การเชื่อมขวางเจลาตินด้วยสารฟีนอลิกจากพืชเพื่อใช้ในการขึ้นรูปเจลาติน

นายธริษตรี พรพิชญา

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต
สาขาวิชาเทคโนโลยีชีวภาพ
คณะวิทยาศาสตร์ จุฬาลงกรณ์มหาวิทยาลัย
ปีการศึกษา 2553

CROSS-LINKING OF GELATIN USING PLANT PHENOLICS FOR GELATIN FABRICATION

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A Thesis Submitted in Partial Fulfillment of the Requirements

for the Degree of Master of Science Program in Biotechnology

Faculty of Science

Chulalongkorn University

Academic Year 2010

Thesis Title	CROSS-LINKING OF GELATIN USING PLANT PHENOLICS FOR GELATIN FABRICATION.
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สารประกอบฟีนอลิกเป็นสารที่เกิดจากกระบวนการเมตาบอลิซึมของพืช สามารถถูกออกซิไดซ์และทำปฏิกิริยากับหมู่อะมิโนของสายเปปไทด์เกิดเป็นสารประกอบที่มีสมบัติที่เปลี่ยนไป งานวิจัยนี้ได้ศึกษาปริมาณสารประกอบฟีนอลิกจากพืชชนิดต่างๆ ได้แก่ เมล็ดกาแฟ ดอกอัญชัน ผลทับทิม ขิง ลูกพลับดำ หัวหอมแดง ผลแก้วมังกร และผลมะเขือเทศ จากนั้นคัดเลือกสารสกัดจากพืชที่มีปริมาณสารฟีนอลิกมากที่สุดสามชนิด ได้แก่ เมล็ดกาแฟ ดอกอัญชันและผลทับทิม มาปรับปรุงสมบัติของเจลาตินโดยการเชื่อมขวาง จากการทดลองพบว่าเจลาตินที่ผ่านการเชื่อมขวางที่อัตราส่วนของโพลีฟีนอลต่ออะมิโนอิสระต่างๆ มีสมบัติต่างไป กล่าวคือ เมื่ออัตราส่วนของสารประกอบโพลีฟีนอลต่ออะมิโนอิสระมากขึ้น ปริมาณอะมิโนอิสระของเจลาตินที่ผ่านการเชื่อมขวางลดลงตามลำดับ การเกิดเจลและการละลายของเจลาตินที่ผ่านการเชื่อมขวางด้วยกรดกาลิก น้ำจากผลทับทิมและน้ำกาแฟ สามารถเกิดได้ที่อุณหภูมิที่ต่ำลง โดยสารสกัดจากดอกอัญชันมีผลต่อการเกิดเจลและการละลายของเจลาตินมากที่สุด ฟิล์มเจลาตินที่ผ่านการเชื่อมขวางมีอัตราการบวมลดลงเมื่ออัตราส่วนของสารประกอบโพลีฟีนอลต่ออะมิโนอิสระมากขึ้น นอกจากนี้ ยังพบว่าฟิล์มเจลาตินที่ผ่านการเชื่อมขวางมีค่าการต้านทานแรงดึงและค่าการยืดตัวสูงกว่าฟิล์มเจลาตินที่ไม่ผ่านการเชื่อมขวาง ในขณะที่ค่าโมดูลัสของยังลดลงอย่างมีนัยสำคัญ ($p \leq 0.05$) ฟิล์มเจลาตินที่ผ่านการเชื่อมขวางมีค่าความสว่าง ค่าสีเหลือง และค่าความขาวลดลง แต่ค่าสีแดงเพิ่มขึ้นอย่างมีนัยสำคัญ ($p \leq 0.05$).

สาขาวิชา เทคโนโลยีชีวภาพ ลายมือชื่อนิสิต ธริษตรี พรพิชญา

ปีการศึกษา 2553 ลายมือชื่ออ.ที่ปรึกษาวิทยานิพนธ์หลัก

ลายมือชื่ออ.ที่ปรึกษาวิทยานิพนธ์ร่วม

5072298023 : MAJOR BIOTECHNOLOGY

KEYWORDS : PLANT PHENOLICS / GELATIN / CROSS-LINKING / GELATIN FILMS

THARITTRI PHONPHITCHAYA : CROSS-LINKING OF GELATIN USING PLANT PHENOLICS FOR GELATIN FABRICATION. ADVISOR: ASST. PROF. JIRARAT TATTIYAKUL, Ph.D., CO-ADVISOR: PROF. PITT SUPAPHOL, Ph.D., 102 pp.

Phenolic compounds, natural plant metabolites, are known to react under oxidizing conditions with side chain amino groups of peptides producing complexes with modified properties. In this study, the total phenolics content, expressed in term of gallic acid equivalent (GAE), of crude extract from several local grown phenolic-rich plants were determined. The three highest phenolics-containing crude aqueous extract, which were light roasted coffee, butterfly pea and pomegranate solution were used in cross-linking of gelatin. The effect of cross-linking degree on gelling properties of gelatin solutions was studied. The viscosity of 6.67% gelatin solutions and free amino groups were found to deceased when increasing polyphenol:NH_2 molar ratio. Cross-linking of gelatin using gallic acid and the extract of pomegranate and light roasted Arabica coffee caused the gelation and melting temperature to decrease. Butterfly pea extract was the most effective cross-linker that caused gelling and melting complex viscosities to decrease. The swelling ratios of cross-linked gelatin films decreased when increasing polyphenol:NH_2 molar ratio. The tensile strength and elongation to break of cross-linked gelatin films increased compared with native gelatin while Young' modulus decreased ($p \leq 0.05$). Lower lightness (L*) value, yellowness (b*) and whiteness but higher redness (a*) were observed in cross-linked gelatin films ($p \leq 0.05$).

Field of Study : Biotechnology Student's Signature Tharittri Phonphitchaya

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ACKNOWLEDGEMENTS

I am especially grateful to my advisor and co-advisor, Assistant Professor Dr. Jirarat Tattiyakul and Professor Dr. Pitt Sulpapol for their valuable guidance and assistance throughout my studies and research at Chulalongkorn University. I wish to thank my graduate committee member, Assistant Professor Dr. Pasawadee Pradipasena, Dr. Chanprapa Imjongjirak and Associate Professor Dr. Manop Suphantharika for their helpful advice and comments.

I would like to thank Graduate School, Chulalongkorn University for financial support. This research work is also part of the project "Development of antimicrobial fiber mat using electrostatic spinning" that was supported by the TARGET 500 (A1B1-2) grant, Faculty of Science, Chulalongkorn University, for fiscal year 2009. I am also thanking Department of Food Technology, Faculty of Science, Chulalongkorn University for laboratory facilities. I thank all my friends, all members in program of Biotechnology and in Department of Food Technology for their friendship and helps during my graduate studies.

Finally, I would like to appreciate my family for their great support and encouragement throughout my education.

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CHAPTER I

INTRODUCTION

Gelatin is water-soluble, hydrophilic, derived colloidal proteins produced by controlled hydrolysis of water-insoluble collagen by breaking of the triple-helix structure of collagen, which is the major protein component in skin, bones, hide, and white connective tissue of animal body. Molecular structure of gelatin produced by hydrolysis is polypeptide with 18 types of amino acids and it contains a large proportion of glycine, proline and 4-hydroxyproline residues (Foegeding et al., 1996). Although widely used in variety of applications, but gelatin gel, as most protein gels, does not have ideal mechanical properties and water vapor barrier, which limited its application. Cross-linking, by means of chemical compounds such as formaldehyde, glutaraldehyde, dextran dialdehyde and carbodiimide (Zhang et al., 2006), is usually carried out to improve the aforementioned properties. However, due to an increasing concern in the cooperation of chemical compounds in food or food contact materials, alternative approaches for cross-linking of galatin are investigated.

Phenolic compounds in food and nutraceuticals originate from one of the main classes of secondary metabolites in plants derived from phenylalanine and, to a lesser extent in some plants, also from tyrosine. Chemically, phenolics can be defined as substances possessing an aromatic ring bearing one or more hydroxyl groups, including their functional derivatives (Shahidi and Naczk, 2006). Polyphenols are a structural class of natural, synthetic and semi-synthetic organic chemicals characterized by the presence of large multiples of phenolics, which are widely distributed as minor components but are functionally important constituents of plant tissues, occur mainly in rigid tissues, such as the hulls of cereal grains, cell walls of fruits (e.g. grapes, apples), coffee beans, tea leaves, and tubers (e.g. potatoes). The most common polyphenols are hydroxylated cinnamic acids such as caffeic acid (3, 4-dihydroxycinnamic acid), chlorogenic acid (its quinic acid ester), caftaric acid (its tartaric acid ester), and flavonols such as quercetin and rutin (its rutinoside). These compounds have an ortho-

diphenol (or a 1-hydroxy-2-methoxy) structure (Strauss and Gibson, 2004). Phenolic compounds have in common multiple phenolic functionalities, high molecular weights, and the ability to bind protein, its can react with some amino acid in proteins such as tyrosine, lysine, hydroxyproline and cross-link protein molecules (Gomez-Guillen et al., 2002; Toshiyuki et al., 2003). Thus, it can be used as a cross-linking agent to improve the properties of protein-based edible gels/films.

The objectives of this study were to investigate the extent of cross-linking by means of oxidative reaction of phenolic compounds with gelatin molecules and to observe the chemical and physical properties of the cross-linked gelatin. The work was divided into two parts. In the first part, total phenolics of some plants were extracted and determined. For the second part, cross-linking of gelatin using various polyphenol: free amino ratios was carried out and the chemical and physical properties of cross-linked gelatin were studied.

CHAPTER II

LITERATURE REVIEW

2.1 Gelatin

Gelatin is denatured collagen, which is the major protein component of cartilage, bone, skin and connective tissue and constitutes the major part of the extracellular matrices in animals. Insoluble collagen is converted to soluble gelatin by acid or alkaline processing. Gelatin type A, with isoionic point of 7 to 9, is processed by an acidic pretreatment before thermal denaturation. Gelatin type B, with isoionic point of 4.8 to 5.2, is processed by an alkaline pretreatment. The alkaline pretreatment is supposed to convert amide residues of glutamine and asparagines into glutamic and aspartic acid, which leads to a 25% higher carboxylic acid content for gelatin type B than for gelatin type A (Veis, 1964; Bailey and Light, 1989). Gelatin is sold with a wide range of special properties, such as gel strength, to suit particular applications. Gelatin forms thermally reversible gels with water, and the gel melting temperature usually below 35°C, is below human body temperature, which gives gelatin products unique organoleptic properties and flavor release.

The relative independence of pH 4 to 9 for gelation makes gelatin particularly useful for a broad reange of applications. Yet, the single largest food application of gelatin is cold desserts because of the unique cold-set gelling and melt-in-mount property of the protein. A typical gelation process will involve heating the gelatin solution to pass its denaturation point and then cooling rapidly to allow renaturation and interaction of gelatin molecules to form a protein gel matrix. Because the hydrogen bond is the predominate force that stabilizes the gel and no inter-peptide covalent linkages are present, the gelatin gel is thermo-reversible and readily melts at body temperature (Xiong, 1997; Yada, 2004). Rapid gel setting is essential to immobilization and an even distribution of other particles in the gel matrix.

2.1.1 Chemical composition and structure

Gelatin is not a single chemical substance. The main constituents of gelatin are large and complex polypeptide molecules of the same amino acid composition as parent collagen. The primary structure, such as amino acid sequence, of collagen and gelatin consist of 18 amino acids. Coils of amino acids are joined together by peptide bonds. The predominant amino acid sequence is Gly-Pro-Hyp (Poppe, 1997). As a result, gelatin contains relatively high levels of these amino acids: glycine (Gly) 26-34%, proline (Pro) 10-18%, and hydroxyproline (Hyp) 7-15% (Veis, 1964). Other significant amino acids include alanine (Ala) 8-11%, arginine (Arg) 8-9%, aspartic acid (Asp) 6-7%, and glutamic acid (Glu) 10-12% (Veis, 1964; Hudson, 1994) (Table 2.1). The secondary structure, such as local configuration of chains, does not favor formation of α-helical chain segments because of the high number of prolyl and hydroxyprolyl residue. Collagen chains are helical macromolecules with a tendency mainly for interchain, more than intrachain, hydrogen bonding (Carver and Blout, 1967). The tertiary structure standing for large-scale folding and helicity of collagen is better understood through the fundamental unit tropocollagen. Tropocallagen is a right handed superhelix with a repeat unit of about 100 Å consisting of three left-hand stands (Rich and Crick, 1961). 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 a small to medium size aggregates of tropocallagen molecules (Figure 2.1).

The primary structure of gelatin is almost identical to that of parent collagen but for same small differences due to the pretreatment a dextraction processes. These differences include removal of amide group of asparagines and glutamine, conversion of arginine to ornithine and removal of telopeptides (Johnston-Banks, 1990). The precise macromolecular constitution of gelatin resulting from a melting process that depends on collagen source and on the extraction method. Gelatin chain can be intertwined back into the collagen helix through an appropriate technique, such as cooling or annealing in solution (Johnston-Banks, 1990).

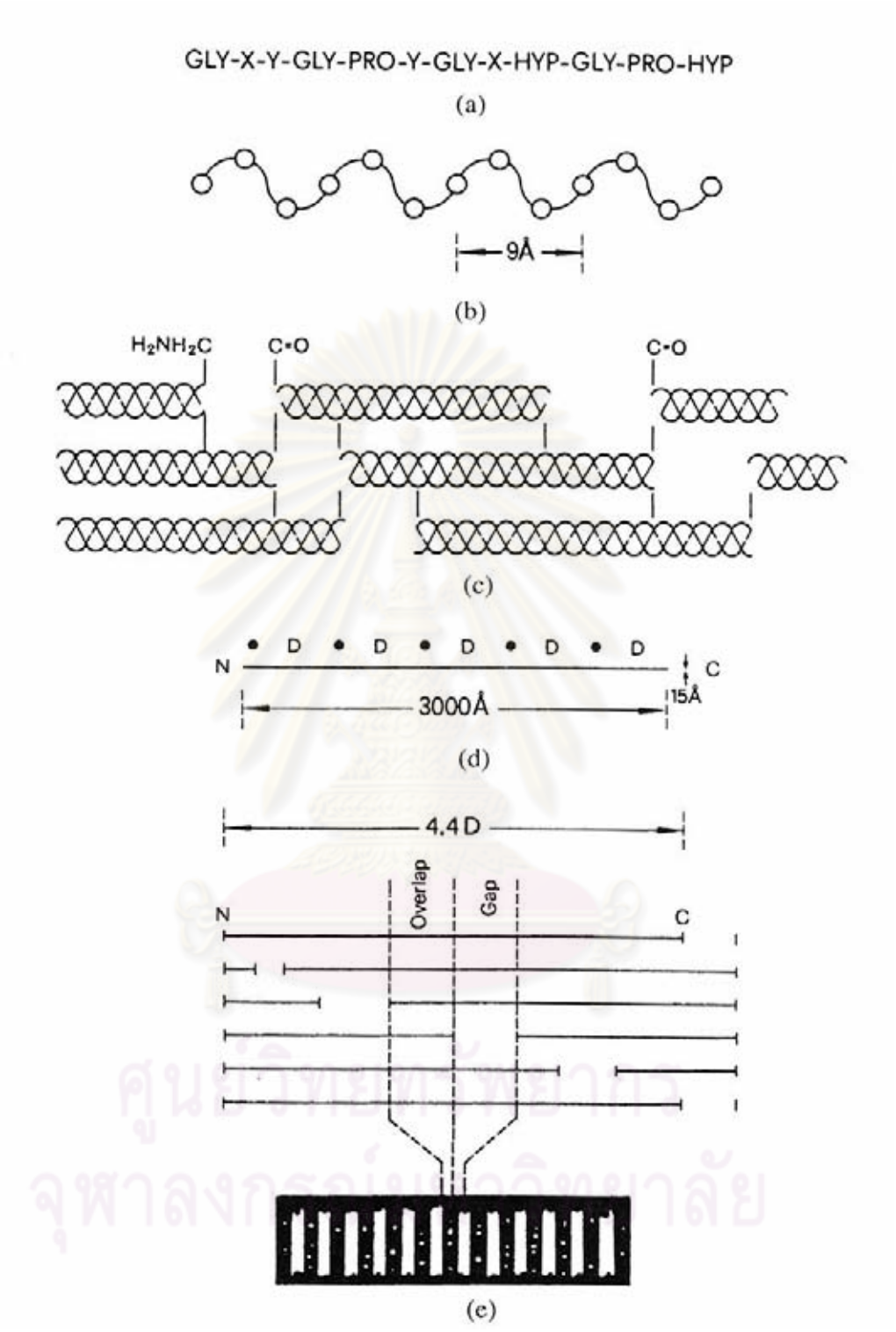


Figure 2.1 The formation of collagen: (a) primary, (b) secondary, (c) and (d) tertiary, and (e) quaternary structure.

Source: Gennadios (2002)

Table 2.1 Amino acid composition of gelatin.

Amino acids	% by weight	Amino acids	% by weight	Amino acids	% by weight
Glycine	26-34	Proline	10-18	Hydroxyproline	7-15
Glutamic acid	10-12	Alanine	8-11	Arginine	8-9
Aspartic acid	6-7	Lysine	4-5	Serine	3-4
Leucine	2-3	Valine	2-3	Phenylalanine	1-3
Threonine*	1-2	Isoleucine	1-2	Hydroxylysine	1-2
Methionine**	< 1	Histidine	< 1	Tyrosine	< 0.5

* Essential amino acid which cloud be eliminated either by hydrolyzing or at the gelatin process

** Essential amino acid which could be eliminates by oxidation

Source: Veis (1964); Hudson (1994)

2.1.2 Gelatin manufacture

Gelatin is a protein obtained by partial hydrolysis of water-insoluble collagen by breaking of the triple-helix structure of collagen, which is the major protein component in skin, bones, hide, and white connective tissue of animal body. Molecular structure of gelatin produced by hydrolysis is polypeptide with 18 types of amino acids and it contains a large proportion of glycine, proline and 4-hydroxyproline residues. A typical structure is -Ala-Gly-Pro-Arg-Gly-Glu-4Hyp-Gly-Pro-. As a partial product of collagen (Figure 2.2), the structure of gelatin is similar to collagen but it has shorter polypeptide chain (Figure2.3).

$$C_{102}H_{149}N_{31}O_{38} + H_2O \text{ ---------- } > C_{102}H_{151}N_{31}O_{39}$$

(Collagen) (Gelatin)

Figure 2.2 Hydrolysis of collagen to gelatin.

Source: Carver and Blout (1967)

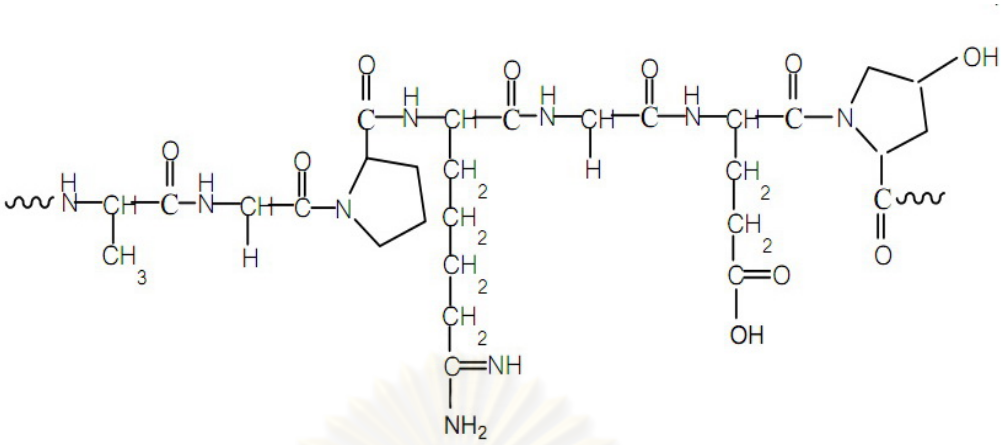


Figure 2.3 The general structure of gelatin: -Ala-Gly-Pro-Arg-Gly-Glu-4Hyp-Gly-Pro-.

Source: Chaplin (2009)

Transforming collagen into gelatin could be done by hydrolysis by means of acid, alkaline, enzyme, or high temperature controlling resulting in breaking of collagen molecule chain and reforming into gelatin. The aim of gelatin production is to control the hydrolysis of collagen from raw material and to convert the resulting product into a solution material with desirable physical and chemical properties, such as gel strength and color (Ockerman and Hansen, 1988). The three changes involved in transforming collagen in to gelatin are:

a) Rupture of a limited number of peptide bonds to reduce the length of the chain.
b) Rupture or disorganization of a number of the lateral bonds between chains.
c) Change in chain configuration.

There are basically two processes by which collagen is processed to gelatin (Johnston-Banks, 1990; GMIA, 1993). In the first process, the collagen in hide or deminerlized bone is partly depolymerized by prolonged liming that breaks down covalent cross-links. The occurring hydrolysis results in extensive release of collagenous material, which is solubilized at near neutral pH at high temperatures. The acid process

involves soaking skin or bone in a dilute acid followed by extraction at acidic pH. Sometimes, acid pretreatment is used for beef ossein, but this is relatively uncommon.

2.1.3 Gelatin properties and uses

2.3.1.1 Solubility Gelatin is soluble in water and in aqueous solution of polydydric alcohols such as glycerol and propylene glycol. Many water-soluble organic solvents are compatible with gelatin, but interfere with its gelling properties. Dry gelatin absorbs water exothermally. The cross-linking of gelatin matrix by chemical means is used extensively in photographic products, and this so called hardening permanently reduces the solubility of gelatin.

In products with limited moisture, as in confectionery, and where there is another polymer, as in glucose syrup, competing for the available water, gelatin can precipitate resulting in loss of gelation and cloudiness. In these cases the gelatin solubility is very dependent on the charge on the protein molecule or the pH of the product. Hence, the further the product pH is from the isoionic pH the better will be the solubility and performance of the gelatin.

2.3.1.2 Adhesive properties. Possibly the oldest use of gelatin was as animal glue. For adhesion to take place a warm gelatin solution must be used and the gelatin must not have gelled before the surfaces to be glued are brought together. Gelatin-based glues are also used as adhesives to put those organic stickers on fruits and vegetables. Gelatin is also used in prepared meat products such as canned ham, luncheon meats, turkey, and chicken rolls where it helps to maintain consistency and moisture.

2.3.1.3 Gelation. The gelation and structure formation are important functional properties of gelatin in fabricated and natural product. Gelatin contribute in

varying degrees to the solid or elastic properties of the food by formation of an orderly, three-dimensional network of associated or aggregated gelatin molecules that are capable of physically entrapping large amounts of water within the matrix (Hermansson, 1979).

The formation of gel from protein is apparently a two-step process. The first step involves a change in conformation, usually heat-induced, or partial denaturation of the protein molecules. As denaturation proceeds, the viscosity of the dispersion increases owing to an increase in molecular dimensions of the unfolding protein. This is followed by a gradual association or aggregation of the individual denatured proteins. During the association step, there is an exponential increase in viscosity as the material approaches a continuous network. This dispersion of protein aggregates then beings to display some of the characteristics of an elastic solid, i.e. the storage modulus (G') of the solution increase. This second step should be below, relative to the first, so that a well-organized gel network is formed. If the second step occurs too quickly, a random network, i.e. a coagulum, that is unable to hold water is formed and syneresis occurs. A critical balance between attractive and repulsive forces must also be present for successful network formation and stabilization (Hermansson, 1979). If attractive forces predominate, a coagulum is formed and water is expelled from the gel matrix. If repulsive forces predominate, no network will be formed.

The gelling properties of gelatin are influenced by the structure and size, their concentration, the source of gelatin, and the various processing conditions such as pH, ionic strength, and heating rate. Gelation may occur during heating and upon cooling depending on the condition of gelation. A thermo-set gel is formed upon heating, and thereafter cannot melting without destroying the primary structure of the original protein molecules (Rodriguez, 1982). The process involves the formation of an elastic solid, a permanently cross-linked three-dimensional solid network.

In confectionery, gelatin is used as the gelling binder in gummy products, wine gums etc. In the manufacture of these products gelatin is combined with

sugar and glucose syrups. Incompatibility between gelatin and glucose syrup can occur (Marrs, 1982) and is a function of the concentration of glucose polymers containing more than 2 glucose units, contained in the syrup. Competition between gelatin and glucose polymers for water in low water content products can result in, at worst, precipitation of the gelatin and at best a marked loss in gelling properties or hardness of the product. It is also known that different gelatins with similar properties in water can have very different properties in confectionery formulations (Marrs, 1982).

