เสถียรภาพต่อการเกิดออกซิเดชันของอิมัลชันน้ำมันทูนาที่เคลือบด้วยสารพอลิอิเล็กโทรไลต์มัลติเลเยอร์

นางสาวเพชรรัตน์ ยงบุตร

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต
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OXIDATIVE STABILITY OF TUNA OIL EMULSION COATED WITH POLYELECTROLYTE MULTILAYER

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A Thesis Submitted in Partial Fulfillment of the Requirements
for the Degree of Master of Science Program in Petrochemistry and Polymer Science
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Thesis Title	OXIDATIVE STABILITY OF TUNA OIL EMULSION COATED WITH POLYELECTROLYTE MULTILAYER
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Field of Study	Petrochemistry and Polymer Science
Thesis Advisor	Luxsana Dubas, Ph.D.

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เพชรรัตน์ ยงบุตร : เสถียรภาพต่อการเกิดออกซิเดชันของอิมัลชันน้ำมันทูนาที่เคลือบด้วยสารพอลิอิเล็กโทรไลต์มัลติเลเยอร์ (OXIDATIVE STABILITY OF TUNA OIL EMULSION COATED WITH POLYELECTROLYTE MULTILAYER) อ.ที่ปรึกษาวิทยานิพนธ์หลัก: อ. ดร.ลักษณา ดูบาส, 116 หน้า.

วัตถุประสงค์ของงานวิจัยนี้คือ การชะลอการเกิดออกซิเดชันของกรดไขมันไม่อิ่มตัวในอิมัลชันน้ำมันทูนาในน้ำ โดยการเตรียมเป็นมัลติเลเยอร์อิมัลชันและการเติมสารต้านอนุมูลอิสระ การเตรียมมัลติเลเยอร์อิมัลชันทำการเตรียมโดยใช้วิธีเคลือบชั้นต่อชั้น อิมัลชันชั้นปฐมภูมิ เตรียมได้โดยการผสมน้ำมันทูนา 5 เปอร์เซนต์โดยน้ำหนัก และสารละลายเวย์โปรตีนความเข้มข้น 0.4 เปอร์เซนต์โดยน้ำหนัก ในสารละลายอะซิติคอะซิเตตบัฟเฟอร์ อิมัลชันทุติยภูมิและอิมัลชันตติยภูมิเตรียมได้ด้วยการเคลือบผิวของน้ำมันด้วยพอลิเมอร์ชีวภาพที่มีประจุ โดยเลือกใช้ไคโทซานเป็นสารพอลิอิเล็กโทรไลต์ที่มีประจุบวก และเลือกใช้อัลจิเนตและคาราจีแนนเป็นสารพอลิอิเล็กโทรไลต์ที่มีประจุลบ ค่าพีเอชของสารละลายอิมัลซิฟายด์เออร์ถูกควบคุมที่ 3.5 และ 6.0 ศึกษาคุณลักษณะทางกายภาพและเสถียรภาพทางกายภาพโดยการวัดการเกิดครีม เปอร์เซ็นต์การส่องผ่านของแสง ขนาดและค่าศักย์เซต้า พบว่าเมื่อเพิ่มจำนวนชั้นของอิมัลชัน ขนาดของอิมัลชันเพิ่มขึ้น ในขณะที่การเกิดครีมและการส่งผ่านของแสงดีขึ้น เสถียรภาพทางกายภาพของอิมัลชันตติยภูมิถูกปรับปรุงเมื่อเปรียบเทียบกับอิมัลชันปฐมภูมิและอิมัลชันทุติยภูมิทั้งสองพีเอช เสถียรภาพต่อการเกิดออกซิเดชันในอิมัลชันทำการติดตามด้วยวิธีเฟอริคไทโอไซด์ยาเนต ผ่านเทคนิคยูวีวิซิเบิล สเปกโตรสโคปี พบว่าหลังจากการเตรียมเป็นอิมัลชันแล้วมีการเกิดลิปิดไฮโดรเปอร์ออกไซด์ในอิมัลชันทุกชนิด ซึ่งอิมัลชันตติยภูมิและอิมัลชันทุติยภูมิสามารถลดการเกิดออกซิเดชันได้เมื่อเปรียบเทียบกับอิมัลชันปฐมภูมิ แสดงว่า มัลติเลเยอร์อิมัลชันสามารถปรับปรุงเสถียรภาพของอิมัลชันน้ำมันได้ นอกจากนี้ได้มีการศึกษาอิทธิพลของสารต้านอนุมูลอิสระซึ่งได้แก่ น้ำมันงา สารสกัดจากพริก เจนนิสทีน และสารสกัดจากกระเจี๊ยบ ต่อการลดการเกิดออกซิเดชันของอิมัลชันปฐมภูมิ โดยทำการเติมน้ำมันงาและสารสกัดจากพริกลงในน้ำมันทูนาโดยตรง พบว่า 0.5 เปอร์เซนต์โดยน้ำหนักของสารสกัดจากพริก มีเสถียรภาพต่อการเกิดออกซิเดชันดีที่สุด ในขณะที่เจนนิสทีน และสารสกัดจากกระเจี๊ยบ เติมโดยตรงลงในสารละลายอิมัลซิฟายเออร์ พบว่า สารสกัดจากกระเจี๊ยบ 200 ppm สามารถปรับปรุงเสถียรภาพต่อการเกิดออกซิเดชันและเสถียรภาพทางกายภาพได้ดี

สาขาวิชา ปิโตรเคมีและวิทยาศาสตร์พอลิเมอร์ ลายมือชื่อนิสิต .....

ปีการศึกษา 2554 ลายมือชื่อ อ.ที่ปรึกษาวิทยานิพนธ์หลัก .....

5172394623: MAJOR PETROCHEMISTRY AND POLYMER SCIENCE

KEYWORDS: POLYELECTROLYTE MULTILAYER / TUNA OIL / EMULSION / STABILITY / LIPID OXIDATION / ANTIOXIDANT

PHETCHARAT YONGBUT: OXIDATIVE STABILITY OF TUNA OIL EMULSIONS COATED WITH POLYELECTROLYTE MULTILAYER. ADVISOR: LUXSANA DUBAS, Ph.D., 116 pp.

The aim of this study is to retard the oxidation of unsaturated fatty acid in tuna oil-in-water emulsion by preparing as multilayer emulsion and adding antioxidants. The multilayer tuna oil-in-water emulsions were prepared using layer-by-layer technique. The primary emulsion was prepared by homogenizing 5 %w/v tuna oil, 0.4 %w/v WPI in 100 mM acetic-acetate buffer solution as emulsifier. The secondary and tertiary emulsions were prepared by coating the oil droplets with the charged biopolymers. Chitosan, a cationic polyelectrolyte, and alginate and carrageenan, anionic polyelectrolytes, were selected. The pH of emulsifier was controlled at either pH 3.5 and 6.0. The physical characteristic and physical stability was investigated by measuring the creaming index, % transmission, particle size and zeta potential. The particle size of emulsions increased, while the creaming index and % transmission were improved when increasing the number of layers around emulsion. The physical stability of tertiary emulsions was improved comparing to the primary and secondary emulsions for both pH conditions. The oxidative stability of emulsions was investigated via the ferric thiocyanate method by using UV-Vis spectroscopy. After storage, all emulsions released lipid hydroperoxide. We found that the secondary and tertiary emulsions decreased the oxidation reaction comparing to the primary emulsion. Thus, the multilayer emulsions could be improved the stability of tuna oil-in-water emulsion. The effect of antioxidants, sesame oil, capsaicin oleoresin, genistein and roselle extract on the decrease in the oxidation rate of primary emulsion were investigated. Sesame oil and capsaicin oleoresin were added directly in tuna oil, which we found that added 0.5 %w/v capsaicin oleoresin the better oxidative stability. While genistein and roselle extract were added directly in emulsifier solutions, we found that the 200 ppm roselle extract could improve the oxidative and physical stabilities.

Field of Study : Petrochemistry and Polymer Science Student's Signature .....

Academic Year : 2011 Advisor's Signature .....

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CONTENTS

PAGE

PAGE

PAGE

PAGE

LIST OF TABLES

LIST OF FIGURES

FIGURE **PAGE**

FIGURE **PAGE**

FIGURE **PAGE**

FIGURE **PAGE**

LIST OF ABBREVIATIONS

PEL	=	Polyelectrolyte
PEM	=	Polyelectrolyte multilayer thin films
LbL	=	Layer-by-Layer technique
ESA	=	Electrostatic self-assembly
p*I*	=	Isoelectric point
pK_a	=	Acid dissociation constant
min	=	Minute
mL	=	Milliliter
μL	=	Microliter
mM	=	Milli molar
nm	=	Nanometer
mV	=	Milli Volt
%w/w	=	% weight per weight
%w/v	=	% weight by volume

CHAPTER I

INTRODUCTION

In the early studies indicating that the omega-3 polyunsaturated fatty acids (n-3 PUFA) are important in human nutrition. Omega-3 fatty acids are essential fatty acids, especially eicosapentaenoic acid (EPA, C20:5), α-linolenic acid (ALA, C18:3) and docosahexaenoic acid (DHA, C22:6). Utilization of tuna oil and marine oil, the source of n-3 PUFA fatty acids into the food is problematic because they are highly susceptible to oxidative degradation, that the structure of omega-3 polyunsaturated fatty acids (n-3 PUFA) is highly polyunsaturated. The oxidation could be changed the quality of food, such as type, concentration, taste, and nutritional profile [1]. The chemical structure, oxygen concentration and metal ions are the factor affecting the lipid oxidation. The decrease in the lipid oxidation could be presented in many methods, such as microencapsulated, addition antioxidant and preparation of the emulsions [2-6].

Oil-in-water emulsions are produced by homogenizing the oil and aqueous phases [7, 8]. Emulsifiers will be adsorbed to the surfaces, that may prevent aggreration of oil droplets under repulsive force. Proteins are a widely used as emulsifiers. However, if the pH of emulsion is close to p*I* of protein, the net electrical charges on the droplets are neutralized. Then, the emulsions were highly unstable to droplet flocculation. The electrostatic repulsion was less to attractive interaction [9]. These limitations have led to the researches to find new methods to improve the oxidative stability of emulsions.

An alternative strategy is to create an interfacial layer around oil droplet, which is consisting of multiple layer of difference material by using the Layer-by-Layer deposition technique (LbL) or Electrostatic self-assembly technique (ESA) [6]. This technique have been create the complex between protein–polyelectrolyte [8], that could be achieved using to prepare better both of physical stability to environmental stresses and oxidative stability of polyunsaturated fatty acids than

single layer oil-in-water emulsions [8-13]. Applications of protein–polyelectrolyte systems have been strongly influenced for investigated and follow up.

The objective of this study, we aim to improve the oxidative stability of omega-3 fatty acids by preparing tuna oil-in-water emulsions stabilized by whey protein isolate (WPI) and multilayer oil-in-water emulsions by using the LbL method. Cationic chitosan and anionic alginate and carrageenan were selected to produce interfacial membrane layer. Moreover, we added Thai's herb, such as capsaicin oleoresin, roselle extract and sesame oil in oil-in-water emulsions to improve the oxidative stability of omega-3 fatty acids. The effects of number of coating layer, pH of emulsion and antioxidant on the oxidative stability of tuna oil-in-water emulsions were also evaluated.

CHAPTER II

THEORIES AND LITERATURE REVIEW

2.1 Omega-3 fatty acids

As we knew in the last 30 years ago polyunsaturated fatty acids (PUFA) had sciencnically significant in human nutrition and disease prevention. These PUFA which are omega-3 (n-3) and omega-6 (n-6) are precursors of hormone like compounds known as eicosanoids. And both are involved in many important biological processes in the human body [1, 8].

Several review articles exhibited that an imbalance of the ratio between omega-3 and omega-6 can emphasize the insufficiency state of the omega-3 fatty acid. The reason of increasing of the ratio of n-6 to n-3 fatty acids was increasing on consumtion of vegetable oils rich in n-6 fatty acids and reducing on consumption of foods rich in n-3 fatty acids in industrialized societies. So the ratio of arachidonic acid (20:4 n-6) to DHA may also be important since both n-3 and n-6 fatty acids are essential [8].

Essential fatty acids likes omega-3 cannot be manufactured from other nutrients by the body and must be obtained from food. The omega-3 can reverse and prevent high blood pressure, decrease pain and inflammation throughout the body, prevent breast and colon cancer, and help reverse and prevent depression and other mental/emotional health challenges. We can summarize that they can help keep heart and blood vessels healthy. The chemical structures of nutritional important omega-3 were shown in Figure 2.1, which are α-linolenic acid (ALA; 18:3 n-3), eicosapentaenoic acid (EPA; 20:5 n-3) and docosahexaenoic acid (DHA; 22:6 n-3) respectively. Primarily found of α-linolenic acid (ALA) was in dark green leafy vegetables, flax seeds, hemp seeds, walnuts, and a variety of vegetable oils. EPA and DHA are found primarily in cold water fish like salmon, cod, mackerel, tuna and in fresh seaweed. Also found in smaller amounts in organically raised animal products like free-range eggs, chickens, and grass-fed beef [8].

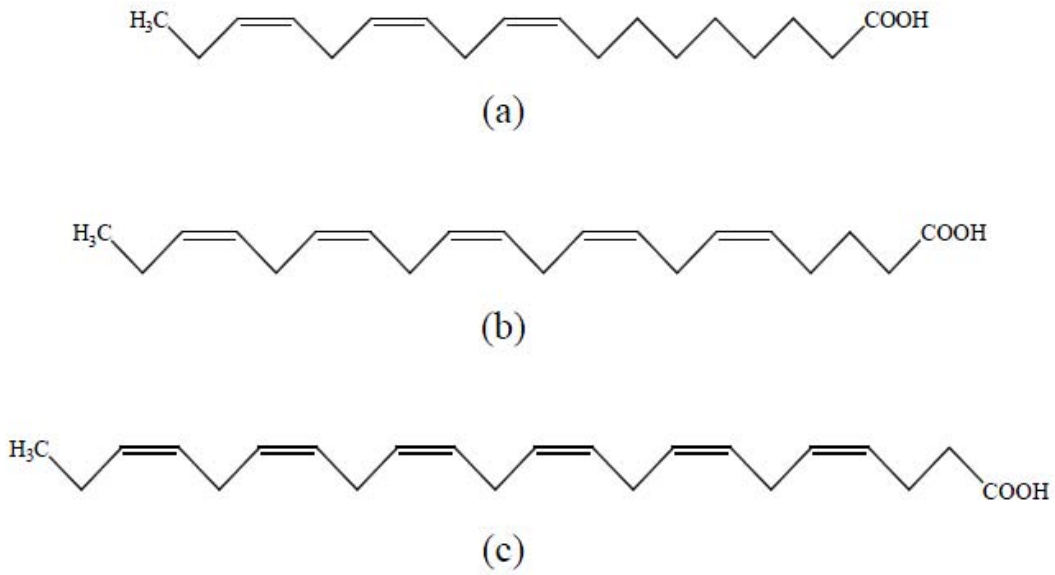


Figure 2.1 Chemical structures of (a) α-linolenic acid (ALA), (b) eicosapentaenoic acid (EPA) and (c) docosahexaenoic acid (DHA).

2.2 Lipid oxidation

2.2.1 Mechanism of lipid oxidation [2, 7, 9]

General term of a complex sequence of chemical changes that result from the interaction of lipids with oxygen-active species is called lipid oxidation which their precise mechanism in a particular food depends on the nature of the reactive species present and their physicochemical environment. Three distinct stages are used to separate lipid oxidation there are initiation, propagation and termination step [2].

Initiation: formation of free radicals

$$RH \longrightarrow R^{\bullet} + H^{\bullet}$$

Propagation: free radical chain reaction

$$R^{\bullet} + O_2 \longrightarrow ROO^{\bullet}$$

$$ROO^{\bullet} + RH \longrightarrow ROOH + R^{\bullet}$$

Termination: formation of non-radical products

$$R^{\bullet} + R^{\bullet} \longrightarrow RR$$

$$ROO^{\bullet} + ROO^{\bullet} \longrightarrow ROOR + O_2$$

$$RO^{\bullet} + R^{\bullet} \longrightarrow ROR$$

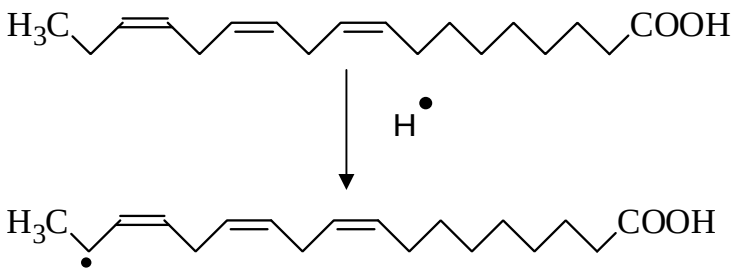
$$ROO^{\bullet} + R^{\bullet} \longrightarrow ROOR$$

$$2RO^{\bullet} + 2ROO^{\bullet} \longrightarrow 2ROOR + O_2$$

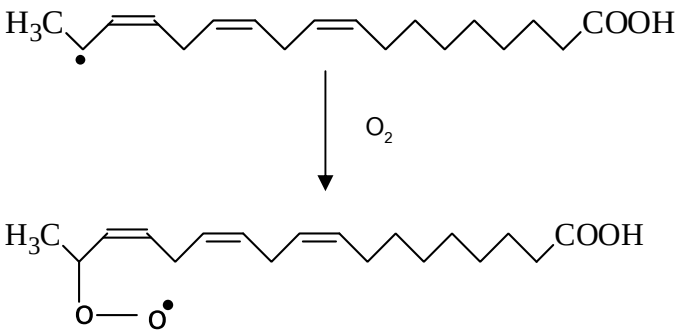
First, the initiation, a radical ($R^{\bullet}$) is form by losing of a hydrogen from unsaturated hydrocarbon (RH) then oxygen adds at double bond to form radical in the propagation. The termination occurs after two radicals interact and no radicals are available for further reaction with oxygen. A new initiation reaction can occur if oxidation is continuing.

Since it relates both the site of attract and to the energy requirement, initiation reaction is of great interest. The energy for radical production by rupture of CH bond is about 80 kcal less energy is necessary for addition of oxygen to form diradical at double bond, but both requirements appear to be so excessive that numerous energy-reducing postulates exits for involvement of metal activation, enzyme catalysis or photooxidation [7]. Figure 2.2 shows the formation of hydroperoxide in the autoxidation of linoleic acid [15].

Initiation:



Propagation:



Termination:

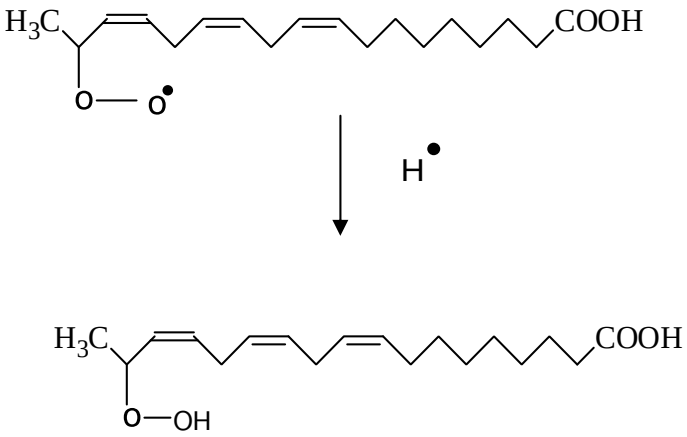


Figure 2.2 The formation of hydroperoxide in the auto-oxidation of linoleic acid [7].

Although at room temperature the primary oxidation products called as lipid hydroperoxides are relatively stable, in the presence of metals or at high temperature they are readily decomposed to alkoxy radicals and then form secondary oxidation products. These products are aldehydes, ketones, acids, esters, alcohols, and short-chain hydrocarbons. A hemolytic cleavage between oxygen and the oxygen bond is the most likely pathway of hydroperoxide decomposition in which alkoxyl and hydroxyl radicals are produce (Figure 2.3). The time for secondary product formation differs for different oils.

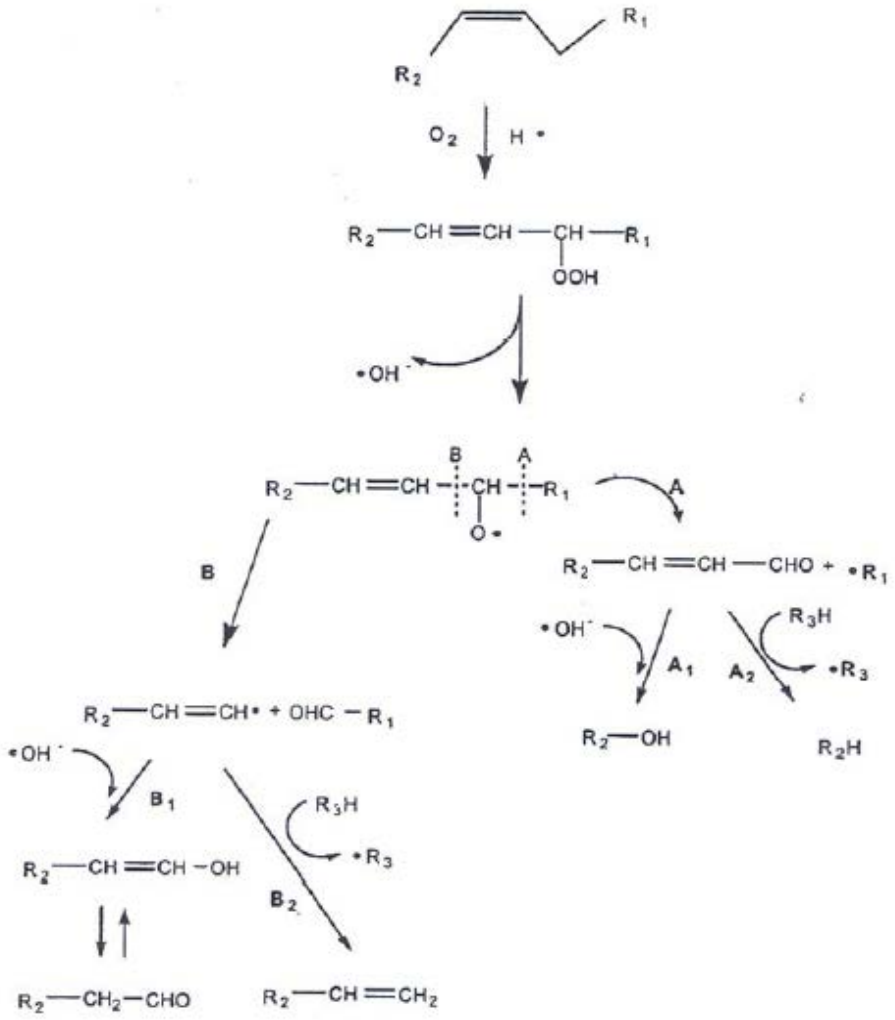


Figure 2.3 Mechanisms of hydroperoxide decomposition to form secondary oxidation compounds [16].

2.2.2 Factors that influence lipid oxidation in emulsions

2.2.2.1 Chemical structure of lipids[16]

The sensitivity to oxidation is depand on chemical structure of a lipid molecule especially the number and the position of the double bonds. Considering about stable to lipid oxidation, saturated lipids are more stable than unsaturated ones for example, saturated lipids are more likely to be crystalline than unsaturated lipids of the same chain length, so utilization of a lipid source that contained little or no unsaturated fats is the most straightforward means of retarding

lipid oxidation in food emulsions. But unsaturated fats have physical and sensory characteristics that cannot be obtained using saturated fats alone so these means are not a feasible strategy.

Case of colloidal dispersions is an example of the significant of double bond position which stability to oxidation is greater when the closer of the double bond with methyl chain end is greater. This was probably because the fatty acids are orientated within the micelles so that the carboxyl group protrudes into the aqueous phase, while the hydrocarbon tail is located in the hydrophobic interior. The sensitivity to oxidation is also depend on the polarity of lipid molecules which in turn determines their location within an emulsion droplet. Experiments with oil-in-water emulsions containing surface active ethyl linoleate show that this lipid is more susceptible to oxidation when it is located at the droplet surface than when it is in the droplet interior.

2.2.2.2 Metal [3, 4, 13]

Thus reaction between transition metals with unsaturated lipids to break down them into alkyl radicals occurs extremely slow, therefore it is not believed to be important in promoting lipid oxidation. The mechanism for the acceleration of lipid oxidation in emulsions is the decomposition of lipid hydroperoxides (ROOH) into highly reactive peroxyl ($ROO^{\cdot}$) and alkoxyl ($RO^{\cdot}$) radicals by transition metals (Eq.1 and 2) or other pro-oxidants. These radicals react with unsaturated lipids (LH) within the droplets or at the oil-water interface, which leads to the formation of lipid radicals ($L^{\cdot}$ and $LOO^{\cdot}$) (Eq. 3 to 5). The lipid oxidation chain reaction propagates as these lipid radicals react with other lipids in their immediate vicinity (Eq. 6). Some of the lipid radicals formed may be terminated when they react with other radicals (Eq. 7).

$$Fe^{3+} + ROOH \longrightarrow Fe^{2+} + ROO^{\cdot} + H^{+} \quad (1)$$

$$Fe^{2+} + ROOH \longrightarrow Fe^{3+} + RO^{\cdot} + OH^{-} \quad (2)$$

$$ROO^{\cdot} + LH \longrightarrow ROOH + L \quad (3)$$

$$RO^{\cdot} + LH \longrightarrow ROH + L \quad (4)$$

$$L^{\cdot} + O_2 \longrightarrow LOO^{\cdot} \quad (5)$$

$$LOO^{\cdot} + LH \longrightarrow LOOH + L^{\cdot} \quad (6)$$

$$LOO^{\cdot} + LOO^{\cdot} \longrightarrow \text{nonradical products} \quad (7)$$

2.2.2.3 Oxygen concentration [3, 5, 6]

When unsaturated lipids react with oxygen, that call lipid oxidation reaction. As we knew oxygen is about three times more soluble in food oils than in water and so there is always likely to be sufficient oxygen present in the oil phase to fuel lipid oxidation [6], unless specific measures are taken to exclude it so the effect of oxygen concentration on the kinetics of lipid oxidation in oil-in-water emulsions has been investigated. The results showed that at low oxygen concentrations, the rate-limiting step for lipid oxidation was the diffusion of oxygen through the aqueous phase. Under these limited oxygen conditions, the rate of lipid oxidation increased because these processes increased the oxygen concentration with mechanical agitation and cooling. At high oxygen concentrations, the rate of oxygen diffusion was much faster than the rate of lipid oxidation, and so it was not limiting. So an effective means to retard lipid oxidation is the reducing of the concentration of oxygen present.

2.2.2.4 Storage temperature and light [7, 12]

The reaction temperature has little effect on O_2 oxidation.Even if the storage temperature increases, the autoxidation of lipids and the decomposition of hydroperoxides will increase too, at low temperature the formation of autoxidation products is slowly. Light is much more important in O_2 oxidation.

2.2.2.5 Antioxidants [4, 17]

Incorporate antioxidants is one of the most effective means of retarding lipid oxidation in fatty foods. Antioxidants work by a variety of different methods, example including control of oxidation substrates (oxygen and lipids), control of pro-oxidants (reactive oxygen species and pro-oxidant metals), and inactivation of free radicals. By the way the term antioxidant must be carefully used because when we change conditions some substances that retard lipid oxidation may promote it under a different set of conditions. This useful for classify antioxidants according to the mechanism of their action as either primary or secondary antioxidants.

Primary antioxidant, also known as a chain-breaking antioxidant, can delay the initiation step or interrupt the propagation step of autoxidation. Because primary antioxidant is a substance that capable of accepting free radicals. Eq.8 to10 showed how chain-breaking antioxidants react with lipid and peroxyl radicals and convert them to more stable, radical, or nonradical products. The antioxidant radicals (A) produced by this process are much less reactive than lipid and peroxyl radicals, and therefore are less effective at promoting oxidation. Chain-breaking antioxidants have a higher affinity for peroxyl radicals than lipids, and therefore they tend to scavenge the free radicals produced during the initiation and propagation steps.

$$ROO^{\cdot} + AH \longrightarrow ROOH + A^{\cdot} \quad (8)$$

$$RO^{\cdot} + AH \longrightarrow ROH + A^{\cdot} \quad (9)$$

$$R^{\cdot} + AH \longrightarrow RH + A \quad (10)$$

Chain-breaking antioxidants are also capable of terminating the lipid oxidation reaction by reacting with peroxyl radicals, alkoxyl radicals and other antioxidants (Eq. 11 to 13).

$$ROO^{\cdot} + A^{\cdot} \longrightarrow ROOA \quad (11)$$

$$RO^{\cdot} + A^{\cdot} \longrightarrow ROA \quad (12)$$

$$A^{\cdot} + A^{\cdot} \longrightarrow AA \quad (13)$$

Secondary antioxidants can retard lipid oxidation through a variety of mechanisms but should be noted that none of these mechanisms involves conversion of free radical species to more stable products [13,16]. Decomposition of lipid hydroperoxide constitutes a very complicated process and produces a maltitude of material may have biological effect [7, 8]. The mechanism of secondary antioxidants include of chelation of transition metals, replenishing of hydrogen to primary antioxidants, oxygen scavenging, and deactivation of reactive species. The most important type of secondary antioxidants are those that chelate transition metal ions from the standpoint of oil-in-water emulsions. A major factor in the promotion of

lipid oxidation is the presence of transition metals, such as iron or copper, in the aqueous phase of oil-in-water emulsions and their effectiveness in promoting lipid oxidation increases dramatically when they are located near droplet surfaces. Because they are then in closer proximity to the lipid substrate. Consequently, we can retard of lipid oxidation by using aqueous phase component that chelates transition metals and removes them from the vicinity of the droplet surface [3, 13].

2.2.2.6 Droplet composition [3, 7, 9]

Emulsions primarily consist of a mixture of polyunsaturated fatty acids, saturated and monounsaturated fatty acids, free fatty acids as glycerol esters and various other minor components. The polyunsaturated fatty acids are highly susceptible to lipid oxidation [3]. In the other hand, the saturated and monounsaturated fatty acids are relatively stable to lipid oxidation. Recently, a study was carried out the influence of droplet composition on the rate of lipid oxidation in emulsion (ratio of poly-unsaturated to saturated oil).

Oil-in-water emulsions were prepared using different ratios of the polyunsaturated substrate and the saturated inert diluent as the oil phase [7]. The rate of lipid oxidation depend on the concentration of oxidizable substrate in the droplets [9]. Initially, oxidation occurred more rapidly in droplets containing low concentrations of substrate. However, during later stages oxidation became more rapid in droplets containing high concentrations. It is hypothesized that oxidation is probably initiated more rapidly at low substrate concentrations because a greater percentage of the surface active substrate molecules is present at the surface of the droplet, and therefore more easily accessible to attack by the free radicals generated in the aqueous phase. The spread of oxidation within a droplet is slower at low substrate concentrations because collisions between reactive species and substrate molecules is less frequent.

2.2.3 Assessment of lipid oxidation

Various factors are effect the relative amounts of reaction products formed by lipid oxidation and a wide range of tests from simple organoleptic evaluation to chemical and physical methods of various complexities have been suggested [8, 10]. Table I summarizes a few chemical assays of lipid peroxidation [8].

From the multitude of assays, the universal method (correlates well with the extent of food deterioration throughout the entire course of autoxidation) is not available. Some of the presented methods give an information about particular stages more than the others which in view of the chemical diversity of the food matrices and of the oxidation pathways. This situation should be expected. Compare between oxidation and time, the loss of lipid substrate or the amount of oxygen can be explain serve as general though nonspecific and usually not sensitive enough, indexes for peroxidation of lipids. Since hydroperoxides are the primary products of lipid oxidation, that can measurement concentration of hydroperoxides for determine the oxidation [12]. The peroxide value test represent the total concentration of peroxides and hydroperoxides depend on time. An alternative approach to the determination of the extent of oxidation is the measurement of products of hydroperoxide degradation. In addition to the peroxide determination, such carbonyl compounds is degradation, that show the composition are change. The application of methods depend on requires a detailed knowledge of the chemistry involved, stability of the compounds assayed.

Further, thiobarbituric acid test (TBA) is one of the most common [13, 14]. This method is based on the color product resulting from the condensation of TBA with malonaldehyde which may be cause from the oxidized fats. However, a large changing suggests that other food components can react with TBA to generate the same chromophore and even the formation of malonaldehyde is dependent on the composition of the initial lipid.

The scope of oxidation can be determined by many methods. Peroxide value is the first and most commonly method used measurements of the extent of oxidation in oils [11, 12]. The measurement of peroxide value is useful for bulk oils that can be analyzed directly. For foods, emulsions or muscle tissues, the lipid is extracted with mixtures of solvents that must be carefully removed by evaporation without decomposition of hydroperoxides. The ferric thiocyanate method is commonly applied to emulsions, when the oxidative retrograde at relatively low peroxide values. Thus, the ferric thiocyanate method was used for determining the extent of lipid oxidation in the research.

The ferric thiocyanate method [11] is involved the reaction of ferrous (Fe^{2+}) reform to ferric (Fe^{3+}) ions, which are determined colorimetrically as ferric

thiocyanate. This method is more sensitive and requires a smaller sample than does the iodometric method.

$$ROOH + Fe^{2+} \longrightarrow Fe^{3+} + RO^{\cdot} + OH^{-}$$

$$Fe^{3+} + 3(NH_4)SCN \longrightarrow Fe(SCN)_3 + 3NH_4^{+}$$

Table 2.1 Compendium of assays for lipid oxidation [8]

Monitored reaction effect	Method of assay
Loss of lipid substrate	Gas chromatography
Oxygen uptake during oxidation	Oxygen uptake
Formation of peroxides, hydroperoxides	Iodometry, enzymatically, chemiluminescence
Formation of malonaldehyde	Direct determination by UV absorption (λ_{max}= 245 nm) or HPLC , derivatization with thiobarbituric acid (absorption at λ_{max}= 532 nm or fluorescence at λ_{em} = 553 nm), Fluorescence of Schiff base (λ_{em} = 455 nm)
Formation of conjugated dienes	Increase in OD at λ = 233 nm.
Formation of carbonyl compounds	Direct determination by GS-MS**,** HPLC, derivatization with 2,4-dinitrophenyl-hydrazine.
Formation of free fatty acids	Titration, electric conductivity.

