ฤทธิ์ต้านออกซิเดชันของสารสกัดจากข้าวเหนียวดำ

นางสาววนิดา เทวารุทธิ์

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ANTIOXIDANT ACTIVITY OF BLACK GLUTINOUS RICE *Oryza sativa* L. EXTRACTS

Miss Wanida Tewaruth

A Thesis Submitted in Partial Fulfillment of the Requirements
for the Degree of Master of Science Program in Food Science and Technology
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Thesis Title	ANTIOXIDANT ACTIVITY OF BLACK GLUTINOUS RICE *Oryza sativa* L. EXTRACTS.
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วนิดา เทวารุทธิ์ : ฤทธิ์ต้านออกซิเดชันของสารสกัดจากข้าวเหนียวดำ (ANTIOXIDANT ACTIVITY OF BLACK GLUTINOUS RICE *Oryza sativa* L. EXTRACTS)
อ. ที่ปรึกษา : อ. ดร. ชนิษฐา ธนานุวงศ์, อ. ที่ปรึกษาร่วม : รศ. ดร. วรรณา ตุลยธัญ, 56 หน้า

งานวิจัยนี้มีวัตถุประสงค์เพื่อศึกษาชนิดของตัวทำละลายและภาวะการสกัดที่เหมาะสมสำหรับการเตรียมสารสกัดหยาบจากข้าวเหนียวดำ และศึกษาการนำสารสกัดหยาบไปใช้เป็นสารต้านออกซิเดชัน และ สารให้สีในผลิตภัณฑ์มายองเนสเสริมน้ำมันปลา ในขั้นแรก ศึกษาการสกัดแป้งข้าวเหนียวดำด้วยตัวทำละลาย 3 ระบบ ดังนี้ สกัดด้วย 50% เอธานอล (v/v) 2 รอบ 70% อะซิโตน (v/v) 2 รอบ และ 50% เอธานอล (v/v) ตามด้วย 70% อะซิโตน (v/v) ในภาวะกรด (pH 2) และภาวะที่เป็นกลาง (pH 6.8) เป็นเวลา 2 4 และ 8 ชั่วโมง และนำสารสกัดหยาบที่ได้มาวิเคราะห์หาปริมาณสารฟีนอลิกทั้งหมด, ปริมาณสารแอนโธไซยานินในรูปโมโนเมอร์ทั้งหมด และฤทธิ์การต้านออกซิเดชัน ด้วยวิธี DPPH และ FRAP จากการวิเคราะห์ทางสถิติ พบว่าปัจจัยหลักทั้ง 3 ปัจจัย กล่าวคือ ชนิดของตัวทำละลาย pH ของตัวทำละลาย และเวลาในการสกัด และอิทธิพลร่วมระหว่าง 2 ปัจจัย และ/หรือ 3 ปัจจัย มีผลอย่างมีนัยสำคัญต่อแต่ละค่าที่ตรวจวัด ($p \leq 0.05$) เนื่องจากระบบการสกัดที่ดีที่สุดสำหรับการสกัดสารฟีนอลิกทั้งหมด แอนโธไซยานินในรูปโมโนเมอร์ทั้งหมด และการศึกษาฤทธิ์ต้านออกซิเดชันนั้นไม่สอดคล้องกัน จึงพิจารณาภาวะการสกัดที่เหมาะสมจากภาวะที่ใช้เวลาในการสกัดที่สั้นที่สุด ที่สามารถสกัดสารฟีนอลิกทั้งหมดและสารแอนโธไซยานินในรูปโมโนเมอร์ทั้งหมดในปริมาณที่ยอมรับได้ และให้สารสกัดหยาบที่มีฤทธิ์ต้านออกซิเดชันสูง ซึ่งภาวะดังกล่าว คือ การสกัดด้วย 50% อะซิโตน ที่ภาวะเป็นกลางจำนวนสองรอบ เป็นเวลาทั้งหมด 4 ชั่วโมง จากนั้นจึงนำสารสกัดหยาบที่ได้จากภาวะนี้ไปศึกษาการใช้ประโยชน์ในผลิตภัณฑ์มายองเนสเสริมน้ำมันปลา โดยเติมสารสกัดหยาบที่ผ่านกระบวนการทำแห้งแบบแช่เยือกแข็งลงในผลิตภัณฑ์ ที่ 2 ความเข้มข้น คือ 500 ppm และ 1000 ppm (โดยน้ำหนักของน้ำมันในผลิตภัณฑ์) ติดตามการเกิดออกซิเดชันของวัฏภาคน้ำมันในตัวอย่างที่เก็บรักษาที่อุณหภูมิ 30°C ทุกๆ 5-7 วัน เป็นเวลาทั้งหมด 30 วัน โดยวิเคราะห์หาปริมาณ conjugated diene hydroperoxides (CDH) และ ปริมาณ thiobarbituric acid reactive substance (TBARs) และวัดสีของผลิตภัณฑ์ในระบบ Hunter (L, a, b) จากผลการทดลองพบว่า สารสกัดหยาบสามารถชะลอการเพิ่มปริมาณ CDH และ TBARs ในวัฏภาคน้ำมันของตัวอย่างระหว่างการเก็บรักษา โดยที่สารสกัดที่ความเข้มข้น 1000 ppm จะมีฤทธิ์การต้านออกซิเดชันสูงกว่า อย่างไรก็ตาม ค่าความสว่าง (L) และค่าสีเหลือง (b) ของตัวอย่างมีค่าลดลง ขณะที่ ค่าสีแดง (a) ของตัวอย่างมีค่าเพิ่มขึ้นระหว่างการเก็บรักษา ซึ่งแสดงถึงความไม่คงทนของสีแอนโธไซยานินในผลิตภัณฑ์ จากการทดลองโดยรวมของการศึกษาถึงการใช้ประโยชน์ของสารสกัดหยาบในมายองเนสเสริมน้ำมันปลา พบว่า แม้ว่าสารสกัดหยาบจะมีฤทธิ์ต้านออกซิเดชันที่สูงในผลิตภัณฑ์ แต่ไม่เหมาะที่จะใช้เป็นสารให้สีในผลิตภัณฑ์นี้ เนื่องจากให้สีชมพูหรือม่วงอ่อนที่ไม่คงตัว

ภาควิชา.... เทคโนโลยีทางอาหาร..... ลายมือชื่อนิสิต.....วนิดา เทวารุทธิ์.....
สาขาวิชา..วิทยาศาสตร์และเทคโนโลยีทางอาหาร.... ลายมือชื่ออาจารย์ที่ปรึกษา.....
ปีการศึกษา...2550... ลายมือชื่ออาจารย์ที่ปรึกษาร่วม.....

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KEY WORD: BLACK GLUTINOUS RICE / NTIOXIDANT / EXTRACTION / APPLICATION

WANIDA TEWARUTH: ANTIOXIDANT ACTIVITY OF BLACK GLUTINOUS RICE *Oryza sativa* L. EXTRACTS. THESIS ADVISOR: KANITHA TANANUWONG, Ph. D. THESIS COADVISOR: ASSOC. PROF. VANNA TULYATUN, Ph. D. 56 pp

The objectives of this research were to determine the appropriate solvent and the optimum extraction condition to prepare crude extract from black glutinous rice and to determine the application of the crude extract as an antioxidant and colorant in fish oil enriched mayonnaise. Firstly, the black glutinous rice flour was extracted twice with 50% ethanol (v/v), 70% acetone (v/v), and 50% ethanol (v/v) followed by 70% acetone (v/v), at neutral (pH 6.8) and acidic (pH 2) conditions, for 2, 4 and 8 hours of total extraction times. Total phenolic content, total monomeric anthocyanin content and antioxidant activities, determined by DPPH and FRAP methods, of the crude extracts were measured. Statistical analysis showed that there were significant effects of all three main factors, which were type of solvent, pH of solvent and extraction time, as well as significant two-way and/or three-way interactions of those main factors on each parameter measured ($p \leq 0.05$). Since the best extraction conditions for total phenolics, total monomeric anthocyanins, FRAP and DPPH assays were not agreeable, the most suitable extraction condition was selected from the shortest total extraction time which gave an acceptable amount of total phenolics and total monomeric anthocyanins, and high antioxidant activity. Therefore, the most suitable solvent and condition was the twice extraction with neutral 50% acetone at 4 hour total extraction time. The crude extract from the selected condition was then used to study its application in fish oil enriched mayonnaise. The freeze-dried extract was added into the mayonnaise at two different concentrations, 500 ppm and 1000 ppm (based on oil weight in the mayonnaise). Lipid oxidation in the oil phase of mayonnaise samples stored at 30°C was determined every 5-7 days up to 30 days by measuring conjugated diene hydroperoxides (CDH) and thiobarbituric acid reactive substance (TBARs). Color of the mayonnaise was also measured in the Hunter (L, a, b) system. The results showed that the crude extract could retard an increase in both CDH and TBARs in the oil phase of the mayonnaise samples during storage. Crude extract at 1000 ppm showed higher antioxidant activity. However, lightness (L) and yellowness (b) of all mayonnaise samples decreased whereas redness (a) of all samples increased during storage which indicated the instability of anthocyanin colorant in the product. The overall results from the application study showed that although the crude extract exhibited great antioxidant activities in fish oil enriched mayonnaise, it could not serve as a good colorant in this product since its pink or light purple color was not stable.

Department...Food Technology..... ..... Student's.....Wanida T.....

Field of study...Food Science and Technology... Advisor's.....

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CHAPTER I
INTRODUCTION

Commercially, the fat deterioration is important for all persons involved in the entire food chain from farmer to consumer. The decomposition of oxidation products results in the decrease in nutrition and sensory quality. But this type of oxidation can be inhibited by many ways such as prevention of oxygen access, storage at lower temperature, inactivation of oxidizing enzymes, use of suitable packaging, and use of antioxidants. Natural antioxidant was firstly used in 1940s. Gum guaiac was the first antioxidant approved for the stabilization of animal fats, especially lard (Madhavi, Deshpande and Salunkhe, 1995). Natural antioxidants have been replaced by synthetic substances which are inexpensive, more purified and have more uniform antioxidant properties. However, consumers still prefer natural antioxidants probably because they believe that the natural forms are safer to use in food. Therefore, there has been an increasing interest in the extraction and use of antioxidants from natural plant sources in food products. Several articles have reported the antioxidant capacity of grains (Velioglu *et al*., 1998; Adom and Liu, 2002; Pérez-Jimenez and Saura-Calixto, 2005; Sun and Ho, 2005). However, there is still limited data on antioxidant activity of pigmented rice extract and their application in food products, which will be determined in this study.

Although a lot of research studies regarding antioxidant activity of plant extracts have been done, there are several limitations in those previous studies. An important limitation is the extraction procedure. For the solvent extraction of phenolic compounds, the solvent most commonly employed is absolute ethanol or ethanol-water mixture in different proportions (Gray *et al*., 2002). Some authors used methanol-water mixture as an extraction solvent (Handelman *et al*., 1999), but it is not often acidified, yet the acidification has been shown to improve the extraction (Awika, Rooney and Waniska, 2004). Therefore, in this research, the optimum extraction condition for the black glutinous rice extract was investigated. Application of the crude extract in food emulsion system was also determined.

The objectives of this research are:

1. To study the effects of extracting solvents and extracting conditions on total phenolic content, total monomeric anthocyanin content and antioxidant activities of black glutinous rice *Oryza sativa* L. extracts and select the optimum extraction system.

2. To study the application of the black glutinous rice crude extract as antioxidant and colorant in fish oil enriched mayonnaise.

CHAPTER II
LITERATURE REVIEW

A. Phenolic compounds in cereal grains

Cereal grains contain various classes of phenolic compound including phenolic acids, flavonoids and polyphenolic compounds. These compounds are mainly located in the pericarp of cereal grain and exist in free and insoluble bound forms. The insoluble bound phenolics had been found to be the parts of cell wall components, presented as phenolic-carbohydrate esters that can be released by alkaline solvents (Sosulki, Krygier and Hogge, 1982). Adom and Liu (2002) investigated the amount of ferulic acid, total flavonoids and total phenolics in the whole grains of the non-pigmented corn, wheat, oat, and rice as free, soluble conjugate, and insoluble bound forms. Free forms of phenolic compounds were extracted from the grain sample with 80% chilled ethanol. The residues after the ethanol extraction were used to digest bound phenolic compounds and soluble conjugate ferulic acid with 2.0 M sodium hydroxide at room temperature for 1 hour. Total phenolic content of grains were determined by Folin-Ciocalteu assay. The ferulic acid content was determined by high performance liquid chromatography (HPLC). The total flavonoids contents were detected by colorimetric method. The results showed that more than 60% of the total phenolics, ferulic acid and total flavonoids in all grains were found in the bound forms. For all grains, free, soluble conjugated and bound ferulic acids were present in the ratio of 0.1:1:100. Corn had the highest amount of total phenolics, ferulic acid and total flavonoids in bound form, followed by wheat, oat and rice, respectively. The results of total phenolic content in a free form showed that corn and rice had the highest amount of these compounds followed by wheat and oat. The results of free ferulic acids were indicated that corn had the highest free ferulic acid content followed by rice, oat and wheat. As for the total flavonoid contents in a free form, oat had the highest amount of these phenolics followed by rice, corn and wheat.

For most of the pigmented cereals, the major type of pigments in these grains was anthocyanins. Anthocyanin pigmentation may be varying from pink to deep purple. The color development is affected by many factors including light intensity and growth

stage feeding. Pigmentation may appear early or late in the life of the plant and may be permanent or temporary. As for pigmented rice, the purple pericarp containing anthocyanin is common among glutinous varieties. The pigmented glutinous rice possesses a dull and soft grain, the cut surface being described as paraffin-wax-like in appearance. The grain is usually large, having varieties of colors as red, purple and black (Grist, 1986).