Some raw fruits like pineapple and papaya contain proteolytic enzymes like bromelain which hydrolyze gelatin and destroy its gelling ability. In such cases it is essential that the fruit is cooked to destroy protease before the fruit is added to gelatin solutions.

2.3.1.4 Film Forming properties. Gelatin's film forming properties are used in the manufacture of both hard and soft (pharmaceutical) capsules. Gelatin films shrink with great force on drying, hence such uses usually involve the addition of polyhydric alcohols to modify the adhesion and flexibility of the dry film. Also, for film forming, a gelatin with a high viscosity is preferred to one with a low viscosity.

2.3.1.5 Emulsifying properties. Gelatin is amphoteric, meaning that it is neither acidic nor alkali, but possesses both properties depending on the nature of the solution. The pH at which gelatin’s charge in solution is neutral is known as the isoelectric point. The isoelectric point of gelatin ranges between 4.8 and 9.4, with acid processed gelatins having higher isoelectric points than alkali processed gelatins (Poppe, 1997). The amphoteric character as well as hydrophobic areas on the peptide chain gives gelatin limited emulsifying and emulsion stabilizing properties used in the manufacture of toffees and water in oil emulsions like low fat margarine, cosmetic and pharmaceuticals..

2.3.1.6 Stability. Dry gelatin has an almost infinite shelf life as long as the moisture content is such as to ensure that the product is stored below the glass

transition temperature. The stability of gelatin in solution depends on temperature and pH. Generally, to minimize loss of gel strength and viscosity with time, the pH of the solution should be in the range 5 to 7 and the temperature should be kept as low as possible, consistent with the avoidance of gelation and the suitability of the solution viscosity to the particular application. Often the cause of degradation or hydrolysis of gelatin in solution is microbial proliferation, so gelatin solutions should not be stored for longer than absolutely necessary. Moreover, after addition of the acid to confectionery formulations, the solution should be used and cooled/gelled with minimal delays.

2.3.1.7 Microencapsulation - Mixed film forming properties. Besides being precipitated by polymers competing for water, gelatin is amphoteric, i.e. it has both positive and negative charges on the molecule. Hence, at a pH where the basic side chains do not carry a charge, acid groups for example from gum arabic can react with the basic groups of gelatin to form an insoluble gelatin-arabate complex which can be precipitated around emulsified oil droplets, forming microencapsulated oil. Hard microcapsules use aldehydes to cross-link and stiffen the structure of gelatin. Formaldehyde and glutaraldehyde are used as hardening agents for microencapsulation of flavors.

2.3.1.8 Nutritional properties. As stated earlier, gelatin is not a complete protein source because it is deficient in tryptophan and low in methionine content. However the digestibility is excellent and it is often used in feeding invalids and the high level of lysine (4 %) is noteworthy. More controversially, studies have shown that the consumption of 7 to 10 g/day can significantly improve nail growth rate and strength (Schwimmer and Mulinos, 1957).

2.2 Phenolic compounds

Phenolic compounds constitute a large group of secondary plants products which differ in chemical; structure and reactivity. Phenolic is used to define substances that possess one or more hydroxyl (-OH) substituent bonds onto an aromatic ring. The name derives from the simple parent substance phenol (Figure 2.4). Compounds that have several or many phenolic hydroxyl substuents are often referred to as polyphenol (Waterman and Mole, 1994).

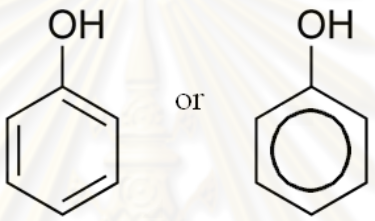


Figure 2.4 Basic structure of phenol.

Source: Waterman and Mole (1994)

2.2.1 The most important groups of plant phenolic compounds

A group of polyphenols, responsible for the color of many fruits, vegetables, and flowers, are known as anthocyanins. There are several important classes of phenolic compounds (Table 2.2). According to the basic skeleton, the structure of natural polyphenols varies from simple molecules, such as simple phenols (volatile phenols), to highly polymerized compounds, such as condensed tannins.

Table 2.2 The classes of phenolic compounds in plants.

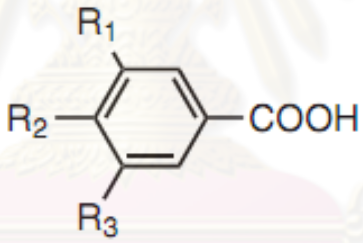
Number of Carbon atom	Basic skeleton	Class	Examples
6	C_6	Simple phenols Benzoquinones	Phenol, hydroquinone 2,6-Dimethoxybenzoquinone
7	$C_6 - C_1$	Hydroxybenzoic acids	Gallic, *p*-hydroxybenzoic
8	$C_6 - C_2$	Acetophenones Phenylacetic acids	3-Acetyl-6-ethoxybenzaldehyde *p*-Hydroxyphenylacetic
9	$C_6 - C_3$	Hydroxycinnamic acids Phenylpropenes Coumarins Isocoumarins Chromones	Caffeic, ferulic, *p*-coumaric Myristicin, engenol Aesculetin Bergenon Eugenin
10	$C_6 - C_4$	Naphthoquinones	Juglone
13	$C_6 - C_1 - C_6$	Xanthones	Mangiferin
14	$C_6 - C_2 - C_6$	Stilbenes Anthraquinoids	Resveratrol Emodin
15	$C_6 - C_3 - C_6$	Flavonoids Isoflavonoids	Quercetin, catechin Genistein
18	$(C_6 - C_3)_2$	Lignans Neolignans	Pinoresinol Eusiderin
30	$C_6 - C_3 - C_6)_2$	Biflavonoids	Amentoflavone
n	$(C_6 - C_3)_n$ $(C_6)_n$ $(C_6 - C_3 - C_6)_n$	Lignins Catechol melanins Condensed Tannins	

Source: Waterman and Mole (1994)

2.2.1.1 Volatile Phenols. Simple phenols such as phenol, *o*-cresol, 4-ethylphenol, guaiacol, 4-vinyl guaiacol and eugenol have been found in the volatiles of fruits and vegetables. The 4-vinyl guaiacol is a major objectionable flavor component of citrus fruits and contributes a rotten flavor to orange juice (Tatum et al., 1975).

2.2.1.2 Phenolic acids. Hydroxybenzoic and hydroxycinnamic acids are predominant phenolic acids found in plants. These derivation differ patterns of hydroxylations and methoxylations of their aromatic rings (Shahidi and Naczk, 1995).

a) Hydroxybenzoic acids have a general structure of C_6-C_1 (Figure 2.5). Hydroxybenzoic acids are commonly present in bound form. They are components of complex structures such as hydrolysable tannins and lignins. Hydroxybenzoic acids are also found in the form of organic acid and sugar derivatives (Schuster and Herrmann, 1985).



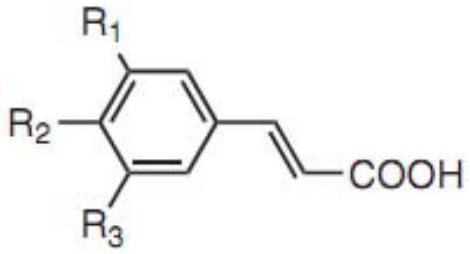
Acid		R1	R2	R3
p-Hydroxybenzoic	4-Hydroxybenzoic	H	OH	H
Protocatechuic	3,4-Dihydroxybenzoic	OH	OH	H
Vanillic	4-Hydroxy-3-methoxybenzoic	OCH_3	OH	H
Syringic	3,5-Dimethoxybenzoic	OCH_3	OH	OCH_3
Gallic	3,4,5-Trihydroxybenzoic	OH	OH	OH

Figure 2.5 Phenolic acids of benzoic acid families found in food and nutraceuticals.

Source: Shahidi and Naczk (1995)

The most important phenol in this class is gallic acid, in which the single carbon side-chain is carboxylic acid. Gallic acid is a trihydroxybenzoic acid, a type of phenolic acid, a type of organic acid, also known as 3,4,5-trihydroxybenzoic acid (Waterman and Mole 1994), found in gallnuts, sumac, witch hazel, tea leaves, oak bark, and other plants. Pure gallic acid is in the form of colorless crystalline organic powder. It is used as a standard for determining the phenol content of various analytes by the Folin-Ciocalteau assay that results are reported in gallic acid equivalents. Gallic acid can also be used as a starting material in the synthesis of the psychedelic alkaloid mescaline.

b) Hydroxycinnamic acids (Figure 2.6) are also commonly found in foods of plant origin. Of these, caffeic acid is the predominant hydroxycinnamic acids in many fruits. Hydroxycinnamic acids are mainly present in bound form and are rarely found in free form. Hydroxycinnamic acids usually occur in various conjugated forms. The conjugated forms are esters of hydroxyacids such as quinic, shikimic and tartaric acid, and their sugar derivatives. Hydroxycinnamic acids may also be linked with cutin, flavonoids suberin and lignin. The free hydroxycinnamic acids can be released from chemical or enzymatic hydrolysis during tissue extraction.



Acid		R1	R2	R3
p-Coumaric	4-Hydroxycinnamic	H	OH	H
Caffeic	3,4-Dihydroxycinnamic	OH	OH	H
Ferulic	4-Hydroxy-3-methoxycinnamic	OCH_3	OH	H
Sinapic	4-Hydroxy-3,5-dimethoxycinnamic	OCH_3	OH	OCH_3

Figure 2.6 Phenolic acids of cinnamic acid families found in food and nutraceuticals.

Source: Shahidi and Naczk (1995)

2.2.1.3 Flavonoids. Flavonoids represent the most common and widely distributed group of plant phenolics. They belong to a group of phenolics having the structure (C_6-C_3-C_6) consists of two aromatic rings (A ring and B ring) linked through a three carbon bridge that is usually an oxygenated heterocycle (C ring). Figure 2.7 shows the basic structure and the system used for the carbon numbering of the flavonoid nucleus.

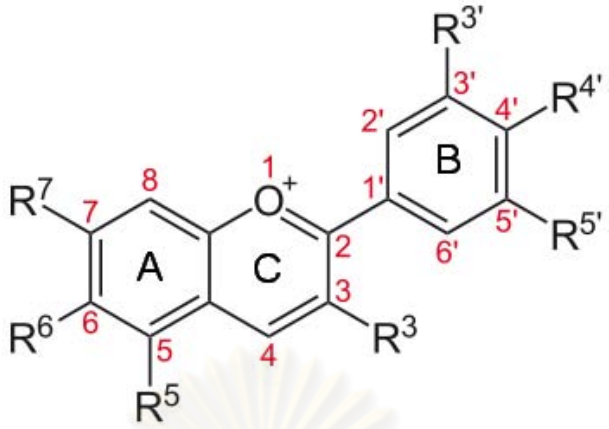


Figure 2.7 Basic structure of flavonoid skeleton.

Source: Pietta (2000)

The major flavonoid classes include anthocyanins, chalcones, flavanols, flavanones, flavones, flavonol, and isoflavones. The variability of the flavonoids is based on the hydroxylation of the pyrone ring, absence or presence of double bond, the number of hydroxyls in the A ring and B ring, and/or a double bonded oxygen atom attached to position 4 of the C ring. Flavonoids may be monomeric, dimeric, or oligomeric. Polymeric flavonoids, known as tannins, are divided into two groups, condensed and hydrolysable. Condensed tannins are polymers of flavonoids while hydrolysable tannins contain gallic acid (Pietta, 2000).

a) Flavones and flavonols are generally present in food as aglycones and glycosides. Flavanols are known as flavan-3-ols, and they are the subunits of proanthocyanidins, which have a hydroxyl group attached to the 3 position of the C ring, no positive charge on the oxygen atom and no double bond in the C ring. The structures of flavonols are very similar to those of flavanols, except that there is a double-bonded oxygen atom attached to position 4 of the C ring and a double bond in the C ring.

b) Flavanones and Flavanonols are characterized by presence of a saturated 3-C chain and oxygen atom in the 4 position.

c) Anthocyanins are widely distributed among fruits and vegetables. They are one of the main classes of flavanoids. They contribute significantly to the antioxidant activities of the flavanoids. Anthocyanins are water soluble pigments responsible for red, blue and violet colors found in most species in the plant kingdom. Blackberries, red and black raspberries, nectarines, peaches, blueberries, bilberries, cherries, currants, grapes, pomegranates, cranberries, and butterfly pea contain anthocyanins. Anthocyanins are glycosylated anthocyanidins with sugars generally attached to the 3-hydroxyl position of the anthocyanidin. The sugar part usually consists of mono-, di-, and trisaccharides of glucose, rhamnose, galactose, xylose and arabinose. The sugar moiety can be additionally acylated with phenolic acids such as p-coumaric, caffeic, or ferulic acids. Anthocyanidin is an aglycone. This means that there is no sugar group or other functional group attached to the flavan nucleus. Also, the oxygen atom on the C ring has a positive charge on it, and there are two double bonds in the C ring. In addition to hydroxylated anthocyanidins, such as delphinidin, cyanidin, and pelargonidin, there are also methylated anthocyanidins (malvidin, peonidin, and petunidin).

2.2.2 Phenolics in Food and Nutraceuticals

Phenolics are secondary metabolites synthesized by plants, both during normal development and in response to stress conditions such as infection, wounding and UV radiation, among others. These compounds are a very diversified group of phytochemicals derived from phenylalanine and tyrosine. Many of food phenolics are soluble in water or organic solvent. Most plant, if not all, contain polyphenols which differentiate them from one another. Phenolic found in food generally belong to phenolic acid, phenylpropanoids, benzoic acid derivatives, flavonoids, stilbenes, tannins, lignans and lignins (Table 2.3). Together with long-chain carboxylic acids, phenolics are also components of suberin and cutin (Harbone, 1993).

Table 2.3 Dietary sources of plant phenolics

Phenolic compounds	Dietary source
Phenolic acids	
Hydroxycinnamic acids	Apricots, plums, carrots, cereals, pears, tomatoes, spinach
Hydroxybenzoic acids	Blueberries, cereals, cranberries, oilseeds
Flavonoids	
Anthocyanins	Butterfly pea, black and red currants, blueberries, strawberries
Chalcones	Apples
Flavanols	Apples, blueberries, grapes, onions, lettuce
Flavanonols	Grapes
Flavanones	Citrus fruits
Flavonols	Apples, beans, blueberries, buckwheat, tomatoes, onions
Flavones	Citrus fruits, celery, parsley, spinach
Isoflavones	Soybeans
Xanthones	Mango, mangosteen
Tannins	
Condensed form	Apples, grapes, peaches, plums, mangosteens, pears
Hydrolyzable form	Pomegranate, raspberries
Other phenolics	
Alk(en)ylresorcinols	Cereals
Arbutin	Pears
Avenanthramides	Oats
Capsaisinoids	Pepper
Coumarins	Carrots, celery, citrus fruits, parsley, parsnips
Lignans	Buckwheat, flaxseed, sesame seed, rye, wheat
Secoiridoids	Olives
Stilbenes	Grapes

Souce: Shahidi and Naczk (2006)

Phenolic compounds are essential for the growth and reproduction of plants and also act as antifeedant and antipathogens (Butler, 1992). Their contribution to pigmentation of plant foods is also well recognized. Injured plants may secrete phenolics to defend them against pathogens. The antifeedant activity of phenolics is greater for those containing oxidized phenolics with quinodial structures.

Many properties of plant products are associated with the presence, type and content of their phenolic compounds. The astringency of foods, the beneficial health effects of certain phenolics or their potential antinutritional properties when present in large quantities in plants, are important to consumers (Butler, 1992; Harborne, 1993). Furthermore, polyphenolic anthocyanins are responsible for the orange, red, blue, violet and purple colors of most plant species and their fruits and products. For example, colorants produced from the skin of grapes, after their extraction with water and concentration or other processes, are used by the food industry (Francis, 1993).

Many plant foods contain polyphenol oxidases which catalyze reactions with phenols in the presence of molecular oxygen (McEvily et al., 1992). Initial oxidation of phenol to quinone followed by the formation of colored pigments results in enzymatic browning of products. Inhibition of enzymatic browning is therefore achieved by changes in the pH, temperature or application of procedures which inhibit the enzymes, substrates or their reaction product. The most widely used anti-browning agent are sulfites. The 4-substituted resorcinols have also been shown to be potent polyphenol oxidase inhibitors.

2.2.3 Effect of phenolic compounds on food quality

Phenolic compounds are closely associated with nutritional and sensory quality of food derived from plant sources. The level of phenolics in plant sources also depends on such factors as cultivation techniques, cultivar, growing conditions, ripening process, as well as processing and storage conditions, among others. While at low concentrations, phenolics may protect the food from oxidative deterioration, at high

concentrations they, or their oxidation products, may participate in discoloration of foods, interactions with proteins, carbohydrates and minerals (Shahidi and Naczk, 2006). In addition, the astringency and bitterness of foods depends on their concentration of phenolic compounds. Many of the phenolics in foods occur as their glycoside and may modify the quality characteristics of foods. In general, the leaves, flower, fruits and other living tissues of the plants contain glycosides while woody tissues contain a glycones. The seed may contain phenolics in either form.

2.3 Cross-linking

Cross-linking is the process of chemically joining two or more molecules by a covalent bonds. This technique involves the formation of covalent bonds between two proteins by using bifunctional reagents containing reactive end groups that react with functional groups, such as primary amines and sulfhydryls, of amino acid residues. Usually, cross-linking refers to the use of cross-links to promote a difference in the polymers such as physical and chemical properties (Bigi et al., 2001). Cross-linking reagents have been used to assist in determination of near-neighbor relationships, three-dimensional structures of proteins, and molecular associations in cell membranes.

Conformational changes of proteins associated with a particular interaction may be analyzed by performing cross-linking studies before and after the interaction occurs. Comparing cross-linkers with different arm lengths for success of conjugation can provide information about the distances between interacting molecules (Means and Feeney, 1971). Homo-bifunctional reagents, specifically reacting with primary amine groups (i.e., **ε**-amino groups of lysine residues) have been used extensively as they are soluble in aqueous solvents and can form stable inter- and intra-subunit covalent bonds.

2.3.1 Protein functional targets and reactive groups

Despite the complexity of protein structure, including composition and sequence of 20 different amino acids, only a small number of protein functional groups comprise selectable targets for practical bioconjugation methods. In fact, just four protein chemical targets account for the vast majority of cross-linking and chemical modification techniques (Means and Feeney, 1971):

- **Primary amines (–NH2):** This group exists at the N-terminus of each polypeptide chain (called the alpha-amine) and in the side chain of lysine (Lys.) residues (called the epsilon-amine). Because of its positive charge at physiologic conditions, primary amines are usually outward-facing (i.e, on the outer surface) of proteins; thus, they are usually accessible for conjugation without denaturing protein structure.
- **Carboxyls (–COOH):** This group exists at the C-terminus of each polypeptide chain and in the side chains of aspartic acid (Asp.) and glutamic acid (Glu.). Like primary amines, carboxyls are usually on the surface of protein structure.
- **Sulfhydryls (–SH):** This group exists in the side chain of cysteine (Cys.). Often, as part of a protein's secondary or tertiary structure, cysteines are joined together between their side chains via disulfide bonds (–S–S–). These must be reduced to sulfhydryls to make them available for cross-linking by most types of reactive groups.
- **Carbonyls (–CHO):** Ketone or aldehyde groups can be created in glycoproteins by oxidizing the polysaccharide post-translational modifications (glycosylation) with sodium meta-periodate.

For each of these protein functional-group targets, there exist one to several types of reactive groups that are capable of targeting them and have been used as the basis for synthesizing cross-linking and modification reagents.

2.3.2 Polyphenol-protein cross-linking reactions

The formation of rigid molecular structures by reactions of ortho-quinones with proteins is demonstrated by Strauss and Gibson (2004) (Figure 2.8). The diphenol moiety of a phenolic acid or other polyphenol (1) is readily oxidized to an ortho-quinone, either enzymatically as in plant tissues, or by molecular oxygen. The quinone forms a dimer (2) in a side reaction, or reacts with amino or sulfhydryl side chains of polypeptides to form covalent C–N or C–S bonds with the phenolic ring, with regeneration of hydroquinone. The latter can be reoxidized and bind a second polypeptide, resulting in a cross-link (3). Alternatively, two quinones, each carrying one chain, can dimerize, also producing a cross-link (4).

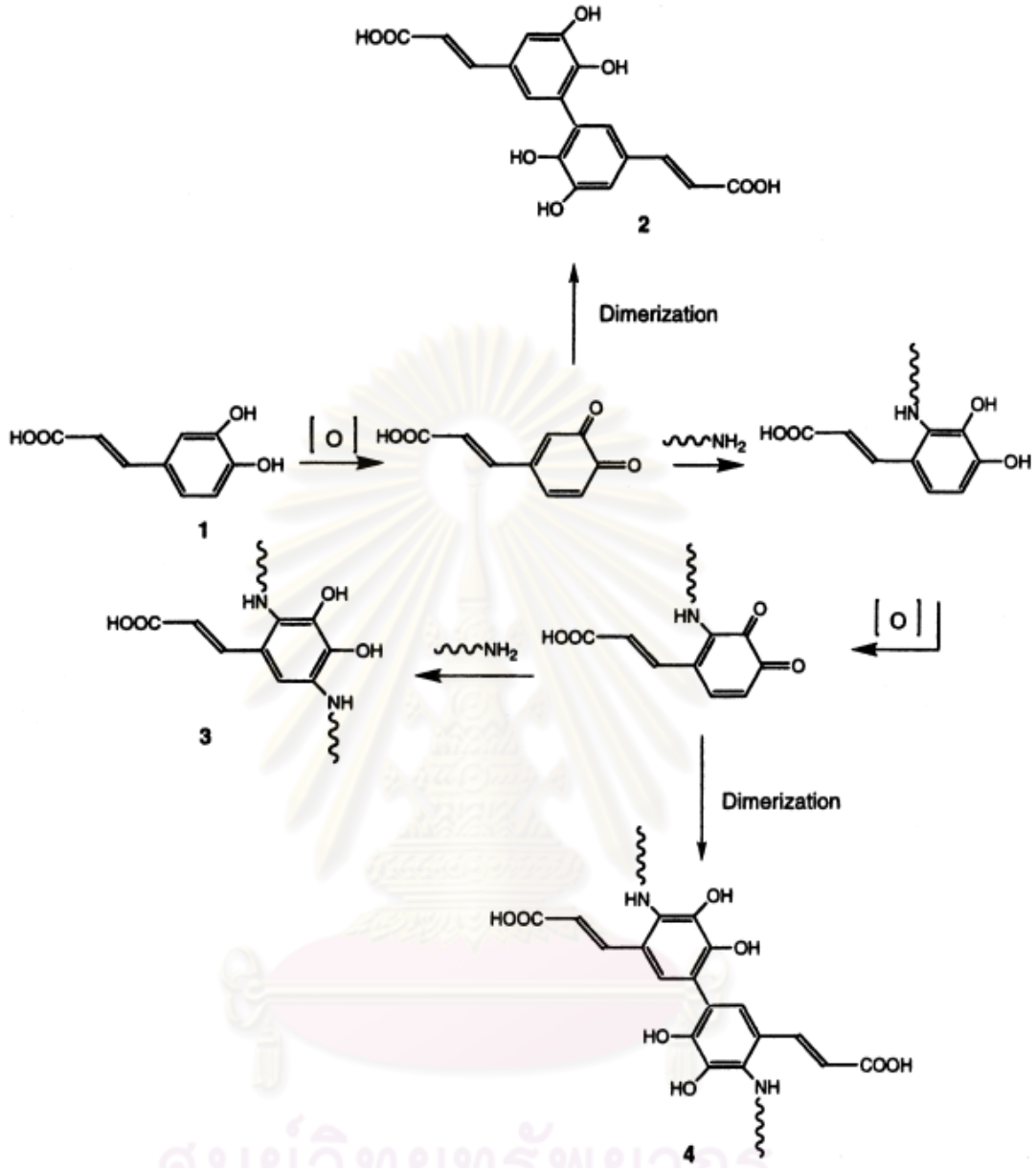


Figure 2.8 Reactions of a phenolic acid with amino side chains of polypeptides.