2.3 Emulsion

An emulsion is a mixture of two or more liquids which are normally immiscible, one is dispersed as droplet in another. That lipid which is broken up into droplet is termed the dispersed phase, while the lipid surrounding the globules is termed as the continuous phase or dispersing medium. In other hand, these

are frequently referred to as the Internal and External phases respectively. Since emulsion is a mixture of two or more fluid bodies which are not soluble in each other but kept is suspension by mechanical means therefore the finer the division of the particles, the better they reach the objective. If these particles can be divided in form a colloidal state, then they will not separate on standing. In the case of an oil-in-water emulsion, water is continuous phase and oil is dispersed in water, while in the water-in-oil emulsion, the reverse is the case [9].

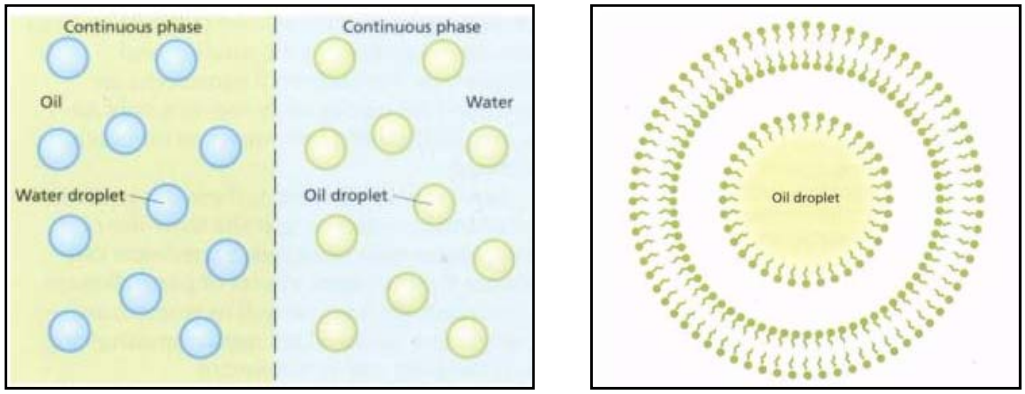


Figure 2.4 Emulsions: (A) the two types of emulsion; (B) an oil droplet in water, surrounded by a ring of stabiliser molecules that prevent it from coalescing with its neighbors [18].

2.3.1 Emulsion Stability [9]

Lipophilic dispersions, that show this condition are not thermodynamically stable. They posses some degree of kinetic stability, and one must respect such things as what degree of change and what time scale. Many different ways can explain the dispersed speices.

1.) Creaming or Sedimentation. The particles float upwards or sink, depending on density difference between the dispersed and continuous phases. Although this is not yet a destabilization of the dispersion, it produces two separate layers of dispersion that have different dispersed phase concentrations.

2.) Aggregation. The droplet was dispersed to form a bigger droplet, that may be cause the different of density of two phase. At this point possibly no change in total surface area.

3.) Coalescence is occur, when mono-particles together to form two or more particles, droplets or bubbles condense. The integration to form a single larger unit and reducing the total surface area.

4.) Breaking, could be form between coalescence and creaming, when the emulsions break into two phase. One phase move upwards to the top and clear phase below by eyes vision.

Sedimentation or creaming will occur, that may be cause concentrate dispersed species and promote aggregation. At this point, we must considered the stability of aggregation, but can be form in an emulsion (Fig 2.5). If the emulsions are not very stable. They needed to addition component that can forms a film around the dispersed droplets to increase the stability and prevent both aggregation and coalescence. The stability of emulsion depend on surfactant and include involves the mechanical properties of the interfacial films. The factors which affect the stability both aggregation and droplet coalescence can be follow [19]:

- Interfacial tension
- Surface viscosity and/or mechanical
- Electric double layer and/or steric repulsions
- Dispersion force attraction
- The volume of dispersed phase.
- Droplet size.
- The density difference between the phase.
- Bulk viscosity.

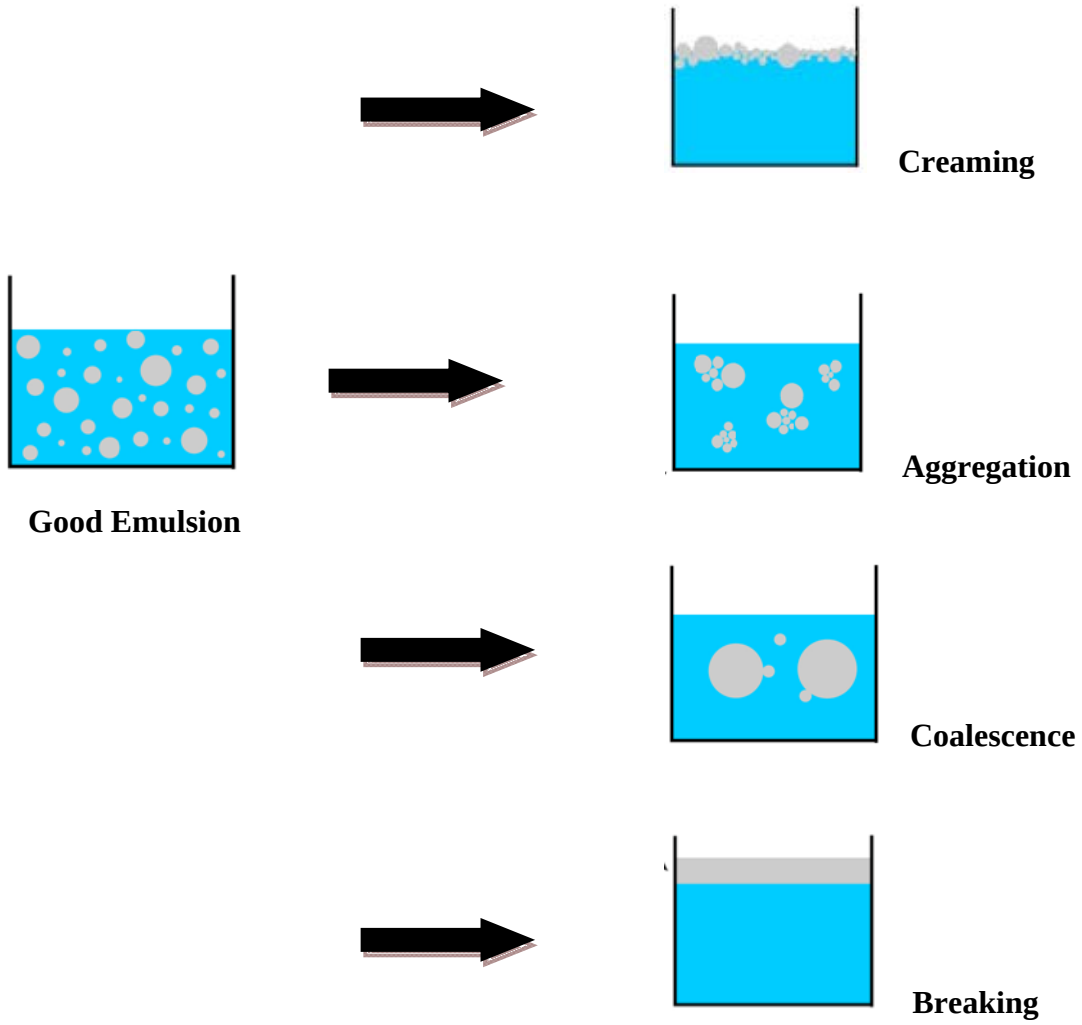


Figure 2.5 Illustration of creaming, aggregation and coalescence in an emulsion, form or suspension [19].

2.4 Polyelectrolytes

Polymers, that have a repeating electrolyte group unitsand dissociate in aqueous solutions are call polyelectrolytes. The properties of polyelectrolyte are both electrolytes (salts) when show the electrically conductive in solution and polymers (high molecular weight compounds) when show the viscous properties [20]. Charged molecular chains, commonly present in many systems ancilliary structure, stability and the interactions of various molecular assemblies. Features the unique properties, their are being exploited in a wide range of technological and industrial fields. The important major roles, polyelectrolyte could

be use in biology and biochemistry. Many biological molecules are polyelectrolytes, such as polypeptides (thus all proteins) and DNA are polyelectrolytes. Both natural and synthetic polyelectrolytes are used in a variety of industries.

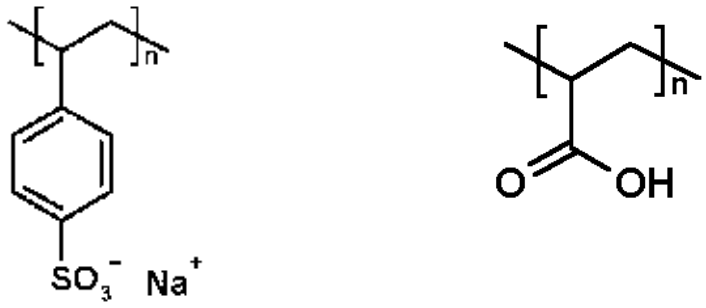


Figure 2.6 Chemical structures of two synthetic polyelectrolytes, as examples. To the left is poly(sodium styrene sulfonate) (PSS), and to the right is polyacrylic acid (PAA) [21].

Polyelectrolytes was decide into 2 type [16, 21]. One polyelectrolyte that can dissociates completely in solution at suitable pH are call a 'strong' polyelectrolyte, by contrast, one polyelectrolyte can dissociates partially in some pH, that call a “weak polyelectrolytes” Thus, weak polyelectrolytes are not fully charged in solution, and can be changing properties by vary the solution pH, counterion concentration, or ionic strength. The degree of charging of polyelectrolyte solutions are affected to physical properties . The Debye length and counter ion are needful in the solution’s. When solutions of two oppositely charged polymers, anionic mixed with cationic, that usaully form a bulk complex (precipitate) because the oppositely-charged polymers attract one another and irreversibly bind together.

Sometime show 2 type of charging in polymer change, there are called polyampholytes, such as protein (some amino acids tend to be acidic while others are basic), that has competition between the acid-base equilibria of these groups leads to additional complications in their physical properties. These polymers show oppositely charged in difference pH [21].

The investigation in the adsorption of charged polymers on charged surfaces and interfaces were studies. At low surface charge density, there are almost constant condition to controlled the thickness of the adsorbed layer by the balance of the energy gain due to electrostatic attraction and confinement entropy loss.

At high surface charge densities, polymers completely cover the surface and the thickness of the adsorbed layer are increases linearly, which can control by the balance between electrostatic attraction of the charged monomers.

In this range of the surface charge densities the electrostatic attraction between polyelectrolyte chains and the oppositely charged substrate can be affected by the controlling the larger thick. The results of these simulations are in qualitative agreement with the prediction of the scaling models of polyelectrolyte adsorption.

The examples of weak polyelectrolyes are shown in Fig. 2.12. The pH is important to behavior of the chain and in turn influences their conformation upon adsorption onto a surface.

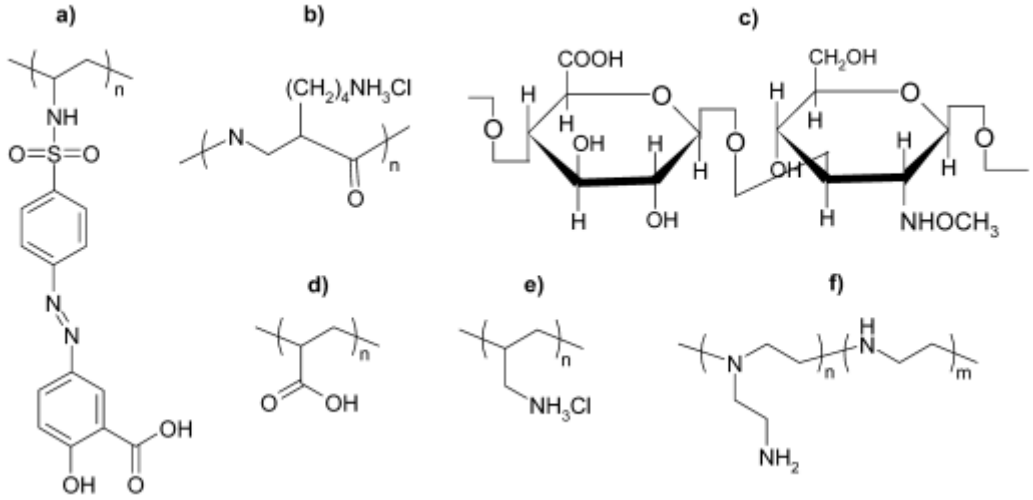


Figure 2.7 Some examples of weak polyelectrolytes (a) poly{1-[4-(3-carboxy-4-hydroxyphenylazo) benzenesulfonamido]-1,2-ethanediyl} (PAZO), (b) poly(L-lysine hydrochloride) (PLL), (c) hyaluronic acid (HA), (d)poly(acrylic acid) (PAA), (e) polyallylamine hydrochloride(PAH) and (f) poly(ethylenimine) (PEI) [16].

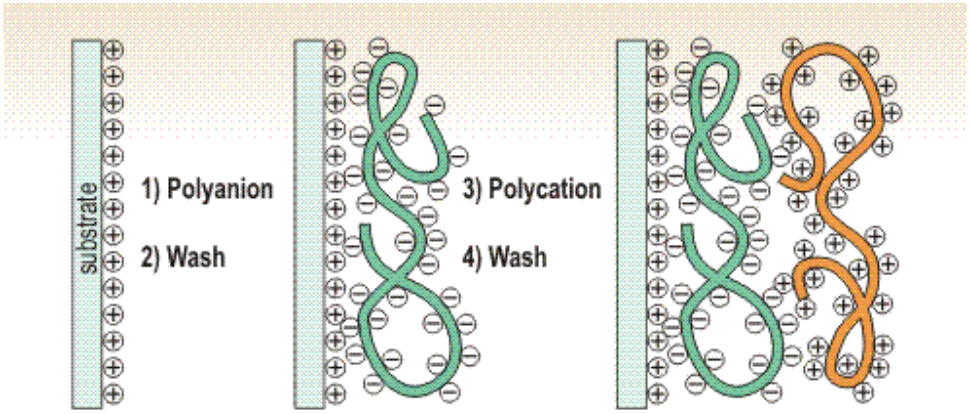
2.5 Layer-by-Layer self-assembly [6, 7, 9, 17, 20]

Self-assembly is a process that can reform uninstructed structural that processes are reversible and held together by non-covalent intermolecular forces. The supramolecular chemistry are call the studies of the area about non-covalent molecular interactions and the formation of thin film technology. The properties of self-assembled surface monolayers, the conductive show in a two dimensional conductive sheet that can be extended to three dimensional structures if a stack or a collection of monolayers can be achieved, there are useful for the fabrication of various devices [17]. This is call layer-by-layer (LbL) self-assenbly. The LbL method can be use in many technique and being rapidly extened to the other technique, such *as* polymeric nanocrystals, metel and semiconductor nanoparticles and dendrimers. The important research in the LBL method is using in biological such as proteins, enzymes, DNA, cell membranes and viruses. Thus, the applications of LbL films has been suggested for optics and optoelectronics, drug delivery and electrochemistry.

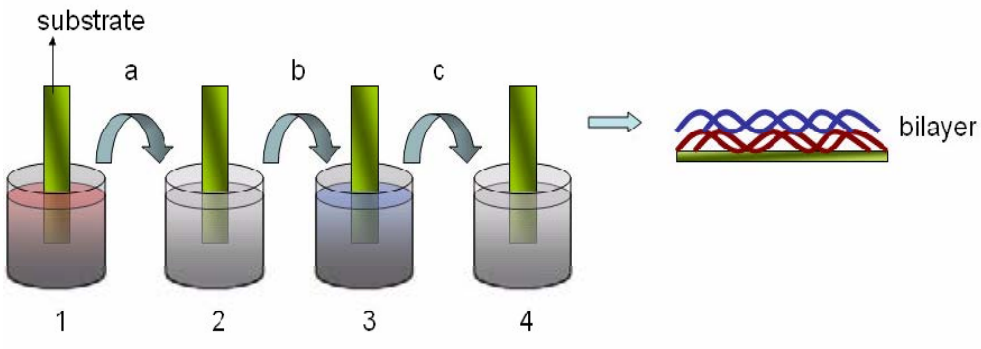
The LbL technique have been detailed in many series, for example in case adsorption in LbL films is governed by electrostatic interactions between species bearing opposite charges and secondary interactions have also been shown to be important. The LbL technique was widely to used in many work, that may be used, which include hydrophilic and hydrophobic glass, mica, silicon, metals, quartz, and polymers. In addition, LbL films may be deposited directly onto colloidal suspensions throughout the cationic and anionic dipping solutions, alternately [20].

In addition, the LbL technology can be adapted to produce multilayer emulsions using a step-by-step method that should be fairly easy, fast and cheap [19]. The basic method of producing multilayer emulsions is shown in Figure 2.8. The primary emulsion is prepared by homogenizing oil and water phases in the presence of a positive or negatively charged emulsifier. Then mixed into an oppositely charged polyelectrolyte solution to create a secondary emulsion. The secondary emulsion is then mixed into another solution containing polyelectrolytes that have an opposite charge to the previous one to create a tertiary emulsion, and so on alternative, that can form the multilayer oil-in-water emulsion [9, 14]. Multilayer emulsions may be have

any excess polyelectrolyte between each adsorption step, that has affected to the properties, thus to wash for remove it for suitable condition.



(A)



(B)

Figure 2.8 (A) Schematic of Layer-by-Layer deposition technique [19]. (B) Schematic fabrication of a 1-bilayer LbL film. A solid substrate, bearing for example negative charges, is initially immersed in the polycationic solution (1). In the following step (a), the excess of molecules can be removed by immersing the substrate in the washing solution (2). The substrate containing the cationic layer is subsequently (b) immersed in the anionic polyelectrolyte solution (3). The molecules not effectively attached can be removed (c) in the washing solution (4) [18].

If in the system have any excess free polyelectrolyte, multilayer systems are poor stability because show the droplet aggregation. Thus the development to produce stable multilayer were studies [20]:

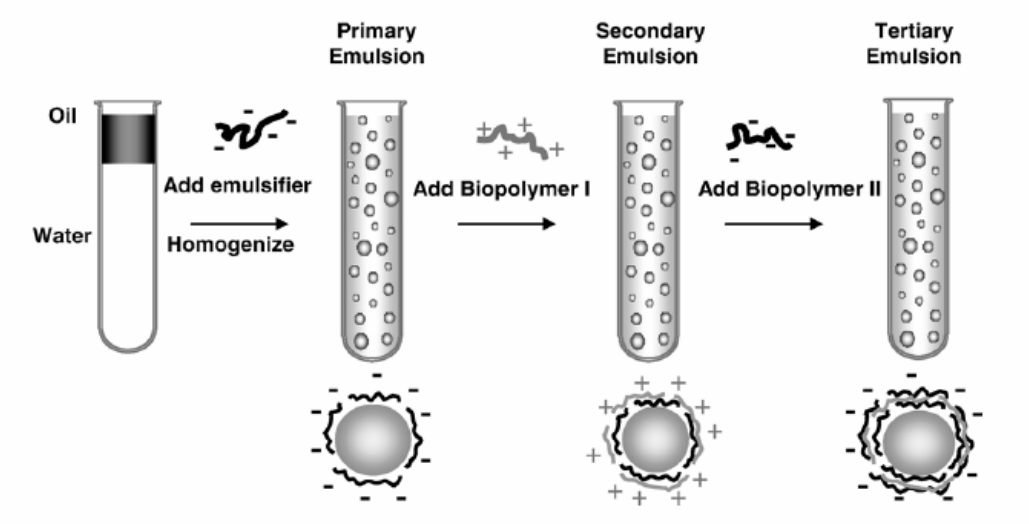


Figure 2.9 Schematic of Layer-by-Layer technique for the production of oil-in-water emulsions [20].

1.) Saturation method. It show the completely coat all of the particles, so that there is little free polyelectrolyte remaining in the aqueous phase. The zeta potential use to be determined the saturation concentration for suitable system.

2.) Centrifugation method. This method, excess polyelectrolyte in solution that contains will be removed by centrifuging the colloidal suspension, collecting the particles, and re-suspending. This procedures can be repeated a number of times to ensure that all of the free polyelectrolyte has been removed. This method will be made before added the next polyelectrolyte solutions. But the big problem of this method is can put on particle aggregation during the centrifugation step because the particles are forced into close proximity.

3.) Filtration method. In this method use membrane filtration to removed the excess non-adsorbed polyelectrolyte molecules, follow by a filter allows the polyelectrolyte molecules to pass through, but not the colloidal particles. The pressure put to forces the aqueous phase containing the excess polyelectrolyte through the filter. A buffer solution add to the system to keep the overall volume of the system constant. Advantage of this method is not necessary to have a density difference between the particles and the surrounding liquid.

For all of the methods, saturation method, centrifugation method and filtration method must carefully control the system composition and preparation conditions to form stable multilayer colloidal particles.

2.6 Zeta Potential [23, 24]

Zeta Potential was applies to the electrical charges on the surface of liquid emulsions or dispersions of a colloidal nature. Around surface droplet has a fluid coated is surrounded by a dense layer of ions having a specific electrical charge. This layer is surrounded by another layer, and diffuse in another layer , that has an electrical charge of its own. The bulk of the suspended liquid also has its own electrical charge. The difference in electrical charge between the dense layer of ions surrounding the particle and the bulk of the suspended fluid is the zeta potential. When ions or polymers are absorbed or dispersed on a particle in a colloidal system and emulsion, the charge of the layer surrounding the particle were change because the changing of the potential difference between the surrounding layer of ions and the bulk of the suspending fluid. This mean zeta potential was changed. The zeta potential was effect to the stability of a colloidal. Thus, the measurement of zeta potential could be used in the control of processes wherein dispersion or agglomeration is important.

2.6.1 Adsorption of charged species (ions and ionic surfactants) [23]

Surfactant ions may be specifically adsorbed onto the surface of a particle**.** Cationic surfactants can produced a positively charged surface, while anionic surfactants can produced a negatively charge surface.

2.6.2 The Electric Double Layer [24]

Development of a net charge on the surface of the particle affects to the distribution of ions in the surrounding interfacial region. If two layer adsorb on the surface (ions of opposite charge to that of the particle), that resulting in an increased concentration of counter ions. Thus an electrical double layer exists round each particle.

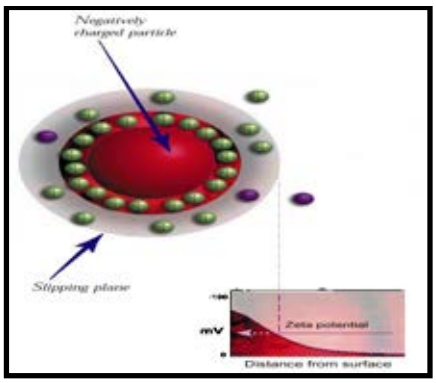


Figure 2.10 Schematic detailing the distribution of ions around a charged particle [24].

From Figure 2.16, the liquid layer surrounding the particle exists as two parts; an inner region (Stern layer) where the ions are strongly bound and an outer (diffuse) region where they are less firmly associated. The layer closest to the surface of the charged particle is composed of condensed or absorbed counter ions. Within this diffuse layer is a notional boundary known as the slipping plane, within which the particle acts as a single entity. The boundary of outer layer is defined as the slipping plane, and it is the summation of charge within the slipping boundary that defines the strength of the electrostatic interaction [25].

2.7 Antioxidant [3, 26-29]

A wide variety of oxygen free radicals and other reactive oxygen species can be formed in the human body and in food systems. Transition metal ions accelerate free-radical damage. Antioxidant defenses, both enzymic and nonenzymic, protect the body against oxidative damage and so free-radical damage must be constantly repaired.

Early research on the role of antioxidants in biology focused on their use in preventing the oxidation of unsaturated fats, which is the cause of rancidity [26]. Antioxidant activity could be measured simply by placing the fat in a closed container with oxygen and measuring the rate of oxygen consumption [27]. The mechanisms of action of antioxidants were first explored when it was recognized that a substance with anti-oxidative activity is likely to be one that is itself readily oxidized.

Antioxidants were devided into two broad divisions, depending on the solubility property in water (hydrophilic) or in lipids (hydrophobic) [28]. The action of one antioxidant may therefore depend on the proper function of other members of the antioxidant system. The amount of protection provided by any one antioxidant will also depend on its concentration, its reactivity towards the particular reactive oxygen species being considered, and the status of the antioxidants with which it interacts [29].

The mechanisms of antioxidant action for inhibition of oxidative reactions was produced by this process are much less reactive than lipid and peroxyl radicals, and therefore are less effective at promoting oxidation according to the initiation and propagation steps, shows that antioxidants interfere with free radical, chain oxidation and that reaction products of antioxidant molecules and oxidized lipid molecule may appear among the final product [3].

$$ROO^{\cdot} + AH \longrightarrow ROOH + A^{\cdot}$$

$$RO^{\cdot} + AH \longrightarrow ROH + A^{\cdot}$$

$$R^{\cdot} + AH \longrightarrow RH + A$$

2.8 Literature reviews

2.8.1 Emulsions

Hu et al. [30] studied the impact of whey protein emulsifier on the oxidative stability of monolayer of salmon oil-in-water emulsions stabilized by whey protein isolate (WPI), sweet whey (SW), ß-lactoglobulin (β-Lg) and α–lactalbumin (α-La) at pH 3.0. They evaluated the effect of pH and temperature on the oxidative stability of emulsions. The result showed the oxidative stability was in the order of β-Lg ≥ SW > α-La ≥ WPI whereas the surface charge was in the order of β-Lg > α-La > WPI > SW. That can be explained that the emulsion droplet charge was not the only factor influencing the oxidative stability of the lipid. The different whey proteins include differences in amino acids and differences in how the proteins impact the thickness or packing of the emulsion droplet interface.

Similarly [31], casein (CAS), whey protein isolate (WPI) and soy protein isolate (SPI) were chosen to stabilize the oil-in-water emulsions at pH 3.0, which was below the isoelectric point (*pI*) the resulting in the cationic oil-in-water emulsion droplets. The oxidative stability of the protein stabilized emulsion was CAS > WPI > SPI, although the electrical charge of WPI higher than CAS. One reason is casein can form a thick interfacial layer around dispersed oil droplets up to 10 nm compared to 1-2 nm whey proteins isolate. Thus, the particle size of emulsion droplet could impact lipid oxidation rate because smaller particle size result in larger surface area and thus the greater possibility for lipid-aqueous phase prooxidant interactions.

Akoh et al. [32], investigated the effect of emulsifier type, droplet size, and oil concentration on lipid oxidation in structured lipid-based oil-in-water emulsions. Whey protein isolate (WPI) and Ryoto sugar ester (SFE) were used to prepared 10 and 30% oil emulsions. The resulting in the oxidative stability were showed, if decreasing oil concentrations, total oxidation were increase. WPI had a significant antioxidant effect on the oxidation rates. Particles size had no effect on lipid oxidation in structured lipid-based oil-in-water emulsions, because the effect of oil concentration and emulsifier type were important on lipid oxidation too.

2.8.2 Multilayer emulsions

Ogawa et al [33], used the LbL method to prepare oil-in-water emulsions containing cationic droplets coated with lecithin-chitosan bilayers. They selected lecithin to prepare primary emulsion and secondary emulsions were used chitosan to coat the droplet. The stability of secondary emulsions were better than primary emulsion, may be cause ionic strength of secondary emulsion can be repulse the flocculation forming. Neverless, secondary emulsions could not be used at higher pH (pH > 5) because chitosan lose the positive charge.

They studied and prepared multilayer emulsions consist three layer interfacial membrane (lecithin-chitosan-pectin) to improve the property of oil-in-water emulsions. The resulting showed the tertiary emulsions had a good stability to droplet aggregation and creaming over a wide range of pH value [34].

Klinkesorn, U et al [35], prepared and studied oil-in-water emulsions by using an electrostatic layer-by-layer deposition technique, the oxidative stability of omega-3 in emulsions coated by lecithin alone and by lecithin-chitosan also the ability of the antioxidants mixed tocopherol and EDTA on the stability of the emulsions at pH 3.0. The oxidative stability of the secondary emulsion (lecithin-chitosan) had a better more than the primary emulsion (lecithin alone) due to the thicker interfacial membrane that could decrease interactions between lipid and aqueous phase prooxidants. The combination of mixed tocopherol and EDTA was effective at increasing the oxidative stability of spray-dried multilayer emulsion, because antioxidant activity of tocopherols and EDTA.

Katsuda et al [36], studied physical properties and oxidative stability of oil-in-water emulsion. β-lactoglobulin, citrus pectin and sugar beet pectin were chosen to stabilize the emulsions at pH 3.5. The zeta potential of all of the emulsions became slightly more negative during storage due to formation of anionic species such as free fatty acids which are generated from the hydrolysis of triacylglycerols at low pH values. The citrus pectin secondary emulsion was more oxidatively stable than the β–lactoglobulin stabilized emulsion even though the surface charge of the secondary emulsion was anionic and thus would attract prooxidative metals due to the thicker emulsion droplet interfacial membrane was able to inhibit metal-lipid interactions and thus decrease lipid oxidation rate. The antioxidant activity of pectin has been postulated due to its ability to chelate metals and possibly scavenge free radicals.

Gudipati et al [37], prepare monodispersed fish oil-in-water emulsions containing large droplets coated by multilayers using the LbL method to study their oxidative stability and lipase digestibility. Citrim, chitosan and alginate were selected to produce infacial membrane layers. The positively charged secondary emulsions (+56.27 ±2.5 mV) were more stable to lipid oxidation compared to negatively charged primary (-45.13 ±1.7 mV) and tertiary emulsions (-24.8 ± 1.2 mV).

2.8.3 Antioxidant in emulsions

G.M. Huber et al [4], studied the properties of natural antioxidant flavonols and quercetin glycoside compared with buthylated hydroxytoluene (BHA) and α–tocopherol in the oxidation of polyunsaturated fatty acids (PUFA) in aqueous emulsion and bulk oil. The effectiveness of quercein exhibited a better antioxidant activity than BHT in bulk fish oil. While in the aqueous emulsion, quercetin and its glycosides were more effective than α-tocopherol.

Silverstre et al [28], studied the factor in the oxidation stability of oil-in-water emulsion by the properties of the interfacial membrane surrounding the lipid core and the properties of α-tocopherol. The emulsion were prepared by using polyoxyethylene 10 stearyl ether (Brij 76) and polyoxyethylene 100 stearyl ether (Brij 700) as emulsifier. The emulsions added with α-tocopherol was prepared as well. They reported that oxidative stability of emulsion prepared using the polyoxyethylene 100 stearyl ether (Brij 700) was better than the other, because the structural of large polar headgroups increased interfacial thickness, that can decrease lipid oxidation. However, there were no differences in the oxidation rate between α-tocopherol and the different surfactant systems. This suggested that free radical concentrations were high enough in both surfactant systems to not limit the rate of free radical α-tocopherol interactions.

Based on our knowledge, there have no reports about multilayer emulsion using WPI, chitosan and alginate. Moreover, the emulsion added with the extract from thai’s herbs have not been reported yet. In this work, the improvement of the oxidative stability of omega-3 fatty acids by preparing multilayer oil-in-water emulsions of tuna oil containing omega-3 fatty acid using Layer-by-Layer technique was studied. The effects of number of layers coated around the droplets on lipid oxidation rate and physical stability were investigated. Thai’s herb extracts, antioxidant, was added in oil-in-water emulsions of tuna oil as well.

CHAPTER III

EXPERIMENTAL

3.1 Chemicals

All chemicals used for emulsion preparation were purchased in analytical grade from suppliers listed in Table 3.1.

Table 3.1 Chemicals list

Chemicals	Supplier
1.Tuna oil, fully refined oil grade (5.0 % EPA and 25.0 % DHA)	T.C. Union Global and T.C. Union Agrotech company
2. Whey protein isolate (WPI), Food Grade	Glanbia nutritionals
3. Chitosan (Mw 57,000)	A.N. Lab
4. Alginic acid sodium salt (Mw 198.0)	Sigma-Aldrich
5. Carrageenen (Mw 788.0)	Nicho
6. Cumene hydroperoxide (Mw 152.5, 80% purity)	Sigma chemical
7. 1-butanol, A.R. Grade	Sigma chemical
8. Sodium acetate trihydrate, A.R. Grade	Merck
9. Glacial acetic acid, A.R. Grade	Merck
10. Hydrochloric acid, A.R. Grade	Merck
11. Sodium hydroxide, A.R. Grade	Merck

Chemicals	Supplier
12. Isooctane, A.R. Grade	Merck
13. Methanol, A.R. Grade	Merck
14. 2-propanol, A.R. Grade	Merck
15. Ammonium thiocyanate	JT Baker
16. Ferrous sulphate	JT Baker
17. Barium chloride	JT Baker
18. Genistein (98% HPLC)	Sigma-Aldrich
19. Roselle extract	Given by Tipco Foods (Thailand) Public Company Limited
20. Capsaicin oleoresin	Prepared by Natural Product Reserch Unit (CUIR), Department of Chemsitry, Chulalongkorn University
21. Sesame oil	Prepared by Natural Product Reserch Unit (CUIR), Department of Chemsitry, Chulalongkorn University

3.2 Apparatus

The apparatuses used in this study were listed in Table 3.2.

Table 3.2 Apparatus lists

Apparatus	Company, model
1. Homogenizer	Polytron, PT 3100
2. Vortex	Scientific Industries, Vortex-Genie 2
3. Centrifuge	Sanyo, Centaur-2
4. UV-visible spectrophotometer	Hewlett Packard 8453
5. Zetasizer	Malvern Instruments, Nano ZS
6. Magnetic stirrer (control tempertue)	IKA, C-MAGHS7

Figure 3.1 Zetasizer Nano ZS, Malvern Instruments.

3.3 Solution preparation

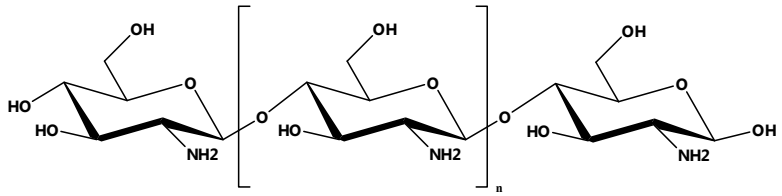
3.3.1 Acetic- acetate buffer solution

A stock buffer solution was prepared by mixing 100 mM acetic acid with 100 mM sodium acetate with volume ratio 0.034 and then adjusting the pH to 3.5 using 100 mM HCl and adjusting the pH to 6.0 using 100 mM NaOH.

