การระบุความสามารถในการต้านอนุมูลอิสระในข้าวเหนียวสีของไทยสายพันธุ์ต่าง ๆ โดยวิธีทางเคมีและวิธีฟูเรียร์ทรานสฟอร์มอินฟราเรดสเปกโทรสโกปี

นางสาวอัมพวัน จันทะสี

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต
สาขาวิชาชีวเวชศาสตร์
มหาวิทยาลัยเทคโนโลยีสุรนารี
ปีการศึกษา 2554

DETERMINATION OF ANTIOXIDANT CAPACITY OF THAI VARIETIES OF COLORED GLUTINOUS RICE BY CHEMICAL METHODS AND FOURIER TRANSFORM INFRARED SPECTROSCOPY

Ampawan Jantasee

A Thesis Submitted in Partial Fulfillment of the Requirements for the Degree of Master of Science in Biomedical Science

Suranaree University of Technology

Academic Year 2011

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Suranaree University of Technology has approved this thesis submitted in partial fulfillment of the requirements for a Master's Degree.

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งานวิจัยนี้มีวัตถุประสงค์เพื่อศึกษาคุณสมบัติต้านอนุมูลอิสระ และปริมาณไฟโตเคมิคอล ซึ่งประกอบด้วยสารประกอบฟีนอลิก ฟลาโวนอยด์ แอนโทไซยานิน และโพรแอนโทไซยานิดิน ของข้าวเหนียวสีของไทยสายพันธุ์ต่าง ๆ จำนวน 50 สายพันธุ์ รวมถึงศึกษาเปรียบเทียบคุณสมบัติและปริมาณสารเหล่านี้ ระหว่างข้าวดิบและข้าวสุก วิธีทางเคมีและฟูเรียร์ทรานสฟอร์มอินฟราเรดสเปกโทรสโกปี (ที่เลขคลื่น 400-4,000 ซม.$^{-1}$) ถูกนำมาใช้ในการวัดคุณสมบัติดังกล่าว เพื่อเปรียบเทียบข้อมูลที่ได้และประเมินความสอดคล้องของข้อมูลจากเทคนิคทั้งสอง จากผลการวัดคุณสมบัติต้านอนุมูลอิสระพบว่า ข้าวสีดำมีศักยภาพในการต้านอนุมูลอิสระสูงสุด ตามด้วยข้าวสีแดง สีน้ำตาล และข้าวสีขาว เช่นเดียวกับสารประกอบฟีนอลิก ปริมาณฟลาโวนอยด์ ปริมาณแอนโทไซยานิน และโพรแอนโทไซยานิดิน เมื่อข้าวถูกทำให้สุกพบว่า คุณสมบัติต้านอนุมูลอิสระ ปริมาณสารประกอบฟีนอลิก ปริมาณฟลาโวนอยด์ ปริมาณแอนโทไซยานิน และปริมาณโพรแอนโทไซยานิดินในข้าวทุกกลุ่มสีลดลงอย่างน้อยร้อยละ 30 ถึงแม้ข้าวสีดำจะคงมีคุณสมบัติต้านอนุมูลอิสระ สารประกอบฟีนอลิก ปริมาณฟลาโวนอยด์ ปริมาณแอนโทไซยานิน และปริมาณโพรแอนโทไซยานิดินเหลืออยู่มากที่สุดหลังถูกทำให้สุก ตามด้วยข้าวสีแดง สีน้ำตาล และสีขาว แต่ข้าวสีดำมีเปอร์เซ็นการลดลงของคุณสมบัติเหล่านี้มากที่สุด ตามด้วย ข้าวสีขาว ข้าวสีแดงและข้าวสีน้ำตาล และจากการเปรียบเทียบสารประกอบฟีนอลิกที่เหลืออยู่ในข้าวสุกพบว่าฟลาโนอยด์มีเปอร์เซ็นต์การลดลงต่ำที่สุด ซึ่งเป็นข้อมูลที่แสดงให้เห็นว่า ฟลาโวนอยด์เป็นสารประกอบฟีนอลิกที่ทนต่อกระบวนการทำให้สุกมากที่สุด ในขณะที่โพรแอนโทไซยานิดินไวต่อกระบวนการทำให้สุกที่สุด เนื่องจากมีเปอร์เซ็นต์การลดลงมากที่สุด คุณสมบัติต้านอนุมูลอิสระและปริมาณสารประกอบฟีนอลิกที่ประเมินได้โดยใช้วิธีทางเคมีและใช้ฟูเรียร์ทรานสฟอร์มอินฟราเรดสเปกโทรสโกปีมีความสอดคล้องกันอย่างดี นอกจากนี้ ผลการวิเคราะห์องค์ประกอบหลักแสดงให้เห็นว่าการดูดกลืนแสงที่เลขคลื่น 1635 ซม.$^{-1}$ ซึ่งแตกต่างกันในข้าวแต่ละกลุ่มสีเป็นลักษณะที่เหมาะสมในการใช้จำแนกข้าวตามคุณสมบัติต้านอนุมูลอิสระ การดูดกลืนแสงที่ค่าเลขคลื่นดังกล่าวเป็นการดูดกลืนแสงที่เกิดจากการสั่นของพันธะในหมู่ไฮดรอกซิลในสารประกอบฟีนอลิกซึ่งเป็นโมเลกุลที่ให้คุณสมบัติต้าน