Source: Strauss and Gibson (2004)

2.4 Relevant Researches

The cross-linking of gelatin with chemical cross-linking agent is being used to extend the uses of gelatin. In particular, treatment of gelatin with phenolic compounds receives considerable study in order to improve their thermal resistance, decrease their solubility in water as well as to improve their mechanical properties, without introducing chemicals which are potentially harmful to health into the structure.

Strauss and Gibson (2004) described that plant phenolics can be used to prepare cross-linked gelatin gels in bulk and cross-linked gelatin–pectin coacervates in the form of microparticles. The gelatin-polyphenol solutions were exposed to oxygen by two methods: oxygen was bubbled through the solution or the tube containing the sample was capped by an oxygen-filled balloon with occasional shaking. Gels cross-linked by these materials had greater mechanical strength, reduced swelling, and fewer free amino groups. Dynamic light scattering analyses showed that such cross-linking results in denser polymeric networks and prevent extension of the peptide chains when the pH of gelatin cross-linking is moved away from the isoelectric point. Coacervated gelatin–pectin microparticles when cross-linked became more lipophilic, and were stable at temperatures up to 200^{o}C, in contrast to un-cross-linked particles that coalesce and/or disintegrate on heating.

Cao et al. (2006) studied mechanical properties of gelatin films cross-linked, respectively, by ferulic acid and tannin acid. The cross-linking reactions were placed under ultrasonic to dispel air bubbles and then spray a very small amount of ethyl alcohol to further eliminate air bubbles. The results showed that two natural cross-linking agents had cross-linking effects on gelatin film and maximal mechanical strength of gelatin film could be obtained when the pH value of film-forming solution was 7 for ferulic acid as cross-linked agent, or when the pH value was 9 as for tannin acid. The cross-linking agents could decrease swelling ratios of the films but there were no obvious effects on water vapor permeability.

Balange and Benjakul (2009) studied effect of oxidized phenolic compounds on the gel property of mackerel surimi. The oxidized phenolic compounds were subjected to oxygenation by bubbling the solution with oxygen. Gels with addition of oxidized phenolics had increases in breaking force and in deformation, compared with the control (without addition of oxidized phenolics). Lowered expressible moisture content without any change in the whiteness of resulting gels was found. Slightly lower myosin heavy chain band intensity of gels added with oxidized phenolics at the optimal level was noticeable compared with that of the control. A sensory evaluation study indicated that addition of oxidized phenolic compounds had no negative impact on the colour and taste of the resulting gels ($P > 0.05$). Gels with addition of all oxidized phenolics had a finer matrix with smaller strands.

Gomez-Estaca et al. (2009) investigated physical properties of gelatin films on adding aqueous extracts of oregano and rosemary, taking into consideration differences related to gelatin source. The bovine-hide gelatin reacted only slightly with the polyphenols in the extracts as shown by the electrophoretic profile and analysis of the dynamic viscoelastic properties, and consequently the attributes (mechanical properties, water solubility, water vapor permeability) of the films were practically unchanged compared with the film made without any added plant extract. Their results also showed that tuna-skin gelatin interacted with the polyphenols in both the oregano and the rosemary extracts, especially for the more concentrated of the two extracts tested, thereby altering the attributes of the corresponding films, namely, a higher glass transition temperature, decreased deformability, and, in particular, increased water solubility.

CHAPTER III

MATERIALS AND METHODS

3.1 Materials

Butterfly Pea (*Clitoria ternatea* L.), tomato (*Solanum lycopersicum* L.), red dragon fruit (*Hylocereus undatus (Haw.) Britt. & Rose*), shallot (*Allium ascalonicum*), ginger (*Zingiber officinale* Roscoe), black plum (*Prunus domestica* L.) and pomegranate (*Punica granatum* L.) were obtained from Klong-Toey Market, Bangkok, Thailand. All raw materials were kept at 4°C for not more than 2 days before further analyses. Dark roasted and medium roasted Arabica coffees were purchased from Golden Cream Company Limited (Caffè D´Oro), Bangkok, Thailand. Light roasted Arabica coffee was purchased from the Doi Tung Development Project, Thailand. The coffee samples were packed in plastic pouches and stored in dry condition at ambient temperature (25 ± 2°C).

3.2 Chemical reagent

Chemicals for total phenolic extraction and determination

Gallic acid monohydrate was purchased from Sigma-aldrich, USA.

Folin-Ciocaltue's reagent (A.R. grade) was purchased from Carlo Erba Reagents, Italy.

Sodium carbonate anhydrous (A.R. grade) was purchased from Mallinckrodt Baker Inc., Canada.

Ethyl alcohol (A.R. grade) was purchased from Sigma-aldrich, USA.

Chemicals for Cross-linking process

Gelatin powder was purchased from Ajax Finechem, New Zealand.

Sodium azide (A.R. grade) was purchased from Sigma-aldrich, USA.

Gallic acid monohydrate was purchased from Sigma-aldrich, USA.

Sodium hydroxide (A.R. grade) was purchased from Mallinckrodt Baker Inc., Canada.

Glacial acetic acid (A.R. grade) was purchased from Sigma-aldrich, USA.

Chemicals for cross-linked gelatin solutions determination

L-Lysine monohydrochloride was purchased from Sigma-aldrich, USA.

Sodium tetraborate decahydrate (A.R. grade) was purchased from Sigma-aldrich, USA.

Fluorescamine (A.R. grade) was purchased from Carlo Erba Reagents, Italy.

Acetone dimethyl ketone (A.R. grade) was purchased from Sigma-aldrich, USA.

Acetic acid glacial (A.R. grade) was purchased from Sigma-aldrich, USA.

Chemical for film-forming solutions

Glycerol (A.R. grade) was purchased from Ajax Finechem, New Zealand.

3.3 Instruments

1. UV-VIS Spectrophotometer (Jasco V530, Jasco Inc., Easton, USA)

2. Centrifuge (EBA21, Hettich-Zentrifuge, Germany)

3. Blender (HR2001, Philips Electronics Thailand Co.,Ltd., Bangkok, Thailand)

4. Weighing balance (SI-234, Denver-Instrument, Germany)

5. Weighing balance (XT920M, Precisa Gravimetrics AG, Dietikon, Switzerland)

6. Water bath (S-water bath GFL-1072, Germany)

7. pH meter (CyberScan pH1000, Eutech Instrument, Singapore)

8. Air pump (PT6800, General Pet Time Co.,Ltd., Tokyo, Japan)

9. Espresso maker and coffee grinder (Model TSK-1825, Buono, Taiwan)

10. Bohlin Rheometer (CVOR-150, Malvern Instruments Ltd., Worcestershire, UK)

11. Spectrofluorometer (Jasco FP-6200, Jasco Inc., Easton, USA)

12. Chroma meter (Model CR-400 series, Minolta, Japan)

13. Micrometer (Mitutota Absolute, Mitutoya Crop., Tokyo, Japan)

14. Universal testing machine (Instron 5565, Canton, MA, USA)

15. Scanning electron microscope (JEOL model JSM-6400 LV, Peabody, MA, USA)

16. Ion sputtering (JEOL model JFC-1100E, Peabody, MA, USA)

17. Vortex mixer (model G-560E, Scientific Industries INC., Bohemia, New York, USA)

18. Hot air oven (UC-30, Memmert GmbH and Co. KG., Western Germany)

3.4 Extraction and determination of total phenolic compounds

This part deals with the extraction and the determination of the total phenolics content, expressed in term of gallic acid equivalent (GAE), of crude extract from several local grown phenolic-rich plants.

3.4.1 Preparation of plant extract

The method used in crude extract preparation from different raw materials is outlined in Figures 3.1 to 3.5

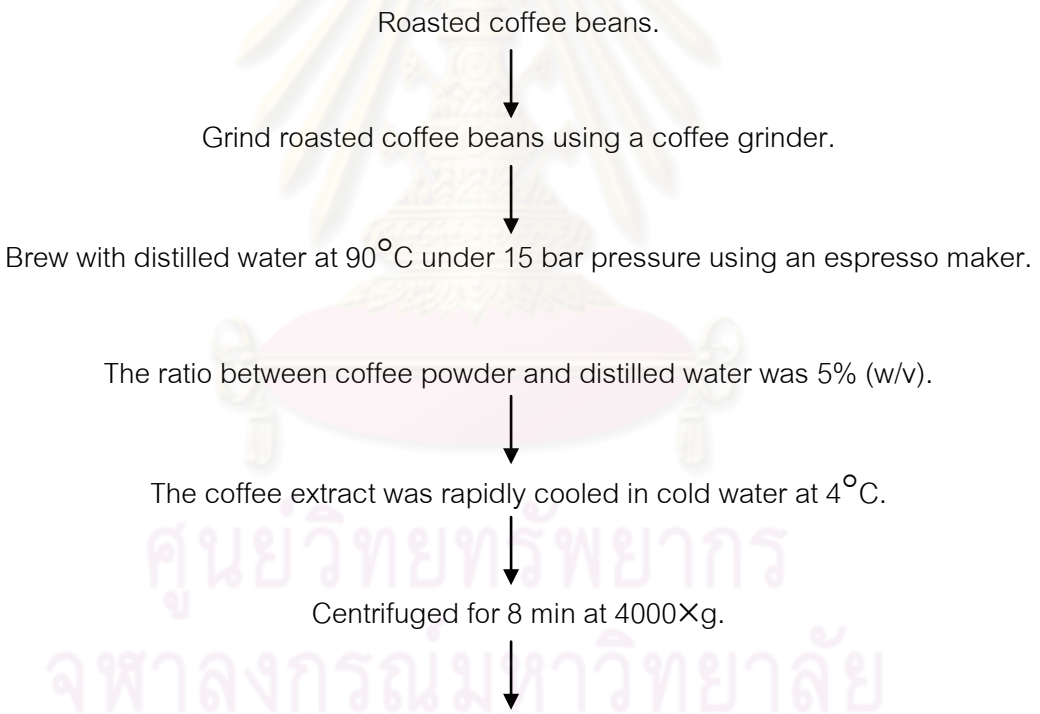


Figure 3.1 The extraction of roasted coffee beans.

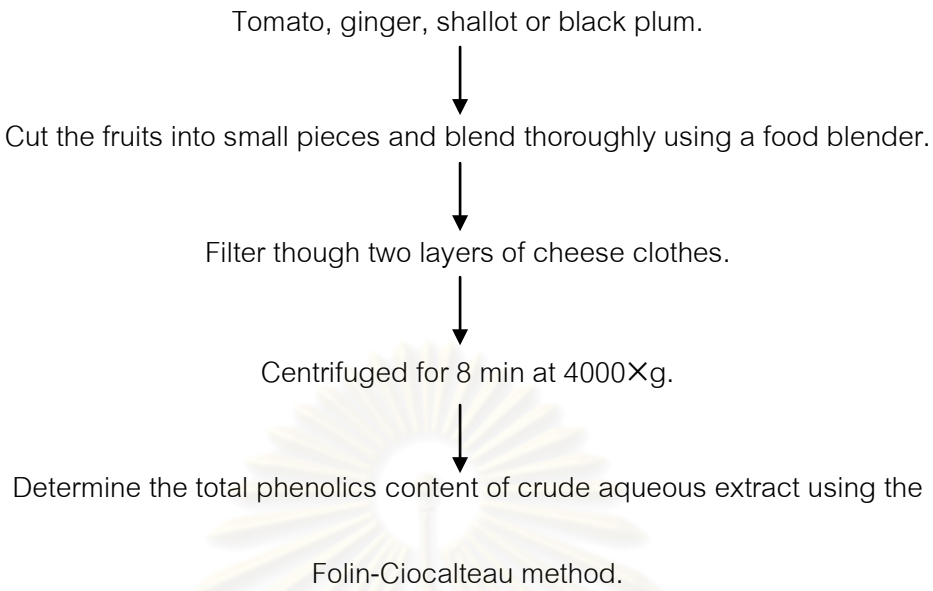


Figure 3.2 The extraction of tomato, ginger, shallot and black plum.

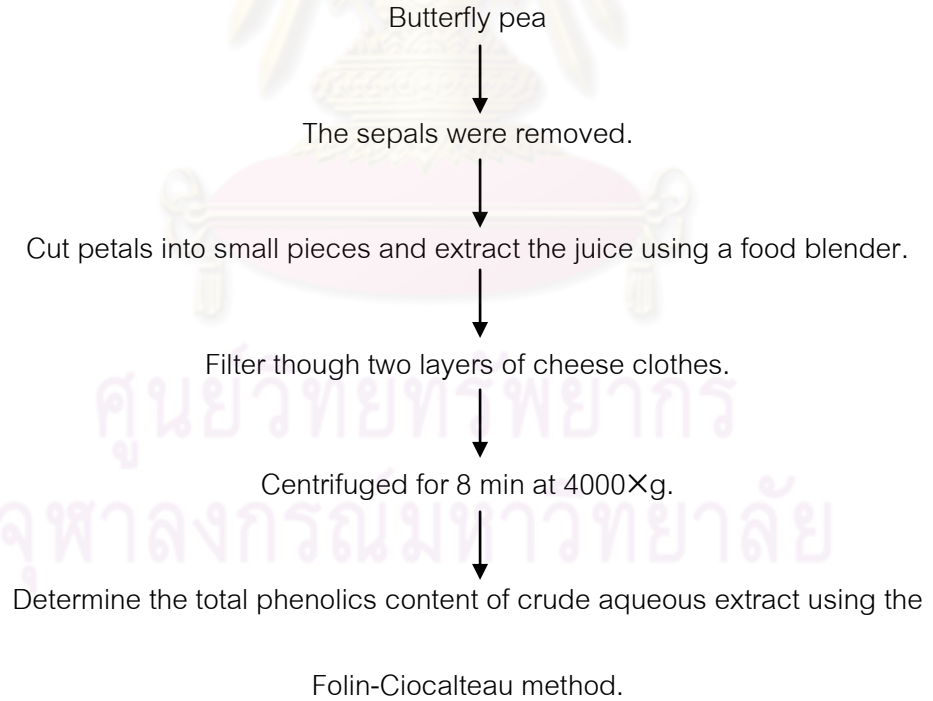


Figure 3.3 The extraction of butterfly pea.

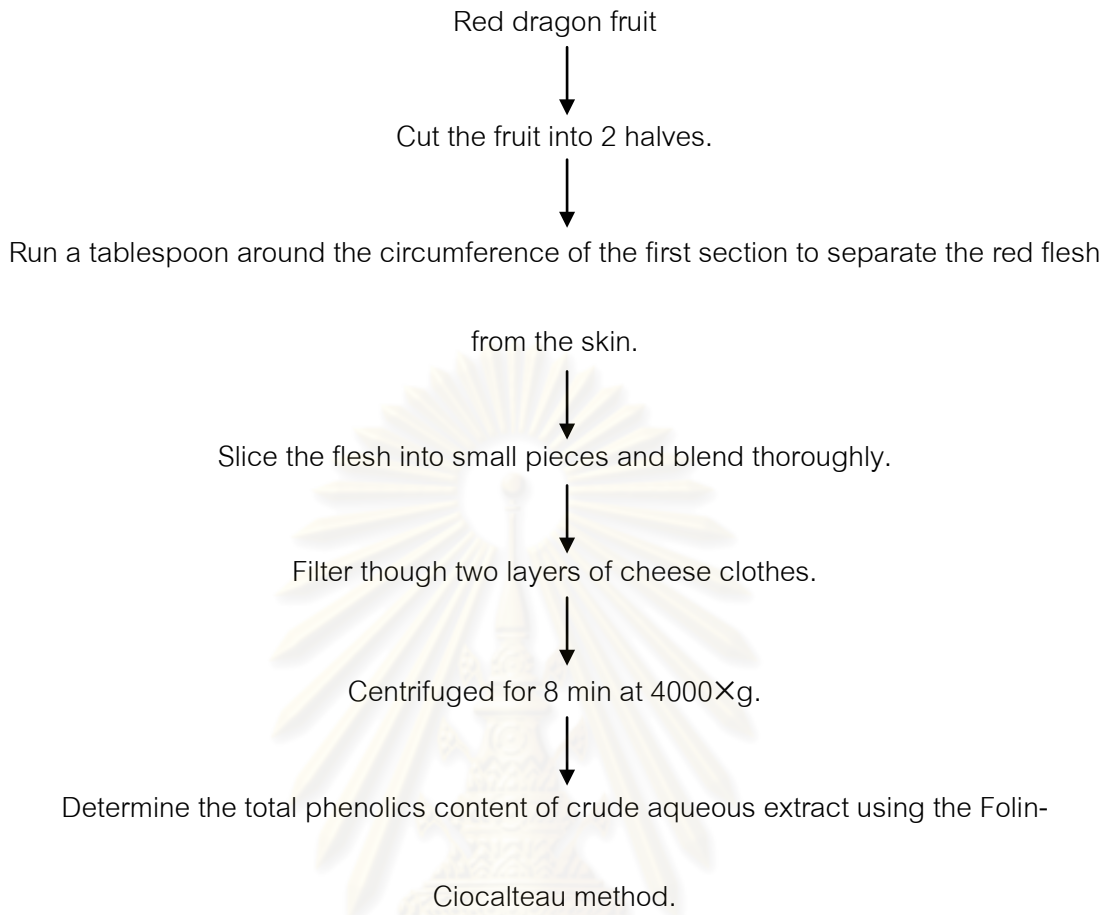


Figure 3.4 The extraction of red dragon fruit.

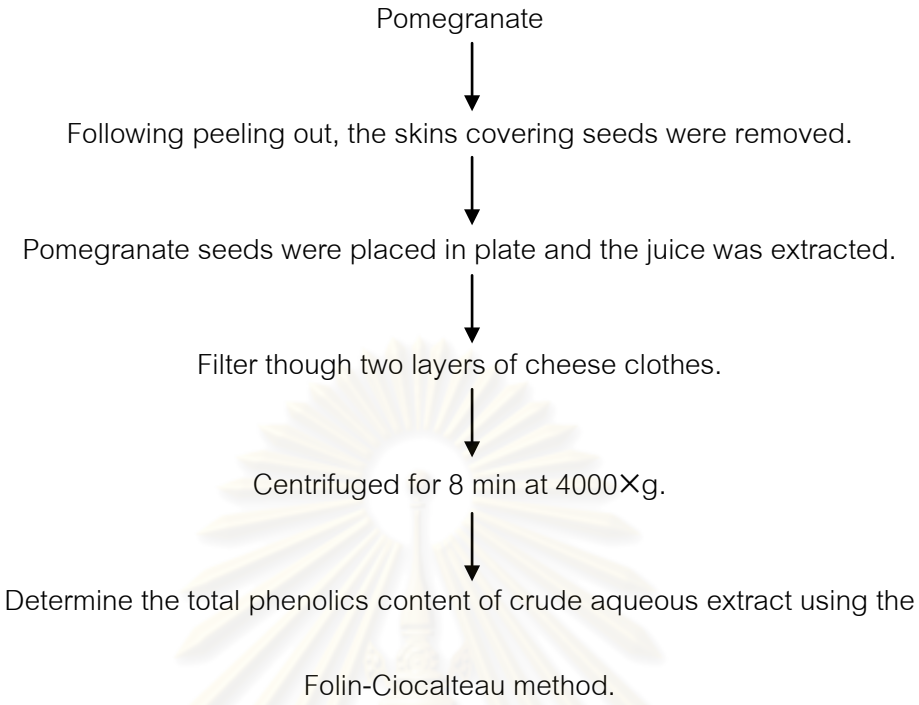


Figure 3.5 The extraction of pomegranate.

3.4.2 Determination of total phenolics content

The total phenolics content of the extract prepared from butterfly pea, tomato, red dragon fruit, ginger, shallot, black plum, pomegranate, Arabica coffee (dark roasted, medium roasted, and light roasted) was evaluated using the Folin-Ciocalteau reagent following the method adapted from Waterman and Mole (1994).

Sixty (60) µL of crude aqueous extract from plant raw materials was added to a test tube containing 4.74 mL of distilled water. Swirl contents to mix. Add 300 µL of 10% (v/v) Folin-Ciocalteau reagent (Appendix A.1) and mix again. After 3 min, add 900 µL of 20% (w/v) sodium carbonate solution (Appendix A.2), record the time as time zero and mix again. Solutions were maintained at room temperature (~25ºC) under dark conditions for 90 min and the total phenolics content was determined at 765 nm using a UV-VIS Spectrophotometer (Jasco V530, Jasco Inc., Easton, USA). Gallic acid standard solutions (Appendix A.3) were used to calibrate the method. A gallic acid standard curve was made available and is shown in appendix B1.

3.5 Cross-linking of gelatin using phenolic compounds

This part aimed to determine the effects of cross-linking on the properties of gelatin solutions. Gelatin solution (6.67% w/v) was cross-linked using gallic acid and the crude plant extracts. The cross-linking reactions were carried out using various polyphenol:NH_2 ratios (0.2:1 to 1:1). The cross-linked gelatin solution was subsequently analyzed for its chemical and physical properties. Figure 3.6 shows the outline for the experiment in this section.

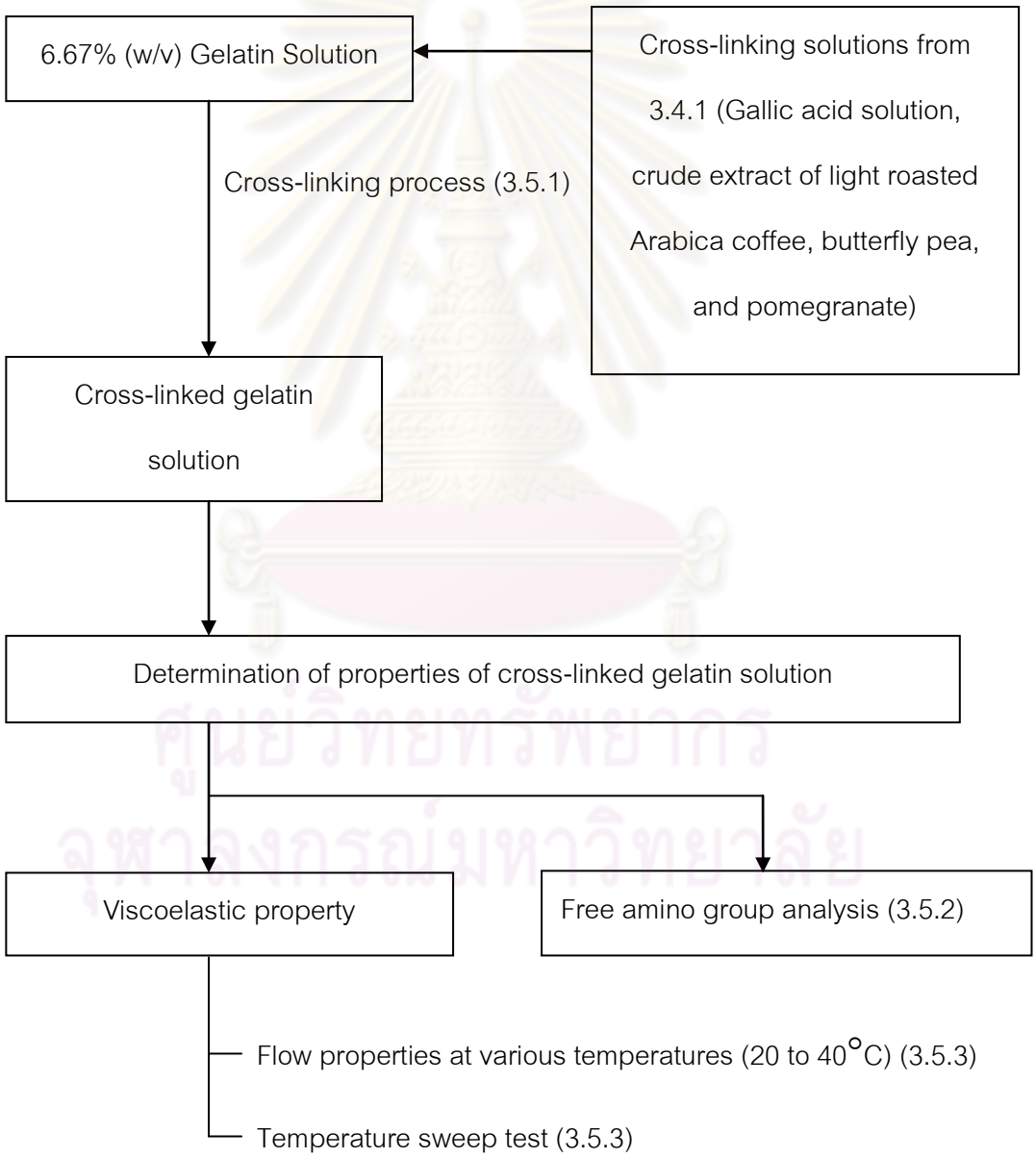


Figure 3.6 Outline for the cross-linking of gelatin and properties determination.