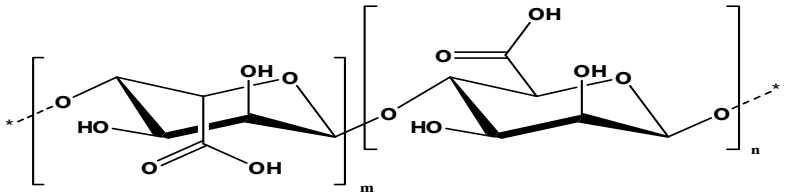
3.3.2 Emulsifier solution

Whey protein isolate (WPI) was used as emulsifier in this experiment. Stock solution of WPI was prepared by stirring 0.4 grams of WPI with 100 mM acetic-acetate buffer solution at pH 3.5 and 6.0, respectively. The emulsifier solution prepared with acetic acetate buffer pH 3.5 was adjusted to pH 3.5 with 100 mM HCl, whereas the emulsifier solution prepared with acetic-acetate buffer pH 6.0 was adjusted to pH 6.0 with 100 mM NaOH. Then, acetic-acetate buffer was added at each pH to obtain 100 mL of solution.

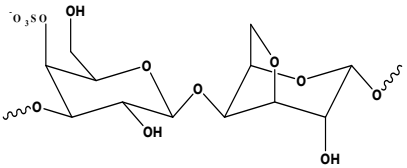
In order to find the suitable primary emulsion, the effect of WPI concentration on the primary emulsion preparation was studied. The 0.2, 0.3, 0.4 and 0.5 %w/v of WPI were used in this study. The emulsifier solution prepared by stirring 0.2, 0.3, 0.4 and 0.5 grams of WPI with 100 mL of acetic-acetate buffer pH 6.0.



(a)



(b)



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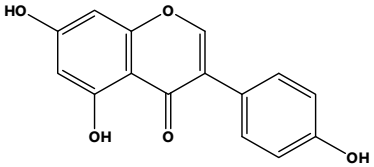

(d)

Figure 3.2 Chemical structure of (a) chitosan, (b) alginate, (c) κ-carrageenan, (d) genistein.

3.3.3 Polyelectrolyte solutions

3.3.3.1 Stock chitosan solution

Chitosan was used as cationic polyelectrolyte for multilayer emulsion preparation. The 1 %w/v chitosan solution was prepared by dissolving powder chitosan 1 gram into 1 M acetic acid 5 mL, and then, the chitosan solution was stirred for 3 hours to ensure complete dissolution. Then, the 10 mL of water was added. The solution was stirred overnight. The pH of chitosan solution was adjusted to pH 6.0 by adding 100 mM of NaOH and to pH 3.5 by adding 100 mM HCl. Then, the solution was adjusted the final volume to 100 mL with water.

3.3.3.2 0.1 %w/v chitosan solution

The 0.1 %w/v chitosan solution could be prepared by diluting 1 %w/v chitosan 10 times using acetic-acetate buffer solution at pH 6.0. In the final step, the pH of solution was checked which the obtained pH was 6.0 by calibrated pH meter.

3.3.3.3 Alginate stock solution

Alginate was used as anionic polyelectrolyte for multilayer emulsion preparation. The 1 %w/v alginate solution was prepared by dissolving alginate 1 gram with acetic-acetate buffer solution pH 6.0 and pH 3.5, then stirred overnight. The pH of alginate solution was adjusted to pH 6.0 by adding 100 mM NaOH and pH 3.5 by adding 100 mM HCl. Then, the acetic-acetate buffer was added till the final volume equal to 100 mL.

3.3.3.4 0.1 %w/v alginate solution

The 0.1 %w/v alginate solution could be prepared by diluting 1 %w/v alginate solution 10 times using acetic- acetate buffer solution. In the final step, the pH of alginate solution was adjusted to pH 6.0 by adding 100 mM of NaOH and to pH 3.5 by adding 100 mM HCl.

3.3.3.5 Carrageenan stock solution

Carrageenan was used as anionic polyelectrolyte for multilayer emulsion preparation. The 1 %w/v carrageenan solution was prepared by dissolving powder carrageenan 1 gram with acetic-acetate buffer solution and heat at 70 °C, 20 min, then stirred to ensure complete dissolution of carrageenan. The acetic-acetate buffer to obtain the total volume equal 100 mL. The pH of solution was checked which the obtained pH was 6.0 by calibrated pH meter.

The 0.8 %w/v carrageenan could be prepared by diluting 1 %w/v carrageenan using acetic-acetate buffer solution. In the final step, the pH of solution was checked and adjusted to the desired values.

3.3.4 Solution for lipid oxidation measurement

3.3.4.1 Cumene hydroperoxide standard solution

Stock solution of cumene hydroperoxide which contained 2.675 mM cumene hydroperoxide was prepared by dissolved 50.00 μL of cumene hydroperoxide, which contained 80% purity, into the mixture solvent of isooctane/2-propanol (3:1, v/v) to obtain total volume of 100 mL. Then, the cumene hydroperoxide standard solutions was prepared by diluting stock solution as 50.00, 100.00, 200.0, 500.00, 750 and 1000 μL with the mixture solvent of isooctane/2-propanol (3:1, v/v) to obtain each total volume of 50 mL. The prepared standard cumene hydroperoxide concentration was 2.68, 5.35, 10.7, 26.8, 40.2 and 53.6 μM, respectively.

3.3.4.2 Ammonium thiocyanate solution

The 50.00 mL of 3.970 M ammonium thiocyanate solution was prepared by dissolving in the range 14.90-15.10 grams of ammonium thiocyante with deionized water.

3.3.4.3 Ferrous iron solution

Ferrous iron solution was prepared by mixing 0.132 M of barium chloride ($BaCl_2$) with 0.144 M of ferrous sulphate ($FeSO_4$) in ratio 1:1. The 50.00 mL of 0.132 M barium chloride was prepared by dissolving in the range 1.58-1.62 grams of barium chloride with 0.4 M HCl. While 50.00 mL of 0.144 M ferrous sulphate was prepared by dissolving approximate 1.98-2.02 grams of ferrous sulphate with 0.4 M HCl. The mixing the ferrous sulphate solution was covered with aluminum foil or paraffin to prevent the auto-oxidation of ferrous ion to ferric ion [30].

3.3.4.4 Antioxidants

74 µM genistein was prepared by dissolve 0.002 grams of genistein in 100 mL methanol, while 100, 200 and 500 ppm roselle extract were prepared by dissolve 10.53, 21.05 and 52.63 mg in 100 mL acetic-acetate buffer before added into the emulsifier solution. Sesame oil was extract from sesame seed by using hexane, while capsaicin oleoresin was extract from chilli by using ethanol.

3.4 Experimental procedures

3.4.1 Preparation of emulsions and multilayer emulsions

3.4.1.1 Preparation of primary emulsion

The 4.47 g (5 mL) of tuna oil was added into the 95 mL of emulsifier solution (WPI), which its pH was adjusted to be either 3.5 or 6. The mixture was homogenized at 20,000 rpm for 2 min per cycle for 5 times. The experimental setup was shown in Figure 3.3. All samples were kept in ice bath during processing. The physical and oxidative stability of primary emulsion were investigated to find a suitable condition for stable primary emulsions. The creaming index, % transmission, the particle size diameter and zeta potential were evaluated for the physical stability study and lipid oxidation was studied to evaluate the oxidative stability. All measurements were repeated three times on three different samples [24].

Figure 3.3 The experimental setup.

3.4.1.2 Preparation of secondary and multilayer emulsions

The layer-by-layer (LbL) electrostatic deposition technique was used to prepare multilayer emulsions by strong electrostatic attraction between the surface droplet charge of emulsion and oppositely charged polyelectrolyte molecule solution. Secondary emulsions were formed by adding the primary emulsions into the oppositely charge polyelectrolyte at suitable ratio, then the mixture was homogenized at 20,000 rpm, for 2 min (1 cycle). Tertiary emulsion were formed by adding the secondary emulsions into the oppositely charge polyelectrolyte at suitable ratio, then the mixture was homogenized at 20,000 rpm, for 2 min (1 cycle). All measurements were repeated two times on three different samples.

In this research, we investigated the effect of pH on the preparation of primary emulsion at pH 3.5 and pH 6.0. Polyelectrolytes were present in table 3.3 and 3.4.

Table 3.3 Polyelectrolyte for multilayer emulsions preparation at pH 3.5

Emulsion	Polyelectrolyte
Primary emulsion	WPI
Secondary emulsions	WPI-Alginate
	WPI-Carrageenan
Tertiary emulsions	WPI-Carrageenan-Chitosan

Table 3.4 Polyelectrolyte for multilayer emulsions preparation at pH 6.0

Emulsion	Polyelectrolyte
Primary emulsion	WPI
Secondary emulsions	WPI-Chitosan
Tertiary emulsions	WPI-Chitosan-Alginate

3.4.1.2.1 Investigate the optimum volume ratio between primary emulsions and cationic polyelectrolyte for secondary emulsions preparation at pH 6.0

The equivalence point could be found by mixing primary emulsion with 20 mL 0.1% w/v chitosan with various volume ratios as listed in Table 3.5. Then, we investigated the optimum volume ratio. The secondary emulsion formation was monitored by measuring % transmission at 550 nm of these mixed solutions. All solutions were diluted 100 times before the measurements.

Table 3.5 The volume ratio between primary emulsion and 0.1% w/v chitosan solution at pH 6.0

Volume of primary emulsion (mL)	Volume ratio
1.00	0.05
2.00	0.10
3.00	0.15
4.00	0.20
5.00	0.25

3.4.1.2.2 Investigate the optimum volume ratio between secondary emulsion and anionic polyelectrolyte for tertiary emulsions preparation at pH 6.0

The equivalence point could be found by mixing secondary emulsions with 20 mL 0.1 %w/v chitosan with various volume ratios as listed in Table 3.6. Then, we investigated the optimum volume ratio as well. The tertiary emulsion formation was monitored by measuring % transmission at 550 nm of these mixed solutions. All solutions were diluted 100 times before the measurements.

Table 3.6 The volume ratio between secondary emulsion and 0.1 %w/v alginate solution for determine the optimum condition at pH 6.0

Volume of secondary emulsion (mL)	Volume ratio
1.00	0.05
2.00	0.10
3.00	0.15

Volume of secondary emulsion (mL)	Volume ratio
4.00	0.20
5.00	0.25
6.00	0.30
7.00	0.35
7.50	0.375

3.4.1.2.3 Investigate the optimum volume ratio between primary emulsions and anionic polyelectrolyte for secondary emulsions preparation at pH 3.5

At pH 3.5, primary emulsion contained WPI coated around tuna oil, that in this condition containing cationic droplets. The equivalence point could be found by mixing primary emulsion with 20 mL 0.1 %w/v alginate and 0.8 %w/v carrageenan with various volume ratios as listed in Table 3.7. Then, we investigated the optimum volume ratio of both polyelectrolytes. All solutions were diluted 100 times before the measurements.

Table 3.7 The volume ratio between primary emulsion and anionic polyelectrolyte solutions to determine the optimum condition at pH 3.5

0.1 %w/v Alginate		0.8 %w/v Carrageenan	
Volume of primary emulsion (mL)	Volume ratio	Volume of primary emulsion (mL)	Volume ratio
1.00	0.05	1.00	0.05
2.00	0.1	2.00	0.1
3.00	0.15	3.00	0.15
4.00	0.2	4.00	0.2
5.00	0.25	5.00	0.25
6.00	0.3	6.00	0.3
7.00	0.35	7.00	0.35
8.00	0.4	8.00	0.4
9.00	0.45	9.00	0.45
10.00	0.5	10.00	0.5
11.00	0.55	10.50	0.525
12.00	0.6	11.00	0.55
12.50	0.625		
13.00	0.65		

3.4.1.2.4 Investigate the optimum volume ratio between secondary emulsions and cationic polyelectrolyte for tertiary emulsions preparation at pH 3.5

The equivalence point could be found as described in section 3.4.1.2.3. In this study, the seconday emulsions (WPI-carrageenan) was mixed with 20 mL 0.1 %w/v chitosan with various volume ratios as listed in Table 3.8. All solutions were diluted 100 times before the measurements.

Table 3.8 The volume ratio between secondary emulsion and 0.1 %w/v chitosan solution for determine the optimum condition at pH 3.5.

Volume of secondary emulsion (mL)	Volume ratio
2.00	0.10
4.00	0.20
6.00	0.30
8.00	0.40
10.00	0.50
12.00	0.60
14.00	0.70
16.00	0.80
18.00	0.90
20.00	1.00

3.4.2 Study on the effect of antioxidant on the stability of primary emulsion

For antioxidant evaluation, we selected four kinds of antioxidant (genistein, sesame oil, capsaicin oleoresin, and roselle extract). Sesame oil and capsaicin oleoresin were added directly in tuna oil. While genistein and roselle extract were added directly in emulsifier solution.

The addition of antioxidant direct in tuna oil as follow; added 0.3 and 0.5 grams of sesame oil, and added 0.1, 0.3, 0.5, 0.7 and 0.9 grams of capsaicin oleoresin into tuna oil, then make up total weight as 5.00-5.10 grams with tuna oil. The addition of antioxidant direct in emulsifier solution as follow; added 5.26 and 10.53 mL of genistein into 0.4 %w/v WPI and make up total volume as 100 mL. The roselle extract were dissolve in various contents 0.0011, 0.0021 and 0.0053 grams in 100 mL acetic-acetate buffer at pH 6.0. Then used to dissolve emulsifier, where the final concenrationb of roselle extract were 100, 200 and 500 ppm.

Similar preparation method was used to produce emulsions that the 5 mL of tuna oil was mix with 95 mL emulsifier solution (0.4 %w/v WPI). The mixture was homogenized at 20,000 rpm for 2 min/cycle for 5 time. All samples were held on ice during processing and on three different samples [38].

3.5 Emulsion characterization

3.5.1 Physical stability

3.5.1.1 %Transmission measurement

The measurement of % transmission is a standard method for study creaming stability of emulsion or multilayer emulsion preparation. The 3.50 mL of samples were transferred into plastic cuvette, covered with parafilm and stored at room temperature for 21 days. %Transmission can be measured at 550 nm by using UV-Vis Spectrophotometer. The light beam passed through the emulsions about 30% height of emulsion, at 10 mm from the cuvette bottom. The acetic-acetate buffer at each pH was used as the background and used to dilute emulsions. All measurements were repeated two times on three different samples.

3.5.1.2 Creaming stability measurement

The creaming stability of emulsions was monitored by transferred 10.00 grams of emulsions into a glass test tube (internal diameter of 15 mm, height of 100 mm) covered by a plastic cap. Then, the test tubes were stored at room temperature and measured the cream phase every day for one week.

3.5.1.3 The particle size diameter and zeta potential of emulsions

The particle size diameter and electrical charge (ζ, zeta potential) of emulsions were measured by using dynamic light scattering measurements and electrophoresis. The particle size diameter and electrical charge (ζ, zeta potential) measurement were reported as the average and standard deviation calculated from measurement of at least three freshly prepared samples, with three readings taken per sample. All measurements were made after preparation emulsion for 24 h at room temperature. The emulsions were diluted 100 times by using the acetic-acetate buffer at each pH for avoiding the multiple scattering effects.

3.5.2 Lipid Oxidation Measurement

Hydroperoxide was the primary product from lipid oxidation reaction that can be followed by using the ferric thiocyanate method [34]. In this study, we selected two different methods to monitor the formation of hydroperoxide. First method, the emulsion 3.00 mL was placed in sealed screw-cap test tubes and allowed to be oxidized at room temperature in the dark for up to 21 days. The amount of lipid hydroperoxide was determined on the 0, 1, 3, 5, 7, 9, 11, 13, 15, 17, 19 and 21 days of storage. The second method, the 3 mL of emulsion was placed in sealed screw-cap test tubes and allowed to oxidize at 50°C (±1) for 12 hours in water bath. The lipid hydroperoxide contents were measured every hour [39].

Based on the ferric thiocyanate method [19], the lipid hydroperoxides in the emulsion were measured by mixing 600 μL of emulsion (vortex before pipet) with 3.0 mL of isooctane/2-propanol (3:1, v/v) by vortexing (10 s, three times). The organic solvent phase was separated by centrifugation at 4000 rpm for 2 min. The organic solvent phase (400 μL) was added to 5.60 mL of methanol/1-butanol (2:1, v/v),

followed by 30.00 µL of 3.94 M ammonium thiocyanate and 30.00 µL of ferrous ion solution (prepared by mixing 0.132 M $BaCl_2$ and 0.144 M $FeSO_4$ in acidic solution). The absorbance of solution was measured at 510 nm with UV-Vis Spectrophotometer, 20 min after the addition of ferrous ion. The blank solution could be prepared follow the above steps except, that the isooctane/2-propanol was used to instead the emulsions.

Hydroperoxide concentrations were determined using a standard curve of cumene hydroperoxide with the concentration of 2.68, 5.35, 10.7, 26.8, 40.2 and 53.6 µM. At each concentration were followed by using cumene hydroperoxide at each concentration instead the organic phases of section the emulsion. Then, follow up by the ferric thiocyanate method. All measurements were repeated two times on three different samples.

CHAPTER IV

RESULTS AND DISCUSSION

In this study, we demonstrated to prepare oil-in-water emulsion by using whey protein isolate (WPI) as emulsifier, and prepare multilayers emulsions by using the LbL method to study their oxidative stability and physical stability. The biopolymer such as cationic chitosan, and anionic alginate and carrageenan were selected to coat droplets.

In this chapter, the physical and oxidative stability studies will be reported in 3 parts. First and second parts, the physical and oxidative stability studies of primary, secondary and tertiary emulsions at pH 6.0 and pH 3.5 were reported, respectively. The third part, the effects of antioxidant on the physical and oxidative stabilities was evaluated.

In previous studies, the most stable primary emulsion using WPI as emulsifier could be prepared at both pH 3.5 and 6.0 in separate studies [37, 40]. Thus, we chose both pHs for this study and compared for the best condition. Because the p*I* of the WPI on the emulsion droplets was found to be 4.8 [30] At pH above 4.8, the emulsion droplets stabilized with WPI are negatively charged, whereas at pH below 4.8, the droplets are positively charged [30].

4.1 The study of the physical and oxidative stabilities of primary and multilayer emulsions at pH 6.0

In order to prepare the suitable primary emulsion by using WPI as emulsifier solution, the factors effecting properties depend on homogenization cycles, pH of emulsifier solution, and WPI concentrations. Thus, in our study, the primary emulsion was prepared by homogenizing 5 %w/v tuna oil and 95 %w/v of emulsifier solution at 20,000 rpm for 2 minutes/cycle for 5 homogenization cycles [38].

4.1.1 The effect of WPI concentration on the stability study of primary emulsion

WPI concentrations are known to influence emulsion droplet size, surface protein concentration and storage stability [32]. Thus, four different concentrations of WPI including 0.20, 0.30, 0.40 and 0.50 %w/v were tested. The physical stability of emulsions was monitored by measuring the creaming stability and % transmission. If the droplet aggregation and creaming occurred, the creaming index would increase which indicated the instability to creaming [41, 42]. Lipid hydroperoxide after storage was also determined to study the oxidation stability.

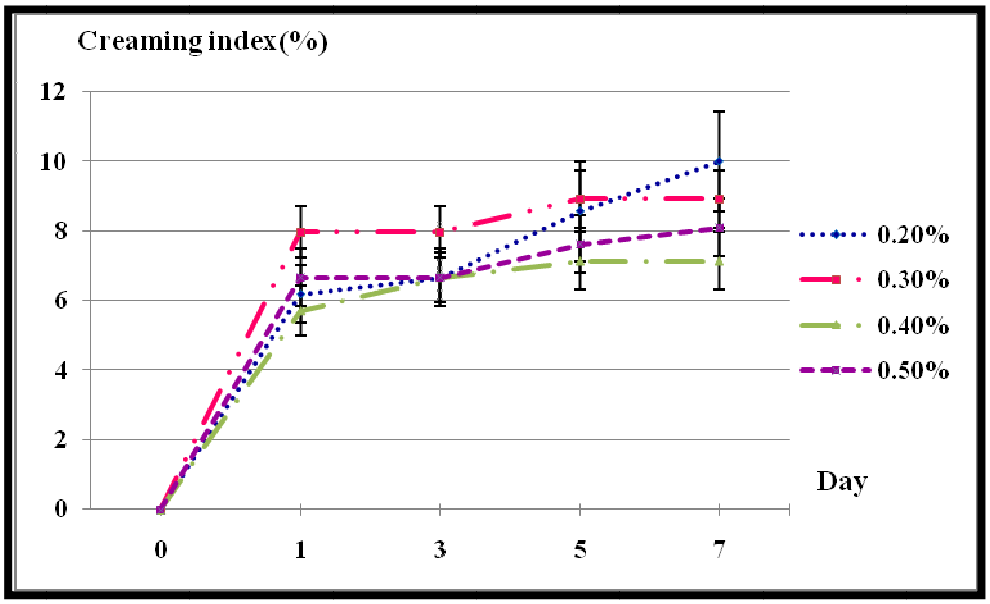

Figure 4.1 Effect of WPI concentrations on creaming index (%) of primary emulsion at pH 6.0.

From Figure 4.1, the changing of creaming index of the emulsions during the storage time was shown. For everyday, emulsions showed the increasing of creaming index. After 1st day, emulsions prepared with 0.3 %w/v WPI showed the highest creaming index, while 0.2, 0.3 and 0.4 %w/v WPI showed similar increasing in creaming index. There were not significant changes in the creaming index when increased WPI concentrations. On the 7th day, emulsions prepared with 0.4 %w/v WPI showed the least increasing in creaming index compared to the other concentrations. After 7th day, the creaming indexs were relatively constant (data not shown).

The emulsifier concentration influenced the formation of the oil droplets and their stability. There was sufficient protein present to cover the surface of the oil droplets formed in the homogenizer, but that the protein were not effective at either creating small droplets within the homogenizer or preventing droplet aggregation during or after homogenization [32]. At 0.5 %w/v WPI showed the higher creaming index more than 0.4 %w/v WPI. In this study, 0.4 %w/v WPI showed the best physical properties. Previous studies have showed the importance of the molecular environment in determining the oxidative stability of an oil-in-water (o/w) emulsion. Emulsifiers play a role in the oxidative stability of oil droplets [32]. The emulsifier concentration influences to the emulsion stability [43]. Consider the effect of concentration WPI on the primary emulsion preparation, that when increased concentration of WPI, the stability was improved. But primary emulsion stabilized by 0.5 %w/v WPI has less stable. It may be the result of the excess of WPI concentration in the emulsion causing the aggregate of WPI molecule and droplets. Thus, in this study, 0.4 %w/v WPI showed the best stability of emulsion.

Effects of WPI concentration on lipid hydroperoxides of primary emulsion at pH 6.0 after the fourteen days of storage were shown in Figure 4.2. Ferric thiocyanate can be used to monitor the lipid hydroperoxide amount. The ferric thiocyanate method is based on the oxidation of ferrous ion (Fe^{2+}) to ferric ion (Fe^{3+}), which are determined as ferric thiocyanate $[FeSCN]^{2+}$ [39]. The absorbance of ferric thiocyanate solution was measured at 510 nm. Hydroperoxide concentrations were determined using a standard curve made from cumene hydroperoxide. If the absorbance was increased, hydroperoxide concentration increased as well.

The good linearity of the standard curve of cumene hydroperoxide was observed over the concentrations of cumene hydroperoxide ($R^2 = 0.990$).

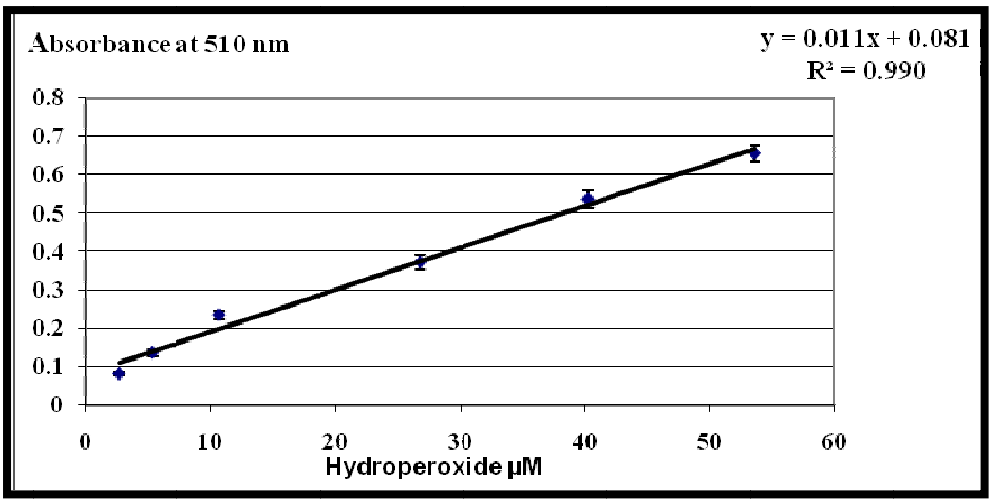

Figure 4.2 The calibration curve of hydroperoxide by using cumene hydroperoxide as standard solutions.

From Figure 4.3, the lipid hydroperoxide content of all emulsions were basically unchanged till the seventh day of storage where the lipid oxidations of tuna oil occurred and increased with longer storage time. After the 7th day, the lipid hydroperoxide of all emulsions were increased rapidly but the emulsion prepared with 0.4 %w/v WPI was slightly increased. During the period from the 7th to 13th day, the emulsion prepared with 0.4 %w/v WPI delivered the least formation of hydroperoxides. This might be the result of WPI was adsorbed to the surfaces of the oil droplets and prevent the attraction lipid from metal [31]. We observed that the emulsion prepared with 0.5 %w/v WPI has less oxidative stability comparing with 0.4 %w/v WPI, which we cannot explain yet.

Thus, in our study, the primary emulsion was prepared by homogenizing 5 %w/v tuna oil and 95 %w/v of emulsifier solution containing 0.4 %w/v WPI at 20,000 rpm for 2 minutes/cycle for 5 homogenization cycles.

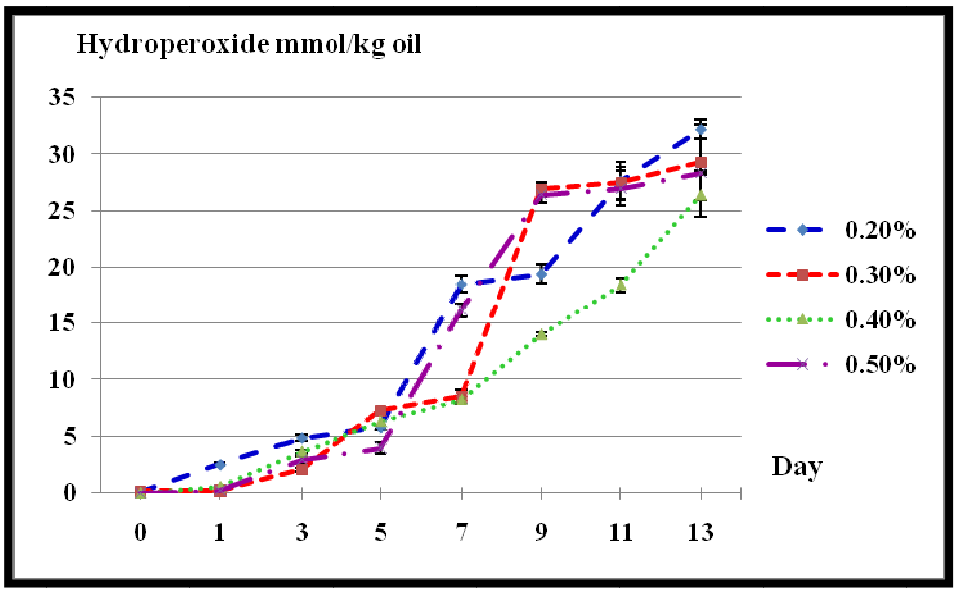

Figure 4.3 Effect of WPI concentrations on lipid hydroperoxides of primary emulsion at pH 6.0.

4.1.2 Preparation of multilayer emulsions

The formation of multilayer emulsion can be prepared by coating emulsion with biopolymers, that have opposite-charged surface. As we have shown before that the layer-by-layer (LbL) deposition method has been used to form multilayer emulsion [7, 33, 34]. However, the main challenge in the preparation of multilayer emulsions is to find the suitable ratio between the volume of emulsion and opposite-charged polyelectrolyte to obtain stable multilayer emulsions.

In this study, the natural food grade ingredients, alginate and carageenan were used as polyanionic polyelectrolytes and chitosan was used as polycationic polyelectrolyte. At pH 6.0, primary emulsion contained 0.40 %w/v WPI showed negative charged droplet [31]. Thus, cationic polyelectrolyte, chitosan, was used for secondary emulsions.

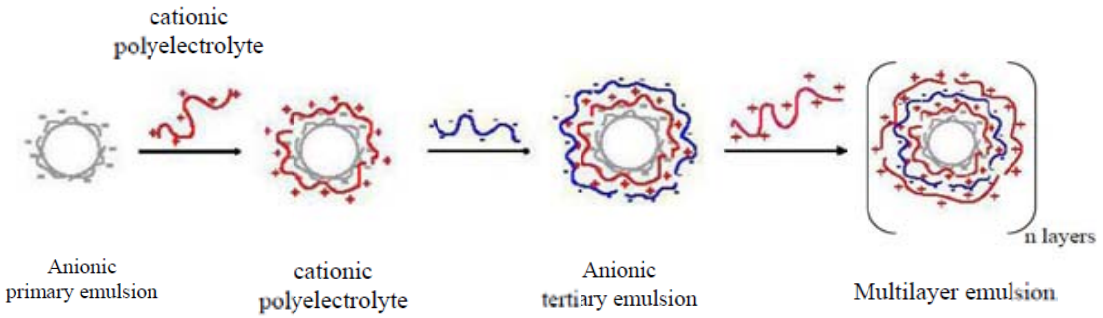

Figure 4.4 Schematic of multilayer emulsion preparation [38].

4.1.2.1 Determination of the optimum volume ratio between primary emulsions and cationic polyelectrolyte for secondary emulsions preparation

Secondary emulsions can be prepared by mixing primary emulsion with opposite charged polyelectrolyte. We investigated the optimum volume ratio, the equivalence point between primary emulsions and cationic polyelectrolyte, where there is no any excess of polyelectrolyte to cause the droplet, resulting in the instability of emulsion. The primary emulsion (mL) was dropped into the polyelectrolyte and monitored by measuring % transmission. Chitosan was selected to produce secondary emulsions.

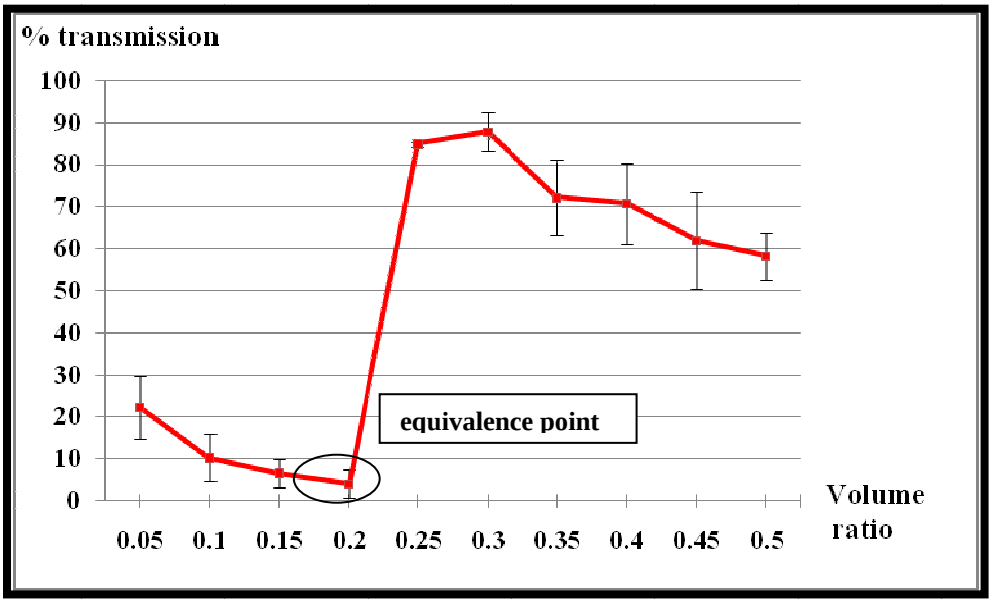

Figure 4.5 The titration curve between primary emulsion and 0.1 %w/v chitosan at pH 6.0.

Figure 4.5 showed the titration curves between primary emulsion and 0.1 %w/v chitosan on %transmission. The graph was separated into 3 sections. First section (volume ratio of 0.05-0.20), the % transmission slowly decreased with increasing the volume of primary emulsion indicating the formation of secondary emulsion [38]. For the second section where the volume ratio covering 0.20 to 0.30, the % transmission was rapidly increasing. This section we could see a precipitation in the solution because the complex between secondary emulsion and the excess primary emulsion. For the last section (volume ratio of 0.30-0.50), the % transmission decreased again due to the addition of primary emulsion making opaque. That the mechanism for preparation secondary emulsion was showed in Figure 4.6.

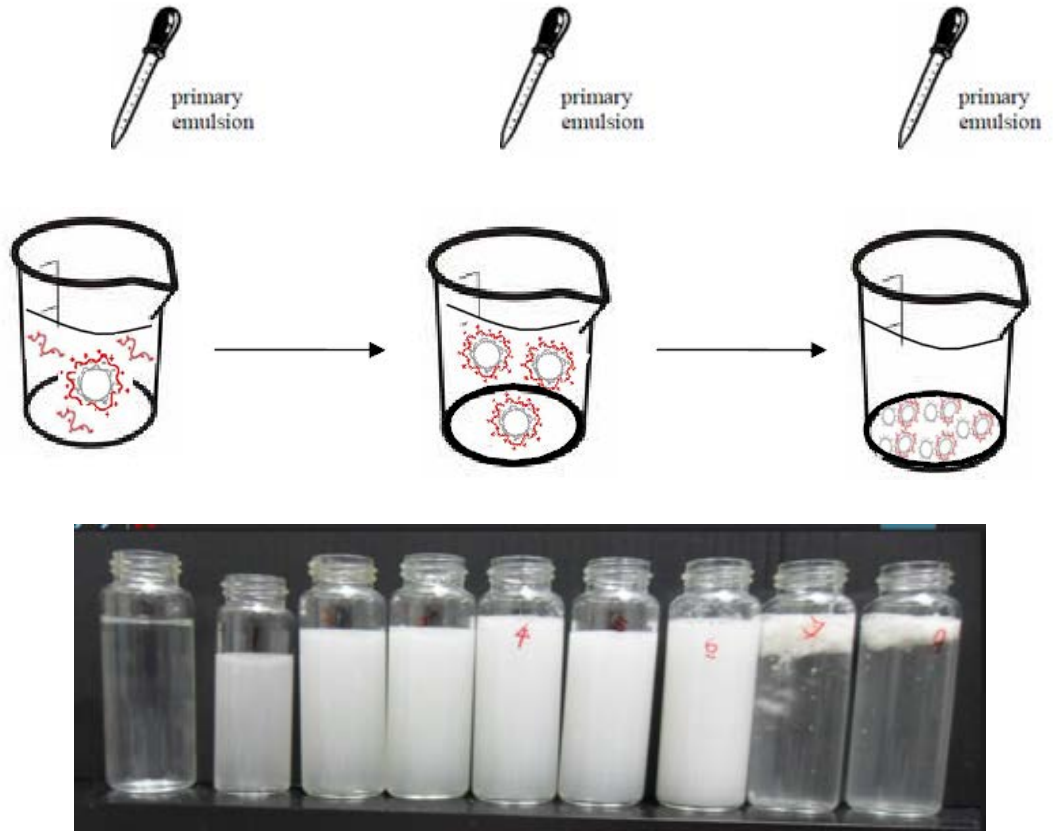

Figure 4.6 The mechanism for preparation secondary emulsion by titration between emulsion and polyelectrolyte.