Abdel-Aal, Young and Rabalski (2006) identified and quantified anthocyanin pigments in blue, purple, red and white wheat, blue barley, blue, pink, purple, red and multicolor corn as well as black and red rice. The ground samples were extracted twice by methanol acidified with 1N hydrochloric acid (85:15, v/v) for 30 minutes. The total anthocyanin content was determined by both spectrophotometric method and HPLC. The results showed that rice had the highest total anthocyanin content followed by corn, wheat and barley. From the colorimetric analysis, black rice, purple corn and blue wheat had the highest total anthocyanin contents among the pigmented rice, corn and wheat, respectively. HPLC results showed that the major type of anthocyanins in the rice, corn, barley and wheat with black, red, blue and/or purple color shades was cyanidin-3-glucoside, except for the case of blue wheat which contained delphinidin-3-glucoside as a main anthocyanin. The peonidin-3-glucoside, pelargonidin and cyanidin-malonylglucoside were also found in these grains.

B. Determination of antioxidant activity in non-lipid systems

Many different methods have been used to determine the antioxidant activity in lipid systems. One of the major disadvantages is that a long period of time, weeks or months, is needed to follow the oxidative changes in bulk oil or emulsion. So the specific chemical test containing free radicals or synthetic oxidants, which are simpler and take shorter time, are widely used to evaluate antioxidant activities.

Prior, Wu and Schaich (2005) reported that the antioxidant could deactivate free radicals by two mechanisms, hydrogen atom transfer and single electron transfer. In the hydrogen atom transfer reaction, the antioxidant activity was measured by the ability of antioxidant to donate hydrogen atom to free radical. Hydrogen atom transfer assays were solvent and pH independent and were rapid. The reaction was completed in

seconds or minutes. Example of the hydrogen atom transfer assays included oxygen radical absorbance capacity (ORAC), total radical trapping antioxidant parameter (TRAP) and total oxidant scavenging capacity (TOSC). On the other hand, the single electron transfer mechanism was based on the reducing power of the antioxidant. The reactions were pH dependent. An example of single electron transfer assay was ferric reducing antioxidant power (FRAP).

The FRAP assay was originally elaborated by Benzie and Strain (1996) for assessing antioxidant power in human plasma by the reduction of ferric tripyridyltriazine complex (Fe(III)-TPTZ) to the ferrous form (Fe(II)-TPTZ) (Figure 2.1) which rapidly completed in 4 minutes. This transformation results in an intense blue color with an absorption maximum at 593-595 nm.

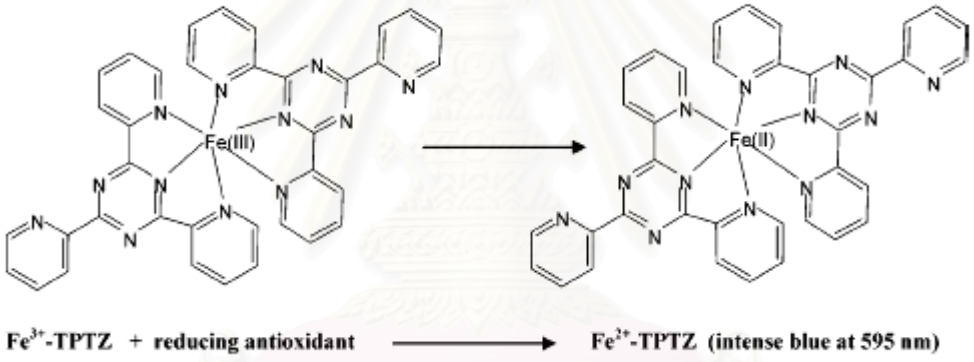

Figure 2.1: The structure of ferric tripyridyltriazine complex and its ferrous form (Prior, Wu and Schaich, 2005)

Another assay which has also been widely used to determined antioxidant activity was DPPH assay. 2,2-Diphenyl-1-picrylhydrazyl (DPPH) assay measures the reducing ability of antioxidants toward DPPH radicals. This assay has usually been classified as single electron transfer reactions. In fact, DPPH radicals may be neutralized by direct reduction via electron transfer or radical quenching via hydrogen atom transfer. In this assay, the loss of the color of DPPH radicals is monitored by a spectrophotometer at 515 nm (Prior, Wu and Schaich, 2005). The reaction can be

interpreted by the percentage of the remaining DPPH (equation 1) (Murakami *et al.*, 2004).

$$\% \text{ inhibition} = 100 \times (1 - (A_{sample} / A_{blank})) \quad \text{equation 1}$$

The concentration of antioxidant that caused the decrease of DPPH radicals to 50% of the initial concentration has been defined as 50% effective concentration (EC50). In general, DPPH assay is simple and rapid but some antioxidants reacted slowly or inert to DPPH. Thus the interpretation of antioxidant capacity from DPPH assay in some antioxidant capacity measurement may be inaccuracy (Prior *et al.,* 2005).

Brand-Williams, Cuvelier and Berset (1995) studied the antiradical power of phenolic compounds by DPPH assay. Nineteen pure compounds were tested. The reaction kinetics of these antioxidants was evaluated by measuring the required time to reach the steady state determined by the constant absorbance which did not change with time in the DPPH reaction. The authors found that there were three types of kinetic behaviors. Firstly, the rapid reaction which could reach the steady state within less than 1 minute. Antioxidants having rapid antiradical reaction were ascorbic acid, isoascorbic acid and iso-eugenol. Secondly, the intermediate reaction, the steady state was reached within 5-30 minutes. Antioxidants with intermediate reaction rate were rosmarinic acid and δ-tocopherol. Thirdly, the slow reaction which took 1-6 hour to reach the steady state. Examples of antioxidants in this category were zingerone, gallic acid, ferulic acid and phenol. However, in most of the researches (Sun and Ho, 2005; Murakami *et al.,* 2004; Pinsirodom, 2006), the antioxidant activity of phenolic compound was determined using the reaction time of 30 minutes in the DPPH assay.

According to the reaction stoichiometry study of the DPPH-antioxidant reaction, the kinetic behaviors of antioxidant depended on the available donating hydrogen atoms on hydroxyl groups. For instance, the mechanism of DPPH-BHT reaction was hypothesized as three possible pathways (Figure 2.2). The BHT radical species could form a ketonic compound (step a) by donation of a second hydrogen atom. The second pathway, a regeneration of the hydroxyl group by dimerization resulted in additional reactions with DPPH radicals to produce a bi-quinonoid structure (step b). The third

pathway (step c) involved a complexation of DPPH radical and BHT radical (Bondet, Brand-Williams and Berset, 1997).

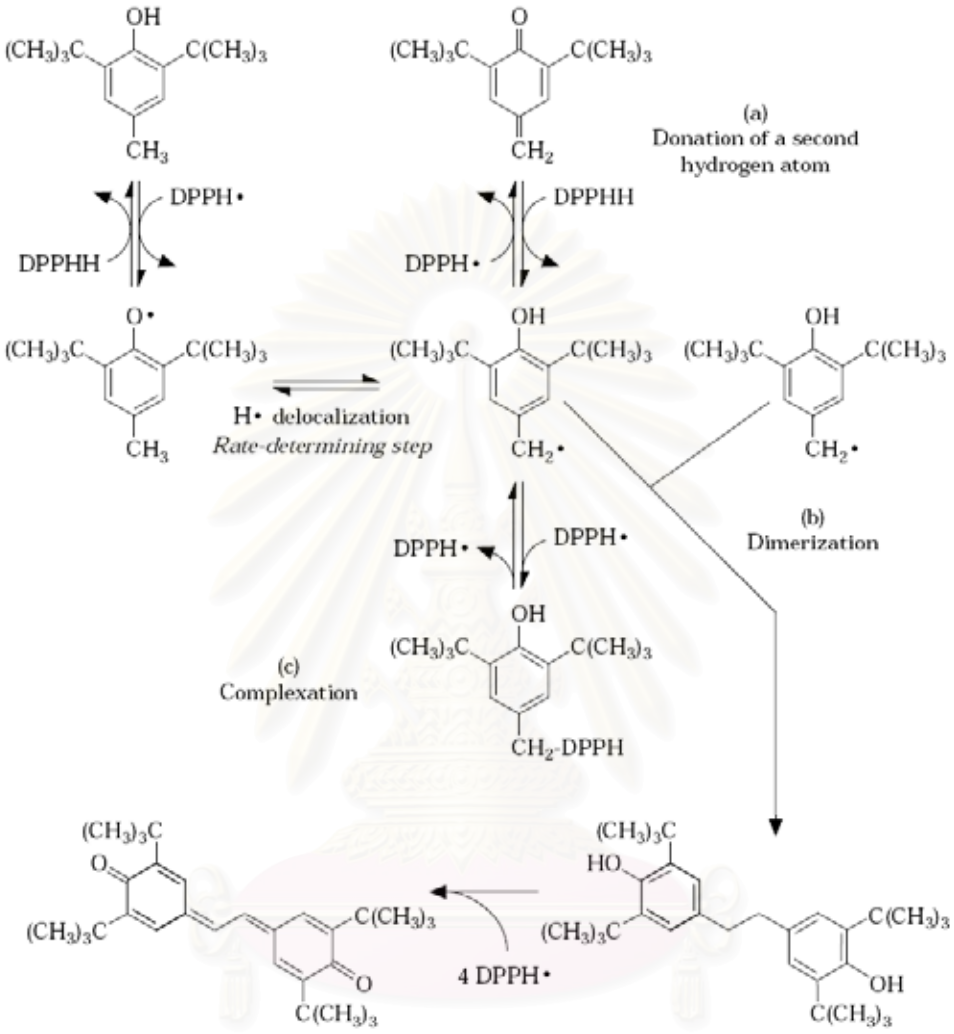

Figure 2.2: The pathways of BHT/DPPH reaction (Bondet *et al.*, 1997)

C. Study of the solvent extraction of phenolic compounds and the evaluation of their antioxidant activities using non-lipid systems

The extractability of polyphenolic compound by organic solvents depends on many factors including polarity of solvents, degree of polymerization of phenolics, and the interactions of phenolics with other constituents resulting in the formations of insoluble complexes. The solvents frequently used for the extraction of phenolic compounds include methanol, ethanol, acetone, water, ethyl acetate, propanol, dimethylformamide and their combinations. It is necessary to optimize an extraction

condition to obtain accurate results on the phenolic contents and their antioxidant activities. Therefore, many researchers have studied the effect of extracting solvents and the extracting condition on the phenolic contents and their antioxidant activities.

Sun and Ho (2005) studied antioxidant activities of buckwheat extracts comparing with butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT) and tertiary butylhydroquinone (TBHQ) using a β-carotene bleaching assay, a DPPH assay and the Rancimat method. Buckwheat was extracted with solvents of different polarities, which were methanol, ethanol, butanol, acetone and ethyl acetate at room temperature for 1 week. The authors found that different extracting solvent greatly affected antioxidant activity. Methanol extract showed the highest yield, highest antioxidant activity coefficient (AAC) by the β-carotene bleaching method, and also longest induction time by the Rancimat method. The acetone extract showed the highest total phenolics and the highest scavenging activity by the DPPH method.

Pérez-Jiménez and Saura-Calixto (2005) assessed the antioxidant capacities of cereals using both chemical extraction and *in vitro* digestive enzymatic extraction of antioxidants in wheat flour, bread, raw and boiled rice, wheat bran, and oat bran. The most efficient antioxidant extraction was achieved by using acidic methanol-water mixture (50:50 v/v, pH 2) followed by acetone-water mixture (70:30 v/v). Samples were extracted for 1 hour at room temperature. FRAP and DPPH methods were used to determine antioxidant activity in part of chemical extraction. For both of chemical extraction and enzymatic extraction, wheat flour had the highest total phenolic content. For antioxidant activity, wheat bran had the highest antioxidant capacity in FRAP method and wheat flour had the highest antioxidant capacity in DPPH method. The result, as determined by *in vitro* digestive enzymatic extracts obtained by enzymatic treatments that mimic conditions in the gastrointestinal tract, showed that the amount of antioxidants released by the cereal matrix into the human intestine may be higher than the one that can be expected from measurements in the usual aqueous-organic extracts.

Anthocyanins are usually extracted with acidic organic solvent especially acidic methanol, which can effectively destroy cell membranes and simultaneously dissolves

the anthocyanins (Shibuya, 1984). The acidic conditions also help stabilize anthocyanin by converting it into flavylium cation form (Figure 2.3).

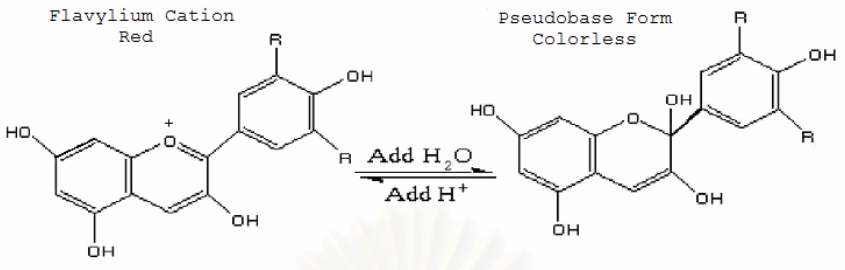

Figure 2.3: Anthocyanin structural forms at different pH (adapted from Brouillard and Delaporte, 1977)

Abdel-Aal and Hucl (2003) used acidified methanol (methanol and 1.0 N hydrolic acid, 85:15, v/v) to extract anthocyanin in blue wheat grain for the stability study at different storage conditions. The effect of temperature and pH on blue wheat anthocyanins were studied during 0-6 hour incubation. Two hundred ppm of freeze-dried anthocyanins were dissolved in 0.1 M HCl-phosphate buffer at pH 1, 3, or 5 and stored at 65, 80 and 95°C. The stability of anthocyanin was evaluated by measuring absorbance at 535 nm. The results showed that, for a given storage temperature and time, anthocyanins from blue wheat were most stable at pH 1, while there was no significant effect on the anthocyanin stability between the storage at pH 3 and pH 5 ($p>0.05$). On the other hand, the degradation of blue wheat anthocyanins increased with the increasing storage temperature and/or storage time for the same pH of the system.