3.5.1 Preparation of cross-linked gelatin solutions

Eight (8) g of gelatin was hydrated at room temperature by suspending in 50 mL of distilled water containing 0.01% (w/v) sodium azide then kept at 40°C for 45 min with occasional stirring. Gallic acid solution (Appendix A.9) and three highest phenolics containing crude aqueous extracts of plant raw materials, which were light roasted Arabica coffee, butterfly pea, and pomegranate, were used in cross-linking of gelatin following the method of Strauss and Gibson (2004) with slight modification. The cross-linking reactions at 0.2:1 to 1:1 polyphenol:NH_2 ratio for gallic acid and light roasted Arabica coffee, 0.2:1 to 0.5:1 polyphenol:NH_2 ratio for butterfly pea, and 0.2:1 to 0.3:1 for pomegranate were carried out. To attain the desired polyphenol:NH_2 ratio, gallic acid and crude plant extracts were mixed with gelatin solution at various proportions. The mixture was made up to 112 mL with distilled water and adjusted to pH 8 using 4M sodium hydroxide (Appendix A.7) or 4M acetic acid (Appendix A.8). The prepared solution was placed in a temperature-controlled water bath at 40°C, under constant stirring at 150 rpm and subjected to oxygenation for 1 hour by bubbling the solution with oxygen to convert the phenolic compounds to quinones which allow subsequent cross-linking reaction. Finally, the cross-linked solutions were determined for their viscoelastic properties, free amino group content and film-forming properties.

3.5.2 Free amino group analysis

Free aliphatic primary amino groups in gelatin, essentially the 1-NH_2 groups of lysine, were determined using the modified method of Strauss and Gibson (2004). Sixty (60) µL of the sample was mixed thoroughly with 2 mL of 0.05 M sodium tetraborate at pH 8.9, followed by the addition of 150 µL of 20% (w/v) fluorescanine solution in acetone. The mixture was well shaken. The reaction was complete in a few seconds. The resulting fluorescence was measured using a Spectrofluorometer (Jasco FP-6200, Jasco Inc., Easton, USA) using 390/475 nm wavelengths for excitation and emission and free amino group content were expressed in term of lysine.

3.5.3 Determination of viscoelastic properties

The rheological behavior of 6.67% (w/v) native gelatin solution and all cross-linked gelatin solutions was characterized by flow tests and oscillatory shear tests using a Bohlin CVOR-150 rheometer (Malvern Instruments Ltd., Worcestershire, UK) using a cone and plate geometry (1° angle, 30 μm gap). The measurement was performed in triplicate.

Shear viscosity of the 6.67% (w/v) gelatin solutions were performed at a constant temperature of 20, 25, 30, 35 and 40°C. Flow test of all samples was also carried out at 25°C in the shear rate range of 0.01 to 100 s^{-1}.

Temperature sweep tests, and oscillatory shear test, were run over the range from 40 to 10°C and followed by temperature ramp of 10 to 40°C at a cooling/heating rate of 1°C/min with constant strain amplitude of 10% that was chosen from the linear viscoelastic region of the samples at 1Hz. Storage modulus (G'), loss modulus (G'') and complex viscosity (G^*) as a function of heating temperature was recorded.

3.6 Determination of properties of cross-linked gelatin films

This part deals with the investigation of film formation properties of cross-linked gelatin with gallic acid and crude plant extracts, which were light roasted Arabica coffee, pomegranate and butterfly pea. The cross-linked gelatin films were determined for the mechanical properties, thickness, Swelling, whiteness and morphology. Figure 3.7 shows the experimental steps in this section.

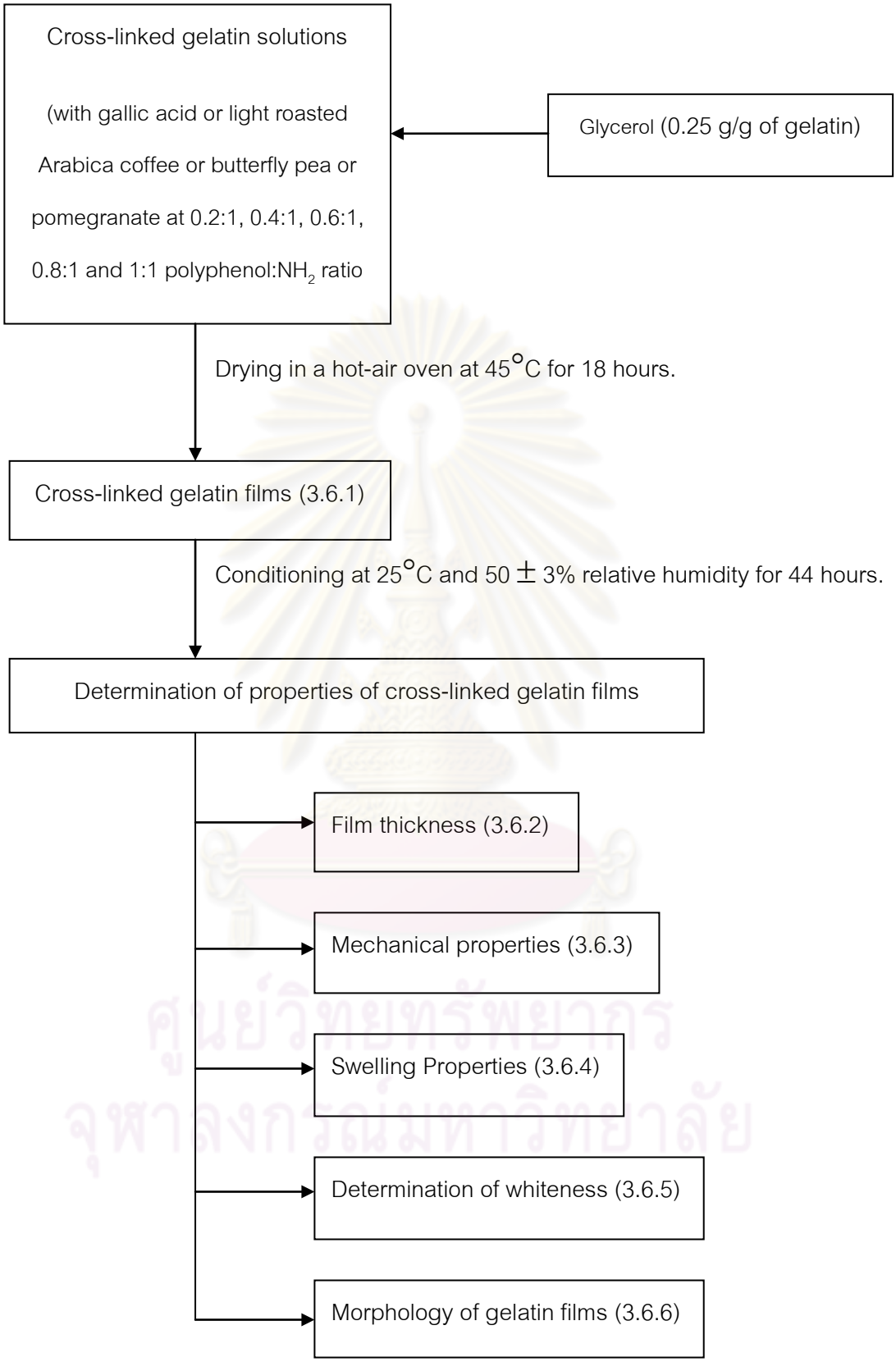


Figure 3.7 Outline for the cross-linking of gelatin films and properties determination.

3.6.1 Preparation of film-forming solutions

The film-forming solutions were made using gelatin, prepared according to the method modified from Gomez-Estaca et al. (2009), at a concentration of 6.67% (w/v) native gelatin and all cross-linked gelatin solutions. Glycerol (0.25 g/g of gelatin) was employed as the plasticizer. All mixtures were warmed and blended by stirring at 40°C for 15 min, and the films were made by casting 105 mL of gelatin solutions on a 12 cm × 28 cm-square plate and drying at 45°C for 18 hours. Before performing the determinations, the films were conditioned in a desiccator at 25°C and 50 ± 3% relative humidity for 44 hours.

3.6.2 Determination of film thickness

The thickness of the film was measured using a micrometer (Mitutota Absolute, Mitutoya Crop., Tokyo, Japan). Five locations around each film (width × length = 50 mm × 80 mm) of five film samples were used for thickness determination.

3.6.3 Determination of mechanical properties

The tensile strength, young's modulus, and elongation of all gelatin films before and after cross-linking were measured using a universal testing machine (Instron 5565, Canton, MA, USA) with a 50 N load cell. The conditioned film samples (width × length = 10 mm × 80 mm) were used. The initial grip separation was set at 50 mm, and crosshead speed was set at 10 mm/min. The measurement was performed immediately when a sample was removed from the desiccator. The thickness of native gelatin and all cross-linked gelatin films was around 88 μm and 90 to107 μm, respectively. Tensile strength, Young's modulus, and elongation measurements for each type of film were replicated at least five. The average values were recorded.

3.6.4 Swelling Properties

Gelatin films were cut into 2.5 × 2.5 cm and weighed in an air-dried condition. They were then immersed in 40 mL of deionized water at 25°C for 2 min. Wet samples were wiped with filter paper to remove excess liquid and reweighed. The amount of adsorbed water was calculated using equation 1 (Bigi, Panzavolta, & Rubini, 2001).

$$\text{Swelling (W\%)} = 100(W_w - W_d) / W_d \quad \text{.....(1)}$$

Where, W_w and W_d are the weights of the wet and the air-dried samples, respectively. The measurement was done in triplicate.

3.6.5 Determination of whiteness

Color of gelatin films was determined using a Chroma meter (Model CR-400 series, Minolta, Japan). CIE L* (lightness), a* (redness/greenness) and b* (yellowness/blueness) were measured and whiteness was calculated as described by Lanier, Hart, and Martin (1991) as follows

$$\text{Whiteness} = 100 - [(100 - L^*)^2 + a^{*2} + b^{*2}]^{1/2} \quad \text{.....(2)}$$

3.6.6 Morphology analysis of gelatin films

Microstructure of upper surface of the films containing oxidized phenolic compounds and the control film (without oxidized phenolic compound) were visualized using a scanning electron microscope (SEM) (JEOL model JSM-6400 LV, Peabody, MA, USA) at an accelerating voltage of 15 kV. Prior to visualization, the film samples were mounted on brass stub and sputtered with gold in order to make the sample conductive, and photographs were taken at 1500× magnification.

3.7 Statistic analysis

Statistical tests were performed using the SPSS® computer program (SPSS for window version 14.0, SPSS Statistical Software, Inc., Chicago, IL, USA). All data were subjected to analysis of variance (ANOVA). Comparison of mean was carried out by Duncan's multiple range tests. The level of significance was set for $p < 0.05$.

CHAPTER IV

RESULTS AND DISCUSSION

4.1 Total phenolic content of selected plant materials

The contents of total phenolic compounds that were measured by Folin-Ciocalteu reagent in terms of gallic acid equivalent (GAE) varied from 1.712 to 163.125 mg GAE /g of dry weight for different plant extracts. Light roasted Arabica coffee had the highest total phenolic content of 163.125 mg GAE /g of dry weight among all samples in this study (Table 4.1). Medium roasted Arabica coffee, dark roasted Arabica coffee, butterfly pea, pomegranate, black plum, red dragon fruit, shallot, tomato and ginger contained 128.471, 108.249, 79.435, 28.385, 8.572, 6.587, 5.159, 4.698 and 1.712 mg GAE /g of dry weight of total phenolic compounds, respectively.

The highest phenolics-containing crude plant aqueous extracts, which was light roasted Arabica coffee contained 31,502 mg GAE /L. Medium roasted Arabica coffee, dark roasted Arabica coffee, butterfly pea, pomegranate, shallot, black plum, ginger, red dragon fruit and tomato contained 24,952, 21,100, 4,039, 2,319, 917, 849, 727, 585, and 325 mg GAE /L total phenolic compounds, respectively (Table 4.2). The three extracts that contained the highest total phenolic compounds were selected for further cross-linking of gelatin.

Table 4.1 Total phenolic content of different plant extracts.

Plant raw materials	Total phenolic compounds*(mg GAE /g of dry weight)
Ginger	$1.712^{i} \pm 0.11$
Tomato	$4.698^{h} \pm 0.47$
Shallot	$5.159^{gh} \pm 0.47$
Red dragon fruit	$6.587^{g} \pm 0.47$
Black plum	$8.572^{f} \pm 0.54$
Pomegranate	$28.385^{e} \pm 1.32$
Butterfly pea	$79.435^{a} \pm 1.30$
Dark roasted Arabica coffee	$108.249^{d} \pm 1.12$
Medium roasted Arabica coffee	$128.471^{c} \pm 1.44$
Light roasted Arabica coffee	$163.125^{b} \pm 1.65$

[a, b, c...] Values with different superscripted letters in the same column are significantly different ($p \leq 0.05$).

* Values are given as average ± standard deviation calculated from three replications.

Table 4.2 Total phenolic content of different crude plant aqueous extracts.

Plant raw materials	Total phenolic compounds*(mg GAE /L)
Tomato	$325^{j} \pm 7.20$
Red dragon fruit	$585^{gj} \pm 10.0$
Ginger	$727^{gj} \pm 9.50$
Black plum	$849^{g} \pm 21.1$
Shallot	$917^{g} \pm 6.50$
Pomegranate	$2{,}319^{f} \pm 84.3$
Butterfly pea	$4{,}039^{d} \pm 68.0$
Dark roasted Arabica coffee	$21{,}100^{c} \pm 315.8$
Medium roasted Arabica coffee	$24{,}952^{b} \pm 408.4$
Light roasted Arabica coffee	$31{,}502^{a} \pm 567.0$

[a, b, c...] Values with different superscripted letters in the same column are significantly different ($p \leq 0.05$).

* Values are given as average $\pm$ standard deviation calculated from three replications.

The level of phenolic compounds in plant materials depends on such factors as cultivation techniques, cultivar, growing conditions, ripening process, as well as processing and storage conditions, among others. It was reported that some processing pretreatments, for example, peeling, chopping, boiling, microwaving and frying onions may reduce the total content of quercetin conjugates from 1% in case of chopping to up to 75% in case of boiling onions in water (Shahidi and Naczk, 1995).

The total flavonol content of tomatoes grown in different countries ranges from 1.3 to 36.4 mg of quercetin/kg of fresh weight (Stewart et al., 2000). According to Crozier et al. (1997), the total quercetin content in tomatoes is between 1.3 and 203 mg quercetin/kg of fresh weight. Flavonols are located mostly in the tomato skin and only

small quantities are found in the flesh and seeds (Stewart et al., 2000). Immature green tomato fruits contain a high level of chlorogenic acid in the pericarp and pulp. The level of chlorogenic acid rapidly declines as the color of the fruit changes from green to pink and then to red. The content of rutin also reaches a maximum in green tomato and then decreases during successive stages of fruit development (Buta and Spaulding, 1997).The bioactive compounds of ginger include gingerols, which are the main pungent component of the rhizome, and shogaol, a monohydrated gingerol, as well as other gingerol-related compounds formed from phenylalanine via ferulic acid, and diarylheptanoids and terpenoids (Kikuzaki and Nakatani, 1996; Wu et al., 1998). Phenolic compounds detected in plums include phenolic acid derivatives, coumarins, flavan-3-ols and anthocyanins. Chlorogenic acids are the major phenolic acid derivatives in plums. Of these, neochlorogenic acid (3-O-caffeoylquinic acid, 3-CQA) is the major chlorogenic acid in plums and prunes (Möller and Herrmann, 1983). Tomás-Barberán et al. (2001) have reported that the total content of flavan-3-ols in plums ranges from 662 to 1,837 and from 138 to 618 mg of catechin equivalents/kg of fresh weight of peel and fruit flesh, respectively, and total content of anthocyanins in the peel and the flesh of ripe plums is 129 to 1,614 and 0 to 28.4 mg/kg of fresh weight, respectively.

The total phenolic content in pomegranate juice ranges from 1,808 to 2,566 mg/L, similar to that found in Cabernet Sauvignon red wine (Gil et al., 2000). Pomegranate is a rich source of hydrolysable tannins and anthocyanins. The total content of anthocyanins and hydrolysable tannins in pomegranate juice ranges from 161.9 to 387.4 mg/L and from 417.3 to 556.6 mg/g, respectively (Gil et al., 2000). A number of hydrolysable tannins have been isolated and identified in pomegranate leaves, bark and fruits. Hydrolysable tannins of pomegranate include gallotannins, ellagic acid tannins and gallagyl tannins such as punicalagin and punicalin. Of these, gallagyl tannins are the major hydrolysable tannins in commercial pomegranate juices because the juice contains 1,500 to 1,900 mg punicalagin/L (Gil et al., 2000). In addition, small quantities of several flavan-3-ols have been detected in pomegranate: catechin (4.0 mg/kg), epicatechin (0.8 mg/kg), gallocatechin (1.7 mg/kg), and

procyanidins B1 (1.3 mg/kg) and B3 (1.6 mg/kg of fresh weight) (de Pascual et al., 2000).

Butterfly pea has several lines with different flower colors: dark blue, light blue, mauve, and white. Of these, 'Double Blue' is well-known to accumulate ternatins, a group of (poly) acylated anthocyanins, in the petals. The total content of anthocyanins varies among also different cultivars of the same flower and is affected by genetic make-up, light, temperature, agronomic and composition factors (Mazza and Miniati, 1993). The basic structure of these anthocyanidins is the flavylium (2-phenylbenzopyrilium) cation substituted with a number of hydroxyl and methoxyl groups. The patterns of hydroxylation and methoxylation affect the color of anthocyanidins. Presence of larger numbers of hydroxyl groups in the molecule tends to deepen the blue hue, while an increase in the number of methoxyl groups enhances the redness (Delgado-Vargas et al., 2000; Mazza and Miniati, 1993). The intensity of color, however, depends also on the pH, presence of metal ions, self-association of anthocyanins, pigment mixtures and copigments such as other colorless phenolic compounds, as well as processing and storage conditions (i.e. temperature, sugar content, presence of ascorbic acid, and presence of oxygen, among others) (Delgado-Vargas et al., 2000; Mazza and Miniati, 1993). The mechanism and thermal degradation of anthocyanins is not well understood. A number of pathways have been proposed. Degradation of anthocyanins depends on the temperature and duration of heat treatment.

Coffee beans may contain at least five major groups of chlorogenic acid isomers as caffeoylquinic acids, dicaffeoylquinic acids, feruoylquinic acids, p-coumaroylquinic acids and caffeoylferuloylquinic acids. The total content of chlorogenic acids in *Coffea arabica L.* is 5 to 8% on a dry weight basis and varies with maturity (Clifford and Kazi, 1987). In roasted coffee bean, phenolic compounds are indigenous or are produced from thermal degradation of chlorogenic acid and lignins. Time and temperature of roasting influence the phenols produced and their composition. Roasting results in a significant decline in the content of chlorogenic acid in green coffee beans; the content of chlorogenic acid decreases rapidly as temperature increases throughout the roasting

cycle (Merrit and Proctor, 1959). Light roasting produces a higher level of caffeoylquinic, dicaffeoylquinic, and feruloylquinic acids in Arabica coffee. The ratio of 5-caffeoylquinic acid to caffeine has been proposed as an indicator for monitoring the roasting process of coffee. During roasting, the content of chlorogenic acid declines while caffeine remains virtually unchanged. Because of this, caffeine may be used as an internal standard, thus allowing measurement of the level of chlorogenic acid in roasted coffee bean independent of weight losses due to formation of volatiles during roasting. This ratio may be useful for measuring the compositional changes of roasted coffee, especially those produced by fast roasting (Purdon and McCamey, 1987). The extraction of phenolics into the coffee juice depends on a number of factors, including the proportion of ground coffee to water, freshness of roasted coffee, grind, method of brewing, water temperature and length of time that water is in contact with ground coffee.

4.2 Effect of cross-linking on the properties of gelatin solutions

This section aimed to investigate the effect of cross-linking of gelatin solutions using gallic acid solution, and selected plant extracts that contain high total phenolics content, which are light roasted Arabica coffee extract, pomegranate extract and butterfly pea extract, on the rheological properties and free amino group content.

4.2.1 Rheological properties

4.2.1.1 Flow behavior

Figures 4.1 to 4.4 shows shear stress-shear rate profiles of 6.67% solution of native and cross-linked gelatin at various polyphenol:NH_2 ratios (0.2:1 to 1:1) at 20°C and pH 8. It is seen that the shear viscosity of native gelatin is the highest among all tested samples. All samples possessed shear-thinning property. From Figure 4.1, it is obvious that cross-linking of free amino groups on the gelatin molecules with

gallic acid solution caused the shear profiles to reduce. This might stem from the reduction in hydration capacity of the cross-linked gelatin molecules which caused the shear stress of the solution to reduce when tested at the same shear rate. The decreased shear stress with increasing polyphenol:NH_2 ratio in the gelatin cross-linking was observed. The same trend was observed for the gelatin cross-linked with light roasted Arabica coffee extract (Figure 4.2), pomegranate extract (Figure 4.3) and butterfly pea extract (Figures 4.4 and 4.5). The cross-linked gelatin with light roasted Arabica coffee extract and butterfly pea extract showed the lowest and highest effect of shear stress decreased, respectively. However, the cross-linking at higher polyphenol:NH_2 ratios was not determined for some plant extracts. This was due to the limitation in total phenolic content of the extracts that was not high enough to reach the desired polyphenol:NH_2 ratio.

Figure 4.2 shows the effect of cross-linking of gelatin using light roasted Arabica coffee extract at 0.2:1 to 1:1 polyphenol:NH_2 ratio. Cross-linking caused the shear stress to reduce gradually up to 0.8:1 polyphenol:NH_2 ratio. At higher polyphenol:NH_2 ratio, such as 1:1 polyphenol/NH_2 , the lower solubility of large phenolic compounds at high concentration causes the difficulty to interact with protein molecules (de Freitas & Mateus, 2001). In addition, the size of the phenolic compound can decrease its conformational flexibility, which is observed to be an important parameter in protein–phenolic compound interactions (Frazier et al., 2003). As a result, the ability to cross-link protein reduced leading to the increase in polymer free volume, hence an increase in shear stress at higher polyphenol:NH_2 ratio.

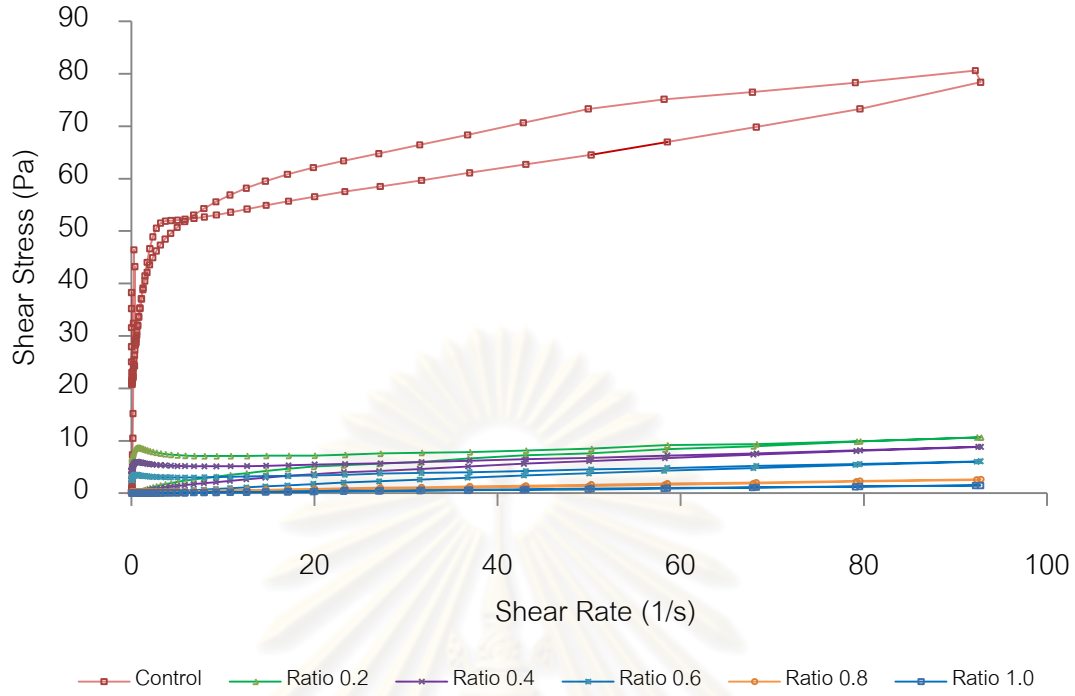


Figure 4.1 Shear stress-shear rate profiles of 6.67% (w/v) native gelatin solution (control) and 6.67% (w/v) gelatin solution cross-linked with gallic acid at polyphenol:NH_2 ratio of 0.2:1 (ratio 0.2), 0.4:1 (ratio 0.4), 0.6:1 (ratio 0.6), 0.8:1 (ratio 0.8) and 1:1 (ratio 1.0) at 20°C and pH 8.