Thus, at pH 6.0 the volume ratio between primary emulsions and 0.1% w/v chitosan equals to 0.20 suitable for secondary emulsions preparation.

4.1.2.2 Determination of the optimum volume ratio between secondary emulsions and anionic polyelectrolyte for tertiary emulsions preparation

Tertairy emulsions were formed by mixing secondary emulsion with oppositely charge polyelectrolyte as same as secondary emulsion preparation. In this research secondary emulsions at pH 6.0 was coated by chitosan, so we chose alginate, anionic polyelectrolytes at pH 6.0 for tertiary emulsions preparation. Alginate are a group of acidic polysaccharides that occur naturally as the major structural polysaccharides of brown marine algae and as extracellular mucilages and pKa of alginate is from 3.38 to 3.65 [37]. The optimum volume ratio between secondary emulsions and this polyelectrolyte was investigated for suitable tertiary emulsions. Figure 4.7 showed the titration curve between secondary emulsions and 0.1 %w/v alginate on % transmission.

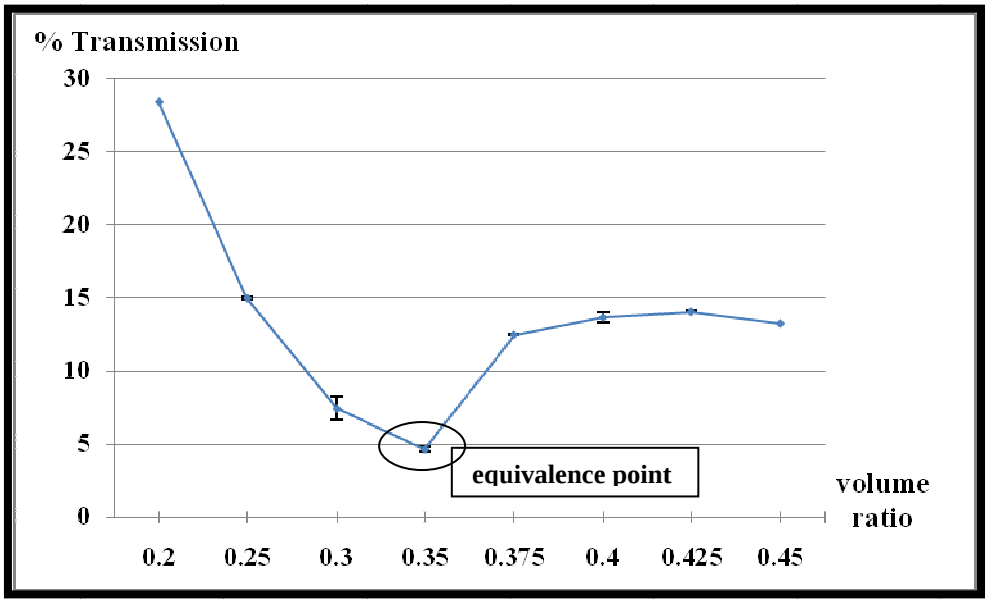

Figure 4.7 The titration curve between secondary emulsions and 0.1% w/v alginate at pH 6.0.

The similar curve as reported in section of the determination of volume ratio for secondary preparation was obtained and shown in Figure 4.7. At the volume ratio of 0.20-0.35, the solution became more opaque where % transmission decreased gradually with increasing the volume of secondary emulsion indicating the

formation of tertiary emulsion. At volume ratio of 0.35, the lowest % transmission was observed. The volume ratio larger than 0.35, %transmission was increased because precipitation of complex in a solution was observed. After this point the % transmission was found to decrease again because of the excess of secondary emulsion.

Thus, to prepare the tertiary emulsions at pH 6.0 the suitable volume ratio between secondary emulsions and 0.1% w/v alginate equals to 0.35.

4.1.2.3 Chracterization and physical stability of primary, secondary and multilayer emulsions

The physical stability of primary, secondary and multilayer emulsions were measured during storage at 30 °C. In this study, we investigated on the zeta-potential, mean particle diameter and creaming stability.

4.1.2.3.1 The particle size diameter and zeta potential of emulsions

The particle size diameter and zeta potential (ζ) measurements indicated the stability of emulsions. We investigated the particle size distribution of all types of emulsions in our studies were bimodal distribution (data was shown in Appendix). This unlike from previous report where WPI-stabilized emulsion exhibited monomodal droplet distributions [30]. Because of the limit of instrumentation in our laboratory, we could not improve the size distribution of our emulsion. We selected to presence the first peak of size distribution that composed of smaller droplet size that exhibited the higher population, which was more than 70% for most cases.

From Figure 4.8, the particle sizes of emulsion were increased with increasing numbers of coating layer around the oil droplets. The particle size of tertiary emulsion was increased comparing with primary and secondary emulsions. These measurements indicated chitosan molecules adsorbed on the surface of the WPI stabilized primary emulsion droplet and indicated the alginate molecule adsorbed on the surface of secondary emulsion (WPI-chitosan) [37, 44].

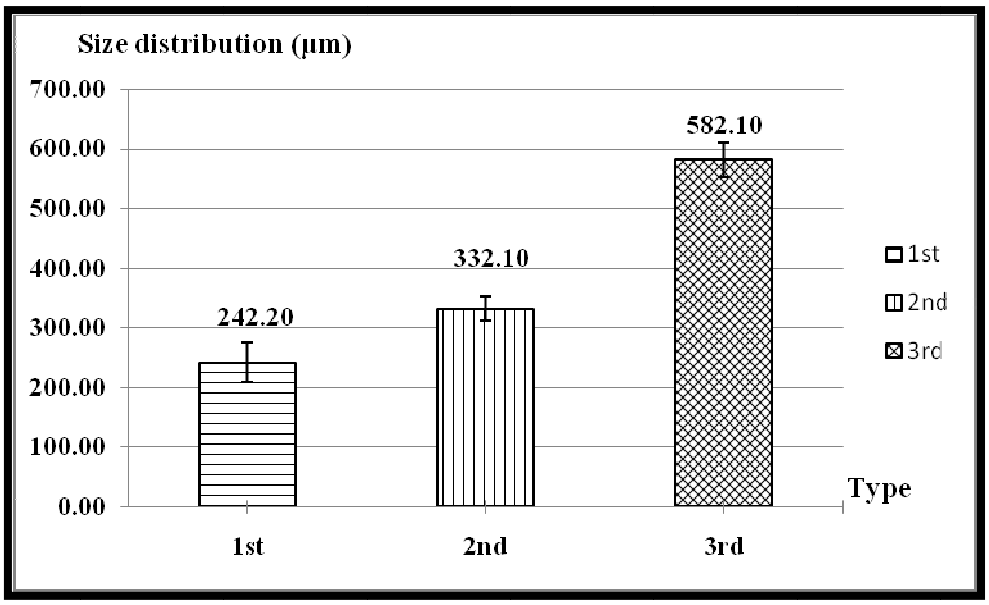

Figure 4.8 The particle size of primary, secondary and tertiary emulsions which diluted 100 times at pH 6. All emulsions were measured after 24 hours of storage.

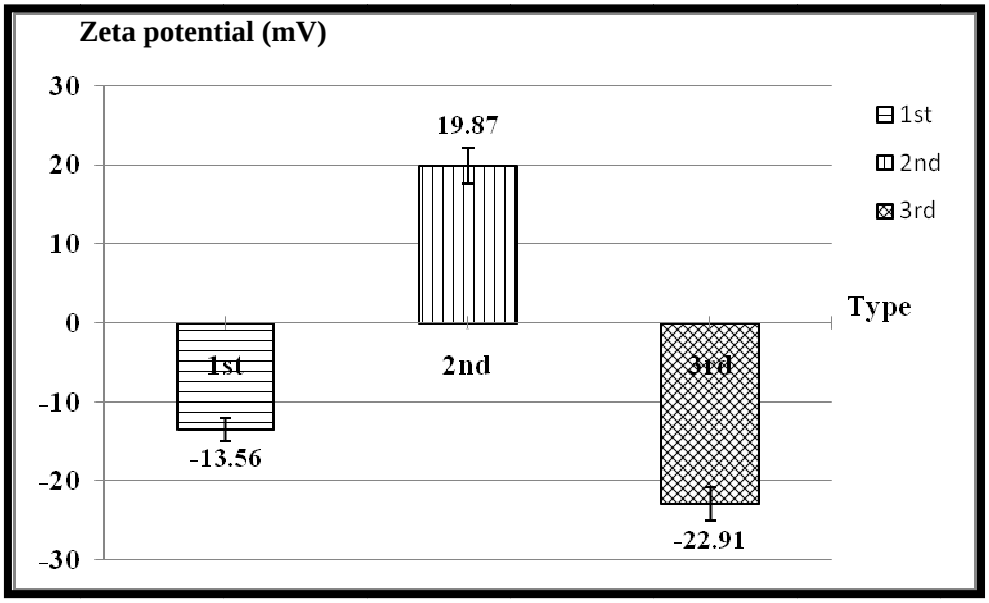

Figure 4.9 The zeta potential of primary, secondary and tertiary emulsions which diluted 100 times at pH 6. All emulsions were measured after 24 hours of storage.

From Figure 4.9, the negative charge on the primary emulsions suggests that the whey proteins have coated around the oil droplets (pH > p*I* (4.8)). The zeta potential of the droplets in the secondary emulsions was positive,

that can be explained by the property of chitosan, which the cationic groups on chitosan have pKa ~6.5 and at pH 6.0 can be exhibited the positive charge [44]. The tertiary emulsions exhibited the negative charges indicating the coating of WPI-chitosan-alginate layer around the oil droplets. It is because the fraction of the anionic groups on the alginate molecule [37, 44].

4.1.2.3.2 Physical stability

% Transmission at 550 nm of emulsions can be used to study the creaming stability of emulsion.

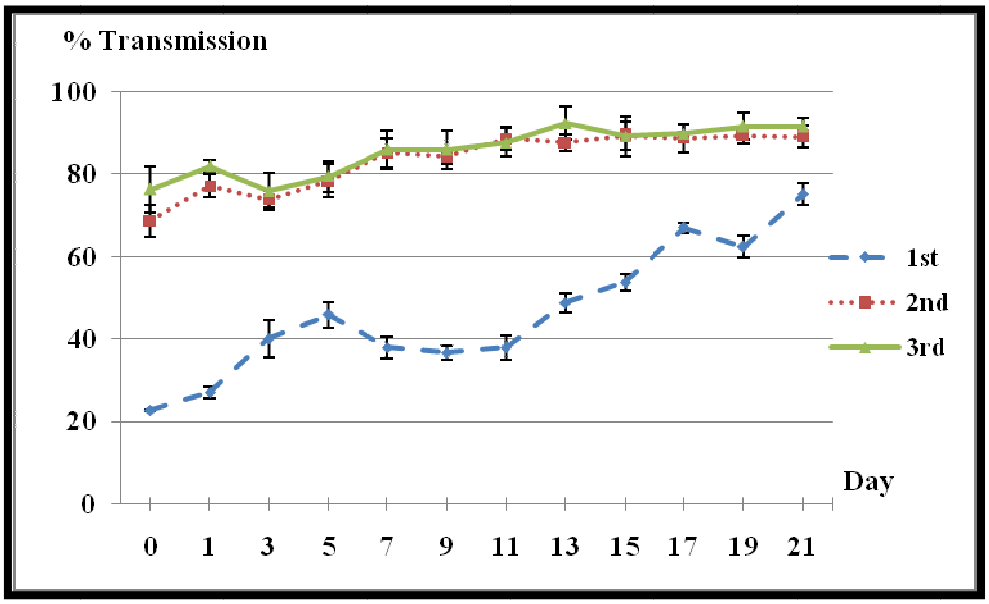

Figure 4.10 % Transmission of primary emulsion (5 %w/v oil stabilized by 0.40 %w/v WPI), secondary emulsions (0.83 %w/v oil stabilized by 0.07 %w/v WPI and 0.083 %w/v chitosan), tertiary emulsions (0.24 %w/v oil stabilized by 0.02 %w/v WPI, 0.029 %w/v chitosan and 0.071% w/v alginate) at pH 6.0, diluted 100 times.

Primary emulsion was highly susceptible to creaming, after five days of storage, and % transmission was dramatically increased more than secondary and tertiary emulsions for 21 days of storage. For multilayer emulsions, we observed similar stability among freshly prepared multilayer emulsions. Figure 4.10 and table 4.1 showed the changes of % transmission during the storage.

Table 4.1 The changing of % transmission

	First day	On the 21th day	% Change*
1st emulsion	22.07	75.19	53.12
2nd emulsions	77.13	89.09	11.96
3rd emulsions	81.92	91.54	9.62

* % changing was the different compared % transmission on the 21th day with % transmission on the first day.

The physical stability was improved when preparing as multilayer emulsions, which is in agreement with previous study [34]. Tertiary emulsions improved the most. For the tertiary emulsions showed similar total charge with secondary emulsions, but more total charge compared with primary emulsion. Thus, the improvement in physical stability might be the result of increase in the thickness of facial layer between the oil and external environment and the ability to repel metal transition from total charge.

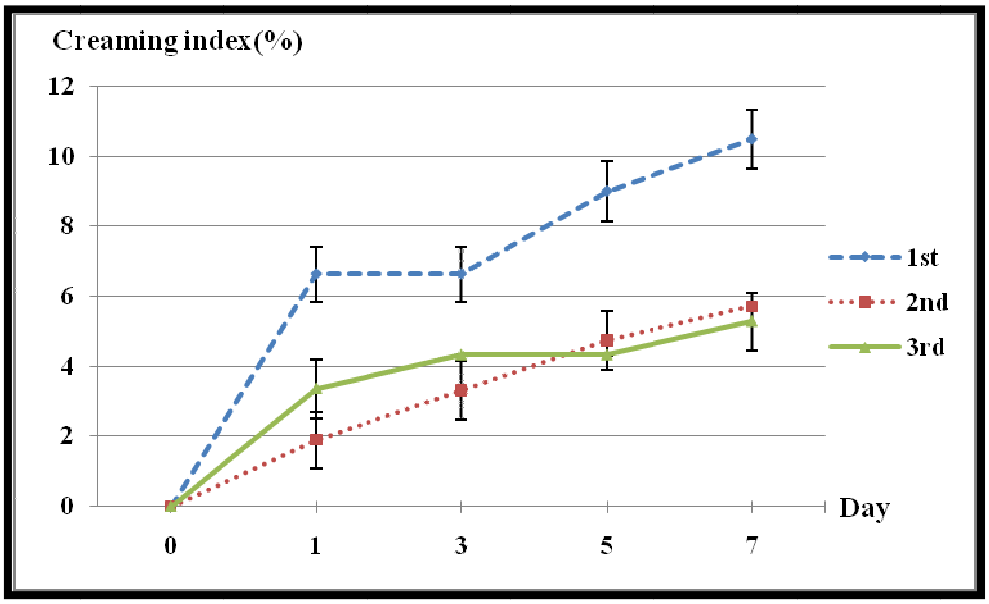

Figure 4.11 The creaming stability of primary emulsion (5 %w/v oil), secondary emulsions (0.83 %w/v oil) and tertiary emulsions (0.24 %w/v oil) at pH 6.0, diluted 100 times.

The changing in % creaming index for 7 days of storage was shown in Figure 4.11. The increase in the creaming index indicates the decrease in the emulsion stability. On the first day, freshly prepared primary emulsion exhibited very similar stability to multilayer emulsions. After the first day of storage, % creaming index of primary emulsion increased more than the others indicating that the primary emulsion was less stable to creaming compare to multilayer emulsions.

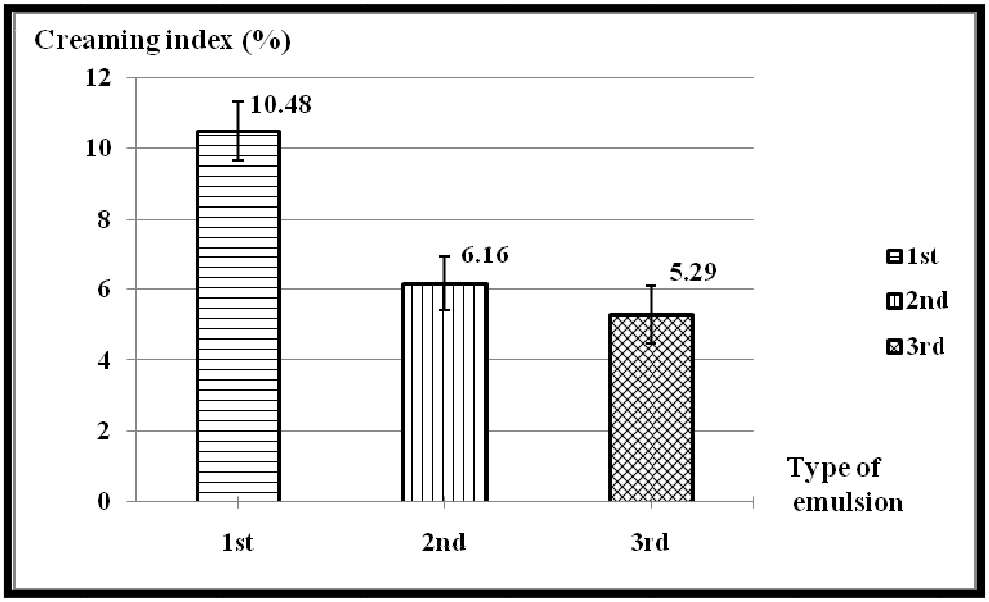

Figure 4.12 The %creaming index on the seventh day of storage of primary emulsion (5 %w/v oil), secondary emulsions (0.83 %w/v oil) and tertiary emulsions (0.24 %w/v oil) at pH 6.0.

This show the similarly trend as in section studied the changing of %transmission that the secondary and tertiary emulsions displayed thebetter creaming stabilities as shown in Figure 4.12. This observation is in agreement with the study of Guzey, and coworker where the tertiary emulsions (lecithin-chitosan-pectin) showed the stability toward the droplet aggregation. This is because the multiple layers decrease the driving force for the gravitational separation and increased the overall density of the droplets (decreased the density difference between the dispersed and continuous phases) [20].

Table 4.2 The summary of particle size mean diameter at the intensity of the first peak and zeta potential of primary, secondary and tertiary emulsion at pH 6.0. The mixture solution was measured after 24 hours of storage then diluted 100 times for the measurement.

Emulsion	% Oil content	Concentration of Polyelectrolyte	Mean diameter(μM)	Zeta potential(mV)
Primary emulsion	5 %w/v	0.4 %w/v WPI	242.20 (±34.03)	-13.41 (±1.71)
Secondary emulsions	0.83 %w/v	0.07 %w/v WPI + 0.83% w/v chitosan	332.1 (±19.87)	19.87 (±2.21)
Tertiary emulsions	0.24 %w/v	0.02%w/v WPI + 0.029 %w/v chitosan+ 0.1% w/v alginate	582.10 (±28.47)	-22.91 (±2.06)

4.1.2.4 Lipid hydroperoxide

The unsaturated lipids could undergo the lipid peroxidation reaction producing the highly reactive and unstable hydroperoxides. [45]. In this study, we chose a analysis method based on the oxidation of Fe(II) to Fe(III) by peroxides, followed by the colorimetric detection of the latter as the thiocyanate complex ($[FeSCN]^{2+}$) [3]. The absorbance of ferric thiocyanate solution was measured at 510 nm. Hydroperoxides concentrations were determined using a standard curve made from cumene hydroperoxide [39].

The good linearity of the standard curve of cumene hydroperoxide was observed over the concentrations of cumene hydroperoxide ranging from 1.34 μM to 53.60 μM (R^2 = 0.990, from Figure 4.2).

The fatty acid compositions of the tuna oils varied in total polyunsaturated fatty acid (PUFA). Figure 4.13 showed the amount of hydroperoxides presents in tuna oil and tuna oil-in-water emulsions for 21 days of storage.

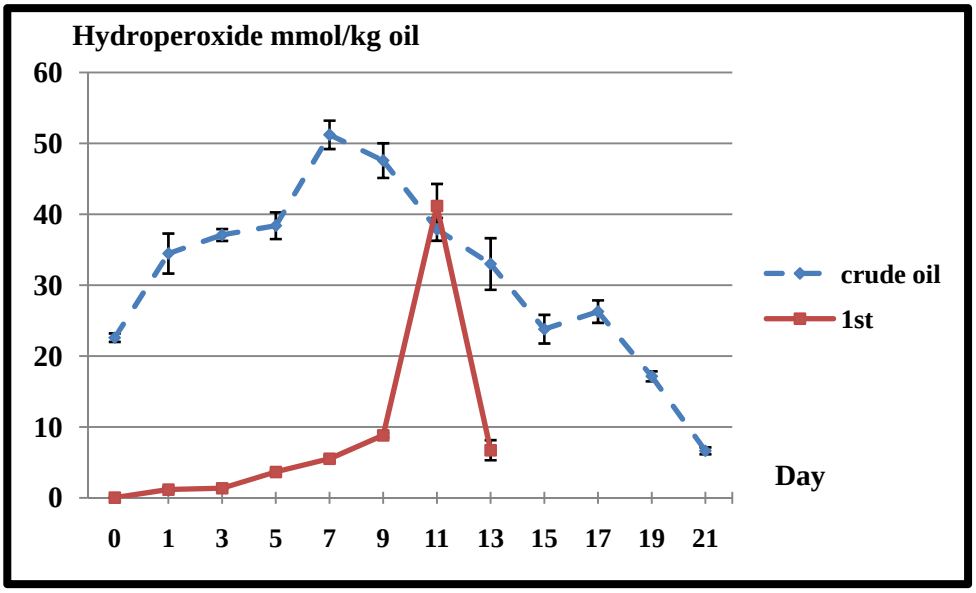

Figure 4.13 Formation of lipid hydroperoxides in crude oil and primary emulsions at pH 6.0 after 21th days of storage.

Figure 4.13 showed a comparison of oxidation rates in tuna oil and primary emulsions. The oxidative stability of tuna oil was improved by preparing as emulsions. Although the charges around the oil droplets are negative, the formation as droplets help decreasing the oil content with the surrounding media. Moreover, the the sulfhydryls group in WPI chain represented the primary antioxidant. It could be useful antioxidant in oil-in-water emulsions [46].

Figure 4.14 showed a comparison of oxidation rates in tuna oil-in-water emulsions stabilized by WPI (primary emulsions), WPI-chitosan (secondary emulsions) and WPI-chitosan-alginate (tertiary emulsions).

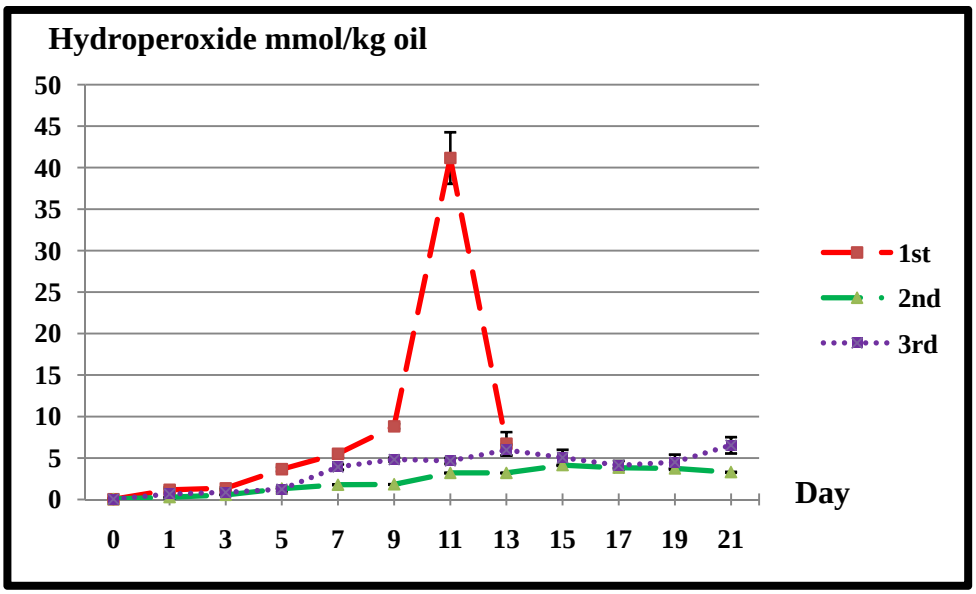

Figure 4.14 Formation of lipid hydroperoxides in primary, secondary and tertiary emulsions at pH 6.0 after 21th day of storage.

On the first seven-days there were little changes on the emulsion oxidation rate in all emulsions. However, primary emulsion showed the higher oxidation rate comparing to the multilayer emulsions. After seven days of storage, the larger amount of lipid hydroperoxides were generated from the primary emulsions and was increased rapidly after the 9th days of storage, whereas the lower content were observed in the secondary and tertiary emulsions on the same day. In primary emulsions, the oxidation rate reached the maximum on the 11th storage date and then decreased. The decrease in the lipid hydroperoxide content might be the result of the degradation of itself to the secondary product, propanal, which we did not monitored this compound in this study. From previous studied, Frankel, reported the formation of secondary oxidation products were various [13]. The secondary products could be monitored by thiobarbituric acid (TBA) method.

Moreover, there was condense as a cube on the top of solution, which might create errorness in the sampling process. The lipid hydroperoxide concentration of primary emulsion was found to be 41.17 mmol/kg oil. After the 13th days of storage, we could not study because mold occurred in the emulsions.

For the secondary and tertiary emulsions stabilized had the lowest level of lipid hydroperoxide concentration compared with primary emulsion.

At the 11th days of storage, we found the maximum amount of lipid hydroperoxide in primary emulsion, but it doesn't occur in both secondary and tertiary emulsions. That showed multilayer emulsions can retard oil droplet from the metal transition and environment.

The greater stability of the secondary emulsions to lipid oxidation can be attributed to the ability of positively charged of WPI-chitosan (+19.03 mV), therefore electrostatically repel prooxidative transition metals and inhibit oxidation [30]. Decker, E. A. showed the experimental that tuna-oil-in-water emulsion droplet coated by lecithin and chitosan more oxidatively stable than emulsions coated by lecithin alone [26]. Nevertheless, the tertiary emulsions had a better oxidative stability than primary emulsion, even though both of them have negatively charges. From the zeta potential, tertiary emulsions had a higher total charge comparing with primary emulsion. There are show the stronger repel prooxidative transition metals. Moreover, the thicker interfacial membrane produced by WPI-chitosan-alginate would impact the interactions between tuna oil and aqueous phase prooxidant, which could be confirmed by the larger particle size of tertiary emulsions (582.10 μM) comparing to the primary emulsions (242.20 μM).

Due to the spoilage of emulsion before we could observe the changes in lipid hydroperoxide content, the heat up method was used to enhance the oxidation reaction of unsaturated fatty acid [4]. The emulsion was allowed to oxidize at 50 °C in the water bath up to 12 hours. Amount of hydroperoxides obtained from tuna oil and primary emulsions was determined by the thyocyanated method.

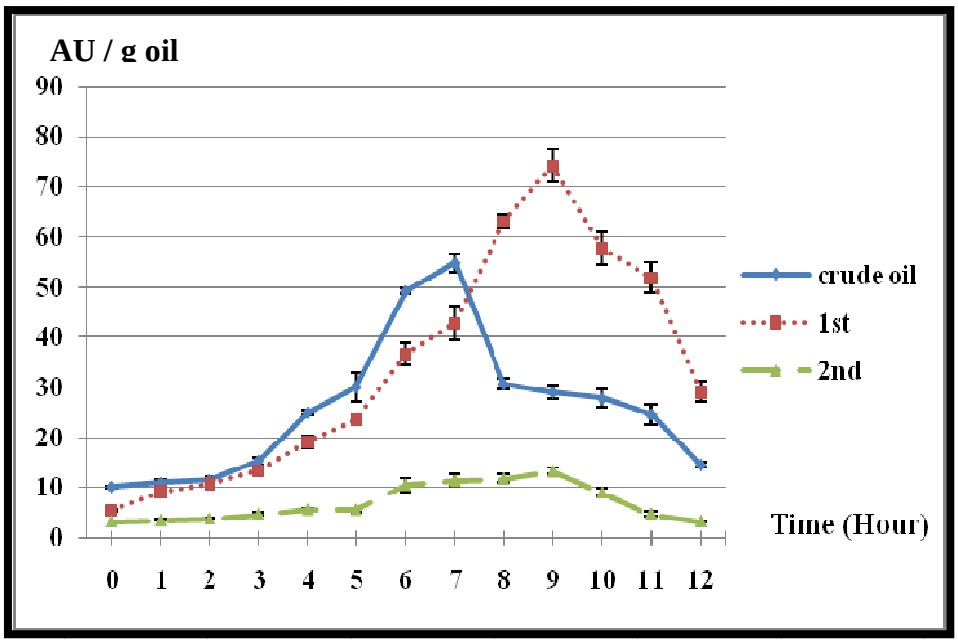

Figure 4.15 Formation of lipid hydroperoxides in crude oil, primary and secondary emulsions at pH 6.0 at 50 °C in the water bath up to 12 hours.

A comparison of oxidation rates in tuna oil and tuna oil-in-water emulsions stabilized by WPI alone (primary emulsions) and WPI-chitosan (secondary emulsions) was presented in Figure 4.15. The rate of oxidation was present as the absorbance at 510 nm per grams oil against time. The level of AU/g oil are related with the formation of lipid hydroperoxide also indicating the oxidative stability of tuna oil. The lipid hydroperoxide of tuna oil was higher comparing to the primary emulsion. The highest level of hydroperoxide generating by crude oil was observed at 7th hour. The level of hydroperoxide obtaining from primary emulsion was increased slowly from 0 hour and up to the highest level hydroperoxide at the 9th hour. We could see the little hydroperoxide in the secondary emulsions. The greater stability of the secondary emulsions can be attributed to the fact that WPI-chitosan coated droplets are a positive charged that can repel prooxidative transition metals. Ogawa et. al [33], showed the tuna oil-in-water emulsions coated by lecithin and chitosan were more oxidatively stable than emulsions coated by lecithin alone. From our study, we observed similar phenomenon as reported by many studies that the lipid oxidation of tuna oil can be improved by preparing as multilayer emulsion [20, 22, 35-38]. The decreasing of oxidation rate of tuna oil and all emulsions, that might be

caused by the decompose of the lipid hydroperoxide (primary oxidation products) to the secondary oxidation products [13].

4.2 The study of the physical and oxidative stabilities of primary and multilayer emulsions at pH 3.5

In this study, we selected protein to use as emulsifier, WPI. When WPI was absorbed onto the surface of emulsion, it is possible showed the electrical charge. The p*I* of the WPI on the emulsion droplets was found to be 4.8 [30]. This means that at pH > 4.8 the emulsion droplets stabilized with WPI are negatively charged, whereas at pH < 4.8 the droplets are positively charged. Thus, at pH 3.5 the emulsion prepared by WPI was positively charged.

4.2.1 Preparation of primary emulsion

The emulsions at pH 3.5 were prepared using similar preparation condition as described in section 4.4.1. By homogenizing 5 %w/v tuna oil and 95 %w/v of emulsifier solution containing 0.4 %w/v WPI at 20,000 rpm for 2 minutes/cycle for 5 homogenization cycles.

The particle size diameter and zeta potential of primary emulsion prepared at pH 3.5 were 297.32 μm and +34.44 mV, respectively. Because pH below the p*I* of WPI showed positive charge [30]. The creaming index of primary emulsion increased after the first day of storage. On 7^{th} days, the creaming index was 9.13. After 7^{th} day, the creaming index were relatively constant (data not shown).

In comparison between primary emulsion prepared at pH 6.0 and 3.5, we could see similar particle size diameter and creaming index. Figure 4.16 showed the formation of lipid hydroperoxides at both pH values. Although on the first nine storage days, the higher content of lipid hydroperoxide was obtained from the emulsion at pH 3.5, the highest amount of lipid hydroperoxide obtaining from this emulsion was reached on the 13^{th} day of storage after emulsion controlled at pH 6. This result is difference from previous study by, Hu and coworker [30]. They reported that the formation of lipid hydroperoxide was much lower at pH values below the p*I* of WPI. Our result might be caused by the difference composition in WPI, which can influence in the formation of lipid hydroperoxide. Moreover, these

two experiments were performed on different period of time that the composition of tuna oil might be different.

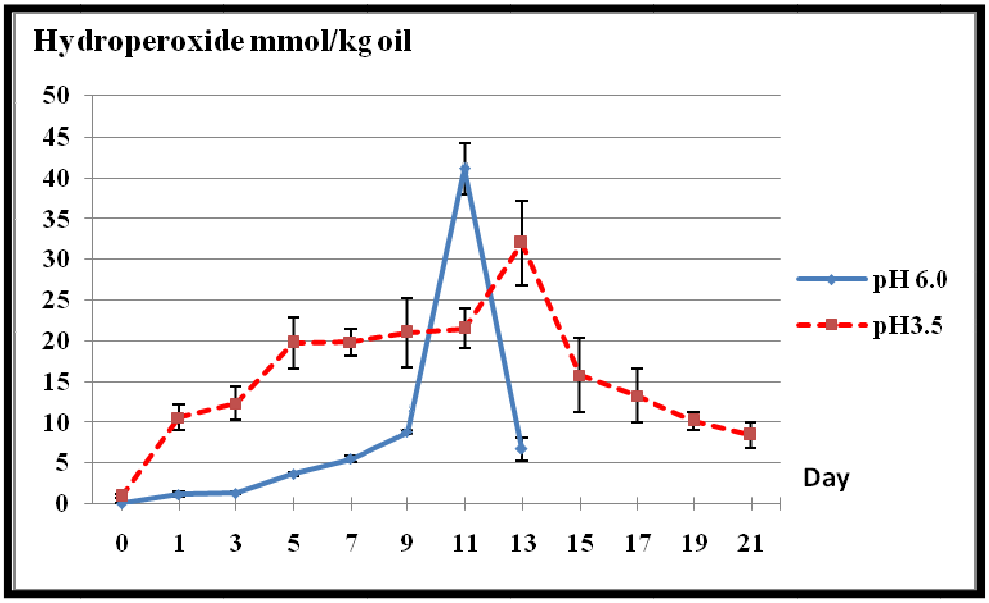

Figure 4.16 Comparison the formation of lipid hydroperoxides in primary emulsions between at pH 6.0 and pH 3.5 after 21 day of storage.

