keratin and egg albumen. Those made from agricultural proteins create new outlets for agricultural products, by-products and waste streams, all of which can positively impact the economics of food processes.

The inherent properties of proteins make them excellent starting materials for films. The distribution of charged, polar and non-polar amino acids along the protein chain creates chemical potential. The shading illustrates the domains of polar and non-polar areas along the protein chain. The resulting interactive forces produce a cohesive protein film matrix. Films form and are stabilized through electrostatic interactions, hydrogen bonding, van der Waals forces, covalent bonding, and disulfide bridges (Krochta, Baldwin & Nisperos-Carriedo, 1994). Protein film-forming capabilities are best demonstrated in emulsified systems in which amphipathic proteins form films at air-water or water-oil interfaces. There are also secondary benefits for using proteins to form films. Proteins have multiple sites for chemical interaction as a function of their diverse amino acid functional groups, which can allow for property improvement and tailoring. Chemical changes can improve the stability of films. Cross-linked proteins films are often more stable than their polysaccharide-based counterparts and have a longer lifetime (Barone & Schmidt, 2006).

Protein-based films are also biodegradable and compostable. As they degrade, they provide a source of nitrogen, which contributes a fertilizer benefit not available with other non-protein-based films. Finally, there is emerging evidence that the bioactive peptides produced upon

digestion of proteins (dairy sources in particular) have antihypertensive and radical scavenging health benefits (Aimutis, 2004).



From Dangaran, K. L., Tomasula, P. M., & Qi, P. (2010). Structure and Function of Protein-Based Edible Films and Coatings. In: M. E., Embuscado, K. C., Huber (Eds). Edible Films and Coatings for Food Applications (pp.25-56). New York, NY: Springer Science Media.

Figure 2.1 Amino Acids and Cross-Linking Reaction of Protein Chains Catalyzed by the Enzyme Transglutaminase

2.3 Collagen and Gelatin

2.3.1 Collagen

Because of the importance of collagen, its structure has been extensively studied. The primary structure (amino acid sequence) of collagen and gelatin consists of 18 different amino acids (Table 2.2). Collagen is rich in glycine (~1/3 of total residues), hydroxyproline, and proline. Also, it is one of few proteins containing hydroxylysine and is devoid of tryptophan. Mammalian collagen is richer in hydroxyproline than fish collagen (Foegeding, Lanier & Hultin, 1996). Collagen is a hydrophilic protein because of the greater content of acidic, basic, and hydroxylated amino acid residues than lipophilic residues. Therefore, it swells in polar liquids with high solubility parameters. The secondary structure (local configuration of chains) does not favor formation of a-helical chain segments because of the high number of prolyl and hydroxyprolyl residues. Collagen chains are helical macromolecules with a tendency mainly for interchain, rather than intrachain, hydrogen bonding (Carver & Blout, 1967). The tertiary structure standing for large-scale folding and helicity of collagen is better understood through the fundamental unit tropocollagen. Tropocollagen is a right hand superhelix with a repeat unit of about 100°A consisting of three left-hand strands. Two or three of these chains can be covalently linked to form a β-chain or a γ-chain, respectively. The quaternary structure refers to formation of small to medium size aggregates of tropocollagen molecules, i.e., complex crystallites first identified by small angle X-ray diffraction (Figure 2.2). Collagen molecules link end to end and adjacently to form collagen fibers. There is a periodicity in the cross-striations of collagen at about 640-700 intervals. The fibers are sometimes arranged in parallel having great strength, as in tendons, or they may be highly branched and disordered, as in skin.

2.3.2 Gelatin

The primary structure of gelatin is almost identical to that of parent collagen but for some small differences due to the pretreatment and extraction processes. These differences include removal of amide groups of asparagine and glutamine, conversion of arginine to ornithine, and (partial) removal of telopeptides (Johnston-Banks, 1990).

Table 2.2 Amino Acid Composition of Collagen and Gelatin (Residues per 100 Total Residues).

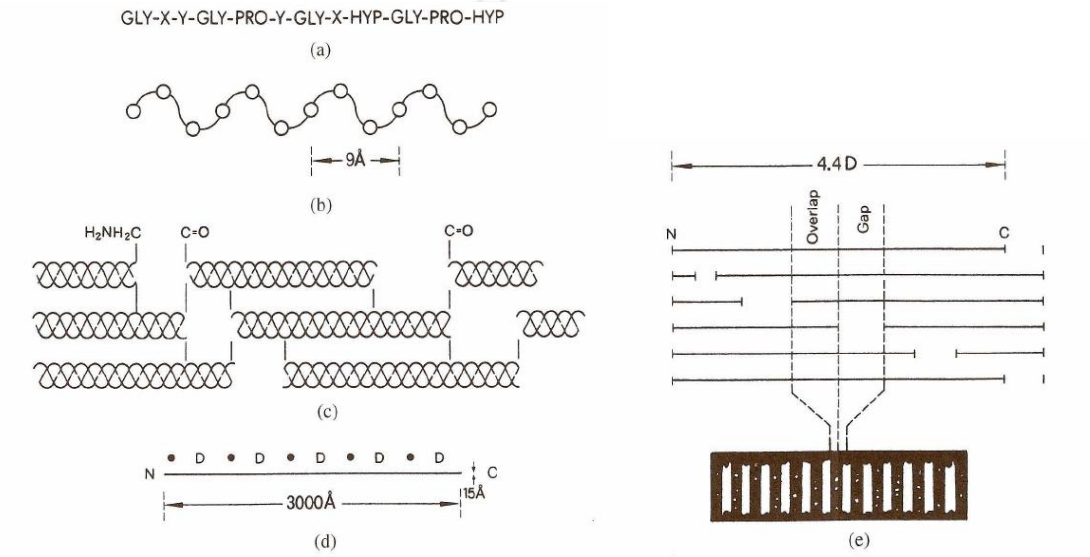
Amino Acid	Bovine Coriurn Collagen*	Acid Pigskin Gelatin**	Limed Calfskin Gelatin**	Limed Bone Gelatin**	Giant Catfish Skin Gelatin***
Alanine	10.7	8.6-10.7	9.3-11.0	10.1-14.2	10.6
Arginine	4.8	8.3- 9.1	8.6- 8.8	5.0- 9.0	6.3
Aspartic acid	4.7	6.2- 6.7	6.6- 6.9	4.6- 6.7	1.5
Glutamic acid	7.2	11.3-11.7	11.l-11.4	8.5-11.6	6.2
Glycine	33.7	26.4-30.5	26.9-27.5	24.5-28.8	35.9
Histidine	0.5	0.8- 1.0	0.7- 0.8	0.4- 0.7	0.4
Hydroxylysine	0.5	1.0	0.9- 1.2	0.7- 0.9	0.5
Hydroxyproline	9.4	13.5	14.0-14.5	11.9-13.4	8.7
lsoleucine	1.1	1.4	1.7- 1.8	1.3- 1.5	1.3
Leucine	2.4	3.1- 3.3	3.1- 3.4	2.8- 3.5	2.3
Lysine	2.5	4.1- 5.2	4.5- 4.6	2.1- 4.4	3.2
Methionine	0.4	0.8- 0.9	0.8- 0.9	0.0- 0.6	1.0
Phenylalanine	1.3	2.1- 2.6	2.2- 2.5	1.3- 2.5	1.3
Proline	12.9	16.2-18.0	14.8-16.4	13.5-15.5	12.4
Serine	3.9	2.9- 4.1	3.2- 4.2	3.4- 3.8	3.6
Threonine	1.7	2.2	2.2	2.0- 2.4	2.4
Tyrosine	0.5	0.4- 0.9	0.2- 1.0	0.0- 0.2	0.3
Valine	2.0	2.5- 2.8	2.6- 3.4	2.4- 3.0	2.2

From * Veis, A. (1960). The Macrornolecular Chemistnr of Gelatin. New York: Academic Press.

** GelatinManufacturers Institute of America (GMIA). (1993). Gelatin. New York: GMIA, pp. 24.

*** Jongjareonrak, A., Rawdkuen, S., Chaijan, M., Benjakul, S,. Osako, K., & Tanaka. M. (2010). Chemical compositions and characterisation of skin gelatin from farmed giant catfish (*Pangasianodon gigas*). LWT - Food Science and Technology, 43(1), 161-165

The precise macromolecular constitution of gelatin resulting from a melting process depends on collagen source and on the extraction method. Gelatin chains can be intertwined back into the collagen helix through an appropriate technique, such as cooling or annealing in solution. Films “cold cast” from gelatin solutions at temperatures below 35°C provided concluding evidence of tertiary structure but not of quaternary structure while "hot cast" gelatin films were amorphous (Bradbury & Martin, 1952).



From Gennadios, A., McHugh, T. H., Weller, C. L., & Krochta, J. M. (1994). Edible Coatings and Films Based on Proteins. In J. M. Krochta, E. A. Baldwin, & M. O. Nisperos-Carriedo (Ed.), Edible Coatings and Films to Improve Food Quality (pp. 201-278). Lancaster, PA: Technomic Publishing Company.

Figure 2.2 Order in Collagen: (a) Primary, (b) Secondary, (c) Tertiary, and (e) Quaternary Structure.

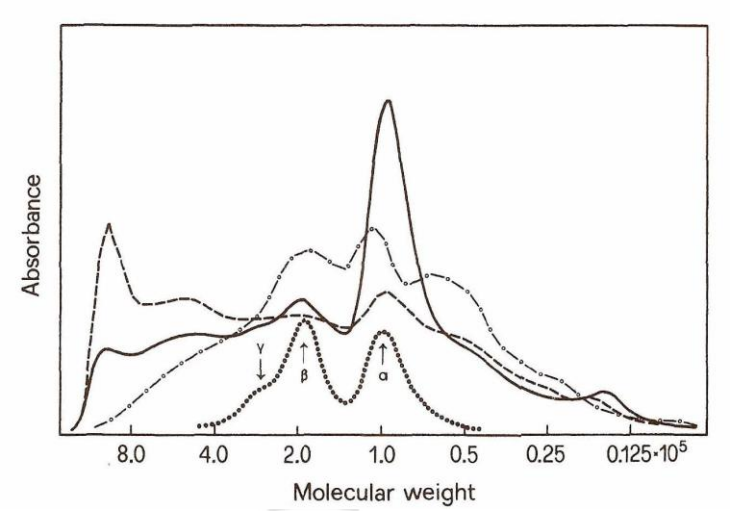
2.3.3 Physical Properties

2.3.3.1 Molecular Weight

The lability of peptide bonds and cross-linkages depends upon temperature, pH, and raw material source. The number average molecular weight *(M,,)* and the weight average molecular weight *(M,)* of non-hydrolyzed gelatins ranged from 5 x 10^4 to 1 x 10^5 Da and from 10^5 to 10^6 Da, respectively (Rose, 1987). Average molecular weight and molecular weight distributions can be determined by osmometry, viscometry, sedimentation, light scattering, filtration chromatography, and gel electrophoresis (Yannas, 1972; Rose, 1987). Gelatin distributions were polydisperse and were resolved into many peaks depending primarily on the measurement method. The proportion of α and β chains or cross-linked aggregates is mainly affected by the pretreatment method (Figure 2.3).

2.3.3.2 Infrared and Holographic Relaxation Spectroscopy

Early infrared (IR) work on proteins was promising and resulted in several proposed models. The IR spectra of all proteins resemble each other closely because protein molecules consist of varying amounts of 20 amino acid residues. The varying abundance of these amino acid residues is reflected in the IR spectrum of each protein. In fact, molecular configuration affects the IR spectrum of a polymer molecule, and different forms of the same protein may have different spectra. Blood proteins, muscle proteins, plant proteins, and sclera-proteins belong to class A**,** whereas collagen, gelatin, and silk fibroin belong to class B. Because the variability of protein IR spectra is rather limited, proteins can fairly easily be distinguished from other biological molecules, such as carbohydrates, lipids, or nucleic acids (Beer, Sntherland, Tanner & Wood, 1959). IR spectra of collagen and gelatin have been extensively investigated over the past 50 years, and several of the initial resolution problems have been resolved. Several changes appearing in the IR spectrum by denaturation of collagen to hot cast gelatin were identified (Yannas, 1972). Collagen in the solid state was also studied with IR spectroscopy by using deuterated samples (Beer, et al., 1959). Structures of gelatin gels, formed by cooling down sufficiently concentrated gelatin solutions, were investigated by holographic relaxation spectroscopy that revealed two peaks, presumably corresponding to “coarse” and “fine” network (Wu, Schrof, Lilge, Luddecke, & Horn., 1991).



From Gennadios, A., McHugh, T. H., Weller, C. L., & Krochta, J. M. (1994). Edible Coatings and Films Based on Proteins. In J. M. Krochta, E. A. Baldwin, & M. O. Nisperos-Carriedo (Ed.), Edible Coatings and Films to Improve Food Quality (pp. 201-278). Lancaster, PA: Technomic Publishing Company.

Figure 2.3 Molecular Weight Distributions of High Bloom Gelatins:·····, Type A (Pigskin); ---, Limed Calfskin; —, Limed Ossein; and ·····, Thermally Denatured Soluble Calfskin Collagen.

The former consisted of aggregations of a collagen-like triple stranded helix, whereas the latter consisted of entanglements between polymer chains. Predominance of the former over the latter depended on cooling rate. This model could possibly be expanded to account for structural differences occurring during gelatin film formation (Wu, et al., 1991).

2.3.4 Thermal Properties of Gelatin

Melting of molecularly dispersed collagen (tropocollagen) in heated, salt-free water solutions proceeded with extremely intensive heat absorption (Privalov, 1982). The high specific heat of collagen denaturation facilitates the thermal study of collagen intramolecular melting, which should be carried out by sensitive instruments because of the artifacts that have occasionally occurred. Flory and Garrett (1958) derived the first order transition (T_m = 145°C)

occurring in collagen and gelatin from an extrapolation of dilatometric data. Subsequent studies on collagen/gelatin samples with or without plasticizers led to substantial deviations. Table 2.3 summarizes reported melting temperature (T_m) and glass transition temperature (T_g) values of collagen and gelatin.

2.4 Gelatin-Based Films

Proteins and polysaccharides are good film formers and have occasionally been used for food preservation. Although they exhibit satisfactory gas barrier behavior for coating applications, their water vapor permeability values are usually high. In general, the behavior of non-chemically interacting, mixed polymer systems is dictated by the enthalpy of endothermic segment-segment interactions leading to repulsive forces and mutual exclusion of each polymer from the domain of the other (Ledward, 1994).

Binary, ternary, or quaternary single-phase blends consisting of gelatin, polysaccharide (i.e., soluble starch, modified starch, or chitosan), and plasticizer (i.e., water, sugars, or polyols) have recently been prepared (Arvanitoyannis, Psomiadou, Nakayama, Aiba, & Yamamoto, 1997; Arvanitoyannis, Nakayama & Aiba, 1998). Plasticizers of low molecular weights lowered both T_m and T_g increased gas permeation (proportionally to plasticizer content).

Cohesion of starch/gelatin or chitosan/gelatin blends increased in the presence of self-associated molecules (i.e., water, polyols). The storage modulus (E') was clearly observed passing through the glassy, leathery, and rubbery states. A shoulder-like p-transition at high sorbitol contents was attributed to the pure sorbitol. Similar behavior was observed for glycerol. The mechanical properties of gelatin-based films depended considerably on film preparation method. Films obtained by evaporation at high temperature (-60°C) were weaker (lower tensile strength and percent elongation) than those prepared by the low temperature process for total plasticizer content (TPC) of up to 25%.

Table 2.3 Melting Temperature T_m (°C) Glass Transition Temperature T_g (°C)and Method for Determining T_m and T_g Values of Collagen (COL) and Gelatin (GEL) from Various Sources.

	T_m (°C)			T_g (°C)		
Source	**COL**	**GEL**	**Method**	**COL**	**GEL**	**Method**
Cow tendon	145	145	Dilatometer	95	95	Dilatometer
Cowhide	145	-	Shrinkage	-	-	
Tendon fibert	265	-	Shrinkage	-	-	
Partially dehydrated tendon	200	218	DTA	120	120	DTA
Undehydrated tendon	-	220	Viscoelastic	-	180	Viscoelastic
Rat tail tendon	220	-	DTA	-	-	
Dehydrated rat tail tendon	-	230	DTA	-	200	DTA
Porkskinllli	-	220	Viscoelastic	-	180-200	DTA
Ossein (cold)	230	230	DSC	-	-	
Tendon	210	220	DTA	-	-	
Cross-linked tendon	-	225	DSC	-	195	DMTA
Non-cross-linked tendon	-	225	DSC	-	214	DSC
Pigskintt	-	230	DSC	-	217	DSC
Calfskin		226	DTA	-	212	DTA
Pigskin	-	237	DSC	-	-	
Rat tail (10% H_2O)	-	-		120	120	Viscoelastic
Limed ossein	-	230	DSC	-	220	DSC
Food grade	-	-		-	215	Microhardness

From Gennadios, A., McHugh, T. H., Weller, C. L., & Krochta, J. M. (1994). Edible Coatings and Films Based on Proteins. In J. M. Krochta, E. A. Baldwin, & M. O. Nisperos-Carriedo (Ed.), Edible Coatings and Films to Improve Food Quality (pp. 201-278). Lancaster, PA: Technomic Publishing Company.

At TPC 25%, percent elongation increased considerably. This increase was more pronounced for films of low crystallinity (high temperature process) because such films were more easily plasticized and were more extendable than the respective films of high crystallinity (low temperature process). The more crystalline films had more constraints to elongation because they contained higher numbers of triple-stranded crystallites (Arvanitoyannis et al., 1998).

Propylene glycol alginate or pectate esters interacted with gelatin, forming covalently bonded gels with high thermal stability (T_m > 100°C). Also, polysaccharide-protein interactions have been applied to microencapsulation (gelatin-gellan), drug release (gelatin-dextran), and tissue adhesion (gelatin-poly-carboxylic acid) (Otani, Tabata & Ikada, 1998).

2.4.1 Mechanical Properties

Thin gelatin films are rich in colloid material and behave similarly to rigid gels exhibiting elastic moduli and time-dependent phenomena. Commercially, gelatin is mainly characterized by Bloom strength and viscosity. However, tensile strength and elongation are equally important physical properties for edible film applications. The main parameters affecting film-forming properties of gelatin are raw material source, extraction method, molecular weight, film preparation method (hot vs. cold casting), and degree of hydration or presence of plasticizer.

Table 2.4 presents measured mechanical properties of gelatin films. Healey Rubinstein and Walters (1974) determined the mechanical properties of films made from several binders used in tableting. Gelatin films showed a slight yielding before fracture and had higher values of Young's modulus and tensile strength than methylhydroxyethyl cellulose, starch, acacia gum, or polyvinylpyrrolidone. High elastic modulus, low plasticity, and relative brittleness were possible limitations of gelatin as a tablet binder (Healey, et al., 1974).

2.4.2 Gas and Water Vapor Permeability of Gelatin Films

There is strong demand for minimally processed foods. Edible films have been applied to processed foods to retard and prevent movement of water and gases. Although commercially available synthetic polymers, such as polyethylene and polypropylene, effectively control gas permeability (GP) and water vapor permeability (WVP), concerns over their non-renewable nature generates interest in more environmentally friendly, renewable biopolymers.

Collagen and gelatin films have been used on meats and sausages to reduce GP and/or WVP. Gelatin is a good gas barrier, but is highly hydrophilic. Cross-linking of gelatin membranes reduces their solubility in water. Another approach to increasing moisture resistance is the use of laminated membranes. GP of films changes considerably below and above the glass transition zone (Arvanitoyannis, Nakayama & Aiba, 1998). Similar behavior was observed for gelatin films with activation energies for oxygen permeability being higher at temperatures above T_m than below T_m At temperatures above T_g gas molecules have to create their own interstitial spaces by separating interchain polymer contacts.

The permeant then diffuses through the polymer matrix along cylindrical voids created by the synchronized rotation of polymer chain segments. On the contrary, at temperatures below T_m gas molecules can diffuse through existing interstitial spaces, thus requiring lower activation energy. Activation energies for oxygen permeability through gelatin films varied from 30 to 90 kJ/mol (Arvanitoyannis, et al., 1998). Similar values (20-120 kJ/mol) have been reported for other types of biopolymer films (Biliaderis, Lazaridou & Arvanitoyannis, 1999).

2.5 Film Preparation

There are several ways to form edible films from agricultural proteins, all of which affect the properties of the final film. Films can be formed via several processes, depending on the starting material. Lipid and wax films can be formed through solidification of the melted material. Biopolymers in solution can form films by changing the conditions of the solution. Applying heat, adding salt or changing the pH may alter conditions in the solution, such that the biopolymers aggregate in a separate film phase.

2.5.1 Solvent Casting

Solvent casting is the preferred method used to form edible protein films for research. Various types of equipment are available for solvent casting of films, from simple casting-plates to more advanced batch and continuous lab coaters.

Table 2.4 Tensile Strength (TS), Young's Modulus (YM), and Percent Elongation (E) of Sorbitol-Plasticized Films Prepared with Low or High Temperature Process from Blends of Gelatin and Chitosan or Hydroxypropyl Starch or Soluble Starch.

Film composition (% w/w)	TS (MPa)	YM (MPa)	E (%)
Low temperature process Gelatin/chitosan/sorbitoI/water			
47.5/47.5/0/15	130.0	2,050	4.1
45/45/5/5	106.0	1,850	8.9
40140115/5	75.0	1,500	27.5
Gelatinlhydroxypropyl starch/sorbitol/water			
47.5/47.5/0/5	95.3	770	2.7
46/46/3/5	79.3	634	6.3
41/41/13/5	71.2	500	19.5
Gelatinlsoluble starch/sorbitoI/water			
44.5/44.5/0/5	60.5	375	1 .8
40/40/4/5	51.3	280	4.6
36/36/14/5	44.9	212	11.5
High temperature process Gelatin/chitosan/sorbitoI/water			
47.5/47.5/0/5	111.0	1,700	3.2
45/45/5/5	77.0	1,300	6.5
40/40/15/5	68.0	1,050	38.7
Gelatinlhydroxypropyl starch/sorbitol/water			
47.5/47.5/0/5	86.7	680	2.4
46/46/3/5	80.4	520	4.8
41/41/13/5	65.8	330	35.0
35.5/35.5/24/5	54.3	270	52.5

From Arvanitoyannis, I., Psomiadou, E., Nakayama, A., Aiba, S., & Yamamoto, N. (1997). Edible film made from gelatin, soluble starch and polyols, Part 3, Food Chemistry, 60, 593-604.

Because it is effective and cost-efficient, the most commonly used method for forming protein film samples for research is by manually spreading dilute film solutions (usually 5-10% solids) of protein and plasticizer into level Petri dishes or plates, and then drying them under ambient conditions or controlled relative humidity. More sophisticated equipment can produce larger protein films by mechanically spreading the solution to a fixed thickness.

Kozempel and Tomasula (2004) developed a continuous process for solvent casting of protein films. This process was demonstrated using casein-based films. Parameters that need to be determined for continuous film production are air temperature, surface properties of the substrate upon which the films are formed, flow rate, and drying time. Films can be dried under ambient conditions, with hot air, infrared energy, or microwave energy. The method of drying can significantly affect the physical properties of the final film, including film morphology, appearance, and barrier and mechanical properties (Perez-Gago & Krochta, 2000).

2.5.2 Extrusion of Edible Films

An alternative to solvent casting is extrusion, which uses elevated temperature and shear to soften and melt the polymer, thus allowing a cohesive film matrix to form. Extrusion of proteins into films has certain advantages over solvent casting. Comparatively, extrusion is faster and requires less energy, due to the fact that more concentrated film solutions can be fed into the extruder. For solvent casting, evaporating ethanol or especially water, is both energy intensive and time consuming, adding to the production costs of edible films. The use of extrusion reduces time and energy inputs to bring the cost of biopolymer film formation into a competitive range with synthetic film production.

2.5.3 Spinning

Wet-spinning is a processing technique most commonly used by the textile industry to form fibers. During fiber formation, a solution of polymer is passed through a pin hold spinneret under pressure. Frinnault, Gallant, Bouchet and Dumont (1997) modified the spinning process to form films from casein, replacing the spinneret with a plate die to form flat films. Protein solutions were extruded into a coagulating bath, and then collected onto a roller. The modified wet-spinning process has also been used to make films from soy protein.

2.6 Effects of Processing on Properties of Protein-Based Films

While there are several methods for forming protein-based films, solvent casting still predominates. Most of the literature reports have investigated the properties of solvent-casted films. The main properties of interest for films are tensile properties (tensile strength, elongation at break and elastic modulus), gas permeability, water vapor permeability and appearance. All of these properties can be affected by the extrinsic conditions used to process and produce the films.

2.6.1 Drying Conditions

The effect of drying conditions (e.g., temperature, relative humidity, type of energy source, etc.) on the properties of protein-based films have been investigated for whey protein and casein as well as other protein sources. In all reported studies investigating the films made from either of the dairy proteins, drying effects on film properties were minor, but still measurable. Using convection drying, they found that an air temperature of 34°C and a low relative humidity drastically reduced the period of constant drying from approximately 1,300 to 400 min compared to ambient air temperature, flow and high relative humidity. Also, increasing the initial total solids of solutions from 6 to 15% dropped the constant drying period from 1,300 to 300 min. While drying time was significantly reduced, differences in film tensile properties were not observed. However, tensile strength and elastic modulus did trend upward as the initial total solids were increased.

2.6.2 Cross-Linking

2.6.2.1 Heat Denaturation

Solvent-cast protein films, for which the solvent is water, are typically formed at room temperature and stabilized through electrostatic interactions, hydrogen bonding, and van der Waals forces among the protein chains. The protein film network may be improved through heat-denaturation, which improves the tensile and barrier properties of solvent casted films by induction of cross-linking between the protein chains. Disulfide bond formation, which occurs with heat-denaturation in protein based films, is often used to modify film properties (Perez-Gago

& Krochta, 1999). Heat-denaturation, and the subsequent polymerization of protein chains, has been studied for two proteins commonly used to make edible films: whey protein and soy protein.

2.6.2.2 Irradiation

There are other methods for inducing cross-linking of protein chains besides heat denaturation. Irradiation has been successfully used to cross-link casein proteins, as well as soy proteins. Water forms hydroxyl radicals when exposed to gamma-irradiation. Aromatic amino acids, such as phenylalanine and tyrosine, are more likely to react with the hydroxyl radicals than aliphatic amino acids. A mechanism hypothesized to explain the radical polymerization process involves the formation of bi-tyrosine linkages between protein chains (Brault, D'Aprano & Lacroix, 1997). When exposed to 32 kGy of energy, soy protein and soy protein-whey protein blends form stronger films than their non-treated counterparts. Brault and toher (1997) did find that caseinate films cross-linked through irradiation became very brittle and required a plasticizer to improve their properties.

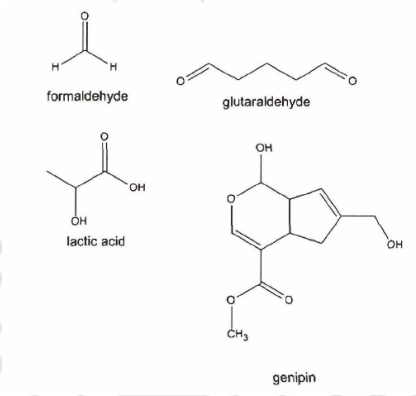
2.6.2.3 Enzymatic and Chemical Cross-linking

Cross-linking of proteins has been induced by both chemical and enzymatic means. Formaldehyde, glutaraldehyde, and lactic acid have been used to cross-link whey proteins through lysine residues (Figure 2.5). However, the cross-linked products are no longer edible, due to the toxicity of the cross-linking agents (Galietta, Gioia, Guilbert & Cuq, 1998). More recently, genipin, a small molecule with no cytotoxicity issues, has been investigated as a protein cross-linking agent.

Transglutaminase is a food grade enzyme that uses the acyl-transferase mechanism to link the gamma-carboxyamide (acyl donor) of a glutamine residue to the gammaamine (acyl acceptor) of lysine residues along protein chains. This enzyme is known to improve elasticity in foods. Originally, transglutaminase was extracted from Guinea pig liver, making it expensive and cost-prohibitive for large-scale production. Transglutaminase is now posible to be obtained at a lower cost from microbial sources, and has been used in both homogenous protein systems and mixtures of proteins to affect tensile and permeability properties. Whey protein, casein, soy, egg albumin and wheat gluten have all been investigated for treatment with this enzyme. The molecular weight of alpha-lactalbumin, beta-lactoglobulin and alpha-lactalbumin beta-lactoglobulin mixtures were shown to increase after transglutaminase treatment, indicative of

cross-linking. Moreover, transglutaminase-treated proteins were also more heat stable relative to untreated ones. The cross-linked protein networks were less soluble, which may improve the water vapor permeability properties of films formed from the cross-linked proteins.

Cross-linking can improve elongation in that the protein chains of the film matrix are associated through covalent bonds instead of relatively weaker van der Waals interactions. After treatment with transglutaminase, solubility dropped to 0.56% under the same experimental conditions. Lower water solubility was expected to lead to lower water vapor permeability; however, WVP actually increased for transglutaminase films.



From Dangaran, K. L., Tomasula, P. M., & Qi, P. (2010). Structure and Function of Protein-Based Edible Films and Coatings. In: M. E., Embuscado, K. C., Huber (Eds). Edible Films and Coatings for Food Applications (pp.25-56). New York, NY:Springer Science Media.

Figure 2.4 Chemical Agents for Protein Chain Cross-Linking

2.6.3 Ionic Strength

The properties of the protein-based films can be affected by the solubility of the protein in a given solvent system. For example, increasing the water solubility of a protein can improve the appearance and increase the elongation of protein films. Similarly, the moisture barrier properties of a film can be improved by decreasing the solubility of the protein in water.

Changing the ionic strength of the solution from which a film is cast represents one of the many ways to affect the solubility of the final film product.

2.6.4 Particle Size

Changes in particle size in relation to either the protein matrix or additives to the film system can affect the properties of the film. Non-soluble particles embedded within a film matrix can affect the permeability of gas through a film. Permeability is affected by the solubility of the diffusing compound in the film and by the diffusion coefficient. Changing the path of travel for a molecule of gas diffusing through the film can change the rate of diffusion. Thus, in a film matrix, insoluble particles create a hindrance to diffusing molecules migrating through the film. For a given mass of an insoluble additive, such as fat, a more tortuous diffusion path can be created by making the particles smaller, thereby lengthening the migration time and improving the barrier properties of the film (Perez-Gago & Krochta, 2001).