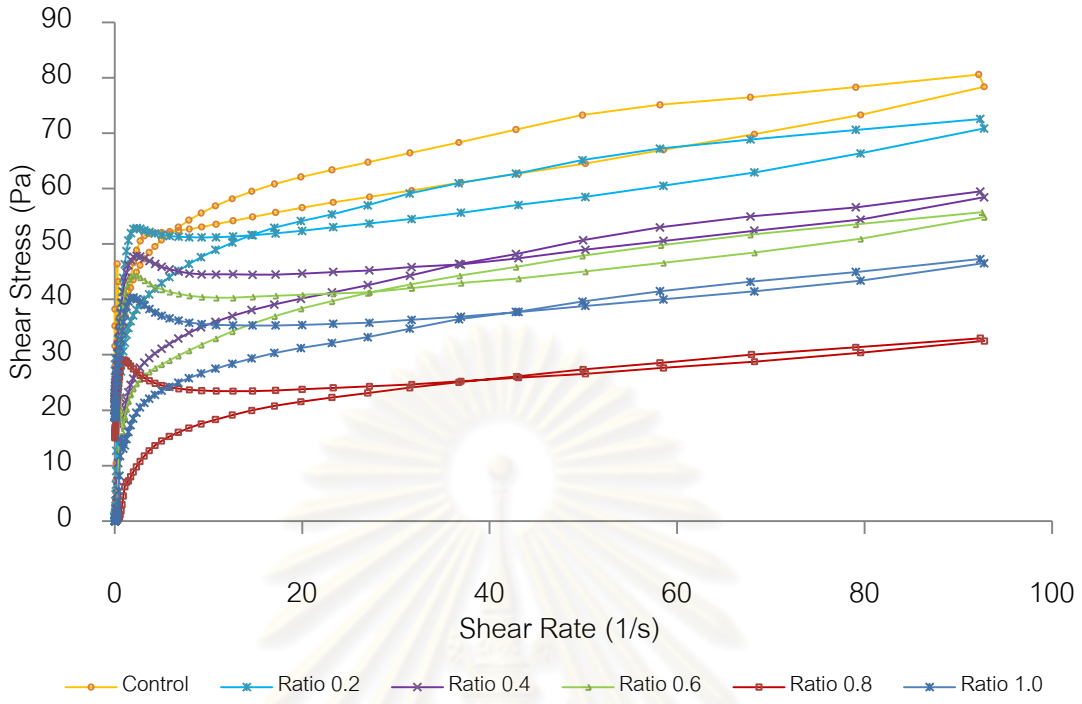


Figure 4.2 Shear stress-shear rate profiles of 6.67% (w/v) native gelatin solution (control) and 6.67% (w/v) gelatin solution cross-linked with light roasted Arabica coffee extract at polyphenol:NH_2 ratio of 0.2:1 (ratio 0.2), 0.4:1 (ratio 0.4), 0.6:1 (ratio 0.6), 0.8:1 (ratio 0.8) and 1:1 (ratio 1.0) at 20°C and pH 8.

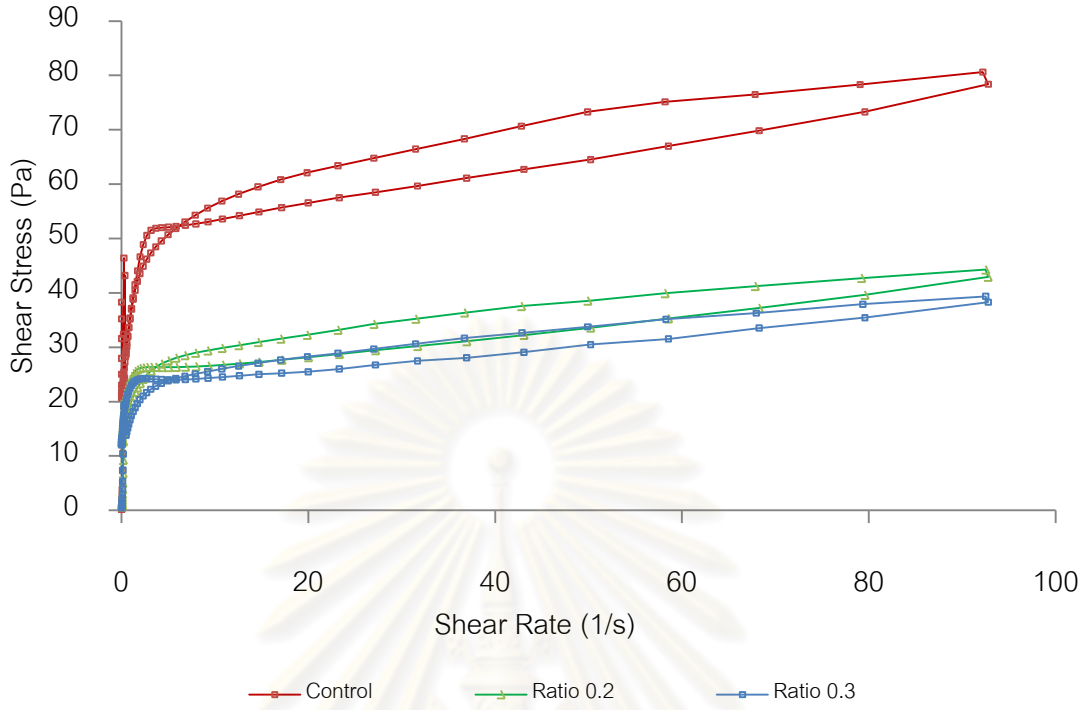


Figure 4.3 Shear stress-shear rate profiles of 6.67% (w/v) native gelatin solution (control) and 6.67% (w/v) gelatin solution cross-linked with pomegranate extract at polyphenol:NH_2 ratio of 0.2:1 (ratio 0.2), 0.3:1 (ratio 0.3) at 20°C and pH 8.

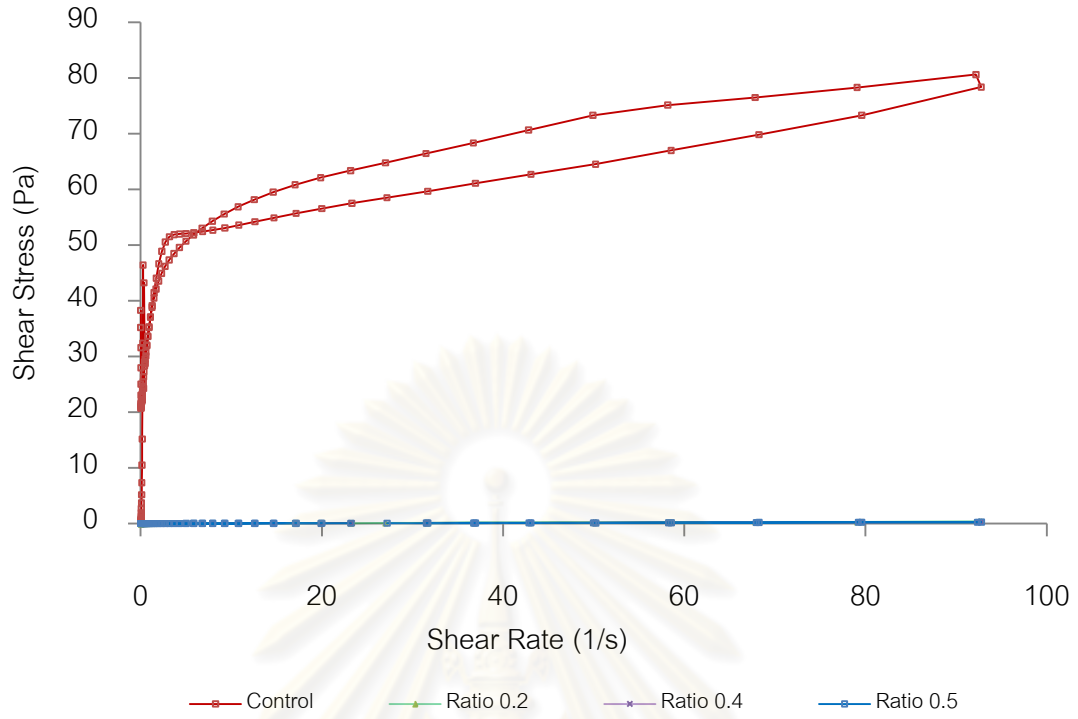


Figure 4.4 Shear stress-shear rate profiles of 6.67% (w/v) native gelatin solution (control) and 6.67% (w/v) gelatin solution cross-linked with butterfly pea extract at polyphenol:NH_2 ratio of 0.2:1 (ratio 0.2), 0.4:1 (ratio 0.4), 0.5:1 (ratio 0.5) at 20°C and pH 8.

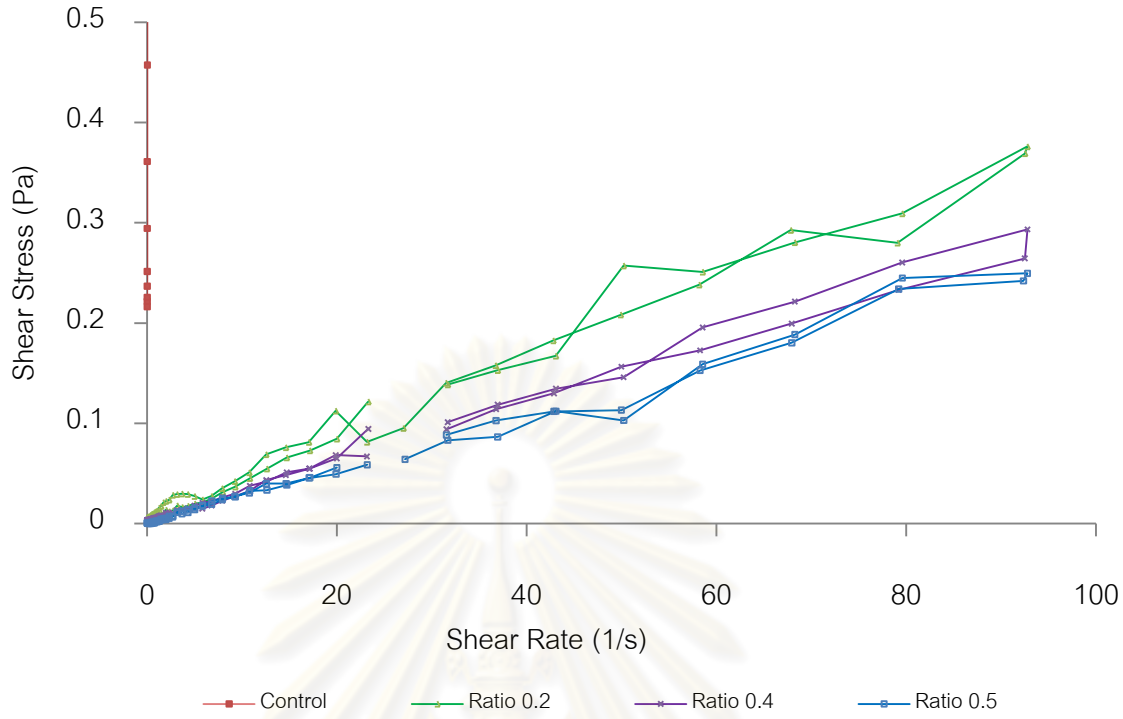


Figure 4.5 Shear stress-shear rate profiles of 6.67% (w/v) native gelatin solution (control) and 6.67% (w/v) gelatin solution cross-linked with butterfly pea extract at polyphenol:NH_2 ratio of 0.2:1 (ratio 0.2), 0.4:1 (ratio 0.4), 0.5:1 (ratio 0.5) at 20°C and pH 8. Shear profiles of cross-linked gelatin solutions in the shear stress range of 0.0 to 0.5 Pa.

Shear stress and shear rate data of native gelatin and all cross-linked gelatin solutions were tested for various rheological models using the software provided along with the rheometer. In the present study, based on standard error data obtained, it was found that the Power-law model was suitable for the explanation of the flow behavior of the cross-linked gelatin solutions.

Table 4.3 - 4.6 shows consistency index (K) value and Flow behavior index (n) of native and cross-linked gelatin solutions at various temperatures (20, 25, 30, 35 and 40°C). In general, K decreased with increasing temperature and polyphenol:NH_2 ratio. The K of native gelatin and cross-linked gelatin with gallic acid, light roasted Arabica coffee extract, pomegranate extract and butterfly pea extract at 20°C were higher than at all temperature (25 to 40°C). At 40°C, the decrease in K value of cross-linked gelatin solution with increasing temperature at all polyphenol:NH_2 ratios compared with native gelatin solution was due to the increase in intermolecular distances, because of the thermal expansion caused by the increase in temperature (Constenella, Lozano, & Crapiste, 1989). Only a marginal decrease in consistency coefficient was observed with an increase in temperature for cross-linked gelatin with gallic acid, light roasted Arabica coffee extract and pomegranate extract. Whereas the decrease was prominent for cross-linked gelatin with butterfly pea extract. The consistency coefficient value at 35°C at cross-linked gelatin added with light roasted Arabica coffee at 1:1 polyphenol:NH_2 was higher than at lower polyphenol:NH_2. This increase in K value is most likely due to complete opening up of polypeptide chain to random coil molecules leading to increased viscosity (Flory & Weaver, 1960). The flow behavior index (n) at 20°C for native gelatin and cross-linked gelatin solution with gallic acid, light roasted Arabica coffee and pomegranate were much lower compared to higher temperatures indicating the viscous nature of gelatin at that temperature (Table 4.3 to 4.6). The flow behavior index (n) for all cross-linked gelatin solutions at 25 to 40°C did not difference. The farther the flow behavior index from 1, the more the deviation from Newtonian behavior (Lewis, 1990).

Table 4.3 Consistency coefficient and flow behavior index of 6.67% (w/v) gelatin solution cross-linked with gallic acid using various polyphenol:NH_2 ratios at different temperatures (pH 8).

Sample	Temperature (°C)	Polyphenol:NH_2 ratio	Consistency index, K (Pa • s^n)	Flow behavior index, n
Native gelatin	20		37.466 ± 1.7696	0.15 ± 0.012
	25		0.011 ± 0.0001	0.97 ± 0.011
	30		0.006 ± 0.0001	0.98 ± 0.034
	35		0.005 ± 0.0002	0.97 ± 0.018
	40		0.004 ± 0.0004	0.98 ± 0.047
Cross-linked gelatin using gallic acid	20	0.2:1	7.571 ± 0.5813	0.03 ± 0.005
		0.4:1	5.362 ± 1.4550	0.05 ± 0.024
		0.6:1	3.263 ± 0.6533	0.06 ± 0.018
		0.8:1	0.264 ± 0.2975	0.59 ± 0.340
		1:1	0.026 ± 0.0063	0.90 ± 0.042
	25	0.2:1	0.008 ± 0.0004	0.98 ± 0.017
		0.4:1	0.008 ± 0.0010	0.97 ± 0.005
		0.6:1	0.008 ± 0.0008	0.97 ± 0.015
		0.8:1	0.009 ± 0.0003	0.99 ± 0.014
		1:1	0.006 ± 0.0008	1.03 ± 0.033
	30	0.2:1	0.006 ± 0.0005	0.98 ± 0.004
		0.4:1	0.005 ± 0.0006	1.01 ± 0.042
		0.6:1	0.006 ± 0.0006	1.00 ± 0.007
		0.8:1	0.007 ± 0.0002	0.98 ± 0.002
		1:1	0.006 ± 0.0012	0.99 ± 0.041
	35	0.2:1	0.005 ± 0.0003	0.98 ± 0.015
		0.4:1	0.004 ± 0.0006	0.98 ± 0.016
		0.6:1	0.006 ± 0.0003	0.95 ± 0.013
		0.8:1	0.005 ± 0.0003	1.00 ± 0.016
		1:1	0.004 ± 0.0009	1.02 ± 0.047
	40	0.2:1	0.004 ± 0.0002	0.99 ± 0.002
		0.4:1	0.003 ± 0.0002	1.02 ± 0.008
		0.6:1	0.005 ± 0.0007	0.95 ± 0.034
		0.8:1	0.004 ± 0.0002	1.01 ± 0.019
		1:1	0.005 ± 0.0008	0.97 ± 0.047

* Values are given as average ± standard deviation calculated from three replications.

Table 4.4 Consistency coefficient and flow behavior index of 6.67% (w/v) gelatin solution cross-linked with light roasted Arabica coffee using various polyphenol:NH_2 ratios at different temperatures (pH 8).

Sample	Temperature (°C)	Polyphenol:NH_2 ratio	Consistency Index*, K (Pa • s^n)	Flow behavior index*, n
Native gelatin	20		37.466 ± 1.7696	0.15 ± 0.012
	25		0.011 ± 0.0001	0.97 ± 0.011
	30		0.006 ± 0.0001	0.98 ± 0.034
	35		0.005 ± 0.0002	0.97 ± 0.018
	40		0.004 ± 0.0004	0.98 ± 0.047
Cross-linked gelatin using coffee extract	20	0.2:1	39.199 ± 1.5563	0.12 ± 0.017
		0.4:1	32.914 ± 2.2590	0.10 ± 0.002
		0.6:1	35.628 ± 3.1959	0.10 ± 0.015
		0.8:1	22.379 ± 0.5607	0.06 ± 0.002
		1:1	29.812 ± 0.6717	0.09 ± 0.003
	25	0.2:1	0.011 ± 0.0001	0.97 ± 0.007
		0.4:1	0.016 ± 0.0004	0.97 ± 0.005
		0.6:1	0.015 ± 0.0016	0.96 ± 0.001
		0.8:1	0.011 ± 0.0000	0.97 ± 0.010
		1:1	0.014 ± 0.0001	0.96 ± 0.001
	30	0.2:1	0.006 ± 0.0001	0.98 ± 0.008
		0.4:1	0.008 ± 0.0013	1.00 ± 0.039
		0.6:1	0.008 ± 0.0006	0.99 ± 0.004
		0.8:1	0.008 ± 0.0002	0.95 ± 0.000
		1:1	0.007 ± 0.0008	1.01 ± 0.026
	35	0.2:1	0.005 ± 0.0001	0.99 ± 0.001
		0.4:1	0.006 ± 0.0001	0.95 ± 0.003
		0.6:1	0.007 ± 0.0009	0.97 ± 0.015
		0.8:1	0.005 ± 0.0002	1.00 ± 0.013
		1:1	0.011 ± 0.0032	0.88 ± 0.005
	40	0.2:1	0.006 ± 0.0012	0.92 ± 0.045
		0.4:1	0.004 ± 0.0012	1.00 ± 0.081
		0.6:1	0.005 ± 0.0006	0.99 ± 0.010
		0.8:1	0.005 ± 0.0006	0.97 ± 0.013
		1:1	0.004 ± 0.0003	1.01 ± 0.008

* Values are given as average ± standard deviation calculated from three replications.

Table 4.5 Consistency coefficient and flow behavior index of 6.67% (w/v) gelatin solution cross-linked with pomegranate using various polyphenol:NH_2 ratios at different temperatures (pH 8).

Sample	Temperature (°C)	Polyphenol:NH_2 ratio	Consistency Index*, K (Pa • s^n)	Flow behavior index*, n
Native gelatin	20		37.466 ± 1.7696	0.15 ± 0.012
	25		0.011 ± 0.0001	0.97 ± 0.011
	30		0.006 ± 0.0001	0.98 ± 0.034
	35		0.005 ± 0.0002	0.97 ± 0.018
	40		0.004 ± 0.0004	0.98 ± 0.047
Cross-linked gelatin using pomegranate	20	0.2:1	20.044 ± 5.5075	0.11 ± 0.002
		0.3:1	21.512 ± 1.5089	0.12 ± 0.005
	25	0.2:1	0.017 ± 0.0044	0.99 ± 0.008
		0.3:1	0.028 ± 0.0214	0.98 ± 0.038
	30	0.2:1	0.010 ± 0.0014	0.98 ± 0.015
		0.3:1	0.013 ± 0.0036	0.98 ± 0.038
	35	0.2:1	0.006 ± 0.0003	0.99 ± 0.004
		0.3:1	0.008 ± 0.0001	0.97 ± 0.028
	40	0.2:1	0.004 ± 0.0001	1.04 ± 0.006
		0.3:1	0.007 ± 0.0008	0.97 ± 0.024

* Values are given as average ± standard deviation calculated from three replications.

Table 4.6 Consistency coefficient and flow behavior index of 6.67% (w/v) gelatin solution cross-linked with butterfly pea using various polyphenol:NH_2 ratios at different temperatures (pH 8).

Sample	Temperature (°C)	Polyphenol:NH_2 ratio	Consistency Index*, K ($Pa \cdot s^n$)	Flow behavior index*, n
Native gelatin	20		37.466 ± 1.7696	0.15 ± 0.012
	25		0.011 ± 0.0001	0.97 ± 0.011
	30		0.006 ± 0.0001	0.98 ± 0.034
	35		0.005 ± 0.0002	0.97 ± 0.018
	40		0.004 ± 0.0004	0.98 ± 0.047
Cross-linked gelatin using butterfly pea	20	0.2:1	0.009 ± 0.0047	0.82 ± 0.173
		0.4:1	0.003 ± 0.0012	1.02 ± 0.088
		0.5:1	0.002 ± 0.0016	1.17 ± 0.241
	25	0.2:1	0.003 ± 0.0001	0.97 ± 0.025
		0.4:1	0.003 ± 0.0005	0.94 ± 0.042
		0.5:1	0.003 ± 0.0011	1.01 ± 0.074
	30	0.2:1	0.002 ± 0.0001	0.99 ± 0.015
		0.4:1	0.002 ± 0.0012	0.98 ± 0.122
		0.5:1	0.003 ± 0.0016	0.93 ± 0.087
	35	0.2:1	0.002 ± 0.0005	0.98 ± 0.009
		0.4:1	0.001 ± 0.0003	1.08 ± 0.055
		0.5:1	0.002 ± 0.0006	0.96 ± 0.012
	40	0.2:1	0.002 ± 0.0006	1.04 ± 0.058
		0.4:1	0.002 ± 0.0008	0.98 ± 0.009
		0.5:1	0.003 ± 0.0006	0.92 ± 0.018

* Values are given as average ± standard deviation calculated from three replications.

4.2.1.2 Gelling and melting of gelatin solutions

Gelling and melting behaviors of solutions from native and cross-linked gelatin were measured using an oscillatory shear tests. Temperature sweep test results indicated that cross-linking did affect gelling and melting temperature of gelatin. Table 4.7 shows that gelation and melting temperature of cross-linked gelatin with gallic acid and light roasted Arabica coffee extract at 0.2:1 to 1:1 polyphenol:NH_2, and pomegranate extract at 0.2:1 to 0.3:1 polyphenol:NH_2. Cross-linking obviously caused gelatin to gel and melt at lower temperatures. The same trend was observed for the gelatin cross-linked with gallic acid, light roasted Arabica coffee extract and pomegranate extract. Of all plant extracts, butterfly pea extract imposed the most significant cross-linking effect on gelling and melting of cross-linked gelatin. The cross-linked gelatin add with butterfly pea extract at 0.2:1 to 0.5:1 polyphenol:NH_2 ratio showed large decreases in gelling and melting complex viscosities (Figure 4.6).

Table 4.7 Temperature sweep test results of 6.67% (w/v) native gelatin solution (control) and 6.67% (w/v) gelatin solution cross-linked with gallic acid, light roasted Arabica coffee extract and pomegranate extract at pH 8.

