CHANGES OF PHENOLIC COMPOUNDS AND ANTIOXIDANT CAPACITY OF KHAOMAK (FERMENTED RICE) MADE FROM 3-UNPOLISHED GLUTINOUS RICE (*Oryza sativa* L. var. indica) DURING PROCESSING AND *IN VITRO* GASTROINTESTINAL DIGESTION

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A Thesis Submitted to the Graduate School of Naresuan University
In Partial Fulfillment of the Requirements
for the Master of Science Degree in Food Science and Technology
May 2017

Thesis entitled "Changes of Phenolic Compounds and Antioxidant Capacity of Khaomak (Fermented Rice) Made from 3-Unpolished Glutinous Rice (*Oryza Sativa* L. var. indica.) during Processing and *In Vitro* Gastrointestinal Digestion"

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ACKNOWLEDGEMENT

First of all, I acknowledge Naresuan University for financial support through ASIAN scholarship for my study. Nevertheless, the completion of my thesis would have not been possible without the support and guidance of several people to whom I would like to deliver my sincere thanks. I would like to express my deepest and high appreciation to my advisor, Associate Professor Dr.Sudarat Jiamyangyuen for her guidance, support and encouragement through my thesis work. I would also express my great gratitude to my co-advisors Assistant Professor Dr. Riantong Siganusong and Assistant Professor Worasit Tochampa for providing the guidance to me to deliver the best version of my thesis work.

I also deliver my special thanks to the committee members, Associate Professor Dr.Khongsak Srikaeo and Associate Professor Dr.Teeraporn Kongbangkerd, for their helpful comments and suggestions to improve my thesis work.

Particular thanks goes to Associate Professor Dr.Anchalee Srichamroen and other Ajarns in Naresuan University for their kind suggestions and encouragement during my study.

I also wish to acknowledge the supports of all friends and staffs at the Department of Agro-Industry, Faculty of Agriculture, Natural Resources and Environment for their helps.

My sincere thanks also goes to my fellow international students, Miss. Citra, Miss. Masyitha and Miss. Amaraa, and all members of Vietnamese students in Naresuan University, who has made me found a family away from home.

I also appreciate the loves from my parents and sisters who always support and believe in me.

Finally, I would like to express my gratitude to many others for their helps since beginning of my study but I cannot name one by one in this acknowledgement.

Nhat Thuan Nguyen Ho

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Academic Paper	Thesis M.S. in Food Science and Technology Naresuan University, 2016
Keywords	Khaomak, Unpolished glutinous rice, Antioxdiant, *In vitro* digestion

ABSTRACT

Khaomak is a Thai traditional dessert, fermented from glutinous rice and a culture of yeasts and molds (Look-bang). Unpolished rice (*Oryza sativa* L. var. indica), ingredients for producing Khaomak, is believed to prevent various cardiovascular diseases because of high content of natural phenolic compounds in rice bran, including anthocyanin and proanthocyanidin. Phenolic compounds in rice have two forms: free and bound. This study aims to analyze the distribution of free and bound phenolic content, total anthocyanin content (TAC), proanthocyanidin content (TPA), and antioxidant activities (AOA) of 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging and ferric reducing ability of plasma (FRAP) of Khaomak during the production steps: raw rice, after cooking and fermentation. A model of *in vitro* gastrointestinal digestion (oral, gastric and small intestinal phase) was applied to figure out the assessable antioxidants of cooked rice and Khaomak in a physiological point of view.

Three cultivars of unpolished glutinous rice (nonpigmented, black and red colours in bran) were used. Three methods of cooking were applied including cooking

without soaking and two ways of steaming where rice were soaked 6 h and 12 h before steaming. The results showed that the phenolic contents (PC), DPPH radical scavenging and FRAP values in free fraction of black and red rice decreased significantly ($p \leq 0.05$) after cooking but not nonpigmented rice ($p > 0.05$). After fermentation, free-PC, DPPH radical scavenging and FRAP values in free fraction significantly improved in nonpigmented rice from 3 methods of cooking. Black rice also showed the increase of free-PC from 2 methods of cooking but red rice was not seen any improvement. However, the antioxidant activity of black and red rice increased after fermentation. Proanthocyanidin was found predominantly in red rice while anthocyanin only existed in black rice. TPA in red rice and TAC in black rice (free fraction) were 1.37 ± 0.01 mg catechin equivalents (CE)/g dry weight (DW) and 1.84 ± 0.75 mg cyanidin-3-glucoside (C3G) equivalents/g DW, respectively. Cooking decreased these values considerably while fermentation exhibited some improvement. The highest content ($p \leq 0.05$) of TAC and TPA in both cooked rice and Khaomak were recorded in the cooking method without soaking. Total phenolic content (total-PC), DPPH radical scavenging and FRAP values (sum of free and bound fraction) of black and red rice in raw rice, cooked rice and Khaomak were significantly higher than nonpigmented rice due to the greater amounts of anthocyanin and proanthocyanidin, respectively. Bound fraction accounted for approximately half of total-PC in nonpigmented rice (raw and cooked rice) but were lower than those in black and red rice.

A study of texture profile analysis was conducted with 3 types of measurement: hardness, adhesiveness and springiness. Soaking reduced significantly ($p \leq 0.05$) the hardness in cooked rice when comparing with without soaking method. Fermentation significantly reduced ($p \leq 0.05$) the adhesiveness in Khaomak while the springiness values of all products were quite fluctuated among treatments.

The *in vitro* digestion were performed with cooked rice and Khaomak made from nonpigmented, red and black rice by the cooking method without soaking. The PC of every treatments generally significantly increased after every digestion step. TAC were highest ($p \leq 0.05$) at the simulated gastric digestion but greatly reduced ($p \leq 0.05$) after intestinal phase. In general, Khaomak may have better bioaccessibility than their cooked rice because of higher antioxidant activities.

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CHAPTER I

INTRODUCTION

Rationale for the study

Rice is one of the most important food crop in the world and it feeds nearly half of world population (Nemat, Yadollah, & Mahdi, 2009; Shen et al., 2009). In Asian countries, rice is considered as the main carbohydrate source in daily diet (S. H. Huang, & Ng, 2012). In a comparison with global average consumption (289 g/day/capita), it witnesses an exceeding consumption by 9 countries, including Bangladesh, Lao, Cambodia, Vietnam, Myanmar, Thailand, Indonesia, Philippines, and Guinea (range: 290 – 475 g/day/capita) (Rothenberg et al., 2015).

Khaomak is believed as a healthy dessert, made from fermented glutinous rice which has a long-standing in Thai traditional cuisine. The starter culture named Look-bang is utilized to ferment the rice starch molecules to monosaccharide, alcohol and lactic acid (Manosroi et al., 2011); therefore, the final product has a sweet and slightly sour taste with a mild alcoholic flavor. This fermented rice is also very popular in other Asian countries, especially in Southeast Asia known under different names. In Indonesia and Malaysia, it is known as "Tapai nasi" meanwhile the Chinese call it "Chiu-niang". "Chao" is a similar product from Cambodia (Gandjar, 2003) and "Com Ruou" is from Vietnam.

Khaomak or similar products are not a new topic for researchers but their applicable values are still various. Many studies on fermented rice or only rice bran with different types of saccharifying organisms revealed that fermentation process improves the quality and nutrition of the final product. The microbial interaction during full rice or rice bran fermentation resulted in good bacteria accumulation and free radical scavenging activity enhancement so it is more likely beneficial for health (Ghosh et al., 2015; Tufan et al., 2013). Another study demonstrated that the quantity of phenolic compounds doubles after fermentation (Schmidt et al., 2014), showing the ability to inhibit the enzymes peroxidase and polyphenol oxidase (Schmidt et al., 2014; Yen, Chang, & Su, 2003). In addition, fermented rice also contributes its value

in cosmetic, functional food development and clinical use. It is considered as non-toxic and has a strong positive relationship between bioactive compounds and bioactive activities (Choi et al., 2014; Manosroi et al., 2011).

Traditionally, Khaomak is prepared from polished glutinous rice which is soaked overnight before steaming the rice for 6 – 12 h (Rojsuntornkitti, 2007). Soaking is a crucial step to provide the starch with sufficient water for gelatinization (Thammapat, Meeso, & Siriamornpun, 2015). However, many studies have reported that it affects on the colour, texture, solid loss, mineral, bioactive compounds and on the antioxidant activities in rice. Research area on antioxidants and their bioactive ability is not at a standstill. A deep looking in procedure to make Khaomak is necessary. To be more precise, soaking time is considered for discussion whether it affects on the antioxidant properties on the final product. Since the common Khaomak is produced from commercial glutinous rice which is already milled and polished, the new approach to make Khaomak with unpolished rice is more appealing due to the rich source of bioactive compounds in the rice bran portion of unpolished rice (Abd Razak et al., 2015).

Besides, multiple studies have been reported about polyphenol constituents in variety of food items and their antioxidant activity based on chemical extraction (Bouayed, Hoffmann, & Bohn, 2011). However, much data have related the reactive oxygen species (ROS), generated in the digestion process, to gastrointestinal (GI) tract diseases (You et al., 2010). *In vitro* GI digestion has developed based on the physiological point of view in which samples are hydrolyzed by enzyme and gastric acid (Gökmen, Serpen, & Fogliano, 2009; Serpen, Gökmen, & Fogliano, 2012). It is a very useful method to access the stability and properties of polyphenol which affect on their biological activity and final metabolic fate (Bermúdez-Soto, Tomás-Barberán, & García-Conesa, 2007). Thus, *in vitro* GI digestion method is carried out to identify and compare the bioactive compounds in cooked rice and Khaomak.

Research objectives

1. Examine the effect of 3 cooking methods on the antioxidant properties and texture profile of cooked rice and fermented glutinous rice or Khaomak made from 3 rice cultivars: nonpigmented, black and red unpolished glutinous rice.

2. Identify the effect of *in vitro* GI digestion model on the change of phenolic components of cooked rice and Khaomak and their antioxidant activity made from 3 cultivars of unpolished glutinous rice.

Research hypothesis

1. Soaking and hydrothermal cooking cause the loss of phenolic components especially anthocyanin and proanthocyanidin in pigmented rice which decreases the antioxidant activity of cooked rice and Khaomak but fermentation gains the quantity of phenolic compounds and improve the antioxidant activity.

2. In physiological point of view, Khaomak is more potential to give a better health benefits than cooked rice for the same variety.

Expected benefits

Khaomak or fermented glutinous rice is a traditional food from many Asian countries. Depending on each country, the product is made from polished or unpolished rice, pigmented or non-pigmented rice. The most popular type for fermenting is polished glutinous rice and following by black (or purple) rice (polished or unpolished) while unpolished-red-glutinous rice has not been studied widely. The current study may bring some expected benefits as following:

1. Giving a background of antioxidant activity of 3 types of unpolished rice after cooking and fermentation and a proper method to produce Khaomak.

2. Predicting health benefits by consuming Khaomak.

3. Contributing an insight of fermented rice which may be useful for other study involves in functional food or clinical purpose.

CHAPTER II

LITERATURE REVIEW

Free radicals as sources of diseases

1. Free radicals and reactive species

Harman (1956) proposed that aging process is the consequence of free radical attack on cell constituents and connective tissues. They also attribute to degenerative diseases like mutations and cancers. In recent years, a number of study in free radicals and antioxidants have been increased in order to comprehend the aging process and promote a better healthcare system (Ratnam et al., 2006).

Reactive species is a phrase collectively describing a variety of highly reactive species or oxidations that are produced in biological systems. They are derived from oxygen, nitrogen and sulfur, thus creating reactive oxygen species (ROS), reactive nitrogen species (RNS) and reactive sulfur species (RSS) (Carocho, & Ferreira, 2013). Some of them are free radicals, some are non-radicals but they share the same potential to cause radical reactions in biological system (Pryor, 1986). Free radicals contain one or more unpaired electrons in their outer shells while non-radical species produce molecules that can be disintegrate to form free radicals (Carocho, & Ferreira, 2013; Halliwell, 1996; Pryor, 1986). Ozone, hydrogen peroxide are examples that create free radicals by a non-radical pathways (Pryor, Prier, & Church, 1983). Examples of ROS, RNS and RSS are displayed in Table 1.

The unpaired electron alters the chemical reactivity of molecule/atom, making it becomes highly unstable and available to react with various organic substrates such as lipids, proteins, DNA (Pham-Huy, He, & Pham-Huy, 2008). Most of biological molecules are non-radicals so an attack of a free radical on a non-radical molecule generates a new free radical which initiate a chain reaction (Halliwell, 1996). For example, $OH^{\cdot}$ radical initiates a propagation of autoxidation chain called lipid peroxidation when it tries to abstract hydrogen atom from fatty acid chains in lipid bilayers (Carocho, & Ferreira, 2013).

Table 1 Reactive oxygen spices (ROS) and reactive nitrogen species (RNS)

Species	Radicals	Nonradicals
ROS	Superoxide, $O_2^{\cdot-}$ Hydroxyl, $OH^{\cdot}$ Peroxyl, $RO_2^{\cdot}$ Alkoxyl, $RO^{\cdot}$ Hydroperoxyl, $HO_2^{\cdot}$	Hydrogen peroxide, H_2O_2 Hypochlorous acid, HOCl Ozone, O_3 Singlet oxygen $^1\Delta g$
RNS	Nitric oxide, $NO^{\cdot}$ Nitrogen dioxide, $NO_2^{\cdot}$	Nitrous acid, HNO_2 Dinitrogen tetroxide, N_2O_4 Dinitrogen trioxide, N_2O_3 Peroxynitrite, $ONOO^-$ Peroxynitrous acid, ONOOH Nitronium cation, NO_2^+ Alkyl peroxynitrites, ROONO
RSS	Thiyl radicals ($RS^{\cdot}$)	Disulfide-S-oxides [$RS(O)_2SR$] Sulfenic acids (RSOH)

Source: Halliwell, 1996; Corpas, & Barroso, 2015

2. Formation of free radicals in human body

In human body, biological free radicals are products from a normal physiological pathway involving in mitochondria metabolism or from an abnormal pathological condition such as inflammation process (Carocho, & Ferreira, 2013; De Filippis et al., 2015). Radiation, environment pollution, industrial chemicals, xenobiotic and smoking are exogenous factors also causing free radicals (Carocho, & Ferreira, 2013; Halliwell, 1996; Pryor, 1986). Nevertheless, RS formation can be cataloged in two mechanism: enzymatic and non-enzymatic reactions (Pham-Huy, He, & Pham-Huy, 2008).

2.1 ROS

Oxygen (O_2), accounting for 21% in the current atmosphere level, is essential for cellular respiration in aerobic organisms (Halliwell, 1996). Though its crucial effect, oxygen is considered poisonous in term of the attribution in formation of free radicals (Halliwell, 2007). At ground state, oxygen molecule (O_2) is a bi-

radical, containing two unpaired electrons and not very reactive with the two electrons in a chemical bond (Turrens, 2003). But when one of two unpaired electron is excited (known as singlet oxygen), it becomes very powerful oxidant.

Mitochondria is the major ROS generator (Orrenius, Gogvadze, & Zhivotovsky, 2007) and uses ~90% of cellular O_2 (Nemat, Yadollah, & Mahdi, 2009). There are two ways to produce ROS either by oxidative enzymes in the cells or by traces of metals such as iron, cobalt and manganese in connective tissues (Harman, 1956). Free radical forming by enzymatic reactions involves in the respiration, the phagocytosis, the prostaglandin synthesis and the cytochrome P450 system (Pham-Huy, He, & Pham-Huy, 2008). The formation and elimination of ROS is chemically described as in Figure 1.

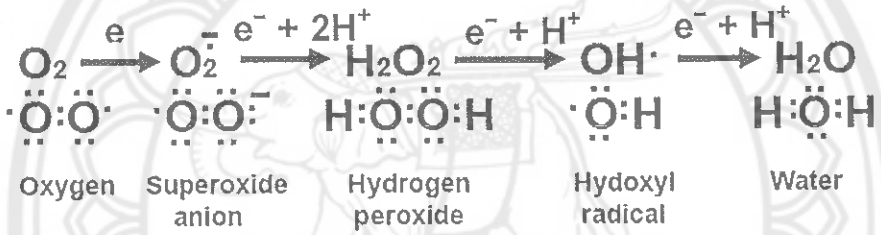


Figure 1 Diagram of ROS formation

Source: Mach et al., 2011; Thomson, & Paton, 2014

Superoxide – Superoxide ($O_2^{\bullet-}$) is the product of the reduction of O_2 during the normal aerobic respiration and physiological intracellular metabolites (Cadenas, & Davies, 2000). It is the precursor of most ROS and a mediator of radical reaction chains (Turrens, 2003). It is believed that 1 – 3% of breathing O_2 are utilized to generate $O_2^{\bullet-}$ and thus, approximately 2 kg of $O_2^{\bullet-}$ are produced every year in a healthy person (Orrenius, Gogvadze, & Zhivotovsky, 2007). Some enzymes take roles in this respiratory such as NADPH oxidase, xanthine oxidase and peroxidases (Pham-Huy, He, & Pham-Huy, 2008). Besides, transition metals including iron (Fe) and copper (Cu) are also catalyzer in O_2 reduction such as:

$$Fe^{2+} + O_2 \rightarrow Fe^{3+} + O_2^{\bullet-} \quad (1)$$

Hydrogen peroxide. Hydrogen peroxide (H_2O_2) is a non-radical, mostly produced by SOD-catalyzed reaction (a non-enzymatic pathway) from $O_2^{\bullet-}$

(Halliwell, 1996) or catalyzed by some oxidative enzymes including amino acid oxidase and xanthine oxidase (Pham-Huy, He, & Pham-Huy, 2008):

$$2O_2^{\bullet-} + 2H+ \rightarrow H_2O_2 + O_2 \quad (2)$$

Hydroxyl radical. There are a few ways to produce hydroxyl radical ($OH^{\bullet}$). Radiation from the environment such as gamma rays can split water in the body to create OH• (Halliwell, 1996). In the body, thevreduce of $O_2^{\bullet-}$ concentration is associated with the reduce transition metals which in turn react with H_2O_2 producing $OH^{\bullet}$ (Turrens, 2003). This is called Fenton-type reaction:

$$O_2^{\bullet-} + Fe^{3+} \rightarrow Fe^{2+} + O_2 \quad (3)$$

$$Fe^{2+} + H_2O_2 \rightarrow OH^{\bullet} + OH + Fe^{3+} \quad (4)$$

Interaction of $O_2^{\bullet-}$ with H_2O_2 by the Haber–Weiss reaction (Kehrer, 2000):

$$O_2^{\bullet-} + H_2O_2 \rightarrow O_2 + OH^{\bullet} + OH^{\bullet} \quad (5)$$

Both $OH^{\bullet}$ and peroxynitrite are strong oxidants which indiscriminately react with nucleic acids, lipids and proteins.

2.2 RNS and RSS

RNS and RSS are the results from the reaction between ROS and nitric oxide and thiols, respectively (Mut-Salud et al., 2016).

Nitric oxide radical ($NO^{\bullet}$) is formed in biological tissues from the oxidation of L-arginine to citrulline by nitric oxide synthase (Zweier, Samouilov, & Kuppusamy, 1999).

$O_2^{\bullet-}$ when meet $NO^{\bullet}$ will make a fast reaction to generate peroxynitrite (Halliwell, 1996):

$$NO^{\bullet} + O_2^{\bullet-} \rightarrow ONOO^- \quad (6)$$

2.3 Oxidative stress, oxidative damage and disease

ROS and RNS are recognized for both physiology effect: deleterious and beneficial (Marian Valko et al., 2007). For example, ROS play a role in defensing against bacterial pathogen by low concentration (Nemat, Yadollah, & Mahdi, 2009). However, when ROS/RNS are overproduced and not neutralized by antioxidants, it becomes injurious and may inactive the proper function of antioxidants (Wiernsperger, 2003). This severe imbalance is called "oxidative stress", leading the biological cells

undergo suffering from "oxidative damage" (Halliwell, 2007). Thus, the alteration or destruction of biomolecules' chemical structure occurs as a result.

Every days, human cells are attacked by around 10,000 – 20,000 free radicals (M Valko et al., 2004). The main targets of ROS, RNS and RSS are proteins, lipids, sugars, RNA (ribonucleic acid) and DNA (deoxyribonucleic acid) (Carocho, & Ferreira, 2013). The machanism is shown in Figure 2.

Lipid modification. Among the cellular molecules, polyunsaturated fatty acid is highly susceptible to oxidation (Orrenius, Gogvadze, & Zhivotovsky, 2007). Free radicals attack the cell membrane fatty acid side chain, forming lipid peroxide. Lipid peroxide accumulation contribute the development of carcinogenesis agent like malondialdehyde (Nemat, Yadollah, & Mahdi, 2009).

Protein damage. Free radicals catalyze the oxidative modification of proteins by several ways: direct oxidation of amino acids, cross-link them and peptide cleavage (Carocho, & Ferreira, 2013). From many investigation, protein oxidation can make cellular and extracellular proteins like enzymes and connective tissue protein lose their normal function permanently (Nemat, Yadollah, & Mahdi, 2009).

Sugar oxidation. During the initial stages of nonenzymatic glycosylation, the short chain species such as glycolaldehyde, products of sugar fragmentation, are prone to autoxidize and produce $O_2^{\bullet-}$ because their carbon chains are too short to permit cyclization. $O_2^{\bullet-}$ is produced as a consequence which lead to another free radical chain reaction to generate mutagenic substances, α,β-dicarbonyls (Benov, & Beema, 2003).

DNA damage. DNA is also highly susceptible to free radical attacks (Nemat, Yadollah, & Mahdi, 2009). Free radicals attack DNA and modify the purine and pyrimidine bases, the deoxyribose backbone, single and double strand–breaks, as well as cross-links to other molecules (Orrenius, Gogvadze, & Zhivotovsky, 2007). Accumulation of DNA lesions with age is the cause for age-associated diseases including cancer (Bohr, 2002).

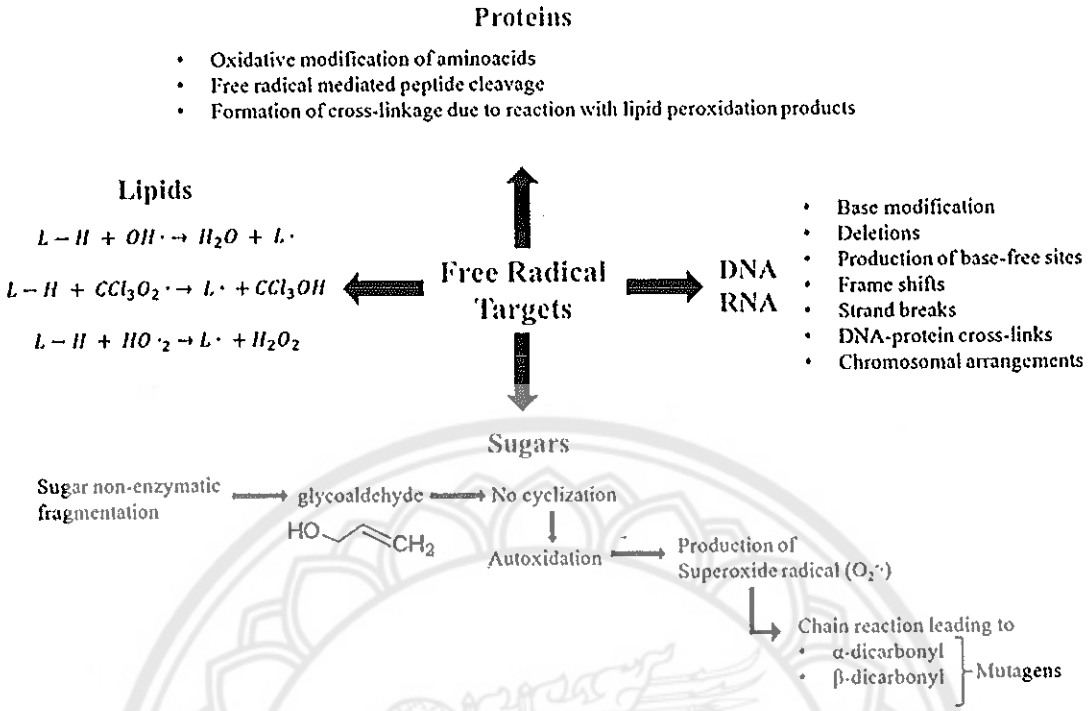


Figure 2 Targets of free radicals

Source: Carocho, & Ferreira, 2013

There are reliable evidences the involvement of radical-mediated reactions and various pathological conditions such as cardiovascular disease, cancer, neurological disorders, arthritis, diabetes, ischemia/reperfusion, other diseases and ageing (Pryor, 1986; Sharma, 2014). These diseases are placed to two categories: "mitochondrial oxidative stress" and "inflammatory oxidative conditions" (Sharma, 2014). The first group is characterized by pro-oxidants shifting the thiol/disulphide redox state and impairing glucose tolerance such as cancer and diabetes mellitus. The second group is characterized by the enhanced activity of either NAD[P]H oxidase which leads to atherosclerosis and chronic inflammation or xanthine oxidase-induced formation of ROS which is implicated in ischemia and reperfusion injury.