อนุมูลอิสระ งานวิจัยนี้เป็นรายงานหนึ่งที่แสดงให้เห็นศักยภาพของฟูเรียร์ทรานสฟอร์มอินฟราเรดสเปกโทรสโกปีในการใช้ระบุคุณสมบัติต้านอนุมูลอิสระของสารจากพืชได้เทียบเท่าวิธีทางเคมี ด้วยข้อได้เปรียบในเรื่องของการเตรียมสารเพื่อตรวจสอบที่ง่าย ใช้เวลาน้อย และการลดค่าใช้จ่ายสำหรับซื้อสารเคมี รวมถึงความรวดเร็วในการทดสอบ ทำให้ฟูเรียร์ทรานสฟอร์มอินฟราเรดสเปกโทรสโกปีน่าจะเป็นวิธีการที่ดีวิธีการหนึ่งในการใช้ตรวจสอบสารประกอบสำคัญในพืชชนิดใหม่ได้อย่างรวดเร็วและแม่นยำ

สาขาวิชาชีววิทยาศาสตร์ ลายมือชื่อนักศึกษา _____

ปีการศึกษา 2554 ลายมือชื่ออาจารย์ที่ปรึกษา _____

ลายมือชื่ออาจารย์ที่ปรึกษาร่วม _____

AMPAWAN JANTASEE : DETERMINATION OF ANTIOXIDANT CAPACITY OF THAI VARIETIES OF COLORED GLUTINOUS RICE BY CHEMICAL METHODS AND FOURIER TRANSFORMED INFRARED SPECTROSCOPY. THESIS ADVISOR : ASST. PROF. DUANGKAMOL MAENSIRI, Ph.D. 130 PP.

GLUTINOUS RICE/ ANTIOXIDANT CAPACITY/ PHENOLIC COMPOUND/ FLAVONOID/ ANTHOCYANIN/ PROANTHOCYANIDIN/ FT-IR

This research aimed to study the antioxidant capacity and phytochemical contents including phenolic compound, flavonoid, anthocyanin and proanthocyanidin of 50 Thai glutinous rice varieties and to compare these qualities between uncooked and cooked rice forms. The chemical methods as well as fourier transform infrared spectroscopy (400-4,000 cm^{-1}) were used to determine these qualities in order to compare the data obtained and evaluate the consistency of data from both methods. The measurement of antioxidant capacity revealed that dark rice had the highest level of antioxidant capacity followed by red rice, brown rice and white rice. This was also true for total phenolic, flavonoid, anthocyanin and proanthocyanidin contents. When rice was cooked, antioxidant capacity, total phenolic, flavonoid, anthocyanin and proanthocyanidin contents reduced for at least 30 percent. Even though dark rice had the highest remaining antioxidant capacity, total phenolic, flavonoid, anthocyanin and proanthocyanidin contents after it was cooked followed by red rice, brown rice and white rice, dark rice showed the highest percentage reduction of these qualities followed by white rice, red rice and brown rice. The comparison of the remaining