2.7 Properties of Edible Films Containing Food Additives

2.7.1 Barrier Properties

Barrier properties of edible films include water vapor permeability, gas permeability (O_2 and CO_2) volatile permeability and solute permeability. Both O_2 and CO_2 permeability is important when respiration or oxidation reactions could affect quality of the food (e.g., fresh or pre-cut fruits and vegetables). Water vapor permeability is an important factor to consider when crispness of a food needs to be maintained during storage. The volatile and solute permeability is an important property to control when diffusion of a compound is to be limited. Barrier properties of edible films prepared from polar polymers (e.g., polysaccharides) are sensitive to humidity. However, protein-based films traditionally have the worst barrier properties. Since lipid is an excellent moisture barrier, incorporation of lipid into a film can reduce water vapor permeability. The structure of the polymer chains and the lipid distribution within the matrix play a significant role in permeability of films. The increase of cohesion between protein polypeptide chains and a uniform dispersion of lipid in the polymer matrix could also be effective for improvement of the barrier properties of films. Molecular crystallinity also significantly affects permeability and the

solubility of films. Lipids can be used in numerous crystalline forms. In general, higher the degree of crystallinity, the lower the permeability of a film

2.7.2 Mechanical Properties

Inclusion of an additive can modify mechanical properties of edible films, because of physical changes induced in the network structure resulting from physicochemical changes in polymer-polymer interactions. Induced changes depend on factors such as the molecular size, polarity, shape, and affinity of the additive to the polymer molecules.

Addition of zein hydrolyzate and use of transglutaminase improved flexibility of a whey protein film without sacrificing water vapor permeability (Oh, Wang, Field & Aglan, 2004). Transglutaminase is effective in introducing covalent bonds into films obtained from slightly deamidated gluten, inducing greater insolubility of treated films, increasing film integrity and the ability to stretch (Larre, Desserme, Barbot & Gueguen, 2000). When combining whey protein isolate and the lactoperoxydase system, tensile properties of whey protein films decreased, limiting its applications to food surfaces. Whey protein isolate films, containing p-aminobenzoic acid as an antimicrobial agent and applied as casing on hot dogs, has similar mechanical properties as those without this additive (Cagri, Ustunol, Osburn & Ryser, 2003).

Salts and organic acids added to edible films might have a cross-linking effect in addition to their known antimicrobial effect. To prevent fungal growth, lactic acid was used to replace glycerol in a pectin film without significant change in dynamic mechanical properties (Hoagland & Parris, 1996). Cross-linking of soy protein isolate with calcium salts resulted in formation of a rigid, three-dimensional film structure with higher mechanical properties than those found in soy protein films without cross-linking. Clucono-glactone contributed to the formation of a homogeneous soy protein-based film structure, due to increased protein-solvent attraction (Park, Rhee, Bae & Hettiarachchy, 2001).

Chemical modification of polymers has a direct effect on mechanical properties. Free cysteinethiol groups of keratin, extracted from chicken feathers, were partially carboxymethylated with iodoacetic acid, after which stable modified keratin dispersions were used for preparation of films by solution casting. The degree of crystallinity in films increased as more cysteine residues

were carboxymethylated, increasing tensile strength, elastic modulus, and elongation at break (Schrooyen, Dijkstra, Oberthiir, Bantjes & Feijen, 2001).

2.7.3 Sensorial Properties

Chitosan and protein-based films showed no effect on the color of salmon and arrow tooth fillets (Sathivel, 2005). Carrots treated with edible cellulose-based coatings had a reduced level of white surface discoloration and higher sensory scores for orange color intensity, fresh carrot aroma, fresh carrot flavor and overall acceptability than non-coated carrots (Howard & Dewi, 1995). When compared to hot dogs prepared with collagen or natural casings, sensory panelists gave hot dogs prepared with whey protein isolate-p-aminobenzoic acid casings superior scores for texture, juiciness, flavor and overall desirability (Cagri, et al., 2003).

2.7.4 Delivery Properties

The movement of a food additive into food and/or between different components of the food could affect its quality. Release kinetics has been modeled for some active packaging systems using food applications. Methylcellulose and hydroxypropyl methylcellulose mixed with lauric, palmitic, stearic and arachidic acid significantly lowered the potassium sorbate release rates as compared to films containing no fatty acids. This approach represents a good option for preserving antimicrobial concentration on food surfaces.

2.7.5 Gas Barrier Properties

Many foods require specific atmospheric conditions to sustain their freshness and overall quality during storage. Packaging a food product under a specific mixture of gases, known as modified atmosphere packaging (MAP), can help maintain quality and safety of such products. To ensure a constant gas composition inside the package, packaging material needs to exhibit certain gas barrier speciticity. Films based on polysaccharides, such as alginate, cellulose ethers, chitosan, carrageenan or pectin, generally exhibit good gas barrier properties (Ben & Kurth, 1995). Addition of lipids or starch to a film formulation can improve both oxygen and oil barrier properties (Wu, Weller, Hamouz, Cuppett & Schnepf, 2002). Addition of a fatty acid through an emulsion with proteins can increase the O_2 and CO_2 permeability of the resulting film, while addition of acetylated monogiycerides actually provides the reverse effect (Wu, et al., 2002).

The conventional approach to producing high-barrier edible films to maintain food in a protective atmosphere involves use of multilayer of different films to collectively obtain the required properties. An example of this multilayer approach might be a biobased laminate, consisting of an outer layer of plasticized chitosan film combined with another layer of polyhydroxyalkanoate (PHA) or alginate, to obtain an appropriate gas barrier.

2.7.6 Water Vapor Permeability (WVP)

The predominantly hydrophophilic character of natural polymers, including many proteins and polysaccharides, result in poor water barrier characteristics (McHugh, 2000). According to Henrique, Tegfilo, Sabino, Ferreira, and Cereda (2007), WVP can be directly related to the quantity of -OH group on the molecule. Also, environmental conditions can significantly affect the WVP. In general, a high relative humidity (90% RH) and a low (-30°C) storage temperature improve WVP. Polysaccharide films are used in Japan for meat products, ham and poultry packaging prior to smoking and steam processing. The film is dissolved during this process, and the coated meat exhibits improved yield, structure and texture, and reduced moisture loss. Lipids have a low affinity for water, and can significantly reduce the WVP of films. For example, it was found that WVP of pure caseinate films could be reduced by over 70% through incorporation of lipid materials (beeswax) (Avena-Bustillos & Krochta, 1993). Solid lipids, such as palm oil, stearic acid, beeswax or paraffin, yielded much smaller WVP values than cellulose ether based films containing liquid lipids, such as oleic acid (Wu, et al., 2002).

Increased cohesion between protein polypeptide chains is thought to improve moisture barrier properties of the protein-based films. For instance, cross-linking of proteins by means of chemical, enzymatic (transglutaminase) or physical treatments (heating, irradiation) was reported to improve water vapor barrier characteristics, as well as the mechanical properties and resistance to proteolysis, of protein films (Brault, et al., 1997).

Improvement of water vapor barrier properties represents a major challenge for the manufacturer of film and coating materials intended for food applications. When comparing water vapor transmittance of various natural polymers, it becomes clear that it is difficult to produce an edible film or coating with water vapor permeability rates compared to those provided by

conventional plastics. If a high water vapor barrier material is required, there are very few biomaterials that can be used.

2.7.7 Solubility

As described previously, most biopolymers in their native states are sensitive to humidity, and are soluble in water. Lipid components of edible origin can be incorporated into films and coatings to alter their susceptibility to water. However, composite films of proteins and lipids can decrease their water-solubility. As for good water vapor properties, a bilayer film would permit to obtain film with better water resistance. However, the process needs to be improved to obtain better stability. Good homogenization will be able to avoid separation of the layers.

Formation of cross-links between polymers can also reduce significantly solubility of edible film constituents. Incorporation of polycaprolactone into alginate-based films following immersion in calcium salt chloride (2 or 20%) decreased water-solubility of the film, exhibiting a recovery yield of 86% after water immersion for 24 h (Salmieri & Lacroix, 2006). It is also expected that polymer-polymer interactions via hydroxyl and carbonyl (H-bonds or covalent reaction) moieties can enhance film insolubility. When applied on beef, bologna or ham, such films were resistant to high humidity conditions, and were able to allow a controlled release of the active compounds (e.g., essential oils from the film to the product during storage.

2.7.8 Thermal Properties

In the packaging industry, heat sealing is widely used to join polymer films. In the heat sealing process, two films are presented together between heated plates or dies. As heat is applied, the surface of the crystalline polymer melts. During cooling, a heat-sealed joint forms due to recrystallization of the polymer (Kim & Ustunol, 2001). Seal strength is an indicator of seal quality. According to Lee (1994), modification of functional groups such as hydroxyl (OH), aldehyde (CHO), and carboxylic acid (COOH) moieties present in polyimide-based films, are responsible for the adhesion strength differences of various films. An increase in interactions between nitrogen- and oxygen-containing functional groups in polyethylene films is responsible for enhancement of seal strength (Possart & Deckhoff, 1999). According to Kim and Ustunol

(2001), hydrogen and covalent bonds involving C-O-H and N-C, may be the main forces responsible for sealed joint formation in films.

2.8 Antioxidant

An antioxidant is a molecule capable of slowing or preventing the oxidation of other molecules. Oxidation is a chemical reaction that transfers electrons from a substance to an oxidizing agent. Oxidation reactions can produce free radicals, which start chain reactions that damage cells. Antioxidants terminate these chain reactions by removing free radical intermediates, and inhibit other oxidation reactions by being oxidized themselves. As a result, antioxidants are often reducing agents such as thiols, ascorbic acid or polyphenols (Sies, 1997).

Although oxidation reactions are crucial for life, they can also be damaging; hence, plants and animals maintain complex systems of multiple types of antioxidants, such as glutathione, vitamin C, and vitamin E as well as enzymes such as catalase, superoxide dismutase and various peroxidases. Low levels of antioxidants, or inhibition of the antioxidant enzymes, cause oxidative stress and may damage or kill cells. As oxidative stress might be an important part of many human diseases, the use of antioxidants in pharmacology is intensively studied, particularly as treatments for stroke and neurodegenerative diseases. However, it is unknown whether oxidative stress is the cause or the consequence of disease.

Antioxidants are widely used as ingredients in dietary supplements in the hope of maintaining health and preventing diseases such as cancer and coronary heart disease. Although initial studies suggested that antioxidant supplements might promote health, later large clinical trials did not detect any benefit and suggested instead that excess supplementation may be harmful (Bjelakovic,Nikolova, Gluud, Simonetti & Gluud, 2007). In addition to these uses of natural antioxidants in medicine, these compounds have many industrial uses, such as preservatives in food and cosmetics and preventing the degradation of rubber and gasoline.

2.8.1 Oxidative Deterioration of Food

Oxidation is initiated by formation of radicals which may be the result of enzyme catalysed reactions like oxygen activation by xanthine oxidase in milk. Light exposure is also

known to induce radical processes in most food products. In milk light absorption thus results initially in protein oxidation followed by lipid oxidation, while in beer free-radical formation has been identified as a primary step in the photodecomposition of isohummulones leading to light struck flavour. In precooked meat and in meat products, iron catalysed cleavage of preformed peroxides is important for formation of hydroxyl (or alkoxyl) radicals initiating oxidative degradation of unsaturated lipids or proteins:

$$Fe^{2+} + H_2O_2 \longrightarrow Fe^{3+} + OH^- + \bullet OH \qquad (2.1)$$

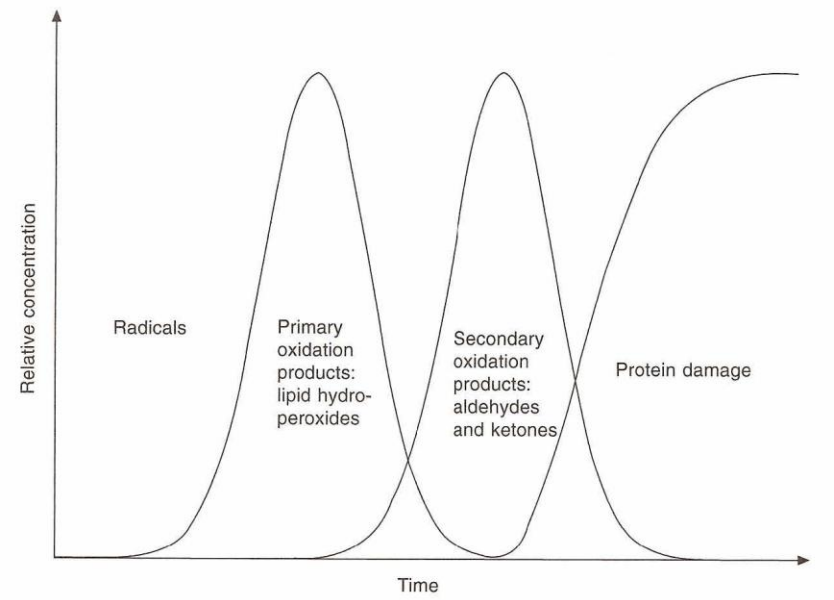
$$Fe^{2+} + ROOH \longrightarrow Fe^{3+} + OH^- + RO\bullet \qquad (2.2)$$

Lipid hydroperoxides are either formed in an autocatalytic process initiated by hydroxyl radicals or they are formed photochemically. Lipid hydroperoxides, known as the primary lipid oxidation products, are tasteless and odourless, but may be cleaved into the so-called secondary lipid oxidation products by heat or by metal ion catalysis. This transformation of hydroperoxides to secondary lipid oxidation products can thus be seen during chill storage of pork. The secondary lipid oxidation products, like hexanal from linoleic acid, are volatile and provide precooked meats, dried milk products and used frying oil with characteristic off-flavours. They may further react with proteins forming fluorescent protein derivatives derived from initially formed Schiff bases (Tappel, 1956).

2.8.2 Classifying Natural Antioxidants

Antioxidants may be active at different stages of the progression of oxidation in food systems. The tocopherols, of which a-tocopherol is vitamin E, are associated with the lipid fraction of food, scavenge radicals and become chain-breaking in the autocatalytic process of lipid oxidation.

Ascorbic acid is water-soluble and may deplete oxygen in the product or regenerate atocopherol from its one-electron oxidised form in the lipid/water interface, as has been exploited in patented protection systems for lard and marine oils. Metal binders like ethylenediamine tetraacetic acid (EDTA) prevent metal catalysis during initiation of oxidation and have been found to be effective in protecting products like mayonnaise by preventing the formation of radicals in the interphase between water and lipid in the food emulsion.



From Dangaran, K. L., Tomasula, P. M., & Qi, P. (2010). Structure and Function of Protein-Based Edible Films and Coatings. In M. E., Embuscado, K. C., Huber (Eds). Edible Films and Coatings for Food Applications (pp.25-56). New York, NY:Springer Science Media.

Figure 2.5 Progression of Oxidation in a Food System from Formation of Radicals Through Primary and Secondary Lipid Oxidation Products to Protein Damage.

Carotenoids yield protection against light-induced oxidation processes, as they deactivate singlet-oxygen formed in sensitized processes, or by absorbing harmful light, as was found for dairy products, and probably also by scavenging of free radicals.

Plant phenolic compounds have antioxidative properties that clearly demonstrated in numerous model systems. It is, however, interesting to note that plant phenols apparently operate as antioxidants by all of the mechanisms described above for the different types of antioxidants. Plant phenols are thus radical scavengers and chain-breaking antioxidants under certain conditions. Plant phenols may also regenerate other antioxidants and act synergistically with chain-breaking antioxidants under other sets of conditions. Polyphenols such as the flavonoids and anthocyanidins have metal chelating properties and bind otherwise redox-active metal ions.

Finally plant phenols protect plants against UV-irradiation and light-induced oxidative damage and may have similar effects in food and beverages.

2.8.3 Nutritive and Non-Nutritive Antioxidants

The chemical formulae for a variety of plant phenols are given, including examples of simpler phenols, such as cinnamic acid derivative, and of tocopherols, flavonoids, flavonoid glycosides and anthocyanidins. The flavonoids include the following subclasses: flavanones (taxifolin), flavones (luteolin), flavonols (quercetin) and flavanols (catechidepicatechin). The flavonoids and the anthocyanidins and proanthocyanins are all 1,3-diphenyl propane derivates, which, together with the cinnamic acid derivatives, are the most widespread non-nutritive antioxidants.

Natural antioxidants may be classified according to their nutritive value or according to their solubility. The hydrophobic vitamin E and the hydrophilic vitamin C are thus important both as nutrients and as antioxidants. The nonnutritive antioxidants may similarly be divided into lipid-soluble and water soluble antioxidants, which will also form the basis for a discussion of exploitation of combinations of antioxidants in order to improve protective effects.

2.9 Analysing Antioxidant Activity in Food

Epidemiological studies have shown an inverse correlation between the intake of fresh fruit and vegetables and the risk of cardiovascular diseases and certain forms of cancer. These effects have been attributed to the antioxidant content of fruits and vegetables, although it has been difficult to show a direct positive effect on markers of oxidative status after dietary intervention. In contrast, positive antioxidant effects of plant extracts rich in polyphenols have clearly been demonstrated in a large variety of foods and beverages using various methods for detection of lipid and protein oxidation. It has often been assumed that the basic antioxidant mechanism of plant phenols is the same in the living organism following intake as it is in foods rich in such antioxidants or enriched with plant extracts. The discussion of antioxidant mechanism will be rather general in order to cover both aspects, although most examples will be related to the better documented effects in model systems and in food and beverages.

Mechanism of Antioxidation

Among the plant phenols, the flavonoids and the anthocyanidins, belonging to the 1,3-diphenylpropans, have been studied in most detail, mainly because of their potential health benefits. With more than 4,000 different flavonoids known, systematic studies of the effects of variation in molecular structure on physico-chemical properties of importance for antioxidative effects have also been possible. Flavonoids were originally found not to behave as efficiently as the classic phenolic antioxidants like a-tocopherol and synthetic phenolic antioxidants in donating a hydrogen atom to a lipid peroxyl radical, the chain-carrying species in lipid autoxidation. Both the inhibition rate constant and the number of radicals quenched by each flavonoid showed a strong dependence on the antioxidant concentration, an observation not in agreement with the classic antioxidant mechanism. Solvent effects are, however, important and hydrogen-accepting 'water-like' solvents induce dramatic decreases in the hydrogen-atom donating capability of the phenols. The inhibition reaction in the autocatalytic lipid oxidation by a phenolic antioxidant occurs in two steps:

$$ROOH\bullet + Ar\text{-}OH \longrightarrow (ROO\bullet \ \ldots HO\text{-}Ar)_{cage} \leftrightarrow (ROOH\ldots O\text{-}Ar)_{cage} \qquad (2.3)$$

$$(ROOH \ \ldots O\text{-}Ar)_{cage} \longrightarrow ROOH + Ar\text{-}O\bullet \qquad (2.4)$$

The initial complex formation of equation (2.3) may be hindered due to a preferential formation of a hydrogen bonded complex between the flavonoid, Ar-OH, and solvent molecule (Foti &Ruberto, 2001). Such an effect was clearly seen for the flavonol quercetin and the flavanol epicatechin, flavonoids which are both widespread in the plant kingdom. The solvent effect between chlorobenzene and tert-butyl alcohol for the inhibition reaction corresponding to the net reaction of equations (2.3) and (2.4) was a factor of 20 for the flavonoids compared to a factor of 4 for atocopherol, with the rate constant being slower in the “water-like” solvent tert-butyl alcohol than in the non-hydrogen-bonding solvent chlorobenzene. In contrast to a-tocopherol, the flavonoids will only be retarders of lipid oxidation without a distinct lag-phase when the phenol is in contact with water or a “water-like” solvent. Notably, certain flavonoids like quercetin are almost as efficient as a-tocopherol in solvents without hydrogen-bonding capabilities. Specific solvent effects in the micro-environment of the flavonoid may accordingly provide the rationale for the variation seen for the antioxidative capacity of the same flavonoids. Flavonoids occur

primarily as glycosides in plants, and in foods these water-soluble forms are not expected to be efficient chain-breaking antioxidants due to the hydrogen-donor accepting capability of water. In aqueous environments, another mechanism may, however, be important, since electron-transfer becomes facilitated through stabilization of an ion-pair (Foti & Ruberto, 2001):

$$ROO\bullet + Ar\text{-}OH \longrightarrow (ROO^{-} \ \ldots + \bullet HO\text{-}Ar)_{cage} \longrightarrow \ ROOH^{-} + H^{+} + ArO\bullet \qquad (2.5)$$

Under aqueous conditions, flavonoids and their glycosides will also reduce oxidants other than peroxyl radicals and may have a role in protecting membranal systems against pro-oxidants such as metal ions and activated oxygen species in the aqueous phase. Rate constants for reduction of superoxide anion show flavonoids to be more efficient than the water-soluble vitamin E analogue trolox.

2.10 Antioxidant Interaction in Food Models

2.10.1 Analysis of Phenols in Foods

Knowledge of the identity of phenolic compounds in food facilitates the analysis and discussion of potential antioxidant effects. Thus studies of phenolic compounds as antioxidants in food should usually by accompanied by the identification and quantification of the phenols. Reversed-phase HPLC combined with UV-VIS or electrochemical detection is the most common method for quantification of individual flavonoids and phenolic acids in foods, whereas HPLC combined with mass spectrometry has been used for identification of phenolic compounds. Normal-phase HPLC combined with mass spectrometry has been used to identify monomeric and dimeric proanthocyanidins. Flavonoids are usually quantified as aglycones by HPLC, and samples containing flavonoid glycosides are therefore hydrolysed before analysis.

The use of HPLC for quantification of phenols is often limited to a single class of phenolics and then often only to low-molecular weight compounds that are available as standards. It is, therefore, often necessary to use colorimetric assays such as the Folin-Ciocalteau assay which rely on the reducing ability of phenols to quantify the amount of total phenolics in a sample. The degree of condensation of polyphenols can be quantified by colorimetric assays such as the acid-butanol assay and the vanillin assay (Schofield, Mbugua & Pell, 2001).

2.10.2 Studying the Actions of Antioxidants in Food

The conclusions about the role phenol plays as an antioxidant in real food systems are often reached by comparing the oxidative behaviour of food samples with differcnt contcnts of phenolic compounds. The variations in phenolics are usually obtained by using products made from different raw materials. However, using different raw materials not only affects the levels of phenols, but also affects the levels of transition metals and enzymes which can have profound effects on the oxidative behaviour of the finished product. It is, therefore, often advantageous to study the oxidative behaviour of samples derived from a single batch of production where the level of phenols has either been increased by addition or decreased by removal (e.g. by fining) (Andersen & Skibsted, 2001).

A number of methods are available for following the oxidative behaviour of food samples. The consumption of oxygen and the ESR detection of radicals, either directly or indirectly by spins trapping, can be used to follow the initial steps during oxidation (Andersen & Skibsted, 2001). The formation of primary oxidation products, such as hydroperoxides and conjugated dienes, and secondary oxidation products (carbohydrides, carbonyl compounds and acids) in the case of lipid oxidation, can be quantified by several standard chemical and physical analytical methods (Honwitz, 2000). It is preferable that the action of phenolic antioxidants should be studied during realistic processing and storage of food; however, the oxidative changes during storage are often too small or too slow to be detected within a convenient timeframe. It is, therefore, often necessary to accelerate the testing of the oxidative behavior by subjecting the food system to high temperatures, adding transition metals, radical initiators such as peroxides or azo compounds, or by irradiation with intense UV or visible light. The accelerated testing can potentially distort the action of antioxidants. Heating will, for example, overestimate the role of reactions with high activation energies, and disturb the distribution of components such as polyphenols between the different phases in complex foods. Accelerated testing has been found to be able to predict the flavour stability of beer.

2.10.3 Antioxidant Assays

The use of real food systems for detailed studies of antioxidants is complicated by a large number of factors which are often unknown or cannot be controlled due to the complex nature of

foods. Using simplified model systems, which mimic the main features of a given food system, or antioxidant assays for quantifying the antioxidant action, can be very helpful in clarifying the action of potential antioxidants (Frankel & Meyer, 2000). The extrapolation of conclusions based on the behaviour of model systems or antioxidant assays to real complex food systems should generally be done with great care, and should ideally be based on results from more than one model system or assay (Frankel & Meyer, 2000).

The terminology describing the action of antioxidants is unfortunately not clear. Terms such as antioxidant power, antioxidant effectiveness, antioxidant ability, antioxidant activity, and antioxidant capacity are often used interchangeably and without discrimination. Here we use the term 'antioxidant activity' as meaning a measure of the rate of antioxidant action, and the term 'antioxidant capacity' as meaning a measure of the extent of antioxidant action, i.e. the amount of radicals or intermediates and products produced during oxidation that are quenched by a given antioxidant. Thus antioxidant activity is related to the kinetics of the antioxidant action and antioxidant capacity to the stoichiometry.

Quantification of antioxidant action usually relies on the reducing ability of antioxidants, measured either by electron transfer, reaction (2.8), or by hydrogen atom transfer reactions, reaction (2.9):

$$R\bullet + Ar\text{-}O^{-} \longrightarrow R^{-} + Ar\text{-}O\bullet \qquad (2.8)$$

$$R\bullet + Ar\text{-}OH \longrightarrow RH + Ar\text{-}O\bullet \qquad (2.9)$$

Electrochemical measurement of redox potentials gives direct information about the ability of antioxidants to donate electrons. The electron transfer antioxidant capacity of antioxidants is commonly quantified by the TEAC assay and the FRAP assay. The TEAC assay (TEAC = trolox equivalent antioxidant capacity) relies on the reduction of the coloured cation radical of 2,2'-azinobis(3-ethylbenzothiazoline-6-sulphonate) (ABTS), and the antioxidant capacity is quantified as amount (mM) of the water soluble-vitamin E analogue trolox that produces the same effect as the test sample (Frankel & Meyer, 2000). For flavonoids with high antioxidative capacity, a stoichiometric factor far larger than 2 is, however, being found in simple solutions of the flavonoid in the TEAC assay; an observation which is difficult to understand. The FRAP assay (FRAP = ferric reducing antioxidant power) measures antioxidant capacity by the

reduction of the ferric 2,4,6-tripyridyl-s-triazine complex to the blue ferrous complex (Benzie & Strain, 1996).

The reduction of the stable 1,l -diphenyl-2-picrylhydrazyl radical (DPPH.) has been used to assess the efficiency of antioxidants in beverages, vegetable oils and of pure phenolic compounds, reaction:

$$DPPH\bullet + Ar\text{-}OH \longrightarrow DPPHH + Ar\text{-}O\bullet \qquad (2.10)$$

DPPH has an intense absorption maximum around 520 nm (Yordanov & Christova, 1997), and antioxidant capacity and activity measured by the reduction of DPPH are easily quantified by VIS-spectroscopy (Brand-Williams, Cuvelier & Berset, 1995). The stable radicals Fremy's salt (potassium nitrosodisulphonate) and galvinoxyl(2,6-di-tert-butyla-(3,5-di-tert-butyl-4-oxo-2,5-cyclohexadiIe-ny-1 idene)-p-tolyloxy radical) have been used in a similar manner but with ESR detection, which can be used with samples that are not optically transparent. The reactivity of antioxidants towards short-lived radicals, such as hydroxyl and peroxyl, can be tested by the ORAC assay (ORAC = oxygen radical absorbing capacity). This assay furthermore allows the testing of pro-oxidant behaviour. The assay is based on quenching the fluorescence from the protein 0-phycoerythrin in the presence of radicals. The activity of antioxidants in retarding lipid oxidation in heterogenous systems can be assessed by measuring the oxygen consumption in linoleic acid oil-in-water emulsions or in methyl linoleate emulsions where the oxidation is initiated by hypervalent myoglobin species (Madsen, Andersen, Jorgensen & Skibsted, 2000).

2.11 Polyphenols in Processed Food

Herbs and spices have been used by mankind since prehistoric time to preserve foods. Around the Mediterranean oregano and rosemary were widely used and in the Nordic countries thyme was added to sausages and lard. The modern development in the use of such plant material for protecting processed food against oxidation started half a century ago. The rich use of a variety of spices, mainly from the plant family Labiatae, has been reviewed and presented in tabulated form. Other substances, such as tea, coffee and waste from the manufacture of various vegetable products and beverages, are now being considered as sources for phenolic compounds

to prevent or retard oxidation processes in food and to replace synthetic antioxidants. Some of these sources have the obvious advantage that they will not dominate the flavour of the food as do some species like clove. For each plant material, extraction conditions need to be optimised with respect to solvent polarity and physical conditions. In agreement with the high antioxidant activity of the tea catechins found in model systems, green tea is among the more promising sources of natural antioxidants. A few examples from recent studies will illustrate some of the developments in the exploitation of various sources of antioxidants for protection of processed foods.

2.11.1 Green Tea

Tang, Kerry, Sheehan and Buckley (2001) found that various raw and cooked meat and fish products had a significantly higher antioxidant capacity when treated with tea catechins than they did when treated with α-tocopherol. Part of the antioxidative effect may be due to protection or regeneration of atocopherol already present in the meat or in the fish muscles. A high affinity of the tea catechins for lipid bilayers of muscles together with the radical scavenging activity was suggested as providing the explanation for the high antioxidant activity. The tea catechins showed a pro-oxidative effect in corn oil-in-water emulsions, but a significant antioxidant effect in liposomes, a difference which also could be understood on the basis of the high affinity of the catechin to liposome surfaces (Huang & Frankel, 1997). In another study, green tea extracts have shown a better antioxidant capacity than rosemary extracts in canola oil, pork lard and chicken fat heated to 100°C (Chen, et al., 1998).

2.11.2 Spices

Based on a review of the numerous studies of a great variety of food products clove, rosemary and sage were concluded generally to be among the most potent antioxidative spices. In studies comparing different spices and extracts thereof, clove seems to have the largest antioxidant potential in oil-in-water emulsions while rosemary and sage have the largest antioxidative effect in lard. Rosemary and sage, in combination with citric acid, seem to improve the oxidative stability of frying oil even more than each of the two spices alone. Extraction of antioxidants from spices depends on the polarity of the solvent. Rosemary and sage mainly contain the polar components, rosmarinic acid, carnosol and carnosolic acid of which rosmarinic

acid is the most water-soluble, and extracts seem more effective in bulk oil systems compared to oil-in-water emulsions, due to affinity for the lipid-air interface.

2.11.3 Olive Oil

Olive oil is renowned for its high oxidative stability and contains polyphenols with high antioxidative capacity. Phenolic substances extracted from extra virgin olive oil have been shown to be effective in protecting minced tuna cooked and stored in brine against oxidation, but to yield less protection of the same product cooked and stored in refined olive oil (Medina, Satue-Gracia, German & Frankel, 1999). The higher antioxidative activity in the aqueous system was explained by the greater affinity of the phenol of the extract towards the polar interphase between water and the fish oil. Isolated phenols, especially myricetin, tannic acid and ellagic acid, showed similar effect on lipid oxidation in steam cooked fish (Ramanathan & Das, 1993).