Antioxidant actions

1. Antioxidant classification

As mentioned above, O_2 is the generator of oxidation. However, at normal atmospheric condition (21% in concentration), aerobic organisms can survive and protect themselves against oxygen toxicity due to their antioxidant defense system (Halliwell, 1996, 2007). Concentration more than 50% of O_2 results in cell damage and death (Thomson, & Paton, 2014).

There are many ways to define antioxidants. At low concentration, antioxidants are compounds that have ability to significantly prevent or delay oxidation of subtracts (Halliwell, 1990) or prevent biomolecules such as proteins, nucleic acids, polyunsaturated lipids, and sugars from enduring oxidative damage through free radical mediated reaction (Bendary et al., 2013). However, Halliwell (2007) simply defines antioxidant is "any substance that delays, prevents or removes oxidative damage to a target molecule". The desirable characteristic of an antioxidant are readily absorbed, quickly neutralize free radicals, chelate redox metals at physiologically relevant levels (Rahman, 2007). In addition, a new stable radical after quenching through intramolecular hydrogen bonding on further oxidation is obtained (Halliwell, 1990).

Antioxidants can be categorized in different ways (Figure 3). The human systems have ability to produce antioxidants (enzymatic and non-enzymatic antioxidants) to retain the balance between the production and neutralization of ROS (Carocho, & Ferreira, 2013). Endogenous antioxidants play a vital role to support cell function and retain systemic health (Rahman, 2007). However, a proper dietary antioxidants are have been proposed to be important free radical scavengers *in vivo* (Halliwell, 1996). Exogenous antioxidants are popularly present in natural food such as fruits, vegetables, grains and cereals (Carocho, & Ferreira, 2013). Plants are the most potential antioxidants and serve as medical herbs (Ghasemzadeh, & Ghasemzadeh, 2011). They can also be synthesized and consumed as supplements (Pham-Huy, He, & Pham-Huy, 2008).

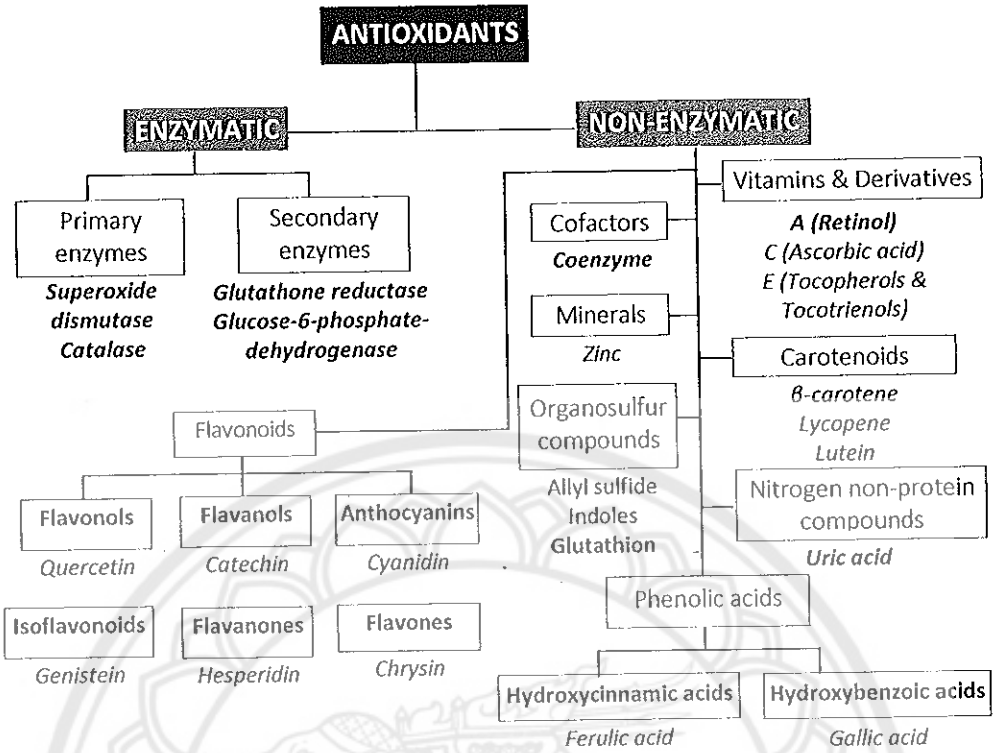


Figure 3 Natural antioxidants separated in classes

Note: Italic words represent exogenous antioxidants, while italic and bold words represent endogenous antioxidants.

Source: Carocho, & Ferreira, 2013

2. Reaction mechanism of antioxidants

According to Mut-Salud et al. (2016), antioxidants can be classified into three lines of defense according to their mechanism of action: (i) preventing the formation of new free radicals, (ii) capturing free radicals and thus prevent oxidative chain reactions, (iii) repairing the damage (including enzymatic antioxidants) caused by free radicals. However, mechanism of phenolic antioxidants can be classified into primary action (Type I, or chain breaking) and secondary action (Type II, or preventive) (Galano et al., 2016).

Type I antioxidants have ability of hydrogen and electron donor as the following.

2.1 Hydrogen-atom transfer (HAT)

$$H_nAntiox + {}^{\bullet}R \rightarrow Hn_{-1}Antiox^{\bullet} + HR \quad (7)$$

This is the key reaction mechanism of phenolic acid and polyphenol family including flavonoids (Galano et al., 2016; Terpinc et al., 2011). The B ring hydroxyl configuration of flavonoid structure is the most significant determinant of ROS scavenging (Procházková, Boušová, & Wilhelmová, 2011). The increase of polymerization of flavonoid monomers, e.g. proanthocyanidins, enhances the antioxidant capacity.

2.2 Proton-Coupled Electron Transfer (PCET)

PCET reactions yield the same products as HAT (equation 7) yet, the transferring of electron and proton in PCET is separated into single steps (Galano et al., 2016). It is an important mode of action of flavonoids.

2.3. Radical Adduct Formation (RAF)

$$H_nAntiox + {}^{\bullet}R \rightarrow [H_nAntiox\text{-}R]^{\bullet} \quad (8)$$

RAF involves the reaction from multiple bonds and albeit electrophilic.

2.4 Single Electron Transfer (SET)

$$H_nAntiox + {}^{\bullet}R \rightarrow H_nAntiox^{+\bullet} + R^{-} \quad (9)$$

and

$$H_nAntiox + {}^{\bullet}R \rightarrow H_nAntiox^{+\text{-}\bullet} + R^{+} \quad (10)$$

SET (equation 9) is an important mechanism of catechin analogs with peroxyl radicals (ROO˙) while SET (equation 10) is involved in the reaction of the nitric oxide radical with Trolox, caffeic acid, uric acid, and genistein.

2.5 Sequential Proton-Loss Electron Transfer (SPLET)

SPLET is involved in a two-step mechanism:

$$H_nAntiox \rightarrow H_{n-1}Antiox^{-} + H^{+} \quad (11)$$

$$H_{n-1}Antiox^{-} + {}^{\bullet}R \rightarrow H_{n-1}Antiox^{\bullet} + R^{-} \quad (12)$$

This is the key mechanism on the protection against oxidative damage with some phenolic phytochemicals such as quercetin, epicatechin, hydroxybenzoic acids, dihydroxybenzoic acids, flavonoids and procyanidins.

2.6 Sequential Electron Proton Transfer (SEPT)

$$H_nAntiox + {}^{\bullet}R \rightarrow H_{n-1}Antiox^{\bullet+} + R^{-} \quad (13)$$

$$H_{n-1}Antiox^{\bullet+} \rightarrow H_{n-1}Antiox^{\bullet} + H^{+} \quad (14)$$

SEPT is also a two-step mechanism involving both electron transfer and deprotonation. This is the main radical-scavenging route for Trolox models when reacting with DPPH and galvinoxyl.

2.7 Sequential Proton-Loss Hydrogen-Atom Transfer (SPLHAT)

$$H_nAntiox \rightarrow H_{n-1}Antiox^{-} + H^{+} \quad (15)$$

$$H_{n-1}Antiox^{-} + {}^{\bullet}R \rightarrow H_{n-2}Antiox^{\bullet-} + HR \quad (16)$$

SPLHAT involves the deprotonation of the antioxidant, followed by an H transfer reaction. It plays a crucial role in the antioxidant activity of anthocyanidins, in the raction of gallic acid with hydroxyl radicals.

The mechanism of Type II antioxidants are to prevent the formation of free radical such as $OH^{\bullet}$, the most reactive radical in ROS, by inhibiting the endogenous production of oxidants (Galano et al., 2016). Fenton reaction and the metal catalyzed Haber-Weiss recombination are mentioned as the dominant intracellular source of $OH^{\bullet}$ production. Some special flavonoids like quercetin can chelate iron and copper thereby removing a causal factor for the development of free radicals (Procházková, Boušová, & Wilhelmová, 2011).

Many flavonoids have a stronger antioxidant capacity *in vitro* than those of vitamins C and E as they can neutralize vitamin E radicals (Procházková, Boušová, & Wilhelmová, 2011). Furthermore, by interaction with vitamin E radical, they possess a great potential to delay the oxidation of low density cholesterols. Flavonoids can also activate endogenous enzymes (e.g. NAD[P]H-quinone oxidoreductase, glutathione S-transferase, and UDP-glucuronosyl transferase), which play an important role in defense mechanism against electrophilic toxicants and oxidative stress. Besides, flavonoids play many roles in inhibition of oxidases, mitigation of oxidative stress caused by nitric oxide, increase in uric levels, and in antioxidant properties of low molecular antioxidants.

3. Biological effects of antioxidants

Epidemiological studies have established inverse correlation between the consumption of fruits, vegetables, cereals, grains and the occurrence of diseases such as inflammation, cardiovascular disease, cancer, Alzheimer's, and aging-related disorders (Bendary et al., 2013). In fact, phenolic compounds take a major amount in

those food. Due to their phenolic ring and hydroxyl substituents, phenolic phytochemicals can function as effective antioxidants due to their ability to quench free electron (D. Vattem, Ghaedian, & Shetty, 2005). A huge amount of phenolic phytochemicals present in wholegrain rice or rice bran, so they are potential sources of food antioxidants.

Flavonoids can work as both redox-active metal chelators and free radical scavengers (Kris-Etherton et al., 2004). They are highly effective to scavenge hydroxyl and peroxyl radicals as well as quench superoxide radicals and singlet oxygen. Some of them can function as anti-inflammatory substance.

Anthocyanins are potent and distinct hydrogen-donating antioxidants showing positive effects on various forms of cancer, cardiovascular disease, obesities and diabetes and eye vision disease (J. He, & Giusti, 2010). Beyond the antioxidant property, anthocyanin takes a positive effect on cell signaling, anti-inflammatory, antimicrobial and gene expression pathways (J. He, & Giusti, 2010; Lila et al., 2016).

The bioactivity of proanthocyanidin depends on the substitution patterns (e.g., catechin and epicatechins to form procyanidins), glycosyls and chain length. Proanthocyanidins with a lower DP have been observed that exhibiting better anti-obesity anti-cancer and anti-inflammatory activities (Y. Zhang et al., 2016). It was also reported that proanthocyanidins help to accelerate the healing of stomach ulcers, demonstrating antifungal, antiseptic, antiviral, antihemorrhagic and to induce apoptosis in some tumor cells (Mut-Salud et al., 2016).

Pigmented rice and phenolic compounds

A class of chemical compounds in organic chemistry which consist of a hydroxyl group (-OH) directly bonded to an aromatic hydrocarbon group is known as phenols or phenolics. Phenolic compounds play a role as chemical protectors in various mechanisms. A number of studies have been done to investigate the bioactive potential of phenolic compounds in rice. It is properly divided into 4 groups: phenolic acids, flavonoids, anthocyanins and proanthocyanidins. Phenolic acids are classified as mono-phenol while others are known as polyphenols because they have more than 1 aromatic hydrocarbon ring (Pereira et al., 2009).

1. Phenolic acids

Phenolic acids are basically made from a phenolic ring and an organic carboxylic acid function (Goufo, & Trindade, 2014). Figure 4 shows two main derivatives of phenolic acids including benzoic acid and cinnamic acid. They are different by the number of hydroxyl and methoxyl groups at the ortho- or para-positions. It was observed that the antioxidant capacity is strongly enhanced by the increase of hydroxyl group (Pereira et al., 2009).

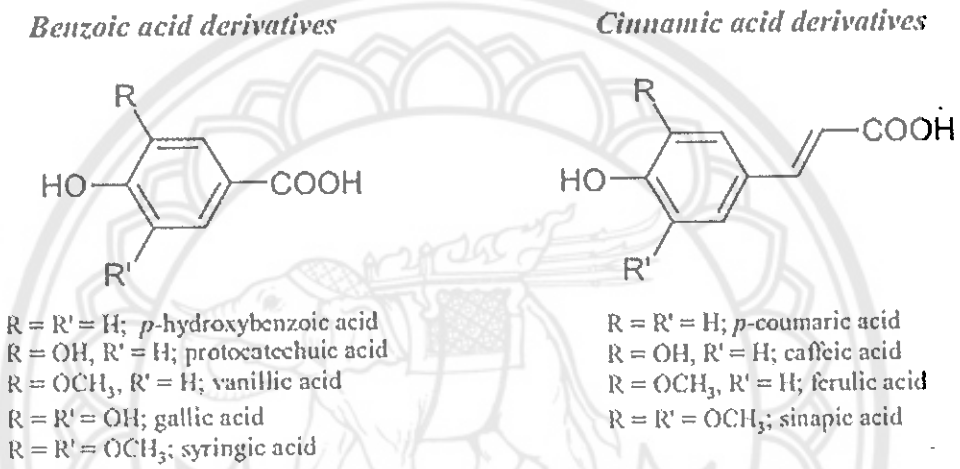


Figure 4 Chemical structures of phenolic acids

Source: Pereira et al., 2009

Phenolic acids are present in most plant food and reported as the prominent phenols in rice (Heleno et al., 2015; Zhou et al., 2004). The structures of phenolic acids are described in the Figure 4. There are usually 12 phenolic acids detected in rice including: gallic acid, protocatechuic acid, ρ-hydroxybenzoic acid, vanillic acid, syringic acid, chlorogenic acid, caffeic acid, ρ-coumaric acid, sinapic acid, ferulic acid, cinnamic acid and ellagic acid. Ferulic acid is the most abundant phenolic acid in rice, accounting for 56 – 77% of total phenolic acids in both free and bound form, followed by ρ-coumaric acid (8 – 24%) (Goufo, & Trindade, 2014; Zhou et al., 2004).

2. Flavonoids

Flavonoids are the largest derivative of polyphenol, having diphenylpropanes (C6-C3-C6) skeleton with two aromatic rings linked by a three-carbon bridge (Acosta-Estrada, Gutiérrez-Uribe, & Serna-Saldívar, 2014; Rice-Evans, Miller, & Paganga, 1996; Ross, & Kasum, 2002). The basic structure of flavonoid is displayed in Figure 5. Flavonoids are widely distributed in plants with glycosylated forms and contribute to the brilliant shades of blue, scarlet, and orange in many types of food and beverage which are originally from fruits, plants, seeds and cereals (Pietta, 2000; Ross, & Kasum, 2002) Major subclasses of flavonoids includes flavonols, flavanols, anthocyanidins, isoflavonoids, flavanones, flavanonols and flavones. Other flavonoid groups are biflavones, chalcones, aurones, and coumarins. Hydrolyzable tannins, proanthocyanidins (flavan-3-ol oligomers), caffeates, and lignans are all plant phenols, and they are usually classified separately (Pietta, 2000).

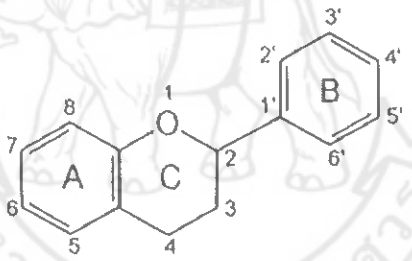


Figure 5 Basic flavonoid structure

Source: Pietta, 2000

A review from Goufo & Trindade (2014) showed that flavones are the most commonly recognized flavonoids in non-pigmented rice. It is also mentioned about 5 flavonoids that are often reported with quantity including tricin, luteolin, apigenin, quercetin and isorhamnetin while tricin appears to be the most abundant in rice bran. Figure 6 describes other flavonoids that have been detected in rice. Anthocyanin is the prominent flavonoid pigment for the colour of black rice while red rice is a rich source of proanthocyanidin.

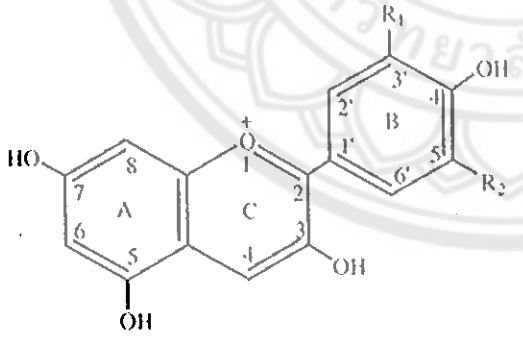
Flavonols	Flavanols	Anthocyanidin	Flavanones	Flavones
•Quercetin •Kaempferol •Isorhamnetin •Myricetin	•Catechin •Epicatechin •Epigallo-catechin	•Cyanidin •Malvidin •Pelargonidin	•Hesperetin •Naringenin •Taxifolin	•Luteolin •Apigenin •Tricin

Figure 6 Common flavonoids in rice

Source: Pietta, 2000; Carocho, & Ferreira, 2013; Goufo & Trindade, 2014

3. Anthocyanins

Anthocyanins are another subclass of flavonoids and naturally found in plants as water-soluble glycosides of anthocyanidins (Goufo, & Trindade, 2014; Wu, & Prior, 2005). The existences of anthocyanin are varied by their hydroxyl, methoxyl substituents in their basic flavylium (2-phenylbenzopyrilium) (Figure 7) (Wu, & Prior, 2005) and sugar moieties locating on carbon 3, 5, 7, 3', and 5' (Harborne, 1973; Kähkönen, & Heinonen, 2003).



R_1=H, R_2=H: pelargonidin (Pg)
R_1=OH, R_2=H: cyanidin (Cy)
R_1=OH, R_2=OH: delphinidin (Dp)
R_1=OCH_3, R_2=H: peonidin (Pn)
R_1=OCH_3, R_2=OH: petunidin (Pt)
R_1= OCH_3, R_2=OCH_3: malvidin (Mv)

Figure 7 Chemical structures of the six common anthocyanidins in nature. Atom numbering and ring nomenclature of the 2-phenylbenzopyrylium (flavylium) core are marked

Source: Zhao et al., 2017

Glucose is the most common prevalent sugar moiety but rhamnose, xylose, galactose, arabinose, and fructose are also available as sugar groups attaching to anthocyanidins (Kähkönen, & Heinonen, 2003; Rice-Evans, Miller, & Paganga, 1996). Among anthocyanin glycosides, the diglycoside and triglycoside forms are more stable than the monoglycoside forms (Tsuruda et al., 2013).Anthocyanins are the most widespread pigment of colouring matters in plants (Harborne, 1973), which are present in the sap of plant cells and responsible for nearly all the pink, scarlet, red, mauve, violet and blue colours in petals, vegetables and fruits (Harborne, 1973; John, 1999). The differences of number and position of the hydroxyl and methoxyl groups make the colour of anthocyanin varied (Rice-Evans, Miller, & Paganga, 1996). As the number of hydroxyl groups increases, the bluish shade tend to be deeper while the rise of methoxyl groups increases redness (John, 1999). Furthermore, the colour and structures of anthocyanins are considerably effected by medium pH which they are dissolved (Alkema, & Seager, 1982). They may exist in a variety of protonated, deprotonated, hydrated, and isomeric forms. At extremely acidic pH (pH 1 – 3), the red flavylium cation is dominant. In aqueous media, when the pH is raised to 4 – 5, hydration reactions create the colourless carbinol pseudo-base, which additionally experience ring opening to the light yellow chalcones, most quickly at pH 2.5 – 5 at higher temperatures. . The flavylium cation can alternatively be transformed to quinonoidal bases through proton transfer reactions and at pH valoues between 6 and 7 be further converted to the blue-purple quinonoid anions (Kähkönen, & Heinonen, 2003). The structure of anthocyanin in different pH is shown in Figure 8.

There are approximately 18 types of anthocyanin determined in rice, meanwhile only four have been quantified (cyanidin-3-O-glucoside, peonidin-3-O-glucoside, cyanidin-3-O-rutinoside, and cyanidin-3-O-galactoside) in the report of Goufo and Trindade (2014). Cyanidin-3-O-glucoside and peonidin-3-O-glucoside are dominant derivatives which make up 51 – 84% and 6 – 16% of total antioxidant capacity, respectively. Antioxidant activity of anthocyanins primarily depends on their phenolic structures. The contribution of more hydroxyl groups in B-ring results in higher antioxidant activity (Haq, Riaz, & Saad, 2016). In the other side, glycosylation decreases the capacity of anthocyanins to delocalize electrons, thus reducing their antioxidant activity. The potent antioxidant of anthocyanins is due to the hydrogen

donor's ability which based on the presence of positive charge O-atom. Anthocyanins also perform an excellent ability to scavenge ROS when comparing with common antioxidants such as vitamins E and C (Hatier, & Gould, 2009)

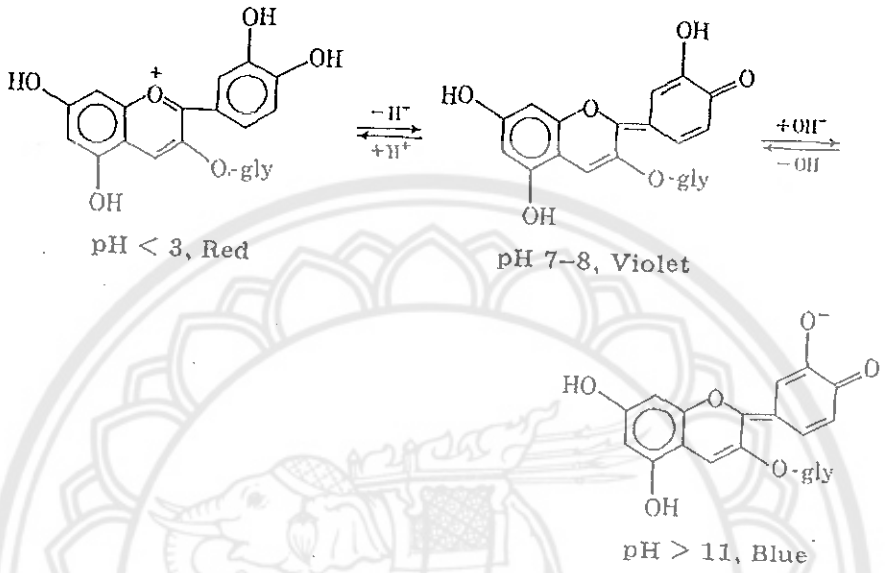


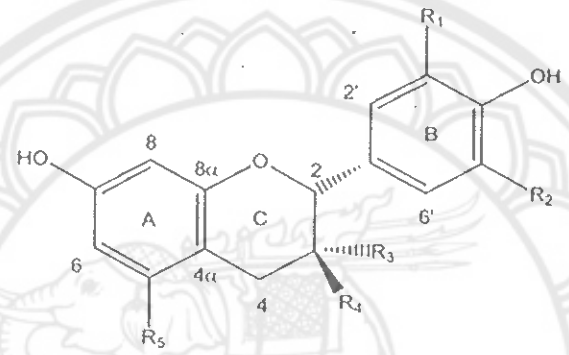
Figure 8 Structure of anthocyanin in different pH

Source: von Elbe, & Schwartz, 1997

4. Proanthocyanidins

Proanthocyanidins belong to another class of flavonoids: tannins. Tannins are special polyphenol which are known for the ability of combining with protein and other polymers such as polysaccharides so that they are able to precipitate alkaloids, gelatin, and other proteins (von Elbe, & Schwartz, 1997). The chemistry of tannins are complex but they are generally divided into two groups: hydrolysable tannins and nonhydrolyzable tannins (Hagerman et al., 1998; John, 1999). Hydrolysable tannins consist of phenolic acids and sugars that can be hydrolyzed by acid, alkaline or enzyme units (Schofield, Mbugua, & Pell, 2001). Condensed tannins, commonly known as proanthocyanidin, are oligomers or polymers of monomeric flavan-3-ols (Rasmussen et al., 2005). They are classified by different degree of polymerization (DP) as 1, 2, 3 or 4 which are corresponding to monomers, dimers,

trimers, or tetramers (Ou, & Gu, 2014). Higher DPs are called polymers or high polymers, which also indicate higher molecular sizes. Proanthocyanidins are classified as "extractable proanthocyanidins" (EPAs) which can be extracted by aqueous-organic solvents and "non-extractable proanthocyanidins" (NEPAs) that are mostly linked to the protein and polysaccharides of the dietary fibre matrix (Saura-Calixto et al., 2010). NEPAs cannot be extracted either by the action of digestive enzymes or by the usual aqueous acidic methanol-acetone solvents.