phenolic compound in cooked rice showed the lowest percentage reduction of flavonoid suggesting that flavonoid was the most resistant phenolic compound to cooking process, whereas proanthocyanidin was the most sensitive since it had the highest percentage reduction. Antioxidant capacity and phenolic compounds contents determined by chemical methods and fourier transform infrared spectroscopy were corresponding well. Moreover, the principal content analysis indicated that the difference of absorbance at wavenumber of 1,635 cm^{-1} between rice varieties was the suitable character to be used classification of rice varieties according to their antioxidant capacity. Absorbance at this wavenumber was the result of bonds vibration within hydroxyl groups in phenolic compound which contributed to antioxidant capacity. This research is one of the reports that suggests the comparable capacity of fourier transform infrared spectroscopy to chemical methods as the tool to determine antioxidant capacity of phytochemicals. With advantages in simple sample preparation, less time required and reduced cost for chemicals, fourier transform infrared spectroscopy is one of the promising fast and precise methods for determination of important compounds in new source plants.

School of Biomedical Science

Academic Year 2011

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ACKNOWLEDGEMENTS

I am sincerely grateful for my thesis advisor, Asst. Prof. Dr. Duangkamol Maensiri for her invaluable helps, encouragement and financial support throughout the course of this research. I will be forever grateful.

Thanks to my co-advisor Asst. Prof. Dr. Nooduan Muangsan and Dr. Kanjana Thummanoo, for their advices as academic mentors in the preparation of this thesis. I would like to thank Asst. Prof. Dr. Wilairat Leeanansaksiri, for all opportunities and her support during the time I was taking course work.

I wish to thank Suranaree University of Technology for the scholarship and research facilities.

I wish to thank Mr. Somjai Sangjun from Rice research center, Nong Khai province, for rice grains that he kindly provided in this research.

Finally, I wish to thank my family for their unfaltering faith in my ability and encouragement throughout the period of this research.

Ampawan Jantasee

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LIST OF ABBREVIATIONS

ABTS	=	2, 2′-azinobis (3-ethylbenzothiazoline-6-sulfonic acid)
cm	=	centimeter
^{o}C	=	degree celsius
EPR	=	electron spin resonance
DPPH	=	2,2-diphenyl-picrylhydrazyl
DFA	=	discriminant function analysis
FRAP	=	ferric reducing antioxidant power
FT-IR	=	fourier transform infrared spectroscopy
g	=	gram
hr	=	hour
HAT	=	hydrogen atom transfer
H_2O_2	=	hydrogen peroxide
OH•	=	hydrogen radical
µg	=	microgram
µl	=	microliter
mg	=	milligram
mL	=	milliliter
mM	=	millimolar
Min	=	minute
nm	=	nanometer

LIST OF ABBREVIATIONS (Continued)

ORAC	=	oxygen radical absorbance capacity
PLSR	=	partial least-squares regression
%	=	percentage
PCA	=	principle component analysis
SET	=	single electron transfer
TAC	=	total antioxidant capacity
TEAC	=	trolox equivalent antioxidant capacity
TRAP	=	total radical-trapping antioxidant parameter
v/v	=	volume by volume
w/v	=	weight by volume

CHAPTER I

INTRODUCTION

1.1 Background/Problem

Rice is a major cereal crop in many Asian countries and staple food of more than 40% of the world's population. Approximately 95% of rice is produced in Asia (Bhattacharjee, P., Singhal, R. S., and Kulkarni, P. R., 2002). Thailand is one of the major rice exporters (www.irri.org, 2011) and Thai people consume rice as staple food. Although white rice is extensively consumed, several varieties of pigmented rice, particularly black, red and brown rice, have been cultivated in Thailand. Among these varieties, black glutinous rice is the most famous one, because it is generally used as an ingredient in snacks and desserts. At present, whole grain pigmented rice has been categorized as one of the potent functional foods since it contains high amount of phenolic compounds, especially anthocyanins in pericarp (Abdel-Aal et al., 2006; Ryu et al., 1998; Yawadio et al., 2007).