2.11.4 Waste from Fruit and Vegetable Products

Evening primrose meal is an example of a by-product with a great potential as a source of natural antioxidants as it has proved effective in meat systems. Other waste products like hulls from a variety of grains have also been considered. The large production in China of peeled rhizomes of edible lotus gives large quantities of waste and the extract of the rhizome knots especially has a high content of polyphenols of great potential for use as food additive.

2.11.4.1 Longan

Longan is an evergreen tree, *Dimocarpus longan* Lour. (syn. *Euphoria longana* Lam.), of the Sapindaceae family, which is widely grown in Southern China, India, and Southeast Asia. Longan fruit is one of the most favoured tropical fruits in China. In recent years, production of longan fruits in Thailand has dramatically increased due to continuous development of plantations and improvement of agricultural management. Currently, longan arils are consumed as fresh and processed fruits while the seeds, which account for about 17% of the fresh weight of whole fruits, are discarded as waste or burned as fuel. However, longan seeds have been found to be a rich source of antioxidant phenolic compounds which are promising as functional food ingredients or natural preservatives. Soong and Barlow (2005) reported longan seeds contained high levels of gallic acid, corilagin and ellagic acid, which have been proven to possess strong free radical-scavenging activity. However, Rangkadilok and other (2007) reported that the

aforementioned three characterised polyphenols might not be the only contributors for the high antioxidant activity of longan seeds. Instead, other phenolic constituents might also play important roles. On the other hand, besides gallic acid and ellagic acid, many other phenolic glycosides such as monogalloyl-glucose, monogalloyl-diglucose, digalloyl-diglucose, penta- to heptagalloyl-glucose, ellagic acid-pentose conjugate, galloyl-hexahydroxydiphenoyl (HHDP)-glucopyranose, pentagalloyl-HHDP-glucopyranose, etc., were found in longan seeds.

2.11.4.2 Lychee

Lychee (*Litchi chinensis* Sonn.), an evergreen tree belonging to the Sapindaceae family, is widely cultivated in subtropical area for fruits. Lychee fruits are favoured by consumers for their juicy and sweet arils and attractive red pericarps. Over the past decade, annual production of lychee in China has increased steadily due to plantation developments and the improvement of agricultural management. Currently, lychee arils are consumed as fresh and processed fruits, while lychee seeds are mainly discarded as waste. Recent work has indicated that lychee seed could be used as a readily accessible source of natural antioxidants, which were promising as functional food ingredients or natural preservatives (Prasad, Wang, Tang, Huang & Jackson, 2009). Furthermore, it was reported that lychee seed possessed antihyperglycemic, antihyperlipidemic, antiplatelet, and antiviral activities (Chen, et al., 2007), as well as inhibitory effect on the tumour growth in sarcoma S-180- and hepatoma-implanted mice (Xiao, et al., 2004). Previous chemical studies have revealed that lychee seed contained high levels of flavan-3-ol derivatives including (-)-epicatechin and proanthocyanidins A1 and A2 (Ding, Wang, Zhao & Du, 2006). As many flavan-3-ol derivatives have been proven to possess antioxidant, cardioprotective, and anticarcinogenic properties in human and many animal models (Prior & Gu, 2005), flavan-3-ol derivatives in lychee seed could be considered as the major constituents responsible for their pharmacological effects accordingly. However, published literature concerning the components out of flavan-3-ol derivatives from lychee seed remain very scarce (Tu, Luo & Zheng, 2002).

2.11.4.3 Passion fruit

Passion fruit is a perennial woody creeper which is indigenous to the tropical regions of America. The two main commercial varieties are purple passion fruit (*Passiflora edulis* L.), and yellow passion fruit (*P. edulis f. flavicarpa*) that mainly cultivated in the northern part of Thailand. The preliminary results showed that one ton of passion fruits produced about 300

kilograms of juice, 110 kilograms of seed, 320 kilograms of inner peel and 225 kilograms outer peel. However, there are considerably higher ratios of seed and other by-products arising from passion fruit juice processing industry and derived products. It would be beneficial, in improving the complete utilization of the seeds and other wastes, if they could be used as a source of natural food additives and ingredients. The extracts of different parts of the passion fruit plant contain flavonoids, alkaloids, cyanogenic compounds, glycosides, vitamins, minerals, and terpenoid compounds (Zibadi & Watson, 2004). As defined in recent reviews, mixtures of bioflavonoids in the diet and as supplements have been recognized to play a protective role by reducing the prevalence of cardiovascular diseases (Slayback & Watson, 2006; Zibadi, Larson & Watson, 2007).

2.11.4.4 Rambutan

Rambutan *(Nephelium lappaceum* L.) is a tropical tree which belongs to the Sapindaceae family and also the fruit of this tree. Rambutan is widely distributed throughout Indonesia, Malaysia and Southeast Asia and is identified in some countries, by different names. Rambutan is a tasty fruit and it is very popular in many countries. The study of Thitilertdecha, Teerawutgulrag and Rakariyatham (2008) showed that the *N. lappaceum* L. peel and seed can be considered as an easily accessible source of natural antioxidants and antibacterial agents (542.2 mg/g peel and 58.5 mg/g seed).

2.12 Methods of Phytochemical Extraction

Extraction is a process whereby the desired constituents of a plant are removed using a solvent. The primary ways for extraction of organic molecules of interest to biologists and medical investigators involve breaking open the cells. Cell rupturing is carried out in a variety of ways. The method used depends on the type of tissue used. For plant cells grown in cell suspension culture or for plant callus tissue, a French press or a sonicator can be used to break open the cell. Plant cells grown in culture can also be ruptured with a glass tissue homogenizer. Silicified or highly lignified plant tissues within the organs such as roots, stems, leaves, fruits and seeds are usually frozen and pulverized using liquid nitrogen in a mortar and a pestle. Softer

tissues can be ground in a small volume of buffer in a mortar, washed white sand and a pestle to rupture the cells.

When the cells have been ruptured, the extraction is performed using the appropriate techniques. Water soluble compounds and proteins are extracted in buffers or water. Organically soluble compounds are extracted with organic solvents. Boiling ethanol is a good all-purpose solvent for preliminary extraction (Harborne, 1998). The success of the extraction with alcohol is directly related to the extent chlorophyll (when isolating substances from green tissue) is removed into the solvent. The most common chemical procedure for obtaining organic constituents from dried plant tissues is to continuously extract powdered material in a Soxhlet apparatus with a range of solvents, starting with petroleum ether and chloroform (tolseparate lipids and terpenoids) and then using alcohol and ethyl acetate (for more polar compounds). This method is useful when working on the gram scale. However, one rarely achieves complete separation of constituents and the same compounds may be recovered (in varying proportions) in several fractions. The extract obtained is clarified by filtration and is then concentrated in vacuum in a rotary evaporator. To prevent fungal growth, a trace of toluene may be added to the concentrated extracts and they should be stored in refrigerator. There are short cuts in extraction procedures which one learns with practice. The concentrated extract may deposit crystals on standing (Raaman, 2006).

2.12.1 Organic Solvent Extraction

Organic solvent extraction is one process for separating the desired substance from plant material. Fresh plants and dried plants are used for extraction. The plants are first ground and then thoroughly mixed with a solvent such as hexane, benzene, or toluene inside a tank. The choice of solvent depends on several factors including the characteristics of the constituents being extracted, cost, and environmental issues. If the end product contains trace amounts of residual solvent, a nontoxic solvent must be used. Once the solvent dissolves the desired substances of the plant, it is called “miscella.” The miscella is then separated from the plant material. There are a number of techniques for solvent extraction, which include maceration, percolation, and countercurrent extraction. The following is a brief description of each (Raaman, 2006).

2.12.1.1 Macerotion

This method involves soaking and agitating the solvent and plant materials together. The solvent is then drained off. Remaining miscella is removed from the plant material through pressing or centrifuging. This method does not totally extract the active ingredients from the plant materials

2.12.1.2 Percolation

With this method, the plant material is moistened with solvent and allowed to swell before being placed in one of a series of percolation chambers. The material is repeatedly rinsed with solvent until all the active ingredients have been removed. Solvent is reused until it is saturated. New solvent is used on plant material that is almost completely exhausted, and then re-used on subsequently less exhausted batches. This method is more effective in obtaining active ingredients than the maceration technique.

2.12.1.3 Cold percolation

This is a traditional method of extraction used by herbalists throughout the world and it is very simple. Above a flask or vessel, a cone or tube is suspended. The bottom of the tube has a perforated base which holds powdered plant material. Solvent is poured into the top of the tube where it soaks through the plant sample leaching out the extract and then falling out the bottom end of the tube into the flask. If desired, the percolation tube can be wrapped in heating tape to help facilitate the extraction.

2.12.1.4 Countercurrent extraction

This is a highly effective process whereby solvent flows in the opposite direction to plant material. Unlike maceration and percolation, which are batch processes, this method is continuous. Screw extractors and carousel extractors are two types of equipments used for countercurrent extraction.

2.12.2 Extraction with Supercritical Gases

This is a method for extracting active ingredients using gases. The plant material is placed in a vessel that is filled with a gas under controlled temperature and high pressure. The gas dissolves the active ingredients within the plant material, then passes into a separating chamber where both pressure and temperature are lower. The extract precipitates out and is removed

through a valve at the bottom of the chamber. The gas is then reused. Gases suitable for supercritical extraction include carbon dioxide, nitrogen, methane, ethane, ethylene, nitrous oxide, sulfur dioxide, propane, propylene, ammonia, and sulfur hexafluoride. An advantage of supercritical extraction is that it can take place at low temperature, thus preserving the quality of temperature-sensitive components.

2.12.3 Steam Distillation

Steam distillation is another method for extracting active ingredients from medicinal plants. The plant material is loaded onto perforated plates inside a cylindrical tank or still, and steam is injected from below. The steam dissolves the desired substances in the plant, then enters a condenser where it is condensed back into a liquid (Stichlmair & Fair, 1998). This condensate then passes into a flask, where the extract either rises to the top or settles to the bottom and is separated from the water. Distillation is complete when there is no more extract present in the condensate. The water may be reused, and the extract is purified through centrifuging and filtering.

2.12.4 Soxhlet Extractor

The original Soxhlet extraction equipment was developed by Franz Von Soxhlet, a German agricultural chemist, in the early part ofthis century. In Soxhlet extractor, within an enclosed flask there is an inverted condenser pointing down into the flask from the top. Just below that condenser will be suspended either what's called a soxhlet basket or a recovery vessel depending on whether you are extracting or recovering solvent. The condenser will have cold liquid circulating through it to keep the condenser cold. In the bottom of the main flask, solvent is placed. To do an extraction, the powered plant material is placed in the soxhlet basket which is a vessel with perforated sides and bottom so that liquid can fall through it. When gentle heat is applied to the main flask, the solvent begins to evaporate and the solvent vapors reach the cold condenser at the top of the flask and begin to liquefy on the sides of the condenser (much the same way that a cold glass of water becomes wet on the outside of itself on a hot day).

The re-condensed solvent on the sides of the condenser begins flowing down the sides of the condenser and begins dripping off of drip points on the end of the condenser. This solvent drips into the top of the soxhlet basket where it saturates the herb being extracted. The solvent

flows through the basket and out of the holes in the bottom of the basket carrying the extract with it into the bottom of the flask. The extract laden solvent falling from the soxhlet basket is dark in color and as it becomes clearer, one can know that the plant material is leached out and the process is finished. At this point one can do one of 3 things: (1) Stop the operation and pour the extract infused solvent out of the main flask. (2) Hook up the recovery vessel and remove the solvent from your extract which generally leaves a paste behind. (3) Dump and squeeze out the spent plant material in the soxhlet basket, then start a fresh basket of herb in the extractor using the same solvent which continually re-distills and extracts regardless of how much extract is infused into it in the bottom of the main flask.

2.12.5 Accelerated Solvent Extractor

To facilitate quick extraction of many samples, Accelerated Solvent Extractors are available. Using this equipment, we can get extracts of 20 samples at a time without much wastage of solvents. Extractions that normally take hours can be done in minutes using Accelerated Solvent Extraction System (ASE). Compared to techniques like Soxhlet and sonication, ASE generates results in a fraction of the time. In addition to speed, ASE offers a lower cost per sample than other techniques by reducing solvent consumption by up to 90%. The advantages of Accelerated Solvent Extractor are extractions in minutes, dramatic solvent reduction, wide range of applications, automated Extraction of up to 24 samples, sample cell sizes: 1, 5, 11, 22, and 33 mL, collection bottle 40 or 60 mL, and operating pressure 500-3000 PSI (35-200 Bar). Time is of the essence in most analytical laboratories. By using conventional liquid solvents at elevated temperatures and pressures, ASE increases the efficiency of the extraction process. Increased temperature accelerates the extraction kinetics, and elevated pressure keeps the solvent below its boiling point, thus enabling safe and rapid extractions. When an extraction takes only 15 min to complete, you can get to the data faster than ever before. Although ASE uses the same aqueous and organic solvents as traditional extraction methods, it uses them more efficiently.

Therefore, the use of solvent volume per sample is less and there is an instant savings for the operation. A typical ASE extraction is done using only 15-45 mL of solvent. ASE operates by moving the extraction solvent through an extraction cell containing the sample. The sample cell is heated by direct contact with the oven. The extraction is performed by direct contact of the

sample with the hot solvent in both static and dynamic modes. When the extraction is complete, compressed nitrogen moves all of the solvent from the cell to the vial for analysis. The filtered extract is collected away from the sample matrix, ready for analysis. By using elevated temperature and pressure, ASE makes more efficient use of extraction solvents, providing lower cost per extraction. When compared to other extraction techniques, ASE provides the most efficient use of costly solvents (Raaman, 2006).

2.13 Purification and Concentration of Miscella

Miscella that has been separated from the plant material generally contains some unwanted substances such as tannins, pigments, microbial contaminants, or residual solvent. Methods such as decanting, filtration, sedimentation, centrifuging, heating, freeze drying, adsorption, precipitation, and ion exchange are used to separate impurities from the miscella. Sometimes the miscella resulting from solvent extraction is used as the final dosage form. This is known as a “fluid extract”. The miscella is sometimes concentrated in order to increase the proportion of the desired substance. This is done through evaporation or vaporization. Solvent is generally recovered and reused. The degree of concentration depends on the desired end product. Equipment for concentrating the miscella may include descending film, thin layer or plate concentrators. Any method used to concentrate the miscella must avoid excessive heat because the active compounds may be subjected to degradation.

Sometimes extracts are dried completely using vacuum freeze dryers, cabinet vacuum dryers, continuously operating drum or belt dryers, microwave ovens, or atomizers. The technique for drying depends on the stability of the product and the amount of moisture that must be removed. The resulting powdered extract is less subject to microbial contamination than are liquid extracts.

The rotary evaporator is a device for gently and efficiently evaporating solvents from a mixture. It consists of a heated rotating vessel (usually a large flask) which is maintained under a vacuum through a tube connecting it to a condenser. The rotating flask is heated by partial immersion in a hot water bath. The flask's rotation provides improved heat transfer to the contained liquid; the rotation also strongly reduces the occupancy of 'bumps' caused by

superheating of the liquid. The solvent vapors leave the flask by the connecting tube and are condensed in the condenser section. The condenser section is so arranged that the condensed vapors drain into another flask where they are collected. It is a very efficient way of rapidly removing large quantities of solvent. The major use in chromatography is the recovery of non-volatile solutes in preparative chromatography and the recovery of solvents for recycling. The device is also used for preparing coated supports for gas chromatography. For this application a weighed amount of support is placed in the flask, the required amount of stationary phase dissolved in excess of solvent is added to the solid and mixture tumble dried. This procedure has two advantages; as well as drying the support; it ensures a very even distribution of the stationary phase throughout the support. Rotary evaporators can accommodate volumes as large as 3 litres. They are found in almost every organic laboratory (Raaman, 2006).

CHAPTER 3

METHODOLOGY

3.1 Materials

3.1.1 Raw Materials

Farmed giant catfish (*Pangasianodon gigas*) with an average total length of 75–80 cm (20-25 kg) were obtained from a Pla Thong farm, Phan district, Chiang Rai.

Food grade gelatin from beef skin (EXPORT A: Gel strength 150-170 Bloom, 20 mesh) was obtained from Nutrition Science Co., Ltd. (Bangkok, Thailand).

Sample of fruits seeds, longan, lychee, passion fruit and rambutan were collected in Chiang Rai, Thailand and immediately transported to the laboratory.

Soybean oil (A-ngun) purchased from connivance store that not contain antioxidant in product.

3.1.2 Chemicals and Reagents

Table 3.1 Chemical Lists

Part	Chemical
1 Extraction of Gelatin	- Sodium hydroxide (NaOH) (Fluka)
	- Glacial acetic acid (CH_3COOH) (J.T. Beaker)
2 Protein Determination	- Bovine serum albumin (BSA) (Fluka)
	- Distilled water
	- Sodium potassium tartate ($KNaC_4H_4O_6$) (Univar)

Table 3.1 (Continued)

Part	Chemical
2 Protein Determination (cont.)	- Copper sulphate pentahydrate ($CuSO_4.5H_2O$) (Uniivar)
	- Sodium citrate ($Na_3C_6H_5O_7.2H_2O$) (Univar)
	- Sodium carbonate (Na_2CO_3) (Univar)
	- Sodium potassium tartrate ($NaKC_4H_4O_6.4H_2O$) (Univar)
	- Folin-Ciocalteu reagent (BDH AnalaR)
3 Proximate Determination	- Sulfuric acid (H_2SO_4) (Merck)
	- Cupric Sulphate ($CuSO_4$) (Fluka)
	- Sodium Sulphate (Na_2SO_4) (Fluka)
	- Sodium hydroxide (NaOH) (Fluka)
	- Hydrochloric acid (HCl) (Merck)
	- Methyl Red (Fluka)
	- Anhydrous Diethyl Ether (Merck)
4 Electrophoresis	-Polyacrylamide (Sigma)
	- Bis-acrylamide (Sigma)
	-*N,N,N',N'*-tetramethyl ethylene diamine (TEMED) (Bio-Rad)
	-Coomasie Brilliant Blue R-250 (Bio-Rad)
	-Methanol (CH_4O) (J.T. Beaker)
	- Bromophenol Blue (Sigma)
	- Beta-mercaptoethanol (βME) (sigma)
	- Glycerol ($C_3H_5(OH)_3$) (Merck)
	- Glacial acetic acid (CH_3COOH) (J.T. Beaker)
	- (hydroxymethyl) aminomethane (Tris) ($(HOCH_2)_3CNH_2$) (Merck)
	- Protein standard marker (Thermo Scientific)

Table 3.1 (Continued)

Part	Chemical
5 Extraction of Antioxidant	- Absolute ethanol (Merck)
	- Distilled water
6 Antioxidant Determination	- 2,2-azinobis (3-ethyl-benzothiazoline-6-sulfonic acid) (ABTS) (Fluka)
	- 2,4,6-tripyridyl-*s*-triazine (TPTZ) (Fluka)
	- Folin–Ciocalteu phenol reagent (Fluka)
	- 2,2′-diphenyl-picrylhydrazyl (DPPH$^{\bullet}$) (Sigma)
	- Gallic acid (Sigma)
7 Film Forming	- Glycerol (Fluka)
	- Distilled water
8 Lipid Oxidation Determination	- Thiobarbituric acid-reactive substances (TBARS) (Fluka)
	- 1,1,3,3-tetramethoxypropane (malonaldehyde; MAD) (Fluka)
	- Methanol (Sigma)
	- Ammonium thiocyanate (Fluka)
	- Ferrous chloride (Sigma)

3.1.3 Instruments

Table 3.2 Instrument Lists

Instrument	Model	Company, Country
Water bath	WNE-22	Memmert, Schwabach, Germany
Spectrophotometer	Libra S22	Biochrom, Cambridge, USA
Micro centrifuge	VS 15000N	Vision, Korea
Table top centrifuge	PLC-05	Industrial Corp., Taipei, Taiwan

Table 3.2 (Continued)

Instrument	Model	Company, Country
Electrophoresis equipment	Miniprotean Tertra cell	Bio Rad Laboratories, Inc, Richmond, CA, USA
Hot Plate with magnetic stirrer	Unimag, AREX2	Scott Instrument, GMBH Germany
pH meter	pH 510	Eutech Instrument, Ayer Rajah Crescent, Singapore
Shaker	KS 4001IC	IKA® Werke GmbH & Co. KG, Germany
Ultrasonic centrifuge	Avanti J-30I	Beckman Coulter, USA
Homogenizer	IKA Ultra Turrax, T25 D	IKA® Werke GmbH & Co. KG, Germany
Panasonic Food Processor	MK5086M	Panasonic, Selangor, Malaysia
Blender	HR 2011	Philips Electronics,China
Vortex	G560E	Scientific Industries Inc, New York, USA
Digital balance 4 digits	Sartorius, ED224S	Sartorius AG, Germany
Digital balance 2 digits	Adventerter, ARC120	Ohaus,New Jercy,USA.
Hot air oven		Memmert, Schwabach, Germany
Climaric Chamber	FBF 115	BINDER, USA
Hand-held micrometer	Mitutoyo	Mitutoyo, Japan
Universal testing machine	Lloyd Instrument,	Hampshire, UK
Sonicator	Sonorex digitex, DT255H	Bandeline electronic, Berlin, Germany
Texture analyzer	TA-XT2	Stable Micro system, Surrey,UK
Refrigerator	P2003	Sanyo, Bangkok, Thailand
Hunter lab color meter	Hunter Lab Color Quest XE	Hunter Lab, Virginia, USA

Table 3.2 (Continued)

Instrument	Model	Company, Country
Freeze dryer	FD-8 drywinner	Heto, Cambridge, UK.
FTIR spectrometer	Spectrum GX,	PerkinElmer, USA
Differential Scanning Calorimetry	Model DSC-7	Norwalk, CT, USA
Scanning electron microscope	LEO01450VP	Cambridge, UK

3.2 Methods

3.2.1 Extraction and Characteristic of Skin Gelatin from Farmed Giant Catfish

3.2.1.1 Fish Skin Preparation and Gelatin Extraction

1. Fish Skin Preparation

Frozen farmed giant catfish skin stored at -18°C was thawed and washed with running tap water. The skin was then scrapped to remove the adhering fat and foreign matter and cut into small pieces (0.5x0.5 cm). Prepared fish skin was kept on ice prior to gelatin extraction.

2. Extraction of Fish Skin Gelatin

Gelatin was extracted from prepared fish skin according to the method of Jongjareonrak, Benjakul, Visessanguan, Prodpran, and Tanaka (2006). Fish skin was soaked in 0.2 M NaOH (1:10) at 4°C with continuous gentle stirring to remove non-collagen protein and pigment. Alkaline-treated skin was then washed with tap water until pH<7.5 of wash water was obtained. To swell the collagenous material in the fish skin matrix, the alkaline-treated skin was soaked in 0.05 M acetic acid with a skin to solution ratio of 1:10 (w/v) for 3 h at room temperature with a continuous gentle stirring. Acid-treated skin was washed as previously described.

For the first extraction, the swollen fish skin was soaked in distilled water with a skin/water ratio of 1:10 (w/v) at 45±1°C for 12 h with continuous stirring. The extract obtained was then filtered and freeze dried and referred to “first extraction gelatin”. The remnant from the first extraction was used as the raw material for the re-extraction process. It was soaked in distilled water as mentioned above and then incubated at 60, 75 or 90°C for 12 h with a

continuous stirring. The temperature that gave the highest gelatin yield from this step was used as the extraction temperature for the following experiment on the extraction time. The extraction times of 1, 3, 5, 7, 9 and 12 h were done in the re-extraction process. The extract obtained was then filtered by using two layers of cheesecloth and freeze dried. The dry matter was ground and referred to as "gelatin powder" and used for further study. The gelatin yield was calculated (3.1):

$$\text{Yield of gelatin (\%)} = \frac{\text{(Weight of freeze dried gelatin (MC ~ 4\%)) x 100}}{\text{Weight of fresh skin}} \qquad (3.1)$$

3.2.1.2 Proximate Composition Analysis

Moisture, ash and fat contents of fish skin, remnant and gelatin from the first and the re-extractions were determined according to the AOAC method numbers 927.05, 942.05 and 920.39 B, respectively (AOAC, 2000). Protein content was measured by the Kjeldahl method according to the method number 984.13 and a nitrogen conversion factor of 5.8 was used.

3.2.1.3 Biochemical Properties of Gelatin

1. Electrophoretic Analysis

Protein patterns of fish skin and gelatin samples were determined by SDS–PAGE according to the method of Laemmli (1970). The samples (1 g) were dissolved in 10 ml of 5% (w/v) SDS solution and then heated at 85°C for 1 h to dissolve the proteins. Supernatants were mixed with sample buffer (0.5 M Tris-HCl, pH 6.8 containing 4% (w/v) SDS, 20% (v/v) glycerol and 10% (v/v) βME) at the ratio of 1:1 (v/v). The mixture was boiled for 3 min. Protein samples (15 μg) were loaded into the polyacrylamide gel made with a 7.5 % (v/v) running gel and a 4 % (v/v) stacking gel and then subjected to electrophoresis at a constant current of 15 mA per gel using a Mini-PROTEAN® Tetra Cell (Bio-Rad Laboratories, Inc. ,Richmond, CA., USA). After electrophoresis, the gel was stained with 0.1 % (w/v) Coomassie blue R-250 in 15 % (v/v) methanol and 5 % (v/v) acetic acid and de-stained with 30 % methanol and 10 % acetic acid.

2. Solubility of Gelatin

Gelatin samples were separately dissolved in 50 mM phosphate buffer pH 7.0, containing different concentrations of NaCl (0, 0.5, 1.0, 1.5, 2.0, 2.5, and 3.0 M) according to the method of Binsi, Shamasundar, Dileep, Badii & Howell (2009). The ratio of gelatin to the buffer used was 1:100 (w/v) with the incubation time of 1 h at room temperature. The gelatin solution

was centrifuged at 8,000g for 15 min. Protein content in the supernatant was determined by the Biuret method (Robinson & Hodgen, 1940) using BSA as a standard. Relative solubility was calculated in comparison with that sample gave the highest solubility.

3.2.1.4 Physical Properties of Gelatin

1. Colour Measurement

The colour of gelatin powder were measured by a Hunter lab colour meter (Color QuestXE, Virginia, USA) and reported by the CIE system. *L*, a** and *b** parameters indicated lightness, redness/greenness and yellowness/blueness of the measured samples, respectively.

2. Turbidity Measurements

The measurement of turbidity/clarity was determined by a transparency of the gelatin solution. The sample solution (6.67%, w/v) was poured into 1 cm cuvettes at room temperature. The turbidity of the solution was measured using a UV/Visible Spectrophotometer (Biochrom Libra S22, England) at 600 nm against distilled water (Hoque, Benjakul & Prodpran, 2010). Each turbidity measurement was carried out in triplicate and calculated as:

$$\text{Transparency} = -\log T_{600}/x,$$

where T_{600} is the transmittance at 600 nm and x is the cuvettes thickness (mm).

3. Fourier Transforms Infrared Spectra Analysis (FTIR)

FTIR spectra of gelatin samples were obtained by using a FTIR spectrometer (Spectrum GX, PerkinElmer, USA). For spectra analysis, gelatin samples were clamped into the mount of the FTIR spectrometer. The spectra in the range of 400–4000 cm^{-1} with automatic signal gain were collected in 16 scans at a resolution of 4 cm^{-1} and were rationed against a background spectrum recorded from the clean empty cell at 25°C (Jongjareonrak et al., 2008).

4. Differential Scanning Calorimetry (DSC)

Thermal properties of gelatin samples were determined using Perkin Elmer Differential Scanning Calorimetry (Model DSC-7, Norwalk, CT, USA). Temperature calibration was performed using the Indium thermogram. The gelatin samples (5 mg) were accurately weighed into aluminum pans, sealed, and scanned over the range of 10–150°C with a heating rate of 10°C/min, in inert atmosphere (100 ml/min of N_2) along with an empty pan as a reference. The

maximum transition temperature was estimated from the endothermic peak of DSC thermogram and transition enthalpy was determined from the area under the endothermic peak (Jongjareonrak Benjakul, Visessanguan & Tanaka, 2006a).

3.2.2 Extraction of Phenolic Antioxidant from Tropical Fruit Seeds

3.2.2.1 Fruits Seeds Preparation

The fruits seeds were dried in hot air oven at 50°C for 1 day and than were powdered with a hammer mill through a 20-mesh (0.84 mm) sieve. The sample powder was stored in a plastic bag and kept in a freezer at -20°C until use for extraction.

3.2.2.2 Extraction of Phenolic Compounds

The influence of the solid-to-liquid ratio on the extraction was investigated, by considering four ratios (1:5, 1:10, 1:15, 1:20, 1:30, 1:40; g:ml). The total volume of the extraction was fixed at 30 ml of an EtOH:H_2O (50:50, v/v) solution. The mixtures were agitated (150 rpm) at room temperature for 4 h. Extraction solution was centrifuge at 8,000 rpm for 15 min and then supernatant was filtrated through filter paper. Residual yield and antioxidant activities of the extract were determined and defined as longan seed extract: LS, lychee seed extract: LCS, passion fruit seed extract: PFS, and rambutan seed extract: RS. The ratio gave the highest value of extractable phenolic content (EPC) and antioxidant activities (AAO) was chosen for response surface methodology.

3.2.2.3 Response Surface Methodology (RSM)

The optimization of the extraction of phenolic compounds from the fruit seeds by RSM was carried out using an experimental plan based on a three factor/five level design referred to as a rotatable central composite design, which consisted of seventeen experimental runs, including three replicates at the center point. The independent variables were the ethanol proportion (X_1; 40-80%, v/v ethanol/water), the extraction time (X_2; 60-180 min), and extraction temperature (X_3; 40-80°C). Five levels of values for the independent variables were expressed in their coded and uncoded forms (Table 3.4). The experimental data were fitted to a second-order polynomial model (Equation 3.1) and the regression coefficients were obtained by multiple linear regression.

$$Y = \beta_0 + \sum_{i=1}^{3} \beta_i X_i + \sum_{i=1}^{3} \beta_{ii} X_i^{\ 2} + \sum_{i-1}^{2} \sum_{j=2}^{3} \beta_{ij} X_i X_j \qquad (3.1)$$

where X_1, X_2 and X_3 are the independent variables affecting the responses Y; β_0, β_i, (i = 1, 2, 3), β_{ii}, (i = 1, 2, 3), and β_{ij}, (i = 1, 2, 3; j = 2, 3) are the regression coefficients for the intercept, linear, quadratic and cross-product terms, respectively.

The optimal extraction conditions were obtained by the desirability function approach using Minitab statistical software. The response surface plots were developed using the STATISTICA Kernel Release 7.0.61.0 EN (StatSoft Inc., Tulsa, OK) for Windows.