Flavan-3-ol Monomers	R1	R2	R3	R4	R5
(+)-afzelechin	H	H	H	OH	OH
(+)-catechin	H	H	OH	H	OH
(+)-epicatechin	H	OH	H	OH	OH
(+)-gallocatechin	H	OH	OH	H	OH
(+)-epigallocatechin	OH	OH	H	OH	OH
(+)-epigallocatechin	OH	OH	OH	H	OH
(+)-fisetinidol	H	OH	H	OH	H
(+)-epifisetinidol	H	OH	OH	H	H
(+)-robinetinidol	OH	OH	H	OH	H

Figure 9 Structures of some flavan-3-ol monomer

Source: Rasmussen et al., 2005

Several monomers of proanthocyanidins are displayed in Figure 9. These units are generally linked through carbons 4 → 8 or 4 → 6, which are called B type linkage or proanthocyanidin dimmers (Aron, & Kennedy, 2008; von Elbe, & Schwartz, 1997). In addition, A type linkage, another group of proanthocyanidin dimmers, is the result of ether bond between carbons 2 → 7 (Aron, & Kennedy, 2008; Rasmussen et al., 2005). Proanthocyanidin trimmers are those possessing the

interfalvan linkage of carbon 4 → 6 and namely C type (Aron, & Kennedy, 2008). Some examples of proanthocyanidins are shown in Figure 10.

Proanthocyanidins are found in various fruits and tissues of apple, grape skin, and bine bark (Ito et al., 2013). With respect to the molecular weights, the main proanthocyanidin compounds in rice are most likely oligomers of epicatechin linked by four to eight carbon–carbon bonds (Goufo, & Trindade, 2014). They are most present in rice bran of pigmented rice (red colour). However, the presence of a significant quantity of proanthocyanidin in plant cell wall residues limits the extractability of polysaccharides, as well as the accessibility of polysaccharide-cleaving enzymes, in particular the highly methylated pectic fraction (Ruiz-Garcia, Smith, & Bindon, 2014).

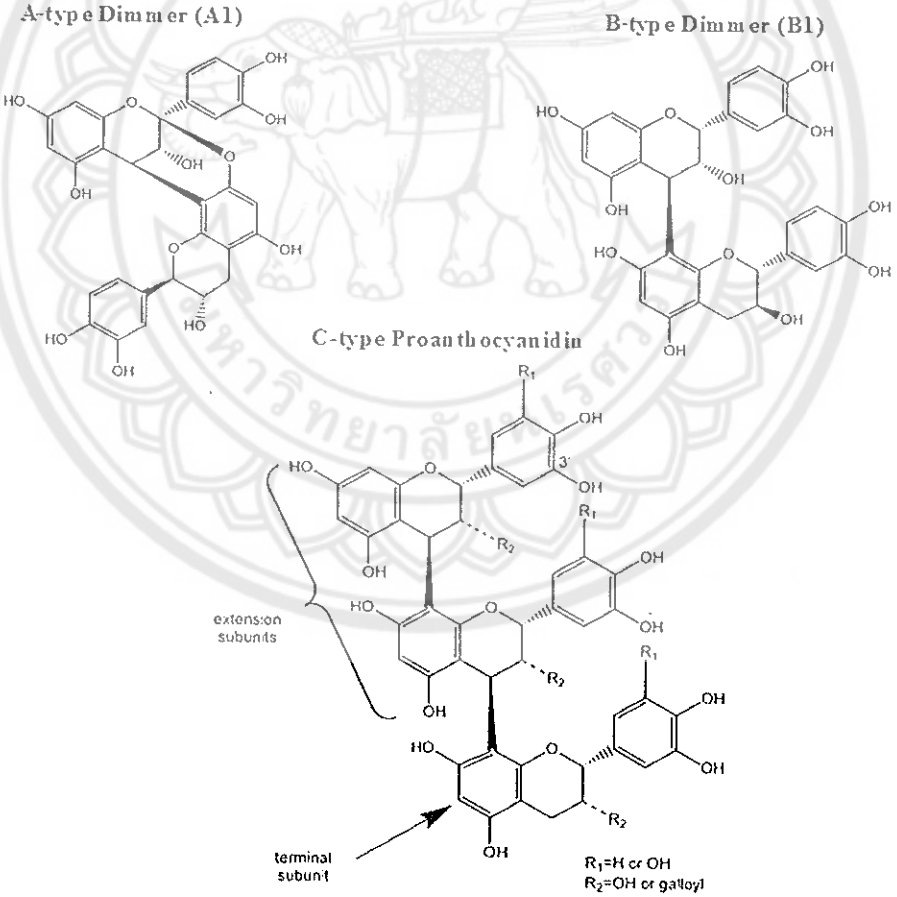


Figure 10 Structures of some proanthocyanidins

Source: Rasmussen et al., 2005

Khaomak and Khaomak-like products

1. Overview

Fermentation is a well-known method of food preservation from ancient time. In Asia-Pacific region, rice paddy is widely cultivated due to its tropical and subtropical humid climate. This climatic condition is also suitable for mold and yeast growth. Aside from soy, rice is another cereal in Asia, which is considered as the most regular substrates for plant-based fermented food (Hutkins, 2006). The rice was first fermented to develop a nutty flavor and to ease hulling (Lan, & Mahapatra, 2007). Moreover, cereal fermentation with molds is the result of the consumption of rice as a staple food, and the limit of animal husbandry practices (Haard, 1999).

Khaomak is a fermented rice product from Thailand. It is traditionally prepared by fermenting Thai polished nonpigmented rice with starter culture (Look-bang) to create a mildly alcoholic flavor, partially juicy rice paste having a sweet and sour taste. The dessert can be found in Thai markets but it is also easy to be manually cooked at home. In Thai folklore wisdom, Khaomak has been considered to boost the growth of malnutrition children and used as a dietary supplement. It also promotes the healthy bacteria gut flora by providing an increasing amount of food for these bacteria (Manosroi et al., 2011). The Figure 11 shows Khaomak products are sold in a local Thai market.

Figure 11 Khaomak sold at a weekend market in Phitsanulok, Thailand

The process of Khaomak is alcohol fermentation because yeasts and molds are utilized as the starter culture. In general, fermentation is defined as a metabolite process in which substrate such as carbohydrates and related compounds are oxidized with the release of energy in the absence of external electron acceptors or simply as a process in which chemical changes are brought about in an organic substrate through the action of enzymes elaborated by microorganisms. This resulted in the alteration of the chemical composition and other characteristics of the final products. Particularly, molds grow on the rice materials and produce microbial amylases during first stage of fermentation (Rhee, Lee, & Lee, 2011). These enzymes convert starch present in the rice into sugars. The acid-forming bacteria then use sugars to produce organic acids, reducing the pH to below 4.5, which is optimum for yeast growth at the later stage of alcohol fermentation (Battcock, & Azam-Ali, 1998b; Rhee, Lee, & Lee, 2011).

Khaomak is a very perishable product because the fermentation continues even after the optimum stage (Merican, & Quee-Lan, 1989). Over fermentation results in a sour taste and strong alcohol aroma that are unaccepted by consumers. Therefore, Khaomak has to be consumed immediately when it reaches the desired flavor. However, if it is chilled, the shelf-life can extend to 2 weeks.

In Asia, similar products to Khaomak are found in many countries but are known under different names. Starters are used in the manufacture of these products and these starters are also known by different names although the product is basically the same (Table 2). They may differ from appearance, texture or way of serving.

Table 2 Khaomak and Khaomak-like products from different countries

No.	Country	Product name	Description
1	Thailand	Khaomak	Fermented glutinous rice
2	Vietnam	Com Ruou	Fermented glutinous rice
3	Indonesia	Taipe	Fermented glutinous rice
4	Malaysia	Taipe	Fermented glutinous rice
5	China	Lao Rhao	Fermented glutinous rice

Source: Adapted from Haard et al., 1999; Hutkins, 2006

2. Glutinous rice

The ingredient of Khaomak and Khaomak-like product is glutinous rice. Other names are glutinous rice and waxy rice. Rice with the remained bran is called unpolished rice while polished rice means rice that has the bran removed. In Thailand and the southern part of Vietnam, polished rice is popularly used to make this fermented dessert. However, according to some international students in Naresuan University, unpolished glutinous rice especially black cultivar is popularly consumed in these regions such as Indonesia, Cambodia and northern part of Vietnam.

Structure of a rice grain is divided into 5 parts (Figure 12) as bellowing.

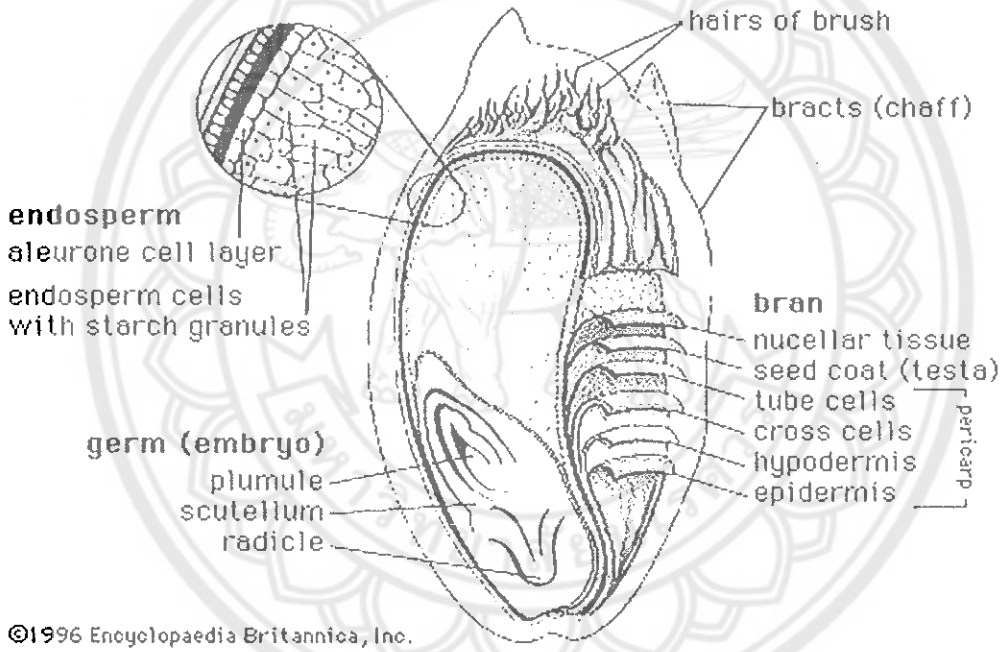


Figure 12 Structure of a rice grain

Source: Kent-Jones, D. W., 1996

Hull – The hull provides protection to the rice caryopsis. It composes of two modified leaves, the palea and the larger lemma.

Bran (pericarp, seed coat, and nucellar tissue) – There are three distinct layers of crushed cells, making up the caryopsis coat, surrounding the endosperm. Pigments in coloured rice are in the pericarp or the seed coat.

Embryo – It is extremely small and located on the ventral side at the base of the grain. Embryo is rich of nutrients which are necessary for rice germination. The two major parts of the embryo are the scutellum (cotyledon) and the embryonic axis. The C-shaped embryonic axis of the embryo is separated from the starchy endosperm by the scutellum proper. The scutellum contains globoid-rich particles resembling aleurone grains.

Aleurone layer – This is the outermost layer of endosperm tissue, differing both in morphology and function from the starchy endosperm. It completely surrounds the rice grain and the outer side of the embryo. Two storage structures are prominent: aleurone grains (protein bodies) and lipid bodies.

Starchy endosperm – The starchy endosperm is divided into two regions, the subaleurone and the central region consisting of the rest of the starchy endosperm. The main structures are starch and protein.

Rice which is still tightly enveloped by husk after threshing is referred to as paddy or rough rice. Brown rice or unpolished rice is which only husk is removed. This means that the rice grain retains its bran. Depending on the consumption market, brown rice can be directly sold to market or milled to peel off the bran to become polished rice. Milled rice is the most common sort of rice consumed. Rice bran is the byproduct after milling which can be utilized in oil industry or feedstock. Bran contains more of the pericarp, seed coat, nucellus, aleurone layer, and germ than the polish, which contains relatively starchy endosperm.

Carbohydrate of rice is predominantly starch, with small portions of pentosan, hemicelluloses, and sugars (Houston, & Kohler, 1970). Starch makes up about 85 – 90% of rice solids. Amylose and amylopectin are two main components occurring in starch. Glutinous rice differ from common rice by the amylose amount. They have very little or no amylose while amylopectin contribute around 12 – 35% of total starch.

Rice bran contains a vast amount of protein and oil. Protein account for 11.3 – 14.9% while oil is about 15 – 19.7% in rice bran (Juliano, & Bechtel, 2004). On the other hand, the milled fraction or polished rice contain only 6.3 – 7.1% of protein and 0.3 – 0.5% of oil (Juliano, & Bechtel, 2004). Rice bran is an abundant source of bioactive compounds that have many positive biological effects on human health.

Vitamin E (refers to any of the 8 naturally occurring forms, α, β, γ, and δ species of both tocotrienols and tocopherols) and γ-oryzanol (ferulic acid ester of phytosterols) are prominent lipophilic antioxidants found in rice bran (Goufo, & Trindade, 2014; Min, McClung, & Chen, 2011). γ-Oryzanol is especially known to be beneficial for lowering cholesterol, reducing menopause problems and increasing muscle mass (Patel, & Naik, 2004). Besides those phytochemicals, rice bran accounts for a number of phenolic compounds such as phenolic acids and flavonoids which are discussed later. From information above, unpolished glutinous rice may be more advantageous in term of health benefits than polished glutinous rice. Hence, Khaomak made by unpolished glutinous rice provoke more interest to study because of antioxidant concern.

3. Starter culture

The starters used for the production of Khaomak are known as Look-bang. Look-bang is a microbial environment which includes a mixture of yeasts and molds for fermentation purpose. The culture is prepared by inoculums in rice flours with desirable microorganism (mother cultures) and herbs such as pepper, garlic and galangal as antibacterial agents (Manosroi et al., 2011). Look-bang is often shaped into dried hemispherical balls to be stored in dried place.

Starter cultures from other countries have different names as shown in the Table 3. The starters used for tapai production are known as "ragi". There are two types of ragi. "Ragi tapai" which probably originated in Indonesia are used for producing tapai. "Ragi samsu" are to make rice wine originated in China (Merican, & Quee-Lan, 1989). Although there are different in ragi production technology from localities to localities, they are generally similar to Look-bang. In Vietnam, the starters are called "banh men". They are classified based on the ingredients putting in "banh men", where "banh men" with Chinese herbs adding, banh men without Chinese herb but antibiotic adding.

Table 3 Starter cultures for Khaomak-like products from different countries

No.	Country	Name
1	Thailand	Look-bang
2	Vietnam	Banh men
3	Malaysia	Ragi
4	Indonesia	Ragi
5	China	Chiu-yeu

Source: Haard et al.,1999; Hutkins, 2006

All the starters are done in a cottage scale so the quality and microorganisms are not consistent. However, the predominant molds are found including *Aspergillus sp., Mucor sp.* and *Rhizopus sp.* while S. *cerevisiae* and *Candida* sp. are two species of yeasts (Manosroi et al., 2011; Plaitho, Kangsadalampai, & Sukprasansap, 2013). Molds and bacteria in the starter cultures are employed in lactic acid fermentation and yeast cells are in responsible for ethanol fermentation, which are the specific characteristic of Khaomak and Khaomak-like fermentation (Shahidi, & Yeo, 2016). However, *S. cerevisiae* are facultative anaerobic microbes; hence, they only take part in producing ethanol when it is lack of oxygen (Bekatorou, Psarianos, & Koutinas, 2006). This point could be taken into consideration to control the production of ethanol in the final product.

4. Traditional preparation of Khaomak

In the traditional method for preparing Khaomak, taipai, com ruou and other Khaomak-like products, the glutinous rice is washed, then soaked overnight or from 6 – 12 h and cooked by steamer pots (Merican, & Quee-Lan, 1989; Rojsuntornkitti, 2007). The texture of cooked unpolished rice are not so adhesive as polished rice and quite separated and rigid due to the bran. Cooked rice, when cooled, is mixed with Look-bang, wrapped in banana leaves or placed in containers, and allowed to ferment at room temperature (28 – 30 °C) for 1-3 days. Look-bang is used with amount of 0.1 – 0.3% based on weight of raw rice (Manosroi et al., 2011; Rojsuntornkitti, 2007).

During Khaomak processing, the soaking and steaming pretreatment of glutinous rice are essential for further fermentation and sensory attributes of the final products (Li et al., 2015). The raw glutinous rice often reaches approximately 30 – 40% in water content after soaking (Li et al., 2015; Thammapat, Meeso, & Siriamornpun, 2015). Beside the normal soaking method as mentioned above, there are other methods also utilized. In the north and north-eastern parts of Thailand, people commonly use salt solution to soak rice in order to reduce soaking time (Thammapat, Meeso, & Siriamornpun, 2015). A study of germinated brown rice have proved that soaking Hom Nil brown rice in salt water (2 tsp/L or 12.95 g/L) for 24 h significantly increased the contents of phenolic compound and total antioxidant activity (Maksup, 2016). Temperature and soaking time are other factors affecting on the quality of cooked rice (Tian et al., 2014). Soaking can cause a huge loss of minerals and protein (Albarracín, González, & Drago, 2013).

According to the legally binding document for fermented sweet rice by Thai Industrial Standards Institute, five indicators are measured in the final including alcohol content, total solid, pH and *Escherichia coli* level, yeast and mold limitation.

Ethyl alcohol content: less than 0.5%

Total solid range: $10 <$ total solid < 50

pH range: $4 <$ pH < 4.5

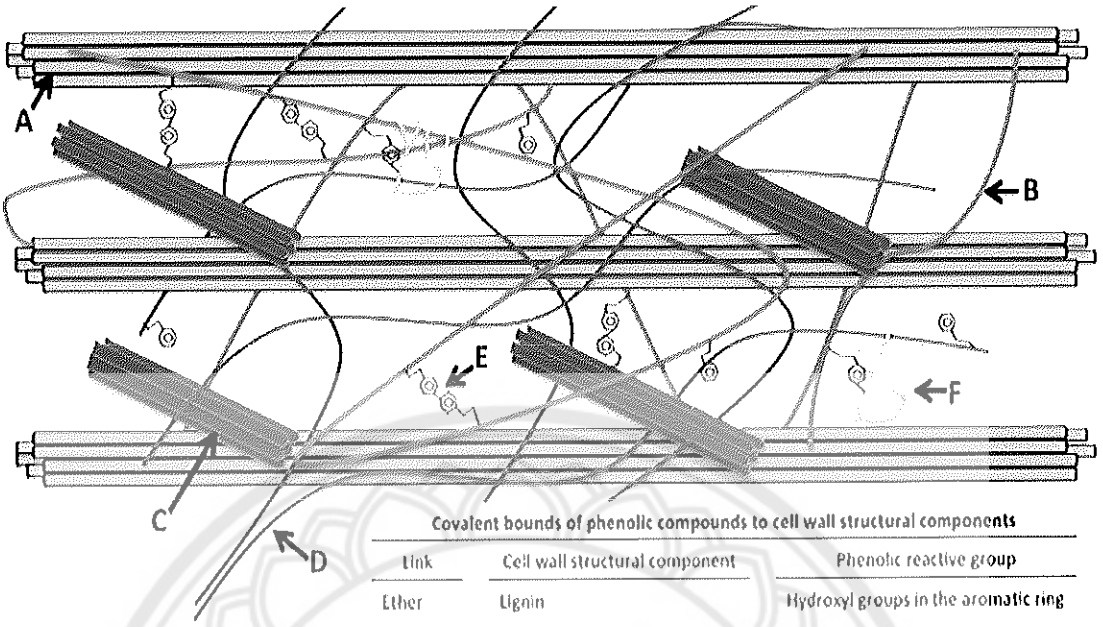
E.coli count: less than 3/g by MPN

Yeast and mold count: less than 100 CFU/g

Performance of fermentation on the structure of phenolic compounds

1. Phenolic compounds in food matrices

Phenolic compounds occur naturally in three major forms, including soluble free form, soluble conjugated form and insoluble bound form (Butsat, Weerapreeyakul, & Siriamornpun, 2009). Phytochemicals of fruits and vegetables largely exist in free or soluble conjugated forms (Adom, & Liu, 2002). On the other hand, a greater part of grain phytochemicals are insoluble bound (Acosta-Estrada, Gutiérrez-Uribe, & Serna-Saldívar, 2014; Gökmen, Serpen, & Fogliano, 2009). Figure 13 gives an examples of phenolic compounds present in a cell wall of a plant material.



Covalent bounds of phenolic compounds to cell wall structural components

Link	Cell wall structural component	Phenolic reactive group
Ether	Lignin	Hydroxyl groups in the aromatic ring
Ester	Structural carbohydrates Proteins	Carboxylic group

Figure 13 Representations of primary cell wall structure of plant material and cross-linking between structural components and phenolic compounds

Note: (A) Cellulose, (B) Hemicellulose, (C) Structural proteins, (D) Pectin, (E) Phenolic acids, (F) Lignin.

Source: Acosta-Estrada et al., 2014

1.1 Chemically and physically free phenolics

Chemically and physically free phenolics are low molecular weight and free from physical and chemical interaction with other macromolecules, which can be extracted with water or hydroalcoholic mixture (Gökmen, Serpen, & Fogliano, 2009).

1.2 Soluble conjugated (glycosides) phenolics

As stated by Bravo (1998), conjugated structure of phenolics have one or more sugar moieties linked to hydroxyl groups, although direct bonds of the sugar unit to an aromatic carbon atom also exist. Flavonoids presents as glycosides with single or multiple sugar moieties linked through an OH group (O-glycosides) or through carbon-carbon bonds (C-glycosides) (Acosta-Estrada, Gutiérrez-Uribe, &

Serna-Saldívar, 2014). Cyanidin 3-glucoside is an example of soluble conjugated phenolics. It is an aglycone of anthocyanidin which links with a sugar moiety.

1.3 Bound phenolics

It is possible to arrange bound phenolics into 2 groups: chemically bound and physically bound. Phenolic acids are commonly found in bound form (Acosta-Estrada, Gutiérrez-Uribe, & Serna-Saldívar, 2014). Many insoluble bound phenolics occur in dietary fiber (Butsat, Weerapreeyakul, & Siriamornpun, 2009).

Physically entrapped phenolics – They give non-covalent interaction with food matrix components as well as those physically entrapped in cellular structure (Gökmen, Serpen, & Fogliano, 2009).

Chemically bound phenolics – They bound to cell walls components such as cellulose, hemicellulose, lignin, pectin and rod-shaped structural proteins though covalent bonds (Figure 15) or to food matrices via ionic bridge (Acosta-Estrada, Gutiérrez-Uribe, & Serna-Saldívar, 2014; Gökmen, Serpen, & Fogliano, 2009). Phenolic acids are commonly found in this form. For example, ferulic acids are esterified to hemicellulose of the cell walls in wheat bran (R. H. Liu, 2007).

1.4 Insoluble polymeric polyphenols

Aside from those structures above, it is not superfluous to mention about another group namely insoluble polymeric antioxidants. Due to their high degree of polymerization, these compounds are poor extractability. They can be partially soluble but mainly insoluble in water as well as in other solvents. For instance, high-tannins are made by flavonoid units joined by carbon-carbon bond with a molecular mass of up to 30,000 Da (Bravo, 1998). High molecular weight tannins are completely insoluble and only extracted by strong acid hydrolysis.

2. Fermentation and bioavailability of phenolic compounds

Bioavailability is the term to refer the amount of bioactive compounds released from food matrix that are available for absorption *in vivo* and function to the target tissue (Lila et al., 2016). Free and some conjugated phenolic acids are thought to be readily available for absorption in the human small intestines. However, a vast amount of phenolic dominantly occurs as insoluble-bound forms, which account for 20 – 60% in vegetable, fruits and legume/seeds, compared with soluble phenolics.

Several food process such as thermal processing, pasteurisation, fermentation, and freezing help to liberate bound phenolics (R. H. Liu, 2007). Some commercial enzymes are utilized to enhance the nutraceutical values but fermentation is more preferable because of low cost (Acosta-Estrada, Gutiérrez-Uribe, & Serna-Saldívar, 2014). Fermentation was described by Nguyen Thai et al. (2014) as a potential method which not only increase the release of bound phenolics from the cell walls, but also improve their bioactivity by converting them into various metabolites. The microbial activity induce is the influential factor to induce the breakdown of vegetal cell walls and thus, release or induce the synthesis of various bioactive compounds, which could be responsible for the increase the antioxidant activity of total phenolics (Muñoz et al., 2017).

A number of studies have demonstrated the role of fungi in the improvement of phenolic profile obtained from plant food. For instance, ellagic acid content increased after 5 days of incubation between cranberry pomace with *Lentinus edodes* (D. A. Vattem, & Shetty, 2003), a remarkable raise of phenolic acids was witnessed during oat fermentation by using *Aspergillus oryzae* var. *effuses*, *Aspergillus oryzae* and *Aspergillus niger* (Cai et al., 2012). During fermentation, the activity of microorganism also helps to enrich nutrition by improving digestibility of protein and carbohydrates; removal of antinutrients, natural toxicants and mycotoxins (Haard, 1999). In the other hand, 24 h of fermentation has ability to eliminate phytic acid, an important compound to protect grains from oxidative damage during storage but less desirable in term of nutrition (M. d. S. Oliveira et al., 2010).