Awika *et al.* (2004) quantified and identified anthocyanins from black sorghum and evaluated their antioxidant properties. Two extracting solvents, 1% HCl in methanol and 70% aqueous acetone were used. Samples were extracted for 2 hours at room temperature and stored at -20°C in darkness overnight. The quantity of total anthocyanins were determined by colorimetric and HPLC method. The antioxidant properties were evaluated by using the 2, 2´-azinobis (3-ethylbenzothiaziline-6-sulfonic acid) method. The results presented that acidified methanol resulted in higher amount of the total anthocyanins as well as higher antioxidant activity of the extract. The authors also reported that sorghum brans had three to four times higher anthocyanin contents

than the whole grains. From HPLC analysis, the 3-deoxyanthocyanidins including luteolinidin and apigeninidin were major anthocyanins in black sorghum.

From the previously mentioned literatures, the extracting solvents were varied and there are different results in different samples. Many researches used acidic extracting solvents and from some researches, the antioxidant activity of acidic solvent extracts is higher especially for the extracts containing high amount of anthocyanin. The condition of extraction such as temperature and time may also affect the antioxidant activity of the extracts. All of these researches showed the extracting method greatly affected the antioxidant activity of plant extracts.

D. Determination of the antioxidant activity in lipid systems

Although the non-lipid systems are often used to evaluate antioxidant activities of many food grade antioxidants because of its simplicity and rapid, the reagent used in non-lipid systems cannot be found naturally, especially in food products. The determination of the antioxidant activity in lipid systems is necessary for an application of antioxidants in food since the test systems can represent the real food products. Therefore, the bulk oil, oil-in-water emulsion model systems and real food system can be used to investigate the antioxidant activities of antioxidants as food additives, following the screening test from non-lipid systems. The lipid oxidation can be measured by two main methods, the sensory methods and chemical test methods. The sensory evaluation is mainly performed to detect the rancid odor in food. The intensity of the rancid odor can be related to the extent of the lipid oxidation. The chemical methods are used to follow the change of primary oxidative products and secondary oxidative products. The primary oxidative products can be quantified by the measurement of peroxide value, conjugated diene and triene hydroperoxides, while the secondary oxidative products can be quantified by the measurement of carbonyl compounds, 2-thiobarbituric acid (TBA) value and volatile oxidative products. The latter compounds can be measured by gas chromatographic methods (Frankel, 2005).

The measurement of conjugated diene hydroperoxides and the TBA analysis are among the mostly used methods to determine the extent of lipid oxidation due to its simplicity and rapid. The conjugated diene hydroperoxides produced from the oxidation

of polyunsaturated lipid can be measured spectrophotometrically by measuring the absorbance of the oil-solvent mixture at 234 nm. This technique is rapid and simple. For the TBA test, 2-thiobarbituric acid can react with the oxidative products of polyunsaturated lipids represented by malonaldehyde and provided the pink color. Absorbance of the sample is then measured at 532-535 nm. TBA does not react with only malonaldehyde (Figure 2.4) but can also react with any reactive secondary oxidative compounds which can be called TBA-reactive substances (TBARs).

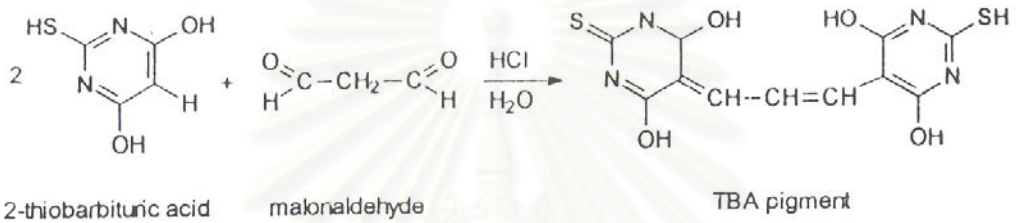

Figure 2.4: The production of TBA pigment from the reaction of 2-thiobarbituric acid and malonaldehyde (Frankel, 2005)

E. Study of the antioxidant activities of phenolic compounds and other types of antioxidant in lipid systems and their application in some food products

Frankel *et al.* (1996) evaluated the antioxidant activity of a rosemary extract and of its constituents, carnosol, carnosic acid and rosmarinic acid, comparing to α-tocopherol in inhibiting the formation and decomposition of hydroperoxides in tocopherol stripped corn oil and corresponding corn oil-in-water emulsion at 60°C. Oxidative stability was evaluated by analyzing samples periodically for conjugated diene hydroperoxides by spectrophotometry and for hexanal by headspace gas chromatography. In bulk corn oil, the rosemary extract, carnosic acid, rosmarinic acid, and α-tocopherol were significantly more active than carnosol. In contrast, in corn oil-in-water emulsion, the rosemary compounds were less active than in bulk corn oil, and the rosemary extract, carnosic acid, carnosol, and α-tocopherol were more active than rosmarinic acid. The difference in antioxidant activity of rosemary extract and its constituents in bulk oil and oil-in-water emulsion may be explained on the basis of interfacial partitioning. Hydrophilic antioxidants (rosemary extract, carnosic acid and

rosmarinic acid) provide better protection than the lipophilic antioxidants (α-tocopherol and carnosol) in bulk oil because those of the latter group remain in solution in the oil phase. In the emulsion system, the surface active lipophilic antioxidants are more protective, by being more evenly oriented in the oil-water interface, than the hydrophilic antioxidants, which remain in solution in the water phase.

Paroz *et al*. (2005) studied the antioxidant activities of grape flavonoids in lipid systems. The grape sample used in this study was a by-product from wine industry. The sample was extracted by 70% ethanol and fractionated by HPLC. The fractions of flavonoids in crude grape extracts were separate into groups of monomers and oligomers. The antioxidant activities of the fractional extracts were studied in fish oil, 10% fish oil-in-water emulsion and frozen mackerel in comparison with a commercial antioxidant, propyl gallate. 0.01% antioxidant (w/w) was added in all systems. The fish oil and 10% fish oil-in-water emulsion were stored at 40°C and 30°C, respectively. The lipid oxidation of fish oil and its emulsion were determined by measuring the conjugated diene hydroperoxides and the conjugated triene hydroperoxides by spectrophotometer every day for 8 days. For the frozen minced mackerel, the samples were kept at -10°C. The lipid oxidation of frozen mackerel was evaluated by measuring peroxide value, conjugated diene hydroperoxides and conjugated triene hydroperoxides, and TBA value every 10-20 days for 6 months. The results showed that the monomeric compounds had more efficiency to retard an increase in hydroperoxides during storage of bulk oil system. However, the oligomeric compounds had higher antioxidant activities in the emulsion system and the frozen mackerel system. Nevertheless, propyl gallate had the highest efficiency to increase the induction period of lipid oxidation in bulk oil, emulsion and frozen fish systems. The difference in the antioxidant activities of monomeric and oligomeric flavonoids in different systems was related to their partitioning coefficients between oil and water phase.

Let, Jacobsen and Meyer (2007) studied the ability of γ-tocopherol, ethylenediaminetetraacetate (EDTA) and ascorbyl palmitate to retard lipid oxidation in fish oil enriched salad dressing. The salad dressing contained 25% oil (15% refined rapeseed oil and 10% refined cod liver oil (w/w)). The effects of γ-tocopherol (22 and 88 mg/kg product), EDTA (10 and 50 mg/kg product) and ascorbyl palmitate (5 and 30

mg/kg product) on the oxidative stability of fish oil enriched salad dressing were studied during 6 week storage at room temperature. The lipid oxidation of salad dressing was evaluated periodically by measuring peroxide value and volatile oxidative products. The latter compounds were analyzed by headspace gas chromatography (HS-GC). The results presented that ascorbyl palmitate at high concentration (30 mg/kg product) promoted higher antioxidant efficiency in the inhibition of peroxide formation but it acted as the prooxidant in the volatile compounds determination. For EDTA and γ-tocopherol, the concentrations of antioxidants did not affect the antioxidant efficiency. Considering the effect of single antioxidant on improving oxidative stability of the product, EDTA was the most efficient. The combination of EDTA and γ-tocopherol at low concentration had less antioxidant efficiency than the combination at high concentration. The combination of all three antioxidants at high concentration (30 ppm ascorbyl palmitate, 50 ppm EDTA and 88 ppm γ-tocopherol) presented the highest antioxidant efficiency in both peroxide and HS-GC determinations. The authors indicated that the prooxidant effects of ascorbyl palmitate were overshadowed by EDTA and γ-tocopherol in this treatment.

CHAPTER III
MATERIAL AND METHODS

A. Scope of the research

This research was divided to 2 parts as listed below.

1. **Study the effects of solvents and extraction conditions on total phenolic content, total monomeric anthocyanin content and antioxidant activities of crude extracts from black glutinous rice**

The objective of this study was to determine the appropriate extracting solvent and the optimum extraction condition to prepare the crude extract from black glutinous rice. The black glutinous rice was extracted by different solvents and different extraction conditions, which were pH and extraction time. The extracting solvents were 50% ethanol and 70% acetone. The pH of the solvent was adjusted to pH 2 (acidic) and pH 6 (neutral). The total extraction times were 2 hours, 4 hours and 8 hours, respectively. Total phenolic content, total monomeric anthocyanin content and antioxidant activities, determined by DPPH and FRAP methods, of crude extract from each extraction system were measured. The most suitable extraction system, yielding the crude extract with the highest antioxidant activity and acceptable total phenolic content as well as total monomeric anthocyanin content, was used for the next part of this research.

2. **Study the application of the black glutinous rice crude extract in fish oil enriched mayonnaise**

This study aimed to determine the application of the crude extract as an antioxidant and colorant in fish oil enriched mayonnaise. The freeze-dried extract was added into the mayonnaise at two different concentrations, 500 ppm and 1000 ppm. Lipid oxidation in the mayonnaise stored at 30°C was determined periodically up to 30 days by measuring conjugated diene hydroperoxides and TBA values. Color of the mayonnaise was also measured in the Hunter (L, a, b) system.

B. Material: black glutinous rice

Black glutinous rice (*Oryza sativa* L.) used in this study was obtained from Chiang Mai province, harvested in November 2005. The hulled black glutinous rice was

hand-milled with mortar and pestle and passed through the 70 mesh sieve (Retsch, Germany). The rice flour was packed in a polypropylene bag, sealed, and kept at -18°C until use.

C. Experimental procedures

1. Extraction: The black glutinous rice was extracted by the method adapted from Pérez-Jiménez and Saura-Calixto (2005).

One hundred ml of extracting solvent was added to 10 g of black glutinous rice flour (known exact weight) in 250 ml Erlenmeyer flask. The flask was shaken in a shaking water bath shaker (Heto-Hollen A.S., Denmark) at 32°C for the desired period of time. The supernatant and pellet was separated by centrifugation (Kubota 5200, Japan) at 1250xg for 15 minutes at room temperature. The pellet was reextracted with 100 ml of extracting solvent using the same extraction condition as the first extraction. The supernatant from first and second extraction was combined and evaporated by a rotary vacuum evaporator (Eyela, N-N series, Japan) at 78.5°C for 12 minutes. The final volume was adjusted to 40 ml. The crude extract was then filtered with Whatman no.4 filter paper, kept in brown glass bottles and stored at -18°C until use. The flow chart of the extraction was shown in Figure 3.1.

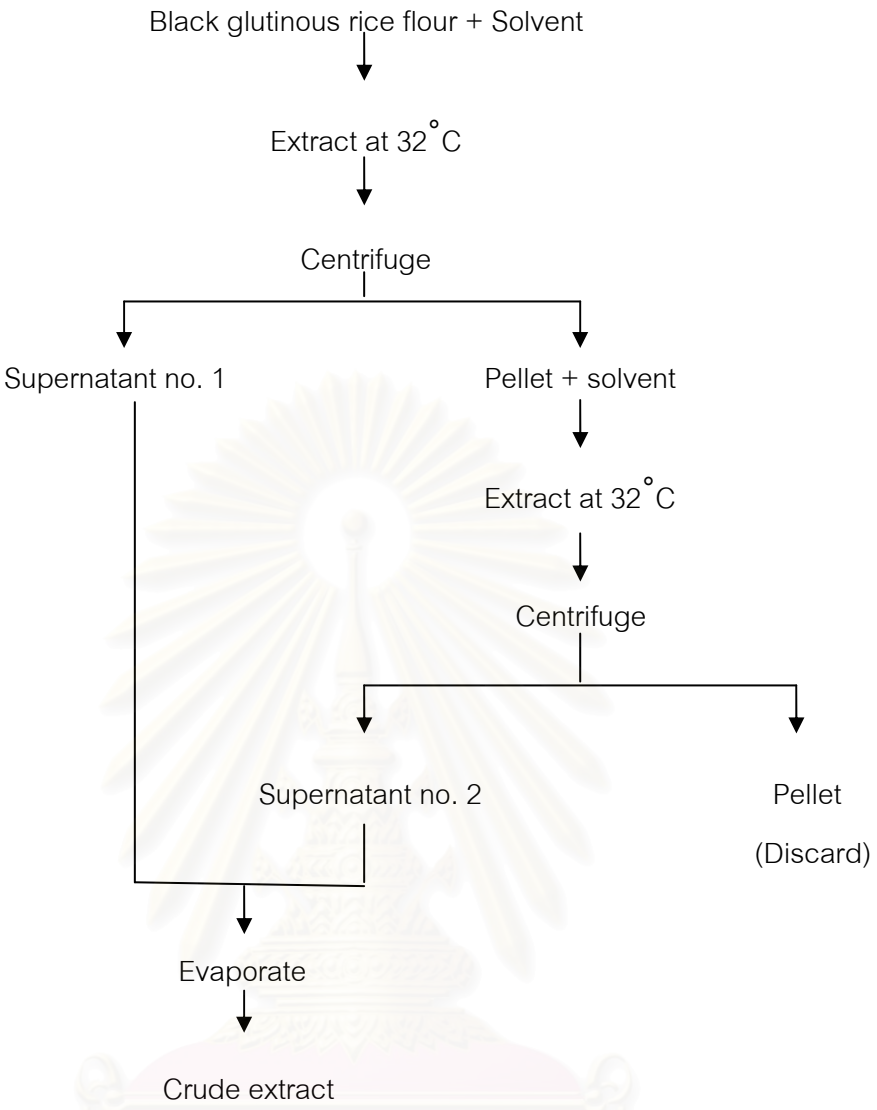

Figure 3.1: Flow chart of the extraction of the crude extract from black glutinous rice (adapted from Pérez-Jiménez and Saura-Calixto, 2005)

The extracting solvents and conditions were varied as following:

a) Type of extracting solvent

Ethanol (Analytical reagent grade, VWR Prolabo, France) and acetone (Analytical reagent grade, Ajax Finechem, Australia) was diluted with water to obtain the final concentration of 50% and 70% respectively. The double extractions with ethanol-ethanol, ethanol-acetone and acetone-acetone, as listed in Table 3.1, were performed.