Sample	Polyphenol:NH_2	Temperature	
		Gelling	Melting
Native gelatin (control)	-	22.0	27.5
Cross-linked gelatin using gallic acid	0.2:1	19.5	25.0
	0.4:1	19.5	25.0
	0.6:1	19.0	24.5
	0.8:1	18.5	24.0
	1:1	18.5	23.5
Cross-linked gelatin using coffee extract	0.2:1	22.0	27.5
	0.4:1	22.0	27.0
	0.6:1	22.0	27.0
	0.8:1	22.0	26.5
	1:1	21.5	26.5
Cross-linked gelatin using pomegranate extract	0.2:1	21.0	26.5
	0.3:1	20.5	26.0

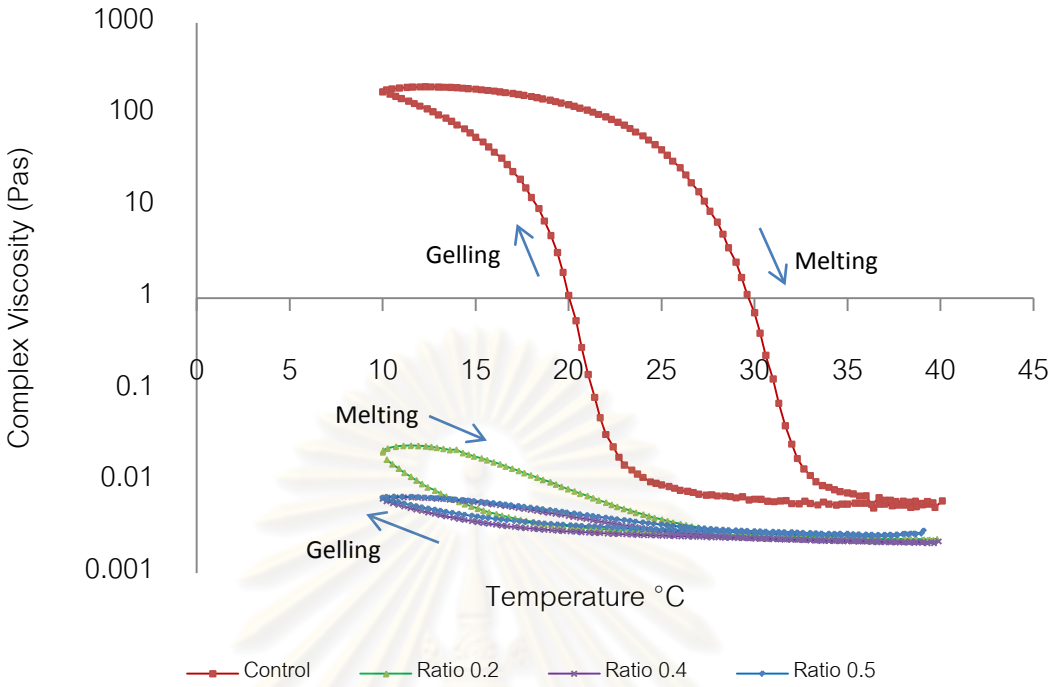


Figure 4.6 Temperature sweep test results of 6.67% (w/v) native gelatin solution (control) and 6.67% (w/v) gelatin solution cross-linked with butterfly pea extract at polyphenol:NH_2 ratio of 0.2:1 (ratio 0.2), 0.4:1 (ratio 0.4), 0.5:1 (ratio 0.5) at pH 8. The profiles show changes in complex viscosity (η^*) of the solution during melting and gelling.

4.2.2 Free amino group content

Free amino group contents of gelatin before and after cross-linking with different plant phenolic compounds are shown in Figure 4.7. At the same polyphenol:NH_2 ratio, the higher loss in free amino groups was observed in gelatin modified by oxidation with butterfly pea extract, followed by that modified with oxidized pomegranate extract, light roasted Arabica coffee extract and gallic acid, respectively. This result is in good agreement with the result presented in the previous section in that cross-linking using butterfly pea extract caused the tremendous reduction in the melt and gel of cross-linked gelatin.

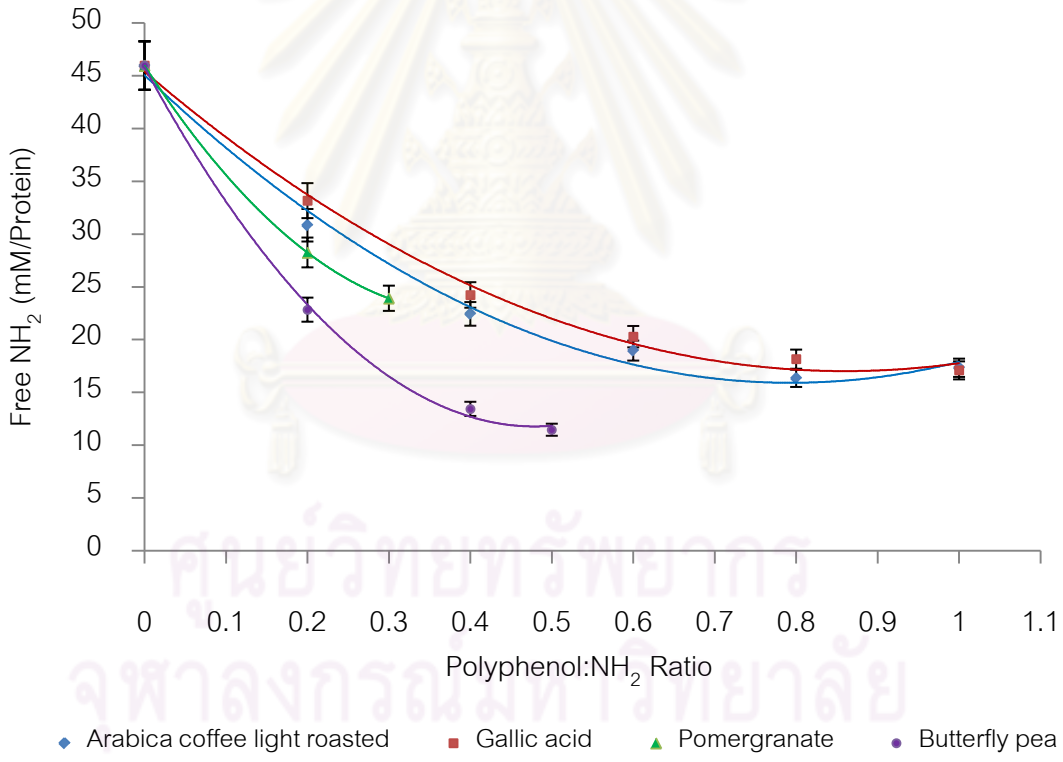


Figure 4.7 Effect of cross-linking using different plant extracts and gallic acid on free amino group content of gelatin. Polyphenol:NH_2 ratio of 0.2:1 (0.2), 0.4:1 (0.4), 0.6:1 (0.6), 0.8:1 (0.8) and 1:1 (1.0).

The rate of loss in free amino group of gelatin depended on the type and concentration of oxidized phenolic compounds used (Rawel, et al., 2001). In general, the loss in free amino groups was increased when the concentration of oxidized phenolic compounds increased. The hydroxyl groups of gallic acid, light roasted Arabica coffee extract, pomegranate extract and butterfly pea extract were presumably converted to quinone, which functioned as cross-linkers. Quinones have been indirectly proven to react with amino acids in a peptide chain. The covalent modification of proteins by phenolic oxidation products generated at alkaline pH has been studied (Rawel et al., 2002). Covalent protein modification by phenols oxidized at alkaline pH induced protein cross-linking and a decrease of the isoelectric pH of proteins (Prigent, 2005). The decrease in free amino is responsible for the decrease in shear viscosity, gelation and melting temperature reported earlier. The result was in agreement with Strauss and Gibson (2004) who found the decrease in bloom strength of gelatin gels incorporated with oxygenated phenolics.

4.3 Effect of cross-linking on the properties of gelatin films

4.3.1 Mechanical properties and thickness of cross-linked gelatin films

Mechanical properties of native gelatin and cross-linked gelatin films with gallic acid and light roasted Arabica coffee extract could provide an indication of expected film integrity under conditions of stress that would occur during processing, handling, and storage. Pomegranate and butterfly pea serve as a rich source of phenolic compounds such as anthocyanins, flavonoids, phenolic acids and proanthocyanidins, and some element such as sugars (i.e. glucose and fructose). These are usually present as glycoside with sugar moiety linked though an OH group or through carbon-carbon bonds. It is obvious that the structure of phenolic compounds and some element of pomegranate extract and butterfly pea extract caused cross-linked gelatin films with pomegranate extract and butterfly pea extract did not film-forming formation.

Tensile strength, elongation to break and Young's modulus of cross-linked gelatin films incorporated with gallic acid at polyphenol:NH_2 ratio of 0.2:1, 0.4:1, 0.6:1, 0.8:1 and 1:1 are presented in Figures 4.8 to 4.10.

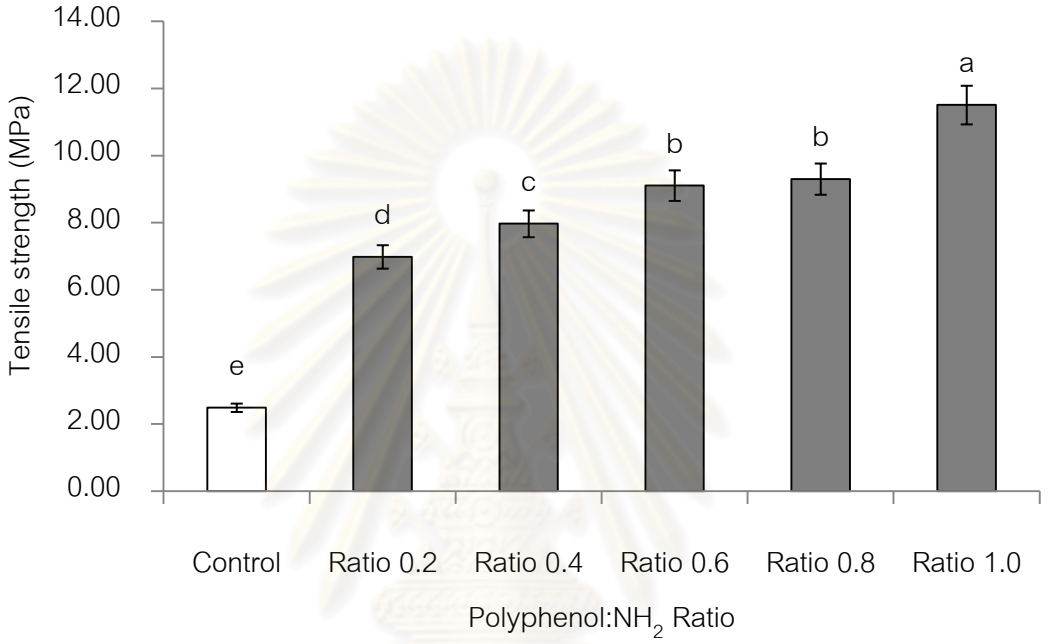


Figure 4.8 Tensile strength of the gelatin films prepared from 6.67% (w/v) native gelatin solution (control) and 6.67% (w/v) gelatin solution cross-linked with gallic acid at polyphenol:NH_2 ratio of 0.2:1 (ratio 0.2), 0.4:1 (ratio 0.4), 0.6:1 (ratio 0.6), 0.8:1 (ratio 0.8) and 1:1 (ratio 1.0). Different letters above different bars denote significant differences ($p \leq 0.05$).

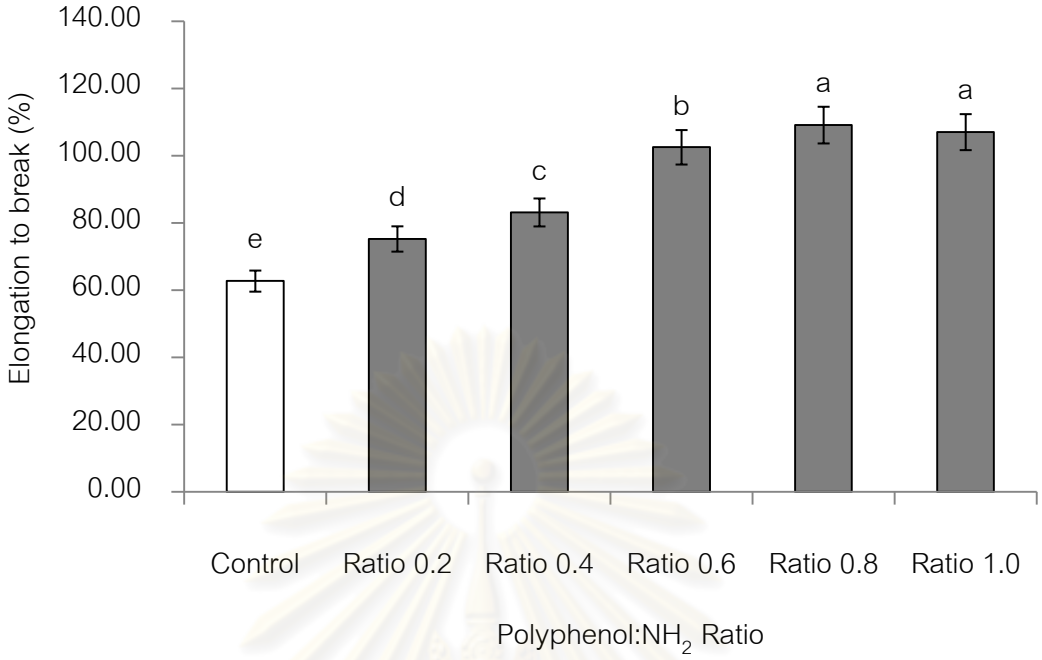


Figure 4.9 Elongation to break of the gelatin films prepared from 6.67% (w/v) native gelatin solution (control) and 6.67% (w/v) gelatin solution cross-linked with gallic acid at polyphenol:NH_2 ratio of 0.2:1 (ratio 0.2), 0.4:1 (ratio 0.4), 0.6:1 (ratio 0.6), 0.8:1 (ratio 0.8) and 1:1 (ratio 1.0). Different letters above different bars denote significant differences ($p \leq 0.05$).

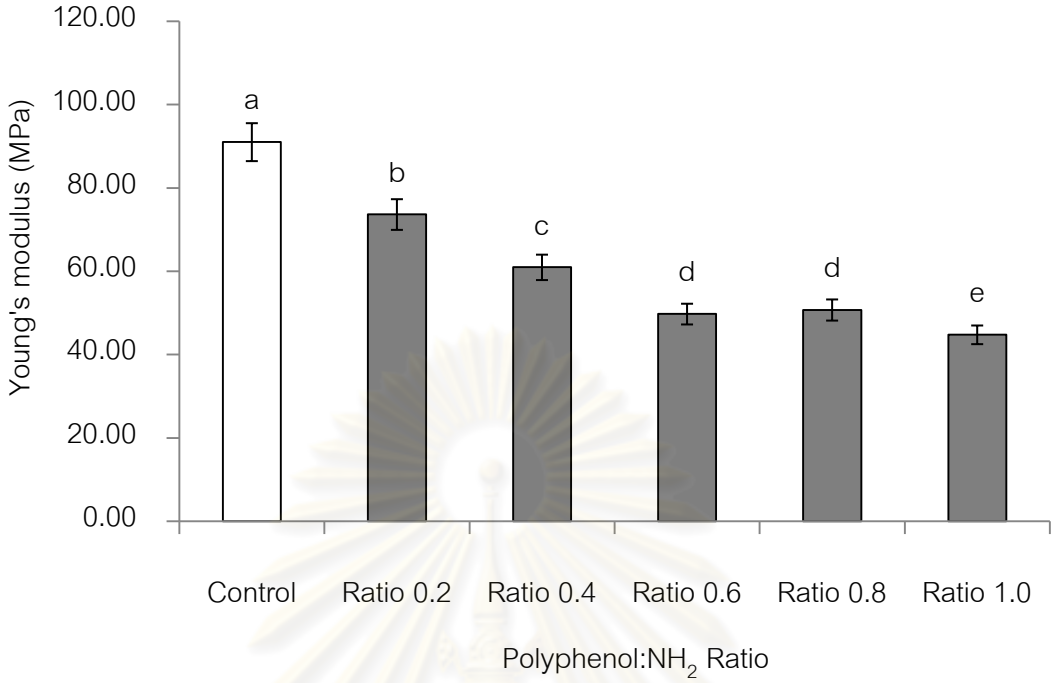


Figure 4.10 Young's modulus of the gelatin films prepared from 6.67% (w/v) native gelatin solution (control) and 6.67% (w/v) gelatin solution cross-linked with gallic acid at polyphenol:NH_2 ratio of 0.2:1 (ratio 0.2), 0.4:1 (ratio 0.4), 0.6:1 (ratio 0.6), 0.8:1 (ratio 0.8) and 1:1 (ratio 1.0). Different letters above different bars denote significant differences ($p \leq 0.05$).

The major advantages of phenolic compound over the aldehydes used for cross-linking of gelatin are that they are non-toxic and entirely biodegradable. Phenolic compound could react with more than one protein site and led to cross-links between proteins (Haslam, 1989). At pH 8, phenolic compounds could be converted to quinone, a protein cross-linker, in which new covalent cross-links could be formed. Quinones react with amino or sulfhydryl side chains of polypeptides to form covalent C–N or C–S bonds (Strauss and Gibson, 2004). From the result in Figures 4.8 to 4.10, Tensile strength and elongation to break of cross-linked gelatin films at all gallic acid concentrations was significantly higher ($p \leq 0.05$) while Young's modulus was significantly lower ($p \leq 0.05$) than that of non cross-linked gelatin film.

Elongation to break of film added with gallic acid concentration at all polyphenol:NH_2 ratios tended to increase with increasing polyphenol:NH_2 ratios. However, there were no obvious changes observed as polyphenol:NH_2 ratio increased from 0.8:1 to 1:1 ($p>0.05$). Young's modulus was seen to decrease with increasing polyphenol:NH_2 ratio in the 0.2:1 to 0.6:1 range and remained unchanged from 0.6:1 to 0.8:1 polyphenol:NH_2 ratio ($p>0.05$). When the concentrations of gallic acid were higher than 0.6:1 polyphenol:NH_2 ratio, no obvious changes in elongation to break and Young's modulus were observed ($p>0.05$). The results indicated that phenolic compound at a polyphenol:NH_2 ratio greater than 0.8:1 could be an excessive concentration, in which intermolecular cross-links could not be formed any further. From the result, the marked increase in both tensile strength and elongation to break was found in the film prepared from cross-linked gelatin using gallic acid. Gallic acid is a large molecule with several functional groups, which can polymerize protein with a longer chain length. This might contribute to the increase in elongation to break of resulting films (Orliac et al., 2002).

Films from gelatin cross-linked with light roasted Arabica coffee extract at various polyphenol:NH_2 ratios had varying tensile strength, elongation to break and Young's modulus (Figures 4.11 to 4.13).

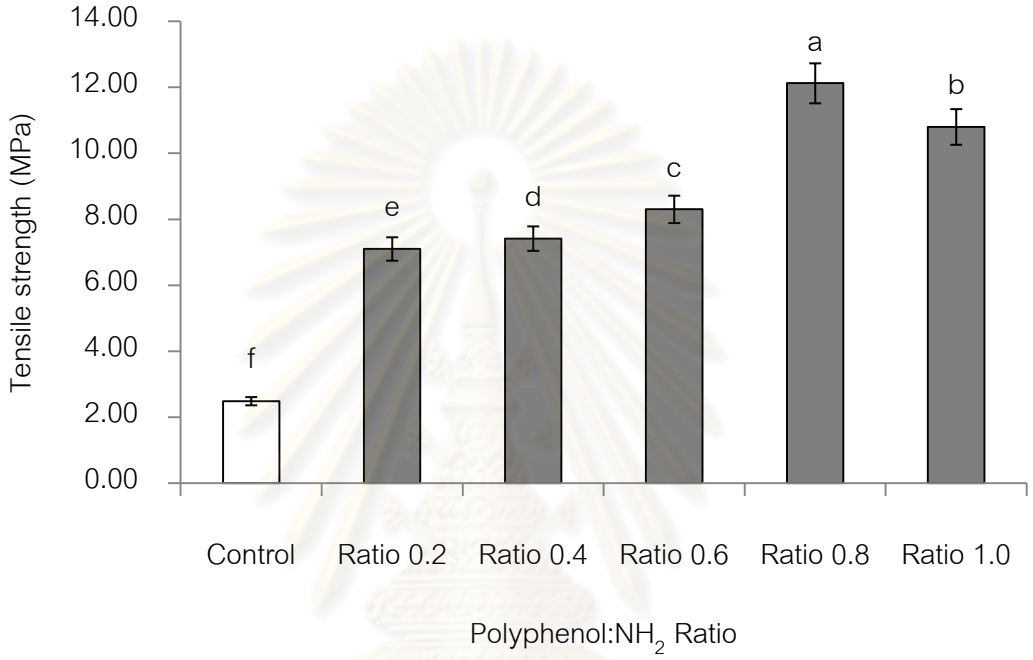


Figure 4.11 Tensile strength of the gelatin films prepared from 6.67% (w/v) native gelatin solution (control) and 6.67% (w/v) gelatin solution cross-linked with light roasted Arabica coffee extract at polyphenol:NH_2 ratio of 0.2:1 (ratio 0.2), 0.4:1 (ratio 0.4), 0.6:1 (ratio 0.6), 0.8:1 (ratio 0.8) and 1:1 (ratio 1.0). Different letters above different bars denote significant differences ($p \leq 0.05$).

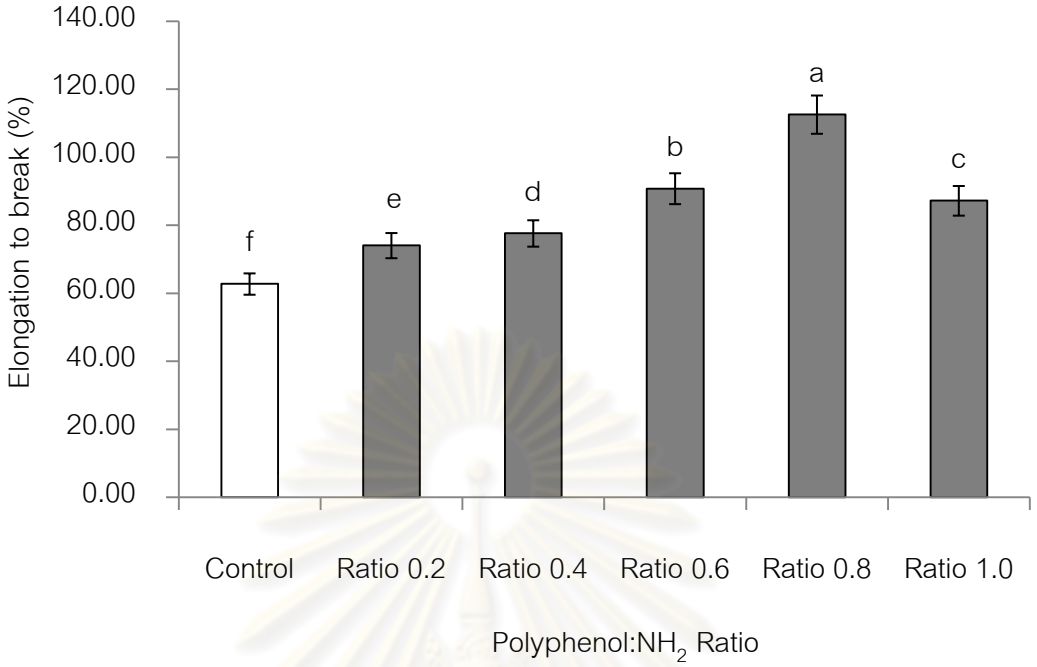


Figure 4.12 Elongation to break of the gelatin films prepared from 6.67% (w/v) native gelatin solution (control) and 6.67% (w/v) gelatin solution cross-linked with light roasted Arabica coffee extract at polyphenol:NH_2 ratio of 0.2:1 (ratio 0.2), 0.4:1 (ratio 0.4), 0.6:1 (ratio 0.6), 0.8:1 (ratio 0.8) and 1:1 (ratio 1.0). Different letters above different bars denote significant differences ($p \leq 0.05$).