4.2.2 Determination of optimum volume ratio between primary emulsions and anionic polyelectrolyte for secondary emulsions preparation.

At pH 3.5, primary emulsions contained 0.40 %w/v WPI showed positive charges (p*I* of WPI ~ 4.8) [30], thus alginate and carrageenan were selected to prepare secondary emulsions. We investigated the optimum volume ratio, the equivalence point between primary emulsion and anionic polyelectrolyte where there is no any excess of polyelectrolyte to cause the droplet aggregation. The % transmission was used to monitor the secondary emulsion formation as shown in Figure 4.17 and 4.18.

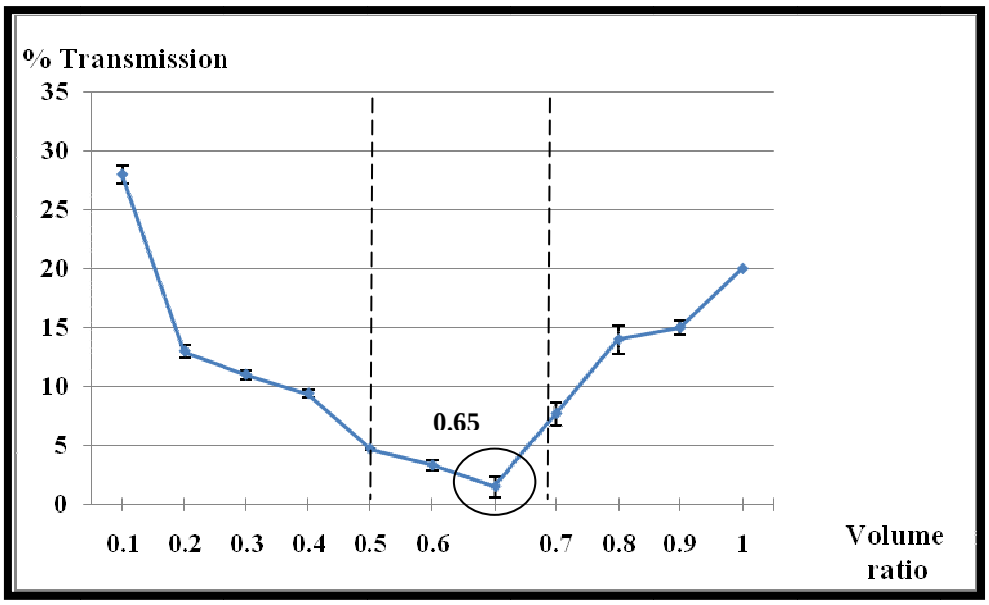

Figure 4.17 The titration curve between primary emulsion and 0.1 %w/v alginate at pH 3.5.

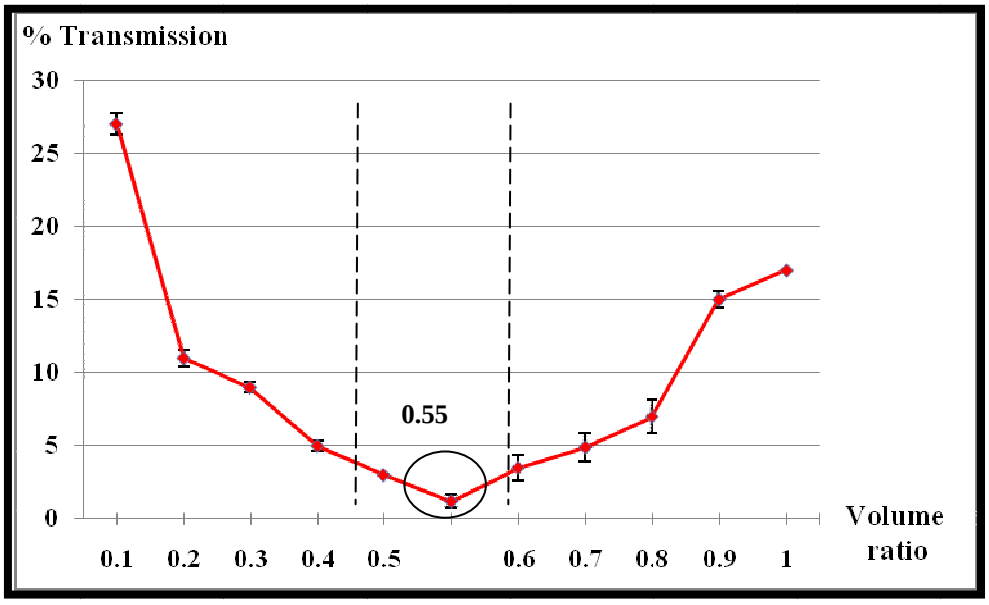

Figure 4.18 The titration curve between primary emulsion and 0.8 %w/v carrageenan at pH 3.5.

We could see that graph was separated into 3 sections. As described in the section 4.1.2.1 that the first part, solution became more opaque where % transmission decreased gradually with increasing the volume of emulsion. In the

second part, the lowest %transmission was observed. For the last part, we observed a precipitation in the solution which resulted in the increase of %transmission.

Therefore, the lowest % transmission is the equivalence point between primary emulsion and anionic polyelectrolytes. The observed equivalence points were in the range volume ratio of 0.50-0.65 for alginate and 0.50-0.60 for carrageenan. We chose to prepare the secondary emulsions between primary emulsion and 0.1% w/v alginate using the volume ratio of 0.65. The volume ratios between primary emulsion and 0.8% w/v carrageenan equal to 0.55 was used to prepare WPI-carrgenan secondary emulsion.

The difference in the optimum volume between these two polyelectrolytes might be due to the different in concentration and less ionized groups presenting in the alginic acid molecule (pKa of alginate = 3.38 to 3.65) [37], because that it is close to pKa. However, the pKa value of the anionic sulfate groups on carrageenan is around 2 [47, 48]. Therefore at pH 3.5 carrageenan may have a higher ionized charges. Thus, a primary emulsion required more amount of 0.1% w/v alginate to bind with.

Hence, the secondary emulsions at pH 3.5 could be prepared by mixing primary emulsion with 0.1% w/v alginate at volume ratio of 0.65 and with 0.8% w/v carrageenan at volume ratio of 0.55.

4.2.3 Determination of optimum volume ratio between secondary emulsions and anionic polyelectrolyte for tertiary emulsions preparation.

Tertairy emulsions was formed by mixing secondary emulsion with oppositely charge polyelectrolyte as same as secondary emulsion preparation. In this research, secondary emulsions coated by carrageenan, was used to prepare tertiary emulsions. Chitosan was selected as cationic polyelectrolyte. The optimum volume ratio between secondary emulsions and cationic polyelectrolyte was investigated for suitable tertiary emulsions.

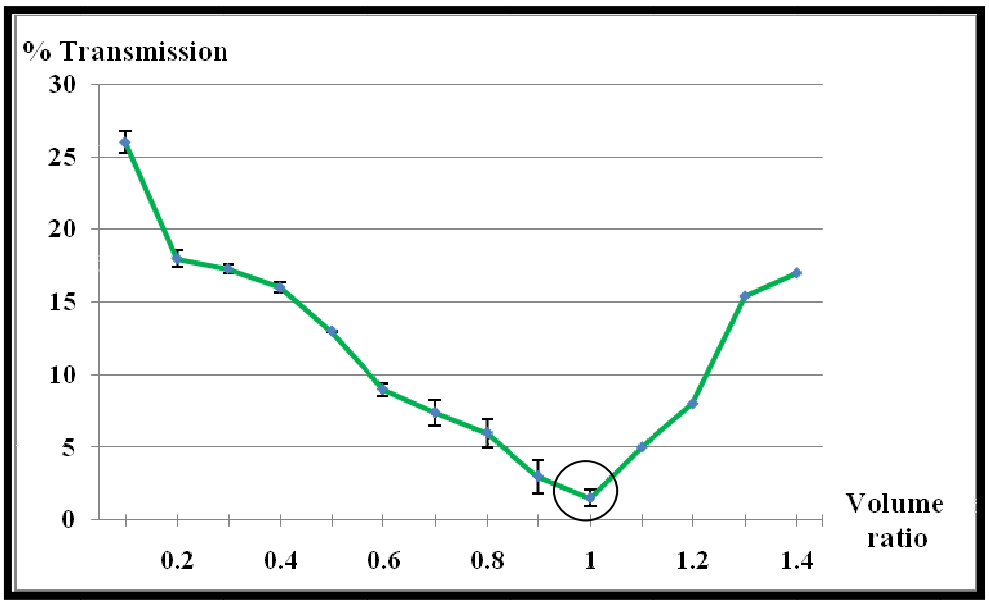

Figure 4.19 The titration curve between secondary emulsions (stabilized by WPI and 0.8 %w/v carrageenan) and 0.1 %w/v chitosan at pH 3.5.

The equivalence point for preparation of the tertiary emulsions was showed in Figure 4.19. The volume ratio of 1.0 showed the lowest %transmission indicating the equivalence point of the titration between secondary emulsions and chitosan.

Thus, the tertiary emulsions at pH 3.5 could be prepared from the mixing secondary emulsion (stabilized by WPI and 0.8% w/v carrageenan) and 0.1% w/v chitosan at volume ratio of 1.0.

4.2.4 Characterization and physical stability of primary, secondary and multilayer emulsions

The physical stabilities of primary, secondary and multilayer emulsions were measured during storage at 30 °C as performing at pH 6.0. In this study, we investigated on the zeta-potential, particle size and creaming stability.

4.2.4.1 The particle size diameter and zeta potential of emulsions

The particle size diameter and zeta potential (ζ) measurements indicated the stability of emulsions. We investigated the particle size distribution of all types of emulsions in our studies were bimodal distribution as observed in pH 6.. We selected to presence the first peak of size distribution that exhibited the higher population, which was more than 70% for most cases.

From Figure 4.20**,** the positive charge on the primary emulsion droplets suggested that WPI could be coated around the oil droplet because the p*I* value of WPI was around 4.8. The zeta potentials of both secondary emulsions were negative charge that could be explained by the pKa values of alginate and carrageenan.. Tertiary emulsions (WPI-carrageenan-chitosan), possess the positive potential indicating the coating of chitosan molecules around the droplets.s [37, 44].

From Figure 4.21**,** the particle size of the primary, secondary (stabilized by 0.1 %w/v alginate), secondary (stabilized by 0.8 %w/v carrageenan) and tertiary emulsions (stabilized by 0.8 %w/v carrageenan and 0.1 %w/v chitosan) were increased were 297.32, 384.79, 499.78 and 505.86 µm, respectively. The particle sizes of emulsions were increased when increasing the coating layer around the droplets. This showed the polyelectrolyte could be coated on the droplets by layer-by-layer technique at the pH 3.5.

Interestingly, the particle size of secondary emulsions prepared by aliginate was smaller than the one prepared by carrageenan. This observation might be that the carrageenan has higher molecular weight (M_w 100,000) [47] and forming helical structures [48]. It should be coated around WPI and showed the bigger droplet size.

In comparison of the particle size between secondary emulsions (WPI-carrageenan) and tertiary emulsions (WPI-carrageenan-chitosan), the relative same sizes were observed, 499.78 and 505.86 µm, respectively. However, the further experiments are needed to understand this phenomenon.

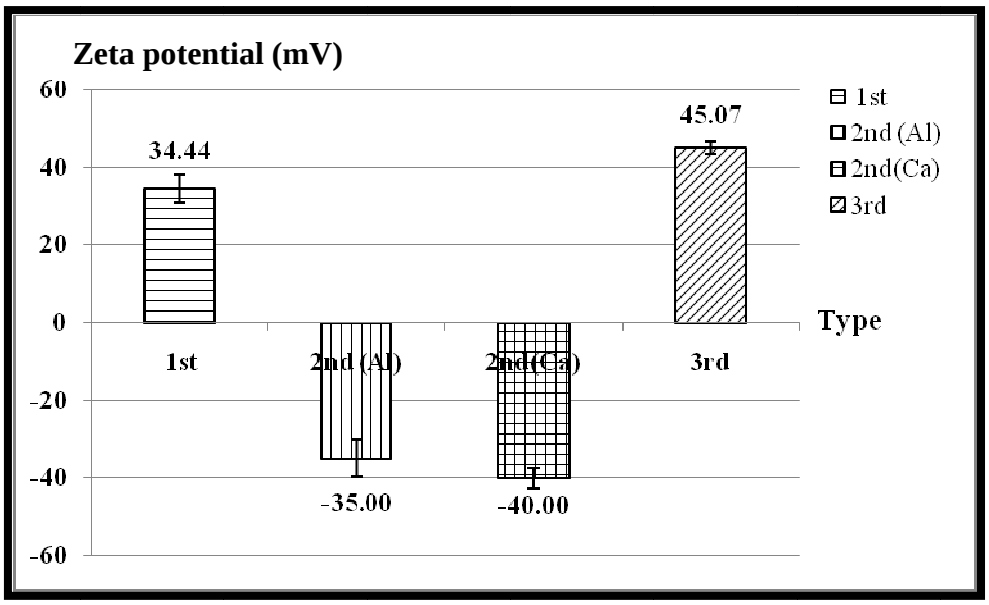

Figure 4.20 The zeta potential of primary, secondary and tertiary emulsions which diluted 100 times at pH 3.5. All emulsions were measured after 24 hours of storage.

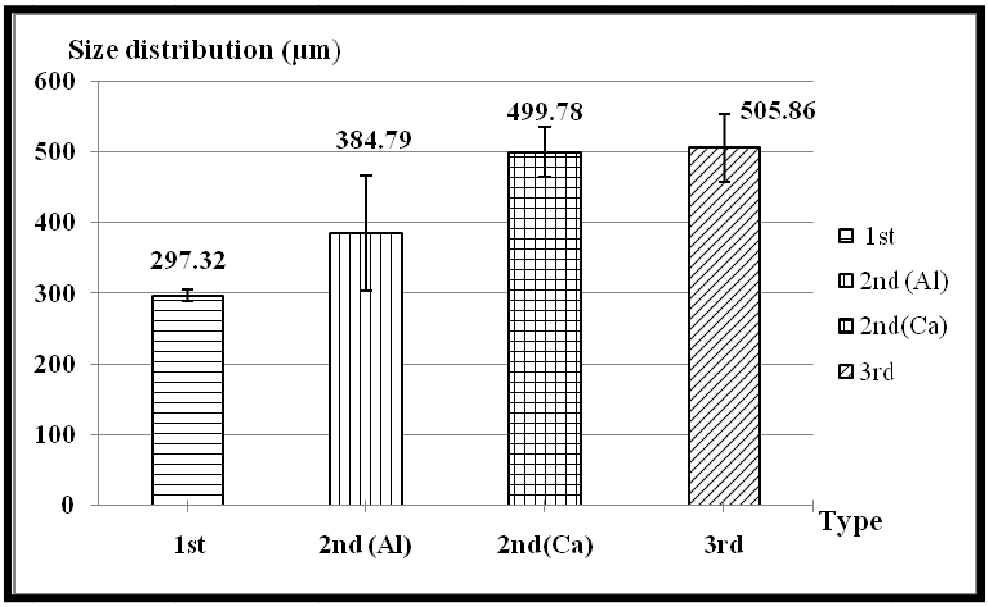

Figure 4.21 The particle size of primary, secondary and tertiary emulsions which diluted 100 times at pH 3.5. All emulsions were measured after 24 hours of storage.

4.2.4.2 Physical stability

% Transmission of diluted emulsions at 550 nm, which diluted by acetic-acetate buffer was used to study the creaming stability of emulsion. [33].

. Figure 4.22 and table 4.3 showed the changing of %transmission of emulsions at pH 3.5 for 21 days of storage. Similar physical stabilities were observed for all emulsions.

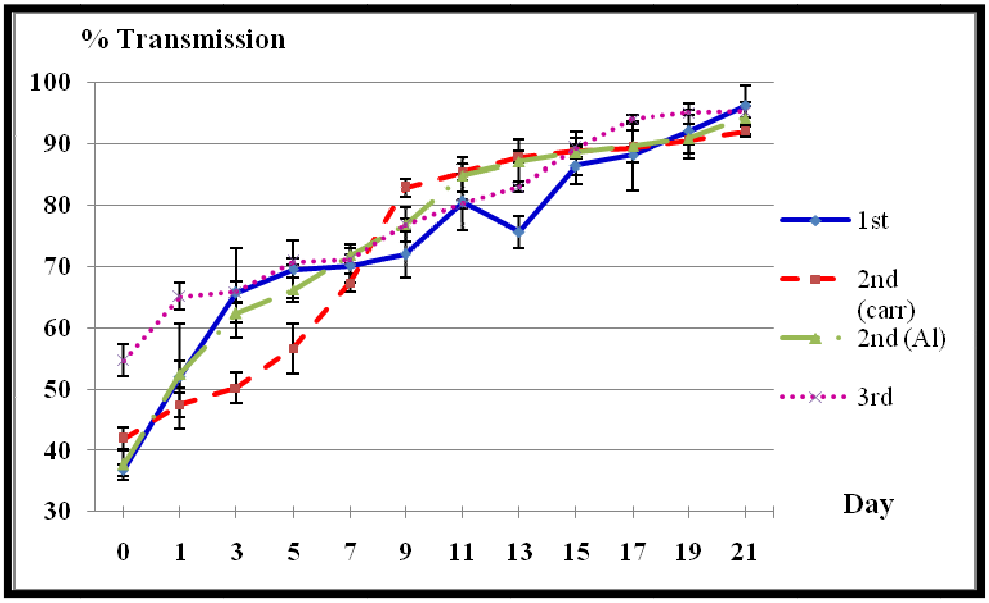

Figure 4.22 % Transmission of primary emulsion (stabilized by 5 %w/v oil),), secondary emulsion (stabilized by 1.77 %w/v oil and carrageenan), secondary emulsion (stabilized by 1.97 %w/v oil and alginate and tertiary emulsion (stabilized by 0.89 %w/v oil) at pH 3.5, diluted 100 time.

Table 4.3 The changing of % transmission

	First day	On the 21th day	% Change*
1st emulsion (5 %w/v oil)	36.84	96.20	59.36
2nd emulsion (1.77 %w/v oil and carrageenan)	37.76	94.19	56.43
2nd emulsion (1.97 %w/v oil and alginate)	41.92	91.97	51.05
3rd emulsion (by 0.89 %w/v oil)	54.77	95.23	40.46

* % changing was the different compared % transmission on the 21th day with % transmission on the first day.

The similar changes in the % transmission were observed. From Figure 4.20, the zeta potentials of all primary, secondary and tertiary emulsions were relatively similar inducing similar strength of repulsive forces among emulsion droplets. In consideration of the particle size of primary, secondary and tertiary emulsions, we found that the similar size in the secondary emulsions and tertiary emulsions but the particle size of primary emulsion was smaller. This result is difference from previous study by Guzey, that increased the thickness of the interfacial membrane may help prevent the droplet aggregation [49]. In this study, the particle size might play a minor role on the physical stability.

The changing of creaming index for 7 days of storage at pH 3.5 was shown in Figure 4.23. At the first day, freshly prepared primary emulsion exhibited very similar stability to multilayer emulsions. Then the % creaming index of primary emulsions increased more than another indicating that the primary emulsion was less stable to creaming compare to multilayer emulsions for all cases. The result at pH 3.5 similar as emulsions prepared at pH 6.0.

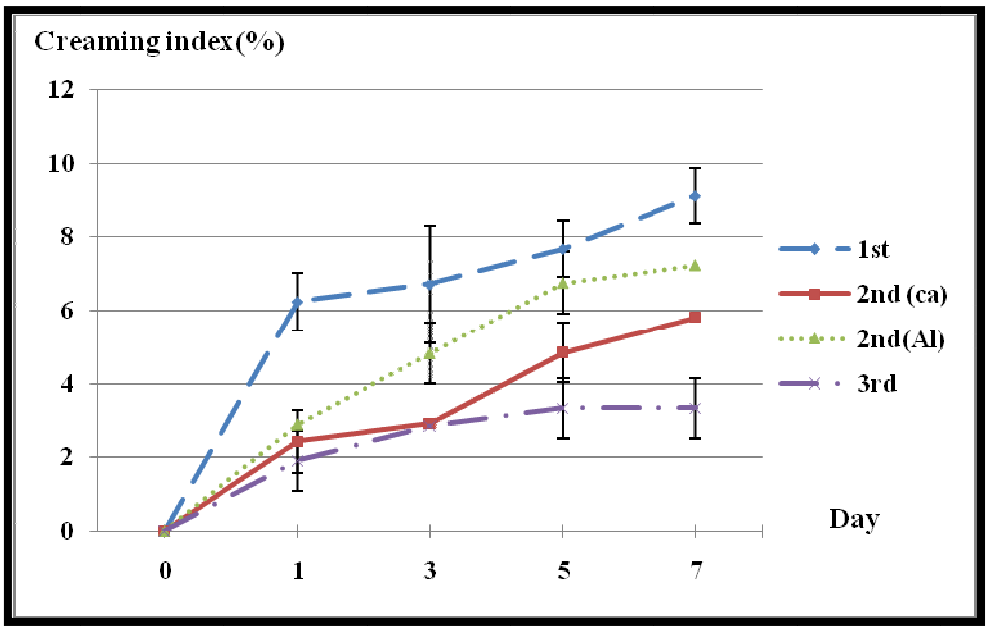

Figure 4.23 The creaming stability of primary emulsion (stabilized by 5 %w/v oil), secondary emulsions (stabilized by 1.77 %w/v oil and carrageenan), secondary emulsions (stabilized by 1.97 %w/v oil and alginate), and tertiary emulsions (stabilized by 0.89 %w/v oil) at pH 3.5.

Figure 4.24 showed the creaming index of emulsions on the 7th days. The creaming index of primary emulsion was 9.13, while secondary emulsions prepared by 0.1 %w/v alginate and 0.8 %w/v carrageenan were 7.25 and 5.82, respectively, and tertiary emulsions was 2.86. These results suggested that the primary emulsion had relatively poor stability to aggregation. For multilayer emulsions, the creaming have been improved even though the thick electrically charged interfacial membrane can repulsive colloidal interactions. In previous work, the tertiary emulsions (lecithin-chitosan-pectin) showed the stability toward the droplet aggregation because of the relatively strong electrostatic and steric repulsion associated with the relatively thick and electrostatically charge three layer interfacial membrane [33].

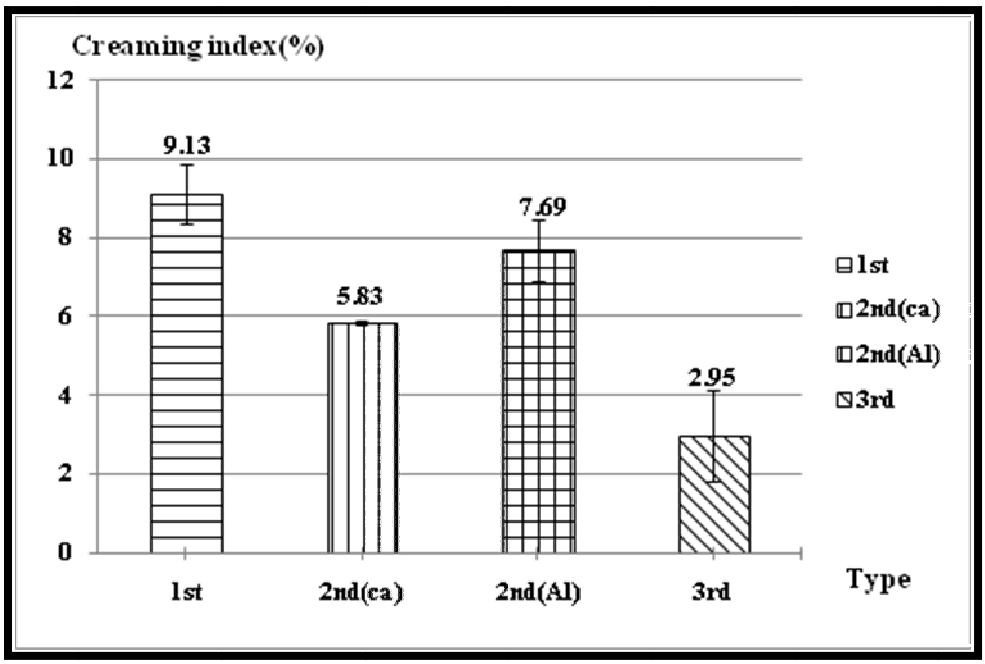

Figure 4.24 The creaming stability of primary emulsion (stabilized by 5 %w/v oil), secondary emulsion (stabilized by 1.77 %w/v oil and carrageenan), secondary emulsion (stabilized by 1.97 %w/v oil and alginate), and tertiary emulsion (stabilized by 0.89 %w/v oil) at pH 3.5, after 7^{th} days of storage.

From Figure 4.22 and Figure 4.24 we observed the different results between % transmission and the %creaming index. The % transmission showed similar increase in all emulsions but the creaming index showed the improving of creaming in tertiary emulsions. This might be due to the different in % oil content in these two experiments.

Table 4.4 The summary of particle size mean diameter at the intensity of the first peak and zeta potential of primary, secondary and tertiary emulsion at pH 3.5. The mixture solution was measured after 24 hours of storage then diluted 100 times for the measurement.

Emulsion	% Oil content	Concentration of Polyelectrolyte	Mean diameter(μM)	Zeta potential(mV)
Primary emulsion	5 %w/v	0.40 %w/v WPI	297.32 (±8.86)	34.44 (±3.56)
Secondary emulsions	1.77 %w/v	0.149 %w/v WPI + 0.28% w/v carrageenan	499.78 (±35.58)	-35.00 (±4.83)
Secondary emulsions	1.97 %w/v	0.165 %w/v WPI + 0.039% w/v alginate	384.79 (±81.70)	-40.00 (±2.75)
Tertiary emulsions	0.24 %w/v	0.075%w/v WPI + 0.0014 %w/v carrageenan+ 0.05% w/v chitosan	505.86 (±47.76)	45.07 (±1.59)

4.2.5 Lipid oxidation

Lipid peroxidation result in the formation of highly reactive and unstable hydroperoxides of unsaturated lipids [39]. In this part, the lipid oxidation in oil-in-water emulsion at pH 3.5 was also studied.

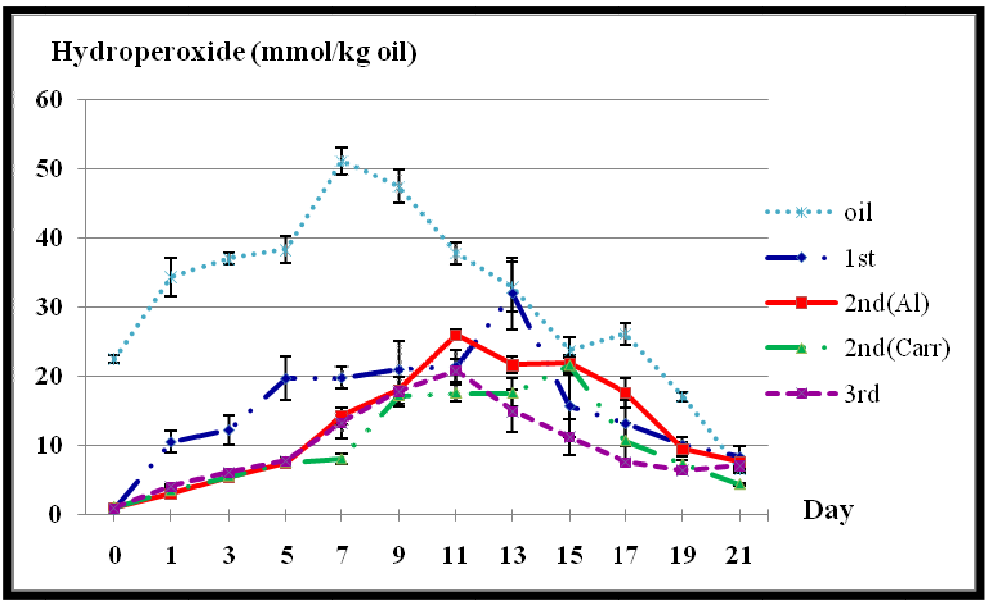

Figure 4.25 Formation of lipid hydroperoxides in tuna oil and primary, secondary and tertiary emulsions at pH 3.5 for 21th days of storage.

Figure 4.25 showed a comparison of oxidation rates in tuna oil and tuna oil-in-water emulsions stabilized by WPI alone (primary emulsions), WPI-alginate, WPI-carrageenan (secondary emulsions) and WPI-carrageenan-chitosan (tertiary emulsions). The maximum of oxidation rate of tuna oil was found to be on the day seventh, while the maximum point of primary emulsion was generated after the 7th date.

The trend of lipid hydroperoxide formation form secondary emulsions (WPI-carrageenan and WPI-alginate) and tertiary emulsions was in the similar fashion. The amount of lipid hydroperoxide of secondary (WPI-alginate and WPI-carrageenan) emulsions were showed the maximum of lipid hydroperoxide at 11th days (26.02 mmol/kg oil) and 15th days (21.67 mmol/kg oil) of storage, respectively. The improving stability of the secondary emulsions to lipid oxidation can be attributed to the increasing the number of layer that influence increase the region from outer layer to the oil droplet [20]. In addition, the electrical charges on secondary emulsions stabilized by both alginate and carrageenan were negatively but there are effect of electrostatically repulsive and can prevent the droplet from the metal catalyst coming into the lipid substrate. Althought the secondary emulsion stabilized by WPI-alginate was reached the maximum of lipid hydroperoxide before

primary emulsion but less amount of lipid hydroperoxide (at 11th days, 26.02 mmol/kg oil).

From Figure 4.25, we found that the secondary emulsion stabilized with WPI-carrageenan delivered better performance comparing to the one stabilized with WPI-alginate. It might be because the difference in the particle size where we found that the size of secondary emulsions (WPI-carrageenan) was larger than the secondary emulsions (WPI-alginate). This thicker interfacial layer could protect droplet from transition metal and environment more than secondary emulsions (WPI-alginate).

Comparing between tertiary emulsion with primary emulsion. The lipid hydroperoxide generated from tertiary emulsions was increased to the maximum on the 11st days (20.86 mmol/kg oil), while the primary emulsion showed the maximum of lipid hydroperoxide on the 13th days (32.04 mmol/kg oil). During the first day to 21th days, the tertiary emulsions was improved the lipid hydroperoxide. The improving stability can be attributed to the increasing the number of layer that influence increase the region from outer layer to the oil droplet [20].

The tertiary emulsions stabilized by WPI-carrageenan-chitosan showed the similar oxidative stability as secondary emulsions. The particle size of tertiary emulsions was quite similar to secondary emulsions (WPI-carrageenan) but showed the difference electrical charge. This result may be explain by the ability of positively charged of the secondary emulsions [38], while the tertiary emulsions can be explain by the region from the outer layer to oil droplet [20].

As explained in section 4.1.2.4, the accelerated method was used to monitor lipid hydroperoxide as well.

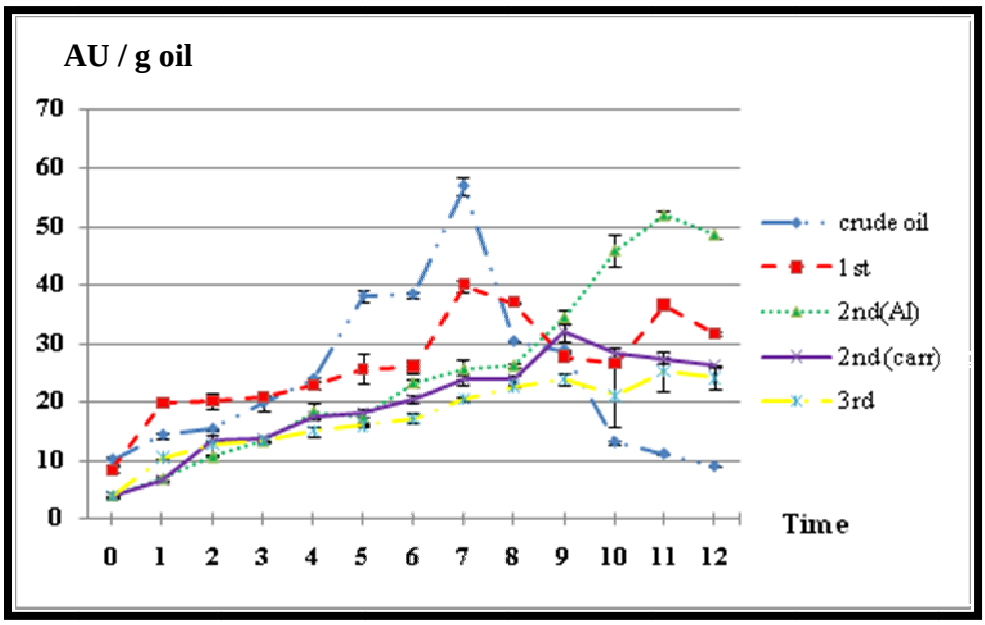

Figure 4.26 Formation of lipid hydroperoxides in crude oil, primary,secondary and tertiary emulsions at pH 3.5 at 50°C up to 12 hours

From Figure 4.26, the similar result was observed that the primary emulsion could improve the lipid oxidation of tuna oil. For the secondary emulsions stabilized by alginate reached to the highest level on the 11th hour, 51.94 AU/g oil. while the secondary emulsions stabilized by carrageenan reached the highest level at the 9th hour with the lower generated level.. These result showed that the secondary emulsion prepared with carrageenan could retard the oxidation reaction better than the secondary emulsion prepared with alginate.

For the tertiary emulsion can retard the oxidation rate slightly more than the secondary and tertiary emulsions as we could see that it took longer time to reach the maximum value of lipid hydroperoxide.

4.3 The effect of antioxidant on the physical and oxidation stabilities

In this part, we studied the ability of antioxidant to inhibit lipid peroxidation of tuna oil-in-water emulsions. Genistein, sesame oil, capsaicin oleoresin and roselle were selected to study at pH 6.0. Two approaches were developed to add the antioxidant into the emulsion. First, the sesame oil and capsaicin oleoresin, oil-soluble mixture, was added directly in the oil before emulsion preparation. Second approach

is adding the genistein and roselle extract directly in emulsifier solution before homogenization.