Table 3.1: The extracting solvent in each condition for double extraction

System	First round	Second round
1	50% Ethanol (v/v)	50% Ethanol (v/v)
2	50% Ethanol (v/v)	70% Acetone (v/v)
3	70% Acetone (v/v)	70% Acetone (v/v)

b) pH of the extraction solvent

The apparent pH of the solvent was varied to 6.8 (unadjusted pH, neutral condition) and 2 (adjusted pH with 0.1 N hydrochloric acid (J.T. Baker Neutrasorb, USA)). The pH of solvent was determined by pH-meter (Cyberscan 2000, Eutech Cybernetics, Singapore) and pH paper (Macherey-Nagel, Germany).

c) Extraction time

The extraction time was varied to 3 levels; 1, 2, and 4 hours for each step in double extraction.

The extractions were done in triplicate. The 2×3×3 factorial in Completely Randomized Design (CRD) was used in this experiment.

2. Evaluation of phenolic substances and antioxidant activities of crude extract

a) Total phenolic content was determined by the method of Folin-Ciocalteu assay (Waterhouse, 2005; Appendix A.1).

b) Total monomeric anthocyanin content was determined by the pH differential method (Wrolstad, Acree and Decker, 2005; Appendix A.2).

c) The antioxidant activity of the crude extract was determined by the following assays:

(a) Ferric reducing ability of plasma, FRAP assay (Benzi and Strain, 1996; Appendix A.3).

(b) Free radical scavenging capacity by DPPH method (Brand-williams *et al.,* 1995; Appendix A.4).

All analyses were done in triplicate. Analysis of variance (ANOVA) of the experimental data was performed and Duncan's New Multiple Range Test was used to evaluate the different between means at the 95% confidence interval.

The most suitable extraction system was the one that provided the crude extract with the highest antioxidant activity and acceptable total phenolic content as well as total monomeric anthocyanin content.

3. Study the application of the black glutinous rice crude extract in fish oil enriched mayonnaise

a) Preparation of the black glutinous rice extract: the crude extract was freeze-dried at -53°C under vacuum (-124 psi) for 48 hour and kept at -18°C before use.

b) Preparation of fish oil enriched mayonnaise (adapted from Let *et al*., 2007)

(a) Ingredients of Mayonnaise

List of the ingredients of the fish oil enriched mayonnaise was shown in Table 3.2

(b) Preparation of the crude extract solution

A known weight of freeze-dried extract of black glutinous rice was added to 10 ml of vinegar and mix well. The final concentration of the crude extract in the oil phase of mayonnaise was varied to 500 ppm and 1000 ppm (or 395 ppm and 790 ppm based on the total weight of mayonnaise). For instance, 0.09 g of crude extract in 10 ml 0f vinegar was added to 228 g of mayonnaise (containing 180 g of total oil) to obtain the concentration of 500 ppm.

Table 3.2: The ingredients of the fish oil enriched mayonnaise

Ingredient	Weight (g) per batch	Percentage (%, w/w)
Soy bean oil (Thai Vegetable Oil Pubic Company Limited, Thailand) containing approximately 100 ppm of α-tocopherol	135	59.21
Fish oil (Tuna Fully refined oil, T.C. Union Global Public Company Limited, Thailand) containing approximately 1000 ppm of α-tocopherol	45	19.74
Distilled vinegar (5.0% acetic acid) (H.J. Heinz Company, USA)	21.5	9.49
Egg yolk (CPF, Thailand)	20	8.86
Iodized table salt (Prung Thip, Thailand)	2.9	1.27
Potassium sorbate	2.28	1
Yellow mustard (H.J. Heinz Company, USA)	1.5	0.66
Total	228	100

(c) Preparation of mayonnaise for oxidative stability test

The egg yolk was mixed to get a homogeneous yolk mixture. Fish oil and vegetable oil were mixed together before use. The homogeneous yolk, vinegar, crude extract solution, salt, mustard and potassium sorbate were beat with an electric mixer (Philips, Japan) for 30 second at medium setting. An approximately 10 ml portion of fish oil and vegetable oil mixture was added and beated at high speed for 60 seconds. This step was done for 6 times to incorporate 60 ml of oil in the mixture. The remaining oil was added in approximately 20 ml portion at a time and beated at high speed and for 120 seconds after each addition. Note that it should not take more than 20 seconds between additions. The mayonnaise was then divided into 20 g portions. Each portion was packed in a plastic bag (Nylon/DL/LLDPE, 70 μm thickness) and heat-sealed. Headspace oxygen in each bag was limited by pressing the headspace area to remove the remaining air before sealing the bag. Therefore, approximate 11 bags of sample were obtained from each batch (228g). The samples were placed in an

incubator (Memmert, Germany) in the dark at 30°C for 30 days for the oxidative stability test. The flow chart of mayonnaise preparation was shown in Figure 3.2.

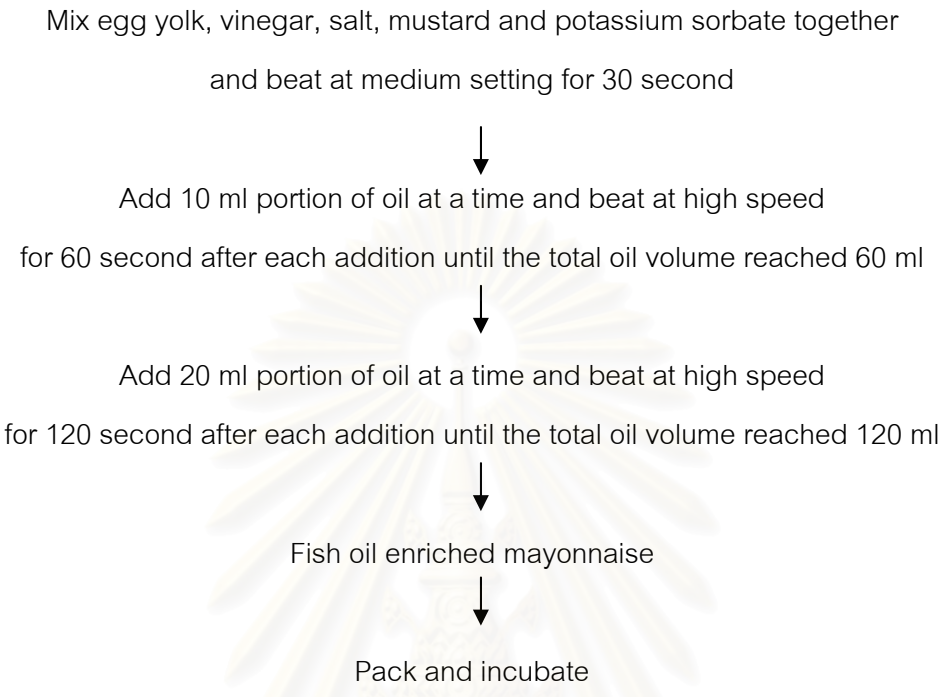

Figure 3.2: Flow chart of the mayonnaise preparation (adapted from Let *et al.,* 2007)

The mayonnaise samples were separated into 3 groups, the samples without antioxidant addition (control), the samples containing 500 ppm and 1000 ppm antioxidant (oil weight basis). For each group, the samples were prepared in triplicate (3 batches). To determine the oxidative stability of the samples, conjugated diene hydroperoxides and thiobarbituric acid reactive substances (TBARs) measurements were done with the sample every 5-7 days for 30 days. Each bag of the samples was used only once for the measurement of both conjugated diene hydroperoxides and thiobarbituric acid reactive substances at a specific storage time.

c) Lipid extraction (adapted from Jacobsen, Meyer and Adler-Nissen, 1998): the mayonnaise was frozen at -40°C for at least 22 hours to destabilize the emulsion and thawed at 5°C for 2 hours. The thawed mayonnaise was centrifuged at 25400xg at 4°C for 10 minutes to separate oil phase from aqueous phase. The oil phase was then used in the following lipid oxidation tests.

d) Lipid oxidation analyses

(a) Measuring conjugated diene hydroperoxides spectrophotometrically (Frankel *et al.*, 1994; Appendix A.6)

(b) Performing thiobarbituric acid reactive substances (TBARs) assay (Pegg, 2005, Appendix A.7).

(c) Color measurement:

Color of the mayonnaise sample was measured by the colorimeter (Minolta CR-300, Japan) in the Hunter (L, a, b) system. Three measurements at 3 different positions were done for each sample.

All analyses were done in triplicate (from (a) to (c)).

CHAPTER IV
RESULTS AND DISCUSSION

A. Study the effects of solvents and extraction conditions on total phenolic content, total monomeric anthocyanin content and antioxidant activities of crude extracts from black glutinous rice

1. Total Phenolic content

Statistical analysis showed that there were significant effects of all three main factors which were type of extracting solvent, acidity of extracting solvent and extraction time as well as significant two-way and three-way interactions of those main factors on the total phenolic contents of black glutinous rice crude extract ($p \leq 0.05$) (Table B.1 in Appendix B). From the Figure 4.1, extraction time and pH of extracting solvent obviously affected the total phenolic content of the crude extract from 50% ethanol - 50% ethanol (EtOH-EtOH) extracting solvent and 50% ethanol - 70% acetone (EtOH-Ace) extracting solvent. For example, for the using of similar extracting solvent and extraction time, the total phenolic content from neutral solvent extraction was higher than acidic solvent extraction except for the 50% ethanol - 70% acetone (EtOH-Ace) extracting solvent at 8 hour extraction where the acidity of solvent had no effect on the total phenolic compound. However, the extraction time and pH of solvent had no significant effect on 70% acetone - 70% acetone (Ace-Ace) extraction ($p > 0.05$) except that the neutral Ace-Ace system could extract more total phenolics than the acidic Ace-Ace system at 2 hour extraction time. Therefore, the twice extraction with neutral Ace-Ace had the highest efficiency in the extraction of total phenolics at any extraction time. The results implied that the polarity of the extraction solvent could greatly affect the efficiency of total phenolics extraction. Seventy percent acetone (v/v) solvent might provide an appropriate polarity for the extraction of wide range of phenolic compounds in our sample.

From this research, the highest total phenolic content of black glutinous rice extract was 1992 µg gallic acid/g flour which obtained from neutral Ace-Ace extraction for 2 hour. This value was closed to the total phenolic content of black glutinous rice reported by Pinsirodom (2006) as 1870 µg gallic acid/ g grain. Moreover, our

pigmented rice contained higher amounts of total phenolics than non-pigmented Spanish raw brown rice which contained the total phenolics of 1340 µg gallic acid/ g flour rice, (Pérez-Jiménez and Saura-Calixto, 2005).

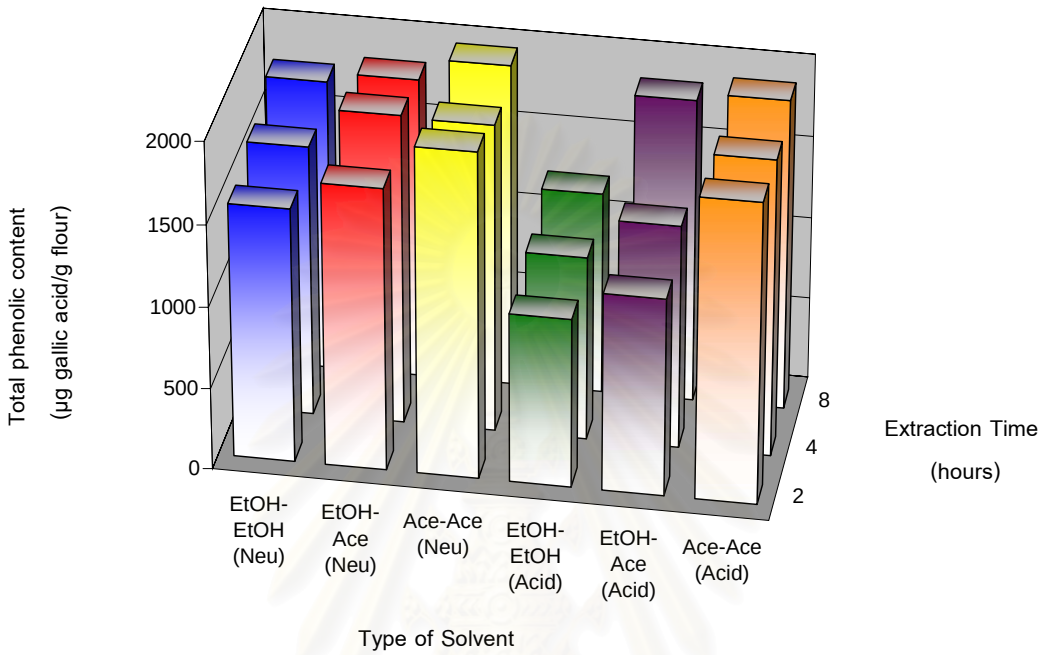

Figure 4.1: Total phenolic content of black glutinous rice crude extract obtained from different solvent systems, solvent pH and extraction times; Neu stands for neutral condition; Acid stands for acidic condition; EtOH stands for 50% ethanol; Ace stands for 70% acetone.