Table 3.3 Independent Variables and Their Coded and Actual Values Used for Optimization

Independent variables	**Units**	**Symbol**	**Code levels**				
			-α	**-1**	**0**	**+1**	**+α**
Ethanol proportion	% (v/v)	X_1	26.36	40	60	80	93.64
Temperature	°C	X_2	26.36	40	60	80	93.64
Time	min	X_3	19.09	60	120	180	220.90

Validation of the Model

Optimal conditions for the extraction of the phenolic antioxidants from the fruit seeds, depending on the ethanol proportions, times and extraction temperatures were obtained using the prediction RSM equations. After the multifactor analysis of variance and the 2nd order model prediction determinations, the optimal extraction conditions were obtained by the desirability function approach.

3.2.2.4 Antioxidative Activity Determinations

1. Extractable Phenolic Content Determination

Extractable phenolics content was determined by the Folin–Ciocalteu method, which was adapted from Swain and Hillis (1959). The 50 µl of extract, 200 µl of de-ionized water, and 50 µl of Folin–Ciocalteu reagent were combined in a quartz vial and then mixed well using a Vortex.

Table 3.4 Three-Factor, Five-Level Face-Centered Cube Design Used for RSM

Standard order[a]	Run order[b]	Factor 1 (X_1)	Factor 2 (X_2)	Factor 3 (X_3)
		Ethanol proportion (%)	Temperature (°C)	Time (min)
1	15	40.00 (-1)	40.00 (-1)	60.00 (-1)
2	11	80.00 (+1)	40.00 (-1)	60.00 (-1)
3	4	40.00 (-1)	80.00 (+1)	60.00 (-1)
4	13	80.00 (+1)	80.00 (+1)	60.00 (-1)
5	1	40.00 (-1)	40.00 (-1)	180.00 (+1)
6	3	80.00 (+1)	40.00 (-1)	180.00 (+1)
7	16	40.00 (-1)	80.00 (+1)	180.00 (+1)
8	6	80.00 (+1)	80.00 (+1)	180.00 (+1)
9	12	26.36 (-α)	60.00 (0)	120.00 (0)
10	2	93.64 (+α)	60.00 (0)	120.00 (0)
11	14	60.00 (0)	26.36 (-α)	120.00 (0)
12	7	60.00 (0)	93.63 (+α)	120.00 (0)
13	9	60.00 (0)	60.00 (0)	19.09 (-α)
14	5	60.00 (0)	60.00 (0)	220.90 (+α)
15	8	60.00 (0)	60.00 (0)	120.00 (0)
16	10	60.00 (0)	60.00 (0)	120.00 (0)
17	17	60.00 (0)	60.00 (0)	120.00 (0)

Note. [a] No randomized.

[b] Randomized.

The mixture was allowed to react for 6 min then 500 µl of 7% (w/v) Na_2CO_3 solution was added and mixed well. The solution was incubated at room temperature in the dark for 90 min. The absorbance was measured at 760 nm using a UV-spectrophotometer (Biochrom/Libra S22, England) and the results were expressed in gallic acid equivalents (mg

GAE /100 g dry mass). Additional dilution was done if the absorbance value measured was over the linear range of the standard curve.

2. Ferric Reducing Antioxidant Power (FRAP)

FRAP was assayed according to Benzie and Strain (1996). Stock solutions included 300 mM acetate buffer (pH 3.6), 10 mM TPTZ solution in 40 mM HCl**,** and 20 mM $FeCl_3 \cdot 6H_2O$ solution. A working solution was prepared freshly by mixing 25 ml of acetate buffer, 2.5 ml of TPTZ solution and 2.5 ml of $FeCl_3 \cdot 6H_2O$ solution. The mixed solution was incubated at 37 °C for 30 min in a water bath (Memmert, D-91126, Schwabach, Germany) and was referred to as FRAP solution. A sample (90 µl) with the concentration range of 0.5–10 mg/l was mixed with 810 µl of FRAP solution and kept for 30 min in the dark at room temperature. The ferrous tripyridyltriazine complex (coloured product) was measured by reading the absorbance at 595 nm. A sample blank at each concentration was prepared by omitting $FeCl_3$ from the FRAP solution and distilled water was used instead. The standard curve was prepared using Gallic acid and expressed as gallic acid equivalents (GAE) mg/100g dry seed fruit.

3. DPPH Radical Scavenging Activity

DPPH radical scavenging activity was determined as described by Brand-Williams et al. (1995) with a slight modification. Samples (600 µl) were added to 600 µl of 0.20 mM (DPPH) in 95% ethanol. The mixture was mixed vigorously and allowed to stand at room temperature in the dark for 30 min. The absorbance of the resulting solution was measured at 520 nm using a UV-spectrophotometer (Biochrom/Libra S22, England). The sample blank at each concentration was prepared in the same manner except that ethanol was used instead of DPPH solution. The standard curve was logarithm between 2 and 25 µg/ml gallic acid. Results are expressed as gallic acid equivalents (GAE) mg/100g dry seed fruit. Additional dilution was needed if the DPPH value measured was over the linear range of the standard curve.

4. ABTS Radical Scavenging Activity

ABTS radical scavenging activity was assayed as per the method of Arnao, Cano, and Acosta (2001) with a slight modification. The stock solutions included 7.4 mM ABTS solution and 2.6 mM potassium persulphate solution. The working solution was prepared by mixing the two stock solutions in equal quantities and allowing them to react for 12-16 h at room temperature in the dark. The solution was then diluted by mixing 5 ml ABTS solution with 50 ml

methanol in order to obtain an absorbance of 1.1 ± 0.02 units at 734 nm using a UV-spectrophotometer (Biochrom/Libra S22, England). A fresh ABTS solution was prepared for each assay. Samples (50 µl) with a concentration range of 0.5–10 mg/l were mixed with 950 µl of ABTS solution and the mixture was left at room temperature for 120 min in dark. The absorbance was then measured at 734 nm using the spectrophotometer. A sample blank at each concentration was prepared in the same manner except that methanol was used instead of ABTS solution. A standard curve of Gallic acid was prepared. The activity was expressed as gallic acid equivalents (GAE) mg/100g dry seed fruit.

3.2.3 Development of Gelatin Film Incorporated with Antioxidant Compounds

3.2.3.1 Preparation of Gelatin Films

Gelatin powder was mixed with distilled water to obtain the film-forming solution (FFS) with the protein concentration of 3% (w/v). Glycerol was used as a plasticizer at the concentration of 25% of protein. The solution was incubated at 60 °C for 30 min in a water bath with an occasional stirring. De-aerated FFS (4±0.01 g) was cast onto a rimmed silicone resin plate (50×50 mm) and dried with a ventilated oven (Environmental chamber model H110K-30DM; Seiwa Riko Co., Tokyo, Japan) at 25±0.5 °C and 50±5% relative humidity (RH) for 24 h. Dried films obtained were manually peeled off.

To study the effects of LS extract and BHT on film properties and antioxidative activity of incorporated film, longan seed (LS) extract and BHT were dissolved in ethanol and added to FFS to obtain a final concentration of 50-500 ppm for LS extract and 50-100 ppm for BHT. FFS was then stirred gradually for 10 min, cast and dried as previously described. Films incorporated without and with LS extract or BHT were subjected to the determinations of properties and antioxidative activity during storage in a humidity control chamber (50% RH) at 25 °C for 0, 2 and 4 weeks.

3.2.3.2 Analyses

The films were conditioned for 48 h at 25±0.5 °C and 50±5% RH prior to analyses. The film thickness was determined without conditioning.

3.2.3.3 Physical Properties

1. Film Thickness

Film thickness was measured to the nearest 5 μm with a hand-held micrometer (Mitutoyo, Tokyo, Japan). Nine measurements were taken at random positions. Precision of the thickness measurements was ±5%.

2. Mechanical Properties

Tensile strength (TS) and elongation at break (EAB) were determined as described by Iwata, Ishizaki, Honda, and Tanaka (2000) with a slight modification using the Universal Testing Machine (Lloyd Instruments, Hamsphire, UK). Nine samples (2×5 cm^2) with initial grip length of 3 cm were used for testing. Cross-head speed was 30 mm/min and load cell used was 100 N.

3. Color and Film Transparency

The colour of gelatin powder were measured by a Hunter lab colour meter (Color QuestXE, Virginia, USA) and reported by the CIE system. L^*, a^* and b^* parameters indicated lightness, redness/greenness and yellowness/blueness of the measured samples, respectively.

The light barrier properties of the films were measured according to Gómez-Guillén, Ihl, Bifani, Silva and Montero, 2007. Films were exposed to light at wavelengths ranging from 570 to 270 nm, and absorbance was measured using a spectrophotometer. Results have been expressed as percentage transmittance. The transparency value of the films was calculated at 600 nm by the following equation:

$$\text{Transparency value} = -\log T_{600}/x, \qquad (3.2)$$

where T_{600} is the fractional transmittance at 600 nm and x is the film thickness (mm).

4. Water Vapour Permeability (WVP)

Water vapor permeability of films was measured using a modified ASTM method as described by Sobral, Menegalli, Hubinger and Roques (2001). Films were attached over the openings of cells (permeation area = 9.62 cm^2) containing desiccated silica gel, and the cells were placed in climatic chamber (90% RH) at 22 °C. The cups were weighed at 1 h intervals over a 10 h period and WVP of films was calculated as follows (McHugh, Avena-Bustillos &

Krochta, 1993). Five samples in total were determined for each film types. WVP was calculated using the equation:

$$WVP = w \bullet x \bullet t^{-1} \bullet A^{-1} \bullet \Delta P^{-1} \qquad (3.3)$$

where *w* was weight gain (g), x film thickness (mm), *t* elapsed time for the weight gain (h), and ΔP the partial vapour pressure difference between the dry atmosphere and pure water (2642 Pa at 22 °C). Results have been expressed as $g \cdot mm \cdot h^{-1} \cdot cm^{-2} \cdot Pa^{-1}$.

5. Scanning electron microscopy (SEM)

Microstructure of active films prepared by the different methods was determined using a scanning electron microscope (LE01450VP, Cambridge, UK). Samples with a size of 20x30 mm^2 were dried auto desiccators. Dried samples were mounted on a bronze stub and sputter-coated with gold (Sputter coater, SC7620, Polaron, UK). The specimens were observed with a SEM at an acceleration voltage of 10 kV.

6. Differential Scanning Calorimetry (DSC)

Thermal properties of film samples were determined by using Perkin Elmer Differential Scanning Calorimetry (Model DSC-7, Norwalk, CT, USA). Temperature calibration was performed using the Indium thermogram. The film samples (5 mg) were accurately weighed into aluminum pans, sealed, and scanned over the range of 10–150°C with a heating rate of 10°C/min, in inert atmosphere (100 ml/min of N_2) along with an empty pan as a reference. The maximum transition temperature was estimated from the endothermic peak of DSC thermogram and transition enthalpy was determined from the area under the endothermic peak (Jongjareonrak Benjakul, Visessanguan & Tanaka, 2006b).

3.2.3.4 Chemical Properties

1. Determinations Performed on the Films

Polyphenols were quantified spectrophotometrically at 750 nm using Folin–Ciocalteau reagent (Montreau, 1972) and gallic acid as standard. Films were first dissolved in 0.5 M acetic acid at 40 °C. The resulting solution was shaken vigorously in a 95% ethanol solution for 30 min to extract the phenols. Phenol–protein interactions were then evaluated on the assumption that extractability decreases with the degree of interaction. The mixture was left to stand for 10 min, and the supernatant was employed as the extract for the total phenol

determination and DPPH radical scavenging activity. All assays were performed at least in triplicate.

3.2.4 Effect of Fish Skin Gelatin Film Incorporated with LS Extract and BHT on the Retardation of Lipid Oxidation

3.2.4.1 Active Film Preparation

The active gelatin film sample that incorporated with LS concentration of 50, 100, 300, and 500 ppm and BHT at concentration of 50 and 1000 ppm were made a sachet (size 2x4 cm^2). Soybean oil (2 mL) was filled into the active gelatin films bag (2x4 cm) and then sealed by desktop poly sealer. The samples were stored in a humidity control chamber (50% RH) at 28 °C for 30 days. The samples were taken after 0, 1, 3, 5, 10, 15, 20 and 30 days for the analysis of Conjugated diene (CD), peroxide value (PV) and thiobarbituric acid reactive substances (TBARS). The oil without film covering were used as the control.

3.2.4.2 Analysis of Active Gelatin Film

1. Peroxide Value (PV)

Peroxide value was determined according to the method of Sakanaka, Tachibana, Ishihara, and Raj Juneja (2004). To 50 µl of the oil sample, 2.35 ml of 75% ethanol, 50 µl of 30% ammonium thiocyanate and 50 µl of 20 mM ferrous chloride solution in 3.5% HCl were added and mixed thoroughly. After 3 min, the absorbance of the coloured solution was measured at 500 nm using a UV-1601 spectrophotometer. An increase in absorbance at 500 nm indicated the formation of peroxide (Yen & Hsieh, 1998).

2. Conjugated Diene (CD)

The conjugated diene was measured according to the method of Frankel, Huang, Aeschbach, and Prior (1996). The oil (0.1 ml) was dissolved in 5.0 ml of methanol and the absorbance was measured at 234 nm using a UV-1601 spectrophotometer. The conjugated diene was measured as the increase in absorbance at 234 nm.

3. Thiobarbituric Acid-Reactive Substances (TBARS)

The thiobarbituric acid-reactive substances (TBARS) assay was performed as described by Buege and Aust (1978). Sample 0.5 ml of oil were mixed with 2.5 ml of a TBA

solution containing 0.375% thiobarbituric acid, 15% trichloroacetic acid and 0.25 N HCl. The mixture was heated in boiling water for 10 min to develop a pink colour, cooled with running tap water and then sonicated for 30 min followed by centrifugation at 5000×*g* at 25 °C for 10 min. The absorbance of the supernatant was measured at 532 nm. A standard curve was prepared using 1,1,3,3-tetramethoxypropane (malonaldehyde; MAD) at a concentration ranging from 0 to 10 ppm and TBARS was expressed as mg of MAD equivalents/kg sample.

3.2.5 Statistical Analysis

All chemical analyses were preformed in triplicate. Colour and turbidity properties, at least 5 determinations at each treatment were conducted. Data were subjected to analysis of variance (ANOVA). Comparison of means was carried out by Duncan's multiple-range test. Analysis was performed by using a SPSS package (SPSS 10.0 for Windows, SPSS Inc, Chicago, IL, USA).

CHAPTER 4

RESULTS AND DISCUSSIONS

4.1 Extraction and Characteristics of Skin Gelatin from Farmed Giant Catfish

4.1.1 Proximate Composition of Fish Skin and Gelatins

The proximate compositions of fish skin, remnant and gelatins from the first and re-extraction are presented in Table 4.1. The compositions of giant catfish skin were 54% moisture, 43% protein, 1.6% fat and 0.35% ash. Compared to all compositions of fish skin, protein is the main remaining component of skin after moisture content. However, fat content had no significant amount compared to 1.1% in Nile tilapia (Songchotikunpan, Tattiyakul & Supapho, 2008), but lower than that of many freshwater fish species, such as 6.8% in Nile perch skins (Muyonga, Cole & Duodu, 2004b) and 10.3% in channel catfish (Liu, et al. 2008). After the first extraction of the skin gelatin with a temperature of 45 °C for 12 h, the remnant obtained was subjected to proximate analysis and then used as the raw material for the re-extraction process. The remaining protein accounted for about 24% in the remnant. This value takes a part of around 50% compared to the original protein content in the fish skin. Rawdkuen, Sai-Ut, Ketnawa, Thitipramote and Benjakul (2010) reported that high compact arrangement of structural bundles in fish skin microstructure gave a lower gelatin yield. Higher amounts of moisture content (74%) was also observed when compared to the fish skin. It is probably due to the water absorption of the denatured fish skin obtained from the first extraction. However, lipid (1%) and ash (0.10%) content were reduced by the extraction process. In different studies, the skin of *Priacanthus tayenus* and *Priacanthus macracanthus* contained protein as the major component (93.66% and 97.34%) and low fat content (0.61-0.64%) (Benjakul, Oungbho, Visessanguan, Thiansilakul & Roytrakul, 2009). Shark skin contained 89.60% moisture, 5.93% crude protein, 0.28% crude lipid, and 0.85% crude ash (Limpisophon, Tanaka, Weng, Abe & Osako, 2009).

The proximate compositions of extracted skin gelatin from farmed giant catfish are also presented in Table 3.1. The first extracted gelatin contained 91% protein as the major constituent, followed by 4% moisture, 1.9% fat, and 2% ash. The farmed giant catfish skin gelatin showed to be higher in ash content (2%), compared with those from grass carp skin (0.12%; Ladislaus, et al., 2007), and channel catfish skin (1%; Liu, Li & Guo 2008), but then to have lower amounts compared to Nile perch skin (6.8%; Muyonga, Cole & Duodu, 2004b). However, the first extracted gelatin still contained high fat content (1.85%). The other components present in the gelatin indicated the efficiency of the extraction process. The result suggested that the extraction process could remove some lipid and inorganic matters during the extraction, resulting in lower amounts of these matters. The re-extraction process still provided high contents of gelatin (85%) compared with the first extraction (91%) process. In addition, lower fat and ash content in the extracted gelatin was also found. These results suggested that large amounts of gelatin remained in the extracted fish skin after passing through the first extraction process at a mild condition (temperature 45 °C). Moreover, the re-extraction process could remove more contaminants during the gelatin extraction, resulting in more gelatin yield.

Table 4.1 Chemical Composition of Fish Skin and Gelatin From Farmed Giant Catfish.

Composition*	**Fish skin**	**Gelatin (First extraction)**	**Remnant**	**Gelatin (Re-extraction)**
Moisture	$53.80 \pm 5.32^{b**}$	4.16 ± 0.44^{a}	73.50 ± 6.45^{c}	3.39 ± 0.43^{a}
Protein	43.00 ± 0.89^{b}	91.14 ± 6.68^{c}	23.60 ± 0.45^{a}	85.27 ± 0.69^{c}
Fat	1.60 ± 0.45^{a}	1.85 ± 0.64^{a}	1.01 ± 0.14^{a}	1.24 ± 0.15^{a}
Ash	0.35 ± 0.09^{b}	2.00 ± 0.19^{c}	0.10 ± 0.01^{a}	0.17 ± 0.02^{ab}

Note. * Mean ± SD (n = 3), expressed as % (wet weight).

**Different superscripts in the same row indicate the significant differences ($P < .05$).

4.1.2 Effect of Temperatures and Time on the Gelatin Extraction Yield

The results of the gelatin extraction yield from farmed giant catfish are shown in Figure 4.1. The first extraction process at 45 °C for 12 h provided 10.14% gelatin yield (Figure 4.1a). With same extraction time (12 h), increasing extraction temperatures during the re-extraction process by using the remnant from the first extraction significantly increased the gelatin yield ($P < .05$). The gelatin yield from the re-extraction process at 90 °C for 12 h showed the highest value (19.5%) compared with the extraction temperature of 60 °C (2.7%) and 75 °C (7.4%). This might be because the re-extraction process with high temperature could influence the release of the polypeptide chains from the fish skin. Ease of extraction of gelatin from fish skin is highly influenced by the environment that the fish lived in (i.e, cold water or tropical) and is totally dependent on the types of pre-treatments. Fish skin is easily extracted with high yields at relatively moderate temperatures, usually below 50 °C (Gómez-Guillén, Giménez & Montero, 2005). The yield of gelatin extraction at 45 °C was 71-75% for fresh salmon skins or cod backbones (Kołodziejska, Skierka, Sadowska, Kołodziejski & Niecikowska, 2008). Wangtueai and Noomhorm (2009) reported that the optimum conditions for the highest gelatin extraction yield in lizardfish scales was 78.5 °C for 3 h. High extraction temperature strongly increased the extraction yield. Subsequent thermal treatment above 40 °C (well above helix-to-coil transition temperatures for fish gelatins) destroys hydrogen bonding and cleaves a number of covalent bonds, which destabilizes the triple-helix via a helix-to-coil transition and therefore results in a conversion to soluble gelatin (Djabourov, Lechaire & Gaill, 1993). The temperature at which this occurs depends upon the amount of proline and hydroxyproline in the α chain, and this temperature is the point of denaturing. From the result, the temperature of 90 °C achieved the highest yield since at that temperature, the collagen in the raw skin was relaxed and the skin was shrunk, leading to the release of more collagen, and then it converted to soluble gelatin (Kołodziejska, et al., 2008).

The extraction time (1–12 h) of the re-extraction process was performed at the extraction temperature of 90 °C. As shown in Figure 4.1b, longer extraction time continuously increased the extraction yield ($P < .05$). The highest gelatin yield of the re-extraction process was achieved when the re-extraction time was 12 h ($P > .05$). During the heating of the extracted fish skins over a long time, most of the collagen was converted into gelatin.

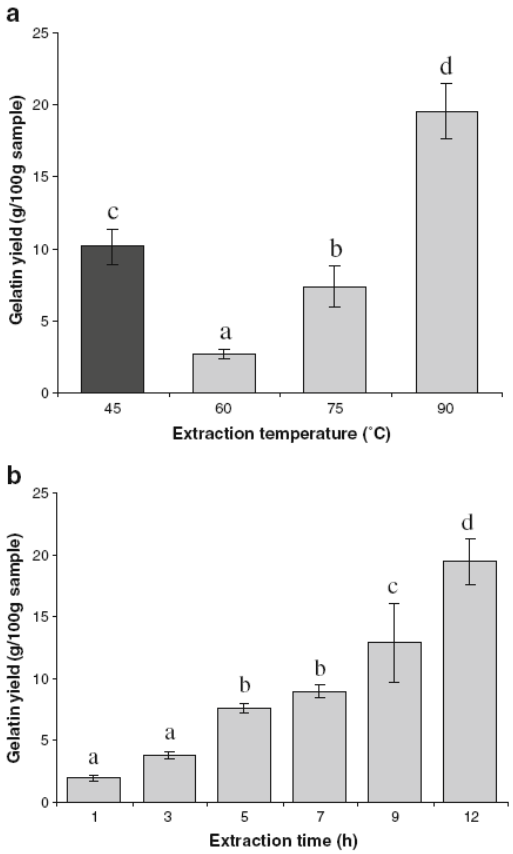


Figure 4.1 Extraction Yield of Gelatin from Farmed Giant Catfish Skin at Various Temperatures for 12 h (a) and Times at 90 °C (b). Extraction Temperature at 45 °C was used for the First Extraction (Black Bar), and 60, 75 and 90 °C for 12 h were used for the Gelatin Re-extraction Process (Gray Bar). Different Letters in the Same Chart Significant Differences ($P < .05$)

As mentioned before, the processing parameters, especially temperature and time, played a major role in this experiment. In addition, the pH, pretreatment, and the properties of raw materials were important during the gelatin extraction process. The preservation method of the raw material was also important. From the result, the extraction time for 12 h at 90 °C was the selected condition for the gelatin re-extraction process. The gelatin obtained from this extraction condition was named “G90” and used for further characterization. Rahman, Al-Saidi, and Guizani (2008) have also reported a higher yield (18%) of gelatin from yellow fin tuna skin using thermal extraction processing.

4.1.3 Electrophoretic Characteristic of Gelatin

The protein patterns of extracted gelatin at different temperatures and time are shown in Figure 4.2a, b. Gelatins from farmed giant catfish skin contained α_1 and α_2 chains as the major constituents. A higher band intensity of the β chain was observed in the fish skin compared with the extracted gelatin at different conditions. In addition, gelatin extracted with the general condition (45 °C, 12 h) showed slightly difference compared with the protein patterns of fish skin resulting from the extraction process. However, slightly decreased band intensity of β chain with the increased α_1 chain was observed when the extraction temperatures were increased from 45 °C to 75 °C in the re-extraction process. All of protein components disappeared with further re-extraction at 90 °C. The protein pattern of the commercial beef skin gelatin (BG) showed no major component on the SDS-PAGE gel. As a result, the gelatin obtained from the re-extraction process, especially at high temperature, gave lower molecular weight (MW) protein components that resulted from the destabilization of the native collagen. However, cross-linking of proteins (on the top of the gel) in the gelatin samples extracted with high temperature (> 60 °C) were clearly observed. High MW of these proteins was possibly generated by thermal treatment and cannot completely solubilized during sample preparation for SDS-PAGE.

The protein patterns of the extracted gelatin with different extraction times are also presented (Fig. 2b). By increasing the extraction time (1-12 h), more degradation of gelatin components (β and α-chains) were observed. A markedly decreased intensity of the major band (β and α-chain) of extracted gelatin was clearly observed when the extraction time was prolonged. Complete disappearance of the protein bands were observed at the extraction duration of 12 h at 90 °C. Gómez-Guillén et al. (2002) reported that damage or partial loss of α_1-chains can occur during the extraction procedure in the presence of a protein band with a MW lower than 100 kDa. Muyonga et al. (2004b) revealed that Nile perch skin gelatins contained low MW peptides, especially in gelatins extracted at higher temperatures. This result might be caused by the degradation induced by the thermal process or by endogenous proteinases during the gelatin preparation. Intarasirisawat et al. (2007) reported that the skin of *P. macracanthus* contained endogenous serine and metallo proteinases, which caused the autolysis of the skin at 50–60 °C.

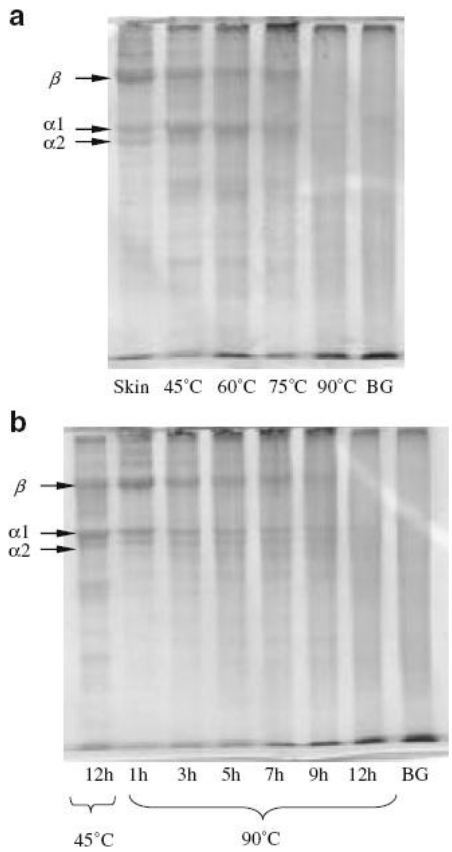


Figure 4.2 Protein Patterns of Giant Catfish Skin and Gelatin Extracted at 90 °C for Different Temperatures (a: 45, 60, 75, and 90 °C) and Different Times (b: 1–12h). BG: Beef Skin Gelatin

During the gelatin extraction process, the conversion of collagen into gelatin yields molecules of varying mass, because of the cleavage of inter-chain covalent cross-links and the unfavorable breakage of some intra-chain peptide linkages. Liu and Guo (2008) reported that more degradation of high MW components (α, β, and γ) in channel catfish skin gelatin. During the conversion of collagen to gelatin, the inter- and intra-molecular bonds that link collagen chains as well as some peptide bonds are broken. The more severe the extraction process, the greater the extent of the hydrolysis of peptide bonds, and therefore, the higher the proportion of peptides with a MW less than α chains are obtained (Muyonga, et al., 2004a). The age of the source skin material may influence the ease with which gelatin can be extracted and the extent of the peptide hydrolysis during the extraction (Muyonga, et al., 2004a).

4.1.4 Solubility

The solubility profiles of the extracted gelatin at 90°C for 12 h (G90) and commercial beef skin gelatin (BG) as an effect of NaCl concentration were reported in Table 4.2. The importance of protein solubility is that it influences other functional properties, such as gelation, water and oil absorption, emulsification, foam formation and also film formation. Generally, relative solubility of both gelatins was greater than 80% at all salt concentrations tested. The result also showed that a maximum solubility was found at the NaCl concentration of 2.5 and 3.0 M for G90 and BG, respectively. Slightly different in relative solubility of G90 and BG was found ($P < .05$) while the relative solubility of BG increased when the salt concentration increase.

G90 solubility decreased when the salt concentration was higher than 2.5 M. The difference in solubility of different gelatins might result from the differences in MW and the content of polar and non-polar groups in amino acids (Benjakul, et al., 2009). Salts can also affect the electrostatic interactions among the macromolecules, contributing through the ionic force. The effect of salts on protein solubility in aqueous solutions is a function of the ionic species present that concentration of salt is sufficient to change the structure of the water and conformation of the proteins. Generally solubility increases with increase in salt concentration up to certain level (salting in) and with further increase in salt concentration, the solubility decreases (salting out) (Damodaran & Kinsella, 1982).

4.1.5 Physical Properties of Gelatin

4.1.5.1 Color and Transparency

The color and transparency (clarity) of gelatin solution from giant catfish skin (G90) and beef skin (BG) at the concentration of 6.67% are presented in Table 4.3. These attributes of gelatin may or may not be important depending on its application. L^* value represent the lightness of the sample. No significant difference of lightness for both G90 and BG was observed ($P > .05$). However, lower a^* and b^* values of G90 compared with BG was found ($P < .05$). Different color attributes between extracted fish skin gelatin and commercial beef skin gelatin are probably due to the differences in gelatin processing.

Commercial gelatin is normally stabilized by using stabilizing agents (protein or carbohydrate), so higher a^* and b^* values are normally observed. Benjakul et al. (2009) reported

that *L**, *a**, and *b** values of gelatin from the skins of bigeye snapper, *P. tayenus* (29.71, -1.61, -3.22), *P. macracanthus* (28.55, -1.33, -3.90), and bovine bone (35.85, -0.77, 18.29) were observed. The color of the gelatin solution was directly affected by the application of gelatin as a coating material or edible gelatin film.

Table 4.2 Solubility of Gelatins as a Function of NaCl Concentration.

NaCl*	Relative solubility (%)**	
(M)	Giant catfish gelatin	Beef gelatin
0.0	80.86 ± 3.01 ^{a}	81.53 ± 0.80 ^{a}
0.5	83.53 ± 5.62 ^{ab}	87.68 ± 2.02 ^{b}
1.0	92.24 ± 2.02 ^{c}	89.02 ± 4.10 ^{bc}
1.5	94.51 ± 5.09 ^{c}	89.83 ± 2.42 ^{bc}
2.0	93.71 ± 1.23 ^{c}	92.24 ± 3.07 ^{bc}
2.5	94.24 ± 4.12 ^{c}	93.44 ± 3.37 ^{cd}
3.0	89.96 ± 3.29 ^{bc}	97.72 ± 2.67 ^{d}

Note. * Values are given as mean ± SD from triplicate determinations.