The release of bound phenolics in gastrointestinal tracts and simulated *in vitro* gastrointestinal digestion

1. Releasing of bound phenolics

Several researchers even investigated that absorption of flavonoids such as anthocyanins starts in the stomach and appear rapidly in the blood plasma after ingested (Lila et al., 2016). By contrast, the absorption of covalently bound phenolic phytochemicals to indigestible polysaccharides depends on digestive enzymes or microorganisms in intestinal lumen (Gutiérrez-Grijalva et al., 2016). As determined by Wang, He, & Chen (2014), the complex matrix of cereal bran severely hinders the

bound phenolics to access the necessary enzymes that participate in their release in human gastrointestinal tract. Figure 14 is a review of the enzymatic and microbial activity on the using of phenolics during gastro-intestinal digestion.

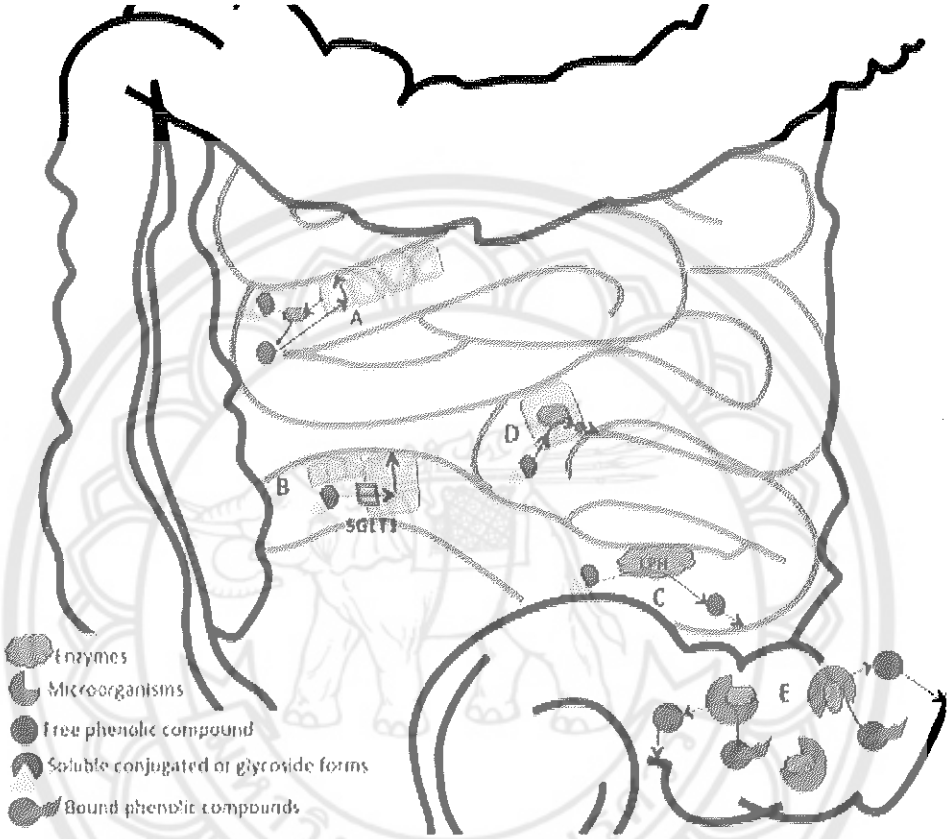


Figure 14 Absorption pathways of bound phenolic compounds in the gastrointestinal tract

Note: (A) Hydrolysis of bound soluble conjugated forms by mucosa cells cinnamoyl esterases. (B) Soluble conjugated forms transport into enterocytes by the sodiumdependent glucose transporter SGLT1. (C) Lactase phloridzine hydrolase (LPH) (glycosidase) of the brush border hydrolysis soluble conjugated phenolic compounds. (D) Epithelial cells cytosolic b-glucosidase hydrolyzes glycosides, and aglycones are formed after absorption. (E) Esterase and xylanase activities of colon microorganism (e.g. *Clostridium spp., Eubacterium spp., and Bifidobacterium adolescentis*).

Source: Acosta-Estrada et al., 2014

In addition, some reports show that phenolics in many plant food exist in conjugated forms either with sugars as glycosides or other moieties. While antioxidant activity of phenolic compounds is influenced by number of free hydroxyl groups, this conjugation, occurring via the hydroxyl groups, may reduce their ability to function (D. Vattem, Ghaedian, & Shetty, 2005).

2. Simulated *in vitro* gastro intestinal digestion

In physiologic perspective, the biological properties of antioxidants such as phenolic compounds will rely on upon their discharge from the food matrix during the digestion process (Pastoriza et al., 2011). Thus, the chemical extraction of phenolic compounds, a popularly method to extract phenolic for testing their antioxidant activity, may not reflex the accuracy amount that human body can response. Meanwhile, simulated *in vitro* digestion method can provide a physiological condition to observe the releasing of bound phenolics. *In vitro* (Latin for within the glass) refers to the technique of biological processes made to occur outside the living organism in an controlled environment ("Differences between *in vitro*, *in vivo*, and *in silico* studies," 2015). *In vitro* simulation of digestion usually consists of 3 stages including oral digestion, gastric digestion and small intestinal digestion, and occasionally large intestinal fermentation. The techniques are broadly utilized to conduct the gastro-intestinal behavior of food or pharmaceuticals because these method are more rapid, less costly, less labour intensive, and overcome some drawbacks of *in vivo* methods in term of ethical issues (Minekus et al., 2014). Various *in vitro* digestion models have been proposed. They are different in sources of enzymes (porcine, rabbit or human origin), pH, mineral type, ionic strength and digestion time (Minekus et al., 2014). Figure 16 is an example flow chart of *in vitro* digestion study.

2.1 Oral digestion

The objective of oral digestion is to create a mass of chewed food known as a bolus (Bornhorst, & Singh, 2014). The bolus must have an adequately small particle size and be enough greased up with salivation so it can be securely gulped. The main enzyme in this phase is α-amylase (ptyalin) in human saliva which has a pH ideal at pH 6.8 (Pedersen et al., 2002). α-amylase is easily inactivated by acidic pH in the stomach; hence, frequently respected to be of lesser significant in the comparison with the pancreatic α-amylase (Minekus et al., 2014). Nevertheless, some

studies showed that 50% starch of bread and 25% starch of spaghetti can be digested within 20 to 30 s.

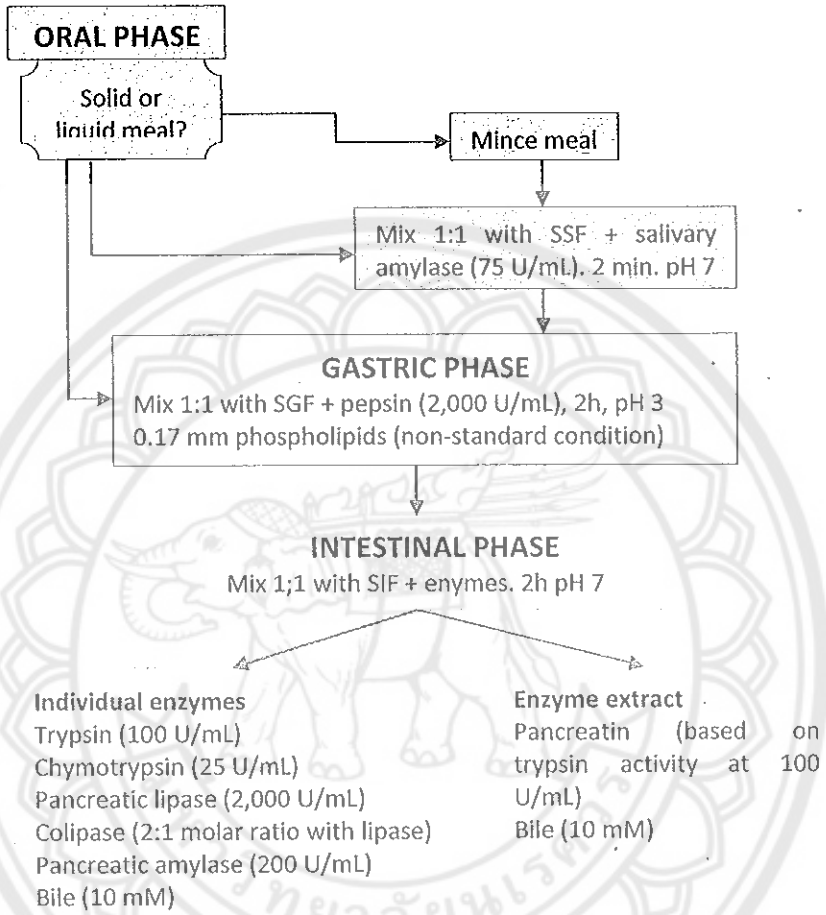


Figure 15 Model of *in vitro* digestion

Source: Minekus et al., 2014

2.2 Gastric digestion

The main role of the stomach is to convey digesta to the duodenum in a managed way to achieve intestinal digestion (Minekus et al., 2014). At the lower part of stomach, pepsinogen, secreted by chief cells, converses into pepsin upon contact with hydrochloric acid, secreted by parietal cells. The pH is relatively low from the beginning of the digestion procedure, without the initial buffering impact of the food. Likewise, the food is exposed to an enzyme – substrate proportion, which is typically

just come to at half-gastric discharging time. Pepsin is the only proteolytic enzyme in human stomach, which is mostly active between pH 2 – 4 (Minekus et al., 2014).

2.3 Small intestinal digestion

The small intestine extends from the pyloric sphincter to the ileocecal junction and is 6–7 m long. It ensures that the partially digested food, or chyme, is broken down into molecules small enough to be absorbed through the epithelial cells and carried into the bloodstream. The duodenum secretes bicarbonate that will neutralize gastric acid and provide an appropriate pH for further enzymatic digestion to occur.

CHAPTER III

RESEARCH METHODOLOGY

Materials

1. Glutinous rice samples

Three cultivars of glutinous rice (*Oryza sativa* L. var. indica) utilized in the research belong to SME group (Nong Ping Kai) grown in Muang Kampangphet Province, Thailand. They were harvested in December 2014. All the samples were unpolished which bran was still maintained. The samples (Figure 16) include: unpolished glutinous rice with nonpigmented colour in bran, unpolished glutinous rice with black colour in bran and unpolished glutinous rice with red colour in bran.

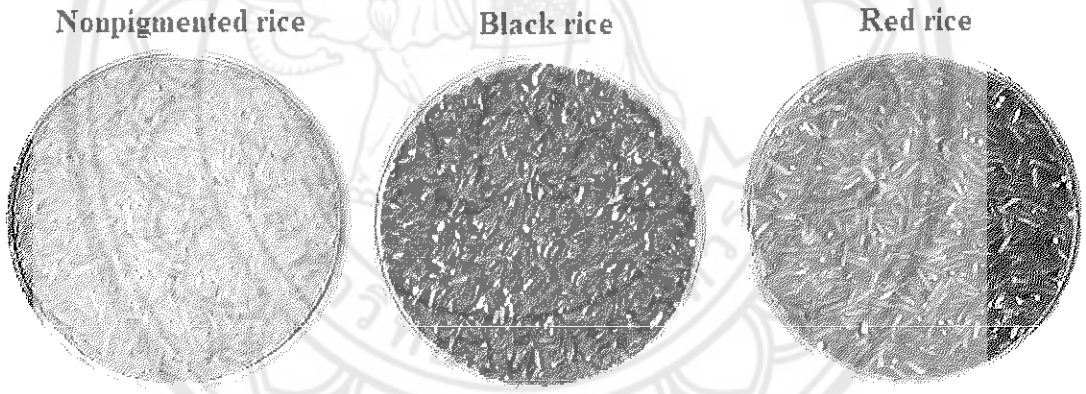


Figure 16 Unpolished glutinous rice used in this study

Unpolished black and red rice were already packed 1 kg in vacuum plastic bag (1 kg per bag) when purchased and stored at room temperature (28 – 30 °C) in a dried place during experiments. Unpolished nonpigmented rice was purchased in paddy form then milled by a lab-scale rice milling machine (NW Turbo 1000). The rice grains were screened at 0.1 mm to remove dust and damaged grains, packed 2 kg in a plastic bag and stored at the same conditions as the other rice samples.

2. Cultures of yeast and molds

Starter culture or Look-bang (Figure 17) was purchased from a local market in Phitsanulok Province, Thailand. Look-bang is used to ferment the unpolished glutinous rice and stored at room temperature in a dry place during study time.

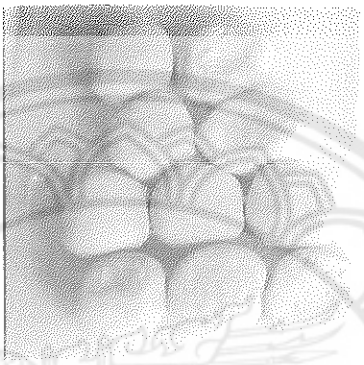


Figure 17 Look-bang

3. Enzymes

3.1 α-Amylase from *Bacillus subtilis*, 10070 Sigma, Thailand

3.2 Pepsin from porcine gastric mucosa, P7000 Sigma, Thailand

3.3 Pancreatin from porcine pancreas, P7545 Sigma, Thailand

4. Chemicals and Reagents

4.1 (+)-Catechin hydrate, ≥96.0%

4.2 (±)-6-Hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid, 97%

4.3 2,2-Diphenyl-picrylhydrazyl

4.4 2,4,6-Tris(2-pyridyl)-s-triazine, ≥99%

4.5 Acetic acid glacial

4.6 Analytical grade solvents (methanol, acetone, hexane and ethyl acetate)

4.7 Bile salt

4.8 Calcium chloride

4.9 Folin-Ciocalteau's phenol reagent

4.10 Gallic acid monohydrate, 98+%

4.11 Hydrochloric acid, 37%

4.12 Iron(III) chloride hexahydrate

4.13 Magnesium chloride hexahydrate

4.14 Monopotassium phosphate

4.15 Potassium chloride

4.16 Sodium bicarbonate

4.17 Sodium hydroxide, 97%

4.18 Sulfuric acid, 98%

4.19 Vanillin, 99%

Overview of the study

This study was to analyze the changes of phenolic compounds and their antioxidant activities during Khaomak production. The assessment of antioxidant response by *in vitro* gastro-intestinal digestion model was also applied. Three types of unpolished glutinous rice with different bran colours (nonpigmented, black and red) were used.

Khaomak traditionally is fermented from cooked Khao-niew (polished glutinous rice) with a starter culture or Look-bang. Glutinous rice was often soaked overnight before cooking by a food steamer. In this research, the cooking process was varied by two methods: direct cooking without soaking and steaming. For steaming treatment, rice was soaked in 6 or 12 h before cooking. On the contrary, soaking step was not necessary when using rice cooker. The fermentation was occurred within 3 days and these specifications were determined: pH and total solid content.

Phenolic compounds, especially anthocyanin and proanthocyanidin in pigmented rice (black and red rice), have considered as natural antioxidants which noteworthy for their antioxidative activities and some pharmacological, medicinal properties. However, high temperature from cooking process is not favorable for those components. In addition, long time of soaking can cause the loss of phenolics due to their hydrophilic characteristic. To address these problems, cooked rice and fermented rice (Khaomak) were identified by these following test: phenolic content (PC) of free and bound phenolics, anthocyanin content (TAC) and proanthocyanidin content (TPC), antioxidant activity (AOA) determination by using DPPH (2,2-diphenyl-1-

picrylhydrazyl) radical scavenging and FRAP (ferric reducing ability of plasma) assays. Besides, texture profile analysis was also applied in cooked rice and Khaomak.

Eventually, cooked rice and Khaomak were passed though *in vitro* gastro-intestinal digestion model to predict the bioaccessibility of phenolic compounds in term of physiology. The model was an imitation of human digestive system within the lumen of the gastro-intestinal tract. It consisted of 3 stages of digestion which were oral, gastric and intestinal stages. At every stage, an amount of the sample was taken out to check the experimental measurement including PC, TAC, TPA, AOA determination of DPPH radical scavenging and FRAP assays. The aim was to check the quality of antioxidants throughout the GI digestion and how potential they can benefit to human health by diet.

The overview of this study is shown in Figure 18.

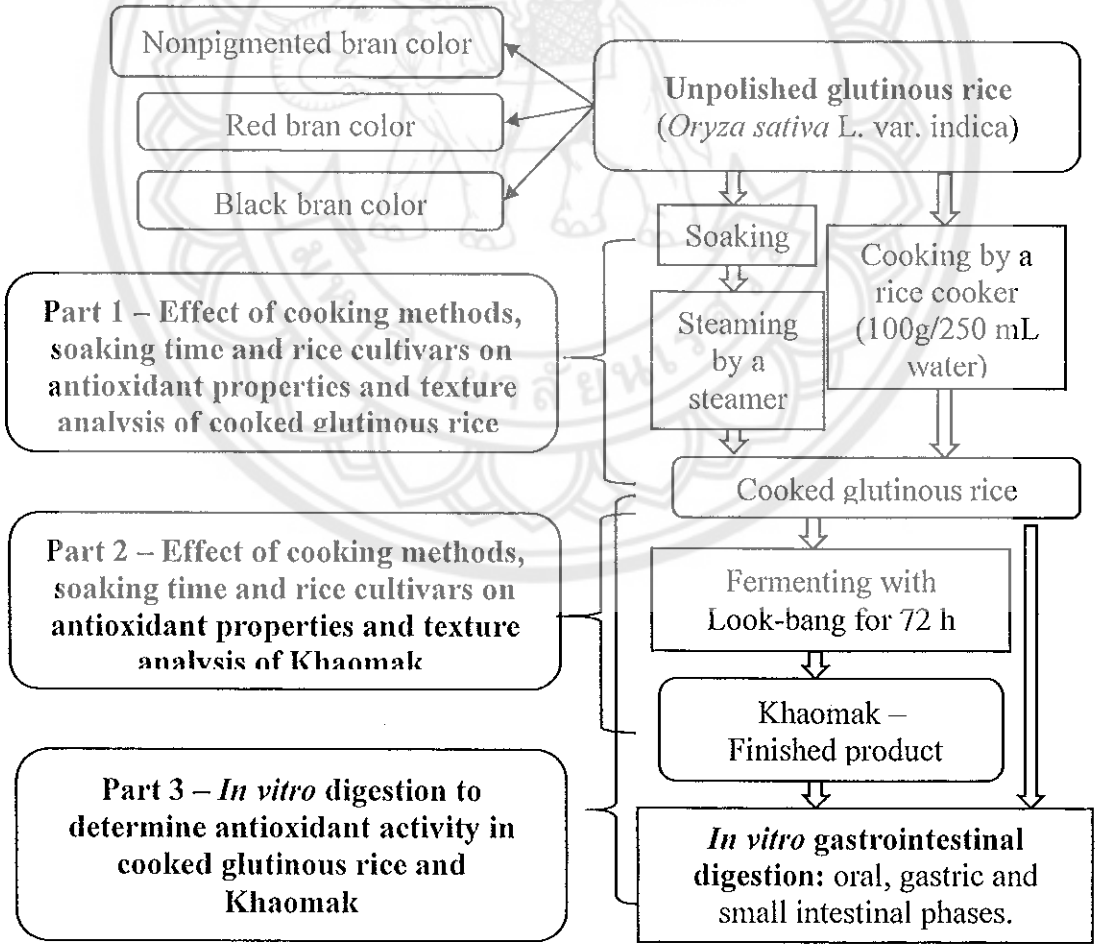


Figure 18 Scope of the study

Place of the research activities

All research activities were conducted at Department of Agro-Industry, Faculty of Agriculture Natural Resources and Environment, Naresuan University, Thailand.

Methodology

1. Part 1 – Effect of cooking methods, soaking time and rice cultivars on antioxidant properties and texture analysis of cooked rice

1.1 Preparation of cooked rice

In traditional preparation, glutinous rice is soaked from 6 to 12 h before steamed. It can be assumed that this is the optimum time for water to be totally absorbed into the grains. Glutinous rice is hydrated by soaking in water before steaming. However, directly cook glutinous rice by using automatic rice cooker without soaking step has been commonly applied by a number of households due to its convenience

In this experiment, rice of each cultivar was cooked by two different methods. The processing steps are described in Figure 19.

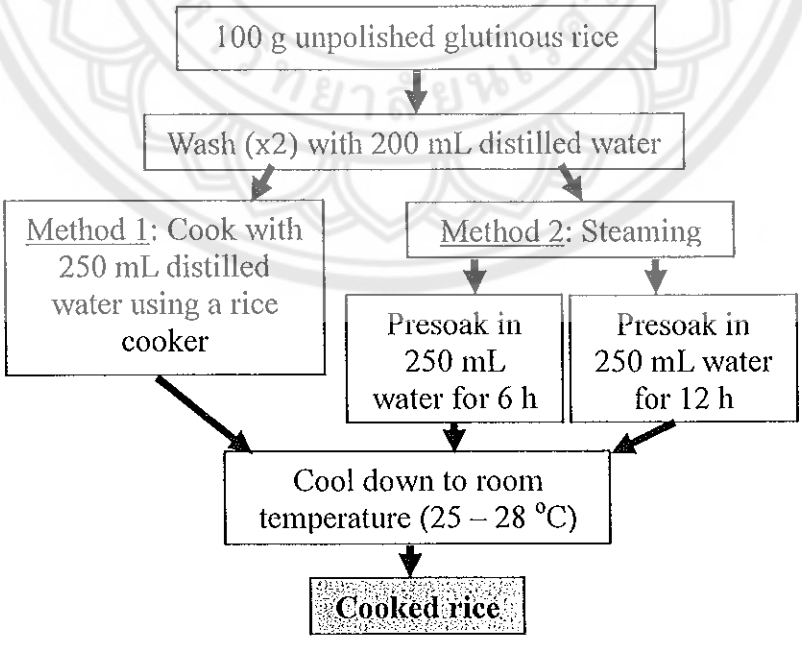


Figure 19 Procedure of rice cooking

Method 1 – Unplolish glutinous rice was directly cooked with an automatic rice cooker without soaking step. A 100 g of unpolished glutinous rice was washed with distrilled water to remove dirt then 250 mL distilled water was added and cooked for approximately 30 min by an automatic rice cooker. After cooked, nonpigmented rice and red rice were quickly cooled at room temperature while black rice were kept inside the cooker for more 15 min at the warm status. . Rice were fully cooked indicated by the complete gelatinization of rice grains.

Method 2 – Unpolished glutinous rice was hydrated in distilled water before steaming. A 100 g of glutinous rice from 3 cultivars was washed with distilled water to remove dirt then soaked into 250 mL of distilled water for 6 h or 12 h. After reaching the time requirement, rice samples were wrapped by a piece of cheesecloth and placed in a steamer for futher cooking process. The steaming time was 1 h 30 min.

There were totally 9 treatments conducted in this experiment which were processed from nonpigmented, red and black unpolished glutinous rice (Table 4). All the treatments were analyzed with the test of antioxidant properties and texture profile analysis.

Table 4 Treatments of the cooking effect on antioxidant properties and texture profile of cooked rice.

	Nonpigmented rice	Red rice	Black rice
Direct cooking	Non-soaking	Non-soaking	Non-soaking
Steaming	6h-soaking 12h-soaking	6h-soaking 12h-soaking	6h-soaking 12h-soaking

1.2 Analysis

1.2.1 Texture of cooked rice

Texture profile analysis (TPA) of the cooked rice were performed using a texture analyzer (QTS-25, Brookfield AMETEK, Massachusetts, USA) with a two-cycle compression (Mohapatra, & Bal, 2006).

In the preparation step, each sample of cooked rice was placed in a plastic container with a lid so that when it closed, the lid fitted the top surface of the rice. The weight of rice samples varies from 13 – 15 g (including the box). The parameters recorded from the test curves were hardness, adhesiveness and springiness.

The program was set up by following:

Probe: 12.7 mm cylindrical with sharp edge

Test seed: 0.5 mm/s

Post – test speed: 0.5 mm/s

Target mode: 50%

1.2.2 Changes in antioxidants of glutinous rice before and after cooking

1) Sample preparation

Raw rice and cooked rice were ground into flour, packed into plastic bags and kept at -20 °C during the experiments. However, cooked rice samples were high in water content so they were lyophilized by using a freeze-dryer to reduce moisture content to nearly 10% before grinding step. These flour samples underwent a process of phenolic extraction. The collected extracts were used for chemical analysis of their bioactive contents and antioxidant activity.

The extraction method was followed by Zhou et al. (2014) with some modification. The extraction was organized at room temperature (25 – 28 °C) in the dark, following by 3 steps as details below.

Step 1: Defatting of rice samples

Before the extraction, rice flour was mixed with pure hexane (1:20 g/v) in an Erlenmeyer flask and stirred at high speed by a magnetic stirrer for 20 min. The residue collected after centrifuged at 8500 x g for 10 min at 25 °C was extracted for the second time with hexane. The defatted samples were dried overnight.