2. Total monomeric anthocyanin content

Significant impacts of each main effect and significant two-way interactions of solvent and time, and solvent and pH were found on total monomeric anthocyanin contents ($p \leq 0.05$) (Table B.2 in Appendix B). The Figure 4.2 presented that, at the same pH, an increasing extraction time resulted in an increasing total monomeric anthocyanin content for the EtOH-EtOH and EtOH-Ace extracting solvent, but did not apparently affect the total monomeric anthocyanin content in the system of acetone-acetone extracting solvent. However, the overall results showed that, for the same extraction time and solvent type used, acidic solvents could extract higher amount of total monomeric anthocyanin than neutral solvents except the Ace-Ace system at 8 hour extraction. This

could be due to the higher stability of anthocyanin in acidic solution (Markakis, 1982). Previous study by Markakis (1982) showed that the rate of anthocyanin breakdown during heat processing was lower at pH 2-4. If there was some degradation of anthocyanin during the extraction and solvent evaporation, the degradation of anthocyanin in acidic condition should be less. However, the best extracting condition for the total monomeric anthocyanins disagreed with that of total phenolics. Acidic EtOH-Ace extraction for 8 hour yielded the crude extract with the highest total monomeric anthocyanin content.

Previous study of anthocyanin content in pigmented rice by Abdel-Aal *et al.* (2006) reported that the Canadian black rice contained 3276 μg total anthocyanins/g flour. This value was much higher than the highest value found in our study, 474 μg monomeric anthocyanin/g flour which obtained from acidic EtOH-Ace solvent at 8 hour extraction. This might be due to the different pigmented rice species and the different analytical techniques used. Although the spectrophotometric determination was used in both studies, the amount of anthocyanin in black glutinous rice crude extract reported in our study was determined only the monomeric form which was cyanidin-3-glucoside while the anthocyanins in Canadian black rice were determined as the total monomeric and polymeric anthocyanin content.

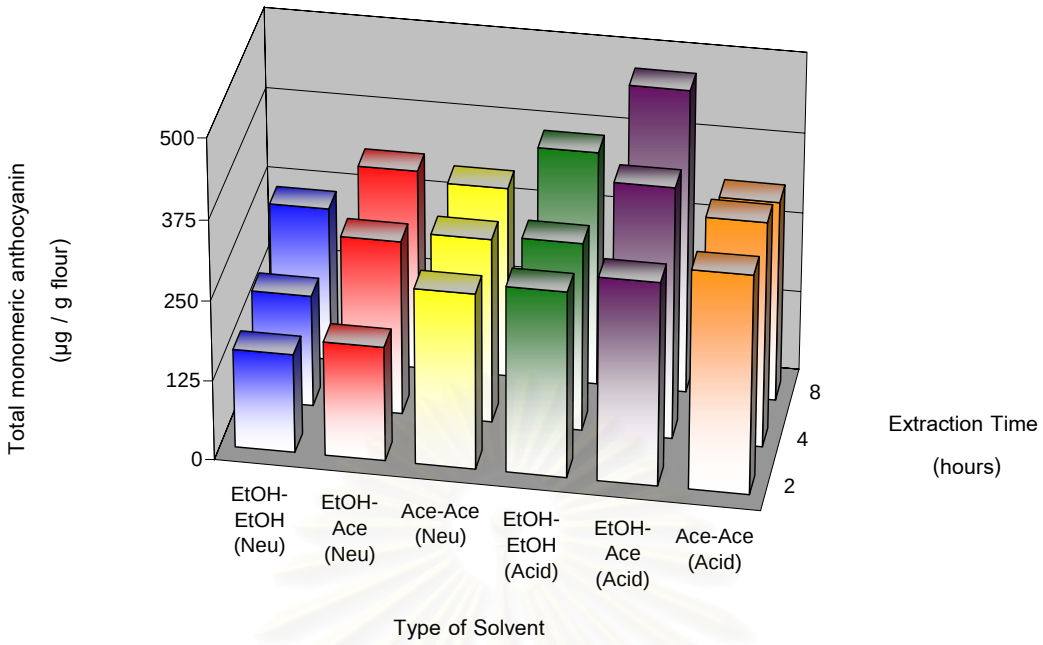

Figure 4.2: Total monomeric anthocyanin content of black glutinous rice crude extract obtained from different solvent systems, solvent pH and extraction times; Neu stands for neutral condition; Acid stands for acidic condition; EtOH stands for 50% ethanol; Ace stands for 70% acetone.

3. Antioxidant activity

a) FRAP assay

The significant effects of each main factor, two-way interaction of pH and solvent and three-way interaction were found on the antioxidant activity by FRAP assay ($p \leq 0.05$)(Table B.3 in Appendix B). Figure 4.3 showed that the increasing extraction time could increase antioxidant activity of crude extract for every extracting solvent in both neutral and acidic condition. The longest extraction time of 8 hours provided the highest antioxidant activity for all extraction system. The results also showed that type of solvents did not apparently affect antioxidant activity of the extracts in acidic conditions while it had greater effect in neutral conditions.

Previous study by Pérez-Jiménez and Saura-Calixto (2005) reported that antioxidant activity of raw Spanish brown rice using FRAP assay was 1.57 µmole trolox/g flour whereas the highest antioxidant activity from FRAP assays of black glutinous rice crude extract was 11.9 µmole trolox/g flour, obtained from neutral EtOH-Ace system at 8

hour extraction. Therefore, the antioxidant activity of black glutinous rice crude extract was much higher than that of the nonpigmented rice which corresponded to the higher amount of total phenolics found in our sample as previously discussed.

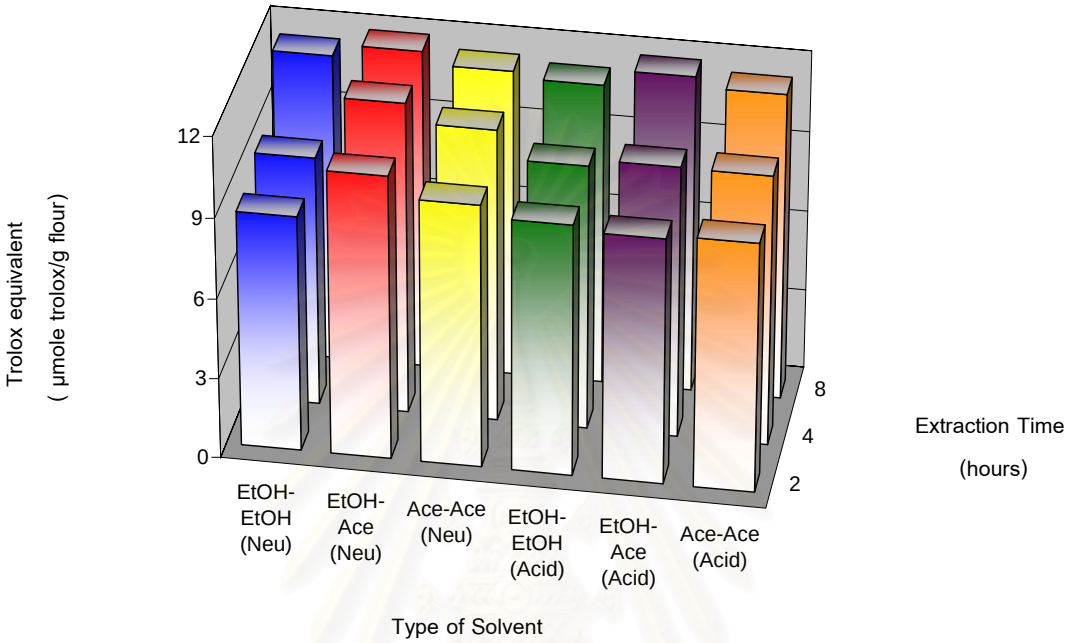

Figure 4.3: Antioxidant activity from FRAP assay of black glutinous rice extract obtained from different solvent systems, solvent pH and extraction times; Neu stands for neutral condition; Acid stands for acidic condition; EtOH stands for 50% ethanol; Ace stands for 70% acetone.

b) DPPH assay

Significant effects of each main factor, two-way and three-way interactions of those main factors were found on antioxidant activity from DPPH assay ($p \leq 0.05$) (Table B.4 in Appendix B). The results of DPPH assay was expressed as EC50 which was the concentration of antioxidant that caused the decrease of DPPH radicals to 50% of the initial concentration. Therefore, lower EC50 value provides higher antioxidant efficiency. Figure 4.4 indicated that the increasing extracting time led to higher antioxidant activity of the extracts for all of the extraction systems except neutral acetone-acetone system. For the similar solvent system and extraction time, the neutral solvent extractions yielded the extracts with the higher antioxidant activity than the

acidic solvent extractions except EtOH-EtOH extracting solvent at 2 hour extraction. The overall data revealed that the using of neutral Ace-Ace extracting solvent presented the highest antioxidant efficiency at all extraction time which was consistent with the results of total phenolic determination. The antioxidant efficiency of black glutinous rice crude extract with DPPH assay in this study (EC50 of 42 g sample/ g DPPH) was higher than that of black glutinous rice reported by Pinsirodom (2006) (EC50 of 61 g sample/ g DPPH). Moreover, the antioxidant efficiency from DPPH assay of our sample was approximately 4 times higher than that of the nonpigmented Spainish rice (EC50 of 183 g sample/ g DPPH) (Pérez-Jiménez and Saura-Calixto, 2005).

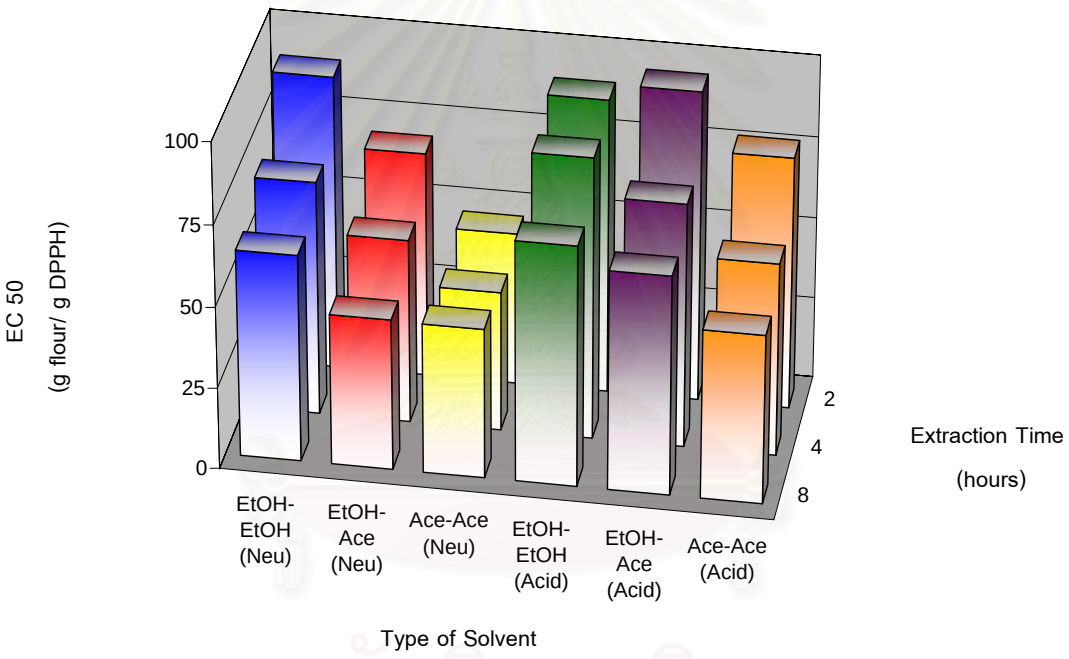

Figure 4.4: Antioxidant activity from DPPH assay of black glutinous rice extract obtained from different solvent systems, solvent pH and extraction times; Neu stands for neutral condition; Acid stands for acidic condition; EtOH stands for 50% ethanol; Ace stands for 70% acetone.

Considering the results from the determination of total phenolics and total monomeric anthocyanins together with the antioxidant activities of the extracts, it was found that the best extracting condition of total phenolics or total monomeric

anthocyanins did not exactly provide the extracts with the highest antioxidant efficiency for both FRAP assay and DPPH assay. The disagreement could be explained as followed. The antioxidant efficiency did not depend only on the amount of phenolic compounds but it also depended on the type and structure of phenolic compounds. Moreover, the results from DPPH assay did not totally agree with the FRAP assay results. The conflicted results of FRAP and DPPH assay were also found in other reports (Deepa *et al*., 2006; Stratil, Klejdus and Kuban, 2006). This might be because the two systems evaluated the different mechanisms of antioxidants. The FRAP assay was based on the electron transfer reactions whereas DPPH assay evaluated both electron transfer and hydrogen atom transfer reactions (Prior *et al*., 2005). FRAP and DPPH assays have usually been used together to evaluate the overall activities of antioxidants (Deepa *et al*., 2006; Jiang *et al*., 2006; Stratil *et al*., 2006; Yan, Teng and Jhi, 2006). Moreover, FRAP and DPPH assays were less interfered by other substances such as amino acids and glucose comparing to ABTS and ORAC assay (Pérez-Jiménez and Saura-Calixto, 2006). Hence, these methods could provide more accurate results especially for the test of antioxidant activities in non-purified samples.

The data in the Table 4.1 revealed that the best extraction conditions for total phenolic content, total monomeric anthocyanin contents and antioxidant activities from FRAP assay and DPPH assay were not agreeable. Therefore, the most suitable extraction system was selected from the shortest total extraction time which gave an acceptable amount of total phenolics and total monomeric anthocyanins, but yielded the highest antioxidant activities. That system was neutral Ace-Ace extract at 4 hour total extraction time. The crude extract from this condition was used to study its application in fish oil enriched mayonnaise as antioxidant and colorant.