**Different superscripts in the same column indicate significant differences ($P < .05$).

The transparency of the gelatin solution was reported as the clarity of the tested sample. The increased transparency is caused by the formation of light scattering aggregates upon processing or by the scattering of light by particles entrapped inside the matrix of the sample. From the result, the G90 sample showed a highest transparency than that of the BG, which may have been a result of a gelatin-gelatin coacervate. Higher transparency was obtained in the G90 (0.22) compared to the BG sample (0.10). Gelatin manufacturing generally has an effective process to clarify the impurities from the gelatin solution, such as by chemical clarification and filtration processes, so a lower of turbidity from BG was found. The G90 prepared by no clarifying process would contain high amounts of interferences that induce aggregation of those molecules in the gelatin sample to form a three-dimensional network. Extensive aggregation

would be expected to increase the number and size of the aggregates, with a subsequent increase in transparency.

Table 4.3 Colour and Turbidity of Gelatin Powder.

Gelatin type*	Colour**			% Turbidity
	*L**	*a**	*b**	
Giant catfish gelatin***	63.07 ± 0.56 [a]	-0.08 ± 0.11 [b]	9.35 ± 0.92 [b]	0.22 ± 0.003 [a]
Beef gelatin	62.13 ± 0.92 [a]	2.17 ± 0.12 [a]	14.21 ± 0.25 [a]	0.10 ± 0.000 [b]

Note. * Values are given as mean ± SD from triplicate determinations.

** Different superscripts in the same column indicate significant differences ($P < .05$).

*** Extract at 90°C for 12 h.

4.1.5.2 FTIR Spectra of Skin Gelatins

FTIR spectroscopy has been used to study the changes in the secondary structure of the gelatin. It has also been used to study collagen cross-linking, gelatin denaturation, and melting. The FTIR spectra of G90 and BG are depicted in Fig. 4.3. Generally, both gelatins showed similar spectra, in which absorption bands were situated in the amide region. Amide I and amide II bands of gelatins from both G90 and BG appeared at around 1,653.10 and 1,553.16 cm^{-1}, respectively. Muyonga et al. (2004a) reported that amide I and II bands of gelatins form Nile perch fish skin at 1,700-1,600 and 1,560-1,500 cm^{-1}, respectively. The spectra of both skin gelatins displayed major bands at 3,320 cm^{-1} (amide A, representative of NH stretching, coupled with hydrogen bonding), 1,653 cm^{-1} (amide I, representative of C = O stretching/hydrogen bonding coupled with COO^{-}), 1,553 cm^{-1} (amide II, representative of NH bending, coupled with CN stretching), and 1,242 cm^{-1} (amide III, representative of NH bending). The FTIR spectra of giant catfish skin gelatin were similar to those found in other gelatins. Amide I and amide II bands of gelatins from both bigeye snapper skins appeared at around 1,630 and 1,545 cm^{-1}, respectively (Benjakul, et al. 2009). The gelatins extracted from Nile perch at higher temperatures

exhibited a much broader amide A than what was observed for the low temperature-extracted gelatins and for the acid-soluble collagens (Muyonga, et al., 2004a).

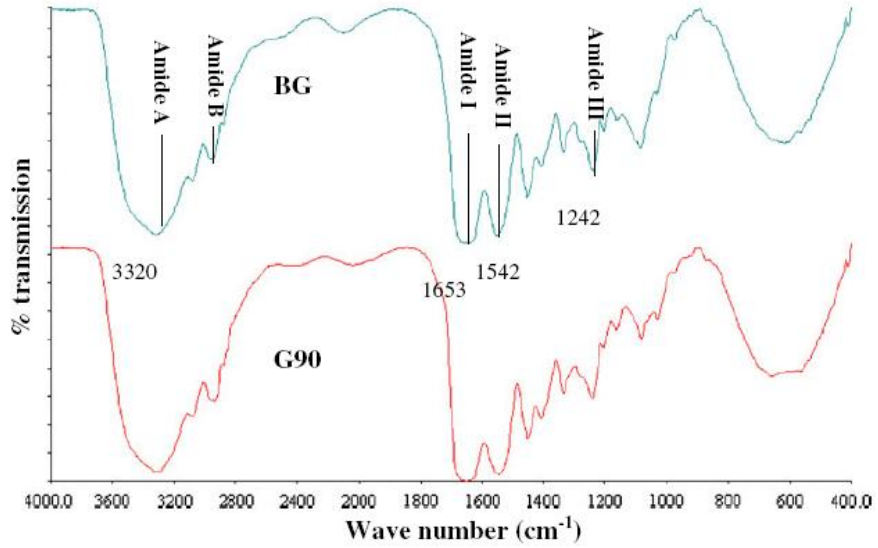


Figure 4.3 Fourier Transform Infrared Spectra of Gelatins from Giant Catfish Skin (GC) and Commercial Beef skin Gelatin (BG) in the Ranges of 400-4,000 cm^{-1} at 25°C.

The absorption in the amide I region is the most useful for the infrared spectroscopic analysis of the secondary structure of proteins. The amide II band is generally considered to be much more sensitive to hydration than to the secondary structure change (Wellner, Belton & Tatham, 1996). The lower peak intensity of amide I was observed in G90 compared with the BG, indicating that the lower protein secondary structure (α helix) was due to the degradation of the gelatin molecules. Small amide III bands of BG and G90 were observed at 1,241 and 1,243 cm^{-1}, which indicated the disorder in gelatin molecules. Amide I band, amide II band and amide III band, which were known to be related to the degree of molecular order and to be involved with the triple helical structure of collagen, were a result of C = O stretching, N-H bending, and CH stretching, respectively (Muyonga, et al., 2004a).

4.1.5.3 Differential Scanning Calorimetry

The DSC traces obtained in the differential calorimetric analysis of the G90 and BG is shown in Table 4.4. BG had two endothermic peaks. The first peak was observed at 67.28 °C with a transition enthalpy of 5.23 J/g, and the second peak was observed at 98.45 °C with 8.26 J/g of transition enthalpy. The G90 had only one endothermic peak at 80.97 °C with a transition enthalpy of 19.80 J/g. The higher transition temperature of BG might be due to the greater amount of amino acids composition (proline and hydroxyproline) of bovine gelatin. They have a direct positive correlation to the thermal stability of gelatin. The molecular weight of the protein chains also contributed to the formation of strong three-dimensional network structure (Jongjareonrak, Benjakul, Visessanguan, Prodpran & Tanaka, 2006). This result indicated that G90 may be composed of proteins with lower molecular weight and the re-crystallization of the α helix structure could not be formed in accordance with the SDS-PAGE protein pattern (Figure 4.2). As a consequence, the melting of G90 occurred at a lower temperature than the BG.

Table 4.4 TransitionTemperature and Transition Enthalpy of Gelatin.

Source of gelatin	Transition temperature (°C) *	Transition enthalpy (J/g) *
Giant catfish gelatin***	$80.79 \pm 2.21^{b**}$	19.80 ± 2.88^{b}
Beef gelatin (1st peak)	67.28 ± 0.09^{a}	5.23 ± 0.92^{a}
Beef gelatin (2nd peak)	98.45 ± 0.75^{c}	8.26 ± 1.17^{a}

Note. * Values are given as mean ± SD from triplicate determinations.

** Different superscripts in the same column indicate significant differences ($P < .05$).

*** Extract at 90°C for 12 h.

From the results, the gelatin recovery was highest at the re-extraction temperature of 90°C for 12 h with the yield of 10.14%. The properties of the re-extraction gelatin were similar to those of commercial beef skin gelatin. However, the thermal properties of giant catfish skin gelatin were greater than those of the beef skin gelatin. Therefore, the developed extraction

process could be applied for the further application for the commercial scale to obtain a higher yield of gelatin from giant catfish skin.

4.2 Extraction of Phenolic Antioxidant from Tropical Fruit Seeds

4.2.1 Longan and Lychee Seeds

4.2.1.1 Effect of Solid-to-Liquid Ratio on Extraction of Phenolic Compounds

The impact of the solid-to-liquid ratio on the extraction of phenolic compounds from lychee and longan seeds were conducted with six ratios (1:5, 1:10, 1:15, 1:20, 1:30 and 1:40; w/v) during 4 h at 25°C by using 50% (v/v) ethanol solution as an extractant. Extraction yield of phenolic compounds from lychee and longan seed at different ratios are shown Table 4.5. Increasing of an extractant volume, the increases of extraction yield was obtained in both seed extracts especially in longan seed.

Extractable phenolic content (EPC) and ferric reducing antioxidant power (FRAP) increased with the decrease of the solid-to-liquid ratio as presented in Figure 4.4 and 4.5. However, at the level higher than 1:20 ratio for lychee, no significant of extraction yield was observed ($P > .05$). Increase in solvent amount lead to significantly increase in EPC from both longan and lychee seeds ($P < .05$). However, there was no significant difference in FRAP from lychee seeds after ratios 1:15 ($P > .05$). EPC and FRAP from the extract of longan had higher than lychee about 4 times. This result had coincided with Guo, Yang, Wei, Li, Xu and Jiang (2003), which longan and lychee had FRAP value of 24.26 and 22.36 (mmol/100g dry weight), respectively.

As the present result, the solid-to-liquid ratio of 1:20 was chosen for further study because of no significant different of extraction yield, EPC and FRAP ($P > .05$) when increase the extractant were obtained. It might be due to dependence of isolated compound yields on theoretical dielectric constant of extraction solvent is well-know from literature (Ondrejovic, Benkovicva & Silhar, 2009). In the extraction of milled berries (Cacace & Mazza, 2003) and milled black currants (Cacace & Mazza, 2002), the yields of total phenolics and anthocyanins were maximized with 19 L of solvent per kg of material, which similar to the extraction of phenolics from *Inga edulis* leaves (Silva, Rogez & Larondelle, 2007). However the extraction of

the phenolic compounds from fruits of *E. oleracea* using an acidified aqueous alcoholic solution (Pompeu, Silva & Rogez, 2009) was the 1:4 solid-to-liquid ratios, which is approximately 5 times higher than the ratio used here.

Table 4.5 Effect of the Solid-to-Liquid Ratio on the Extraction Yield (mg Crude Extract/100g Dry Sample) from Lychee and Longan Seeds.

Ratio (g:ml)	Lychee seed**	Longan seed
1: 5	$6.52 \pm 0.04^{a*}$	8.76 ± 0.17^{a}
1:10	9.12 ± 0.13^{b}	11.04 ± 0.13^{b}
1:15	9.34 ± 0.13^{b}	11.94 ± 0.07^{c}
1:20	9.77 ± 0.06^{c}	12.39 ± 0.21^{d}
1:30	9.81 ± 0.06^{c}	13.30 ± 0.16^{e}
1:40	9.80 ± 0.16^{c}	13.87 ± 0.42^{f}

Note. * Different superscripts (a, b, c, d, e, f) in the same column indicate significant differences ($P < .05$).

** Triplicate determinations

4.2.1.2 Fitting the Models

In RSM, natural variables are transformed into coded variables that have been defined as dimensionless with a mean zero and the same spread or standard deviation (Myers & Montgomery, 2002). Multiple regression equations were generated relating response variable to coded levels of the independent variables. Multiple regression coefficients were determined by employing least squares technique (Myers & Montgomery, 2002) to predict quadratic polynomial models for extraction yield and antioxidant activities of longan and lychee seed extracts. Analysis of variance (ANOVA) shows that the selected quadratic models adequately represented the data obtained for extraction yield and antioxidant activities.

The results of ANOVA analysis for extraction yield and antioxidant activities with corresponding coefficients of multiple determinations (R^2) for fruit seeds fractions are shown in

Table 4.6 and 4.7. The model was adequate and explained most of the variability for both fruits seed extracts. For the model fitted, software generates model coefficients, R^2-values; F-values and significant probabilities and hence one can justify the significance of each experimental variable.

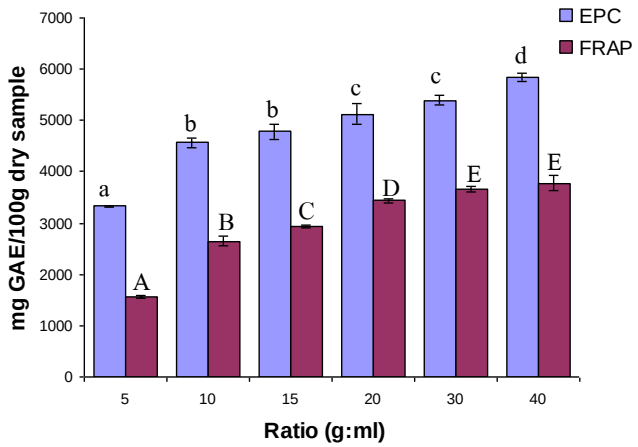


Figure 4.4 Effect of the Solid-to-Liquid ratio on the Extraction of Extractable Phenolics Content (EPC) and FRAP from Longan Seeds, using Aqueous Ethanol (50:50, v/v), at 25°C for 4 h.

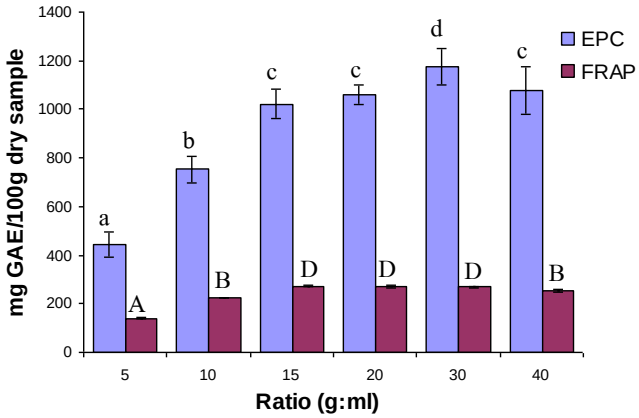


Figure 4.5 Effect of the Solid-to-Liquid ratio on the Extraction of Extractable Phenolics Content (EPC) and FRAP from Lychee Seeds, using Aqueous Ethanol (50:50, v/v), at 25°C for 4 h.

The maximum predictable response for extraction yield and antioxidant activities were obtained based on a total of 17 experiments required for determining 10 regression coefficients of the model. In general, proceeding with exploration and optimization of a fitted response surface may produce poor or misleading results unless the model exhibits an adequate fit (Myers & Montgomery, 2002). This makes the checking of model adequacy essential. A plot of experimental and theoretical values indicated an excellent fit ($R \geqslant 0.94$, $P < .01$) for longan and lychee seed extracts.

4.2.3 Response Surface Analysis

The results of extraction yield during the process optimization for the longan and lychee seed (100 g powder) phenolics extraction varied between 8.09-15.49 mg/100g sample, and 5.71-8.88 mg/100g sample, respectively (data not show). ABTS values were 2-3 times higher than DPPH and FRAP values. This might be due to the significant differences in their response to antioxidants which ABTS and DPPH radicals show similar bi-phase kinetic reactions with many antioxidants and FRAP method is based on the reduction of a ferric analogue (Amadou, Yong-Hui, Sun & Guo-Wei, 2009).

The experimental data were fitted to the second-order polynomial model and the equation obtained was tested to determine if it could describe the variability in the responses, by evaluating the coefficients of multiple determinations and performing analyses of variance. As Table 4.6 and 4.7 shown by an ANOVA of the regression parameters of the surface response analysis of the models, the linear, quadratic, and interaction terms have significant effects ($P \leqslant .001$, $P \leqslant .01$ or $P \leqslant .05$).

The quadratic model fitted well ($R^2 > 0.94$) with the experimental data (extraction yield and antioxidant activities), giving evidence for a low dispersion of the experimental data. Control of model parameters, R^2, Adj-R^2, confirmed the model adequacies (Table 4.6 and 4.7). The lack of fit test at the level of .05 indicates no evidence for lack of fit for both of the extraction yield and the antioxidant activities models.

4.2.4 Effects of Treatment Variables on Phenolic Antioxidants Extraction

Variables in their coded form permitted a direct interpretability of the effects (linear, quadratic, and interaction) of the independent variables, and the surface and contour plots

facilitated the visualisation of the statistically significant factors derived from the statistical analysis. Response surface model plot are depicted in Figure 4.6 and 4.7.

Regarding the ethanol proportion (X_1), linear and quadratic effects were verified to be statistically significant for extraction yield and antioxidant activities, as indicated by the P value (Table 4.6 and 4.7). A negative quadratic effect of X_1 was obtained for extraction yield and antioxidant activities from longan and lychee seed extracts ($P < .001$), indicating that there is a maximum in the longan and lychee extraction at a certain ethanol proportion (60-70%). The extraction yield and antioxidant activities in longan and lychee starts to decrease above this proportion (> 80%). According to Escribano-Bailon and Santos-Buelga (2005) a maximum of phenolic glycosides of most plants are extracted using 50% methanol.

Similar results have been found for phenolic antioxidant extraction of wheat (maximum between 50 and 60% of ethanol) (Liyana-Pathirana & Shahidi, 2005), apple pomace (56% ethanol and 65% acetone) (Wijngaard & Brunton, 2010), grape cane (40 % and 55% of ethanol) (Karacabey & Mazza, 2010), and maximal total phenolic and total anthocyanin contents from milled berries were obtained using 60% ethanol (Cacace & Mazza, 2003). With regard to longan and lychee, the quadratic effect of X_1 was negative, indicating that the extraction yield and antioxidant activities increases with the increase in ethanol proportion theoretically up to medium level and then decrease above.

For the temperature of extraction (X_2), a linear effect was detected for the extraction yield and DPPH activity for longan seed, and antioxidant activities ($P < .05$ and $P < .001$) for lychee seed, which confirms that the increase in temperature improves the phenolic compound yields. Indeed, a higher temperature increases the solubility and diffusion coefficient of polyphenols, allowing higher extraction rate (Cacace & Mazza, 2003). Thus, an increase in the temperature would increase the diffusion coefficient and hence the rate of diffusion, leading to a consequent reduction in extraction time. A negative quadratic effect of X_2 was obtained for extraction yield and antioxidant activities of both fruits seed. The results indicated that the mobilization of active compounds from the solid matrix may occur up to a certain level, followed by a loss. An upper limit must however be respected to avoid degradation of thermo-sensitive phenolic compounds (Silva, et al., 2007). This is possibly due to their decomposition at high temperatures.

Table 4.6 Regression Coefficients of Predicted Models for the Investigated Responses of Longan Seed Extracts and Independent Effects of Factors.

Variables[a]	Yield coefficient	EPC coefficient	DPPH coefficient	ABTS coefficient	FRAP coefficient
β_0	14.042^{***,b}	6015.86***	2337.98***	8339.34***	3567.43***
β_1	-1.062***	-714.44***	-213.78***	-496.76*	-181.88**
β_2	0.676*	42.34^{ns,c}	110.56*	-83.18^{ns}	-67.09^{ns}
β_3			94.54*	-207.84^{ns}	
β_{11}	-1.270***	-771.02***	-254.23***	-1090.15***	-354.75***
β_{22}	-0.670*	-381.96**	-207.99***	-1002.56***	-343.61***
β_{33}					
β_{12}	0.701*		112.05*	716.37*	254.89**
β_{13}					
β_{23}			107.24*	-598.95*	
R^2	0.85	0.86	0.93	0.88	0.89
Adj-R^2	0.77	0.82	0.88	0.79	0.84

Note. [a] Polynomial $Y = \beta_0 + \sum_{i=1}^{3} \beta_i X_i + \sum_{i=1}^{3} \beta_{ii} X_i^{\,2} + \sum_{i-1}^{2} \sum_{j=2}^{3} \beta_{ij} X_i X_j$ model adjusted by backward elimination at the level of 0.05% with the lack-of-fit test, where β_0 is the constant coefficient, β_i is the linear coefficient (main effect), β_{ii} is the quadratic coefficient, and β_{ij} is the two factors interaction coefficient.

[b] *, significant at $P \leqslant .05$; **, significant at $P \leqslant .01$; ***, significant at $P \leqslant .001$.

[c] ^{ns}, not significant ($P > .05$).

For the time of extraction (X_3), only the DPPH activity response variables exhibited significant positive linear effects for longan seed and only the extraction yield can detect positive linear effect for lychee seed (Table 4.6). There were no significant in quadratic effects for X_3 indicated that time is not useful to extract more phenolics.

Table 4.7 Regression Coefficients of Predicted Models for the Investigated Responses of Lychee Seed Extracts and Independent Effects of Factors.

Variables[a]	Yield coefficient	EPC coefficient	DPPH coefficient	ABTS coefficient	FRAP coefficient
β_0	7.586^{***,b}	939.26***	394.37***	1134.67***	387.25***
β_1	-0.718***	-82.34**	-35.32**	-90.88***	-27.28***
β_2	0.100^{ns,c}	60.85*	23.15*	110.54***	50.29***
β_3	0.147*	29.76^{ns}	3.91^{ns}	18.96^{ns}	11.63^{ns}
β_{11}	-0.166*	-129.05***	-66.64***	-135.22***	-55.39***
β_{22}		-83.64**	-47.88***	-110.73***	-31.93***
β_{33}					
β_{12}					
β_{13}	-0.333**				
β_{23}	-0.189*	-75.69*	-36.59*	-71.37*	-30.04*
R^2	0.94	0.91	0.93	0.92	0.92
Adj-R^2	0.91	0.85	0.88	0.88	0.87

Note. [a] Polynomial $Y = \beta_0 + \sum_{i=1}^{3} \beta_i X_i + \sum_{i=1}^{3} \beta_{ii} X_i^{\,2} + \sum_{i-1}^{2} \sum_{j=2}^{3} \beta_{ij} X_i X_j$ model adjusted by backward elimination at the level of 0.05% with the lack-of-fit test, where β_0 is the constant coefficient, β_i is the linear coefficient (main effect), β_{ii} is the quadratic coefficient, and β_{ij} is the two factors interaction coefficient.

[b] *, significant at $P \leqslant .05$; **, significant at $P \leqslant .01$; ***, significant at $P \leqslant .001$.

[c] ^{ns}, not significant ($P > .05$).

Interestingly, a positive interaction (cross-effect) between ethanol porprotion and extraction temperature (Table 4.6) was obtained for the extraction yield, DPPH, ABTS and FRAP assay of longan seed, which may be imputed to a degradation of phenolic compunds upon longer extraction times and higher temperatures. This is likely due to a difference in the composition of the extract which is affected by solvents and process temperature. It has been reported that the

polarity of solvent used in extraction directly affects not only the quantity of total phenolics, but also the composition and potency of phenolics (Yu, Ahmedna & Goktepe, 2005).

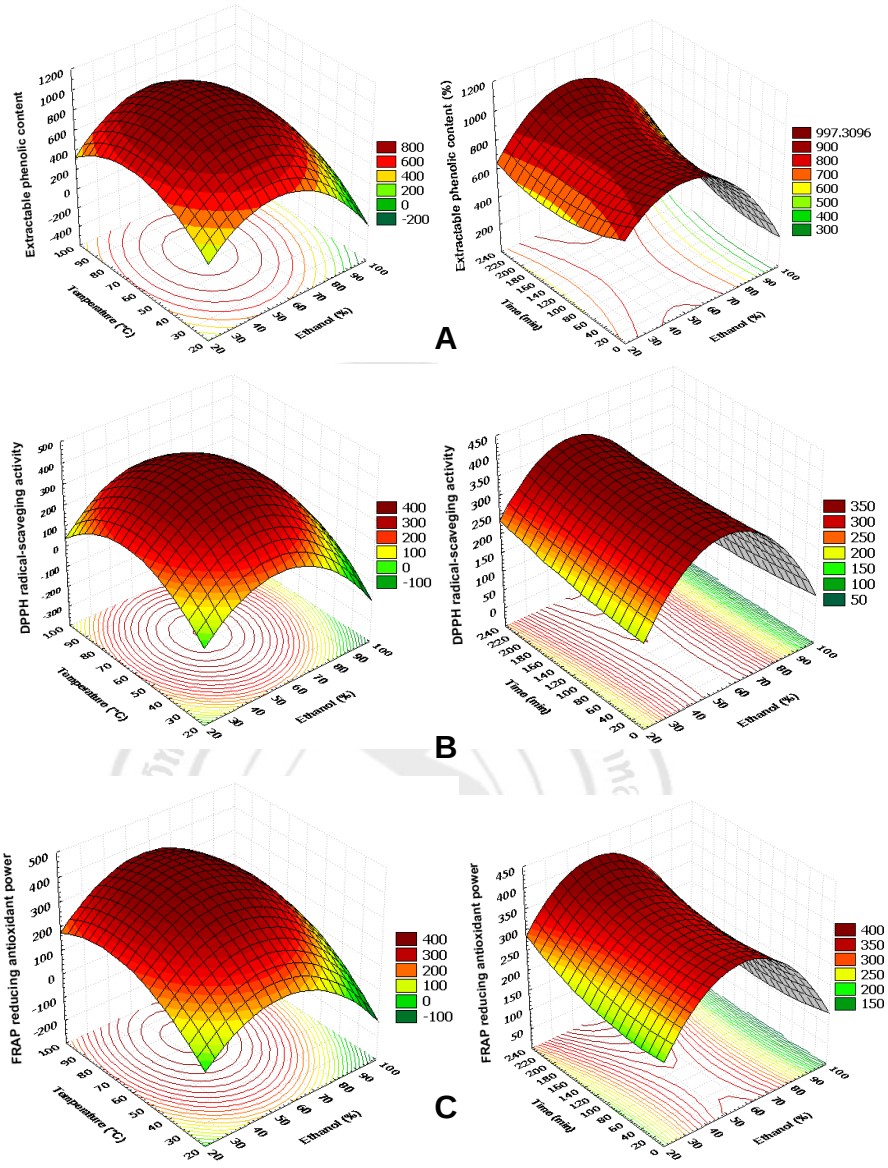


Figure 4.6 Response Surface Model Plot of Predicted Models Showing the Effects of Temperature and Ethanol Proportion (Left) and Effects of Time and Ethanol Proportion (Right) on EPC (A), DPPH (B), and FRAP (C) (mg GAE/100g Dry Sample) from Lychee Seeds.

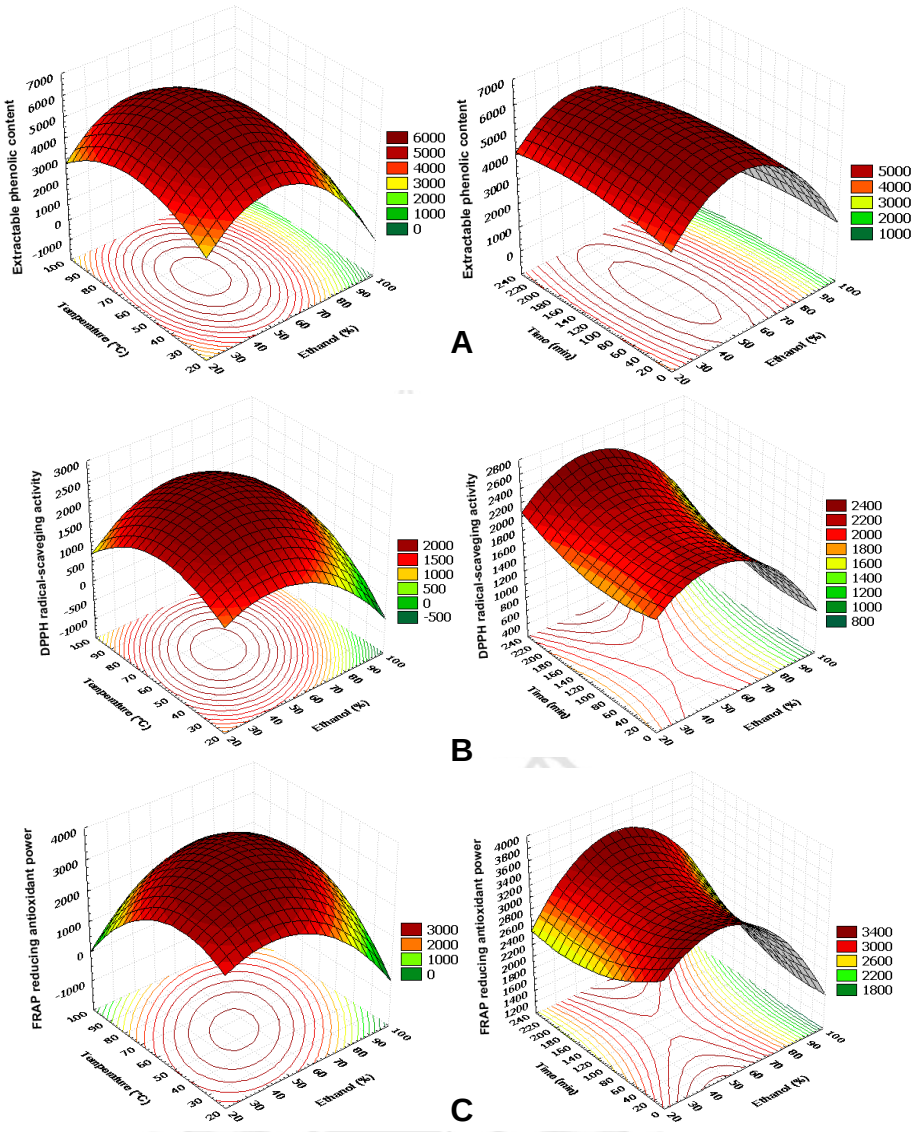


Figure 4.7 Response Surface Model Plot of Predicted Models Showing the Effects of Temperature and Ethanol Proportion (Left) and Effects of Time and Ethanol Proportion (Right) on EPC (A), DPPH (B), and FRAP (C) (mg GAE/100g Dry Sample) from Longan Seeds.

Ethanol- temperature and ethanol- time interactions were found negative for lychee seed, which means that the impacts of the factors temperature or time depends on the level of ethanol proportion being used, and they tended generally to increase at higher levels of ethanol

proportion. Thus, ethanol concentration and/or temperature contributed significantly to the response. On the other hand, the extraction time had no significant effect on phenolic compounds for fruits seed extracts. By considering the regression coefficients obtained for independent and dependent variables, ethanol concentration and temperature were perhaps the most important factors that may significantly influence phenolic compound (Table 4.6 and 4.7). Park, Lee, Jeong and Kwon (1998) found that solvent concentration plays a critical role in the extraction of soluble solids from various natural products. Similarly Kwon, Belanger, and Pare (2003) reported that solvent concentration was the most important factor contributing to the extraction of ginseng components using RSM. Also extraction of phenolic compounds from wheat (Liyana-Pathirana & Shahidi, 2005) and grape cane (Karacabey & Mazza, 2010).

4.2.5 Experimental Validation of the Optimal Conditions

In order to verify the predictive capacity of the model, an optimum condition was determined using the simplex method and the maximum desirability for extraction yield, EPC, DPPH, ABTS and FRAP antioxidant activity from lychee and longan seeds. The optimized condition for each seed extract for antioxidant compound extraction is presented in Table 4.8.