4.3.1 The preparation of emulsion with the addition of sesame oil and capsaicin oleoresin

The extraction of sesame oil and capsaicin oleoresin has the solubility properties in oil. Then, 0.3, 0.5 %w/v sesame oil and 0.3, 0.5 %w/v capsaicin oleoresin was added directly in tuna oil.

(a) (b)

Figure 4.27 Primary emulsion (a) added capsaicin and (b) added sesame oil.

4.3.1.1 Characterization and physical stability of emulsion added sesame oil and capsaicin oleoresin

4.3.1.1.1 The particle size diameter and zeta potential of emulsions

We selected to presence the first peak of size distribution that exhibited the higher population, which was more than 70% for most cases.

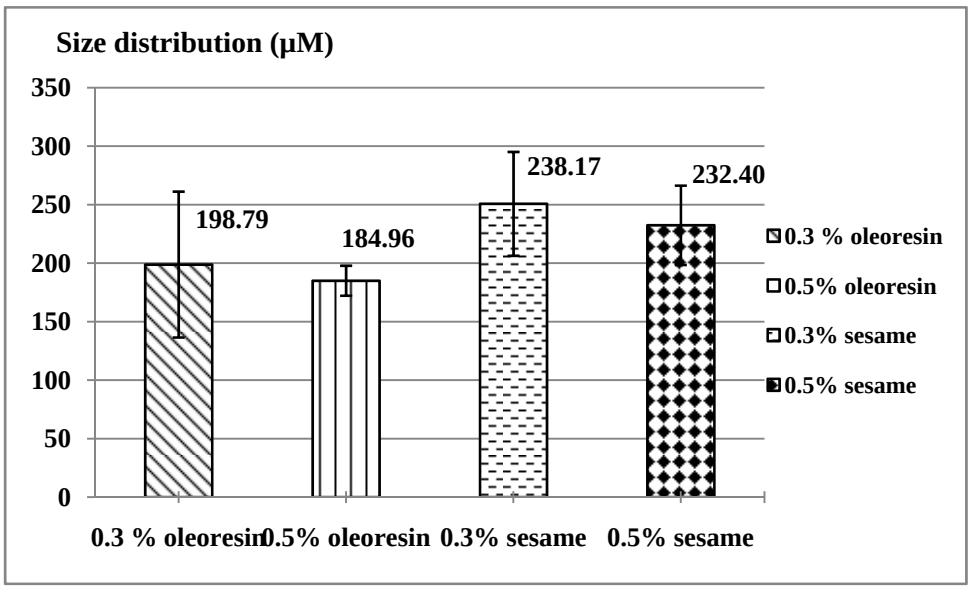

Figure 4.28 The particle size of emulsion added 0.3, 0.5% w/v sesame oil and 0.3, 0.5% w/v capsaicin oleoresin which diluted 100 times at pH 6.0. All emulsions were measured after 24 hours of storage.

The effect of antioxidant on the particle size of emulsion was investigated. The particle size of primary emulsion added sesame oil was bigger than the one added with capsaicin oleoresin.

The zeta-potentials of the droplets of the primary emulsion added with antioxidants at pH 6.0 were negative as expected, which assured that WPI was coated around the oil droplets. However, the zeta potentials of tuna oil added sesame oil in primary emulsion are lower than the one with capsaicin oleoresin. This result may be the effect of the preparation, that affected to the particle size and the zeta-potentials.

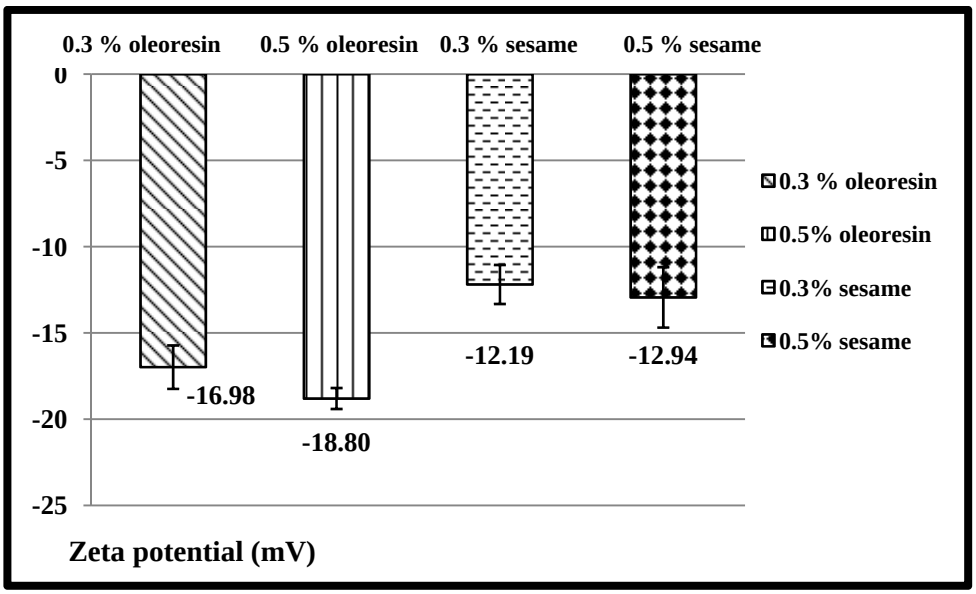

Figure 4.29 The zeta potential of emulsion added 0.3, 0.5% w/v sesame oil and 0.3, 0.5% w/v capsaicin oleoresin which diluted 100 times at pH 6. All emulsions were measured after 24 hours of storage.

4.3.1.1.2 Physical stability

The physical stability of primary emulsions added sesame oil and capsaicin oleoresin were measured during storage at 30 °C.

After storage, we could see the droplet aggregation (showed in previous study). Thus we would expect to improve the changing of % transmission. Figure 4.30, show the change in % transmission for 21 days of storage. The overall of emulsions showed the changing of % transmission in Table 4.5.

From Table 4.5, the changing % transmission during the first day to the 21th days of primary emulsion added 0.3, 0.5% sesame and 0.3, 0.5% capsaicin oleoresin were 62.17, 59.21, 57.82 and 55.59, respectively.

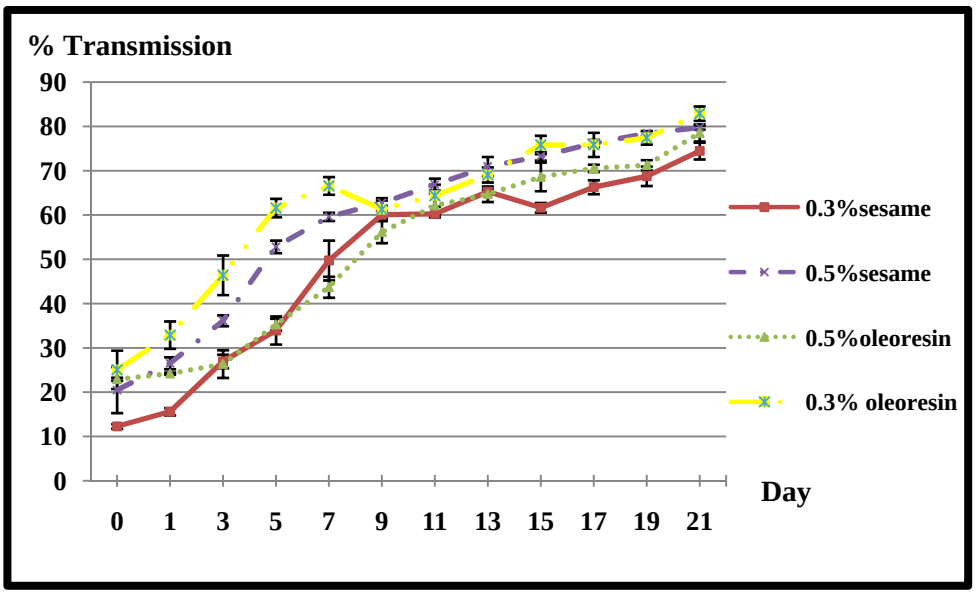

Figure 4.30 % Transmission of primary emulsion added 0.3, 0.5 %w/v sesame oil and 0.3, 0.5 %w/v capsaicin oleoresin at pH 6.0, diluted 100 time.

Table 4.5 The changing of % transmission

	First day	On 21th days	% Change*
1st emulsion + 0.3% sesame oil	12.28	74.45	62.17
1st emulsion + 0.5% sesame oil	20.5	79.71	59.21
1st emulsion + 0.3% capsaicin oleoresin	25.06	82.88	57.82
1st emulsion + 0.5% capsaicin oleoresin	22.93	78.52	55.59

* % changing was the different compared % transmission on the 21th day with % transmission on the first day.

The higher change of % transmission showed a less stability of emulsions. From the result, the primary emulsion added with capsaicin oleoresin showed the better physical stability comparing to the primary emulsion blending with sesame oil. This might be the effective of the particle size of 1st emulsions added 0.5% capsaicin oleoresin was smaller, that can prevent the aggregation of emulsion

droplet. While the zeta potential was higher, that had a higher electrostatic repulsive force.

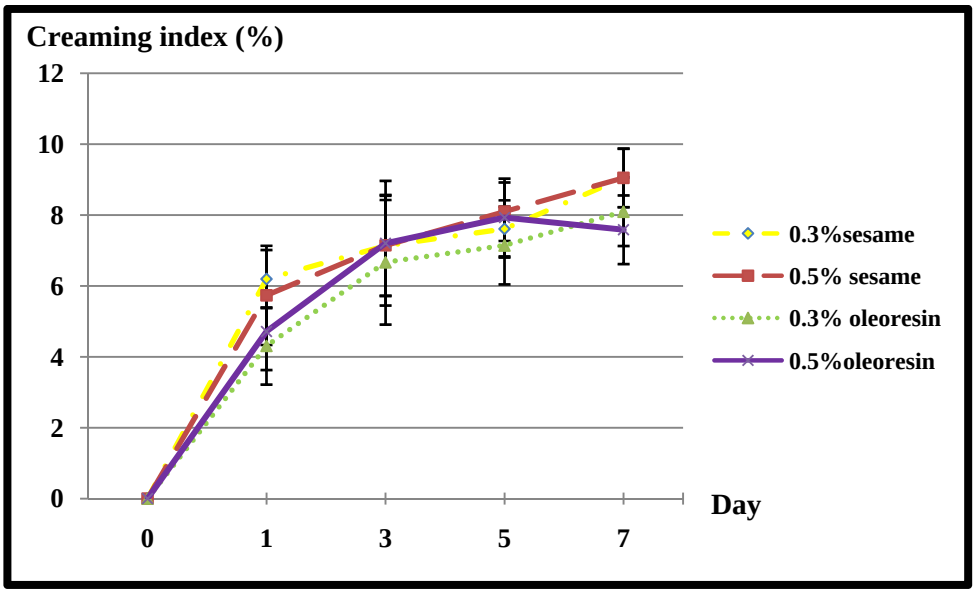

Figure 4.31 The creaming stability of primary emulsion added 0.3, 0.5% w/v sesame oil and added 0.3, 0.5% w/v capsaicin oleoresin at pH 6.0 after storage.

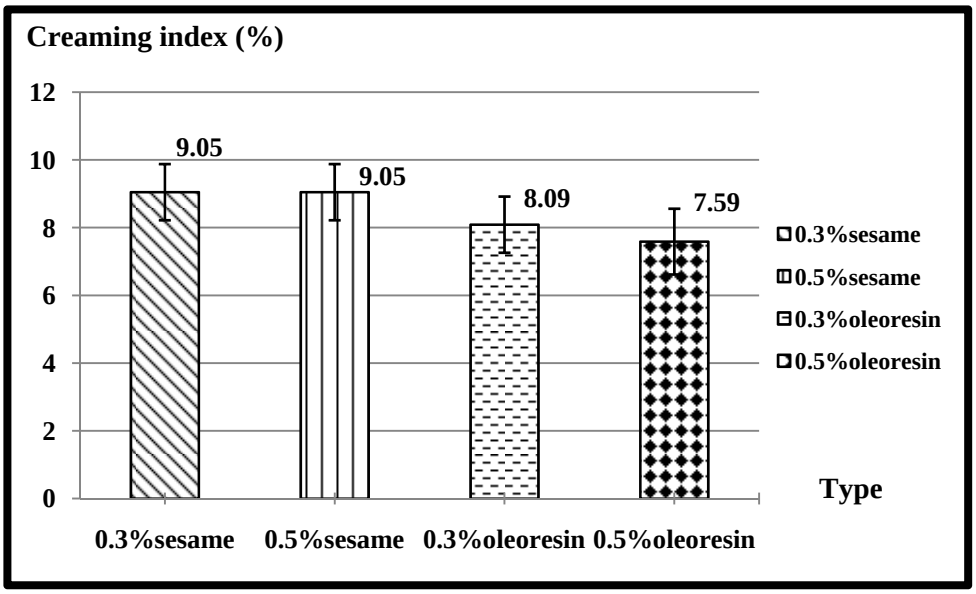

Figure 4.32 The creaming stability of primary emulsion added 0.3, 0.5% w/v sesame oil and 0.3, 0.5% w/v capsaicin oleoresin at pH 6.0 on the 7th day.

From Figure 4.31 and Figure 3.2, all freshly prepared emulsions did not show any creaming. Then, the creaming index was slowly increase in the similar fashion for all primary emulsions added with antioxidants. We observed that the creaming indexes of primary emulsion blending with capsaicin oleoresin were smaller than the other. This result is the result of the smaller particle size and higher zeta potential values gaining from the primary emulsions added with 0.3 and 0.5 %w/v capsaicin oleoresin.

In comparison of the creaming index between the difference contents of capsaicin oleoresin, we found that there weren't obviously difference. That may be because of the similar zeta potentials and size of primary emulsion added with 0.3 and 0.5 % w/v capsaicin oleoresin.

4.3.1.2 Lipid hydroperoxide

The process for study lipid hydroperoxide of primary emulsion added 0.3, 0.5% w/v sesame oil and 0.3, 0.5% w/v capsaicin oleoresin at pH 6.0 were followed to oxidize at room temperature (30 °C) in the dark for up to 21th days.

Antioxidant activities of sesame oil and capsaicin oleoresin incorporated into the oil droplet were compared. From Figure 4.33 showed a comparison of oxidation rates in primary emulsion added with different antioxidants. The oxidation rate of primary emulsion added antioxidant was increased after the storage. From the first day to the 5th date, there was no significant change in lipid hydroperoxide. Then, the oxidation rates of all emulsions were increased. The lipid oxidation of primary emulsion added with 0.3, 0.5%w/v sesame oil were reached the maximum value on the date of 15th and 13th, respectively, while the primary emulsion added with 0.3, 0.5%w/v capsaicin oleoresin were reached to the maximum values on the date of 15th and 19th , respectively. The comparison between the primary emulsion added antioxidant in this part showed the result of 0.5 %w/v sesame oil was better oxidative stability than 0.3 %w/v sesame oil, while the primary emulsion add capsaicin oleoresin showed 0.5 %w/v capsaicin oleoresin was better oxidative stability than 0.3 %w/v capsaicin oleoresin.

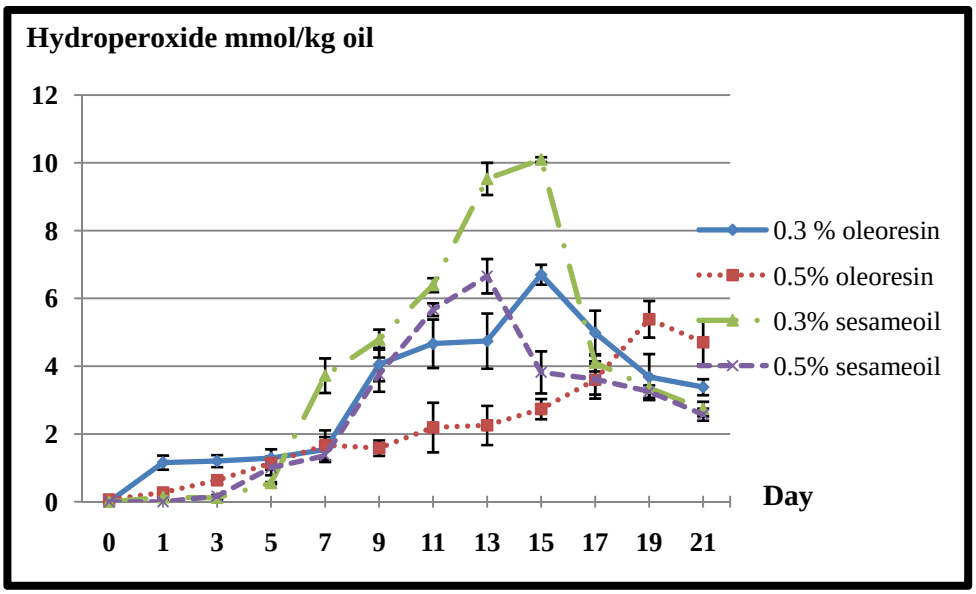

Figure 4.33 Formation of lipid hydroperoxides in primary emulsion added 0.3, 0.5% w/v sesame oil and 0.3, 0.5% w/v capsaicin oleoresin at pH 6.0 for 21th days of storage.

The antioxidant activity of emulsion added sesame oil was improved. This can explain by the properties of function of sesame oil that may be sesamin and sesamolin. They showed the significant scavenging action on free radicals, which could inhibitthe peroxidation reaction. Some literatures have reported the effect extract of sesame on oxidation [50]. There are present the effect extract of sesame can prevented LDL from the activation. These results implied that the extract of sesame have shown remarkable antioxidant activity, including scavenging oxygen radicals, chelating metal ions and protecting liposome from peroxidation reaction. The result of emulsion added capsaicin oleoresin was improved. Capsaicin oleoresin may be contained β-carotene and capsaicinoid fraction [51]. The capsaicinoid fraction was a family of flavonoid, that shows the strong antioxidant activity. All capsinoids and their analogues showed, at noncytotoxic concentrations for vanillyl nonanoate, a noteworthy efficacy as chain-braking antioxidants in scavenging lipid peroxyl radicals, an effect due to their capacity to donate hydrogen atoms and delocalize the resulting radical sites. They can inhibit both its autooxidation and its iron- or EDTA-mediated oxidation in linoleic acid [52].

Compare lipid oxidation between primary emulsion add sesame oil and capsaicin oleoresin. The oxidative stability of primary emulsion added capsaicin oleoresin was found to be better. Thus, it suggests that antioxidant activity of capsaicin oleoresin would be sufficiently higher than sesame oil in this part.

From the result of capsaicin oleoresin could retard lipid oxidation. Thus, we study the effect of content of capsaicin oleoresin in tuna oil-in-water emulsion in oxidation rate. The contents of capsaicin were varied 0.1, 0.3, 0.5, 0.7 and 0.9 % w/v.

The effect of capsaicin oleoresin was studied and the result was shown in Figure 4.34. The emulsion added with 0.9% %w/v capsaicin oleoresin delivered the best performance on the oxidation retardant. However, the physical stability of emulsion added 0.9 %w/v capsaicin oleoresin was the least as shown in Figure 4.35, that show capsaicin oleoresin separated into upward layer. The separation of capsaicin oleoresin may be caused by the lower density than surrounding phase, thus showed move upward after storage. Thus, in this part, the best physical and oxidative stabilities were 0.5% w/v capsaicin oleoresin.

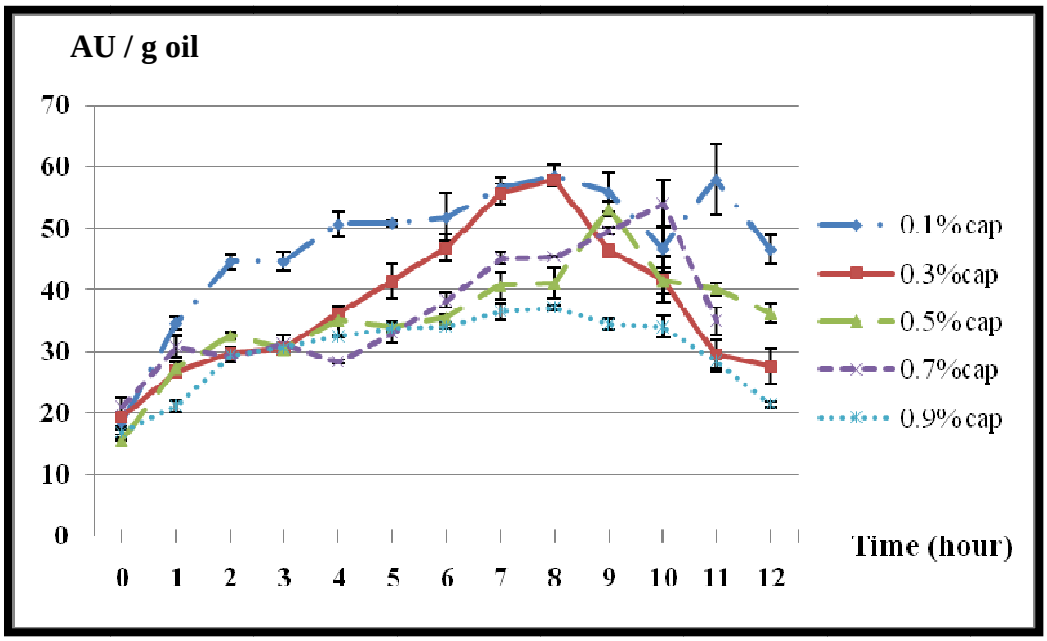

Figure 4.34 Formation of lipid hydroperoxides in primary emulsions with various content of capsaicin oleoresin at pH 6.0, 50 °C in the water bath for up to 12 hours.

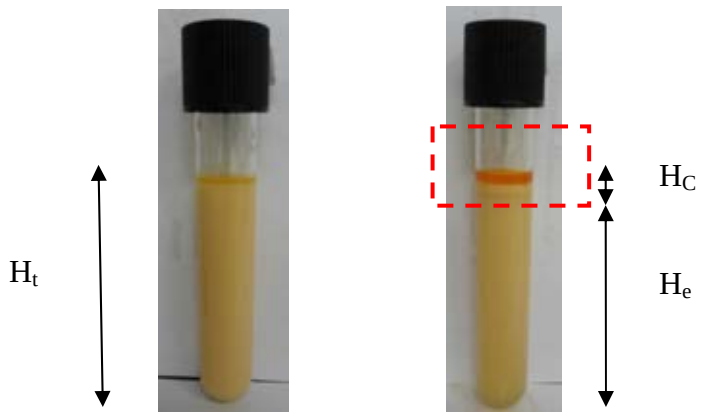

Figure 4.35 The creaming index of 0.9 %w/v capsaicin oleoresin after storage (a) 0 day and (b) 7th days. H_t was the height of emulsion. H_c was the height of creaming.

4.3.2 The preparation of emulsion with the addition of roselle extract and genistein

Roselle extract and genistein has the solubility properties in water. Therefore, we selected to add these two antioxidants directly in the acetic-acetate buffer before emulsification. Roselle extract was dissolved in acetic-acetate buffer pH 6.0, while genistein was dissolved in methanol and then added to emulsifier solution.

4.3.2.1 The study of the effect of antioxidant on the physical stability of primary emulsion

To test the effect of concentration antioxidant, the various concentrations of antioxidants added in emulsifier solution are 100, 200 and 500 ppm roselle and 5 and 10 % w/v of 74 µM genistein, respectively. The physical stability of emulsions was monitored by measuring the creaming stability [49], and lipid hydroperoxide was also determined to study the oxidation stability.

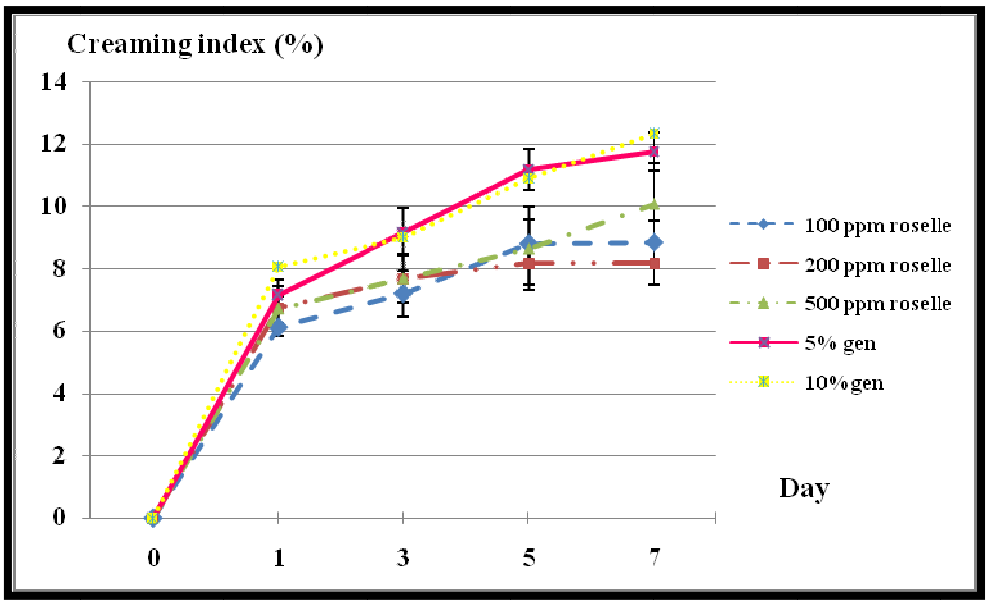

Figure 4.36 The creaming stabilities of primary emulsions added with 100, 200, 500 ppm of roselle extract and 5, 10% of 74 µM genistein at pH 6.0.

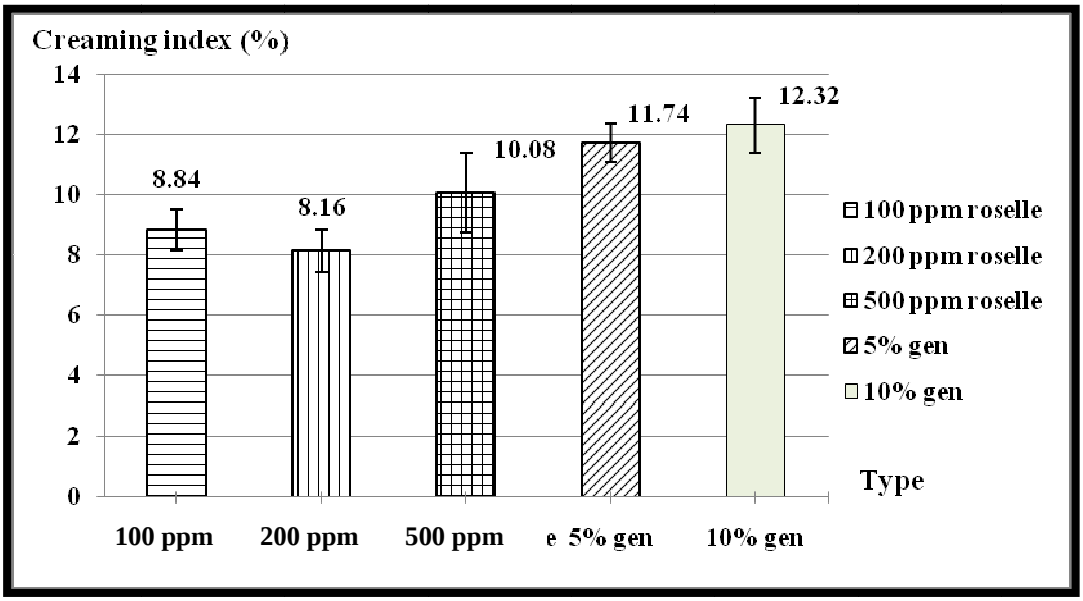

Figure 4.37 The creaming stability of primary emulsion added 100, 200, 500 ppm of roselle extract and 5, 10% 74 µM genistein at pH 6 at 7th day.

From Figure 4.36 and 4.37, the increase in the creaming index of all emulsions was observed for the whole seven days. However, there were no differences in the physical stabilities among the emulsion added with different content of roselle. There are similar in the emulsion added genistein. The emulsion prepared with 5 and 10 %w/v genistein was similar increasing in creaming index. This result were similar, may be cause the little difference amount of antioxidant added in emulsion.

The physical stability of primary emulsion added with roselle extract was improved comparing to the genistein, which will be discussed later.

4.3.2.2 Lipid hydroperoxide

Effect of added antioxidant concentration on lipid hydroperoxide of primary emulsion was studied and result was shown in Figure 4.38. The oxidation rate of primary emulsion added roselle was better than the primary emulsion. Roselle extract showed the contain of ascorbic acid, β-carotene, phenolic compound and anthocyanin [54], that showed reducing power and scavenging effect on free radical [54]. Thus, primary emulsion added roselle extract has improved the oxidative stability of emulsion. When the content of roselle was increased from 100 to 200 ppm, the lipid hydroperoxide content was decreased. However, the emulsion with 500 ppm roselle extract showed the decrease in the ability to retard the oxidation reaction of the unsaturated fatty acid in tuna oil. This result can be explained by the change in pH of emulsifier solution after added roselle extract. We found that the the pH of emulsifier solution after adding the500 ppm roselle is 5.72, which is quite close to the p*I* of WPI (4.8) [30]. The zeta potential around the droplets under this pH might be lower than the emulsion added with 100 and 200 ppm of roselle. Although, the zeta potentials of these emulsions were not measured, the worst physical stability was obtained from the emulsion added with 500 ppm roselle (Figure 4.37). This result could be an evidence to support our hypothesis for the least performance on the oxidation retardant of emulsion added with 500 ppm.

Thus, 200 ppm of roselle extract was the best oxidative stability of emulsion and could inhibit lipid hydroperoxide around 63.96 %.

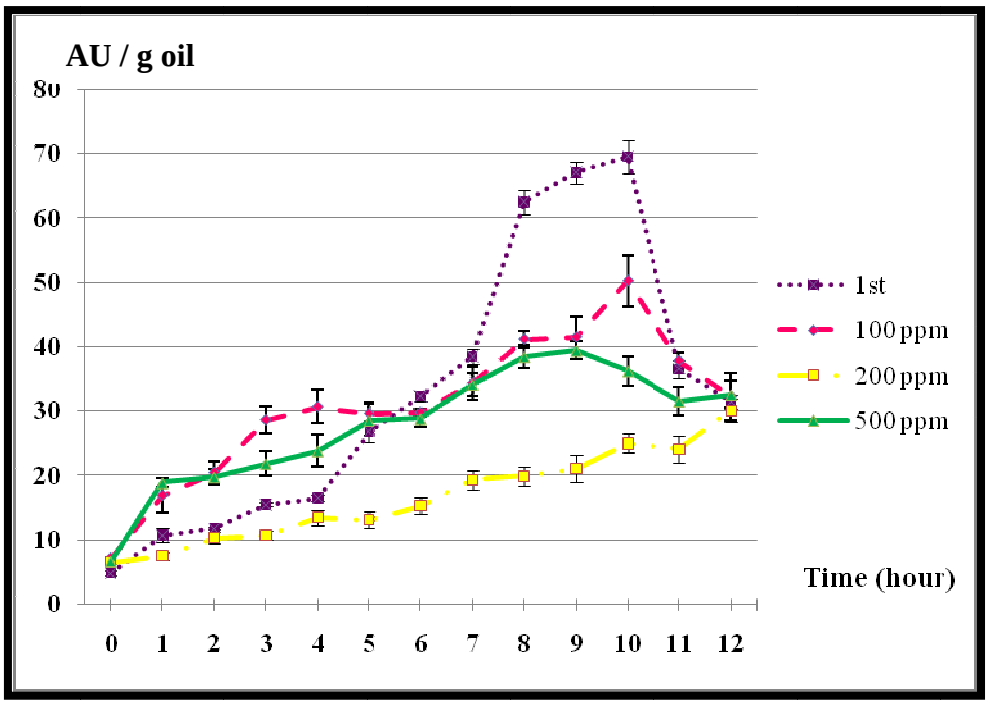

Figure 4.38 Formation of lipid hydroperoxides in primary emulsions with various contents of roselle at pH 6.0, 50 °C in the water bath.

From Figure 4.39, the lipid oxidation of primary emulsion added with 10 %w/v genistein was lower comparing to the one added with 5% genistein based on the delay of the number of days required to reach the maximum value of lipid hydropreoxide. The lipid oxidation of 10 %w/v genistein was higher compared with 5 %w/v genistein, that may be from the physical propertiy that 5 %w/v genistein show a littltle better in creaming index showed in Figure 4.37.

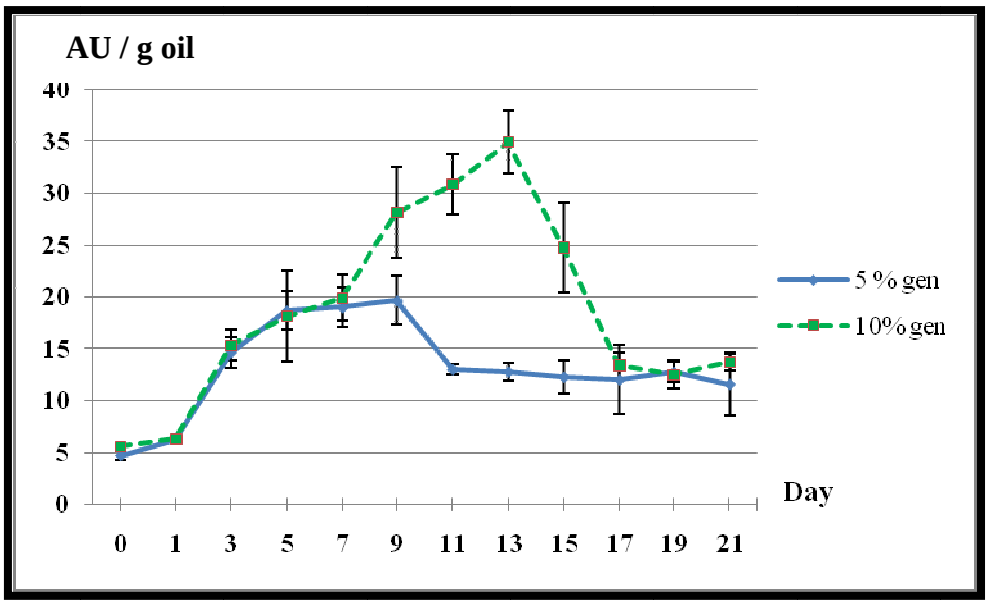

Figure 4.39 Formation of lipid hydroperoxides in primary emulsion added 5 and 10 %w/v genistein at pH 6.0 after 21th day of storage.

4.3.2.3 Characterization and physical stability of emulsion add antioxidant direct in emulsifier solution

From the result in the section 4.3.2.1 and 4.3.2.2the primary emulsion added 200 ppm roselle extract and 5 %w/v genistein were good stability. Thus, we selected both conditions to study the physical stability and characterization the particle size diameter and zeta potential.