Table 4.1: The total phenolic content, total monomeric anthocyanin content and antioxidant activities by FRAP assay and DPPH assay of black glutinous rice crude extract at the different extraction conditions

Conditions (pH/Solvent/time)	Total phenolics (μg gallic acid/ g flour)	Total monomeric anthocyanins (μg / g flour)	FRAP (μmol trolox / g flour)	EC50 (g flour / g DPPH)
Neu./EtOH-EtOH/2h	1554±35 ^{d}	155±5 ^{f}	8.79±0.10 ^{d}	90.4±0.1 ^{b}
Neu./EtOH-EtOH/4h	1650±78 ^{cd}	174±21 ^{f}	9.30±0.43 ^{d}	71.8±1.6 ^{ef}
Neu./EtOH-EtOH/8h	1775±70 ^{bc}	241±24 ^{e}	11.48±0.17 ^{ab}	63.6±2.0 ^{g}
Neu./EtOH-Ace/2h	1724±98 ^{bc}	179±21 ^{f}	10.58±0.1 ^{c}	69.2±1.6 ^{ef}
Neu./EtOH-Ace/4h	1889±47 ^{ab}	272±11 ^{de}	11.62±0.28 ^{ab}	56.5±0.6 ^{h}
Neu./EtOH-Ace/8h	1834±32 ^{bc}	313±15 ^{cd}	11.92±0.36 ^{ab}	46.4±0.7 ^{j}
Neu./Ace-Ace/2h	1992±83 ^{a}	275±26 ^{de}	9.81±0.41 ^{cd}	46.9±2.0 ^{j}
Neu./Ace-Ace/4h	1878±104 ^{ab}	288±22 ^{d}	10.92±0.54 ^{bc}	42.9±2.7 ^{k}
Neu./Ace-Ace/8h	1968±75 ^{a}	298±11 ^{d}	11.46±0.13 ^{ab}	46.0±1.8 ^{j}
Acid/EtOH-EtOH/2h	1035±118 ^{g}	291±22 ^{d}	9.38±0.74 ^{d}	90.5±2.5 ^{b}
Acid/EtOH-EtOH/4h	1123±22 ^{f}	295±35 ^{d}	9.88±0.37 ^{c}	86.6±1.6 ^{c}
Acid/EtOH-EtOH/8h	1232±86 ^{f}	366±18 ^{bc}	11.24±0.62 ^{b}	74.0±1.9 ^{e}
Acid/EtOH-Ace/2h	1209±66 ^{f}	318±36 ^{cd}	9.17±0.21 ^{d}	95.4±3.4 ^{a}
Acid/EtOH-Ace/4h	1364±53 ^{e}	393±32 ^{b}	10.13±0.28 ^{c}	75.0±2.7 ^{de}
Acid/EtOH-Ace/8h	1852±103 ^{b}	474±42 ^{a}	11.82±0.66 ^{ab}	67.4±0.6 ^{f}
Acid/Ace-Ace/2h	1838±48 ^{bc}	342±6 ^{c}	9.31±0.31 ^{d}	77.6±0.5 ^{d}
Acid/Ace-Ace/4h	1815±96 ^{bc}	352±35 ^{bc}	10.10±0.13 ^{c}	59.3±0.5 ^{h}
Acid/Ace-Ace/8h	1898±48 ^{ab}	313±30 ^{cd}	11.46±0.01 ^{ab}	51.9±0.5 ^{i}

Note: 1) Neu. stands for neutral solvent (pH=6); Acid stands for acidic solvent (pH=2); EtOH stands for 70% ethanol ; Ace stands for 50% acetone

2) The data showed the average value ± standard deviation

3) Data with different superscript were significantly different ($p \leq 0.05$)

4) The lower EC50 value presented the higher antioxidant activities

B. Study the application of the black glutinous rice crude extract in fish oil enriched mayonnaise.

1. Lipid oxidation analysis

The addition of black glutinous rice extract at both concentrations could retard an increase in both conjugated diene hydroperoxides (primary oxidative products) and thiobarbituric acid reactive substances (TBARs, secondary oxidative products) in mayonnaise as shown in Figure 4.5. The higher concentration of the extract showed the higher antioxidant efficiency.

The antioxidant activities of phenolic compounds can occur from 3 mechanisms as chain-breaking antioxidant, hydroperoxide destroyer and metal chelator (Heim, Tagliaferro and Bubliya, 2002; Frankel, 2005). The chain-breaking antioxidants act as the hydrogen atom donation toward the lipid peroxyl radicals during propagation or initiation step of lipid autoxidation. Therefore, the formation of hydroperoxides can be reduced. The hydroperoxide destroyers can change the hydroperoxides into the stable or inactive products by non-radical processes such as reduction or hydrogen donation. Hence, the volatile oxidative products will decrease. For instance, phenolic compounds can act as weak hydroperoxide destroyers by reducing linoleate hydroperoxides into stable hydroxylinoleates. The metal chelators can deactivate metal ions. Therefore, the formations and decoposition of hydroperoxide can be retarded (Frankel, 2005). Phenolic compounds in black glutinous rice crude extract might act as both hydrogen atom donor and hydroperoxide destroyer which could retard the formation of both primary and secondary oxidative products in fish oil enriched mayonnaise. Although black glutinous rice crude extract composed of hydrophilic phenolic compounds as main antioxidant which should mainly dissolved in an aqueous phase in mayonnaise, some of them could diffuse to the oil-water interface. Therefore, the antioxidant activity could still be observed. The effect of partitioning of antioxidant on its activities was described elsewhere (Frankel, 2005).

As shown in Figures 4.5 and 4.6, after 18 day-storage of the control sample, the conjugated diene hydroperoxides decreased while the TBA value abruptly increased. This occurrence corresponded to the theory that the increasing of secondary oxidative

product was the result of hydroperoxides degradation. However, this change could not be found in the samples containing the rice extract.

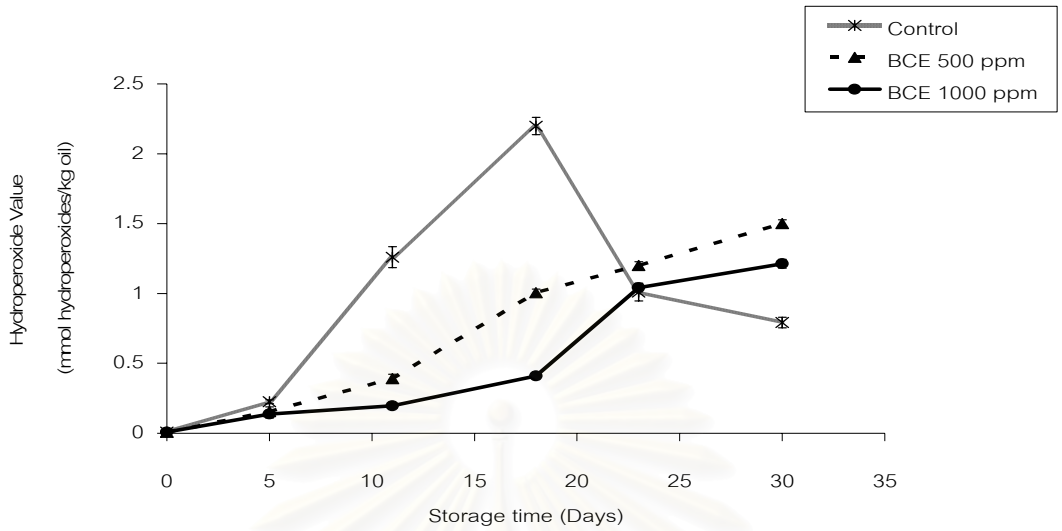

Figure 4.5: The conjugated diene hydroperoxides in oil phase extracted from fish oil enriched mayonnaise during storage; BCE stands for black glutinous rice crude extract; Error bars extend one standard deviation above and below the mean.

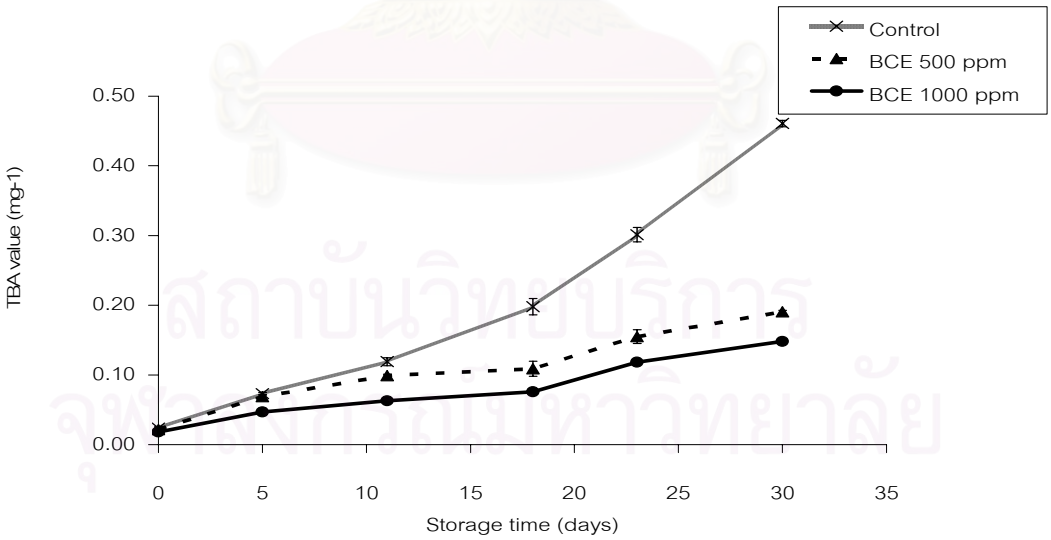

Figure 4.6: The TBA value of oil phase extracted from fish oil enriched mayonnaise during storage; BCE stands for black glutinous rice crude extract; Error bars extend one standard deviation above and below the mean.

The overall results showed that black glutinous rice crude extract could efficiently increase the oxidative stability of the mayonnaise. However, the oxidative changes in the samples could be influenced from other antioxidant. As previously mentioned, the soy bean oil and fish oil contained α-tocopherols. Moreover, egg yolk also contained some carotenoids. So there were three categories of antioxidants in this system which were phenolic compounds from black glutinous rice crude extract, α-tocopherols and carotenoids. The synergistic effect of all antioxidant might improve the antioxidant efficiency of black glutinous rice crude extract in fish oil enriched mayonnaise.

2. Color measurement

The possibility of the application of black glutinous rice crude extract as a colorant, providing pink to purple color, in fish oil enriched mayonnaise was also evaluated. The change of product color was determined by the measurement of color in Hunter (L, a, b) system as shown in Figure 4.8 (a), (b) and (c). The appearance color of mayonnaise after black glutinous rice crude extract adding, it presented the light pink-violet color whereas the color of normal mayonnaise was light yellow color.

The changes in L, a, b values of mayonnaise samples were found during storage. The 'L' value represented lightness, white (+L) to black (–L), 'a' value represented the shade of red (+a) to green (–a) color and 'b' value represented the shade of yellow (+b) to blue (–b) color. During the storage, lightness (L) and yellowness (b) of all mayonnaise samples decreased whereas redness (a) of all samples increased. The color changes might partly result from Maillard reaction. The Maillard reaction or non-enzymatic browning reaction is the reaction between carbonyl group of reducing sugars and amino group of amino acids or other compounds (BeMiller and Whistler, 1996). Since egg yolk contained small amounts of reducing sugars and free amino acid, the Maillard reaction could slowly occurred at acidic condition in the sample (pH 3-4). However, the color change occurred greater in the sample containing black glutinous rice crude extract especially with 1000 ppm crude extract addition. This might partly be due to additional sugar molecules from anthocyanins which could participate in the Maillard reaction. Other reasons for the color change could be due to the oxidative

degradation of anthocyanin and some irreversible transformation of anthocyanin to its colorless forms at low acidic pH of mayonnaise (Delgado-Vargas and Peredes-López, 2002)

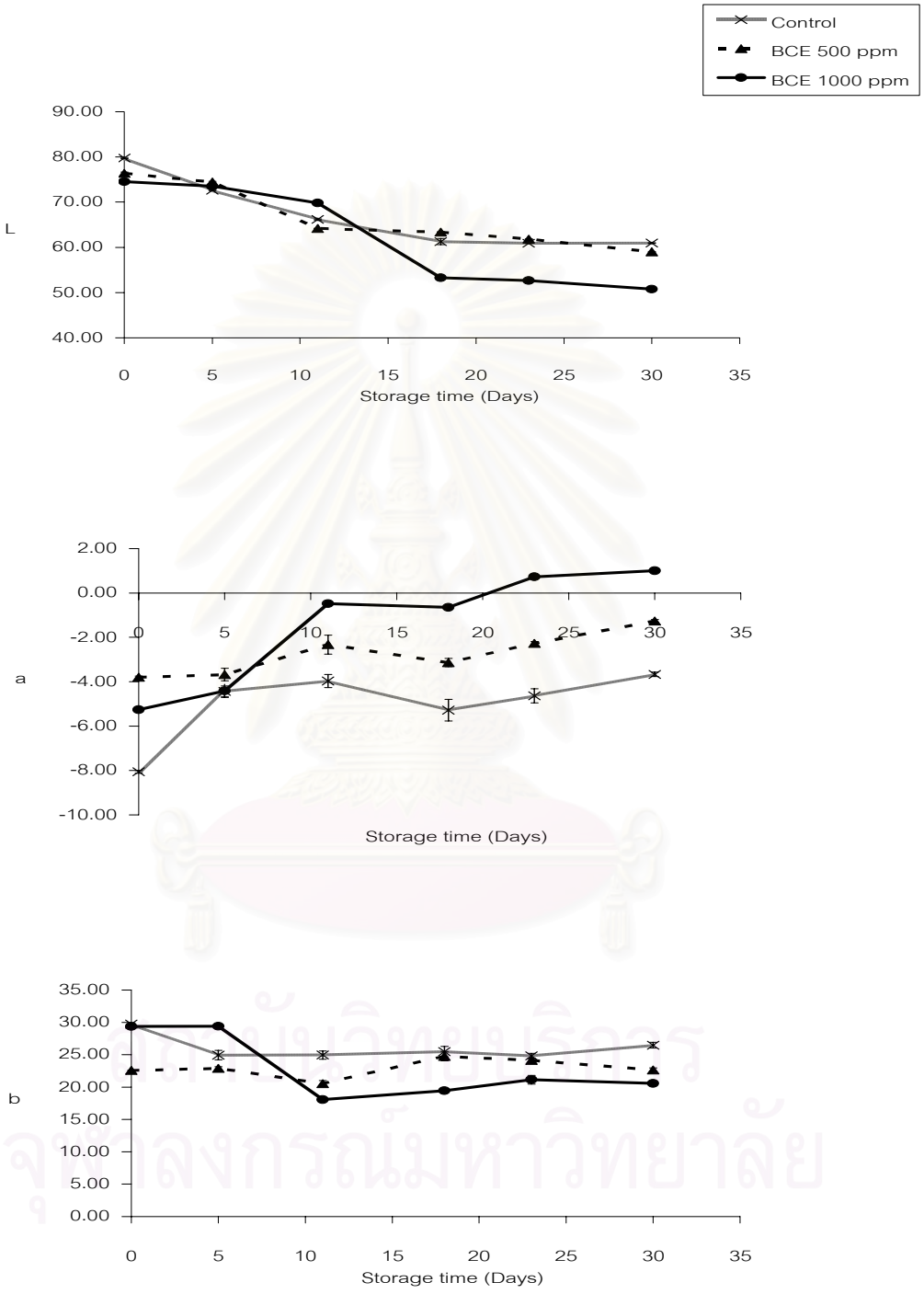

Figure 4.7: The L, a, b value of fish oil enriched mayonnaise during storage; BCE stands for black glutinous rice crude extract; Error bars extend one standard deviation above and below the mean.