Step 2: Extraction of free phenolics

In this step, water and organic solvent including methanol and acetone were utilized for free and soluble phenolic extraction. Soluble conjugated phenolics were possibly extracted in this step. However, free fraction was general term

to imply the extract containing both free and soluble conjugated phenolics in this study.

First extraction – A 1 g of defatted sample was weighed and mixed with 25 mL of 80% methanol in an Erlenmeyer flask covered with a plastic film. The mixture were stirred at high speed for 2 h then centrifuged at 8500 x g for 15 min at 4 °C and the supernatant was collected.

Second extraction – The residue from first extraction was mixed with 25 mL of acetone/water/acetic acid (70/29.5/0.5, v/v/v) and the procedure above was repeated.

Third extraction – The residue from the second extraction was mixed with 25 mL of 80% methanol (chilled and acidified to pH 2.0 by HCl). The procedure was repeated.

Finally, the three supernatants were combined and concentrated to dryness by using a rotary evaporator at 55 °C, and reconstituted with methanol to reach 25 mL, which was used in the analysis tests of free phenolics.

Step 3: Extraction of bound phenolics

The remained residue from free phenolic extraction or step 2 was washed twice with 20 mL acetone, air-dried and followed by a process of bound phenolic extraction. Briefly, the dried residue was mixed with aqueous NaOH (4M; 30 mL) and stirred at a medium speed to prevent foaming at room temperature (25 – 28 °C) for 4 h. The sample was then acidified to pH 1.5 – 2.5 by ice-cold 6M HCl and centrifuged at 8500 x g at 4 °C.

The collected aqueous supernatants were mixed well with 70 mL ethyl acetate in a separatory funnel (Figure 24). It required 1 – 2 min to separate two layers of two immiscible liquids after shaking. As shown in the Figure 23, the bottom layer was the aqueous supernatant and ethyl acetate fraction was in the top as it was a less dense solvent in comparing with aqueous supernatant. At this step, phenolic compounds from aqueous phases transferred to ethyl acetate phase. After the first ethyl acetate fraction was collected, another 70 mL pure ethyl acetate was used to extract the phenolic from aqueous phase. This extraction was repeated 5 times and all collected ethyl acetate fractions were combined. Anhydrous sodium sulfate was added in the ethyl acetate fraction as a drying agent. Then, this supernatant was filter by

Whatman® qualitative filter paper, Grade 2. Ethyl acetate was completely evaporated by using rotary evaporator at 40 °C and the crude extract was reconstituted with methanol to reach 25 mL, which was used in the analysis tests of bound phenolics.

2) Determination of phenolic content (PC)

PC from free and bound fractions were quantified by following method of Kumar, Rajkapoor, & Perumal (2012) with a small modification. In this study, free-PC was the term used for the summary of free and soluble conjugated PC.

Briefly, 100 µL of the extract was mixed thoroughly with 0.5 mL of Folin–Ciocalteu reagent for 3 min and made up to 4 mL with distilled water, followed by the addition of 1 mL of 15% (w/v) sodium carbonate. The mixture was allowed to stand for further 2 h in the dark, and the absorbance was measured at 750 nm.

PC was calculated from the gallic acid standard curve. The results were expressed as mg of gallic acid equivalent (GAE)/g dry weight (DW). All analyses were performed in triplicates.

3) Determination of total anthocyanin content (TAC)

TAC was determined by the pH differential method as described by Shao et al. (2014). Only extracts from free fractions were used in this experiments because anthocyanins are well-known susceptible at alkaline pH so that they were completely degraded after 4 h extraction of bound phenolics (Castañeda-Ovando et al., 2009).

Briefly, two solutions were prepared, one buffer at pH 1.0 (1.49% KCl water buffer, acidified with HCl) and the other buffer at pH 4.5 (1.64% sodium acetate water buffer, acidified with HCl). Each extract was diluted 10 times with 2 buffers and incubated for 15 min in the dark. The absorbance of each sample was measured at both 520 nm and 700 nm.

TAC was expressed as cyanidin-3-glucoside (C3G)/g DW, and calculated as follows:

$$Monomeric\ anthocyanin\ pigment\ \left(\frac{mg}{L}\right) = A \times MW \times DF \times 1000/(\varepsilon \times L)$$

Where

A is the absorbance calculated as:

$A = (A_{520nm} - A_{700nm})\ pH_{1.0} - (A_{520nm} - A_{700nm})\ pH_{4.5}$.

MW is the molecular weight for cyanidin- 3-glucoside (449.2 g/mol)

DF is the dilute factor.

ε = 26,900 is the molar absorbance of cyanidin-3-glucoside

L is cell path length (1 cm).

1000 conversion factor from milliliter to liter.

4) Determination of total proanthocyanidin content (TPA)

TPA was measured by modifying the method of H. Huang et al. (2014) with a small modification. Only extracts from free fractions were used in this experiments because proanthocyanidins were susceptible at strong alkaline pH so that they were completely degraded after 4 h extraction of bound phenolics (Xu et al., 2015).

In brief, 0.5 mL extract was mixed with 1.25 mL of 1% vanillin reagent prepared in methanol. Then, 1.5 mL of 9 N H_2SO_4 solution in methanol was added to the mixture and shaken well. All the samples were incubated in 15 min to allow the maximal reaction. The absorbance for measurement was 500 nm. A blank was prepared by replacing with methanol instead of extract. The measurement

The calibration curve was plotted from different concentration of the standard (+)-catechin. The difference in absorbance between the sample and blank was used to determine total proanthocyanidin concentration, which was expressed as mg (+)-catechin equivalents (CE)/g DW. All analyses were performed in triplicates.

5) Measurement of antioxidant activities

In the experiments, extraction of both fractions of free and bound forms were utilized.

FRAP assay – The FRAP method was determined using the method described by Butsat & Siriamornpun (2010). A fresh working solution was prepared from 0.3 M acetate buffer, pH 3.6, 10 mM TPTZ solution in 40 mM

HCl, and 20 mM $FeCl_3.6H_2O$ solution which was warmed at 37 °C before use. 50 mL of each extracts was allowed to react with 3 mL of FRAP solution. The pure FRAP assay was used as blank. All the test tubes were incubated at 37 °C in 30 min. The absorbance for measurement was 595 nm. The FRAP values were expressed as mg Trolox equivalents (TE)/g DW. All analyses were performed in triplicates.

DPPH radical scavenging assay – The radical scavenging activity was assayed using the method of Gao & Xiao (2012) with slight modifications. Briefly, 2.8 mL of 0.1 mM DPPH in methanol was mixed with 200 µL extract of rice. The mixture was vigorously shaken and maintained in dark for 30 min. Then, the absorbance was measured at 517 nm against a blank (methanol). DPPH radical scavenging values were calculated from the calibration curve and expressed as mg Trolox equivalents (TE)/g DW. All analyses were performed in triplicates.

1.2.3 Treatment selection for *in vitro* digestion

Only cooked rice would be considered for *in vitro* digestion test. From every type of rice, the treatment which displayed the highest antioxidant activity would be chosen. The greatest content of phenolic or anthocyanin and proanthocyanidins would be another supporting consideration.

2. Part 2 – Effect of cooking methods, soaking time and rice cultivars on antioxidant properties and texture analysis of Khaomak

2.1 Preparation of Khaomak

In this study, the diagram of Khaomak production is represent in Figure 20. Khaomak were made by cooked rice as the results from Part 1. There were total 9 treatments of rice fermentation (Table 4). Unpolished glutinous rice from 3 varieties (nonpigmented, red and black colour) was cooked by two methods as described in Part 1. The fermentation was held within 72 h then the Khaomak were kept at -20 °C for further processing.

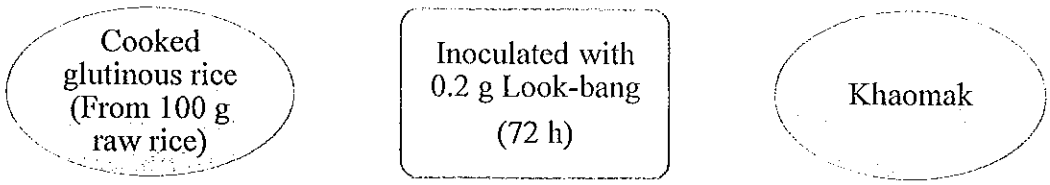


Figure 20 Procedure of Khaomak making

2.2 Analysis

2.2.1 Texture analysis of Khaomak

(Details are shown in Chapter 3, Part 1, Section 1.2.1)

2.2.2 Khaomak properties

Fresh Khaomak were measured for total solid by using refractometer and pH by using pH meter.

2.2.3 Antioxidants in Khaomak

1) Sample preparation

Khaomak made from 3 rice cultivars were lyophilized by freeze-dryer then pulverized into powder. They were kept at -20 ^{o}C during the experiment time. The phenolic extraction of Khaomak was implemented as the description in Part 1, Section 1.2.2. The free and bound phenolic extracts were analyzed for the below tests.

2) Determination of total phenolic content

(Details in Chapter 3, Part 1, Section 1.2.2, Subsection 2)

3) Determination of total anthocyanin content

(Details in Chapter 3, Part 1, Section 1.2.2, Subsection 3)

4) Determination of total proanthocyanidin content

(Details in Chapter 3, Part 1, Section 1.2.2, Subsection 4)

5) Measurement of antioxidant activities

FRAP assay (Details in Chapter 3, Part 1, Section 1.2.2, Subsection 5)

DPPH radical scavenging assay (Details in Chapter 3, Part 1, Section 1.2.2, Subsection 5)

2.2.4 Treatment selection for *in vitro* digestion

From every type of rice, the treatment of Khaomak which displayed the highest antioxidant activity would be chosen. The greatest content of phenolic or anthocyanin and proanthocyanidins would be another supporting consideration.

3. Part 3 – *In vitro* digestion to determine antioxidant activity in cooked rice and Khaomak

There were totally 6 treatments in this study, 3 treatments of cooked rice made from 3 rice cultivars, selected from Part 1, and 3 treatments of Khaomak made from 3 rice cultivars, selected from Part 2. An application of *in vitro* gastrointestinal digestion model was utilized to evaluate the alteration of phenolic compounds and antioxidant activities in the physiological view.

3.1 *In vitro* digestion procedure

The simulated gastrointestinal model was followed the method described by Galano et al. (2016) with some modifications. *In vitro* digestion methods typically include the oral, gastric and small intestinal stages, and large intestinal fermentation. However, there were three phases were implemented in this study, including oral, gastric and small intestinal digestion.

The simulated digestion fluids were made up of the corresponding electrolyte stock solutions, enzymes, $CaCl_2$ and water. Three phases were utilized different fluids: Simulated Oral Fluid (SOF), Simulated Gastric Fluid (SGF) and Simulated Intestinal Fluid (SIF). Table 5 shows the preparation of each simulated digestion fluids.

Table 5 Preparation of stock solutions of simulated digestion fluids

Stock solution	Conc. in SOF (mmol/L)	Conc. in SGF (mmol/L)	Conc. in SIF (mmol/L)
KCL	15.1	6.9	6.8
KH_2PO_4	3.7	0.9	0.8
$NaHCO_3$	13.6	25	85
NaCl	-	47.2	38.4
$MgCl_2(H_2O)_6$	0.15	0.1	0.33

In vitro digestion step in this study was processed as following:

Oral digestion – Five grams of Khaomak (or cooked rice) was mixed with distilled water up to 10 ml. A 7 mL of SOF was added then followed by 1 mL α-amylase (from 1500 U/mL α-amylase solution made up in SOF electrolyte stock solution), 50 µL 0.3M $CaCl_2$ and 1.95 mL water. The mixture was incubated in a shaking water bath at 37 °C, 110 rpm for 20 min. At the end of this stage, 2 mL of digested aliquot was removed for analysis. Another 2 mL of SOF solution (diluted 2 times with water) was added.

Gastric digestion – Twenty milliliter of oral bolus was mixed with 15 mL SGF stock solution. A 3.2 mL porcine pepsin stock solution of 25 000 U/mL made up in SGF, 10 µL 0.3 M $CaCl_2$ were added. 1 M HCl was used to reduce pH to 3. Distilled water was added to make up the final solution to 40 mL. Then, the mixture was shaken evenly at 37 °C, 110 rpm for 1 h by a water bath. A 2 mL of solution was withdrawn at the end of the incubation time for analysis. Another 2 mL of SGF (diluted 2.67 times with water) was refilled.

Intestinal digestion – Forty milliliter of gastric chyme was mixed with 22 mL of SIF electron stock solution, 10 mL of pancreatin solution 1600 U/mL made up in SIF electrolyte stock solution based on pancreatin α-amylase activity (25 USP amylase units per 1X pancreatin x 8), 5 mL bile salt (160 mM in fresh bile), 80 µL 0.3 M $CaCl_2$. 0.1 M NaOH was used to adjust pH to 7. Distilled water was filled up to reach 80 mL. The mixture was incubated at 37 °C for 2 h in a shaking water bath (110 rpm). After incubation time, 2 mL of solution was taken out for measurements.

All the digesta withdrawn from each digestion phase were placed in boiling water for 5 min to inactivate the enzyme activity. After that, they were centrifuged at 8000 x g at 4 °C in 15 min to discard the residues. The collected supernatants were filtered by a Nylon Syringe Filter (25 mm x 0.45 um) then diluted with methanol to 10 mL. Those samples were kept at -20 °C during time of analysis.

3.2 Analysis

GI digesta were evaluated for the below.

3.2.1 Determination of total phenolic content

(Details in Chapter 3, Part 1, Section 1.2.2, Subsection 2)

3.2.2 Determination of total anthocyanin content

(Details in Chapter 3, Part 1, Section 1.2.2, Subsection 3)

3.2.3 Determination of total proanthocyanidin content

(Details in Chapter 3, Part 1, Section 1.2.2, Subsection 4)

3.2.4 Measurement of antioxidant activities

FRAP assay (Details in Chapter 3, Part 1, Section 1.2.2, Subsection 5)

DPPH radical scavenging assay – The details in Chapter 3, Part 1, Section 1.2.2, Subsection 5 with a small modification. DPPH radical scavenging assay was prepared with methanol in water (1:1 v/v).

Statistical analysis

All experiments were conducted in triplicates and the results were expressed as mean ± standard deviation (SD). The statistical examination of the data were performed, using the SPSS program. A different of mean values was analyzed, using an analysis of the variance (ANOVA) test. These means were compared, using the Ducan New Multiple Range Test (DMRT) and $p \leq 0.05$ were applied, in order to establish significant differences.

CHAPTER IV

RESULTS AND DISCUSSION

Parts 1 and 2 – Effect of cooking methods, soaking time and rice cultivars on antioxidant properties and texture analysis of cooked rice and Khaomak

1. Khaomak properties

Table 6 shows pH and total solid values of Khaomak after 72 h fermentation.

Table 6 Physical properties of Khaomak.

Khaomak	pH	Total solid (%)
Nonpigmented rice		
Khaomak-unsoaked	3.81	40.0
Khaomak-6h-soaked	3.98	46.0
Khaomak-12h-soaked	4.32	47.2
Red rice		
Khaomak-unsoaked	3.78	39.2
Khaomak-6h-soaked	4.48	48.0
Khaomak-12h-soaked	4.15	45.0
Black rice		
Khaomak-unsoaked	3.92	38.0
Khaomak-6h-soaked	4.08	43.0
Khaomak-12h-soaked	4.26	42.0

At the early stage of fermentation, fungal enzymes were produced by molds in Look-bang (*Aspergillus sp., Mucor sp. and Rhizopus sp.*) including α-amylase and amyloglucosidase, which hydrolyzed starches to dextrin, maltotriose, maltose and glucose, and alkaline proteases, which hydrolyze proteins to peptides and amino acids (Aidoo, Rob Nout, & Sarkar, 2006; Chuenchomrat, Assavanig, & Lertsiri, 2008). After that, acid-forming bacteria produced organic acids from sugars, which reduced pH of products to below 4.5 and activated yeast fermentation (Rhee, Lee, & Lee, 2011). At this stage, S. cerevisiae fermented sugars to ethanol under the anaerobic condition (Battcock, & Azam-Ali, 1998b). The remained sugars after using by bacteria and yeasts after 3-day-fermentation were considered as the total soluble

content of Khaomak. They were determined with refractive index measurements as °Brix, what expresses the mass percentage of total soluble solid in the Khaomak aqueous solution. From Table 6, it is seen that Khaomak which had lower pH, the total solid was also lower when comparing in the same rice variety. The results are plausible because it indicates the microbial activity in using sugar to produce lactic acids and ethanol. According to TISI criteria, total solid should be less than 50% but greater than 10%. Therefore, Khaomak made from 3 types of unpolished glutinous rice met the general requirement of the total solid content for industrial production.

There should be more studies to concentrate on Khaomak criteria for products made from unpolished glutinous rice if their industrial production are considered. In this current study, the attention was to focus on the aspects of phenolic compounds and their antioxidant activity.

2. Effect of cooking methods, soaking time and rice cultivars on texture analysis of cooked rice and Khaomak

The results of texture profile analysis of cooked rice and Khaomak from 3 varieties of unpolished glutinous rice are shown in the Table 7. Overall, there were significant changes ($p \leq 0.5$) in hardness, adhesiveness and springiness of cooked rice and Khaomak.

According to TTC (2017), hardness is measured as the peak force that occurs during the first compression. Among 3 methods of cooking, cooked rice without soaking showed the highest values ($p \leq 0.05$) in all 3 cultivars. Soaking was described as a supporting step for the hydration of rice grains which enhance the gelatinization (Oko, Ubi, & Dambaba, 2012). Thus, soaking rice before cooking contributed the softness in cooked rice. However, different soaking time did not significantly influence the hardness of cooked rice ($p > 0.05$). After fermentation, it was seen a decrease ($p \leq 0.05$) of hardness in Khaomak without soaking and 6-h-soaking and no significant change ($p > 0.05$) in Khaomak without 12-h-soaking (nonpigmented and red rice). On the other hand, black rice only showed a significant decline ($p \leq 0.05$) of hardness value in non-soaking Khaomak. The other treatments of did not showed any change ($p > 0.05$) in the texture. The level of hardness and softness of the grains seemed to associate with the cellulose structure in the bran (Siesler et al., 2008). *Saccharomyces cerevisiae*, the main yeast strain in Look-bang, has ability to utilize

different carbon sources for energy metabolism and synthesis of cell materials (Hauf, Zimmermann, & Müller, 2000). The cellulase activity might have been the reason of cell wall degradation and thus, Khaomak texture became softer (Deshpande, 1992). The reason that the hardness values of cooked and Khaomak were not significantly different may involve in the low activity of cellulase enzymes. Additionally, method of cooking may also effect on the hardness. Steaming is commonly used to prevent the rice grains from breaking into pieces. Bran breaking may ease the hydrolysis of cellulase enzymes.

Table 7 Textural properties of cooked rice and Khaomak made from unpolished glutinous rice

Treatment		Texture parameters		
		Hardness (g)	Adhesiveness (g.s)	Springiness (mm)
Nonpig-mented rice	Cooked-unsoaked	417.83±47.13^{a}	-111.34±20.80^{b}	4.53±0.83^{d}
	Cooked-6h-soaked	353.43±34.86^{b}	-194.95±36.23^{c}	5.39±0.80^{bc}
	Cooked-12h-soaked	349.00±22.22^{b}	-147.20±18.28^{b}	5.38±0.84^{bc}
	Khaomak-unsoaked	277.33±30.36^{c}	-4.58±0.64^{a}	5.61±0.23^{b}
	Khaomak-6h-soaked	188.80±13.71^{d}	-6.10±0.90^{a}	6.67±0.27^{a}
	Khaomak-12h-soaked	385.75±27.27^{b}	-6.95±0.34^{a}	4.77±0.39^{cd}
Red rice	Cooked-unsoaked	528.83±64.18^{a}	-350.44±56.52^{d}	5.36±0.68^{b}
	Cooked-6h-soaked	453.70±60.75^{b}	-177.99±32.71^{b}	5.61±0.94^{b}
	Cooked-12h-soaked	410.25±51.70^{bc}	-244.2540.06^{c}	5.24±0.61^{b}
	Khaomak-unsoaked	252.00±28.67^{d}	-5.29±3.71^{a}	5.54±0.10^{b}
	Khaomak-6h-soaked	241.83±26.94^{d}	-11.04±1.38^{a}	6.47±0.22^{a}
	Khaomak-12h-soaked	410.40±77.34^{c}	-16.11±3.24^{a}	5.11±0.20^{b}
Black rice	Cooked-unsoaked	516.13±54.74^{a}	-176.94±32.79^{b}	4.35±0.83^{bc}
	Cooked-6h-soaked	413.00±43.79^{bc}	-207.42±34.97^{b}	5.83±0.85^{a}
	Cooked-12h-soaked	391.18±41.10^{bc}	-302.98±18.85^{c}	5.51±0.50^{a}
	Khaomak-unsoaked	363.00±39.16^{c}	-6.90±2.81^{a}	4.51±0.35^{bc}
	Khaomak-6h-soaked	401.20±63.27^{bc}	-9.14±1.89^{a}	3.87±0.26^{c}
	Khaomak-12h-soaked	447.00±8.50^{b}	-10.24±1.28^{a}	4.58±0.24^{b}

Number of replications $n \geq 3$.

Values with the same letters in same variety and column are not significantly different within columns ($p > 0.05$).

Adhesiveness refers to the negative work between the two cycles compression (TTC, 2017). Regarding to the high force used in adhesiveness parameter of cooked rice , high amylopectin content (60% to 90%) in glutinous rice (Nakamura, 1996) was the explanation. Cooked rice from red cultivar was generally stickier than the other two (from -177.99 to -350.44 g.s) as shown in Table 7. Despite of difference in amylopectin content presented in each cultivar, the more dissolved amylopectin in cooking water, the more stickiness of cooked rice were (Nussinovitch, 2016). When comparing cooked rice and Khaomak, cooked rice of nonpigmented, red and black rice had 15 – 60 times more adhesive than their fermented ones. No significant differences ($p>0.05$) of adhesiveness were found in 3 types of Khaomak made from the same cultivar. The initial phrase of fermentation involved in the saccharification of the substrates (Tamang, & Thapa, 2006). Starch was broken into sugar by enzymes via the glycolytic pathway which resulted in the degradation of high amylopectin starch. The extremely low force of adhesiveness on Khaomak of 3 varieties indicated that the structure of gelatinized starch were destroyed completely or they were too less to contribute the stickiness of Khaomak.

Springiness is called "Elasticity" in the original TPA measurement and it indicates how an absolute distance in millimeters of the sample performs in analysis (TTC, 2017). The breaking of gel structure into large pieces results in high springiness during the first TPA compression while low springiness results from a gel breaking into many small pieces (Gayin et al., 2017). On the other hand, springiness is defined as the degree to which cooked rice returns to its original shape once it has been compressed between the teeth (Marshall, & Wadsworth, 1994). The higher degree indicates the more rubbery texture of cooked rice. In the current study, no significant difference ($p>0.05$) of springiness values was found among the treatments of red cooked rice. Meanwhile, cooked rice without soaking of nonpigmented and black rice showed lower springiness values than other treatments of soaking. Thus, it suggested that steaming method can keep the rice grains (nonpigmented and black rice) more rubbery than direct cooking by rice cooker. The results of springiness of Khaomak was fluctuated and did not point the similar trend for 3 rice cultivars. The attributes for this criteria need to be determined for further understanding.

3. Effect of cooking methods, soaking time and rice cultivars on the antioxidant properties of cooked rice and Khaomak

In this section, comprehensive information on the alteration of extractable (free) and cell-wall bound (bound) fractions of phenolic compounds and antioxidant capacities in the unpolished glutinous rice during Khaomak production were determined. Free fraction was the extract collected from organic solvent extraction (Step 2, Section 1.2.2.1, Part 1, Chapter 3) while bound fraction was the result of phenolic extraction by using sodium hydroxide (Step 3, Section 1.2.2.1, Part 1, Chapter 3). Therefore, every measurement from the combination of two fractions reflexed the total value of each treatment.

3.1 Phenolic content (PC)

In this experiment, PC was measured without distinguishing between chemical structures of phenolic compounds. According to the method of analysis, all available phenolics in the extract reacted with Folin-Ciocalteu reagent to form a blue complex that can be quantified by visible-light spectrophotometry. The PC in free and bound fractions of raw, cooked and fermented rice from unpolished glutinous rice are shown in Table 8.

In nonpigmented rice cultivar, free-PC of raw rice was 1.179 ± 0.061 mg GAE/g DW. After cooking, free-PC significantly loss ($p \leq 0.05$) in all 3 types of cooking method. The results were in agreement with the study of Ti et al. (2015), which also revealed a great reduction of free-PC in brown rice after cooking. It is well-documented that many phenolic compounds are unstable with hydrothermal treatment such as cooking and the degradation can accelerate more under the presence of oxygen (Halliwell, 1996). When comparing among cooked rice made from 3 ways of cooking, no significant difference ($p > 0.05$) of free-PC was found. Thus, it indicates the methods of cooking in this study are not the factor influencing the free-PC in nonpigmented cooked rice.