4.3.2.3.1 The particle size diameter and zeta potential of emulsions

From Figure 4.40, the particle size of primary emulsion added 200 ppm roselle extract are smaller than primary emulsions added 5%w/v genistein. Suggesting the roselle extract may be clearly soluble in the emulsifier solution.

From Figure 4.41, the zeta potential of primary emulsion added 200 ppm roselle shown a higher zeta potential than 5% w/v genistein, which affect the physical stability.

From section 4.3.2.1physical stability of primary emulsions added genistein was worst than primary emulsion add roselle extract, showed the higher creaming index in. Because primary emulsion added genistein has a lower zeta potential and larger size comparison to the primary emulsions added roselle extract. Then, the emulsion containing genistein has a lower electrostatic repulsion between the droplets and the aggregation of emulsions was not improved.

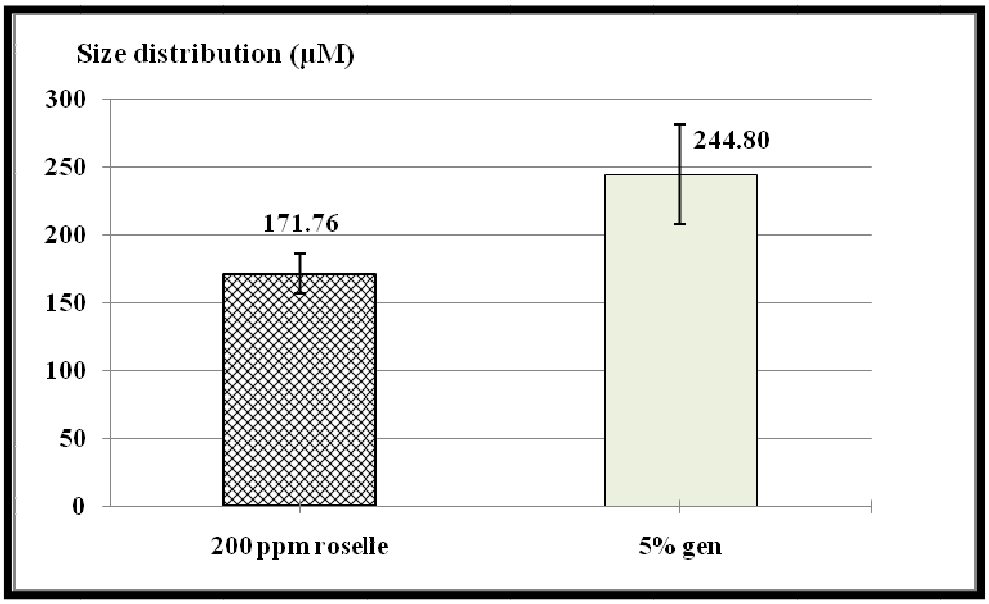

Figure 4.40 The particle size of emulsion added 200 ppm roselle extract and 5%w/v genistein. All emulsions were measured after 24 hours of storage.

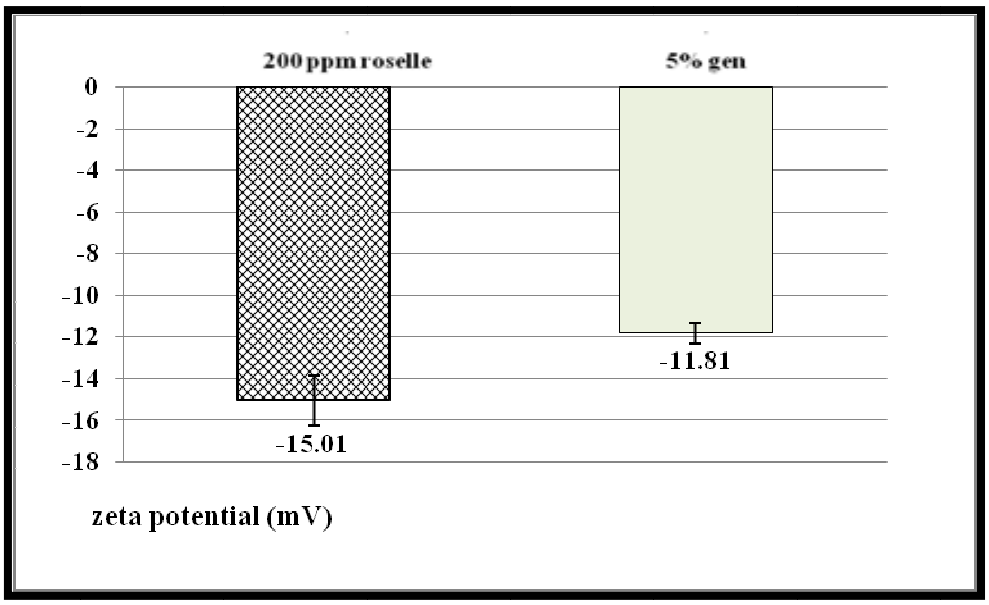

Figure 4.41 The zeta potential of emulsion added 200 ppm roselle extract and 5%w/v genistein. All emulsions were measured after 24 hours of storage.

In this study, we select to study the effect of antioxidants, sesame oil, capsaicin oleoresin, genistein and roselle extract on the decrease in the oxidation rate of primary emulsion. All result could be compared with another study. We were summarized in Table 4.6.

Table 4.6 Comparison the effect of antioxidants to improve the oxidation reaction

Authors	**Antioxidant**	**Role**
Tee, P. L. and coworker. 2002	Roselle	Inhibit 85% of conjugated compound
K. Kogure et al. 2002	α-Tocopherol Capsaicin	:: Inhibit radical chain reaction :: Inhibited both the formation of active oxygen species and the radical chain reaction

Authors	Antioxidant	Role
Naderi et al., 2003		Improve oxidation
	Morin	~75%
	Genistein	~50%
	Apignin	~90%
	Biochanin	~65%
Osborn-Barnes and Akoh, 2003		Improve oxidation
	α-Tocopherol	< 10
	β-Carotene	<10
	Genistein	No effect
	daidzein	No effect
N. Mohd-Esa et al. 2010		Improve TBAR
	Roselle seed extract	62.5%
	BHT	18.75
	Alpha-tocopherol	31.25

CHAPTER V

CONCLUSION

This research has shown that stable monolayer and multilayer emulsions could be prepared by using simple and effective method. The parameters controlling formation of stable primary emulsions such as pH of emulsifier solution, emulsifier concentrations (0.2-0.5 %w/v of WPI) were investigated. Primary emulsion was prepared by homogenizing for 2 minutes/cycle at 20,000 rpm for 5 homogenization cycles. The study to optimize the %WPI, the various emulsifier concentrations. The physical stability of emulsions was monitored by measuring the creaming stability and % transmission.While the oxidative stability was monitored by measuring lipid oxidation. Although the primary emulsion contained 0.4 %w/v WPI concentration exhibited the bimodal distribution, they showed the best stability. The secondary and tertiary emulsions were prepared by coating the oil droplets with the charged biopolymers. The pH of all emulsions were controlled at either pH 3.5 and 6.0. Chitosan, a cationic polyelectrolyte, and alginate and carrageenan, anionic polyelectrolytes were selected. The physical stability was investigated by measuring the creaming index, % transmission, particle size distribution and zeta potential. The creaming index and % transmission were decreased when increasing the number of coating layer around emulsion. The particle size mean diameter obtaining from the peak, that exhibited the higher population, which was more than 70% for most cases, and zeta potential of primary, secondary and tertiary emulsions at pH 6.0 and pH 3.5 were summarized in table 5.1.

The physical stability of all emulsion were monitored by using the creaming index and % transmission. Both pH showed the multilayer emulsion was improved the physical stability.

Table 5.1 The summary of particle size mean diameter at the intensity of the first peak and zeta potential of primary, secondary and tertiary emulsions at pH 6.0 and pH 3.5

Emulsion	% Oil content	Concentration of Polyelectrolyte	Mean diameter (μM)	Zeta potential (mV)
Primary emulsion pH 6.0	5 %w/v	0.4 %w/v WPI	242.20 (±34.03)	-13.41 (±1.71)
Secondary emulsions pH 6.0	0.83 %w/v	0.07 %w/v WPI + 0.83% w/v chitosan	332.1 (±19.87)	19.87 (±2.21)
Tertiary emulsions pH 6.0	0.24 %w/v	0.02%w/v WPI + 0.029 %w/v chitosan+ 0.1% w/v alginate	582.10 (±28.47)	-22.91 (±2.06)
Primary emulsion pH 3.5	5 %w/v	0.40 %w/v WPI	297.32 (±8.86)	34.44 (±3.56)
Secondary emulsions pH 3.5	1.77 %w/v	0.149 %w/v WPI + 0.28% w/v carrageenan	499.78 (±35.58)	-35.00 (±4.83)
Secondary emulsions pH 3.5	1.97 %w/v	0.165 %w/v WPI + 0.039% w/v alginate	384.79 (±81.70)	-40.00 (±2.75)
Tertiary emulsions pH 3.5	0.24 %w/v	0.075%w/v WPI + 0.0014 %w/v carrageenan+ 0.05% w/v chitosan	505.86 (±47.76)	45.07 (±1.59)

The oxidative stability was investigated via the ferric thiocyanate method by using UV-Vis spectroscopy. At pH 6.0, the maximum level of lipid hydroperoxide of primary emulsion was reached on the 11^{th} days. The lipid hydroperoxide content of secondary and tertiary emulsions on the 11^{th} days were 95.58 and 88.7% lower than primary emulsions. At pH 3.5, the lipid hydroperoxide of primary emulsion was reached to the maximum on the 13^{th} days. The lipid oxidation generating from both secondary emulsions (WPI-carrageenan and WPI-alginate) and tertiary emulsions showed the similar increasing after storage. The amount of lipid hydroperoxide of secondary emulsions (WPI-alginate and WPI-carrageenan) were showed the maximum of lipid hydroperoxide on the11^{th} days (26.02 mmol/kg oil) and 15^{th} days (21.67 mmol/kg oil) of storage, respectively. The lipid hydroperoxide generated from tertiary emulsions was increased to the maximum on the 11^{st} day (20.86 mmol/kg oil).

The improvement in the creaming and oxidative stabilities of multilayer emulsion may be due to the ability of multilayered interfacial membranes to either increase the repulsive colloidal interactions between the droplets such as electrostatic and steric repulsive forces, or to block the cations interact with the oil droplets. Therefore, the increasing number layer of polyelectrolyte was increased the region between the outer droplet with oil droplet, that can prevent transmission metal to interact with the lipid.

Hence, multilayer emulsion may have a number of potential applications in the food industry for example protection of lipid oxidation, improving the stability of emulsion to environmental stresses and also for controlled flavor release application.

Addition antioxidants in primary emulsion were investigated to retard the rate of oxidation. Sesame oil and capsaicin oleoresin were added directly in tuna oil, we found that primary emulsion added with 0.5 %w/v capsaicin oleoresin was the better oxidative stability. The genistein and roselle extract were added directly in emulsifier solution, we found that that primary emulsion added with 200 ppm roselle extract was the better oxidative stability.

The study of the oxidative stability of emulsion, we selected two method for monitor the lipid oxidation. First, the emulsion store at the room temperature (30°C) for 21days. We found the spoilage of emulsion before we could observe the changes in lipid hydroperoxide content. But in this method could be compared with the real

situation. Then, we selected to studied the another method, the emulsion was allowed to oxidize at 50 °C in water bath up to 12 hours. This method, we could observe the changes in lipid hydroperoxide content. But cannot use in the real life.

Suggestion for future research

The increasing of oxidative stability of tuna oil-in-water-emulsion, which contained omega-3 is important for the usage in food application. We can enhance the oxidative stability of oil-in-water emulsions by increasing the number of layer and adding natural antioxidant as mentioned above. Many natural antioxidants can be used in this research food industry such as Thai's herb, capsaicin, β-carotene, carotenoid, curcumin, etc. The amount of antioxidants can be varied to exhibit the effect of antioxidant quantity on the lipid oxidation rate. The suggestion for the further work is study in term of add antioxidant in the functional food, especially the extract from Thai's herb, example, roselle extract and curcumin for improve the stability of food and add value.

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APPENDIX

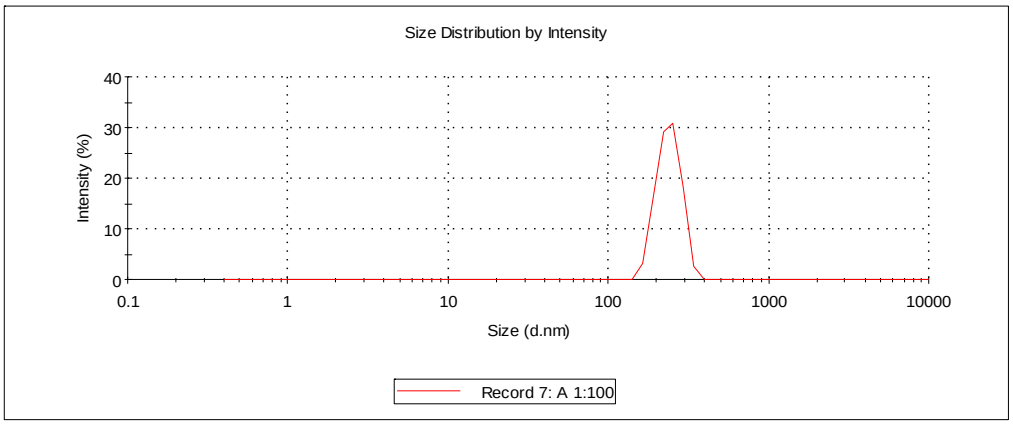

Figure 1 The particle size distribution of primary emulsion (5 %w/v tuna oil and 95 %w/v emulsifier solution (0.4 %w/v WPI)) at pH 6.0 (No.1).

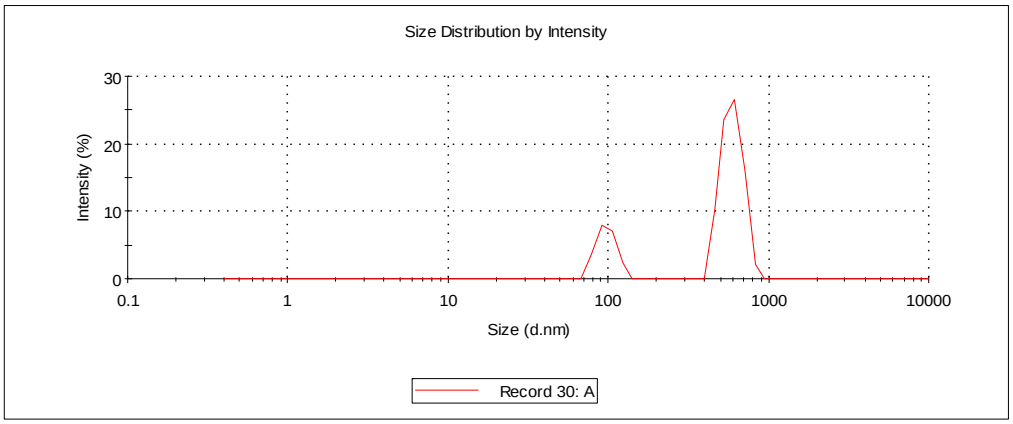

Figure 2 The particle size distribution of primary emulsion (5 %w/v oil and 95 %w/v emulsifier solution(0.4 %w/v WPI)) at pH 6.0 (No.2).

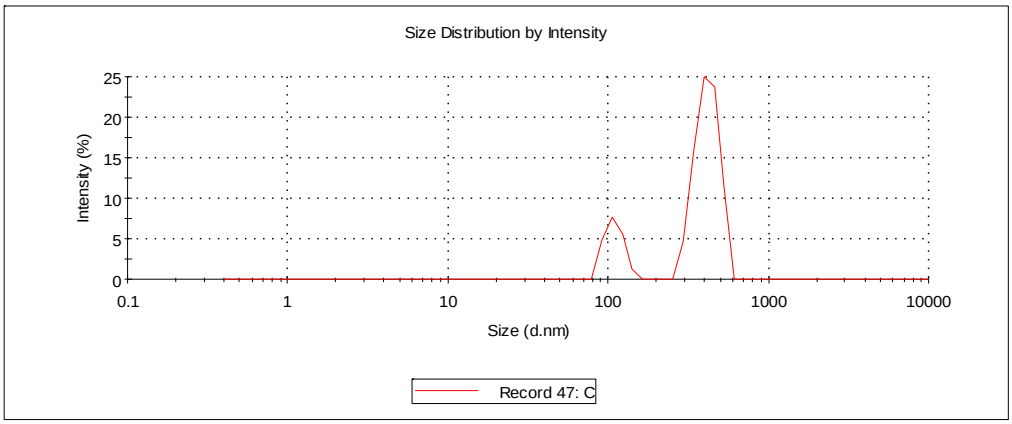

Figure 3 The particle size distribution of primary emulsion (5 %w/v oil and 95 %w/v emulsifier solution (0.4 %w/v WPI)) at pH 6.0 (No.3).

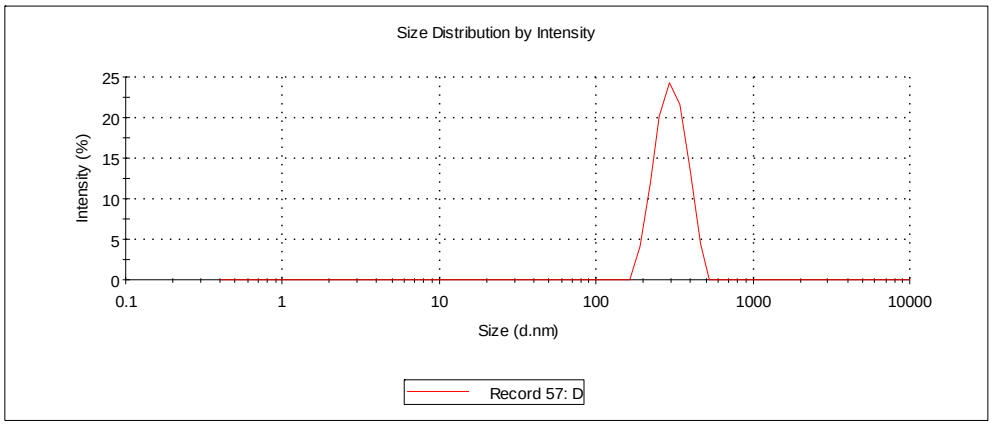

Figure 4 The particle size distribution of secondary emulsion (0.83 %w/v oil and 0.07 %w/v WPI + 0.83% w/v chitosan) at pH 6.0 (No.1).

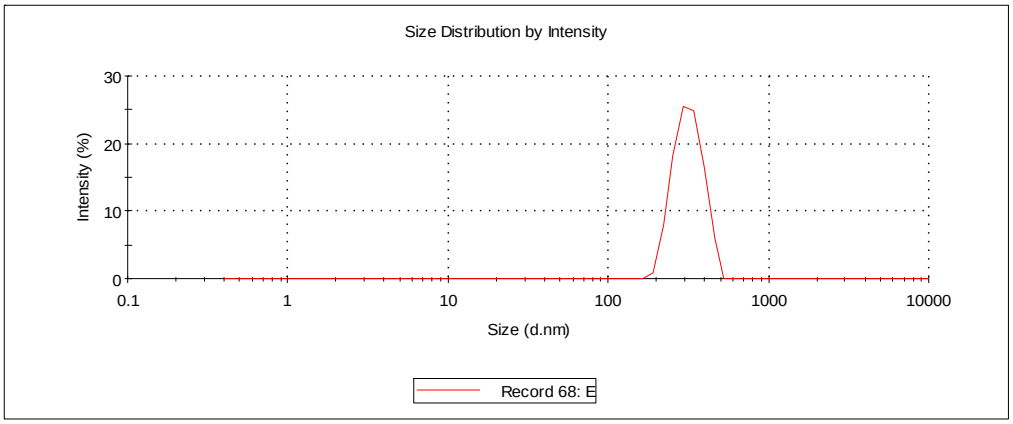

Figure 5 The particle size distribution of secondary emulsion (0.83 %w/v oil and 0.07 %w/v WPI + 0.83% w/v chitosan) at pH 6.0 (No.2).

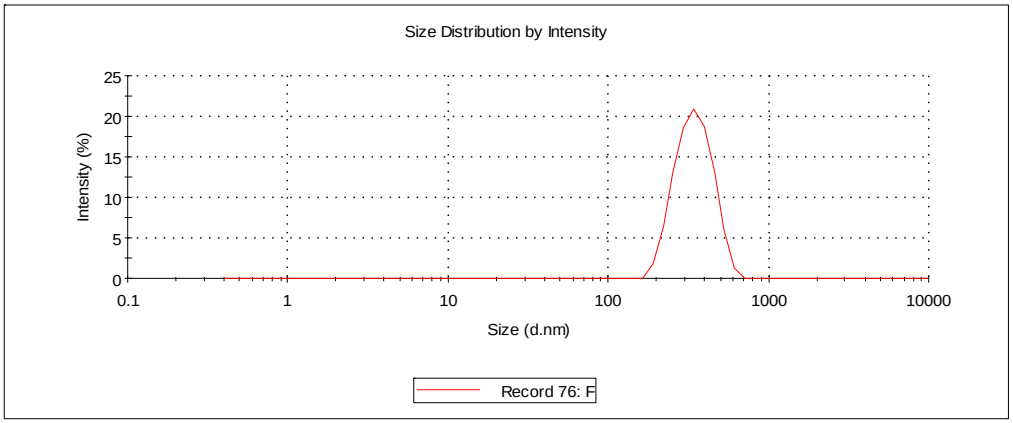

Figure 6 The particle size distribution of secondary emulsion (0.83 %w/v tuna oil and 0.07 %w/v WPI + 0.83% w/v chitosan) at pH 6.0 (No.3).

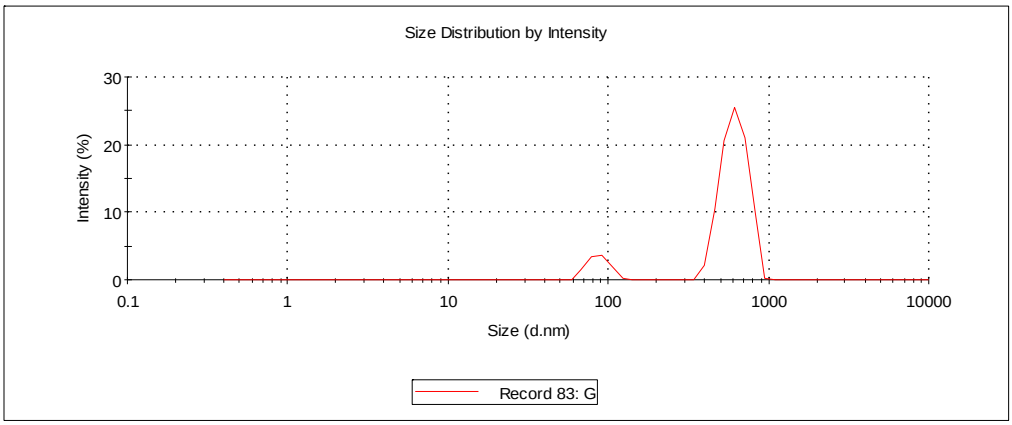

Figure 7 The particle size distribution of tertiary emulsion (0.24 %w/v tuna oil, 0.02%w/v WPI , 0.029 %w/v chitosan and 0.1% w/v alginate) at pH 6.0 (No.1).

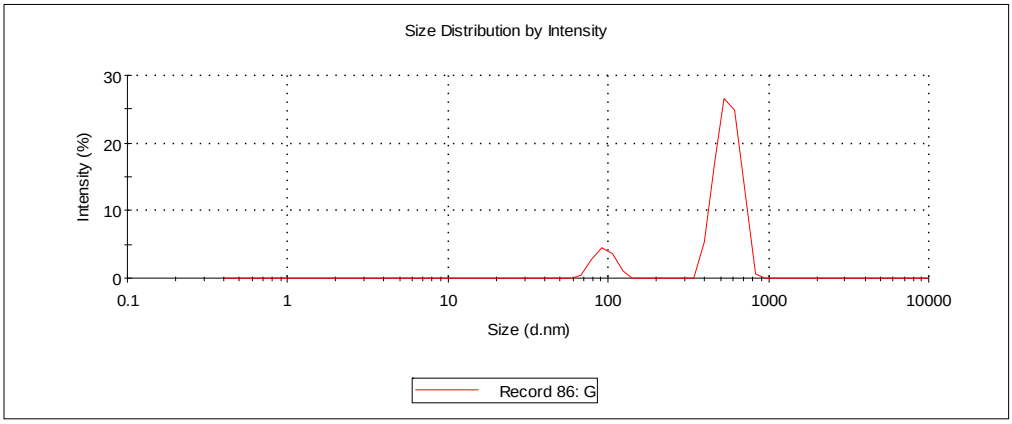

Figure 8 The particle size distribution of tertiary emulsion (0.24 %w/v tuna oil, 0.02%w/v WPI , 0.029 %w/v chitosan and 0.1% w/v alginate) at pH 6.0 (No.2).

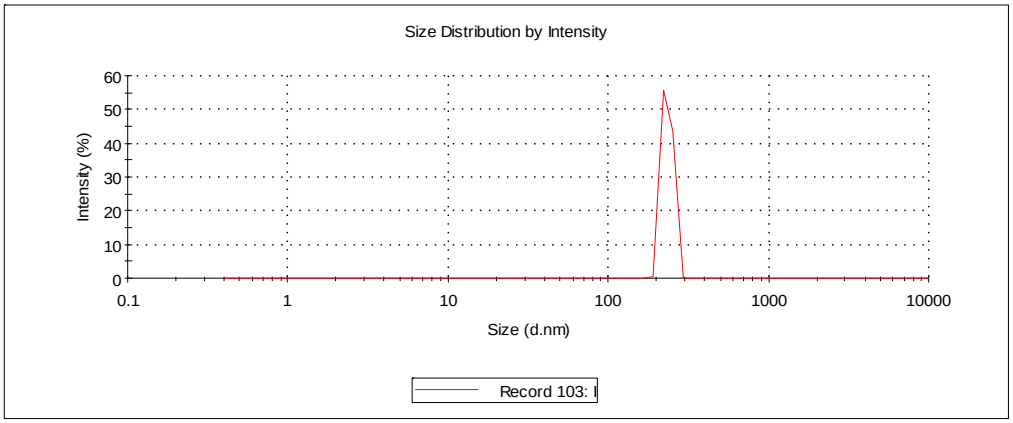

Figure 9 The particle size distribution of tertiary emulsion (0.24 %w/v tuna oil, 0.02%w/v WPI , 0.029 %w/v chitosan and 0.1% w/v alginate) at pH 6.0 (No.3).

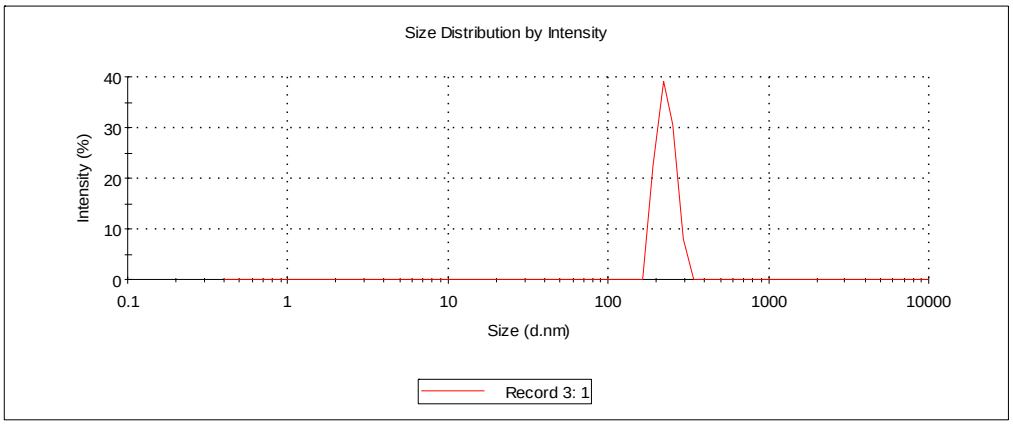

Figure 10 The particle size distribution of primary emulsion (5 %w/v oil and 95 %w/v emulsifier solution (0.4 %w/v WPI)) at pH 3.5 (No.1).

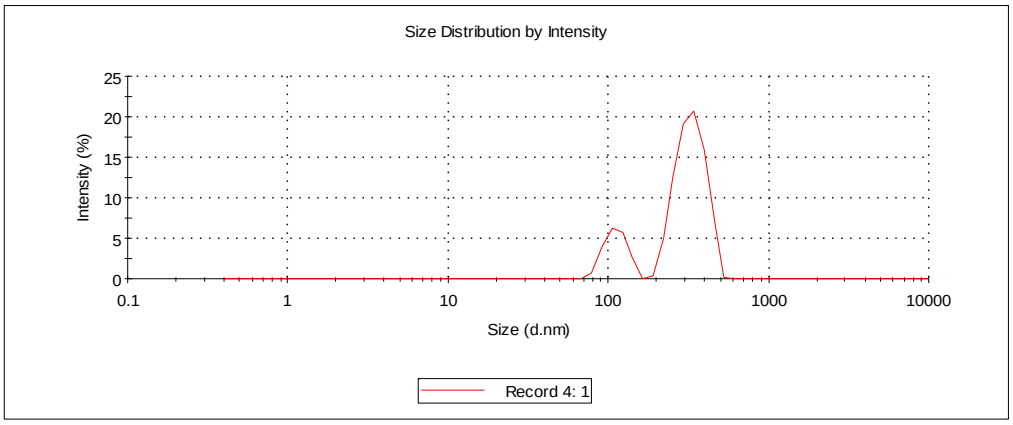

Figure 11 The particle size distribution of primary emulsion (5 %w/v oil and 95 %w/v emulsifier solution(0.4 %w/v WPI)) at pH 3.5 (No.2).

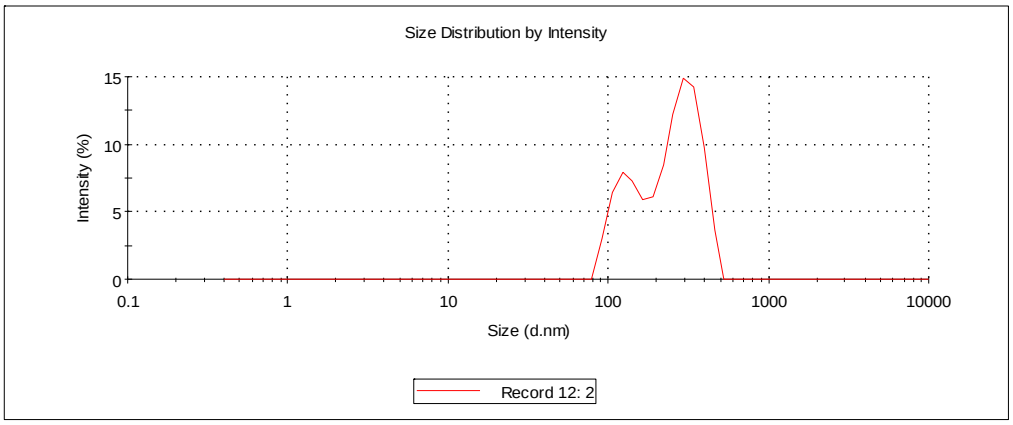

Figure 12 The particle size distribution of primary emulsion (5 %w/v oil and 95 %w/v emulsifier solution(0.4 %w/v WPI)) at pH 3.5 (No.3).

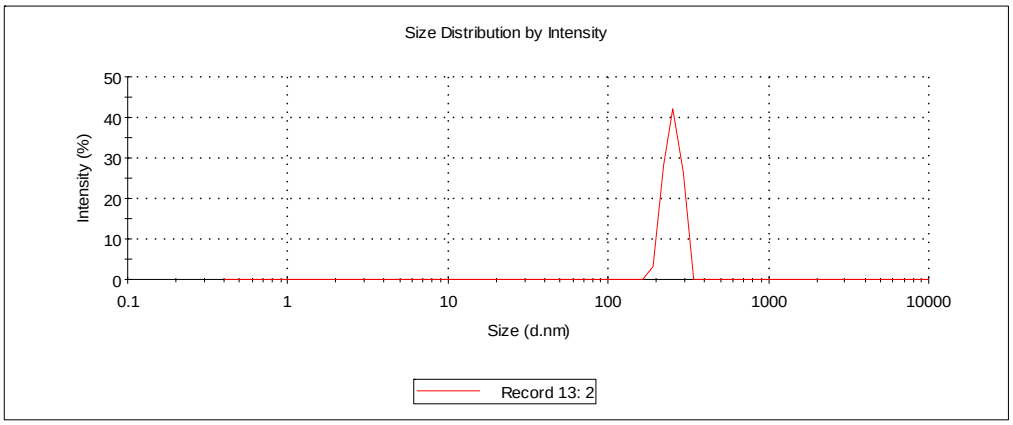

Figure 13 The particle size distribution of secondary emulsion (1.97 %w/v tuna oil, 0.165 %w/v WPI, and 0.039% w/v alginate) at pH 3.5 (No.1).

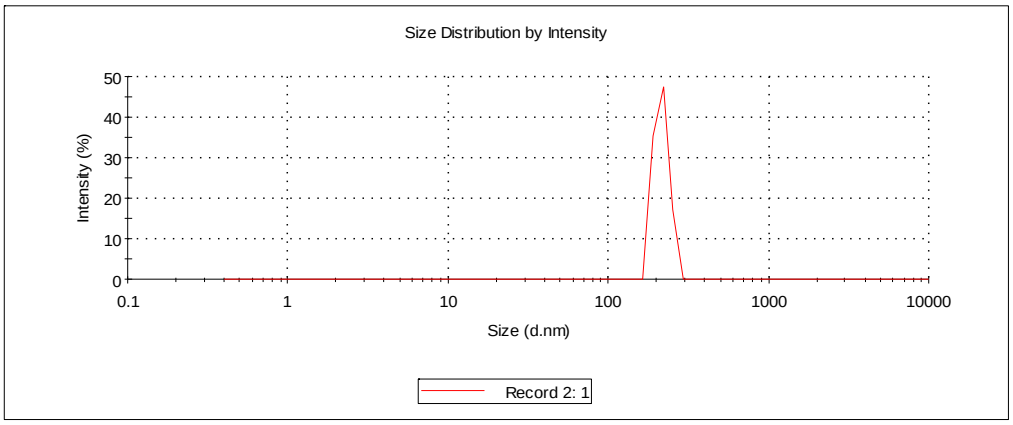

Figure 14 The particle size distribution of secondary emulsion (1.97 %w/v tuna oil, 0.165 %w/v WPI, and 0.039% w/v alginate) at pH 3.5 (No.2).