These results confirm the predictability of the model for the extraction of extraction yield, EPC, DPPH, ABTS and FRAP antioxidant activity from lychee and longan seeds in the experimental condition used. The optimized conditions that maximized the extraction yields, EPC and antioxidant activities from longan and lychee seeds were: ethanol proportion of 53% and 41%, temperature of 58°C and 51°C, and time of 220 min and 139 min, respectively. Under the optimum conditions the corresponding predicted response values for yield, EPC, DPPH, ABTS and FRAP were 14.2, 8.9 (g/100g sample); 6,144, 967; 2,401, 383; 8,353,1,117 and 3,609, 382 (mg GAE/100 g sample) for longan and lychee seeds, respectively.

Table 4.8 Optimization Condition for Extracting Phenolic Compounds with Antioxidant Activity of Longan and Lychee Seeds Obtained by the Simplex Method.

Samples	Optimized Condition		
	Ethanol proportion (%)	**Temperature (°C)**	**Time (min)**
Longan seed	53.15	58.20	139.37
Lychee seed	41.31	51.51	220.90

Table 4.9 Comparison between the Predicted and Experimental Values for Phenolic Antioxidants from the Extract of Longan and Lychee Seeds.

Samples	Condition	Response Values				
		Extraction yield	**EPC**	**DPPH**	**ABTS**	**FRAP**
Longan	Predicted[a]	14.21	6,144	2,401	8,353	3,599
seed	Experimental[b]	14.27 ± 0.24	5,804 ± 26	2,442 ± 227	8,620 ±70	3,609 ± 54
Lychee	Predicted	8.97	967	383	1,117	382
seed	Experimental	8.42 ± 0.25	994 ± 16	425 ± 53	1,036 ± 85	366 ± 6

Note. [a] Predicted using ridge analysis of response surface quadratic model.

[b] Mean ± standard deviation of triplicate determinations from different experiments.

4.2.2 Passion Fruit and Rambutan Seeds

4.2.2.1 Effect of Solid-to-Liquid ratio on Extraction of Phenolic Compounds

Preliminary studies were performed in order to determine the required solid-to-liquid ratio for the extraction of the phenolic compounds from passion fruit and rambutan seeds using an aqueous alcoholic solution. The results showed that the extraction yield of the phenolic compounds was dependent on the solid-to-liquid ratio (Table 4.10) which increasing in solid-to liquid ratio could increase the extraction yield in both of passion fruit and rambutan seeds. EPC and FRAP increased with the decrease of the solid-to-liquid ratio as presented in Figure 4.8.

However, at the solid-to-liquid ratio of 1:40 for passion fruit, decreasing of EPC and FRAP value was observed. There were no significant different at the ratio of 1:30-40 for passion fruit ($P >$ .05). The yield of phenolic compounds increased with the decrease in the solid-to-liquid ratio. The high solubility of polyphenols in hydroalcoholic solution, especially when they are in a glycoside form, may explain the absence of variability for the higher ratios. Thus, as a economic point of view, the 1:20 solid-to-liquid ratio was chosen.

Table 4.10 Effect of the Solid-to-Solvent Ratio on the Extraction Yield of Total Phenolics (mg /100 g of Dry Fruits) from Passion Fruit and Rambutan using Aqueous Ethanol (50:50, v:v), at 25 °C for 4 h.

Ratio	**Extraction Yield (g/100g sample)**	
(g:ml)	**Passion fruit**	**Rambutan**
1: 5	6.87 ± 0.90^{a}	7.46 ± 0.37^{a}
1:10	7.91 ± 0.47^{b}	8.88 ± 0.03^{b}
1:15	9.02 ± 0.40^{c}	10.02 ± 0.59^{bc}
1:20	9.56 ± 0.17^{cd}	10.50 ± 0.22^{c}
1:30	10.16 ± 0.27^{d}	12.05 ± 0.68^{d}
1:40	11.59 ± 0.08^{e}	14.55 ± 1.67^{e}

Note. *Values are given as mean ± SD from triplicate determinations.

**Different superscripts in the same column indicate significant differences ($P <$ .05).

According to our results, whichever the ratio chosen above 1:20, the quantity of phenolic compounds extracted will increase in both fruits seeds, but the energy that consumes to remove solvent in drying process would be increase. This allows choosing any value above this limit, but one should avoid the use of an excessive quantity of solvent and energy consuming in the design of a process.

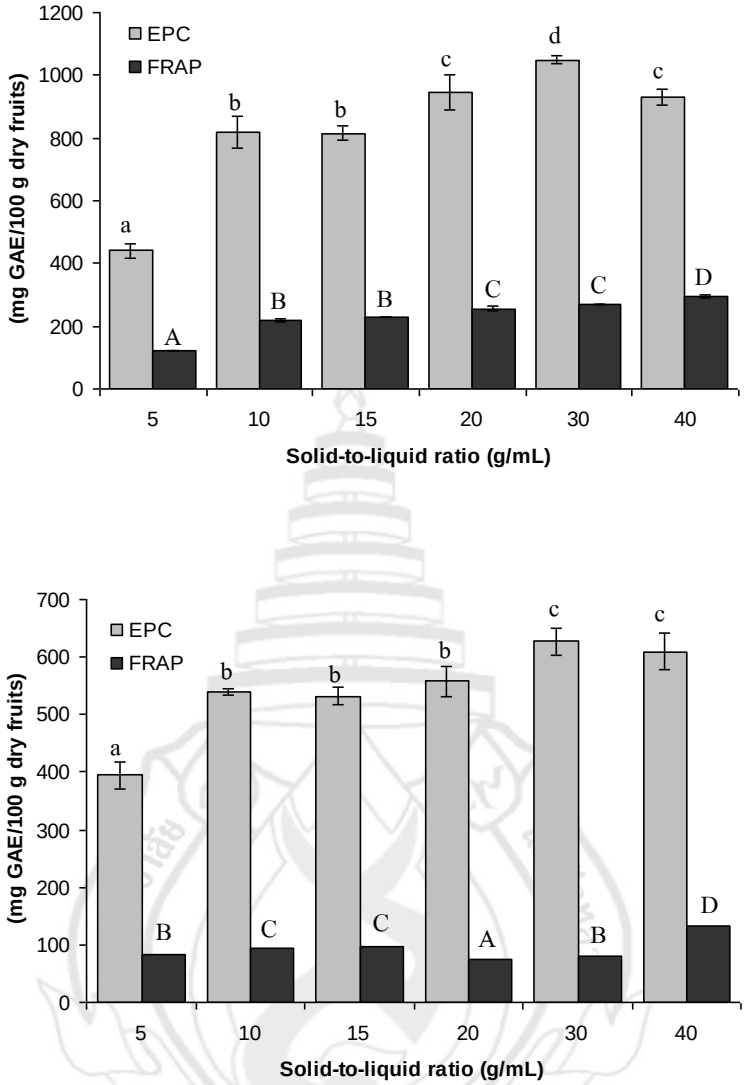


Figure 4.8 Effect of the Solid-to-Solvent Ratio on the Extraction of EPC and FRAP (mg Gallic Acid Equivalent/100 g of Dry Fruits) from Passion Fruit (A) and Rambutan (B) Seed, using Aqueous Ethanol (50:50 v:v), at 25 °C for 4 h.

4.2.2.2 Modeling of the Antioxidant Extraction

In order to the extraction process with reference to the extraction of extraction yield, extractable phenolic content, and antioxidant activities from passion fruit and rambutan seeds using alcoholic extraction, a central composite design was develop as represented in Table 3.2. The result of the analysis of variance, goodness of fit, and the adequacy of the models are summarized in Table 4.11 and 4.12. The data showed g good fit with equation 3.1, which was

statistically acceptable at $P < .05$ and adequate with satisfactory R^2 value. The values of coefficients presented in Table 4.11 and 4.12 were used in final predictive equations after neglecting non-significant terms. On the basic of these equations, three-dimensional plots were constructed to predict the relationships between independent variables and dependent variables.

4.2.2.3 Effect of Process Variables on Extraction Yield

Extraction yield from passion fruit and rambutan seeds obtain under various conditions using alcoholic extraction are presented in Table 3.2. Experimental data was subjected to regression analysis, and the coefficients of estimate are presented in Table 4.11 and 4.12. Statistical analysis revealed that the most relevant variable with $P < .001$ was ethanol concentration and extraction temperature found in passion fruit and ethanol concentration for rambutan seed. The ethanol concentration has this kind of effect of antioxidant activities because increasing the contact temperature and time of the solvent with to solid may improve the diffusion of the compounds (Ghafoor, Choi, Jeon & Jo, 2009).

The result of multiple regression analysis showed that the antioxidant activities value of passion fruit seed extracts were significantly ($P < .05$) affected by the linear, quadratic, and interaction term of ethanol concentration, extraction temperature, and extraction time and rambutan seed extracts only affected by ethanol concentration (Table 4.11). The regression equation was used to calculate the contents' variation through the response surface analysis for passion fruit and rambutan seed extracts as follows:

$$Y = 12.6416 - 0.1030X_1 + 1.1285X_2 + 0.2531X_3 - 0.3768X_1^2 - 0.1901X_2^2 - 0.2668X_3^2 + 0.2498X_1X_2 \quad (4.1)$$

$$Y = 12.0299 - 0.7144X_1 + 0.1852X_2 + 0.1001X_3 \quad (4.2)$$

On the basis of equation (4.1) and (4.2), three-dimension plots to represent the effects of extraction process variables on extraction yield from passion fruit seeds are present in Figure 4.9A. Figure 4.10A shows the effect of ethanol concentration and extraction temperature on extraction yield. The extraction yield increased with the increase of ethanol concentration at a fixed extraction temperature and nearly reached a peak at the highest ethanol concentration test (60%). However, the increase in extraction temperature at a fixed of ethanol concentration did not

effect to the extraction yield. Similarly, there was no change in extraction yield affected by extraction time.

Table 4.11 Regression Coefficients of Predicted Models for the Investigated Responses of Passion Fruit Seed Extracts and Independent Effects of Factors.

Variables[a]	Yield coefficient	EPC coefficient	DPPH coefficient	ABTS coefficient	FRAP coefficient
β_0	12.6416^{***,b}	1507.8***	681.79***	2615.6***	668.35***
β_1	-0.1030^{ns,c}	303.0***	86.81^{ns}	544.0**	161.58***
β_2	1.1285***	633.3***	287.56***	1251.6***	353.84***
β_3	0.2531***	136.7^{ns}	38.64^{ns}	293.3^{ns}	73.47^{ns}
β_{11}	-0.3768***				
β_{22}	-0.1901*				
β_{33}	-0.2668***				
β_{12}	0.2498*				
β_{13}					
β_{23}					
R^2	97.01%	80.66%	79.58%	88.40%	80.73%
Adj-R^2	87.75%	76.19%	74.87%	85.72%	76.28%

Note. [a] Polynomial $Y = \beta_0 + \sum_{i=1}^{3} \beta_i X_i + \sum_{i=1}^{3} \beta_{ii} X_i^{\,2} + \sum_{i-1}^{2} \sum_{j=2}^{3} \beta_{ij} X_i X_j$ model adjusted by backward elimination at the level of 0.05% with the lack-of-fit test, where β_0 is the constant coefficient, β_i is the linear coefficient (main effect), β_{ii} is the quadratic coefficient, and β_{ij} is the two factors interaction coefficient.

[b] *, significant at $P \leqslant .05$; **, significant at $P \leqslant .01$; ***, significant at $P \leqslant .001$.

[c] ^{ns}, not significant ($P > .05$).

Table 4.12 Regression Coefficients of Predicted Models for the Investigated Responses of Rambutan Seed Extracts and Independent Effects of Factors.

Variables [a]	Yield coefficient	EPC coefficient	DPPH coefficient	ABTS coefficient	FRAP coefficient
β_0	12.0299^{***,b}	701.828***	85.0217***	534.786***	103.218***
β_1	-0.7144***	-22.847**	-3.4057**	-21.687**	-2.670^{ns}
β_2	0.1852^{ns,}	9.698^{ns}	1.3486^{ns}	8.714^{ns}	4.487**
β_3	0.1001^{ns,c}	12.845^{ns}	0.1784^{ns}	0.666^{ns}	1.875^{ns}
β_{11}		-51.476***	-7.4113***	-47.425***	-8.686***
β_{22}		-24.714**	-7.1013***	-44.936***	-5.219**
β_{33}			-3.6666*	-21.971^{ns}	
β_{12}					
β_{13}					
β_{23}					
R^2	70.05%	76.90%	86.64%	87.16%	78.36%
Adj-R^2	63.14%	66.40%	78.63%	79.45%	68.53%

Note. [a] Polynomial $Y = \beta_0 + \sum_{i=1}^{3} \beta_i X_i + \sum_{i=1}^{3} \beta_{ii} X_i^{\,2} + \sum_{i-1}^{2} \sum_{j=2}^{3} \beta_{ij} X_i X_j$ model adjusted by backward elimination at the level of 0.05% with the lack-of-fit test, where β_0 is the constant coefficient, β_i is the linear coefficient (main effect), β_{ii} is the quadratic coefficient, and β_{ij} is the two factors interaction coefficient.

[b] *, significant at $P \leqslant .05$; **, significant at $P \leqslant .01$; ***, significant at $P \leqslant .001$.

[c] ^{ns}, not significant ($P > .05$).

For rambutan seed extract, the extraction yield was a strongly linear increase with increase in ethanol concentration at a constant temperature and time. The plot of extractable phenol content as affected by ethanol concentration and extraction temperature (Figure 4.11A) demonstrates a marked increase in extractable phenol content with increase of ethanol concentration at a fixed extraction temperature, while an increase in extraction temperature at a

fixed ethanol concentration also led to a marked increase in phenolic content. A similar slowly linear increase in extractable phenolic content with the extraction time at fixed of ethanol concentration or extraction temperature, while showing a non-important factor, was observed.

4.2.2.4 Effect of Process Variables on Extractable Phenolics Content

The extractable phenolics content of passion fruit and rambutan seed extracts obtained by alcoholic extraction based on the central composite design. Multiple regression analysis was performed on the experimental data, and the coefficients of the model were evaluated for significance. The effect of extraction time was highly significant on the extraction of phenolics ($P < .05$).

Regarding the ethanol proportion (X_1), linear and quadratic effects were verified to be statistically significant for extraction yield and antioxidant activities, as indicated by the P value in Table 4.11 and 4.12 for passion fruit and rambutan seeds, respectively. A positive linear effect of ethanol concentration and extraction temperature was found in passion fruit seeds extract (Table 4.11 and 4.12). A negative linear, and quadratic effect of ethanol concentration and quadratic effect of temperature was obtained for extractable phenolic contents from rambutan seed extracts ($P < .01$)(Table 4.12). These results indicated that there is a maximum in the passion fruit and rambutan extraction at a certain ethanol proportion. The extractable phenolic content for passion fruit seeds start to decrease above this proportion. The value of the coefficients for total phenols as presented in Table was used for a final predictive equation neglecting the non-significant cross-trem as given below:

$$Y = 1507.8 + 303.0X_1 + 633.3X_2 + 136.7X_3 \quad (4.3)$$

$$Y = 701.828 - 22.847X_1 + 9.698X_2 + 12.845X_3 - 51.476X_1^2 - 24.714X_2^2 \quad (4.4)$$

To determine the optimal levels of variables for the extractable phenolics from passion fruit and rambutan seed, three-dimensional surface plots (Figure 4.9-4.12) were constructed according to equation (4.3) and (4.4). Extraction process variable significantly effected ($P < .05$) the extraction of total phenols from the seeds of passion fruit and rambutan seeds.

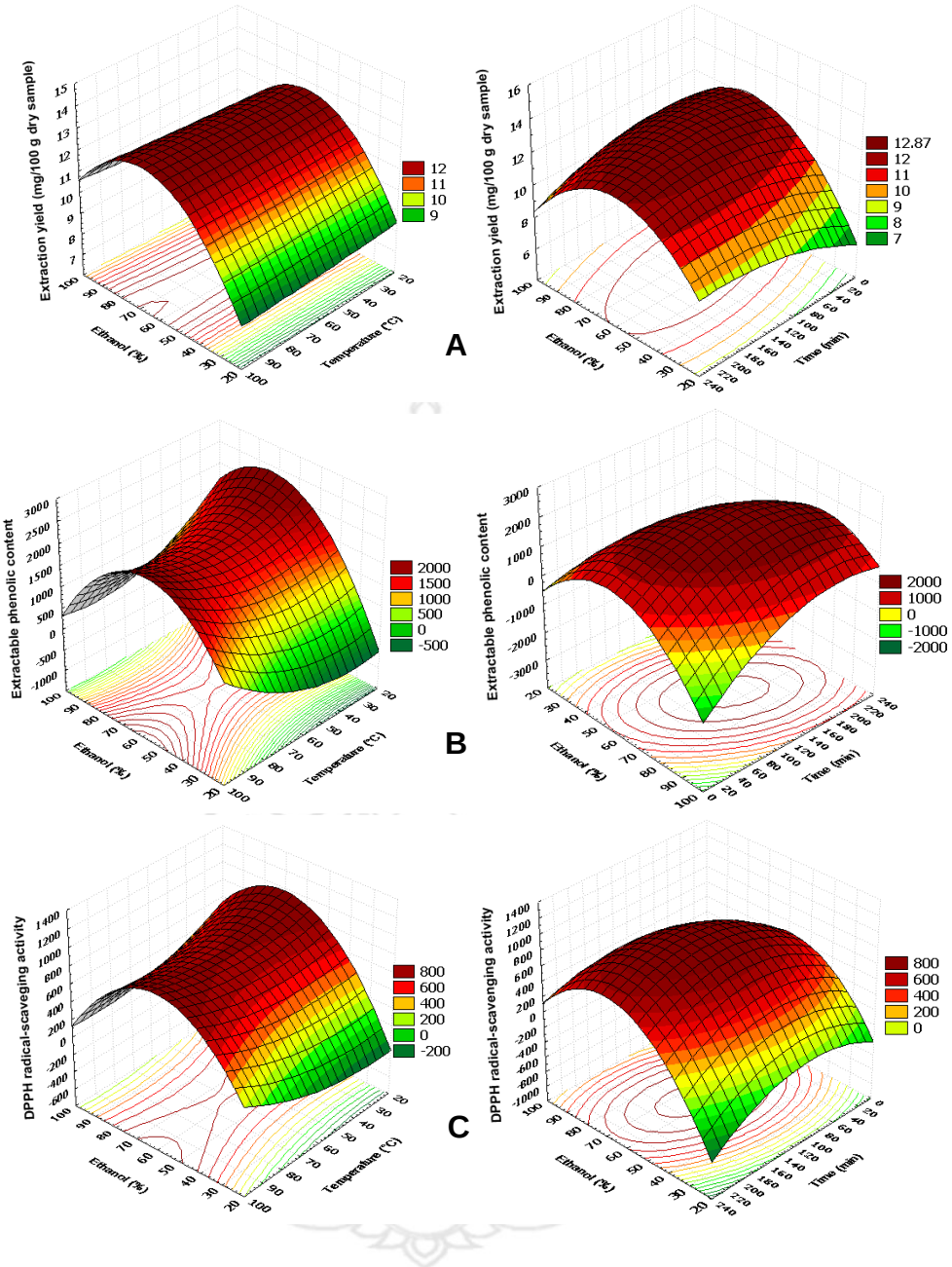


Figure 4.9 Response Surface Model Plot of Predicted Models Showing the Effects of Ethanol Proportion and Temperature (Left) and Effects of Ethanol Proportion and Time (Right) on Extraction Yield (mg/100 g Dry Fruit), EPC and DPPH (mg GAE/g Dry Sample) from Passion Fruit Seed Extracts.

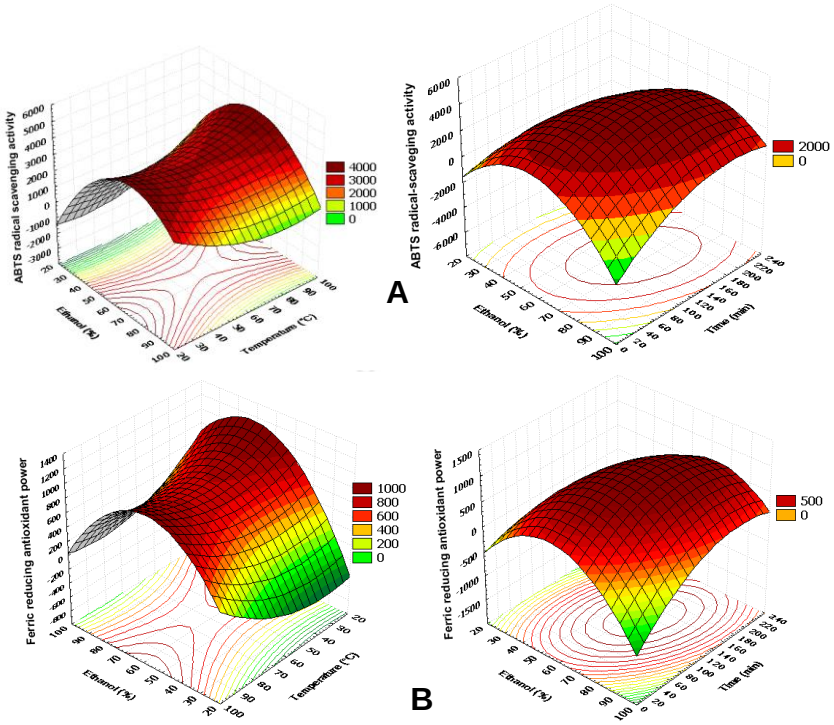


Figure 4.10 Response Surface Model Plot of Predicted Models Showing the Effects of Ethanol Proportion and Temperature (Left) and Effects of Ethanol Proportion and Time (Right) on ABTS and FRAP (mg GAE/g dry sample) from Passion Fruit Seed Extracts.

Figure shows the effect of ethanol concentration extraction temperature and extraction time on the extractable phenolic content. The plot of extractable phenol content as affected by ethanol concentration and extraction temperature demonstrates a marked increase in extractable phenol content with increase of ethanol concentration at a fixed extraction temperature and reached a peak at the middle ethanol concentration test, while an increase in extraction time at a fixed ethanol concentration also led to a marked increase in phenolic content (Figure 4.9-4.12).

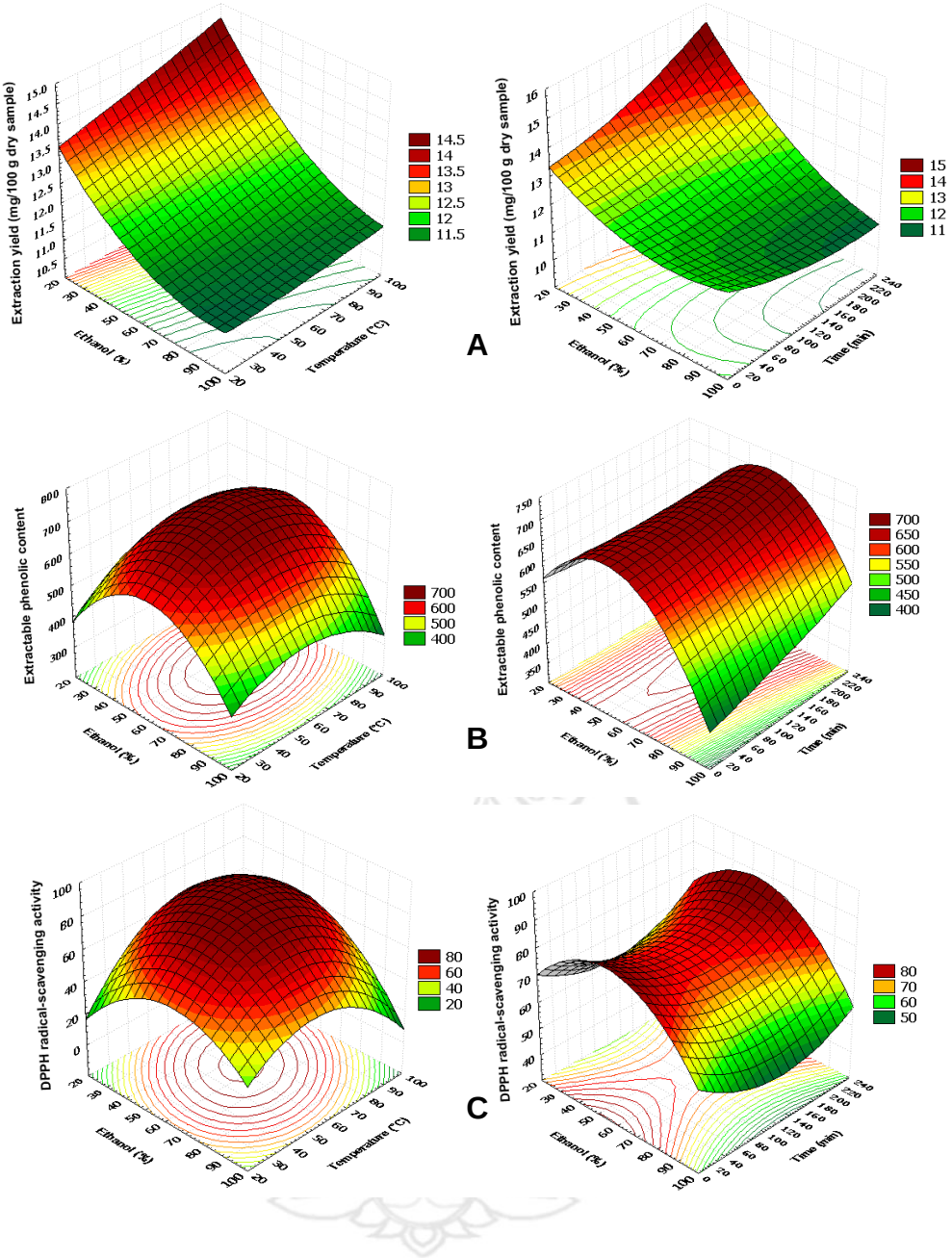


Figure 4.11 Response Surface Model Plot of Predicted Models Showing the Effects of Ethanol Proportion and Temperature (Left) and Effects of Ethanol Proportion and Time (Right) on Extraction Yield (mg/100 g dry fruit), EPC and DPPH (mg GAE/g dry sample) from Rambutan Seed Extracts.

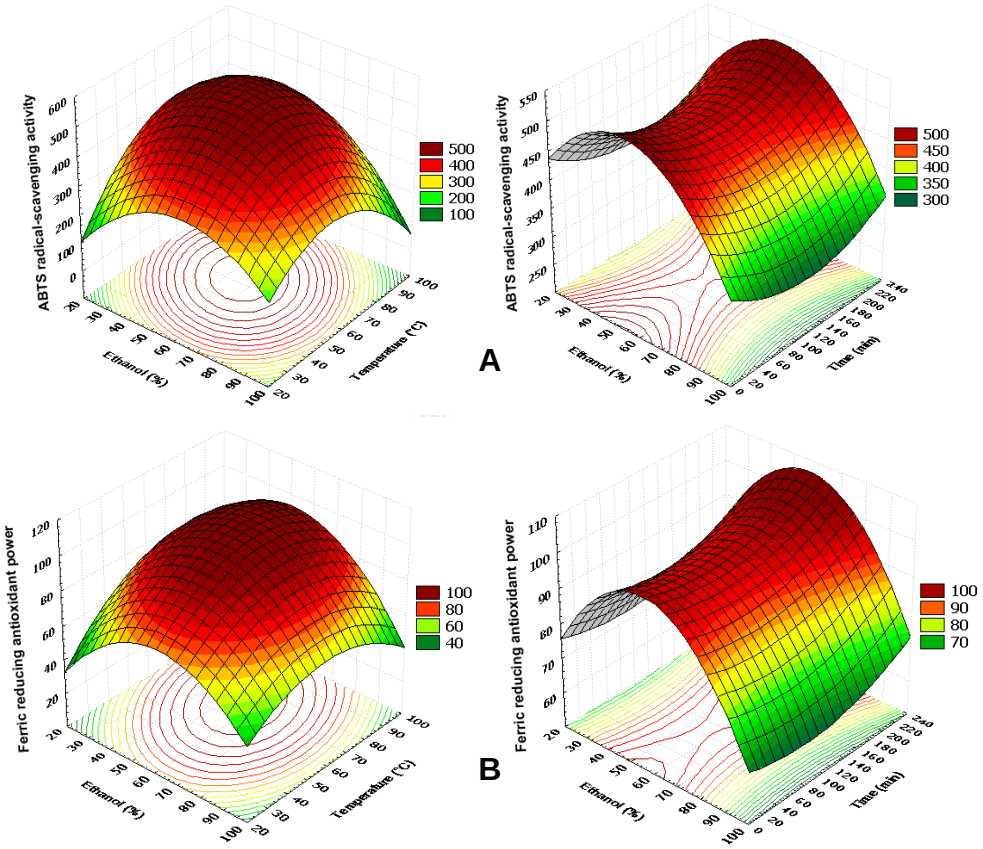


Figure 4.12 Response Surface Model Plot of Predicted Models Showing the Effects of Ethanol Proportion and Temperature (Left) and Effects of Ethanol Proportion and Time (Right) on ABTS and FRAP (mg GAE/g Dry Sample) from Rambutan Seed Extracts.

However extraction temperature did not effect to EPC that indicating a non-important factor (Figure 4.11B). Similarly, EPC of rambutan seed extract increased with increase in ethanol concentration and reached a peak at the middle ethanol concentration test (Figure 4.11B). The EPC for passion fruit seed extracts slowly increased with increase in the extraction time at a fixed ethanol concentration and reached a maximum at the highest extraction temperature tested (Figure 4.12B).

4.2.2.5 Effect of Process Variables on Antioxidant Activities

Statistical analysis revealed that the most relevant variable with $P < .001$ was ethanol concentration and temperature for passion fruit seeds and extraction temperature and time for rambutan seeds (Table 4.11 and 4.12). The ethanol concentration has this kind of effect of antioxidant activities because increasing the contact temperature and time of the solvent with to solid may improve the diffusion of the compounds. The result of multiple regression analysis showed that the antioxidant activities value of passion fruit and rambutan seeds were significantly ($P < .05$) affected by the linear and quadratic term of ethanol concentration, extraction temperature, and extraction time.

The final predicative equation for antioxidant activities of passion fruit and rambutan seeds extract by using significant terms showing in Table 4.11 and 4.12. As presented in the three-dimension plots for antioxidant activities values (Figure 4.9-4.12) the extraction process variables affected the extraction of antioxidants in a way similar to that in the case of extractable phenolic content. This is due to the fact that antioxidant activities of passion fruit and rambutan seeds are closely associated with the phenolic compounds.

For passion fruit seeds, antioxidant activities increased with the increased of ethanol concentration at a fixed temperature and also increased significantly ($P < .05$) with the increase of extraction temperature at a fixed ethanol concentration (Figure 4.11 and 4.12). Similarly, antioxidant activities value of rambutan seeds increased with the increase of extraction time at a fixed of ethanol concentration as represent in Figure 4.11 and Figure 4.11.