In opposition to cooking, fermentation was seen to release more free phenolics in Khaomak made from nonpigmented unpolished glutinous rice ($p \leq 0.05$) and the highest quantity was recorded in the cooking method without soaking (2.122 ± 0.042 mg GAE/g DW). Khaomak underwent different soaking times, 6 h and 12 h, did

not show any dissimilarity ($p>0.05$) in free-PC (1.605 ± 0.033 and 1.619 ± 0.026 mg GAE/g DW, respectively).

Table 8 Phenolic content of raw, cooked rice and Khaomak

Rice sample	Phenolic content (mg GAE/g DW)		
	Free fraction	Bound fraction	Total
Nonpigmented rice			
Raw	1.179 ± 0.061^{cC}	1.438 ± 0.045^{aA}	2.616 ± 0.077^{bC}
Cooked-unsoaked	0.965 ± 0.013^{dC}	1.164 ± 0.07^{cA}	2.129 ± 0.064^{eC}
Cooked-6h-soaked	0.994 ± 0.014^{dC}	1.357 ± 0.015^{bA}	2.351 ± 0.007^{cdC}
Cooked-12h-soaked	0.942 ± 0.048^{dC}	1.134 ± 0.043^{cA}	2.075 ± 0.075^{eC}
Khaomak-unsoaked	2.122 ± 0.042^{aC}	0.862 ± 0.028^{dB}	2.984 ± 0.069^{aC}
Khaomak-6h-soaked	1.605 ± 0.033^{bC}	0.837 ± 0.022^{dB}	2.442 ± 0.055^{cC}
Khaomak-12h-soaked	1.619 ± 0.026^{bC}	0.712 ± 0.015^{dC}	2.330 ± 0.040^{dC}
Red rice			
Raw	4.808 ± 0.243^{aB}	0.876 ± 0.045^{cB}	5.684 ± 0.198^{aB}
Cooked-unsoaked	3.672 ± 0.099^{bB}	1.173 ± 0.009^{bA}	4.845 ± 0.091^{cdB}
Cooked-6h-soaked	2.692 ± 0.147^{cB}	1.075 ± 0.016^{bB}	3.767 ± 0.163^{cdB}
Cooked-12h-soaked	2.552 ± 0.032^{cB}	1.155 ± 0.058^{abA}	3.707 ± 0.028^{dB}
Khaomak-unsoaked	3.768 ± 0.009^{bB}	0.874 ± 0.099^{cB}	4.643 ± 0.108^{bB}
Khaomak-6h-soaked	2.130 ± 0.113^{eB}	0.688 ± 0.066^{dB}	2.818 ± 0.097^{eB}
Khaomak-12h-soaked	2.729 ± 0.078^{cB}	1.225 ± 0.015^{aA}	3.954 ± 0.079^{cB}
Black rice			
Raw	6.853 ± 0.145^{aA}	0.826 ± 0.123^{cdB}	7.679 ± 0.125^{aA}
Cooked-unsoaked	5.815 ± 0.163^{bA}	0.661 ± 0.058^{dB}	6.476 ± 0.125^{bA}
Cooked-6h-soaked	3.784 ± 0.252^{dA}	0.764 ± 0.028^{cdC}	4.549 ± 0.254^{eA}
Cooked-12h-soaked	4.170 ± 0.277^{cdA}	0.928 ± 0.028^{bcB}	5.098 ± 0.278^{dA}
Khaomak-unsoaked	6.495 ± 0.348^{aA}	1.104 ± 0.038^{aA}	7.599 ± 0.382^{aA}
Khaomak-6h-soaked	4.564 ± 0.194^{cA}	1.170 ± 0.174^{aA}	5.734 ± 0.277^{cA}
Khaomak-12h-soaked	4.026 ± 0.150^{dA}	1.054 ± 0.089^{abB}	5.080 ± 0.203^{dA}

Different lowercase superscript letters indicate statistical difference of the results in the same variety and column analyzed; different uppercase superscript letters indicate statistical difference of the results in the same column and treatment for 3 varieties ($p \leq 0.05$).

To explain the increase of free-PC in Khaomak, an insight of microbial activity should be taken into consideration. During fermentation period, bacteria, molds and yeasts from the starter cultures secreted various enzymes such as carbohydrases, proteases and other types of enzymes, employed in lactic acid fermentation, ethanol fermentation and protein hydrolysis (Shahidi, & Yeo, 2016). These processes lead to the disruption of cell wall matrix or hydrolysis of covalent bonds of bound phenolics; hence, it resulted in the releasing of free phenolics. The outcomes were accordant with another research about Khaomak, in which fermented rice made from pigmented unpolished rice contained higher amount of free-PC than the unfermented rice (Plaitho, Kangsadalampai, & Sukprasansap, 2013). The study from Sadabpod, Kangsadalampai, & Tongyonk (2010) also revealed the same trend. On the contrary, Singanusong, Ngamdee, & Jiamyangyuen (2016) established different results. In their study, the PC of Khaomak made from the same variety of nonpigmented unpolished glutinous rice used in this research, subsequently decreased by fermenting. The various methods of phenolic extraction could be a possible reason to explain the dissimilarity.

Total-PC was the summary of free-PC and bound-PC. In term of bound phenolics cooked rice made from nonpigmented cultivar, bound-PC accounted for a major amount (more than 50%) in the total-PC in every treatment (Table 8). The bound-PC of raw rice, cooked rice without soaking, cooked rice with 6 h and 12 h soaking were 1.438 ± 0.045, 1.164 ± 0.07, 1.357 ± 0.015 and 1.134 ± 0.043 mg GAE/g DW, respectively. It was said that hydrothermal treatment could free insoluble and cell-wall bound phenolic (Min, McClung, & Chen, 2014). Therefore, cooked rice of nonpigmented cultivar underwent a decrease of bound-PC ($P \leq 0.05$). After fermentation, bound-PC reduced nearly a half ($p \leq 0.05$) after fermentation in the comparison with cooked rice. It is plausible to expect a decline of bound-PC in Khaomak due to the theory that microbial activities of fermentation help to converse bound phenolics into free forms. It means that when free-PC increase, bound-PC decrease.

In red rice cultivar, free-PC of raw rice was 4.808 ± 0.243 GAE/g DW and significantly decreased ($p \leq 0.05$) to 3.672 ± 0.099, 2.692 ± 0.147 and 2.552 ± 0.032 GAE/g DW in cooked rice without soaking, cooked rice with 6 h and 12 h

soaking, respectively. As mentioned in the case of nonpigmented rice, high temperature was the factor influencing the reduction of free phenolics. However, cooked rice underwent soaking step showed significantly lower ($p \leq 0.05$) free-PC than that of the method without soaking. Different soaking time did not result in different free-PC in two rice samples cooked by steaming method. Phenolics are hydrophilic so soaking provoked more free phenolics to dilute in water. By visual observation, the soaking water of red rice turned reddish as the colour of red rice when the soaking time was completed. The results indicated that a major of phenolics in red rice are possibly available as free and soluble conjugated forms. The data from Table 8 also confirmed this point. In the comparison with bound-PC, free-PC from 7 treatments of red rice made up a vast quantity in total-PC which ranged from 68.8 to 84.6%. Thus, the former way of cooking displayed the advantage of preventing the loss of phenolics into water.

Contrast to Khaomak made from nonpigmented rice, Khaomak made from red rice did not show the significant increase of free-PC in the comparison with cooked rice. Even there was seen a decline of free-PC in the Khaomak subjected to 6 h soaking step. The results are fairly similar to the experiment of Singanusong, Ngamdee, & Jiamyangyuen (2016). A study of Q. He, Lv, & Yao (2007) proved that tea polyphenols are able to inhibit the activity of enzymes including α-amylase, pepsin, trypsin and lipase by binding and precipitating protein, the main component of enzyme structure. Moreover, tea polyphenols are built from catechin units, which were similar to proanthocyanidins largely in red rice (discuss later in Section 3.2.2, Part 1 & 2, Chapter 4). Thus, proanthocyanidins may be related to the shortage of free-PC results in Khaomak made from red rice. It required more evidences to conclude whether proanthocyanidins are capable to weaken the efficiency of enzyme activity, which also lead to the limitation in releasing bound phenolics. Additionally, free form of proanthocyanidins could be potential to bind with proteins hydrolyzed from Khaomak production, which may have interfered the detection of phenolic compounds by Folin-Ciocalteu.

In the case of bound-PC of Khaomak made from red rice, they were fluctuated among treatments. After cooking, the quantity of bound-PC considerably increased. Meanwhile, fermentation process reduced bound-PC ($p \leq 0.05$) in the

Khaomak samples which was soaked in water for 6 h or unsoaked before cooking (Table 8). However, it was seen an increase of bound-PC after fermentation ($p \leq 0.05$) in the treatment with 12 h soaking. The explanation could be due to the complex structure of different phenolic compounds in this cultivar. Hydrothermal treatment may help to unwind some chemical bonds of bound phenolics that if only using method of bound extraction in this study was not able to complete for measurement.

In black rice cultivar, raw black rice showed the highest amount of free-PC (6.853 ± 0.145 GAE/g DW) but not significantly different ($p > 0.05$) from Khaomak without soaking. After cooking, free-PC significantly decreased ($p \leq 0.05$) but greatly gained after fermentation for Khaomak of non-soaking and 6 h soaking treatments ($p \leq 0.05$). High temperature of cooking and enzymatic activities were the explanation in this case, according to previous discussion of nonpigmented and red rice. There was seen an increase of free-PC after fermentation when making Khaomak from a type of black rice from another study (Plaitho, Kangsadalampai, & Sukprasansap, 2013). As same as red nonpigmented, free phenolics were the dominant phenolics present in black rice because free-PC accounted for 79.6 to 89.8% in the total-PC. When comparing 3 ways of cooking, cooking rice without soaking retained the highest quantity of free-PC in both cooked rice and Khaomak. Khaomak made from steaming method with 6 h soaking gained more benefit than that of 12 h soaking in term of free phenolics. In term of bound phenolics, there was no significant difference of bound-PC between raw rice and cooked rice ($p > 0.005$). However, the bound-PC gradually increased after fermentation ($p \leq 0.05$) and no significant difference was found among different Khaomak samples ($p > 0.05$). In this case, the insoluble-bound phenolics in black rice may tremendously bond with cell wall substance via ester linkages and the alkaline hydrolysis in this research was not strong enough to break down the links. Then fermentation may have contributed to loosen the chemical bonds which eased the release of bound phenolics during the bound-phenolic extraction (Shahidi, & Yeo, 2016).

Among 3 cultivars, black rice showed the highest quantity of total-PC in raw rice, cooked rice and fermented rice. Total-PC of raw black and red rice was approximately 3 and 2 times greater than raw nonpigmented rice, respectively. From previous discussion, phenolic compounds in pigmented rice were mostly present as

free form which was accordance with a study of free and bound fractions in five black and four red rice types (Sumczynski et al., 2016). Meanwhile, both free-PC and bound-PC took a major amount in total-PC in nonpigmented rice, which was the same as a study of Ti et al. (2015).

3.2 Total anthocyanin and total proanthocyanidin content (TAC and TPA)

In this study, only free extraction were used to measure TAC and TPA (Table 9) because bound fraction was extracted by 4 M NaOH, which destroyed anthocyanin and proanthocyanidin structures. It is well-documented that anthocyanins and proanthocyanidins are unstable and completely degraded at high pH (Castañeda-Ovando et al., 2009; Xu et al., 2015).

3.2.1 Total anthocyanin content (TAC)

According to the current results, anthocyanins were only available in black rice which was responsible for the dark violet colour of this cultivar (Table 9). TAC underwent a significant loss after cooking, especially the treatment using soaking before steaming. In this case, TAC reduced by approximately 78% and different soaking time did not show effect on TAC. In another case of cooking without pre-soaking, TAC was far higher than steaming method which reduced only 34% after cooking (1.212 mg C3G/g DW). It has been well-studied that anthocyanins are susceptible to degrade from heat severity (Cavalcanti, Santos, & Meireles, 2011). Another primary factor that caused the substantial decline of TAC was soaking step. Anthocyanins are water-soluble so a huge amount of anthocyanin were leached-out into process water during soaking (X. Zhang et al., 2013). Indeed, the soaking water of black rice turned to dark pink colour during soaking period, indicating leaching of anthocyanin in water.

On the contrary, a significant amount of free anthocyanins were released in the Khaomak cooked by rice cooker without soaking (1.869 mg C3G/g DW). After fermentation, TAC slightly increased in the Khaomak treated with soaking before steaming (0.492 and 0.481 mg C3G/g DW for 6 h and 12 h-soaking treatment, respectively. Another study of TAC also confirmed that fermentation could help to improve the quantity of anthocyanins in Khaomak made from the same cultivar of black glutinous rice (Singanusong, Ngamdee, & Jiamyangyuen, 2016).

Table 9 Total anthocyanin content and proanthocyanidin content of free fractions in cooked rice and Khaomak.

	Total anthocyanin content (mg C3G Eq/g DW)	Total proanthocyanidin content (mg CE Eq/g DW)
Nonpigmented rice		
Raw	ND	ND
Cooked-unsoaked	ND	ND
Cooked-6h-soaked	ND	ND
Cooked-12h-soaked	ND	ND
Fermented-unsoaked	ND	ND
Fermented-6h-soaked	ND	ND
Fermented-12h-soaked	ND	ND
Red rice		
Raw	ND	1.371 ± 0.012^{a}
Cooked-unsoaked	ND	0.736 ± 0.008^{c}
Cooked-6h-soaked	ND	0.435 ± 0.005^{d}
Cooked-12h-soaked	ND	0.398 ± 0.009^{e}
Fermented-unsoaked	ND	0.953 ± 0.028^{b}
Fermented-6h-soaked	ND	0.447 ± 0.044^{d}
Fermented-12h-soaked	ND	0.473 ± 0.008^{d}
Black rice		
Raw	1.837 ± 0.065^{a}	0.481 ± 0.055^{a}
Cooked-unsoaked	1.212 ± 0.040^{b}	0.097 ± 0.015^{bc}
Cooked-6h-soaked	0.400 ± 0.048^{d}	0.081 ± 0.016^{c}
Cooked-12h-soaked	0.405 ± 0.048^{d}	0.093 ± 0.010^{bc}
Fermented-unsoaked	1.869 ± 0.050^{a}	0.129 ± 0.005^{b}
Fermented-6h-soaked	0.492 ± 0.014^{e}	0.115 ± 0.003^{bc}
Fermented-12h-soaked	0.418 ± 0.025^{cd}	0.103 ± 0.003^{bc}

Different superscript letters indicate statistical difference of the results in the same variety and column analyzed ($p \leq 0.05$). ND: not detected.

In addition, many bubbles appeared in the process water during soaking time which implied the production of microbial activity (Battcock, & Azam-Ali, 1998a). It was comprehended that soaking in food preparation can activate some hydrolytic enzymes, which initiates exo- and endo-cleaving pathways in starch (Ray et al., 2016). Thus, it may help to free some phenolics that trapped in food matrix. From

these results, it was supported that soaking not only caused the loss of free-TAC but also degraded the bound-TAC in the raw rice.

3.2.2 Total proanthocyanidin content (TPA)

In case of proanthocyanidin, it was found in both pigmented rice (Table 9). However, the quantity of proanthocyanidins in black rice was minor in the comparison with red rice. Unprocessed red rice constituted 1.371 mg CE/g DW of TPA which was 2.8 times higher than that of black rice.

As same as anthocyanins, proanthocyanidins were unstable with hydrothermal process (Xu et al., 2015) so proanthocyanidins were significantly degraded ($p \leq 0.05$) after cooking. Among 3 methods of cooking, direct cooking without soaking showed the greatest advantage in preventing the loss of TPA in both types of glutinous rice, especially in red rice. The highest TPA was observed in the treatment without soaking which accounted for 0.736 and 0.097 CE/g DW in red and black rice, respectively. Monomer form to oligomer forms of proanthocyanidins were seen to be water-soluble (S. X. Liu, & White, 2012). Thus, the low values of TPA in pre-soaking treatments may be due to the loss of free proanthocyanidins into water.

Proanthocyanidins may also be available as bound forms in rice, which was the reason of TPA increasing after fermentation. It was clearly seen in Khaomak processed from red rice without soaking step (0.953 mg CE/g DW), which was nearly 50% higher than that of Khaomak processed with soaking step (for both 6 and 12 h soaking). It also witnessed a significant increase of TPA ($p \leq 0.05$) of Khaomak made from red rice with 12 h soaking but not the one with 6 h soaking when compared to cooked rice. The enhancement of TPA in Khaomak made from black rice was not significant ($p > 0.05$).

Look-bang used in Khaomak fermentation contains several yeasts and fungi such as *Aspergillus oryzae* (Manosroi et al., 2011). It is known to be able to produce α-galactosidase, amylase, invertase, lignin peroxidase and tannase (Shahidi, & Yeo, 2016) which can digest the ester linkages of proanthocyanidins and other subtracts in food matrix, leading more TPA after fermentation.

3.3 Antioxidant activity (AOA)

The change of antioxidant activity of 3 rice cultivars during Khaomak roduction are illustrated in Figure 21.

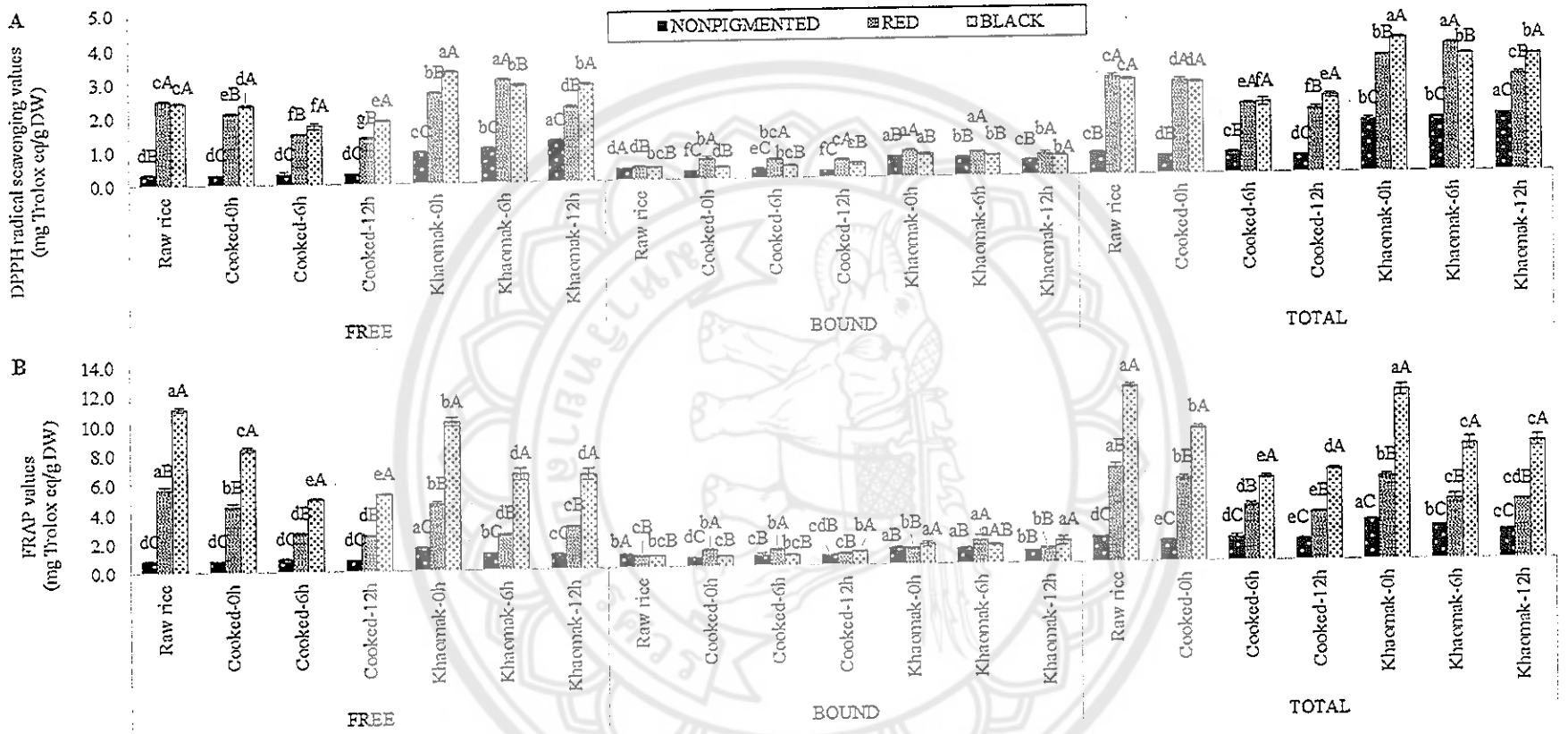


Figure 21 DPPH radical scavenging (A) and FRAP activity (B) of cooked glutinous rice and Khaomak

Different lowercase superscript letters indicate statistical difference of the results in the same rice cultivar and group (free, bound or total); different uppercase superscript letters indicate statistical difference of the results in the same treatment ($P \leq 0.05$).

3.3.1 DPPH radical scavenging activity

Free-DPPH radical (Figure 24) scavenging was in the range of 0.294 – 1.242 mg TE/g DW in the nonpigmented rice. No effect of hydrothermal treatment was seen in AOA values of nonpigmented rice but a significant amount ($p \leq 0.05$) of free-DPPH radical scavenging values increased after fermentation. Longer soaking time showed higher values of DPPH radical scavenging in fermented nonpigmented rice (0.925, 1.032 and 1.242 mg Trolox/g DW for non-soaking, 6 h soaking and 12 h soaking treatment, respectively). In theory, the loss of phenolic content leads to the reduction of AOA. Therefore, the results were not parallel with the decreasing of free-PC in accordance with soaking time. Although the extracts for analysis were processed from phenolic extraction, the extracts may be not pure phenolic compounds because the knowledge is not fulfilled. Long water soaking time can promote the antioxidant activity and bioactive compounds in brown rice (Lin et al., 2015) so a combination of soaking time and fermentation can be a considered to be a reason of antioxidant activity enhancement.

In red rice and black rice, free-DPPH radical scavenging values were in the range of 1.726 – 3.048 and 1.358 – 3.284 mg TE/g DW, respectively (Figure 24). Similar to nonpigmented rice, free-DPPH radical scavenging values significantly reduced ($p \leq 0.05$) after cooking and recovered after fermentation. They provided a huge value in the total-DPPH radical scavenging which was at least 72.3% and 82.7% in red and black rice, respectively. In the comparison of the identical process treatment, red and black rice also displayed a significant greater ($p \leq 0.05$) values than nonpigmented rice for this measurement of antioxidant activity. Great quantity of TAC and TPA in black and red rice may play an important role in showing off the high values of DPPH radical scavenging assay. In the study of Sadabpod, Kangsadalampai, & Tongyonk (2010), cooked rice made from Hom Nil and black rice also showed a slight decrease of AOA after cooking but fermentation even enhanced more AOA. Another experiments of Plaitho, Kangsadalampai, & Sukprasansap (2013) was also compactable with our results when DPPH radical scavenging values of Khaomak were significantly higher ($p \leq 0.05$) than unfermented rice. However, unlike nonpigmented rice, longer soaking time did not show a clear trend of higher AOA in pigmented rice.

In case of bound phenolics, bound-DPPH radical scavenging values made up from 26.8 – 52.5%, 13.6 – 27.7% and 12.9 – 17.6% in nonpigmented, red and black rice, respectively. They underwent an increase of values after fermentation in spite of bound-PC's abatement. According to Sánchez (2009), fungi are not only responsible for polysaccharide degradation but also degrade lignin and open phenyl rings. Therefore, phenolic compounds may escape from food matrix and lose benzene rings at the same time. The alteration of chemical structures of phenolic compounds or more unknown substances released after enzymatic hydrolysis may affect on the AOA.

3.3.2 FRAP activity

Generally, the alteration of FRAP values during Khaomak production reflexed almost the same trend of PC but not DPPH scavenging values. The highest values of FRAP activity were observed in pigmented rice (Figure 21). Free-FRAP values were in the range of 0.776 – 1.586 mg TE/g DW in nonpigmented rice, 2.465 – 5.664 mg TE/g DW in red rice and 2.459 – 11.072 mg TE/g DW in black rice. The extract of free fraction constituted more than 60% in total value of AOA from red rice and 80% from the black rice. Meanwhile, bound fraction from nonpigmented rice contributed a significant value of total-FRAP values.

As the same for DPPH radical scavenging, free-FRAP values in pigmented rice significantly decrease ($p \leq 0.05$) after cooking and recovered after fermentation while the values from nonpigmented rice witnessed a minimal change after heat treatment. The difference was the AOA values of Khaomak made from red rice was a half lower than that of black rice in FRAP assay but the gap in DPPH radical scavenging assay was much lower. The results illustrated that free fraction from Khaomak made from black rice responded in FRAP assay better than Khaomak made from red rice. According to the results of TAC, anthocyanins constituted of a dominant source of free-PC in black rice and may represent for the main antioxidant in black rice. D. Huang, Ou, & Prior (2005) indicated that both DPPH radical scavenging and FRAP assays measured the reducing capacity of antioxidants which involved in electron atom transfer. However, pH of FRAP working assay was around 3.6, more suitable for rich anthocyanin extract because anthocyanins were well-documented to be very unstable in neutral pH as performed in DPPH radical scavenging assay. It may

be the leading reason to explain why black rice extract did not show the tremendous AOA values in DPPH radical scavenging assay.