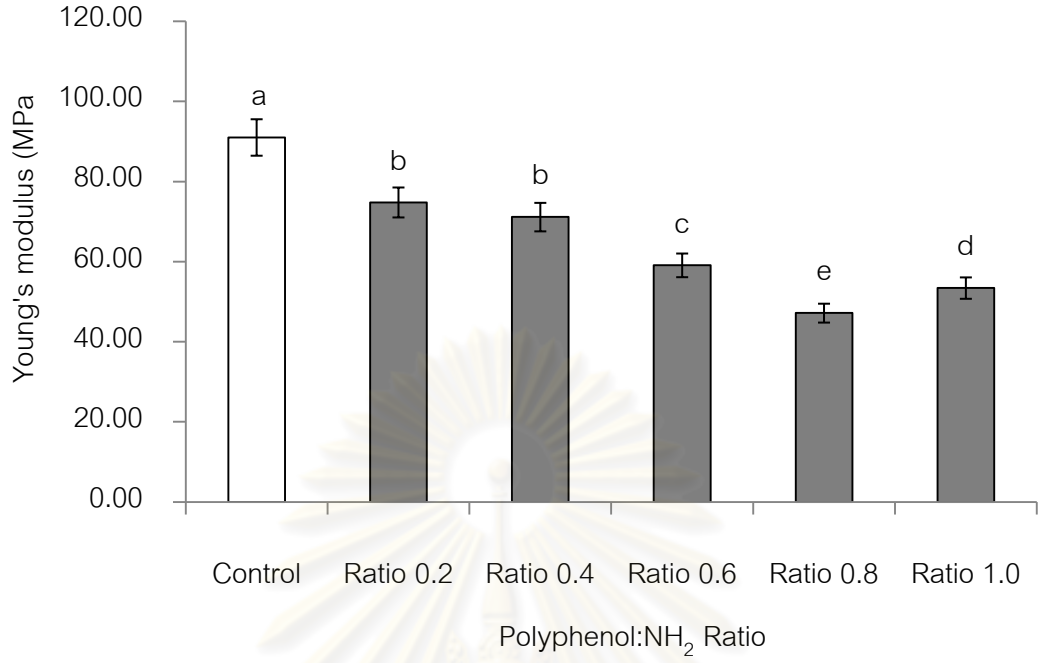


Figure 4.13 Young's modulus of the gelatin films prepared from 6.67% (w/v) native gelatin solution (control) and 6.67% (w/v) gelatin solution cross-linked with light roasted Arabica coffee extract at polyphenol:NH_2 ratio of 0.2:1 (ratio 0.2), 0.4:1 (ratio 0.4), 0.6:1 (ratio 0.6), 0.8:1 (ratio 0.8) and 1:1 (ratio 1.0). Different letters above different bars denote significant differences ($p \leq 0.05$).

Tensile strength of cross-linked gelatin films at all polyphenol:NH_2 ratios for light roasted Arabica coffee extract was significantly higher ($p \leq 0.05$) than that of non cross-linked gelatin film. Elongation to break of the film prepared from gelatin cross-linked at the polyphenol:NH_2 ratio of 0.2:1 to 0.8:1 increased with increasing ratio and slightly deceased as the polyphenol:NH_2 ratio increased to 1:1 ($p \leq 0.05$). Young's modulus decreased with increasing polyphenol:NH_2 ratio from 0.2:1 to 0.8:1 and increased afterwards ($p \leq 0.05$). These results indicate that oxidised phenolic compounds showed the enhancing effect on mechanical properties of gelatin film.

Thickness of all films prepared from gelatin cross-linked with gallic acid and light roasted Arabica coffee extract ranged from 87.7 μm to 107.6 μm (Table 4.8). The thickness of the films prepared from cross-linked gelatin with gallic acid and light roasted Arabica coffee extract tended to increase with an increase in polyphenol:NH_2 ratio ($p \leq 0.05$). At higher polyphenol:NH_2 ratios, the increase in thickness of film incorporated with gallic acid and light roasted Arabica coffee extract was apparent. Quinones from gallic acid and light roasted Arabica coffee extract at a high amount might cross-link the unfolded proteins and were localized between protein molecules resulting in the increased in film thickness.

Table 4.8 Thickness of gelatin films cross-linked with gallic acid and light roasted Arabica coffee extract at various polyphenol:NH_2 ratios.

Type	Polyphenol/NH_2 Ratio	Thickness* (μm)
Native gelatin		$87.7^{d} \pm 4.2$
Gallic acid	0.2:1	$90.7^{d} \pm 6.0$
	0.4:1	$98.7^{bc} \pm 4.5$
	0.6:1	$98.1^{bc} \pm 3.3$
	0.8:1	$90.4^{d} \pm 3.4$
	1:1	$104.5^{ab} \pm 4.8$
Light roasted Arabica coffee	0.2:1	$90.4^{d} \pm 2.8$
	0.4:1	$90.6^{d} \pm 2.2$
	0.6:1	$93.8^{cd} \pm 8.5$
	0.8:1	$94.8^{cd} \pm 7.9$
	1:1	$107.6^{a} \pm 3.7$

a, b, c... Values with different superscripted letters in the same column are significantly different ($p \leq 0.05$).

* Values are given as average ± standard deviation calculated from five replications.

4.3.2 Swelling properties

The effect of gallic acid and light roasted Arabica coffee extract cross-linkers on swelling properties of films is shown in Figure 4.14.

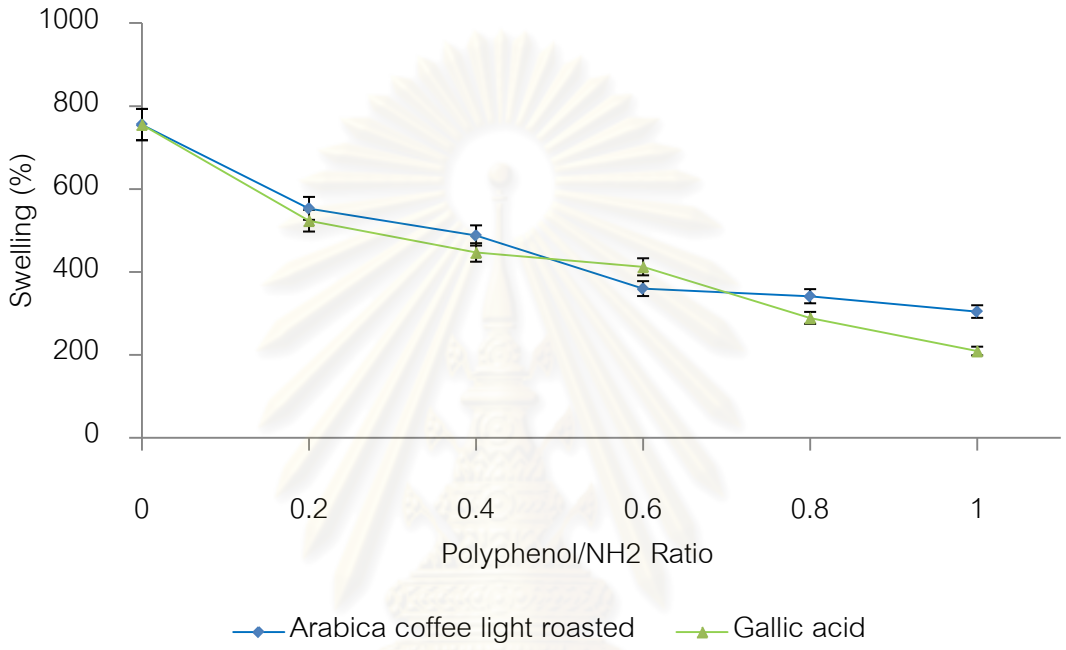


Figure 4.14 Effect of cross-linking agents on swelling ratio of gelatin films. Polyphenol:NH_2 ratio of 0.2:1 (0.2), 0.4:1 (0.4), 0.6:1 (0.6), 0.8:1 (0.8) and 1:1 (1.0).

Figure 4.14 shows that swelling percentage of cross-linked gelatin films decreased with an increase in polyphenol:NH_2 ratio for both light roasted Arabica coffee extract and gallic acid. The increase of cross-linking degree obviously resulted in the decrease of gelatin combination with water. In general, cross-linking and/or hydrogen interactions with other components decrease water uptake by proteins since polar-side-chain groups become less exposed to bind water (Cao et al., 2007; Kim et al., 2005; Rhim et al., 1998; Bigi et al., 2001). Gelatin is a true alternative to many of today's synthetic materials (Schrieber and Gareis, 2007). Completely biodegradable transparent

films for foodstuff packaging for example have already been successfully produced from physically hardened gelatin films (Schrieber and Gareis, 2007). Since one of the major drawbacks in the use of gelatin films in technical applications is their water absorption tendency, any improvement in water resistance as that shown in this work can be highly important.

4.3.3 Whiteness of cross-linked gelatin films.

Table 4.9 shows that color and whiteness of films from cross-linked gelatin with gallic acid and various plant extracts at different polyphenol:NH_2. The lightness (L*) value and whiteness of the cross-linked gelatin films decreased while redness (a*) value and yellowness (b*) values increased as compared to native gelatin film, except for the case of cross-linked gelatin using butterfly pea extract where yellowness (b*) values were found to decrease with cross-linking. Cross-linked gelatin film had the decreased lightness (L*) value and yellowness (b*) values when higher polyphenols:NH_2 ratios of gallic acid, light roasted Arabica coffee extract and pomegranate extract were used. Redness (a*) value of film added with gallic acid and light roasted Arabica coffee extract increased with increasing polyphenols:NH_2 ratio and slightly deceased as the polyphenols:NH_2 ratio increased when pomegranate extract and butterfly pea extract were used as cross-linkers. Whiteness of all cross-linked gelatin films with gallic acid and light roasted Arabica coffee extract decreased while that of gelatin films cross-linked with pomegranate extract increased as the polyphenols:NH_2 ratio increased. Films from cross-linked gelatin with butterfly pea extract had the lowest whiteness (negative value) and showed the lowest yellowness (b*) values (negative value) due to the vivid deep blue color of the flowers.

Table 4.9 Expressible color and whiteness of films from cross-linked gelatin with gallic acid and various plant extracts at different polyphenol:NH_2 ratios.

Polyphenol/NH_2 Ratio	L* [1]	a* [1]	b* [1]	Whiteness [a]
Native gelatin	$96.13^{a} \pm 0.25$	$-0.32^{j} \pm 0.09$	$4.93^{g} \pm 1.11$	$93.71^{a} \pm 1.03$
Gallic acid cross-linking				
0.2:1	$78.37^{b} \pm 2.39$	$3.59^{i} \pm 1.46$	$42.33^{cd} \pm 3.53$	$52.31^{b} \pm 4.32$
0.4:1	$69.01^{d} \pm 2.12$	$11.08^{h} \pm 1.87$	$48.30^{b} \pm 1.98$	$41.50^{c} \pm 1.34$
0.6:1	$64.13^{e} \pm 1.35$	$14.12^{g} \pm 1.19$	$48.48^{b} \pm 1.10$	$38.04^{c} \pm 0.20$
0.8:1	$61.07^{e} \pm 0.32$	$14.52^{g} \pm 0.65$	$45.24^{bc} \pm 0.09$	$38.57^{c} \pm 0.24$
1:1	$52.21^{h} \pm 0.51$	$21.20^{f} \pm 0.38$	$33.27^{e} \pm 0.81$	$38.02^{c} \pm 0.19$
Arabica coffee cross-linking				
0.2:1	$72.72^{c} \pm 3.44$	$9.80^{h} \pm 2.58$	$37.74^{de} \pm 0.61$	$52.34^{b} \pm 2.83$
0.4:1	$59.18^{f} \pm 0.46$	$20.82^{f} \pm 0.79$	$39.43^{d} \pm 7.58$	$39.37^{c} \pm 5.10$
0.6:1	$55.91^{g} \pm 3.96$	$21.62^{f} \pm 2.31$	$37.41^{de} \pm 4.39$	$38.06^{c} \pm 0.90$
0.8:1	$45.26^{i} \pm 0.92$	$26.87^{e} \pm 1.15$	$24.91^{f} \pm 0.27$	$34.12^{d} \pm 1.15$
1:1	$46.57^{i} \pm 2.87$	$27.24^{e} \pm 0.76$	$27.01^{f} \pm 3.89$	$34.11^{d} \pm 0.96$

Table 4.9 (Continue) Expressible color and whiteness of films from cross-linked gelatin with gallic acid and various plant extracts at different polyphenol:NH_2 ratios.

Polyphenol/NH_2 Ratio	L* [1]	a* [1]	b* [1]	Whiteness [1]
Native gelatin	$96.13^{a} \pm 0.25$	$-0.32^{j} \pm 0.09$	$4.93^{g} \pm 1.11$	$93.71^{a} \pm 1.03$
Pomegranate cross-linking				
0.2:1	$47.26^{i} \pm 1.18$	$50.69^{a} \pm 0.50$	$53.57^{a} \pm 1.33$	$9.31^{f} \pm 0.42$
0.3:1	$46.20^{i} \pm 0.32$	$47.28^{b} \pm 1.16$	$48.02^{b} \pm 1.51$	$13.76^{e} \pm 1.63$
Butterfly pea cross-linking				
0.2:1	$17.18^{j} \pm 0.48$	$47.41^{b} \pm 1.03$	$-80.17^{j} \pm 0.91$	$-24.64^{i} \pm 0.86$
0.4:1	$15.43^{j} \pm 2.80$	$39.52^{c} \pm 0.91$	$-65.89^{j} \pm 1.59$	$-14.28^{h} \pm 1.66$
0.5:1	$8.27^{k} \pm 0.30$	$31.79^{d} \pm 1.46$	$-56.61^{h} \pm 1.36$	$-12.39^{g} \pm 0.91$

[a, b, c...] Values with different superscripted letters in the same column are significantly different ($p \leq 0.05$).

[1] Values are given as average $\pm$ standard deviation calculated from three replications.

4.3.4 Effect of cross-linking on morphological of gelatin films

SEM photographs of gelatin films prepared from native gelatin and gelatin cross-linked with gallic acid and light roasted Arabica coffee at various polyphenol:NH_2 ratios of 0.2:1.0, 0.4:1, 0.6:1, 0.8:1 and 1:1 are shown in Figures 4.15 and 4.16. The results show that the gelatin film without cross-linking has smooth surface. The gelatin films cross-linked with gallic acid and light roasted Arabica coffee at 0.2:1 to 1:1 polyphenol:NH_2 ratios show rough protruded uneven surface containing some crystal-like structures in case of the samples treated with gallic acid solutions and some particles in case of those treated with coffee extract. The crystals and particles could possibly be the excessive gallic acid used for the cross-linking process and the fine coffee particles. As the polyphenol:NH_2 ratio increased, more well-defined structures became apparent on the film surface. This might stem from that quinone formed during oxidation of phenolic compounds reacted with amino side chain on polypeptide chain, leading to the reduction of free volume in the polymer matrix. Some cracks were also apparent in films formed from cross-linked gelatin using high polyphenol:NH_2 ratios. Haslam (1989) stated that quinone could induce the aggregation of protein via multidentate mechanism. The discontinuous zones on the film surface could also be related to the formation of preferential channels during the process of film drying (de Carvalho & Grosso, 2004).

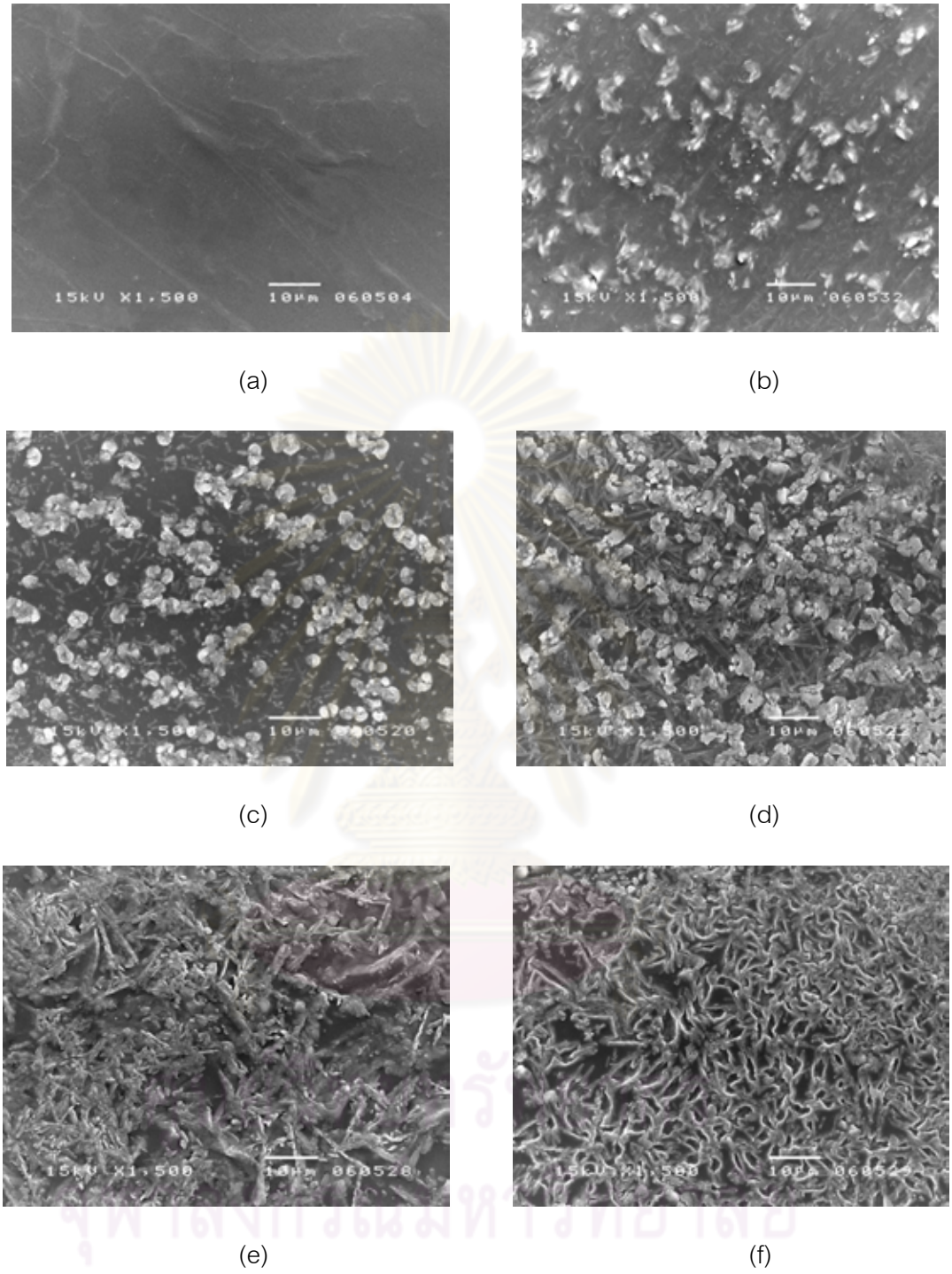


Figure 4.15 SEM images of gelatin films prepared from (a) native gelatin (control), gelatin cross-linked with gallic acid at various polyphenol:NH_2 ratios (b) 0.2:1, (c) 0.4:1, (d) 0.6:1, (e) 0.8:1 and (f) 1:1. All SEM images were taken at 1500x magnification.

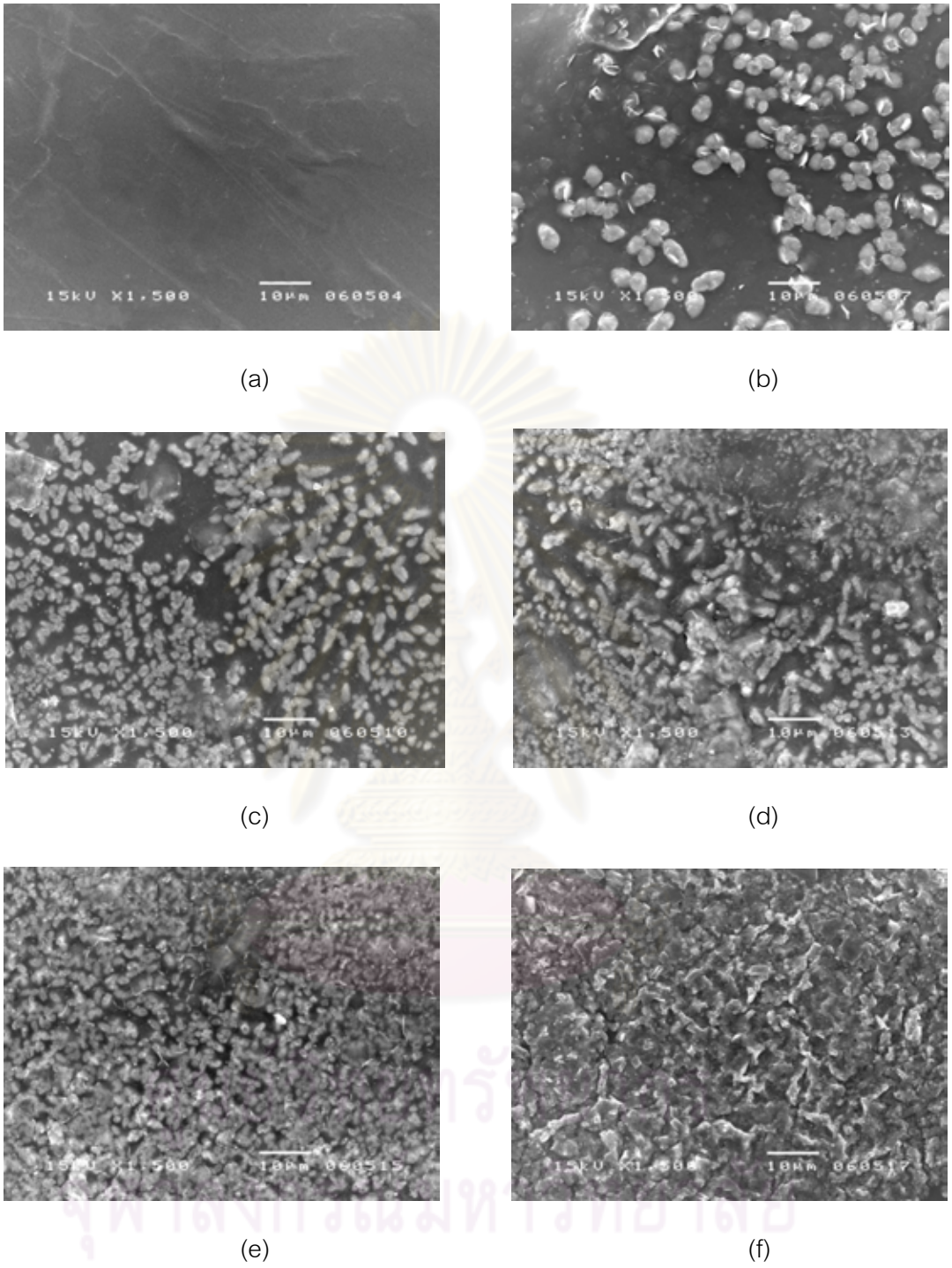


Figure 4.16 SEM images of gelatin films prepared from (a) native gelatin (control), gelatin cross-linked with light roasted Arabica coffee extract at various polyphenol:NH_2 ratios (b) 0.2:1, (c) 0.4:1, (d) 0.6:1, (e) 0.8:1 and (f) 1:1. All SEM images were taken at 1500x magnification.

CHAPTER V

CONCLUSIONS

This work demonstrated that cross-linking of gelatin could possibly be done using plant extracts that were high in total phenolics content.

The total phenolics content, expressed in term of gallic acid equivalent (GAE), varied from 1.712 to 163.125 mg GAE /g of dry weight for different selected plants extracts. The three highest phenolics-containing crude plant aqueous extracts, which were light roast coffee extract, butterfly pea extract and pomegranate extract were used in subsequent cross-linking of gelatin.

In all gelatin cross-linking using different (0.2:1 to 1:1) polyphenol:NH_2 ratios, the viscosity of gelatin solutions (6.67% w/v) and free amino groups decreased when cross-linking polyphenol:NH_2 ratio increased. Cross-linking using gallic acid and the extract of pomegranate and light roasted Arabica coffee caused the gelation and melting temperature to decrease. Butterfly pea extract was the most effective cross-linker that caused gelling and melting complex viscosities to decrease. The thickness of the films prepared from native and cross-linked gelatin varied from 87 to 107 μm. The swelling percentage of cross-linked gelatin films decreased with the increase in polyphenol:NH_2 ratio. In all samples, when 1:1 polyphenol:NH_2 ratio were used, the swelling percentage decreased 59.7% and 61.9%, respectively for light roast coffee and gallic acid cross-linking compared to native gelatin. The films prepared from cross-linked gelatin had lower lightness (L*), yellowness (b*) and whiteness values but higher redness (a*). Except for the films prepared from gelatin cross-linked with butterfly pea extract that showed slightly blue (negative b*) color that is the typical color of the flower of butterfly pea. The tensile strength and elongation to break of cross-linked gelatin films increased while the Young's modulus decreased when added with light roast coffee and gallic acid.