4.2.2.6 Optimum Condition for Maximum Extraction of Antioxidant Compounds

In order to verify the predictive capacity of the model, an optimum condition was determined using the simplex method and the maximum desirability for extraction yield, EPC, DPPH, ABTS and FRAP antioxidant activity from passion fruit and rambutan seeds. The measured values lay within a 95% mean confidence interval of the predicted value for those responses. Thus, the optimized conditions chosen and experiment value were shown in Table 4.13. These results confirmed the predictability of the model for the extraction of extraction yield, EPC, DPPH, ABTS and FRAP antioxidant activity from passion fruit and rambutan seeds in the experimental condition used. The R^2 and R^2-adjusted values for extraction yield, extractable

phenolic content, and antioxidant activites shown in Table 4.12 and 4.13 shows that the model had adequately represented the real relationship between the parameters chosen.

Under those optimum conditions, the corresponding predicted response values compared to experimental values for extraction yield, EPC, DPPH, ABTS and FRAP of the extracts were 12.41% (12.68%), 715 (707), 85 (81), 539 (522) and 107 (108) mg GAE/100 g dry rambutan seed, and 13.18% (14.41%), 3,140 (3,235), 1,328 (1,344), 5,793 (5,564) and 1,567 (1,509)mg GAE/100 g dry passion fruit seed, respectively. The values agreed with those predicted, thus indicating suitability of the model employed and the success of RSM in optimizing the extraction conditions.

The investigated results showed that longan seed extract had highest extraction yield, EPC, and all antioxidant activities determined in comparison with passion fruit and rambutan seed. Therefore, longan seed extracts was used for the further study on the development of active gelatin film incorporated with antioxidant longan seed extract.

Table 4.13 Optimal Extraction Conditions for Phenolic Antioxidants from the Extracts of Passion Fruit and Rambutan Seeds

Factor	Unit	Passion fruit	Rambutan seeds
Ethanol	% (v:v)	87.52	54.90
Temperature	°C	93.64	63.06
Time	min	186.25	220.91

4.3 Development of Gelatin Film Incorporated with Antioxidant Compounds

4.3.1 Thickness, Mechanical Properties, and Water Vapor Permeability

Thickness, mechanical properties, and water vapor permeability (WVP) of giant catfish skin gelatin films without and with longan seeds (LS) extract having 50 ppm to 500 ppm and BHT (50 and 100 ppm) are shown in Table 4.15. Thickness of the active gelatin films were ranged of 35-37 μm. Generally, thickness of film are distribution from 10-50 μm based on its application. All gelatin films had similar tensile strength (TS), elongation at break (EAB) and

thickness ($P > .05$). TS and EAB of gelatin films incorporated with and without LS extract or BHT at different concentrations were no significant different which these active film had TS and EAB in rang of 48-52 MPa and 16-18%, respectively. This result was similar to other report that TS and EAB of fish gelatin films were 40-60 MPa and 10-20%, respectively (Jongjareonrak, Benjakul, Visessanguan & Tanaka, 2008a; Gómez-Estaca, Bravo, Gómez-Guillén, Alemán & Montero, 2009). However, some studies showed that films added with antioxidant, a slight decrease in TS could be probably caused by incompatibility of antioxidant and gelatin molecules, resulting in the lessened integrity of film structure (Jongjareonrak, et al., 2008b). The study of Rattaya, Benjakul and Prodpran (2009) reported that fish skin gelatin film incorporated with seaweed extract showed that EAB of gelatin films incorporated with oxygenated seaweed extract was approximately 2-fold greater than that of the control. Polyphenol, which contained hydrophobic groups entered into hydrophobic district of protein by hydrophobic interaction. Furthermore, phenolic hydroxyl group of polyphenol combined with polar group of protein by hydrogen bonds (Shi & Di, 2000). Increased tensile strength and EAB were reported in soy protein isolate film cross-linked with ferulic acid (Ou, Wang, Tang, Huang & Jackson, 2005).

Table 4.14 Comparison between the Predicted and Experiment Values for Phenolic Antioxidant from the Extracts of Passion Fruit and Rambutan Seed.

Samples	Condition	Response Values				
		Yield	**EPC**	**DPPH**	**ABTS**	**FRAP**
Passion fruit	Predicted	13.68	3,141	1,327	5,793	1,567
	Experimental	14.41 ± 0.66	3,235 ± 109	1,344 ± 46	5,564 ± 375	1,509 ± 92
Rambutan	Predicted	12.41	715	85.89	539	107
	Experimental	12.68 ± 0.77	707 ± 11	80.94 ± 2.14	522 ± 11	108 ± 7

WVP of the gelatin films incorporated with/without LS or BHT is shown in Table 4.15. WVP of this result showed higher value when compare with other reports which ranged from 1.04×10^{-8} g mm h^{-1} cm^{-2} Pa^{-1} in the film containing LS to 1.38×10^{-8} g mm h^{-1} cm^{-2} Pa^{-1} in the film with BHT. Increasing in antioxidant compounds in the gelatin film significantly decrease in WVP

($P > .05$). Phenolic compounds, either in reduced or oxidized form, might enhance the cross-linking of gelatin.

Table 4.15 Mechanical[††] Properties, Water Vapor Permeability and Thickness of Gelatin Films Incorporated without and with LS Extract and BHT at Different Concentrations.

Sa,mles	Thickness (μm)	TS (MPa)	EAB (%)	WVP (10^{-8}·g·mm·h^{-1}·cm^{-2}·Pa^{-1})
Control	36.07 ± 2.46 ^{ab}	52.84 ± 7.59 ^{a†}	17.42 ± 9.68 ^{a}	1.45 ± 0.25 ^{c}
LS 50 ppm	36.13 ± 2.20 ^{ab}	52.65 ± 7.84 ^{a}	18.09 ± 7.88 ^{a}	1.23 ± 0.12 ^{abc}
LS 100 ppm	37.60 ± 2.29 ^{b}	50.13 ± 5.41 ^{a}	17.57 ± 6.99 ^{a}	1.09 ± 0.20 ^{ab}
LS 300 ppm	36.67 ± 2.02 ^{ab}	50.90 ± 5.15 ^{a}	16.48 ± 7.94 ^{a}	1.16 ± 0.14 ^{ab}
LS 500 ppm	35.93 ± 2.52 ^{ab}	49.53 ± 5.05 ^{a}	18.62 ± 7.51 ^{a}	1.04 ± 0.06 ^{a}
BHT 50 ppm	35.60 ± 2.20 ^{a}	49.89 ± 1.93 ^{a}	16.42 ± 7.22 ^{a}	1.38 ± 0.15 ^{bc}
BHT 100 ppm	37.13 ± 2.17 ^{ab}	48.17 ± 2.63 ^{a}	16.99 ± 7.14 ^{a}	1.35 ± 0.03 ^{bc}

Note. † Different superscripts in the same column indicate significant differences ($P < .05$).

†† Mean ± SD ($n = 7$).

LS: Longan seed extract

Increasing reticulation of the network could decrease the free volume of the polymeric matrix and increase the tortuosity of the pathway of the water molecules through the network, thus decreasing diffusion rate of water molecules through the films (Cao, Yang & Fu, 2007). The greater decrease in WVP was noticeable in films added with LS, compared with those incorporated with BHT. The differences in size and the hydrophobicity between LS and BHT might result in the different WVP of the films. Additionally, the chemical nature of the macromolecule, structural/morphological characteristics of the polymeric matrix, chemical nature of the additives and degree of cross-linking affect the barrier characteristics (Rattaya et al., 2009). Regarding water vapour permeability, significant differences were found in LS extract add rather

than BHT incorporated into edible films. The WVP values recorded were comparable to those reported in bigeye snapper (~1.71 × 10^{-8}), brownstripe red snapper (~1.93 × 10^{-8}) or tuna gelatin films (~2 × 10^{-8}) (Gómez-Guillén, Ihl, Bifani, Silva & Montero, 2007; Jongjareonrak, et al., 2008a), although these authors only employed glycerol as the plasticizer (0.5 g/g protein and 0.25 g/g gelatin, respectively). However, taking into account the amino acid composition of squid gelatin, higher WVP values were expected since this gelatin has fewer hydrophobic residues, thus decreasing its hydrophobicity as compared to fish gelatins.

4.3.2 Color, Light Transmission, and Film Transparency

Color of fish skin gelatin films incorporated with/without LS or BHT show in Table 4.16. Incorporation of LS or BHT extract had significant effect on *L**-value, *a**-value and *b**-value of resulting film, regardless of final antioxidant concentration of FFS used for film preparation ($P <$ .05). With increasing LS, a clear decreasing trend in *L**-values was observed. The *L**-values dropped from an initial of 89.6–83.9 units signifying an increase in the color strength. As we aspect, the redness and yellowness of the film incorporated with LS extract increased, as indicated by the increase in *a** and *b**-values, respectively, compared with those of the control film (Table 4.16). *a** and *b**-values showed an increasing trend; from an initial value of -2.20 and 11.72 units in control to -0.89 and 26.43 units in LS added.

However, the film that incorporated with BHT shown higher *L**-value (90.96) than that of the gelatin incorporated with/without LS extract. Pigments remaining in LS extract most likely contributed to the changes in colors of resulting films. The result was in agreement with Cao et al. (2007) and Rattaya et al. (2009) who reported that gelatin film incorporated with phyto compounds had the changes in color. Anthocyanin, the pigments responsible for the characteristic brown color of plants, most likely result in color changes in food. This could be associated with the different color characteristics of the gelatin film from fish skins.

The transparency of the gelatin films incorporated with LS or BHT is showed in Table 4.16. The gelatin films incorporated with LS at 500 ppm were more transparent than the control film and that incorporated with BHT ($P <$ .05), respectively, as indicated by the lower value. This observation was coincidental with the greater changes in transmission of visible light which more LS added more transmission. However, no differences in transparency were observed between

films incorporated with LS and BHT at concentration of 50-100 ppm ($P > .05$). Regardless of antioxidant addition, LS added was generally more transparent than BHT added in the gelatin film ($P < .05$).

Table 4.16 *L**, *a** and *b** values[†] of Gelatin Films Incorporated without and with LS Extract and BHT at Different Concentrations.

Film samples	*L**	*a**	*b**	Transparency value
Control	89.57 ± 0.29 ^{e††}	-2.20 ± 0.02 ^{a}	11.72 ± 0.32 ^{b}	3.32 ± 0.01 ^{b}
LS 50 ppm	89.01 ± 1.01 ^{d}	-1.66 ± 0.13 ^{c}	12.67 ± 2.60 ^{b}	3.24 ± 0.01 ^{a}
LS 100 ppm	87.59 ± 0.26 ^{c}	-1.60 ± 0.11 ^{cd}	16.30 ± 0.69 ^{c}	3.24 ± 0.02 ^{a}
LS 300 ppm	86.24 ± 0.36 ^{b}	-1.54 ± 0.14 ^{d}	20.38 ± 0.98 ^{d}	3.32 ± 0.01 ^{b}
LS 500 ppm	83.86 ± 0.35 ^{a}	-0.89 ± 0.21 ^{e}	26.43 ± 0.96 ^{e}	3.36 ± 0.01 ^{c}
BHT 50 ppm	90.84 ± 0.19 ^{f}	-1.87 ± 0.02 ^{b}	7.02 ± 0.28 ^{a}	3.30 ± 0.02 ^{a}
BHT 100 ppm	90.96 ± 0.28 ^{f}	-1.68 ± 0.03 ^{c}	6.20 ± 0.32 ^{a}	3.24 ± 0.01 ^{a}

Note. [†] Mean ± SD ($n = 10$).

[††]Different superscripts in the same column indicate the significant differences ($P < .05$).

Transmission of UV and visible light at selected wavelength in the range of 200-800 nm of gelatin film incorporated with LS extract at different concentrations is shown in Figure 4.14. Light transmission of all films tested was negligible at 200 nm and was low at 280 nm. In general, film incorporated with LS extract showed the more light transmission in visible range (350-800 nm) than the control. With phenol-protein interaction, the unfolding of gelatin was more intense. As a result, the uniform network could be formed and the light could pass through such a network more easily. No differences in transparency of gelatin films incorporated with and without LS extract or BHT were found, irrespective of concentration of FFS used for film preparation ($P > .05$). The films with added LS or BHT extracts, although maintaining their transparency, were darker, and this could affect the light transmission properties of the films.

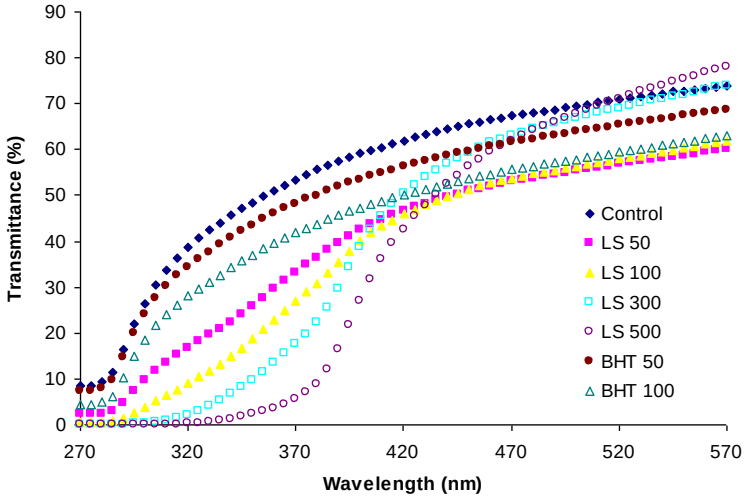


Figure 4.13 Changes in Percentage of Transmittance at Wavelengths Ranging from 570 to 270 nm for Giant Catfish Skin Gelatin Films either Unsupplemented or Supplemented with an Added LS Antioxidant Extract or BHT (50-500: Number Indicated the Concentration in ppm).

On adding extract, the transmittance values became inversely proportional to the amount of extract added. These findings suggest that, in addition to the potential antioxidant benefits afforded, films supplemented with added LS extracts may also possess good light barrier properties, especially to ultraviolet light, a powerful lipid-oxidising agent. These light barrier attributes are in part ascribable to the polyphenol composition of the extracts added to the films, since the transmittance results for the extracts were indicative of a similar pattern of light absorption. These results are in agreement with previous findings that reported better light barrier properties when antioxidant extract from murta was added to gelatin-based edible films, and tuna-skin and bovine-hide gelatin films (Gómez-Guillén & Montero, 2007; Gómez-Estaca et al., 2009). Other protein-made films from gelatin (Jongjareonrak, et al., 2006a,b), myofibrillar proteins (Shiku, Hamaguchi, Benjakul, Visessanguan & Tanaka, 2004), and milk whey proteins (Fang, Tung, Britt, Yada & Dalgleish, 2002) have also displayed good light barrier properties. As a general rule, light transmission by this type of food packaging is much lower than that of synthetic polymers (Shiku, Hamaguchi, Benjakul, Visessanguan & Tanaka, 2004).

4.3.3 Thermal Properties

Transition temperature and transition enthalpy of gelatin incorporated with LS extract and BHT at different concentration are shown in Table 4.17. Transition temperatures (T_{max}) of all gelatin films were in the range of 87.99-94.52°C. Transition temperature indicated the temperature causing the disruption of the polymer interaction formed during film preparation. For transition temperature, a significant difference in T_{max} was found between the control film and film incorporated with LS extract or BHT. The films incorporated with BHT had higher transition temperature than control. For transition enthalpy, the higher enthalpy was shifted to a higher level after the endothermic peak, indicating a change in the film's heat capacity. The shift in baseline was a clear indication of the glass transition temperature. The large endothermic peak appering during this baseline shift represented the aging enthalpy of glassy film. The films did not exhibit any melting event attributable to crystallization of the gelatins.

Adding the LS extract raised the glass transition temperature (T_g) in fish gelatin films. The increase was relatively higher as the amount of added antioxidant extract. Futthermore, the LS extract seemed be bring about a larger increase in T_g than BHT at the same concentration. The enhanced cross-linking induced by phenolic compound in LS extract might contribute to lower molecular mobility. The transition enthalpy of proteins increase with the chian rigidity and the intensity of both inter- and intra-molecular interaction, including hindrance to interal rotation along the macromolecular chain (Barreto & Meyer, 2006). A reduction in the number of hydrogen bonds with a simultaneous increase in the extent of covalent cross-linking resulted in the increase in thermal stability (de Carvalho & Grosso, 2004). Hydrogen bonds or other weak stabilizing the network might be lower in film prepared from film forming solution with antioxidant solvent. This was possibly caused by the increased repulsion force between gelatin molecules in present of antioxidant solvent. This resulted in the low enthalpy used for breaking those bondings (Rattaya, et al., 2009). Nevertheless, the non-disulfide covalent bond stabilizing the network of film incorporated with LS extract and BHT could contribute to the increased transition temperature, reflecting the more rigid structure of film network. Thus, the addition of LS extract had the impact on the thermal property of resulting film. The studied of Gómez-Estaca et al. (2009) adding the herb extracts raised the glass transition temperatures in both the bovine-hide and tuna-skin gelatin films. The increase in T_g was relatively higher as the amount of added extract rose to

bovine and tuna-skin gelatin film. Furthermore, the rosemary extract seemed to bring about a larger increase in the T_g than the oregano extract, despite the similar phenolic contents.

Table 4.17 Transition Temperature (T_{max}) and Transition enthalpy (T_g)[†] of Fish Skin Gelatin Films as Affected by Antioxidant Concentrations.

Film Samples	Transition Temperature (°C)	Transition Enthalpy (J/g)
Control	87.99 ± 0.96 [a††]	65.70 ± 4.99 [c]
LS 50 ppm	89.48 ± 1.22 [ab]	54.92 ± 3.55 [abc]
LS 100 ppm	92.64 ± 1.87 [c]	48.88 ± 17.07 [abc]
LS 300 ppm	93.11 ± 1.18 [c]	42.35 ± 4.66 [ab]
LS 500 ppm	94.52 ± 1.96 [c]	38.67 ± 2.28 [a]
BHT 50 ppm	92.16 ± 1.40 [bc]	60.50 ± 17.57 [bc]
BHT 100 ppm	92.83 ± 2.56 [c]	56.11 ± 7.11 [abc]

Note. [†] Mean ± SD (n = 3).

[††] Different superscripts in the same column indicate the significant differences ($P < .05$).

4.3.4 Water solubility and Protein Solubility of Gelatin Film

Water solubility and protein solubility of gelatin films incorporated without and with LS or BHT is shown in Table 4.18. Water solubility of the gelatin film incorporated with LS extract was 52-54 % which significant higher than control film and the gelatin film added BHT. Addition of the antioxidant extracts to the giant catfish skin gelatin brought about a pronounced increase in film solubility. Kim et al. (2006) also reported increased film solubility on adding green tea extract to soya-protein films. However, the presence of the phenolic extracts or BHT at the same level did not produce any significant differences in the solubility of the giant catfish skin gelatin films. This finding was in consonance with the corresponding electrophoretic profiles, which likewise were barely affected by the different blends. This effect observed in the electrophoretic profile was more apparent in the films containing the more concentrated LS extract, but adding the more concentrated LS extract also exerted an influence, reducing the quantities of HMW-a

present to some extent. The differences in water solubility due to qualitative differences in the polyphenolic composition were observed (Gómez-Estaca, et al., 2009).

Protein solubility of the gelatin film was over 95%, with similar result among different treatments (Table 4.18). No significant different in protein solubility ($P > .05$) was detected in the film incorporated with LS extracts or BHT. Protein solubility over 90–95% has been also reported for other reports (Gbogouri, Linder, Fanni & Parmentier, 2004; Klompong, Benjakul, Kantachote & Shahidi, 2007). The result indicated the presence of hydrogen bond and hydrophobic interaction of protein in the film matrix. This high solubility of film was due to the generation of low molecular weight peptides by hydrolysis, which are expected to have proportionally more polar residues than the parent proteins, with the ability to form hydrogen bonds with water and increase solubility (Gbogouri, et al., 2004). Shi and Di (2000) reported that the formation of phenolic-protein was governed in part by hydrophobic interaction and hydrogen bonds. Polyphenol contained hydrophobic groups and hydroxyl group, which could bind with proteins by hydrophobic interaction and hydrogen bonds, respectively.

Table 4.18 Film Solubility and Protein Solubility[†] of Gelatin Films Incorporated without and with LS Extract and BHT at Different Concentrations.

Film Samples	Film Solubility (%)	Protein Solubility (%)
Control	41.83 ± 2.01 [a††]	97.79 ± 0.26 [ab]
LS 50 ppm	52.47 ± 6.82 [ab]	98.26 ± 0.08 [b]
LS 100 ppm	52.81 ± 2.05 [ab]	98.19 ± 0.11 [b]
LS 300 ppm	53.57 ± 6.30 [b]	97.66 ± 0.21 [a]
LS 500 ppm	53.77 ± 8.30 [b]	97.59 ± 0.24 [a]
BHT 50 ppm	44.44 ± 5.74 [ab]	97.61 ± 0.22 [a]
BHT 100 ppm	48.51 ± 6.06 [ab]	97.82 ± 0.54 [ab]

Note. [†] Mean ± SD ($n = 3$).

[††] Different superscripts in the same column indicate the significant differences ($P < .05$).

4.3.5 Electrophoretic Analysis

The electrophoretic analysis of the active gelatin films showed a molecular weight distribution very similar to the original giant catfish gelatin, with predominance of α-chains (≈100 kDa) and their dimmers (β-components) (Figure 4.14). In the active gelatin film added with LS extract or BHT at various concentrations, a protein fraction of lower molecular weight than the α-chains appeared, as well as a trace fraction at the end of the polyacrilamide gel. The presence of LS extract and BHT in the gelatin film had hardly any effect on the electrophoretic profiles as compared to the control gelatin solution (without incorporated), irrespective of extract concentration. Adding LS extract to the gelatin also brought about increase in the amount of HMW-a present. In contrast, the gelatin film incorporated with LS extract at higher concentrations registered appreciable decreases in β-components and high-molecular-weight aggregates (HMW-a) and a certain increase of polypeptides with molecular weights below 100 kDa. When looking at the molecular weight composition of the active gelatin films, the incorporation of LS extract modified perceptibly the electrophoretic profile compared to that of the control film. The γ-trimmers traces slightly disappeared and the presence of β components and α_2 chains are notably reduced. These results suggested a high degree of interaction between the phenolic substances in the LS extract and the fish-gelatin polypeptides, giving rise to cleavage or disruption of the covalently associated α-chains (β-components and HMW-a)

4.3.6 FTIR Spectra of the Gelatin Film Incorporated with LS Extract or BHT

FTIR spectra of farmed giant catfish skin gelatin films without and with LS extract or BHT incorporated are depicted in Figure 4.15. Generally, slight changes in absorption band intensity at each peak were observed in the fish skin gelatin films when LS extract or BHT were incorporated in different concentrations. The absorption bands in the spectra were situated in the amide band region. In general, the amide I and amide II bands of fish skin gelatin films from giant catfish appeared at the 1630 cm^{-1}. The gelatin films incorporated with/without LS extract or BHT represented similar peaks patterns. From FTIR, it is evident that addition of LS extract, polyphenols could form hydrogen bonding and covalent bonding and thus occupied the functional group of gelatin 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 gelatin films incorporated with LS extract.

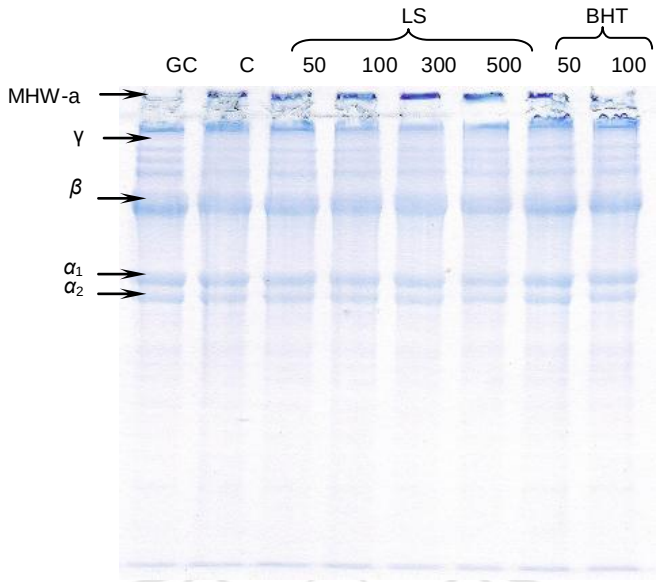


Figure 4.14 Electrophoretic Profiles of the Giant Catfish Skin Gelatins with Added LS Extract or BHT. GC: Giant Catfish Skin Gelatin, C: No Added Extract, Number Indicated Concentration (ppm).

Amide I and amide II of gelatins observed at 1700-1600 and 1560-1500 cm^{-1} were reported by Muyonga et al. (2004), and Yakimes et al. (2005). The amide I vibration mode is primarily a C=O stretching vibration coupled to contributions from the CN stretch, CCN deformation and in-plane NH bending modes (Jongjareonrak, Benjakul, Visessanguan & Tanaka, 2008). The absorption in amide I region is the most useful for infrared spectroscopic analysis of the secondary structure of proteins (Surewicz & Mantsch, 1988). Yakimes et al. (2005) reported that the absorption peak at 1633 cm^{-1} was the characteristic for the coil structure of gelatin. The interaction of those additives with gelatin molecules most likely caused the alteration of their secondary structure.

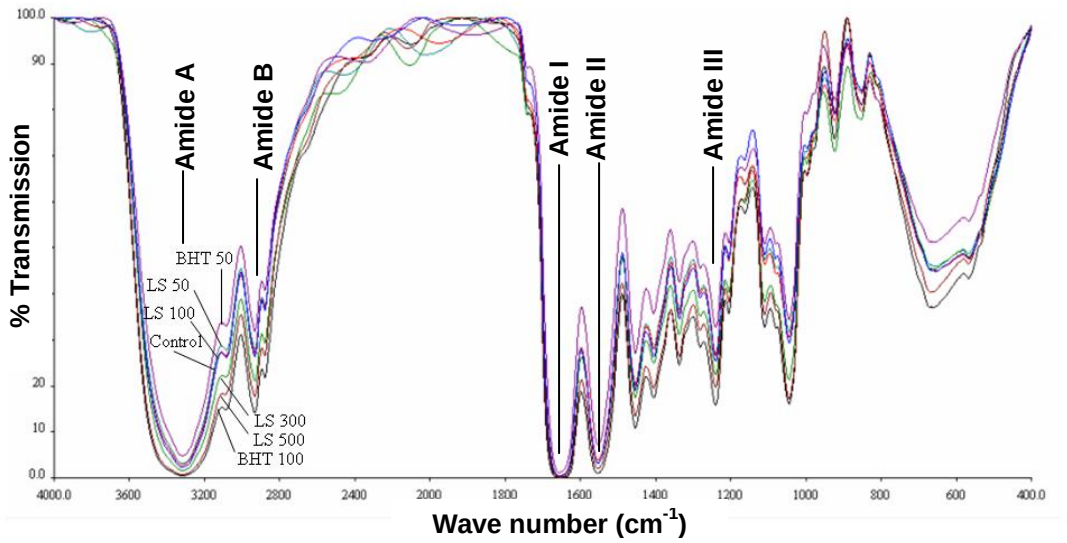


Figure 4.15 Fourier Transform Infrared Spectroscopy (FTIR) of Gelatin Film Incorporated without and with LS Extract and BHT at Different Concentrations.

The amide II vibration mode is attributed to an out-of-phase combination of CN stretch and in-plane NH deformation modes of the peptide group (Jongjareonrak, et al., 2008). The amide II band is generally considered to be much more sensitive to hydration than to secondary structure change (Wellner, et al., 1996). Increase in LS extract or BHT in the film did not change in absorption peak in this study. However, the report of Jongjareonrak et al. (2008) showed that the different FTIR spectra of films from both gelatins as affected by the incorporation of BHT or α-tocopherol revealed that the interaction of gelatin from both sources with additives was different. This might be associated with the different dynamics of gelatin backbone involving flexibility, rigidity or mobility of molecules. These interaction and structural alteration might be related to the mechanical properties of resulting film.

4.3.7 Changes in TPC and Antioxidative Activity

TPC and antioxidative activity of fish skin gelatin films incorporated with LS extract or BHT at the concentrations of 50 - 500 ppm during storage was monitored (Figure 4.16 and 4.17). The gelatin film added with LS showed higher TPC value than the film added with BHT at the same concentration. TPC value of gelatin film incorporated with LS extract or BHT was increase

when storage for long time. Therefore, interactions between the antioxidants and gelatin film matrix might be formed via hydrogen bonding, particularly during film formation. As a result, a small amount of antioxidants could be extracted by methanol (Jongjareonrak, et al., 2008). These results were similar to DPPH radical scavenging activity value.

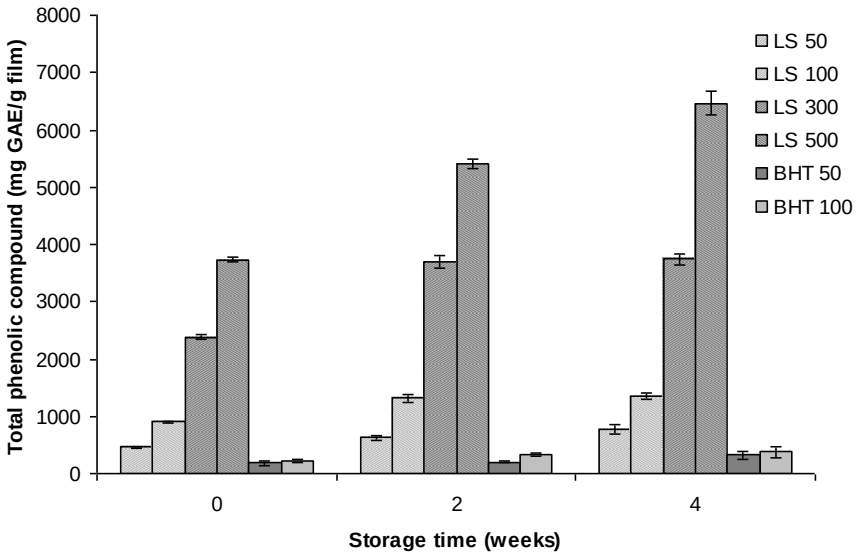


Figure 4.16 Total Phenolic Compound of Gelatin Film Incorporated with LS Extract or BHT During Storage (50-500: Number Indicated the Concentration in ppm).