The overall results of the study of the application of the black glutinous rice extract in fish oil enriched mayonnaise showed that, although the crude extract exhibited great antioxidant activities in mayonnaise, it could not serve as a good colorant in this product since its pink or light purple color was not stable. Moreover, the crude extract could induce non-enzymatic browning in the product.

CHAPTER V
CONCLUSIONS AND SUGGESTIONS

A. Conclusions

1. Study the effects of solvents and extraction conditions on total phenolic content, total monomeric anthocyanin content and antioxidant activities of crude extracts from black glutinous rice

Statistical analysis showed that there were significant effects of all three main factors which were type of extracting solvent, acidity of extracting solvent and extraction time as well as significant two-way and/or three-way interactions of those main factors on the total phenolic contents, total monomeric anthocyanin content and antioxidant activities of black glutinous rice crude extract ($p \leq 0.05$). The results indicated that the use of neutral 50%acetone-50%acetone solvent extraction provided the highest total phenolic content in the crude extract and the extraction time had no significant effect for this solvent system ($p \leq 0.05$). The acidity of the solvents greatly affected the extraction of total monomeric anthocyanins. The extraction with acidic 70%ethanol-50%acetone for 8 hours yielded the extract with the significantly highest total monomeric anthocyanins ($p \leq 0.05$). FRAP analysis presented that the extraction time had the greatest effect on the antioxidant activity of the crude extract. The longest extraction time (8 hours) provided the extracts with the highest antioxidant activity. However, the solvent systems had much greater effects on the antioxidant activity in DPPH assay. The highest antioxidant activity of the crude extracts in DPPH assay was obtained from the extraction with neutral 50%acetone-50%acetone solvent at 4 hours. The results from DPPH and FRAP assay suggested the different optimum extraction conditions because the two systems evaluate different antioxidant mechanisms.

Since the best extraction conditions for total phenolics, total anthocyanins, FRAP and DPPH assays were not agreeable, the most suitable extraction condition was selected from the shortest total extraction time which gave an acceptable amount of total phenolics and total monomeric anthocyanins, and high antioxidant activity of the extract. Therefore, the most suitable system was the twice extraction with neutral 50% acetone at 4 hour total extraction time.

2. Study the application of the black glutinous rice crude extract in fish oil enriched mayonnaise

The black glutinous rice crude extract had good antioxidant activity in food system; fish oil enriched mayonnaise. The crude extract could retard the formation of both primary oxidative products, conjugated diene hydroperoxides and secondary oxidative products, TBARs during storage of the mayonnaise. The crude extract at higher concentration exhibited greater antioxidant efficiency in the product. Although the crude extract efficiently increased the oxidative stability of the mayonnaise, its color was unstable in the product, as seen from the decreasing of lightness (L) and yellowness (b) of all mayonnaise samples whereas redness (a) of all samples increased during the entire storage. This might be the result of Maillard reaction and anthocyanin degradation during storage.

B. Suggestion

1. The types of phenolic compounds in black glutinous rice extract should be fractionated and identified at the different extracting time. The antioxidant activities of these fractions should also be evaluated.

2. Applications of the black glutinous rice crude extract and its fractions should be studied in other food systems.

3. For further studies of the black glutinous rice extract as the colorant in food, the chemical modifications such as acylation, which is the attachment of acyl group onto the glucosidic part of anthocyanin, should be applied. The acylated anthocyanins are more resistant to color loss at high temperature and high pH (pH 4-7). (Delgado-Vargas and Peredes-López, 2002)

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APPENDICES

APPENDIX A
ASSAY TECHNIQUES

Appendix A.1: Total phenolics determination, Folin-Ciocalteu assay (Waterhouse, 2005).

1. Preparation of gallic acid standard solution

a) To prepare a stock solution, 0.500g of gallic acid (Fluka, Spain) was dissolved in 10 ml of ethanol (analytical reagent grade, VWR Prolabo, France) and diluted to 100 ml with water in a volumetric flask.

b) A 0, 1, 2, 3, 5 and 10 ml aliquot of gallic acid stock solution was added to a volumetric flask and diluted to 100 ml with water. The final concentration of gallic acid will be 0, 50, 100, 150, 250, and 500 mg/l.

2. Preparation sodium carbonate solution

The 200 g of sodium carbonate (analytical reagent grade, Ajax Finechem, Australia) was dissolved in 800 ml of water and boiled. After the solution was cooled down, a few crystals of sodium carbonate was added. After 24 hours, the solution was filtered with Whatman no. 1 filter paper and the volume of the solution was made up to 1000 ml in a volumetric flask.

3. Folin-Ciocalteu assays

1 ml of sample or standard solution was added to 100 ml volumetric flask and approximately 70 of ml water was added. 5 ml of Folin-Ciocalteu reagent (Carlo Erba, France) was added, then swirled to mixed and incubated 1-8 minutes at room temperature. 15 ml of sodium carbonate solution was subsequently added and made up the final volume with water to 100 ml. The solution was mixed and incubated at room temperature for 2 hours. The absorbance was measured by a spectrophotometer (Lambda 25 UV-VIS Spectrometer, Perkin Elmer instrument, USA) at 765 nm.

The standard curve was shown in Figure A.1

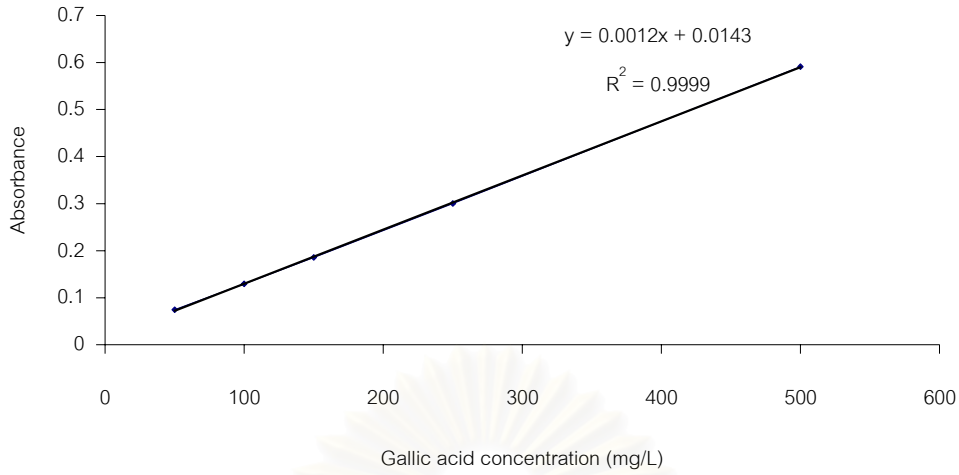

Figure A.1: Gallic acid standard curve for total phenolics determination

4. Calculation of total phenolic content

The amounts of total phenolics in crude extract were calculated using gallic acid standard curve. The value was expressed as µg gallic acid/ g flour.

Appendix A.2: Total monomeric anthocyanins determination: pH differential method (Wrolstad, Acree and Decker, 2005)

The structure of anthocyanin pigments can reversibly transform with a change of pH between pH1 and pH 4.5. The colored oxonium (flavylium cation) form presented at pH 1.0 and the colorless hemiketal (carbinol pseudo-base) form presented at pH 4.5 as shown in Figure A.2.

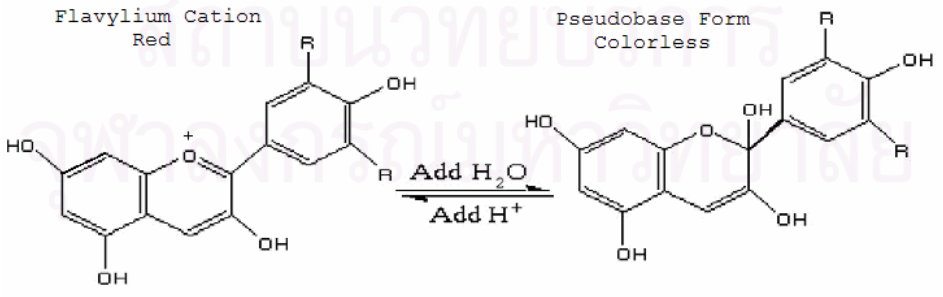

Figure A.2: Anthocyanin structural forms at different pH (adapted from Brouillard and Delaporte, 1977)

1. Preparation of pH 1.0 and 4.5 buffer solutions

Potassium chloride buffer at the concentration of 0.025 M, pH 1.0, was prepared by dissolving 1.86 g of potassium chloride (Ajax Finechem, Australia) in 980 ml of water, adjust pH to 1.0 by concentrated HCl (J.T. Baker Neutrasorb, USA), and made up volume to 1000 ml with water in a volumetric flask. Sodium acetate buffer at the concentration of 0.4 M, pH 4.5, was prepared by dissolving 54.43 g of sodium acetate (Sigma-Aldrich, Germany) in 960 ml of water, adjusted pH to 4.0 with conc. HCl and made up the volume to 1000 ml with water in a volumetric flask.

2. Anthocyanin measurement

The major anthocyanin in the crude extract from black glutinous rice was expected to be cyanidin-3-glucoside (Figure A.3). The $\lambda_{vis\text{-}max}$ of cyanidin-3-glucoside was approximately 510 nm (Wrolstad, Acree and Decker, 2005). To test this assumption, the crude extract was diluted with pH 1.0 potassium chloride buffer to determine $\lambda_{vis\text{-}max}$ in an absorption spectrum by a spectrophotometer (Lambda 25 UV-VIS Spectrometer, Perkin-Elmer instrument, USA). From the experiment, the spectrum of the crude extract showed that $\lambda_{vis\text{-}max}$ was 513 nm which was close to $\lambda_{vis\text{-}max}$ of cyanidin-3-glucoside. Therefore, the total monomeric anthocyanin content of crude extract was calculated as cyanidin-3-glucoside.

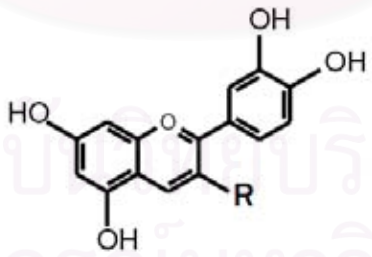

Figure A.3: The structure of cyanidin-3-glucoside; R represents a glucose molecule (adapted from Delgado-Vargas and Peredes-López, 2002)

To start the measurement, the spectrophotometer was set zero with distilled water at both 513 nm and 700 nm. 0.5 ml of crude extract was diluted to 10 ml with pH

1.0 potassium chloride buffer and with pH 4.5 sodium acetate buffer to 10 ml in a volumetric flask (dilution factor was 20) and let them equilibrate for 15 minutes.

a) The absorbance was calculated by:

$$A = (A\lambda_{vis\text{-}max} - A_{700})_{pH\ 1.0} - (A\lambda_{vis\text{-}max} - A_{700})_{pH\ 4.5}$$

Where: A = absorbance

$A\lambda_{vis\text{-}max}$ = absorbance at 513 nm

A_{700} = absorbance at 700 nm

Note: A_{700} was measured and subtracted off in order to eliminate the effect of haze or sediments in the sample.

b) The concentration of monomeric anthocyanin pigment was calculated by:

$$\text{Monomeric anthocyanin pigment (mg/L)} = (A \times MW \times DF \times 1000)(\varepsilon \times 1)$$

Where: A = absorbance

MW = molecular weight of cyanidin-3-glucoside = 449.2

DF = dilution factor = 20

ε = molar absorptivity of cyanidin-3-glucoside = 26900 $M^{-1}cm^{-1}$

Appendix A.3: FRAP assay (Benzi and Strain, 1996)

1. Preparation of trolox standard curve

To prepare a 10000 µM trolox solution, 2.5 g of trolox (Fluka, Denmark) was diluted with 100 ml of methanol (Fisher Scientific, UK) and mixed well. The serial dilution was prepared as listed in Table A.1.

Table A.1: Preparation of standard trolox solution

Initial concentration (μM)	Trolox volume (ml)	Methanol volume (ml)	Final concentration (μM)
10000	5	5	5000
5000	5	5	2500
2500	5	5	1250
1250	5	5	625
625	4	2	417
417	4	2	278
278	4	2	185
185	4	2	123
123	4	2	82

2. Solution preparation

a) Acetate buffer: three g of $CH_3COONa{\cdot}3H_2O$ (Fisher Scientific, UK) was added to 16 ml of CH_3COOH (Analytical reagent grade, J.T. Baker Neutrasorb, USA) and made volume up to 1000 ml of water in a volumetric flask.

b) Tripyridyltriazine (TPTZ) solution: a 0.312 g portion of TPTZ (Fluka, Switzerland) was added to 100 ml of 0.4 M HCl (Analytical reagent grade, J. T. Baker Neutrasorb, USA).

c) $FeCl_3$ solution: a 0.54 g portion of $FeCl_3$ (POCH S.A., Poland) was added to 100 ml of H_2O.

d) FRAP solution: a 25 ml aliquot of acetate buffer was added with 2.5 ml of ferric chloride solution and followed with 2.5 ml of TPTZ solution (the solution must be added in this order).