Besides, there was another noticeable difference in performance between two antioxidant activity assays. The highest FRAP values of free fraction was observed at the treatment of non-soaking during Khaomak production from 3 rice cultivars. The results seem to be in accordance with free-PC.

In case of bound-FRAP values, they fluctuated during Khaomak production but mostly increased after fermentation. The changing trend of FRAP values was generally similar to DPPH radical scavenging values.

3.4 Correlation cofficient

The Pearson's correlation coefficients between phenolic compounds and their antioxidant activity are shown in Table 10.

Table 10 Pearson's correlation coefficients between phenolic compounds and their antioxidant activity

Samples	DPPH	FRAP
Nonpigmented rice		
Free-phenolic	0.824*	0.966**
Bound-phenolic	-0.701*	-0.521
Red rice		
Free-phenolic	0.194	0.982**
Bound-phenolic	-0.314	-0.500
Proanthocyanidins	0.394	0.963**
Black rice		
Free-phenolic	0.445	0.997**
Bound-phenolic	0.927**	0.908**
Anthocyanins-Black	0.450	0.967**
Proanthocyanidins-Black	0.059	0.719*

* Correlation is significant at the 0.05 level (1-tailed).

** Correlation is significant at the 0.01 level (1-tailed).

According to Rumsey (2016), the correlation coefficient r measures the strength and direction of a linear relationship between two variables on a scatterplot. The value of r is always between +1 and –1.. In a positive relationship, the

values can be interpreted based on these range: 0.00 – 0.19 "very weak"; 0.20 – 0.39 "weak"; 0.40 – 0.59 "moderate"; 0.60 – 0.79 "strong"; 0.80 – 1.0 "very strong" (Statstutor, 2017). In free fraction of phenolic compounds, it demonstrated a strong positive linear relationship between free-PC and FRAP activity. Free-phenolics well responded in both DPPH radical scavenging and FRAP assay because the correlation coefficient value was very high (0.824 and 0.966, respectively). However, the free fraction of phenolic compounds of red and black rice only revealed a strong positive trend with FRAP activity but low – moderate results with DPPH radical scavenging activity. The high correlation of anthocyanin and proanthocyanidin content in FRAP assay may indicate they were the dominant bioactive compounds which play an important role of the total antioxidant capacity in red and black rice.

Difference in the ability of adaptation of different rice phenolics in two antioxidant assays can be explained by their activity mechanisms. According to Galano et al. (2016), antioxidant performance of phenolics are known as hydrogen and electron donor. The acidic pH of FRAP assay was an advantage for those phenolics sensitive with neutral pH as anthocyanin. That may explain why free-TAC of black rice were observed in very strong relationship with FRAP assays but moderate in DPPH radical scavenging assay. Additionally, the main antioxidant capacity came from free fraction of TAC in black rice. Therefore, it could be assumed that anthocyanins were the dominant antioxidants in black rice and available mostly in free form, which were in an agreement with the results reported by Goufo & Trindade (2014). They claimed that anthocyanins were primarily stored in the vacuole and not bound to the cell walls.

Proanthocyanidins in black rice also performed better in FRAP assay than DPPH radical scavenging assay because strong positive correlation was found (R = 0.719). It disclosed their profound contribution in total antioxidant activity of black rice extract. In comparison proanthocyanidins in red rice, the value of correlation coefficient of TPA and FRAP activity was even higher than that of black rice. A very strong positive correlation was found (R = 0.963) in this study, which referred to the primary role of proanthocyanidins in total antioxidant activity in red rice.

In the case of bound phenolics, only black rice showed a strong positive correlation between the content of bound-PC and AOA values from both DPPH radical scavenging and FRAP assays. Thus, it implied a good contribution of bound phenolics in the total antioxidant capacity of bound fraction. However, it seemed that bound phenolics were not the main substances in AOA values in nonpigmented and red rice. It was clearly observed by the negative correlation between phenolic compounds and their antioxidant activity. Especially, a strong negative relation was found in the case of nonpigmented rice (R = -0.701 in DPPH radical scavenging).

More assays from other working mechanism such as hydrogen atom transfer should be studied to confirm the relationship. The results inferred that the change of antioxidant activity of rice cultivars during Khaomak production were in accordance with their amount of PC, TAC and TPA. Several studies have reported a close relationship between PC and high antioxidant activity and demonstrated that anthocyanins and proanthocyanidins were the most important antioxidants in pigmented rice such as red and black rice (Goufo, & Trindade, 2014; Min et al., 2012).

4. Remark conclusions for parts 1 and 2

From the results above, it is explicit that cooking was the main reason of the loss of PC. At the cooking step, the highest amount of free-PC were recorded in the treatment of non-soaking in 3 cultivars. The results implied that direct cooking with rice cooker can preserve more free phenolic compounds.

Phenolic compounds and antioxidant activity in cooked rice and Khaomak processed from black and red rice were tremendously higher than that of nonpigmented rice, due to the great content of anthocyanin and proanthocyanidin, respectively. Most of the phenolics present in pigmented rice as free forms while bound form contributed a major part in nonpigmented rice.

In general, the reduction and improvement of two phenolic compounds anthocyanin and proanthocyanidin in free fraction were in similar trend during Khaomak production. From the results above, there was seen a profound effect of the processing steps but rice cultivars also yielded various results depending on quantity and types of phenolic compounds.

In term of phenolics, direct cooking without water soaking showed the most efficient method to reserve those bioactive compounds in both rice cooking and Khaomak production. However, steaming method is commonly practiced in Thai culture. In other to process Khaomak with nonpigmented rice, soaking in water for 6 h or 12 h did not give any significant difference in PC. With regard to rice variety which has high content of free phenolics like black rice, long soaking time should be limited to prevent the loss of anthocyanin. In red rice, extractable proanthocyanidins have potential to be loss into water during soaking period with a great amount of proanthocyanidins was present as bound phenolics. Thus, 12 h soaking may be long enough for promoting natural enzymatic activity which may support other steps including cooking and fermentation to break down the strong ester bonds, which resulted in a higher free-PC than that of 6 h soaking.

When considering the total antioxidant activity, Khaomak made from nonpigmented rice should be soaked for 12 h before processing other steps. For the pigmented rice, producing Khaomak without soaking the rice in water exhibited the best results of antioxidant capacity.

Part 3 – *In vitro* digestion to determine antioxidant activity in cooked rice and Khaomak

In this section, three cultivars of rice including nonpigmented, red and black unpolished glutinous rice were directly cooked with water without soaking and fermented. A comparison of cooked rice and Khaomak in releasing of phenolic compounds and antioxidant activity during *in vitro* digestion in each type of rice was implemented. Effect of rice cultivars on the change of phenolics and antioxidant activity were also conducted in this part.

The *in vitro* gastrointestinal digestion in this study consisted of three sequential steps, an initial α-amylase digestion for 20 min to simulate human saliva conditions, a digestion with pepsin/HCl for 1 h to simulate gastric conditions and a treatment with pancreatin-bile salts for 2 h to simulate small intestine conditions.

1. Effect of *in vitro* digestion and rice cultivars on the phenolic content of cooked rice and Khaomak

Figure 22 illustrates the effect of *in vitro* digestion and rice cultivars on phenolic content.

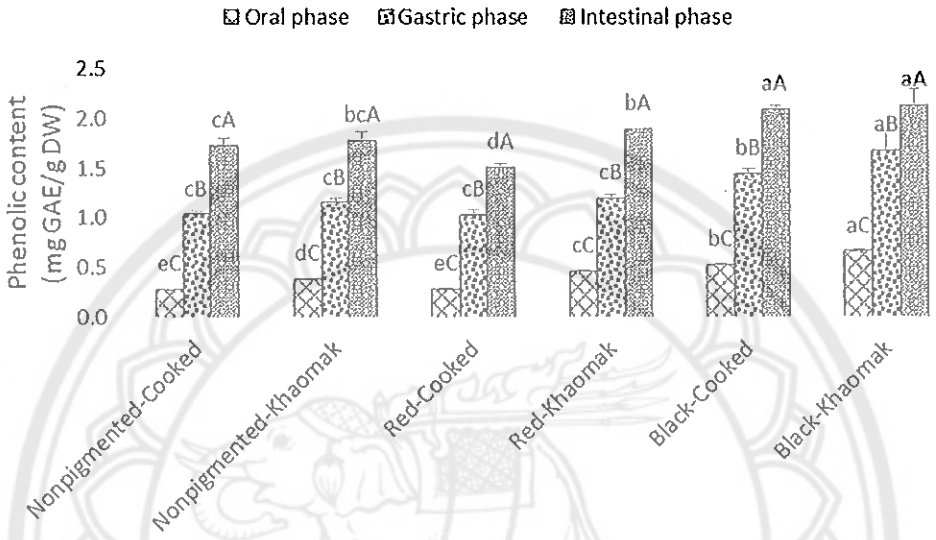


Figure 22 Phenolic content of cooked rice and Khaomak during *in vitro* digestion

Different lowercase superscript letters indicate statistical difference of the results in a same phase among 3 cultivars and stage of food process; different uppercase superscript letters indicate statistical difference among 3 phases within 1 type of treatment ($P \leq 0.05$).

After oral digestion, the PC of Khaomak made from nonpigmented rice were 0.386 ± 0.009 mg GAE/g DW (Figure 22), which was significantly higher ($p \leq 0.05$) than that of cooked rice (0.277 ± 0.005 mg GAE/g DW). The amount of phenolics obtained from this digestion phase were free and conjugated soluble phenolics available in cooked rice and Khaomak. Furthermore, α-amylase enzyme also helped to hydrolyze α-linked polysaccharides, which eliminated more bound phenolics (Shahidi, & Yeo, 2016). The importance of α-amylase activity at oral phase was

highly confirmed in this study. Another *in vitro* model was conducted with only 2 steps gastric and small intestinal digestion. Without α-amylase, the gastric fluid of the cooked rice became gel due to the swelling starch when it was mixed with water. However, this problem was overcome by applying the oral digestion step before the gastric phase.

After pepsin/HCl digestion at gastric phase, the PC of cooked and fermented rice made from nonpigmented rice were 2.9 and 3 times greater ($p \leq 0.05$) than that of the oral phase (Figure 22). The increase of PC continued at intestinal phase to reach the top values ($p \leq 0.05$), around 1.7 mg GAE/g DW, in both cooked rice and Khaomak. In simulated gastric digestion, pepsin digested proteins to produce peptides which may be possible break down the linkage between some phenolic compounds and rice protein. Meanwhile, the enzyme mixtures of pancreatin (trypsin, amylase and lipase, ribonuclease, and protease) at intestinal phase played an important role to release a major of bound phenolics. Ferulic acid, a common bound phenolic in rice (Goufo, & Trindade, 2014), often esterifies with sterols to become phytosteryl ferulates or γ-Oryzanol (Fardet, Rock, & Rémésy, 2008; Mandak, & Nyström, 2012). It suggested that that small intestinal enzymes can hydrolyze steryl ferulates to release ferulic acids into free form (Mandak, & Nyström, 2012). The PC of rice increased after the intestinal digestion as a result. Additionally, the PC of cooked rice and Khaomak made from nonpigmented rice were not at significantly different ($p > 0.05$) when comparing in the same phase (gastric and intestinal digestion).

For red rice, it also witnessed a releasing of bound phenolics from oral to intestinal phase of digestion in both cooked and fermented rice (Figure 22). The PC of Khaomak made from red rice at oral phase was 0.464 ± 0.010 mg GAE/g DW which was 1.6 folds greater than that of cooked rice. After gastric digestion, the PC considerably become greater in quantity but there was no significant difference ($p > 0.05$) between cooked and fermented rice. However, Khaomak made from red rice yielded a higher amount ($p \leq 0.05$) than that of cooked rice at the simulated intestinal digestion. The explanation in the change of PC in red rice could be referred from nonpigmented rice.

As for other treatments, the PC of cooked and fermented black rice gained after each phase of *in vitro* digestion. The highest PC was observed at the intestinal

stage (2.091 ± 0.040 and 2.131 ± 0.161 mg GAE/g DW in cooked and fermented rice, respectively). No significant difference ($p>0.05$) of PC was found between these two treatments. The PC of Khaomak made from black rice obtained after gastric and oral phases were significantly greater ($p\leq0.05$) than that of cooked rice.

When comparing among 3 cultivars, the PC of black rice (both cooked rice and Khaomak) at each stage were the highest ($p\leq0.05$). In part 1, red rice overcame nonpigmented rice in term of phenolic compounds but from this *in vitro* digestion model, the amount of PC from nonpigmented and red rice at gastric stage were not significantly different ($p>0.05$). After intestinal digestion, PC of Khaomak made from nonpigmented rice was found to be significantly greater ($p\leq0.05$) than red cooked rice but no significant difference ($p>0.05$) in Khaomak.

A study of Ti et al. (2015) showed that digested cooked rice from a unpolished nonpigmented cultivar contained more phenolic compounds than that from solvent phenolic extraction. Thus, it was expected to get a higher PC from *in vitro* digestion than that from the solvent extraction method in this work. However, the PC of cooked rice and Khaomak made from nonpigmented, red and black rice after *in vitro* digestion were observed in a lower amount than that of parts 1 and 2. Several studies reported about the inhibition effects of phenolic compound on enzymatic starch hydrolysis by α-amylase and amyloglucosidase, an impact which contributes to the capacity of polyphenols to decrease enzyme activity by binding enzymes/proteins (Kandil et al., 2012). It could be a potential reason to interfere the results of the phenolic amounts after using *in vitro* digestion.

2. Effect of *in vitro* digestion on total anthocyanin and proanthocyanidin content of cooked rice and Khaomak

2.1 Anthocyanin content

Table 11 illustrates the TAC of cooked rice and Khaomak during the simulated *in vitro* digestion.

Anthocyanins were found in black cooked and fermented rice only. After oral digestion, TAC were 0.651 ± 0.038 and 0.639 ± 0.051 mg C3G/g DW for cooked rice and Khaomak, respectively. According to the results of part 1, it can be assumed that the detected anthocyanin amount was mostly free anthocyanin which was easily dissolved in aqueous simulated oral fluid (SOF) at oral phase. In addition,

the enzyme α-amylase may take part in releasing more bound anthocyanin by digesting carbohydrate matrix of rice.

Table 11 Total anthocyanin content of cooked rice and Khaomak during *in vitro* digestion

Treatments	TAC (mg C3G/g DW)		
	Oral phase	Gastric phase	Intestinal phase
Nonpigmented-Cooked rice	ND	ND	ND
Nonpigmented-Khaomak	ND	ND	ND
Red-Cooked rice	ND	ND	ND
Red-Khaomak	ND	ND	ND
Black-Cooked rice	0.651 ± 0.038^{bA}	0.856 ± 0.009^{aA}	0.403 ± 0.038^{cA}
Black-Khaomak	0.639 ± 0.051^{bA}	0.781 ± 0.074^{aA}	0.170 ± 0.000^{cB}

Different lower superscript letters indicate statistical difference of the results in a same row analyzed; different uppercase superscript letters indicate statistical difference in a same column ($p \leq 0.05$). ND: not detected.

The TAC significantly increase ($p \leq 0.05$) after gastric stage. It may be due to the bioactivity of pepsin enzyme which plays the main role in digesting protein. The increase of TAC indicated that bound anthocyanin may be present in a complex by binding with protein molecules.

However, a decline of TAC in cooked rice by half and in Khaomak by 4.5 times at intestinal phase was found. The results was in an agreement with a study of D. Sun et al. (2015), which also showed the high stability of anthocyanin in a black rice during simulated gastric digestion but degraded 76% at intestinal digestion. There are many factors affecting in the degradation of anthocyanin including chemical structures, pH, food matrix, digestive enzymes (Galvano et al., 2004) but the chemical structures and pH are the major factors influencing their stability (Kırca, Özkan, & Cemeroğlu, 2007). The pH 7 at intestinal phase was not flavorable for anthocyanin structures while anthocyanins are stable at the acidic environment at gastric stage (pH 3) (Castañeda-Ovando et al., 2009).

Oral and gastric phases, there were no significant difference ($p>0.05$) of TAC between cooked rice and Khaomak. However, TAC of cooked rice was more than 2 times higher than that of Khaomak at simulated intestinal phase. This could be due to the fact that most of natural anthocyanins are available as glycosides. But the fermentation process has ability of deglycosylation that converses anthocyanins into aglycone forms (Nguyen Thai et al., 2014), and decreased the stability of anthocyanins in alkaline pH that was present in the intestinal phase (Wiczkowski, Szawara-Nowak, & Topolska, 2015).

During *in vitro* digestion part, the aqueous buffers were the solvent for soluble anthocyanin extraction and pH condition were varied from 3 different digestion phases. In addition, enzymatic activities was initially hypothesized to support for releasing bound and conjugated anthocyanins into free forms. From the assumption of parts 1 and 2, anthocyanins in black rice were mostly in free and soluble forms. Thus, they were potentially degraded in oral and intestinal digestion phases, especially Khaomak because they contained more free and soluble anthocyanins than cooked rice. Many studies also reported that anthocyanins from fruits like blueberry, chokeberry, black raspberry, red grape and strawberry severely degraded in the condition of *in vitro* simulated digestion (10 – 80%), especially oral and intestinal phases, where pH were around 7 (Kamonpatana et al., 2012). This finding supported the results found in the current study.

2.2 Proanthocyanidins content

Table 12 illustrates the TPA of cooked rice and Khaomak during the simulated *in vitro* digestion.

During *in vitro* digestion, proanthocyanidin were detected in red and black rice only (Table 12). Only in Khaomak made from red rice, the change of TPA was clearly seen. TPA show the highest ($p\leq0.05$) at gastric stage (0.447 ± 0.016 mg CE/g DW). However, environment of intestinal phase cause the significant reduction of TPA ($p\leq0.05$). In contrast with anthocyanins, proanthocyanidins in red rice are mostly available in bound forms which associated with protein molecules or linked to cell walls (Goufo, & Trindade, 2014). A sharp increase of TPA after simulated gastric digestion strongly related to pepsin, the only available enzyme in this phases, which took the action of protein hydrolysis and released proanthocyanidin molecules. Most

of proanthocyanidins were found in rice as oligomers and the dominant components were dimers (Goufo, & Trindade, 2014). According to Q. Y. Zhu et al. (2002) and Xu et al. (2015), gastric juice caused the change of high polymerized proanthocyanidin to more simple proanthocyanidins such as dimers and trimers. However, dimers of proanthocyanidins were discovered to be unstable with physiological pH conditions, especially pH 7 which was found in the oral and intestinal phases in this study (Xu et al., 2015).

Table 12 Total proanthocyanidin content of cooked rice and Khaomak during *in vitro* digestion

Treatments	TPA (mg CE/g DW)		
	Oral phase	Gastric phase	Intestinal phase
Nonpigmented-Cooked rice	ND	ND	ND
Nonpigmented-Khaomak	ND	ND	ND
Red-Cooked rice	0.037 ± 0.009^{aB}	0.147 ± 0.096^{aA}	0.128 ± 0.045^{aA}
Red-Khaomak	0.119 ± 0.000^{cA}	0.447 ± 0.016^{aA}	0.255 ± 0.024^{bA}
Black-Cooked rice	0.058 ± 0.024^{aB}	0.212 ± 0.131^{aA}	ND
Black-Khaomak	0.037 ± 0.019^{aB}	0.253 ± 0.038^{aA}	ND

Different lower superscript letters indicate statistical difference of the results in a same row analyzed; different uppercase superscript letters indicate statistical difference in a same column ($P \leq 0.05$). ND: not detected.

There was no any significant difference ($p > 0.05$) in red cook rice during *in vitro* digestion. In order to discover the change of proanthocyanidin in this treatment, other methods to detect proanthocyanidin molecules such as high-performance liquid chromatography should be considered. The current method using vaniline assay calculate TPA was not suitable for the extract taken from *in vitro* digesta because it was hydrophilic aqueous media while vaniline assay are said to be best perform in methanol (B. Sun, Ricardo-da-Silva, & Spranger, 1998). That may be the reason of unstable results of PAC in other treatments.

The same explanation was for black cooked rice and fermented rice. No change of TPA was found ($p>0.05$) between oral and gastric phases. At intestinal phase, the data of TPA was not applicable due to very low quantity of the digesta.

Additionally, most of proanthocyanidins in red rice are non-extracted proanthocyanidins which are not easily extracted even by the action of digestive enzymes (Saura-Calixto et al., 2010). Thus, it assumed that a large amount of bound proanthocyanidins were still maintained after gastrointestinal digestion before reaching fermentation step at the colon.

3. Effect of *in vitro* digestion on the antioxidant activity profile of cooked rice and Khaomak

Samples for analysis from each phase of simulated *in vitro* digestion were fresh digesta witdrawn from digested cooked and fermented rice. The difference between digesta and solvent extract (Part 1) was that the digesta contained other bioactive compounds than phenolics while solvent extract was aimed to collect only phenolics whose method milimized the mix of other bioactive compounds such as oryzanol (Goufo, & Trindade, 2014). Therefore, the results of antioxidant activity reflexed all the bioactive compounds present in digesta.

The change of antioxidant activity of cooked rice and Khaomak during *in vitro* digestion is described in Figure 23.

3.1 DPPH radical scavenging

DPPH radical scavenging values of nonpigmented rice cultivar were 0.393 ± 0.043 and 1.042 ± 0.104 mg TE/g DW in cooked and fermented rice, respectively, at simulated oral stage (Figure 23(A)). Then, the values increased 4 and 2 folds ($p\leq0.05$) at gastric stage but dramatically loss ($p\leq0.05$) after simulated intestinal digestion, even lower than that at the oral stage. The GI digesta of cooked rice and Khaomak contained not only phenolic compounds but some other substrates which also own the charactestics as antioxidants. After pepsin treatment at simulated gastric digestion, several hydrophobic peptide were exposed that became more accesible by DPPH radicals (L. Zhu et al., 2008). Thereby, it stabilized DPPH radicals by transfering electron from peptides. Another antioxidants which may be available at this phase were steryl ferulates, an esterification of ferulic acid to a sterol, which

posses the ability of hydrogen donation from ferulic group but they tend more less polar than ferulic acids in free form (Nyström et al., 2007).

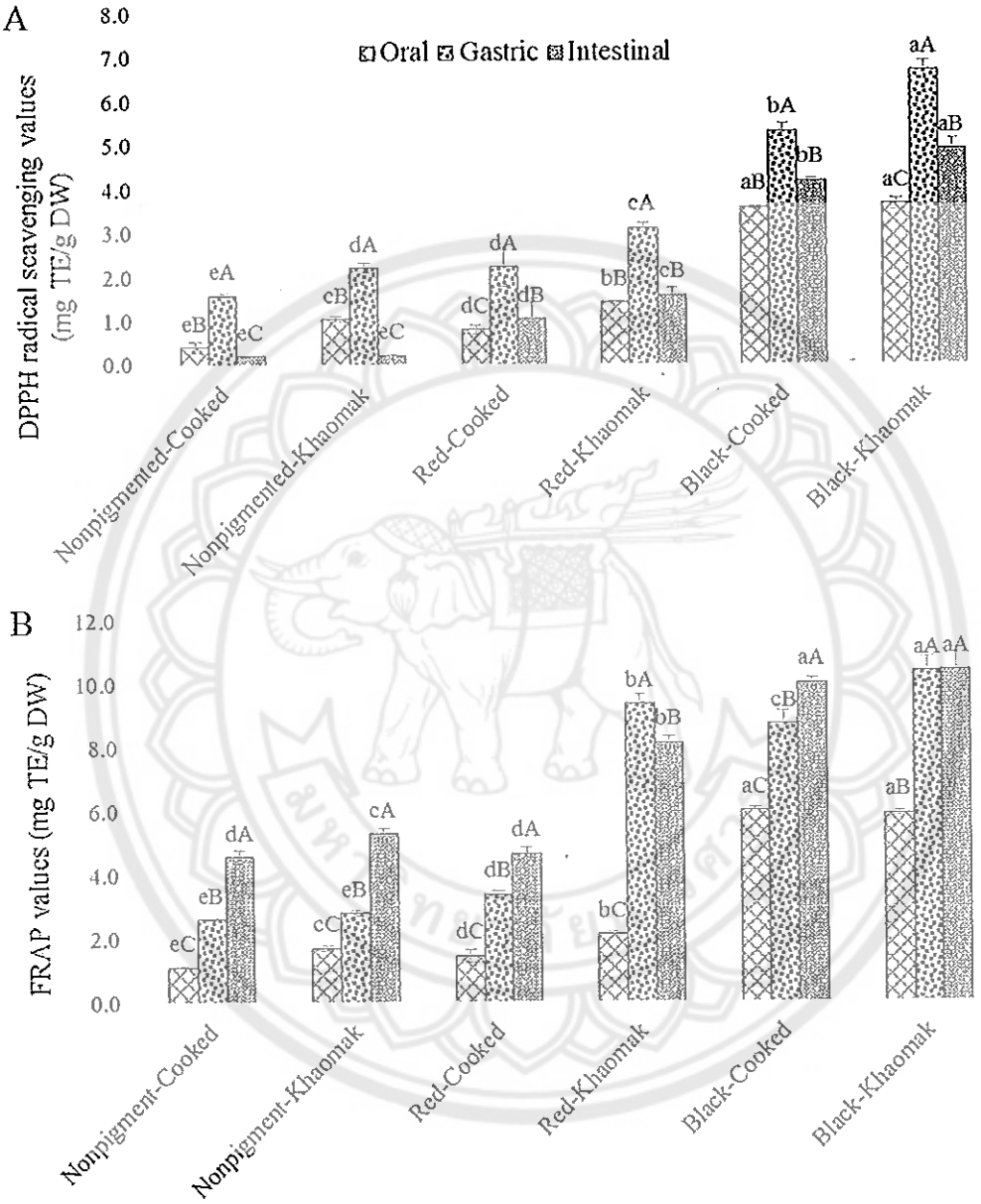


Figure 23 (A) DPPH radical scavenging and (B) FRAP activity of cooked rice and Khaomak during *in vitro* digestion

Different lower superscript letters indicate statistical difference of the results in a same digestion phase; different uppercase superscript letters indicate statistical difference in different phases of digestion in a same treatment ($p \leq 0.05$).