Also DPPH radical scavenging activity of the methanol extracts from the gelatin films of LS or BHT increased with increasing storage time ($P < .05$). The results were in agreement with Jongjareonrak et al., (2008) who study in changes in antioxidative activity of fish skin gelatin films incorporated with BHT or α-tocopherol during storage. The renaturation of gelatin chains into the helix coil structure during storage might cause the release of antioxidant from the film matrix. Consequently, a greater amount of LS or BHT could be extracted from the film when the storage time increased. Wessling, Nielsen and Leufven (2000) reported that BHT-impregnated HDPE film was free from BHT and 19% of the original amount incorporated to the film remained in the cereal after 6 weeks. Due to the greater reactivity of proteins in interacting with antioxidants added, a greater amount of antioxidants should be incorporated to obtain films with

an excessive amount of antioxidants in the free form. The releasing characteristic of antioxidants incorporated as well as properties of the films should be further investigated.

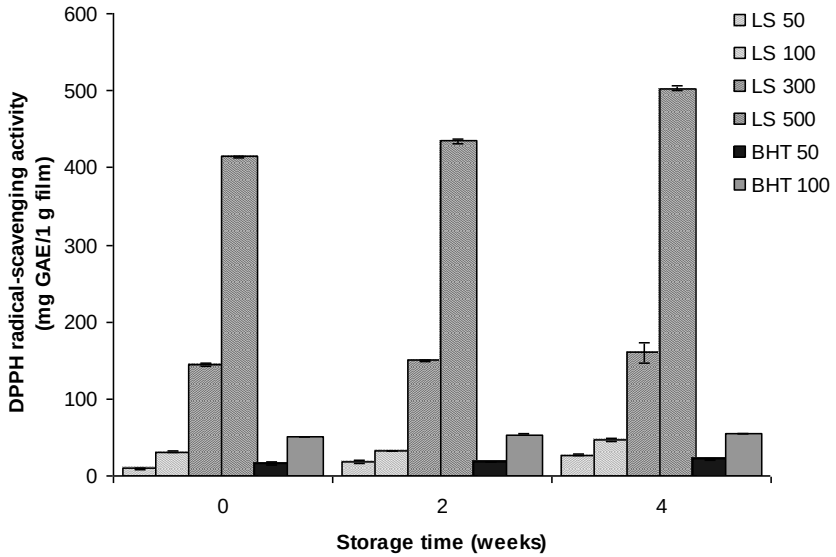


Figure 4.17 DPPH Radical Scavenging Activity of Gelatin Film Incorporated with LS Extract or BHT During Storage (50-500: Number Indicated the Concentration in ppm).

4.3.8 Changes in Lipid Oxidation Products

The formation of CD, PV and TBARS values of soybean oil keep in fish skin gelatin films bag with and without LS extract or BHT at a level of 50-500 ppm during dark storage at 30°C are depicted in Figure 4.18-20. The impact of antioxidant compounds in film on conjugated diene (CD) in oil during the storage is shown in Figure 4.18. After the initiation stage, oxidation is propagated *via* hydrogen subtraction in the vicinity of double bonds. This propagation step implies the formation of isomeric hydroperoxides that frequently carry conjugated diene groups. This is the mechanism of formation of CD. CD levels increased continuously in all samples throughout the 30 day of storage. All samples added with antioxidant compounds showed lower CD formation, compared with the control ($P < .05$). At the same concentration, the effect of LS extract and BHT on CD formation showed not significant different in the prevention ($P > .50$). Five hundred ppm of LS extract exhibited the highest efficacy in preventing of lipid oxidation.

This was due to the higher radical scavenging activity of phenolic acid, thereby lowering the subsequent generation of reactive lipid radicals, which can undergo further chain reactions. The increase in CD was coincidental with the increase in PV.

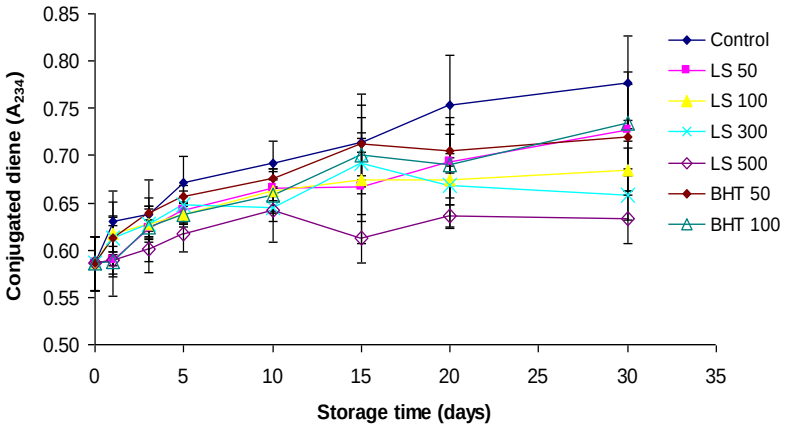


Figure 4.18 Effect of Antioxidant in Gelatin Film at Different Concentration on the Formation of Lipid Oxidation Products in Soybean Oil During Storage for a Period of 30 Days

PV of the control sample was higher than that of samples added with LS extract or BHT throughout the storage ($P < .05$) (Figure 4.19). Samples added with 500 ppm of LS extract contained a lower PV than that of the control and those added with lower concentration of the extract or BHT. The results indicated the efficient antioxidative activity of LS extract in the oil keep in gelatin film bag system. Five hundred ppm of LS extract also showed higher antioxidant activity in most of antioxidant assays (Figure 4.17). Free radical scavenging antioxidants including phenolic compound in LS extract interfere with the initiation or propagation steps of lipid oxidation reactions by scavenging lipid radicals and forming low-energy antioxidant radicals that do not readily promote oxidation of unsaturated fatty acids (Frankel, 1998). The reducing capacity of phenolic antioxidants was realised as a key function for retarding and inhibiting lipid oxidation. Many research showed that phenolic compounds in plant had efficiency in preventing the lipid oxidation in various products. Tea catechin addition (300 mg/ kg meat) effectively reduced lipid oxidation in cooked beef and chicken meat (Tang, et al., 2001). The tannic acid was a more effective antioxidant in the fish mince system (Maqsood & Benjakul, 2010; Alghazeer,

Saeed & Howell, 2008). Jongjareonrak et al. (2008) also report that incorporated gelatin film with α-tocopherol exhibit preventing the oxidation of lard.

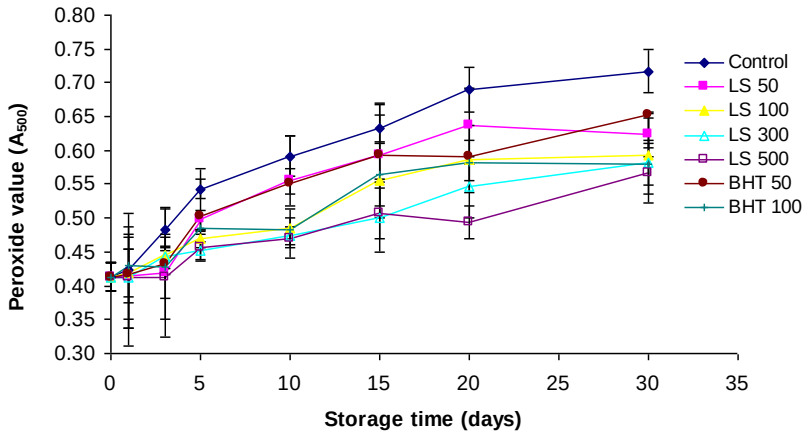


Figure 4.19 Effect of Antioxidant in Gelatin Film at Different Concentration on the Formation of Lipid Oxidation Products in Soybean Oil During Storage for a Period of 30 Days

In general, TBARS values of all treatments increased continuously up to 30 days of storage ($P < .05$) (Figure 4.20). However, the increase in TBARS value of oil keep in fish skin gelatin films bag with antioxidant was at a much lower rate, compared with control sample. Commonly, TBARS assay is a method used to measure secondary lipid oxidation products, specifically aldehydes (Jardine, Antolovich, Prenzler & Robards, 2002). Regardless of antioxidant addition, TBARS value of oil keep in fish skin gelatin films bag from both LS extract and BHT slightly increased during storage. Fish skin gelatin films might function as a barrier to oxygen permeability at the oil surface. Therefore, only a small amount of oxygen could penetrate to the oil, leading to the lower oxidation rate (Jongjareonrak, et al., 2008). From the results, no differences in TBARS were observed among oil keep in gelatin films added with LS extract or BHT.

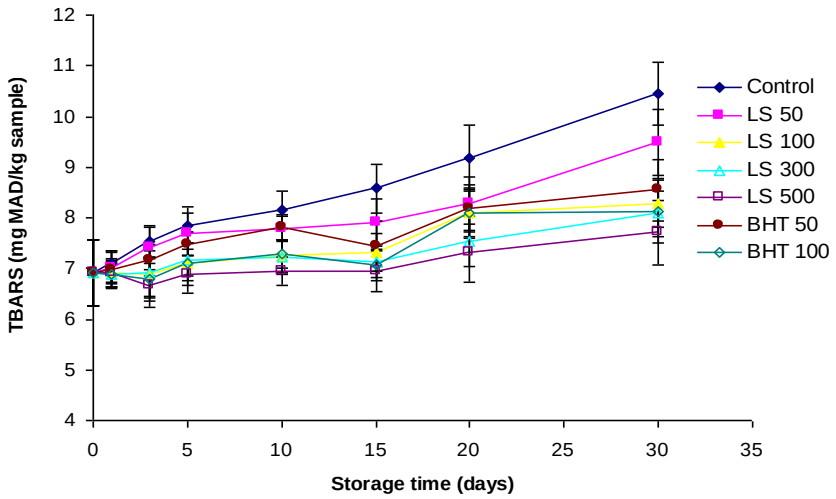


Figure 4.20 Mean TBARS Values (mg of Malonaldehyde/kg Sample) of Soybean Oil Contain in Gelatin Film Incorporated Antioxidant Package, at Dark Storage for 0, 1, 3, 5, 10, 15, 20 and 30 Days (50-500: Number Indicated the Concentration in ppm).

Interaction between antioxidant and film matrix via some bondings possibly resulted in the lowered release of those antioxidants to the oil. As a consequence, no differences in TBARS value were found between samples keep in the gelatin film bag with antioxidants at the same concentraction. It has been reported that the incorporation of hydrophobic compounds into films caused negative effects on oxygen transmission rate (Wessling, et al., 2000). When the TBARS values of oil keep in skin gelatin films bag with low and high concentration of antioxidant were compared, oil keep in the latter films bag had a slightly greater TBARS values at the same storage time determined.

4.3.9 Microstructure of Active Gelatin Films

SEM micrographs of the morphology of the surface and cross-sections of gelatin film with or without LS extract and BHT. It displayed that the untreated films have smooth surface. However, the smooth surface became more roughness when increase in antioxidant concentration (Figure 2.21-2.22). Compared the cross-section of unsupplement with supplement films, it can be seen that after LS extract or BHT were added, the small bubble in the matrix of gelatin film appeared as the addition concentration of antioxidant was increased. This was in accorded with

gelatin modified with hydroxyl group, which because of forming some network structures. Interaction reaction between lysine side chain of protein and quinine form of polyphenol leading to polymerisation, complex formation occur (Rawel, Kroll & Rohn, 2001).

Giant catfish skin gelatin active films incorporated with antioxidant extracts from fruit seeds had similar properties to films without extracts. The increase in the fruit seed extracts concentration in the active films intendance some properties of the films. However, the incorporation of the fruit seeds into the films at higher concentration exhibited the higher retardation effect against the oxidation of soybean oil inside the active film pouch.

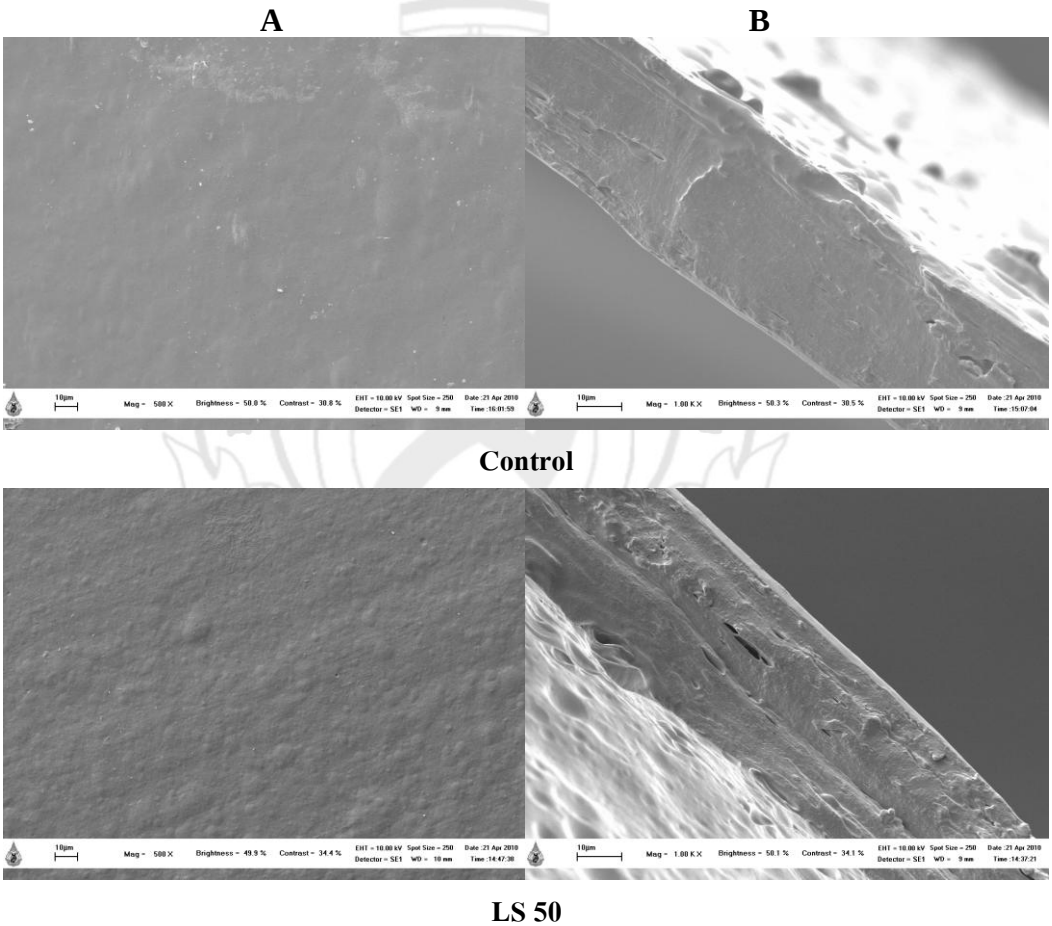


Figure 4.21 SEM Micrographs of Gelatin Films. Surface (A) and Cross Section (B) of Gelatin Film without Antioxidant and Film Incorporated with LS Extract 50 ppm.

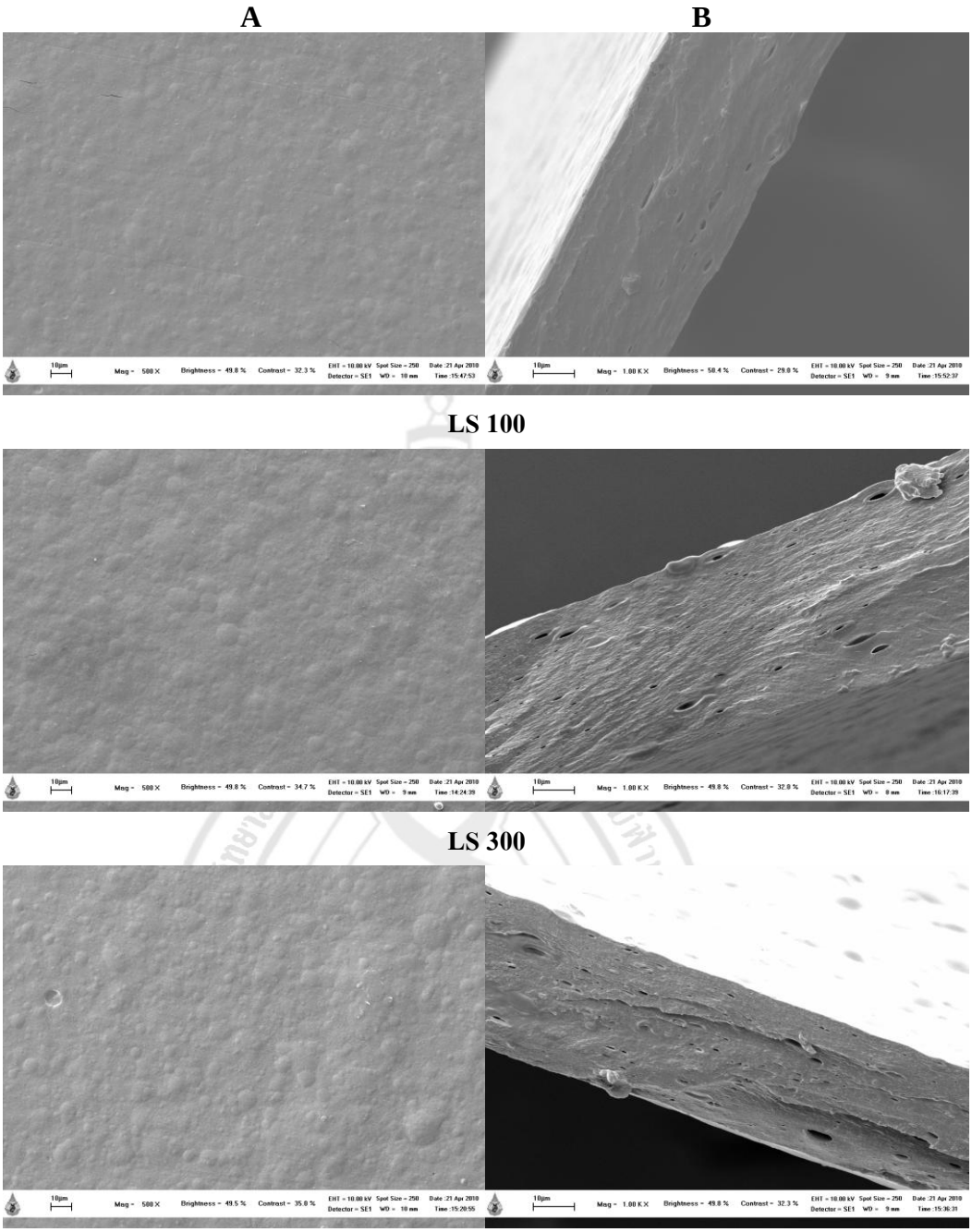


Figure 4.22 SEM Micrographs of Gelatin Films. Surface (A) and Cross Section (B) of Gelatin Film Incorporated with LS Extract 100-500 ppm.

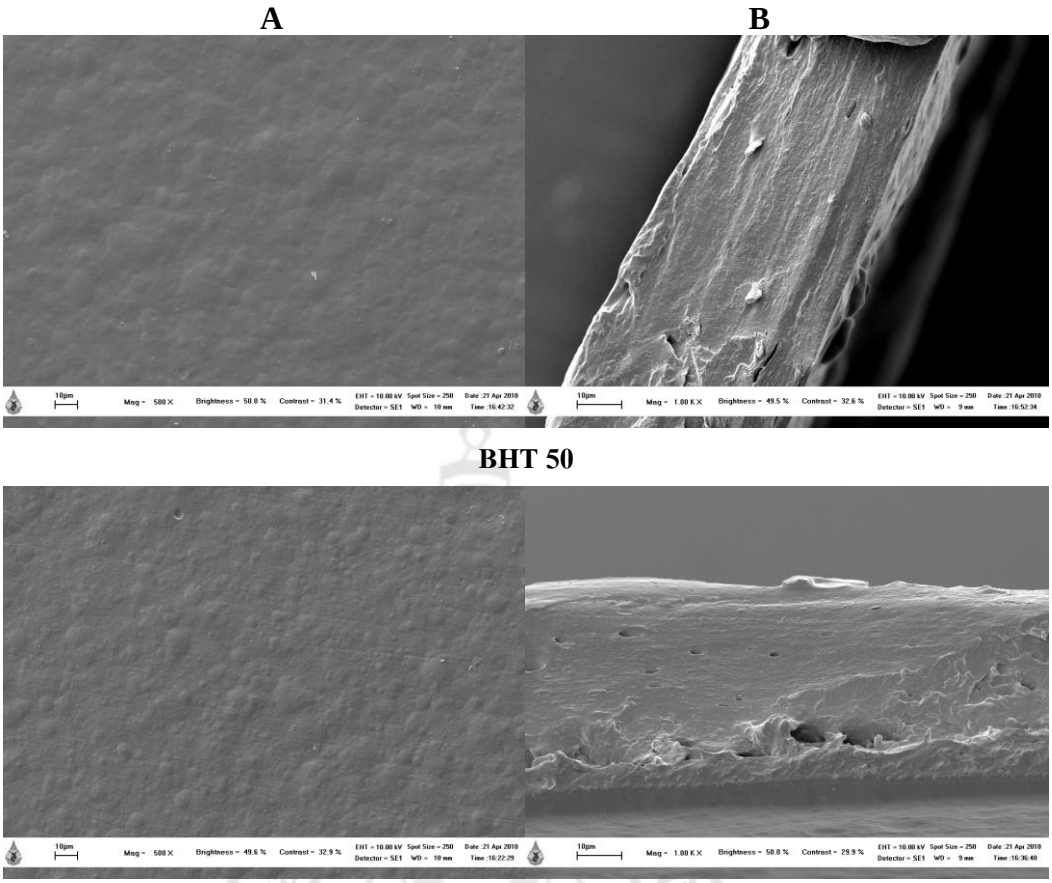


Figure 4.23 SEM Micrographs of Gelatin Films. Surface (A) and Cross Section (B) of Gelatin Film Incorporated with BHT 50-100 ppm.

CHAPTER 5

CONCLUSION

Re-extraction process to obtain the highest recovery was successfully developed. Higher temperatures and longer time during the re-extraction process had significant effects to the gelatin yield. The highest extraction yield was achieved at 90 °C for 12 h. The gelatin obtained from the re-extraction process showed comparable properties to the commercial beef skin gelatin in terms of physicochemical properties.

Longan seed was the most potential source for antioxidants extraction among lychee, passion fruit, and rambutan seeds. RSM was effectively used to design and obtain the optimal conditions for antioxidants extraction from fruit seeds. Under optimized conditions (53% ethanol, 58°C, and 139 min) the experimental values were well agreed with the values predicted by ridge analysis.

The giant catfish skin gelatin active film was successfully developed by incorporation of phenolic compounds rich extract from longan seed or BHT. Auto-oxidation of soybean oil was effectively retarded when the oil was filled in the gelatin active film pouch, and the greater effect was observed when the antioxidants were incorporated at the higher concentration.

5.1 Suggestion

5.1.1 Improvement of re-extraction process of gelatin from giant catfish should be investigated by using some protease enzymes that can loosen the giant catfish skin structure and therefore gelatin might be more extracted from the fish skin.

5.1.2 Analysis of antioxidant compound of the fruit seed extracts should be done for more data and further explanation on the antioxidant activities.

5.1.3 Application and improvement of giant catfish skin gelatin active films should be extensively studied for more understanding and full utilization of the films.

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APPENDICES

APPENDIX A

Schematic DSC Curve

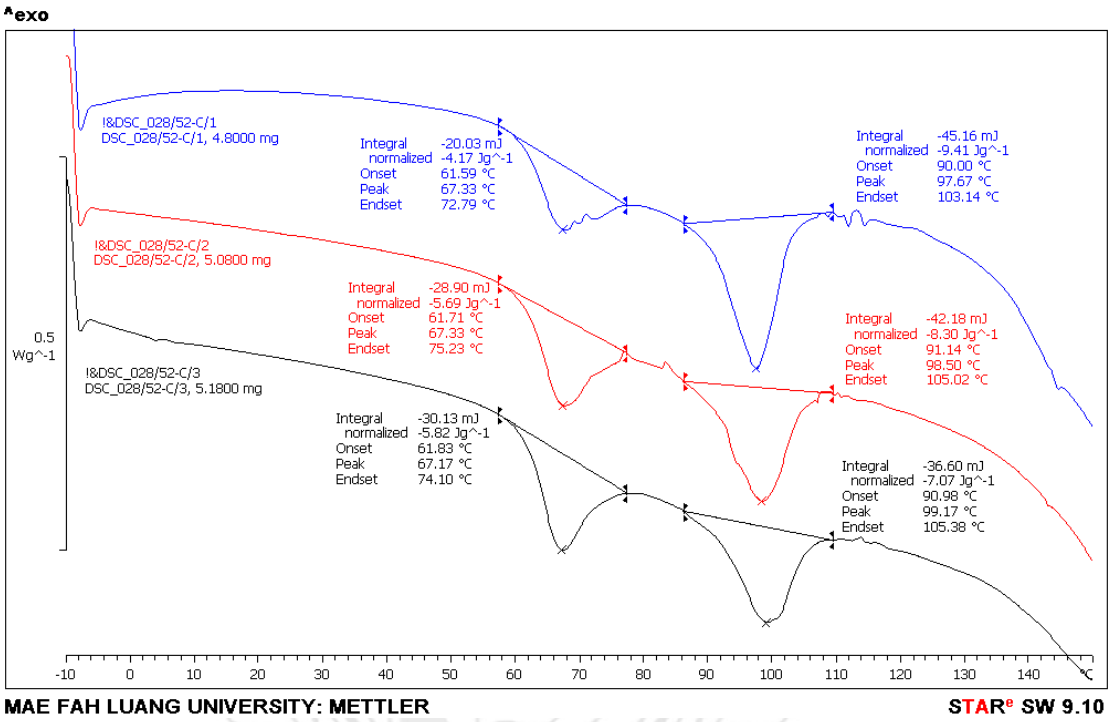

Figure A.1 Schematic DSC Curve of Beef Gelatin.

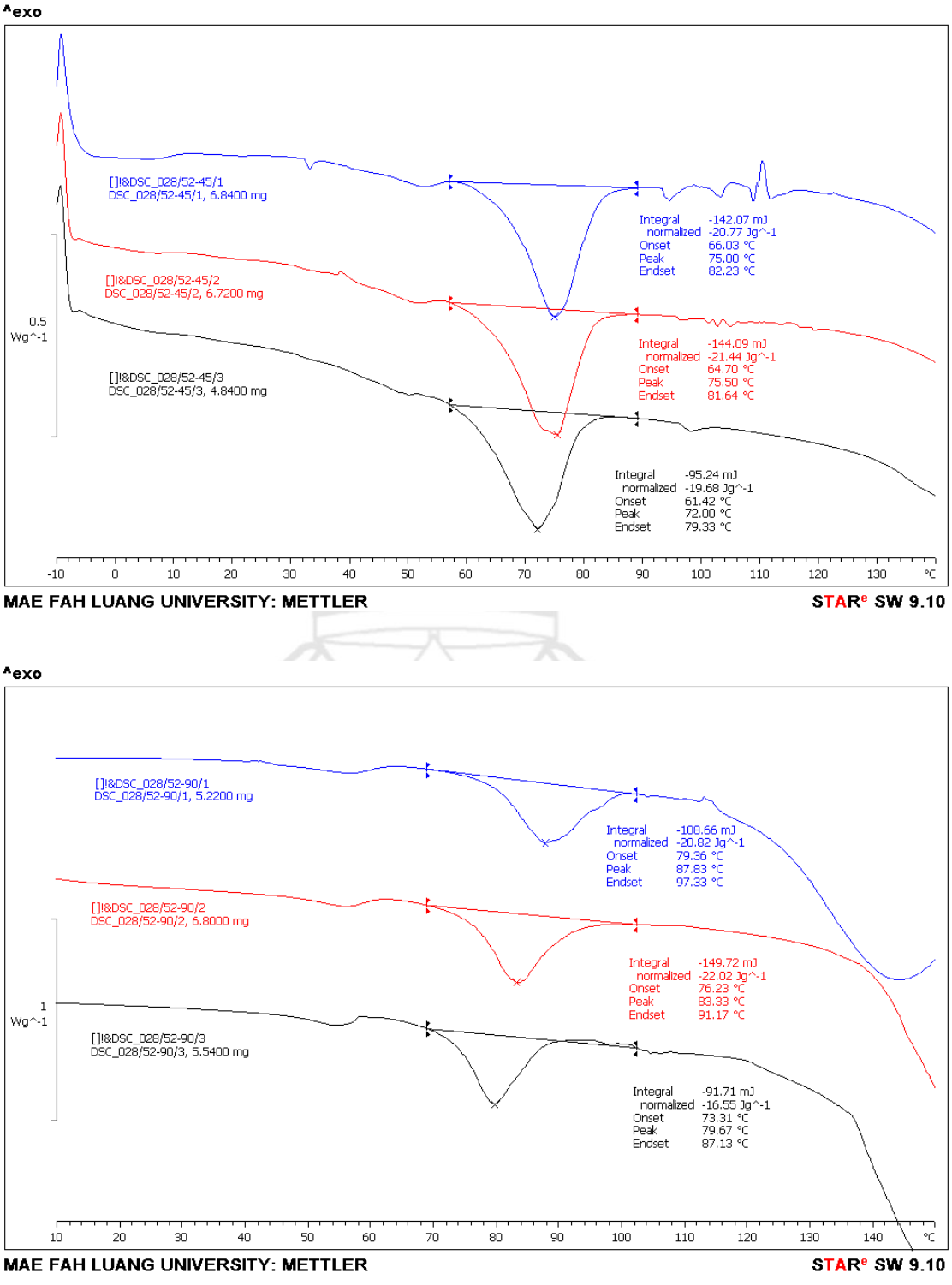

Figure A.2 Schematic DSC Curve of Giant Catfish Skin Gelatin Extracted at 45°C (Top) and 90°C (bottom) for 12 h.

CURRICULUM VITAE

CURRICULUM VITAE

NAME Mr. Samart Sai-Ut

DATE OF BIRTH 13 August 1985

ADDRESS 31 Moo 5 Thung Hong sub-district, Mueang district, Phrae 54000

EDUCATION BACKGROUND

2008 Bachelor of Science
Major in Food Technology, Mae Fah Luang University

WORK EXPERIENCE LEO Food Co., Ltd.

INTERNATIONAL PUBLICATIONS

Rawdkuen, S., Sai-Ut, S., Khamsorn, S., Chaijan, M., Benjakul, S. & Tanaka, M. (2008). Biochemical and gelling properties of tilapia surimi and protein recovered using acid-alkaline process. **Food Chemistry, 112**, 112-119.

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AWARDS

Best Paper on the Topic Agricultural Engineering and Food Technology. **The 3rd Technology and Innovation for Sustainable Development International Conference (TISD2010)**, Royal Mekong Nongkhai Hotel, Nong Khai, Thailand, 4-6 March 2010.

Best Oral Presentation. **The 3rd Technology and Innovation for Sustainable Development International Conference (TISD2010)**, Royal Mekong Nongkhai Hotel, Nong Khai, Thailand, 4-6 March 2010.