3. FRAP assay

FRAP solution was warmed at 37°C in a hot water bath (DT-1 Heto-Holten, Heto Lab Equipment, Japan). To prepare a standard curve, 50 μl of trolox solution was added to 950 μl of FRAP solution in a cuvette. However, for the analysis of crude extract, 10 μl of sample was added to 990 μl of FRAP solution in a cuvette. The mixture was held for 4

minutes at room temperature before measuring the absorbance. The color of the mixture was changed from golden brown to deep blue purple. The absorbance was measured at 593 nm. Acetate buffer was used as blank. The corrected absorbance was calculated as followed:

$$A_{corrected} = A_{final} - A_{initial}$$

Where: $A_{corrected}$ = corrected absorbance

A_{final} = absorbance of the sample after 4 minute holding time

$A_{initial}$ = absorbance of 1000 µl FRAP solution

The corrected absorbance of the samples were compared with the corrected absorbance in trolox standard curve. The trolox standard curve being shown in Figure A.4. The antioxidant activity was calculated as µmol trolox/ g flour.

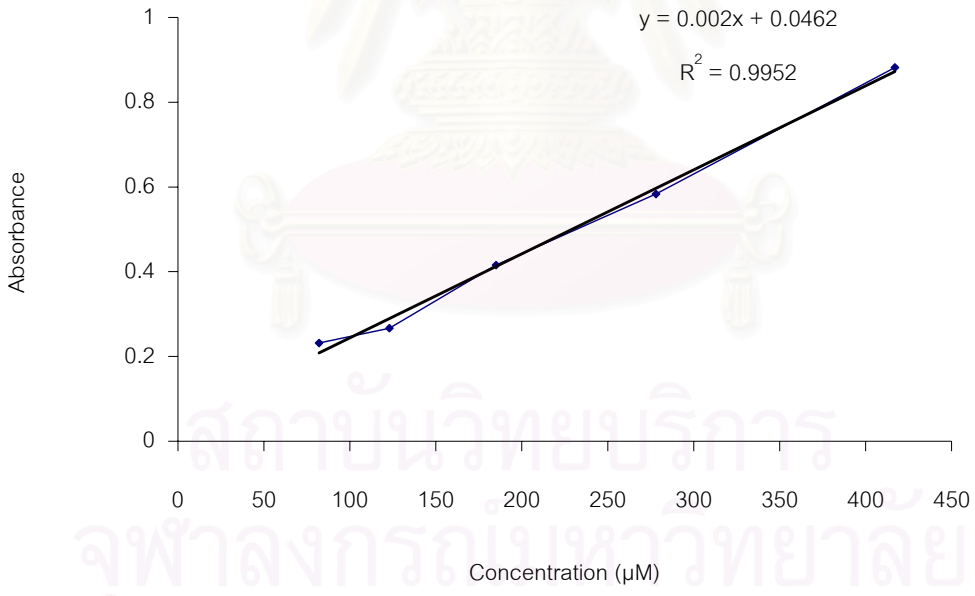

Figure A.4: Trolox standard curve for FRAP assay

Appendix A.4: DPPH assay (Brand-Williams *et al.* 1995; Pérez-Jiménez and Saura-Calixto, 2005; Murakami *et al*., 2004)

1. DPPH solution preparation

Twelve mg of 2,2-Diphenyl-1-picrylhydrazyl (DPPH) was dissolved in 100 ml of methanol to obtain the 3×10^{-4} M DPPH solution. The solution can be kept at 4°C not more than 5 days. Note that the molecular weight of DPPH is 394.33 g/mol

2. DPPH assay

Nine-hundred and fifty µl of DPPH solution was mixed with 50 µl of crude extract in a cuvette (solution A) and held for 30 minutes at room temperature. The absorbance was then read at 515 nm. The deep blue color of DPPH solution was bleached to yellow color. In order to calculate EC50, amount of sample was varied to 4 levels as listed in Table A.2.

Table A.2: The calculation of the equivalent amount of the flour sample in DPPH solution

Number of dilution	1	2	3	4
Volume of crude extract (µl)	20	30	40	50
Volume of methanol (µl)	30	20	10	0
Total volume of sample in solution A (µl)	50	50	50	50
Weight of sample in 50 (µl) (g flour)[(1)]	5×10^{-3}	7.5×10^{-3}	1×10^{-2}	1.25×10^{-2}
Weight of DPPH in solution A (g DPPH)[(2)]	1.14×10^{-4}	1.14×10^{-4}	1.14×10^{-4}	1.14×10^{-4}
Equivalent amount of flour sample (g flour/g DPPH)	43.86	65.79	87.72	109.65

(1) Calculated from the basis that 40 ml of crude extract was obtained from 10 g of flour sample

(2) 950 µl of 3×10^{-5} M DPPH solution

The absorbance at 30 minutes was plot in the graph of the equivalent amount of the sample in DPPH solution (g flour/g DPPH) and % inactivation (Figure A.5) to calculate the EC 50, which was expressed as g sample/ g DPPH.

The % inactivation can be calculated by:

$$\% \text{ inactivation} = (1 - (A_{sample} / A_{blank})) \times 100$$

Where: A_{sample} = absorbance of sample at 30 minutes

A_{blank} = absorbance of blank solution (1000 µl of DPPH solution)

= 3.13

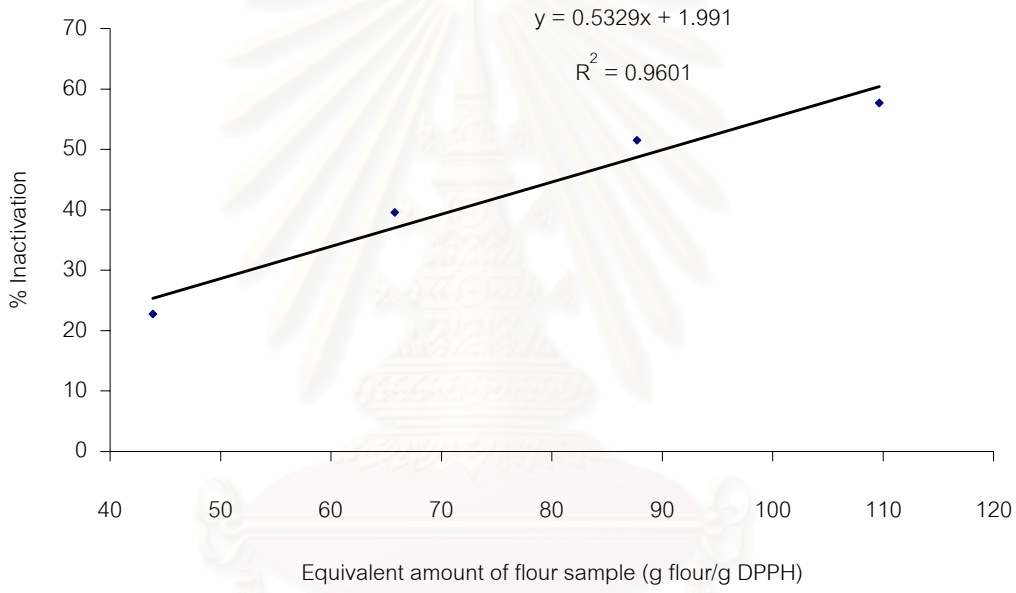

Figure A.5: The percent inactivation of the crude extracts obtained from different amounts of flour sample

Note that the data in Figure A.4 was obtained from the following extraction system, twice extraction with 50% ethanol at pH 6 for 2 hours. The EC50 was calculated from the linear equation of Figure A.4 as the equivalent amount of flour sample that could deplete the DPPH radical to 50%.

Appendix A.5: Measurement of conjugated diene hydroperoxides (Frankel *et al*., 1994)

Fifty mg of oil phase from mayonnaise sample was added to 5 ml of isooctane. The absorbance was measured at 234 nm (Lambda 25 UV/VIS spectrometer, Perkin-Elmer, USA). The result was expressed as mmol hydroperoxides/kg oil as following equation:

$$A = \varepsilon bc$$

Where: A = absorbance

ε = molar absorptivity = 26000 $M^{-1}cm^{-1}$

b = 1-cm cuvette path length

c = concentration of conjugated diene hydroperoxides in oil sample

Appendix A.6: Thiobarbituric acid reactive substances assay (TBARs) (Pegg, 2005).

1. Preparation of sample

Fifty mg of oil phase from mayonnaise sample was weight into 25 ml volumetric flask. The oil sample was dissolved in a small amount of 1-butanol and made volume up with 1- butanol. The solution was mixed thoroughly.

2. Evaluation of TBA reaction

Five ml of sample solution was added with 5.0 ml of 0.2% TBA in 1-butanol in a dry screw cap glass test tube. The solution was vortexed and incubated for 2 hours in 95°C hot water bath (Heto Lab equipment, DT-1, Heto –Holten, Japan). The heated solution was cooled down with the running tap water until reaching the room temperature. The absorbance was read at 532 nm with a spectrophotometer (Lamda 25 UV/VIS spectrometer, Perkin-Elmer instrument, USA). 0.2% TBA in 1-butanol was used as a blank.

3. Calculation of TBA value

TBA value (mg^{-1}) was expressed as the increasing absorbance due to the reaction of the equivalent 1 mg of sample per 1 ml volume with TBA which was calculated by the following equation.

$$\text{TBA value} = (50 \times A_{532})/m$$

Where: A_{532} = Absorbance of sample solution (already corrected by blank)

m = mass of oil sample (mg)

Note: Factor of 50 was based on the volume from 25-ml volumetric flask and 1-cm cuvette path length

Appendix B

Statistical Analysis

Table B.1: The ANOVA table showing the effect of type of solvent, pH of solvent and extraction time and their interactions on total phenolic content at the 95% confidence interval

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	4969099.887[(a)]	17	292299.993	47.325	.000
Intercept	142056536.969	1	142056536.969	22999.800	.000
pH	1332467.734	1	1332467.734	215.734	.000
Solvent	2289696.583	2	1144848.291	185.358	.000
Time	344874.843	2	172437.421	27.919	.000
pH * Solvent	456694.346	2	228347.173	36.971	.000
pH * Time	109357.592	2	54678.796	8.853	.001
Solvent * Time	234480.250	4	58620.062	9.491	.000
pH * Solvent * Time	165889.922	4	41472.481	6.715	.000
Error	222351.292	36	6176.425		
Total	151524506.836	54			
Corrected Total	5191451.179	53			

[a] R Squared = 0.957 (Adjusted R Squared = 0.937)

Table B.2: The ANOVA table showing the effect of type of solvent, pH of solvent and extraction time and their interactions on total monomeric anthocyanin content at the 95% confidence interval

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	301939.617[(a)]	17	17761.154	17.992	.000
Intercept	4712526.630	1	4712526.630	4773.686	.000
pH	159416.586	1	159416.586	161.486	.000
Solvent	55914.359	2	27957.179	28.320	.000
Time	59159.340	2	29579.670	29.964	.000
pH * Solvent	18983.594	2	9491.797	9.615	.000
pH * Time	500.418	2	250.209	.253	.777
Solvent * Time	30604.820	4	7651.205	7.751	.000
pH * Solvent * Time	2165.467	4	541.367	.548	.701
Error	35538.775	36	987.188		
Total	5090938.764	54			
Corrected Total	337478.392	53			

[a] R Squared = 0.895 (Adjusted R Squared = 0.845)

Table B.3: The ANOVA table showing the effect of type of solvent, pH of solvent and extraction time and their interactions on antioxidant activity from FRAP assay at the 95% confidence interval

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	55.524[(a)]	17	3.266	21.037	.000
Intercept	5915.461	1	5915.461	38100.590	.000
pH	1.926	1	1.926	12.408	.001
Solvent	6.759	2	3.380	21.768	.000
Time	38.663	2	19.331	124.511	.000
pH * Solvent	3.865	2	1.933	12.448	.000
pH * Time	.506	2	.253	1.628	.210
Solvent * Time	1.290	4	.323	2.078	.104
pH * Solvent * Time	2.514	4	.629	4.048	.008
Error	5.589	36	.155		
Total	5976.574	54			
Corrected Total	61.113	53			

[a] R Squared = 0.909 (Adjusted R Squared = 0.865)

Table B.4: The ANOVA table showing the effect of type of solvent, pH of solvent and extraction time and their interactions on antioxidant activity from DPPH assay at the 95% confidence interval

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	1718143.113[(a)]	17	101067.242	115.747	.000
Intercept	23985100.950	1	23985100.950	27468.929	.000
pH	455243.312	1	455243.312	521.367	.000
Solvent	668343.521	2	334171.761	382.710	.000
Time	401470.164	2	200735.082	229.892	.000
pH * Solvent	44476.597	2	22238.298	25.468	.000
pH * Time	12742.474	2	6371.237	7.297	.002
Solvent * Time	63549.952	4	15887.488	18.195	.000
pH * Solvent * Time	61734.204	4	15433.551	17.675	.000
Error	31434.194	36	873.172		
Total	26261451.239	54			
Corrected Total	1749577.307	53			

[a] R Squared = 0.982 (Adjusted R Squared = 0.974)

VITA

Ms. Wanida Tewaruth was born on March 24, 1981, in Bangkok, Thailand. She attended Satriwitthaya 2 School from 1993 to 1999. Then, she went on to study at the Faculty of Biotechnology, Assumption University (ABAC) and received Bachelor of Science in 2003. In 2004, she enrolled in the master degree program in Food Science and Technology (English program) at Department of Food Technology, Faculty of Science, Chulalongkorn University. She completed her master degree program in the 2007 academic year. Her present address is 49/1/22 Tiwanon 11, Tiwanon road, Bangkrasor subdistrict, Maung district, Nonthabury province. Her telephone number is 025899223.