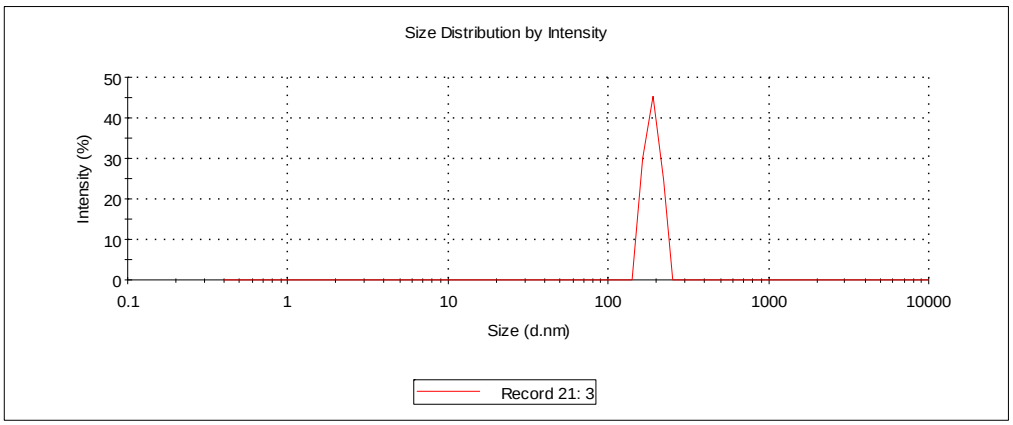

Figure 15 The particle size distribution of secondary emulsion (1.97 %w/v tuna oil, 0.165 %w/v WPI, and 0.039% w/v alginate) at pH 3.5 (No.3).

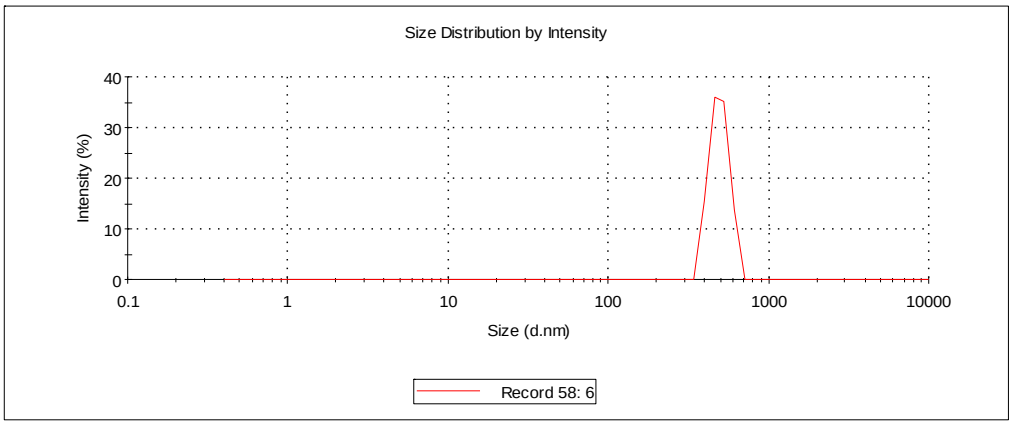

Figure 16 The particle size distribution of secondary emulsion (1.77 %w/v oil, 0.149 %w/v WPI, and 0.28% w/v carrageenan) at pH 3.5 (No.1).

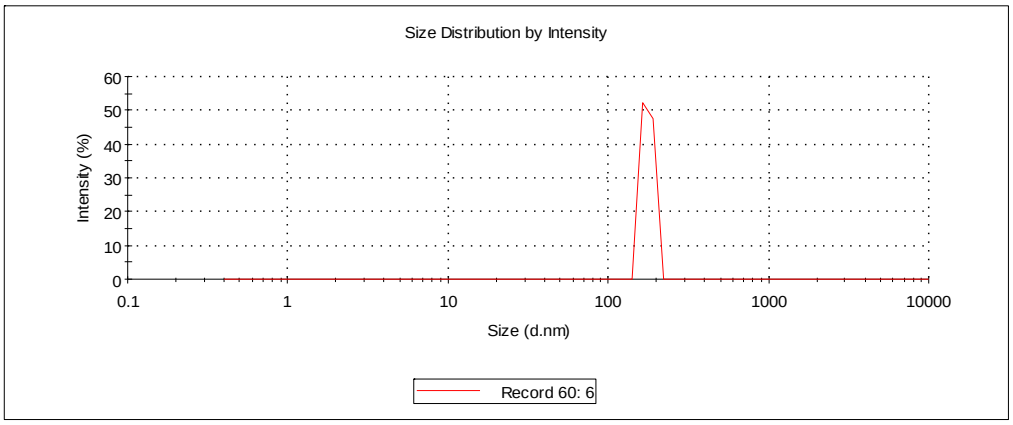

Figure 17 The particle size distribution of secondary emulsion (1.77 %w/v oil, 0.149 %w/v WPI, and 0.28% w/v carrageenan) at pH 3.5 (No.2).

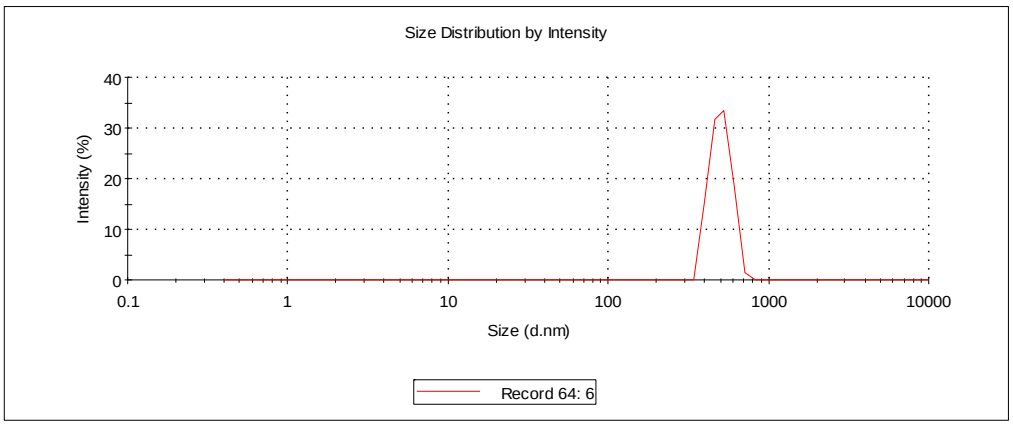

Figure 18 The particle size distribution of secondary emulsion (1.77 %w/v oil, 0.149 %w/v WPI, and 0.28% w/v carrageenan) at pH 3.5 (No.3).

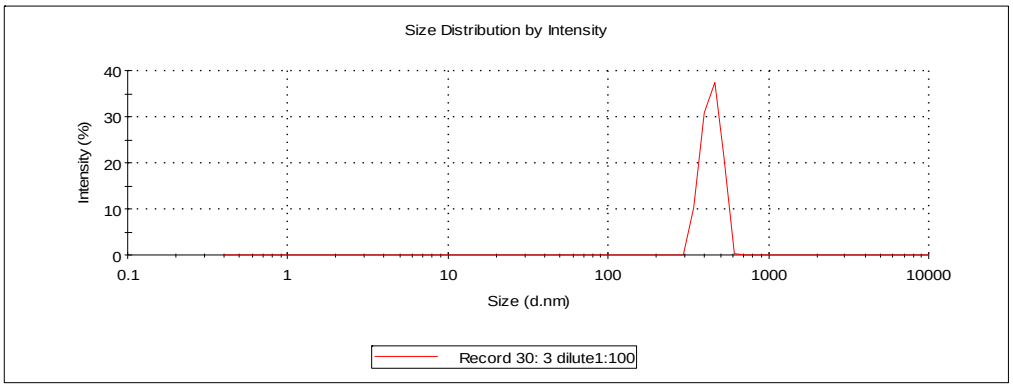

Figure 19 The particle size distribution of tertiary emulsion (0.24 %w/v oil 0.075%w/v WPI, 0.0014 %w/v carrageenan, and0.05% w/v chitosan) at pH 3.5 (No.1).

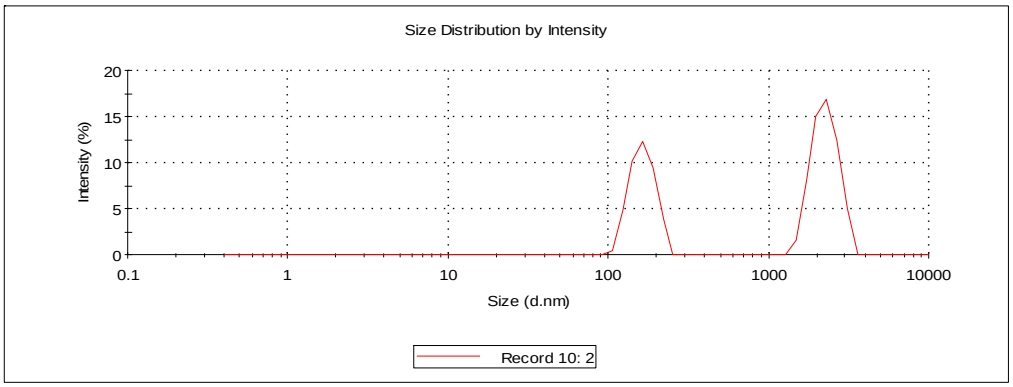

Figure 20 The particle size distribution of tertiary emulsion (0.24 %w/v oil 0.075%w/v WPI, 0.0014 %w/v carrageenan, and0.05% w/v chitosan) at pH 3.5 (No.2).

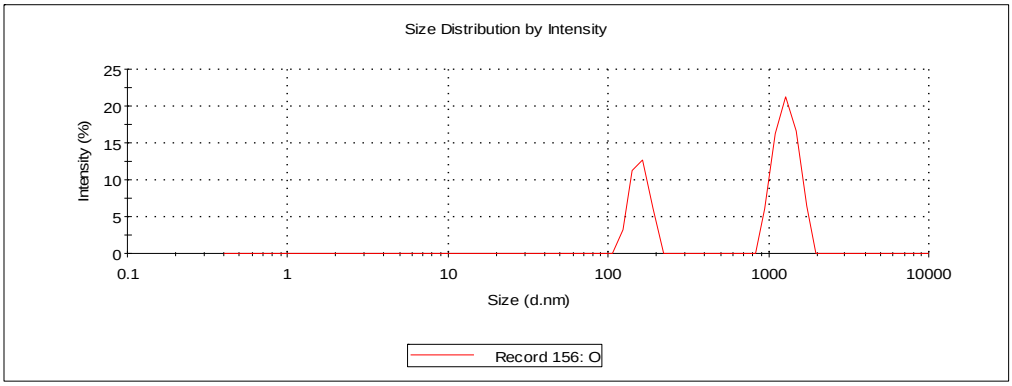

Figure 21 The particle size distribution of tertiary emulsion (0.24 %w/v oil 0.075%w/v WPI, 0.0014 %w/v carrageenan, and0.05% w/v chitosan) at pH 3.5 (No.1).

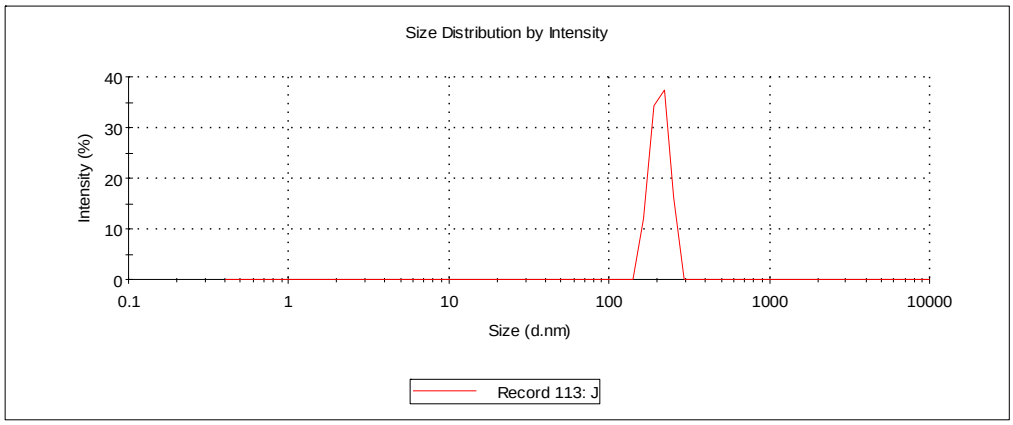

Figure 22 The particle size distribution of primary emulsion add 0.3 %w/v sesame oil at pH 6.0 (No.1).

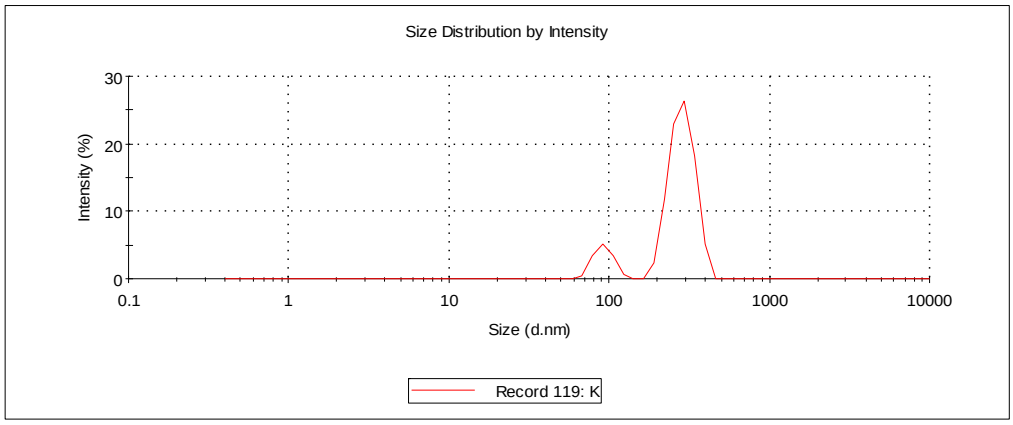

Figure 23 The particle size distribution of primary emulsion add 0.3 %w/v sesame oil at pH 6.0 (No.2).

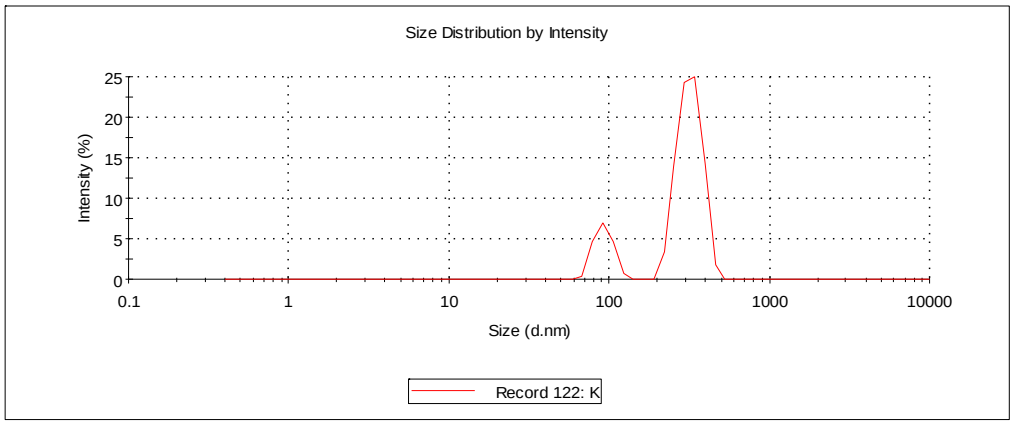

Figure 24 The particle size distribution of primary emulsion add 0.3 %w/v sesame oil at pH 6.0 (No.3).

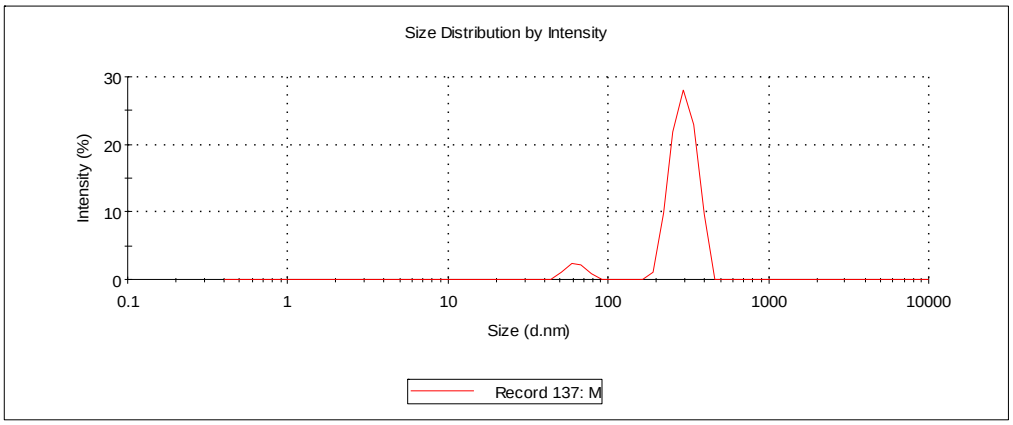

Figure 25 The particle size distribution of primary emulsion add 0.5 %w/v sesame oil at pH 6.0 (No.1).

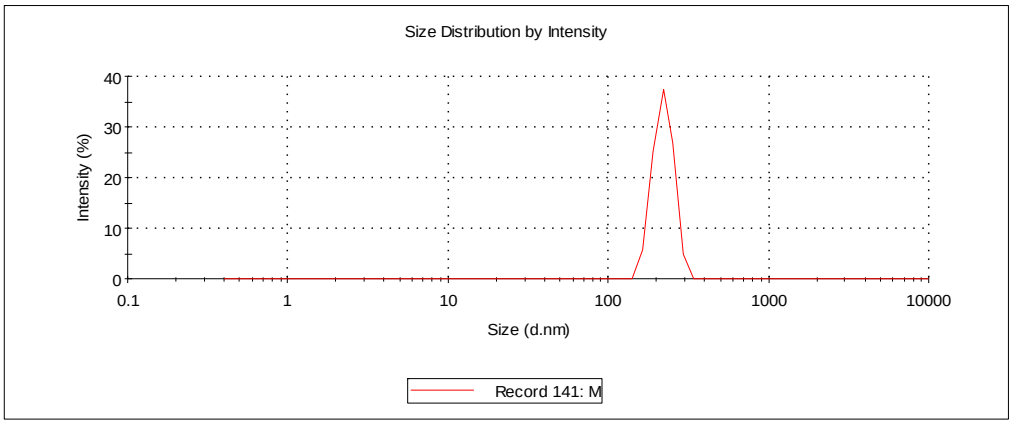

Figure 26 The particle size distribution of primary emulsion add 0.3 %w/v sesame oil at pH 6.0 (No.2).

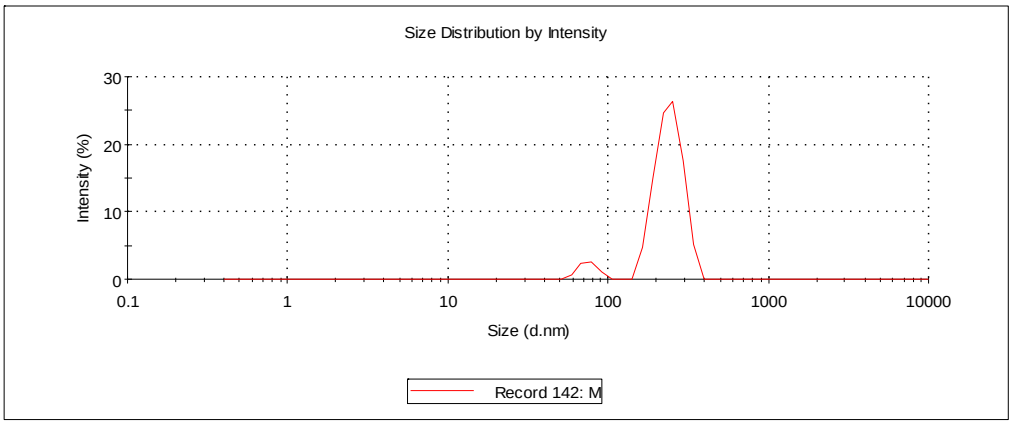

Figure 27 The particle size distribution of primary emulsion add 0.3 %w/v sesame oil at pH 6.0 (No.3).

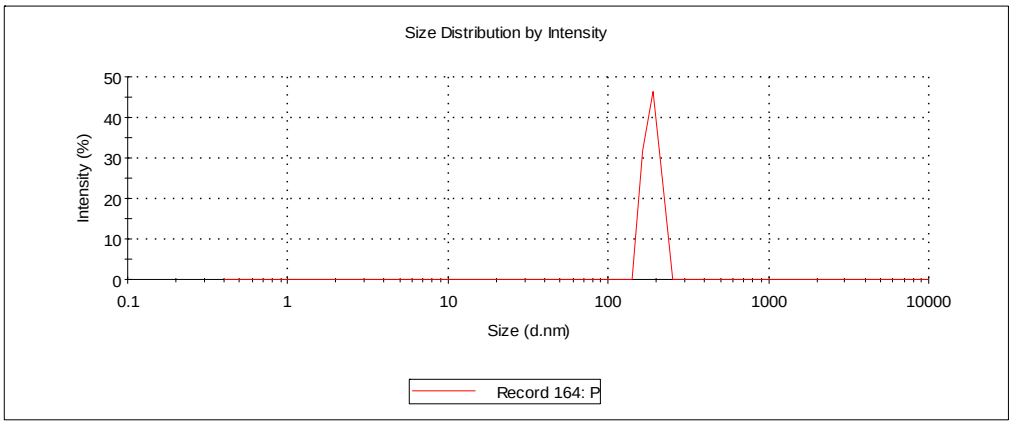

Figure 28 The particle size distribution of primary emulsion add 0.3 %w/v capsaicin oleoresin at pH 6.0 (No.1).

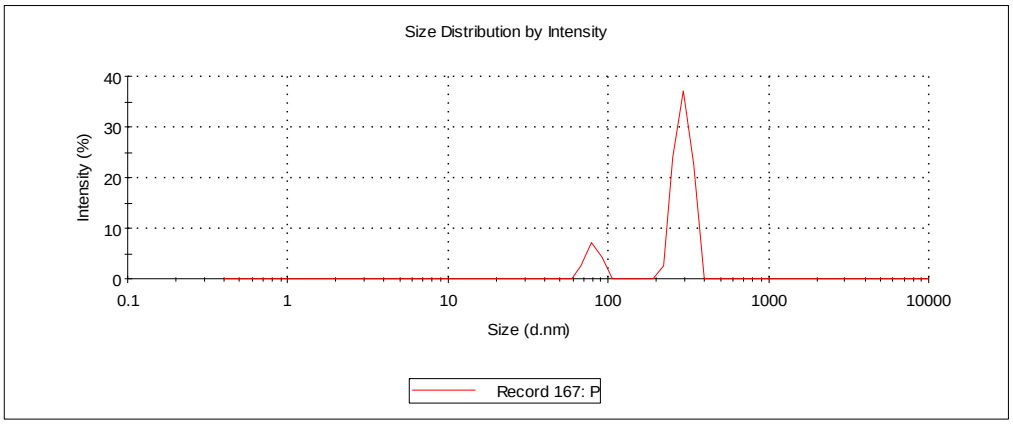

Figure 29 The particle size distribution of primary emulsion add 0.3 %w/v capsaicin oleoresin at pH 6.0 (No.2).

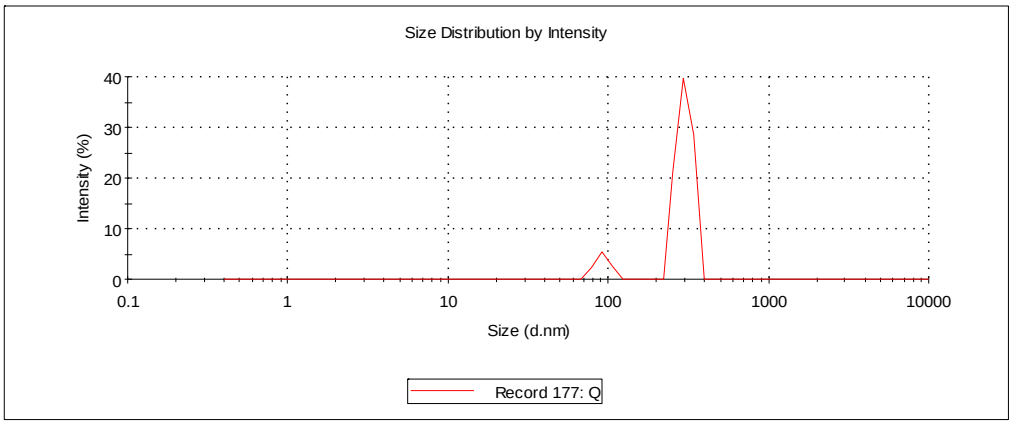

Figure 30 The particle size distribution of primary emulsion add 0.3 %w/v capsaicin oleoresin at pH 6.0 (No.3).

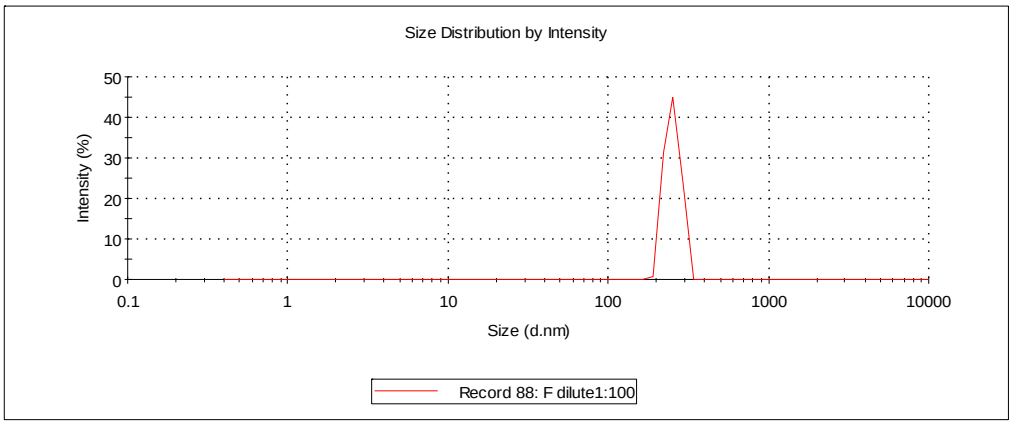

Figure 31 The particle size distribution of primary emulsion add 200 ppm roselle extract at pH 6.0 (No.1).

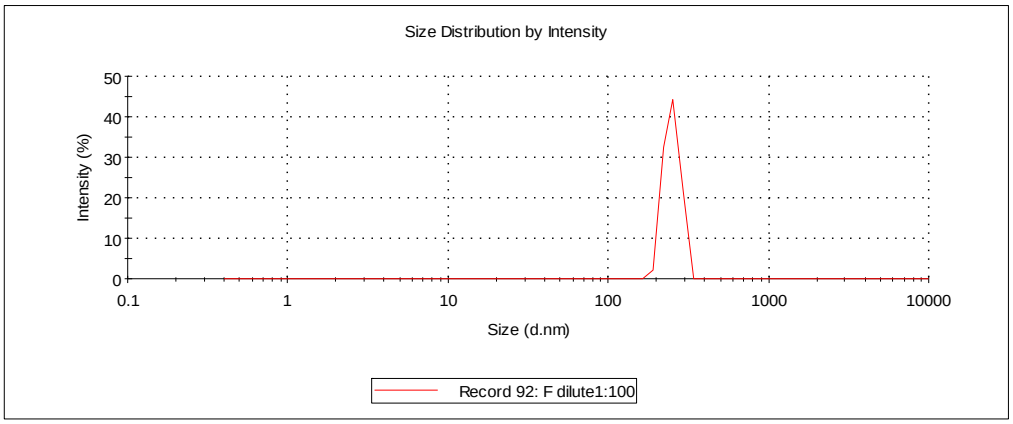

Figure 32 The particle size distribution of primary emulsion add 200 ppm roselle extract at pH 6.0 (No.2).

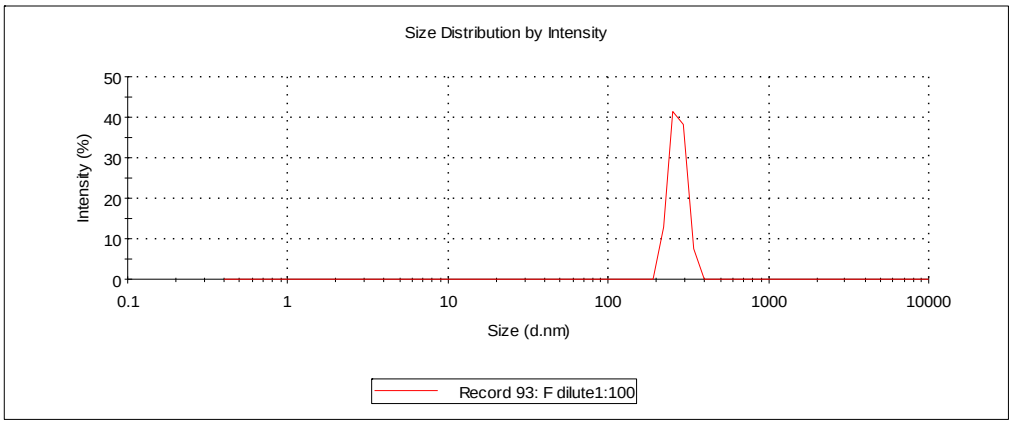

Figure 33 The particle size distribution of primary emulsion add 200 ppm roselle extract at pH 6.0 (No.3).

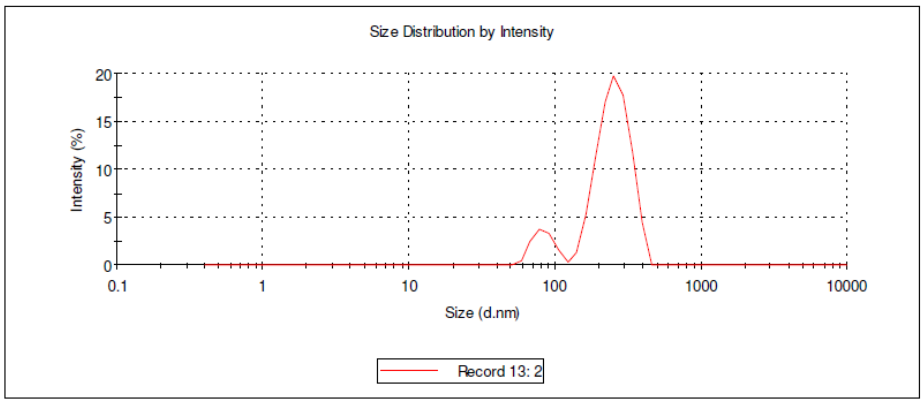

Figure 34 The particle size distribution of primary emulsion add 5 %w/v genistein at pH 6.0 (No.1).

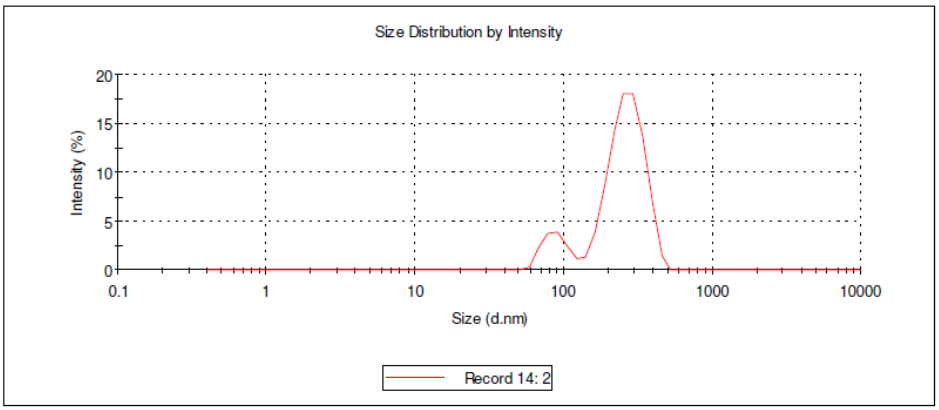

Figure 35 The particle size distribution of primary emulsion add 5 %w/v genistein at pH 6.0 (No.2).

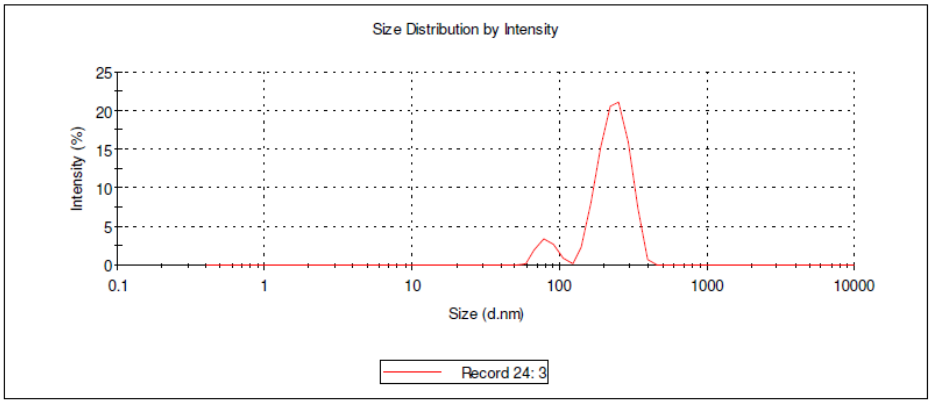

Figure 36 The particle size distribution of primary emulsion add 5 %w/v genistein at pH 6.0 (No.3).

VITAE

Miss Phetcharat Yongbut was born on January 26th, 1985 in Phetchabun, Thailand. She received a Bachelor's Degree of Engineering, majoring in Petrochemicals and Polymeric Materials from Faculty of Engineering and Industrial Technology, Silpakorn University in 2007. She has been a graduate student studying Petrochemistry and Polymer Science as her major course from Faculty of Science, Chulalongkorn University. Throughout master study, she had a great opportunity to present her work in poster session in the topic of "Oxidative stability of tuna oil-in-water emulsion stabilized by chitosan-alginate" (Poster Session) at the 22nd Annual Meeting of the Thai Society for Biotechnology "International Conference on Biotechnology for Healthy Living" Prince of Songkla University, Trang Campus, Thailand, October 20-22, 2010 and the topic of "Physical and oxidative stability of tuna oil-in-water emulsion stabilized with casein and whey protein isolate" (Poster Session) at Pure and Applied Chemistry International Conference (PACCON2011). The finance for joining the conference was supported by National Center of Excellence for Petroleum and Petrochemicals and Advanced Materials (NCE-PPAM), Chulalongkorn University. She graduated in October 2011.

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