By contrast, pancreastin enzymes increased polarity of GI digesta by accumulating more hydrophilic peptides (You et al., 2010) and release bound phenolics such as polar ferulic acids (Nyström et al., 2007). Thus, it gained the difficulty to react with the lipid-soluble DPPH radicals. These informations may help to explain the higher results of DPPH radical scavenging antivity at the gastric phase than that of the intestinal phase. Additionally, no significant difference ($p>0.05$) of DPPH radical scavenging activity was found between cooked rice and Khaomak made from nonpigmented rice at intestinal stage but the gastric phase ($p\leq0.05$).

The same trend was recorded in red cultivar. The significantly higest amount ($p\leq0.05$) of DPPH radical scavenging value was observed in gastric digested samples (2.226 ± 0.110 and 3.155 ± 0.166 mg Trolox/g DW in cooked rice and Khaomak, respectively). Then, it declined by half at intestinal stage for both treatments. There was no statistical difference ($p>0.05$) bewteen DPPH radical scavenging activity between oral and intestinal stages of Khaomak made from red rice but red cooked rice. It was assumed the similar explanation with nonpigmented rice for the changing of DPPH radical scavenging activity in red rice. However, proanthocyanidins which were found in Khaomak made from red rice were degraded in the intestinal condition leading to the decrease of total antioxidant capcity of the GI digesta.

DPPH radical scavenging activity of black cooked rice were 3.575 ± 0.103, 5.318 ± 0.448, 4.181 ± 0.474 mg TE/g DW at oral, gastric and intestinal phases, respectively. It indicated that DPPH radical scavenging activity was highest at the gastric phase. Oral and intestinal digested cooked rice did not show any significant difference ($p>0.05$) in the antioxidant activity. Khaomak also showed the highest DPPH radical scavenging activity at gastric stage (6.688 mg TE/g DW), following by intestinal and oral digestion. It was supported that DPPH radical scavenging activity of cooked rice and Khaomak made from black rice during *in vitro* digestion were generally in accordance with other cultivars, nonpigmented and red rice. On the other hand, pH condition of DPPH radical scanvenging assay was not suitable for anthocynins to fully perform their antioxidant capacity (Sui, Dong, & Zhou, 2014).

Besides the discussion above, it was interesting that not only anthocyanin and proanthocyanidin, but other natural polyphenolic in plants are also damaged when exposed to high pH (Friedman, & Jürgens, 2000).

3.2 FRAP

FRAP activity of nonpigmented rice was 1.096 ± 0.032 and 1.689 mg TE/g DW in cooked rice and Khaomak at oral phase, respectively (Figure 23(B)). In contrast with DPPH radical scavenging, a significant increase in FRAP activity was detected at simulated gastric digestion by half and the values continued to increase to 4.548 ± 0.220 and 5.270 ± 0.194 mg TE/g DW in cooked and fermented rice after intestinal digestion. The results were in accordance with the increase of PC after every phase of *in vitro* digestion. FRAP assay providing low pH (~ 3.6) an polar conditions (D. Huang, Ou, & Prior, 2005); thereby, those antioxidant compounds which did not well perform in DPPH radical scavenging assay could expose their antioxidant activity in FRAP assay by electron-transfer mechanism.

It was found a similar trend of FRAP activity in red cooked rice during simulated *in vitro* digestion. FRAP values of cooked rice started at 1.450 ± 0.083 mg TE/g DW at oral digestion, following by a significant rise ($p \leq 0.05$) at gastric and intestinal phases. Khaomak made from red rice showed a greater antioxidant activity than that of cooked rice (2.103 ± 0.087 mg TE/g DW). Then it witnessed a significant increase ($p \leq 0.05$) of FRAP activity (4.5 times) in gastric digested Khaomak. However, the value declined 12% ($p \leq 0.05$) at intestinal stage.

Digested cooked and fermented rice of black cultivar did not show significant difference ($p > 0.05$) after oral digestion (5.997 ± 0.204 and 5.890 ± 0.90 mg TE/g DW). Then, it was an increase in both gastric and intestinal phase. It was proved in the current study that TAC significantly reduced after simulated intestinal digestion. However, the degradation products of anthocyanins into several benzoic acid derivatives were reported to have higher antioxidant activity (C. Oliveira et al., 2010). Thus, they may contribute to the total antioxidant activity of black rice in FRAP assay.

4. Correlation cofficient

The correlation coefficients between phenolics in cooked and fermented rice from 3 cultivars during *in vitro* digestion with DPPH radical scavenging and FRAP activity are identified in Table 13.

Table 13 Pearson's correlation coefficients between phenolic compounds in digesta and their antioxidant activity

	DPPH	FRAP
Nonpigmented rice		
Phenolic	-0.212	0.973**
Red rice		
Phenolic	0.248	0.766*
Proanthocyanidins	0.864*	0.924*
Black rice		
Phenolic	0.517	0.963**
Anthocyanin	0.291	-0.350
Proanthocyanidins	0.792*	0.204

* Correlation is significant at the 0.05 level (1-tailed).

** Correlation is significant at the 0.01 level (1-tailed).

A strong positive correlation was observed between PC from the extract of 3 types of rice and FRAP activity. However, DPPH radical scavenging activity showed low and moderate correlation with PC from red and black rice, respectively. Especially, PC of nonpigmented rice has negative correlation with DPPH radical scavenging.

From the results of parts 1 and 2, anthocyanins were assumed to be the dominant antioxidants in black rice. However, the correlation coefficient of TAC and DPPH radical scavenging or FRAP activity was not in an agreement with former assumption (0.291 and -0.350, respectively). It seems that anthocyanins did not play a main role of antioxidant activity in the physiologic point of view. Indeed, a vast of anthocyanins were degraded after reaching intestinal digestion, where the most bioactive compounds could be absorbed into blood plasma to function as a health protector. Nevertheless, the metabolites, the products from anthocyanin degradation should be considered for their contribution of total antioxidant activity in the rice samples. On the contrast with anthocyanins, proanthocyanidin contents expressed a strong positive relationship with antioxidant activity, especially in red rice. It indicated that proanthocyanidins in rice are potential antioxidants in physiological condition.

5. Remark conclusions for part 3

5.1 Total phenolic content

In vitro digestion illustrated that a major amount of bound phenolics present in cooked rice and Khaomak and more soluble phenolics were released after every single stage of simulated digestion by enzymatic activity. It was also seen that in the same cultivar, cooked rice had the same ability to release bound phenolics compounds as for Khaomak by those enzymes in the digestive tracts (except for red rice, which Khaomak gave more positive advantage).

5.2 Total anthocyanin and proanthocyanidin content

Anthocyanins were the dominant phenolics of black rice but it was seen a severe degradation after completed *in vitro* gastro-intestinal digestion (results at small intestinal phase). They were not stable in oral and intestinal fluid where pH was too high comparing to acidic pH at gastric stage. On the other hands, many researchers found that anthocyanins are able to permeate through mucous stomach and transfer to blood plasma. The expected result of current study were to see a higher TAC of Khaomak than that of cooked rice at gastric digestion, which may be potential source for anthocyanin absorption. However, there were no significant difference ($p>0.05$) of TAC between the two treatments.

Proanthocyanidins were the highest of content after gastric digestion of Khaomak made from red rice but they degraded after intestinal phase. However, the acidity of gastro-intestinal environment play an important role in modifying high degree polymerization proanthocyanidins to monomers and oligomers which are absorbable in small intestines.

5.3 Antioxidant activity

When comparing cooked rice and Khaomak, it is apparent that Khaomak in every cultivar showed a higher antioxidant activity ($p\leq0.05$) than that of cooked rice, except for cooked rice and Khaomak made from black rice at intestinal digestion ($p>0.05$). The results indicated that Khaomak could provide higher accessible antioxidants than their cooked rice in a physiological point of view, especially from the intestinal phase. In addition, Khaomak made from black rice may give the best AOA values, followed by red rice and nonpigmented rice.

CHAPTER V

CONCLUSION

Conclusion

The results of the present study have clarified the contributions of free and bound forms of phenolics to total content and their antioxidant activities in raw, cooked rice and Khaomak made from 3 rice cultivars: nonpigmented, red and black unpolished glutinous rice. Different methods of cooking significantly affected the results of phenolic compounds and antioxidant activity of cooked rice and Khaomak. However, cooking generally reduced free-PC in 3 cultivars but fermentation improved their quantity which was observed in nonpigmented and black rice. Phenolic compounds in free fraction showed the strong positive relationship with the antioxidant activity. The study also revealed that the most powerful antioxidant was related to black rice, following by red and nonpigmented rice.

Anthocyanins and proanthocyanidins were the major phenolic compounds in black and red rice, respectively. In free form, they were not stable with heat treatment so significantly decreased after cooking. Fermentation also contributed in the higher results of anthocyanins and proanthocyanidins after fermentation. Among 3 methods of cooking, direct cooking with rice cooker without soaking gave the most positive effect to retain these compounds in both cooked rice and Khaomak.

Texture profile analysis of cooked rice were shown that cooked rice processed with soaking method were significantly lower than that of without soaking method. Fermentation considerably reduced the adhesiveness in Khaomak as it destroyed the gel structures of cooked rice by microbial activity.

Cooked rice and Khaomak processed from method 1, cooking without soaking were selected to undergo the *in vitro* digestion and a physiological view was discovered.

The mixtures of enzymes from 3 phases of digestion contributed in releasing bound phenolics to free form. Most of nutrients are absorbed in the intestines so PC at the small intestinal digestion were considered as the most important source for phenolic

absorption. At this point, Khaomak made from nonpigmented and red rice were more potential to provide a larger amount of phenolics than that of cooked rice. However, no significant different of PC at the intestinal digestion was found in cooked and Khaomak made from black rice.

Anthocyanins were the dominant phenolics in black which was assumed to highly support in the antioxidant capacity when consuming its cooked rice or Khaomak.

However, anthocyanins significantly decreased at the intestinal condition. Despite that fact, anthocyanins were the highest in the amount at the gastric digestion, which may still function as diet antioxidants because many studies have proved that anthocyanins are able to permeate through mucus layers of stomach. Additional, Khaomak and cooked rice made from black rice did not show significant difference in TAC at the gastric phase. Hence, if the purpose of consumption based on the quantity of anthocyanins, Khaomak and cooked rice made from black rice may not give much different benefits.

The study of *in vitro* digestion also revealed another physiological aspect of total antioxidant activity in cooked rice and Khaomak. If the values of AOA at the small intestinal is considered as the most important, Khaomak made from pigmented rice provided higher antioxidant activity for radical scavenging than that of cooked rice. Meanwhile, Khaomak made from nonpigmented and red rice resulted in a greater values of FRAP activity when comparing with cooked rice. However, this physiology study disovered that cooked rice or Khaomak made from black rice may give the most efficient antioxidant activity in the comparison with other cultivars.

Suggestion

1. Phenolic profiles of rice, especially anthocyanins and proanthocyanidins should be studied to explain their degradation at intestinal digestion and figure out their potential absorption in stomach and small intestine, respectively.

2. Another antioxidant assays which provide the similarity of human condition should be conducted for comparison.

3. Colon fermentation for the residues of rice samples after pass though intestinal digestion should be conducted to discover more the ability of antioxidant

role of bound phenolics, which are not digested by digestive enzymes. The focus should be one proanthocyanidins because they are mostly available as insoluble-bound phenolics in rice.

4. Another approaching methods need to be evaluated to analyze the structures anthocyanins and proanthocyanidins in order to fulfill the insight of their activity in the physiological point of view.

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APPENDIX

APPENDIX A PROXIMATE ANALYSIS

Determination of moisture content

The method is following AOAC (1999). The crucibles were weighed after drying in oven at 105 °C for 30 min and cool room temperature in desiccator. 2 – 4 g of sample were weight into dried crucibles and placed into 105 °C for 24 h. Then, crucibles with dried samples were cool down until room temperature in desiccator before calculating. Moisture content was calculated based on percentage of wet-weight as following:

$$Moisture\ content\ \% = (W - W_d) \times 100/W$$

W = weight of sample used (g)

W_d = weight of dried sample (g)

APPENDIX B ANTIOXIDANT ACTIVITY OF RAW RICE, COOKED RICE AND KHAOMAK

Table 14 DPPH radical scavenging activity of raw rice, cooked rice and Khaomak

Rice sample	DPPH radical scavenging activity (mg Trolox/g DW)		
	Free fraction	Bound fraction	Total
Nonpigmented rice			
Raw	0.305 ± 0.023^{dB}	0.337 ± 0.014^{dA}	0.642 ± 0.032^{cA}
Cooked-unsoaked	0.304 ± 0.012^{dC}	0.226 ± 0.018^{fC}	0.530 ± 0.011^{dA}
Cooked-6h-soaked	0.322 ± 0.038^{dC}	0.282 ± 0.008^{eC}	0.603 ± 0.042^{fA}
Cooked-12h-soaked	0.294 ± 0.012^{dC}	0.215 ± 0.005^{fC}	0.508 ± 0.011^{eA}
Khaomak-unsoaked	0.925 ± 0.034^{cC}	0.616 ± 0.012^{aB}	1.541 ± 0.045^{aA}
Khaomak-6h-soaked	1.032 ± 0.028^{bC}	0.560 ± 0.014^{bB}	1.591 ± 0.041^{bB}
Khaomak-12h-soaked	1.242 ± 0.018^{aC}	0.455 ± 0.007^{cB}	1.697 ± 0.012^{bA}
Red rice			
Raw	2.475 ± 0.042^{cA}	0.391 ± 0.030^{dB}	2.865 ± 0.059^{bcB}
Cooked-unsoaked	2.118 ± 0.044^{eB}	0.593 ± 0.049^{bA}	2.712 ± 0.081^{dB}
Cooked-6h-soaked	1.488 ± 0.023^{fB}	0.549 ± 0.021^{bcA}	2.037 ± 0.029^{bcB}
Cooked-12h-soaked	1.358 ± 0.029^{gB}	0.521 ± 0.031^{cA}	1.878 ± 0.051^{cB}
Khaomak-unsoaked	2.666 ± 0.013^{bB}	0.766 ± 0.007^{aA}	3.431 ± 0.014^{aB}
Khaomak-6h-soaked	3.048 ± 0.032^{aA}	0.714 ± 0.019^{aA}	3.762 ± 0.015^{bB}
Khaomak-12h-soaked	2.214 ± 0.028^{dB}	0.603 ± 0.063^{bA}	2.816 ± 0.089^{bA}
Black rice			
Raw	2.427±0.019cA	0.368 ± 0.018^{cA}	2.794 ± 0.022^{cA}
Cooked-unsoaked	2.349 ± 0.039^{dA}	0.348 ± 0.030^{dA}	2.697 ± 0.022^{dA}
Cooked-6h-soaked	1.726 ± 0.074^{fA}	0.356 ± 0.022^{eA}	2.082 ± 0.097^{fA}
Cooked-12h-soaked	1.866 ± 0.033^{eA}	0.399 ± 0.042^{fB}	2.264 ± 0.072^{eA}
Khaomak-unsoaked	3.284 ± 0.011^{aA}	0.647 ± 0.019^{bB}	3.931 ± 0.023^{aA}
Khaomak-6h-soaked	2.858 ± 0.027^{bB}	0.598 ± 0.023^{aA}	3.456 ± 0.021^{bB}
Khaomak-12h-soaked	2.854 ± 0.040^{bA}	0.580 ± 0.024^{cB}	3.434 ± 0.017^{bA}

Different lowercase superscript letters indicate statistical difference of the results in the same variety and column analyzed; different uppercase superscript letters indicate statistical difference of the results in the same column and treatment for 3 varieties ($p\leq0.05$).

Table 15 FRAP activity of raw rice, cooked rice and Khaomak

Rice sample	DPPH radical scavenging activity (mg Trolox/g DW)		
	Free fraction	Bound fraction	Total
Nonpigmented rice			
Raw	0.818±0.043^{dC}	0.914±0.053^{bA}	1.732±0.048^{dC}
Cooked-unsoaked	0.824±0.019^{dC}	0.655±0.034^{dC}	1.479±0.049^{eC}
Cooked-6h-soaked	0.861±0.071^{dC}	0.760±0.099^{cB}	1.621±0.158^{dC}
Cooked-12h-soaked	0.776±0.013^{dC}	0.695±0.041^{cdB}	1.471±0.040^{eC}
Khaomak-unsoaked	1.586±0.078^{aC}	1.130±0.049^{aB}	2.716±0.069^{aC}
Khaomak-6h-soaked	1.212±0.005^{bC}	1.088±0.035^{aB}	2.300±0.030^{bC}
Khaomak-12h-soaked	1.079±0.033^{cC}	0.892±0.012^{bB}	1.971±0.033^{cC}
Red rice			
Raw	5.664±0.168^{aB}	0.797±0.035^{cB}	6.461±0.202^{aB}
Cooked-unsoaked	4.474±0.181^{bB}	1.160±0.039^{bA}	5.634±0.158^{bB}
Cooked-6h-soaked	2.618±0.128^{dB}	1.108±0.061^{bA}	3.726±0.168^{dB}
Cooked-12h-soaked	2.459±0.035^{dB}	0.780±0.078^{cB}	3.239±0.081^{eB}
Khaomak-unsoaked	4.515±0.135^{bB}	1.101±0.014^{bB}	5.615±0.148^{bB}
Khaomak-6h-soaked	2.465±0.035^{dB}	1.600±0.286^{aA}	4.065±0.315^{cB}
Khaomak-12h-soaked	2.913±0.028^{cB}	1.069±0.014^{bB}	3.982±0.016^{cdB}
Black rice			
Raw	11.072±0.138^{aA}	0.832±0.009^{bcB}	11.904±0.135^{aA}
Cooked-unsoaked	8.330±0.149^{cA}	0.738±0.012^{cB}	9.068±0.138^{bA}
Cooked-6h-soaked	4.889±0.088^{dA}	0.767±0.039^{bcB}	5.656±0.126^{eA}
Cooked-12h-soaked	5.245±0.025^{bA}	0.956±0.051^{bA}	6.201±0.070^{dA}
Khaomak-unsoaked	10.066±0.273^{dA}	1.377±0.141^{aA}	11.443±0.354^{aA}
Khaomak-6h-soaked	6.514±0.407^{dA}	1.284±0.043^{aAB}	7.798±0.404^{cA}
Khaomak-12h-soaked	6.441±0.379^{dA}	1.485±0.255^{aA}	7.926±0.396^{cA}

Different lowercase superscript letters indicate statistical difference of the results in the same variety and column analyzed; different uppercase superscript letters indicate statistical difference of the results in the same column and treatment for 3 varieties ($p \leq 0.05$).

APPENDIX C PHENOLIC CONTENT AND ANTIOXIDANT ACTIVITY OF COOKED RICE AND KHAOMAK DURING DURING *IN VITRO* DIGESTION.

Table 16 Phenolic content of cooked rice and Khaomak during *in vitro* digestion

	Oral	Gastric	Intestinal
Nonpigment-Cooked	0.277 ± 0.005^{eC}	1.037 ± 0.029^{cB}	1.729 ± 0.074^{cA}
Nonpigment-Khaomak	0.386 ± 0.009^{dC}	1.154 ± 0.037^{cB}	1.780 ± 0.000^{bcA}
Red-Cooked	0.281 ± 0.009^{eC}	1.034 ± 0.043^{cB}	1.509 ± 0.091^{dA}
Red-Khaomak	0.464 ± 0.010^{cC}	1.199 ± 0.059^{cB}	1.899 ± 0.033^{bA}
Black-Cooked	0.530 ± 0.009^{bC}	1.439 ± 0.047^{bB}	2.091 ± 0.040^{aA}
Black-Khaomak	0.663 ± 0.015^{aC}	1.678 ± 0.194^{aB}	2.131 ± 0.161^{aA}

Different lowercase superscript letters indicate statistical difference of the results in the same column analyzed; different uppercase superscript letters indicate statistical difference of the results in the same row ($p\leq0.05$).

Table 17 DPPH radical scavenging activity of cooked rice and Khaomak during *in vitro* digestion

	Oral	Gastric	Intestinal
Nonpigment-Cooked	0.393 ± 0.137^{eB}	1.570 ± 0.043^{eA}	0.200 ± 0.00^{eC}
Nonpigment-Khaomak	1.042 ± 0.037^{cB}	2.202 ± 0.104^{dA}	0.208 ± 0.168^{eC}
Red-Cooked	0.807 ± 0.054^{dC}	2.226 ± 0.110^{dA}	1.039 ± 0.016^{dB}
Red-Khaomak	1.407 ± 0.027^{bB}	3.115 ± 0.166^{cA}	1.570 ± 0.059^{cB}
Black-Cooked	3.575 ± 0.103^{aB}	5.318 ± 0.448^{bA}	4.181 ± 0.474^{bB}
Black-Khaomak	3.644 ± 0.126^{aC}	6.688 ± 0.225^{aA}	4.896 ± 0.246^{aB}

Different lowercase superscript letters indicate statistical difference of the results in the same column analyzed; different uppercase superscript letters indicate statistical difference of the results in the same row ($p\leq0.05$).

Table 18 FRAP activity of cooked rice and Khaomak during *in vitro* digestion

	Oral	Gastric	Intestinal
Nonpigment-Cooked	1.096 ± 0.032^{eC}	2.589 ± 0.035^{eB}	4.548 ± 0.220^{dA}
Nonpigment-Khaomak	1.689 ± 0.082^{cC}	2.783 ± 0.275^{eB}	5.270 ± 0.194^{cA}
Red-Cooked	1.450 ± 0.083^{dC}	3.376 ± 0.107^{dB}	4.650 ± 0.172^{dA}
Red-Khaomak	2.103 ± 0.087^{bC}	9.345 ± 0.412^{bB}	8.136 ± 0.168^{bA}
Black-Cooked	5.997 ± 0.204^{aC}	8.719 ± 0.109^{cB}	10.008 ± 0.206^{aA}
Black-Khaomak	5.890 ± 0.090^{aB}	10.344 ± 0.456^{aA}	10.385 ± 0.706^{aA}

Different lowercase superscript letters indicate statistical difference of the results in the same column analyzed; different uppercase superscript letters indicate statistical difference of the results in the same row ($p\leq0.05$).

APPENDIX D LABORATORY EQUIPMENT

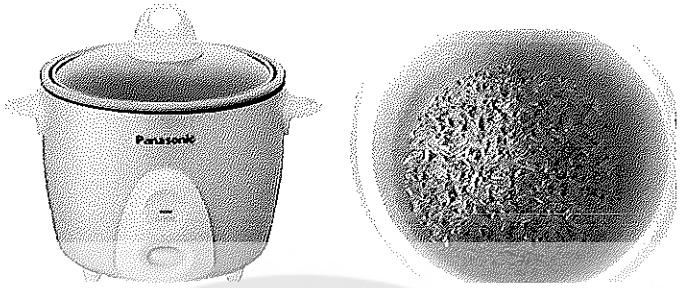

Figure 24 Rice cooker and cooked rice used in method 1

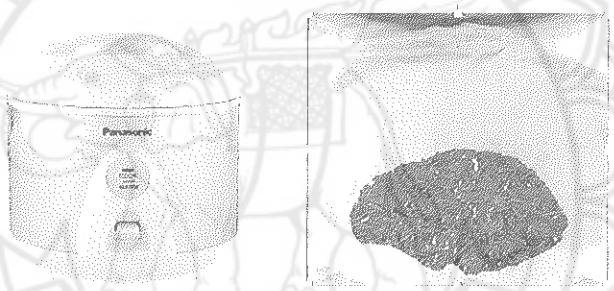

Figure 25 Rice cooker with steamer and rice wrapped by a piece of cheesecloth in method 2

Figure 26 Separatory funnel with two immiscible liquids