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APPENDICES

Appendix A

Sample and Reagent Preparations

A.1 Preparation of 100 mL 10% (v/v) Folin-Ciocalteu Reagent

Chemical

1. Folin-Ciocalteu Reagent
2. Distilled water

Method

Mix 10 mL of Folin-Ciocalteu Reagent and distilled water in a 100 mL volumetric flask.

A.2 Preparation of 100 mL 20% (v/v) Sodium carbonate

Chemical

1. Sodium carbonate anhydrous
2. Distilled water

Method

Dissolve 20 g of anhydrous sodium carbonate in distilled water and bring to a boil. After cooling, add a few crystals of sodium carbonate, and after 24 hr, filter and add distilled water to 100 mL.

A.3 Preparation of 100 mL Gallic acid standard solution

Chemical

1. Gallic acid monohydrate
2. Ethyl alcohol
3. Distilled water

Method

In a 100 mL volumetric flask, dissolve 0.500 g of dry gallic acid in10 mL of ethanol and dilute to volume with distilled water. Can be opened daily, but to store, keep closed in a refrigerator up to 2 weeks.

A.4 Preparation of 25 mL 20% (w/v) Fluorescamine

Chemical

1. Fluorescamine
2. Acetone dimethyl ketone

Method

Mix 5 mg fluorescamine and 25 mL acetone dimethyl ketone in a 25 mL volumetric flask. Can be opened daily, but to store, keep closed in a refrigerator up to 1 week.

A.5 Preparation of 100 mL, 0.05M Sodium tetraborate, pH 8.9

Chemical

1. Sodium tetraborate decahydrate
2. Distilled water
3. Acetic acid glacial

Method

In a 100 mL volumetric flask, dissolve 1.9359 g of sodium tetraborate decahydrate in distilled water. Adjusted to pH 8.9 using 4M sodium hydroxide or 4M acetic acid.

A.6 Preparation of 100mL, 0.2M L-Lysine monohydrochloride stock solution

Chemical

1. L-Lysine monohydrochloride
2. Distilled water

Method

In a 100 mL volumetric flask, dissolve 3.689 g of L-Lysine monohydrochloride in distilled water and adjusted to volume with distilled water in 100 mL volumetric flask. Before performing the determinations, the stock solution were diluted to 0.02 mM L-Lysine monohydrochloride.

A.7 Preparation of 100 mL of 4M Sodium hydroxide

Chemical

1. Sodium hydroxide
2. Distilled water

Method

16.327 g of sodium hydroxide was dissolved in distilled water. The distilled water was addes to reach 100 mL final volume in volumetric flask.

A.8 Preparation of 100 ml, 4M Acetic acid

Chemical

1. Acetic acid glacial
2. Distilled water

Method

Mix 22.9 mL of acetic acid glacial and distilled water in a 100 mL volumetric flask.

A.9 Preparation of 60 mL, 2.812M Gallic acid solution

Chemical

1. Gallic acid monohydrate
2. Ethyl alcohol
3. Distilled water

Method

In a 60 mL volumetric flask, dissolve 32.3844 g of dry gallic acid in 30 mL of ethanol and dilute to volume with distilled water. Can be opened daily, but to store, keep closed in a refrigerator up to 2 weeks.

Appendix B

Standard Curve

B.1 Preparation of gallic acid standard curve

These solutions will have phenol concentrations of 0, 50, 100, 150, 250, and 500 mg/L gallic acid, the effective range of the assay (Table B1).

Table B1 Preparation of gallic acid standard solution for calibration curve.

Gallic acid standard solution (mg/L)	Blank	50	100	150	200	300	450	650
Gallic acid stock solution (µL)	0	60	120	180	240	360	540	780
d.H_2O (mL)	6.0	5.94	5.88	5.82	5.76	5.64	5.46	5.22
Total (mL)	6.0							

From each calibration solution or blank, pipet 60 µL into separate cuvettes, and to each add 4.74 mL distilled water, and then add 300 µL of the Folin-Ciocalteu reagent, and mix well. Wait for 3 min, and then add 900 µL of the sodium carbonate solution , and shake to mix. Leave the solutions at room temperature under dark conditions for 90 min and determine the absorbance of each solution at 765 nm using a UV-VIS Spectrophotometer against the blank (the 0 mL solution) and plot absorbance vs. concentration. Results are reported at Gallic Acid Equivalent, GAE (Figure B1).

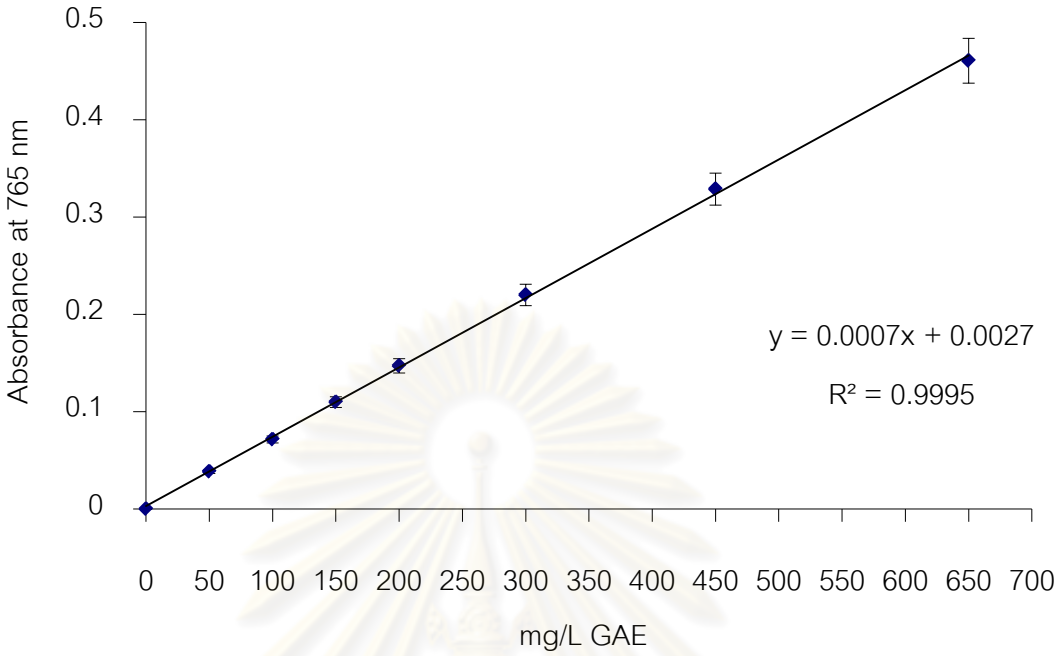

Figure B1 Standard curve of gallic acid, relation between absorbance at 765 nm and gallic acid concentration (mg/L GAE).

B.2 Preparation of L-Lysine monohydrochloride standard curve

These solutions will have Lysine concentrations of 0, 0.02, 0.04, 0.06, 0.08, 0.1, 0.12, 0.16 and 0.2 mM L-Lysine monohydrochloride, the effective range of the assay (Table B2).

Table B2 Preparation of L-Lysine monohydrochloride standard solution for calibration curve.

L-Lysine monohydrochloride standard solution (mM)	Blank	0.02	0.04	0.06	0.08	0.10	0.12	0.16	0.2
L-Lysine monohydrochloride stock solution (mL)	0	0.5	1.0	1.5	2.0	2.5	3.0	4.0	5.0
d.H_2O (mL)	5.0	4.5	4.0	3.5	3.0	2.5	2.0	1.0	0
Total (mL)	5.0								

From each calibration solution or blank, Add exactly 50 µL into separate cuvettes, and to each add 2 mL sodium tetraborate, and then add 150 µL of the fluorescamine, and mix well. The reaction was complete in a few seconds. Determine the absorbance of each solution at 390/475 nm wavelengths for excitation and emission using a spectrofluorometer against the blank (the 0 mL solution) and plot absorbance vs. concentration. It is shown in Figure B2.

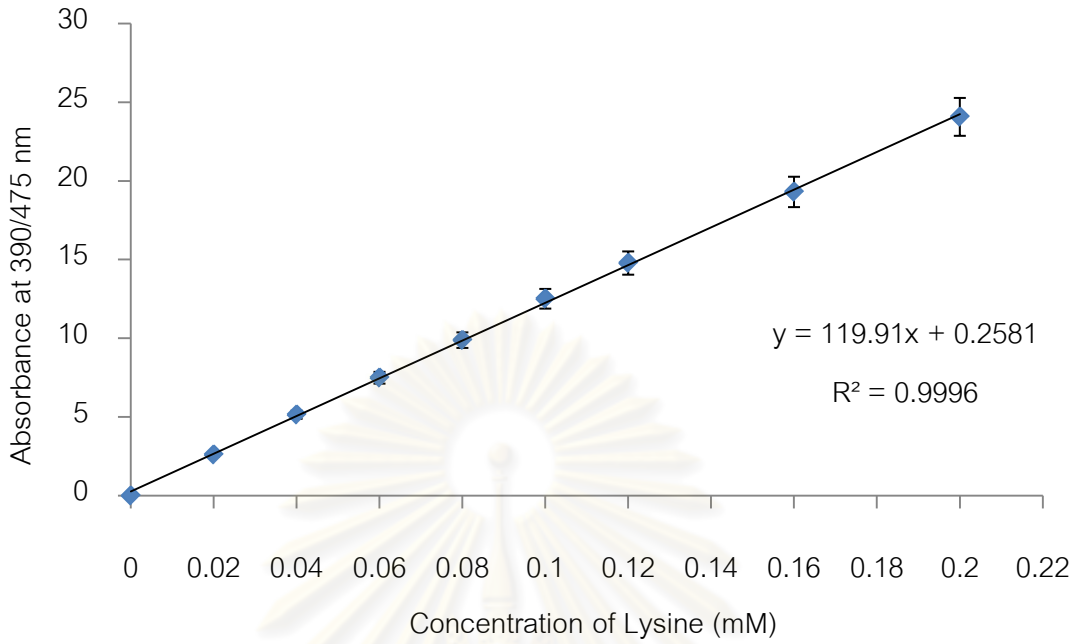

Figure B2 Standard curve of L-Lysine monohydrochloride, relation between absorbance at 390/475 nm and Lysine concentration (mM).

Appendix C

The properties of cross-linked gelatin solutions and films

Table C1 Effect of cross-linking using gallic acid and different plant extracts on free amino group content of gelatin.

	Free NH_2 (mM/Protein)			
Native gelatin	$45.96^{a} \pm 0.1$			
Polyphenol:NH_2 Ratio	Light roasted Arabica coffee*	Gallic acid*	Pomegranate*	Butterfly pea*
0.2:1	$30.83^{c} \pm 0.8$	$33.16^{b} \pm 0.3$	$28.25^{d} \pm 0.5$	$22.83^{e} \pm 0.6$
0.3:1	-	-	$23.91^{e} \pm 0.4$	-
0.4:1	$22.43^{f} \pm 0.5$	$24.22^{e} \pm 0.5$	-	$13.43^{k} \pm 0.5$
0.5:1	-	-	-	$11.45^{l} \pm 0.8$
0.6:1	$18.95^{h} \pm 0.9$	$20.27^{g} \pm 0.3$	-	-
0.8:1	$16.32^{j} \pm 0.6$	$18.13^{hi} \pm 0.8$	-	-
1:1	$17.32^{ij} \pm 0.5$	$17.07^{j} \pm 0.4$	-	-

$^{a, b, c...}$ Values with different superscripted letters in the same column are significantly different ($p \leq 0.05$).

* Values are given as average ± standard deviation calculated from three replications.

Table C2 Effect of cross-linking agents on swelling ratio of gelatin films.

	% Swelling	
Native gelatin	$755.02^{a} \pm 22.1$	
Polyphenol:NH_2 Ratio	**Light roasted Arabica coffee***	**Gallic acid***
0.2:1	$552.93^{b} \pm 20.7$	$523.21^{bc} \pm 61.9$
0.4:1	$487.59^{cd} \pm 27.3$	$446.60^{de} \pm 10.6$
0.6:1	$359.48^{f} \pm 43.9$	$412.00^{e} \pm 28.6$
0.8:1	$341.10^{fg} \pm 2.8$	$288.82^{h} \pm 9.1$
1:1	$304.08^{gh} \pm 8.5$	$208.95^{i} \pm 14.2$

[a, b, c...] Values with different superscripted letters in the same column are significantly different ($p \leq 0.05$).

* Values are given as average ± standard deviation calculated from three replications.

Table C3 Mechanical properties of the gelatin films prepared from 6.67% (w/v) native gelatin solution (control) and 6.67% (w/v) gelatin solution cross-linked with gallic acid.

	Polyphenol:NH_2 Ratio	Tensile strength* (MPa)	Elongation* (%)	Young's modulus* (Mpa)
Native gelatin	Control	$2.49^{e} \pm 0.09$	$62.75^{e} \pm 1.08$	$91.03^{a} \pm 3.71$
Gallic acid	0.2:1	$6.98^{d} \pm 0.07$	$75.29^{d} \pm 4.55$	$73.66^{b} \pm 6.32$
	0.4:1	$7.97^{c} \pm 0.21$	$83.18^{c} \pm 2.04$	$60.98^{c} \pm 1.83$
	0.6:1	$9.11^{b} \pm 0.15$	$102.56^{b} \pm 1.00$	$49.76^{d} \pm 1.92$
	0.8:1	$9.30^{b} \pm 0.12$	$109.17^{a} \pm 1.48$	$50.74^{d} \pm 1.51$
	1:1	$11.51^{a} \pm 0.28$	$107.08^{a} \pm 1.74$	$44.78^{e} \pm 2.67$

[a, b, c...] Values with different superscripted letters in the same column are significantly different ($p \leq 0.05$).

* Values are given as average ± standard deviation calculated from three replications.

Table C4 Mechanical properties of the gelatin films prepared from 6.67% (w/v) native gelatin solution (control) and 6.67% (w/v) gelatin solution cross-linked with light roasted Arabica coffee extract.

	Polyphenol:NH_2 Ratio	Tensile strength* (MPa)	Elongation* (%)	Young's modulus* (Mpa)
Native gelatin	Control	$2.49^{f} \pm 0.09$	$62.75^{f} \pm 1.08$	$91.03^{a} \pm 3.71$
Light roasted Arabica coffee	0.2:1	$7.10^{e} \pm 0.15$	$74.06^{e} \pm 2.55$	$74.80^{b} \pm 3.20$
	0.4:1	$7.42^{d} \pm 0.17$	$77.64^{d} \pm 1.88$	$71.15^{b} \pm 3.25$
	0.6:1	$8.30^{c} \pm 0.25$	$90.80^{b} \pm 2.06$	$59.10^{c} \pm 5.72$
	0.8:1	$12.12^{a} \pm 0.10$	$112.59^{a} \pm 1.53$	$47.17^{e} \pm 4.45$
	1:1	$10.80^{b} \pm 0.19$	$87.24^{c} \pm 3.68$	$53.42^{d} \pm 3.48$

$^{a, b, c...}$ Values with different superscripted letters in the same column are significantly different ($p \leq 0.05$).

* Values are given as average $\pm$ standard deviation calculated from three replications.

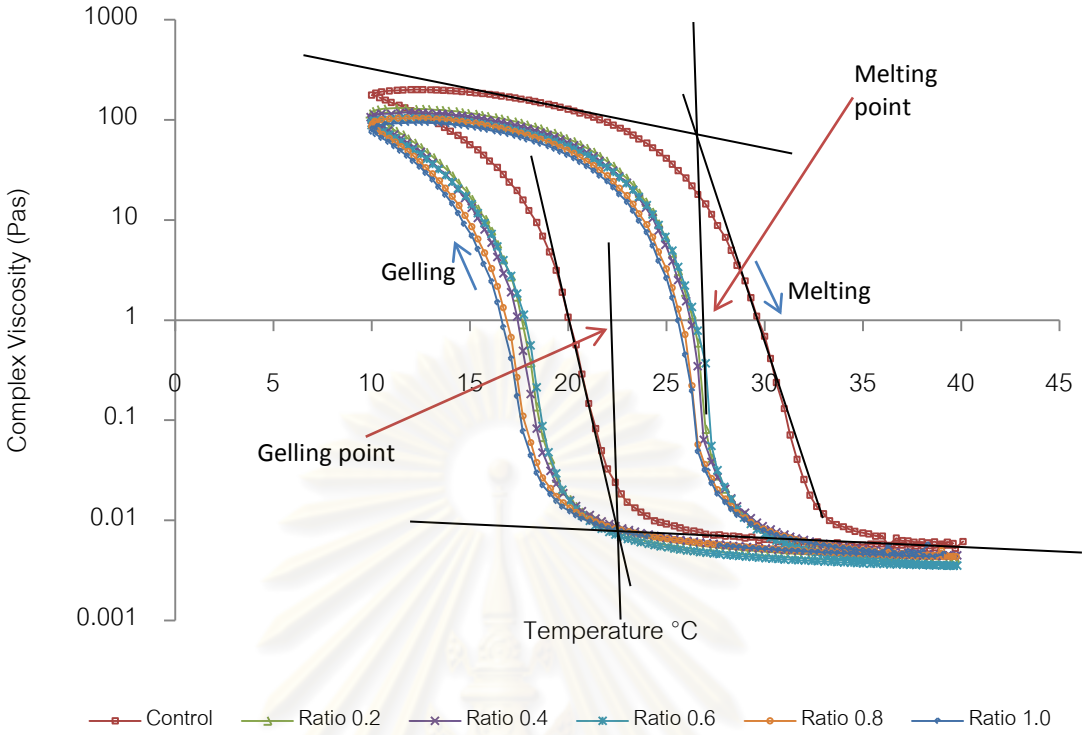

Figure C1 Temperature sweep test results of 6.67% (w/v) native gelatin solution (control) and 6.67% (w/v) gelatin solution cross-linked with gallic acid at polyphenol:NH_2 ratio of 0.2:1 (ratio 0.2), 0.4:1 (ratio 0.4), 0.6:1 (ratio 0.6), 0.8:1 (ratio 0.8) and 1:1 (ratio 1.0) at pH 8. The profiles show changes in complex viscosity (η^*) of the solution during melting and gelling.

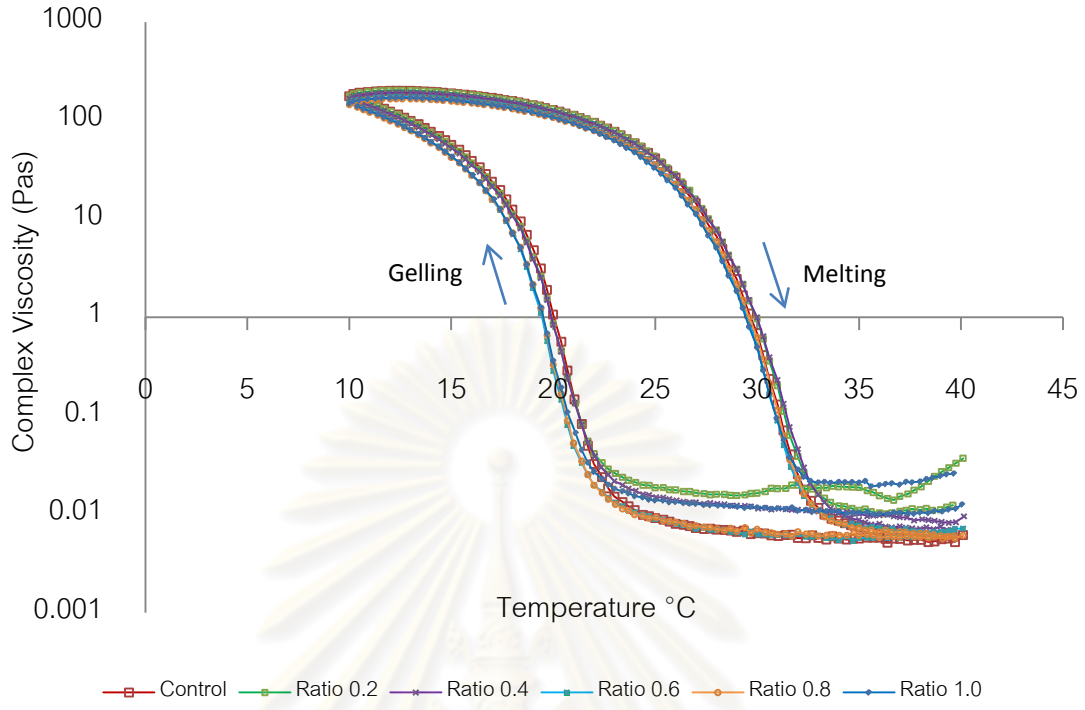

Figure C2 Temperature sweep test results of 6.67% (w/v) native gelatin solution (control) and 6.67% (w/v) gelatin solution cross-linked with light roasted Arabica coffee extract at polyphenol:NH_2 ratio of 0.2:1 (ratio 0.2), 0.4:1 (ratio 0.4), 0.6:1 (ratio 0.6), 0.8:1 (ratio 0.8) and 1:1 (ratio 1.0) at pH 8. The profiles show changes in complex viscosity (η^*) of the solution during melting and gelling.

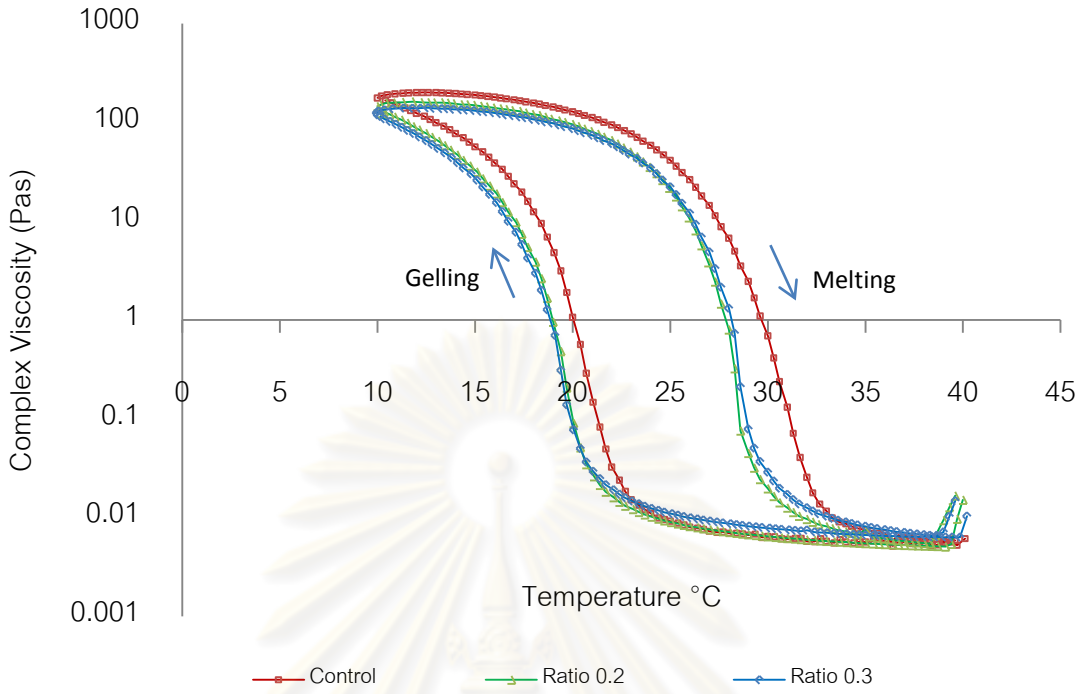

Figure C3 Temperature sweep test results of 6.67% (w/v) native gelatin solution (control) and 6.67% (w/v) gelatin solution cross-linked with pomegranate extract at polyphenol:NH_2 ratio of 0.2:1 (ratio 0.2), 0.3:1 (ratio 0.3) at pH 8. The profiles show changes in complex viscosity (η^*) of the solution during melting and gelling.

CURRICULUM VITAE

Mr. Tharittri Phonphitchaya was born on February 15, 1983 in Nakhon Sawan, Thailand. He graduated with Bachelor's Degree in Agro Industry from Faculty of Agriculture Natural Resources and Environment in 2006. He continued his study in Program in Biotechnology, Faculty of Science, Chulalongkorn University in 2007 and completed in 2010.

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