Many colored rice varieties have been reported as potent sources of antioxidant. They are increasingly recognized as sources of antioxidant (Yawadio et al., 2007). Red rice has become popular in Japan as functional food because of the high contents of anthocyanin and polyphenols (Itani and Ogawa, 2004). Black rice has also gained much attention due to its nutritional advantages over common rice. It provides higher vitamins, protein and minerals with some variations between cultivars and location in which it is grown (Suzuki et al., 2004). Anthocyanin pigments are responsible for the

red, purple, and blue colors of many fruits, vegetables, cereal grains, and flowers. Anthocyanins are one class of flavonoid compounds, which are widely distributed plant polyphenols. There is experimental evidence that certain anthocyanins and flavonoids have anti-inflammatory properties, and there are reports that orally administered anthocyanins are beneficial for treating diabetes and ulcers and may have antiviral and antimicrobial activities (Liu, 2004; 2007). Phenolics are compounds possessing one or more aromatic rings with one or more hydroxyl groups. Phenolic compounds in diet may provide health benefits associated with reduced risk of chronic disease (Liu, 2007). Chinese medicinal plants have high levels of phenolics and potent antioxidant capacity, which might contribute to the protective effects against cancer (Cai et al., 2004).

Rice is the staple food of Thai and Asian people from past to present. The key component in various types of rice is rice flour. However, there are other elements which are important and beneficial to the human body, in particular, elements in the rice with black or red pigment (Awika et al., 2005). Black or red pigment found in rice is responsible for the antioxidant capacity of rice. The study by pharmaceutical research team in Japan found that the pigment extracted from purple black rice had the ability to eliminate free radicals in the superoxide radical and the hydroxyl radical (Ichikawa et al., 2001). In China, the effects of rice with red and black pigments on blockages in the arteries in rabbit were assessed. The results revealed 50% less artery blockages in rabbits fed with colored rice than in those fed with white rice (Ling et al., 2001). Therefore, consumption of rice with red or black pigment should be useful for maintaining health in good condition. Colored rice would be a good choice for consumers who have more concern about their health. With the changing lifestyle in

today's world, risk of illness and disease increases. Food with nourishing substance has its role in the preventive nutrition that will keep people distant from disease.

Almost every Thai person eat rice every day. Being a biodiversity hot spot, there are many varieties of colored rice available in Thailand. To promote consumption of colored rice in the preventive nutrition program, the information on the antioxidant capacity of each colored rice variety is crucial for the start. The objective of this study was therefore to compare total antioxidant activity, total phenolics, flavonoids, proanthocyanidins and anthocyanins contents of Thai glutinous rice varieties (nineteen dark, twenty-seven red, two brown rice and two white rice varieties). Since the nutritional value may be changed during cooking process, the antioxidant activity, total phenolics, flavonoids, proanthocyanidins and anthocyanins contents in cooked rice were as well be measured. The results from the study were expected to be useful in evaluating Thai black rice varieties in terms of their nutritional value, which might be applied in various aspects. Rice varieties with higher levels of antioxidants should be interested by commercial rice farmers. Data on antioxidant activity, total phenolics, flavonoids, proanthocyanindins and anthocyanin contents in cooked rices could be considered together with those in uncooked ones when commercial and nutritional values of them are evaluated.

Another aspect the research focused on was Fourier Transformed Infrared (FT-IR) Spectroscopy. FT-IR is most useful for identifying chemicals that are either inorganic or organic. It can be used to quantitate the components of the unknown mixture. It can be applied to the analysis of solids, liquids and gases. FT-IR is a fairly recent development in the way that information is collected and converted to spectral interference pattern. Computer program has been integrated in the FT-IR system, which makes it faster and more sensitive than older instruments. FT-IR is a powerful

tool for identifying the types of chemical bonds (functional groups). The wavelength of light that is absorbed into the chemical bonds can be seen in the spectrum. The interpretation of the infrared absorption spectrum of chemical bonds in molecules can be identified by FT-IR spectra which are so unique that they can be seen as the fingerprints of molecules.

Antioxidant capacity and total phenolic contents of vegetables have been studied extensively using different antioxidant assays (Stratil et al., 2006). But these assays are time consuming and the development of alternative methods to replace or, at least, monitoring the traditional wet chemical methods is essential if large number of samples are to be screened. Infrared spectroscopy (IR) has unique advantage in the simple preparation of samples, while maintaining accuracy and satisfactory sensitivity (Naumann, 2001; Movasaghi et al., 2008). It is gaining wider use in the analysis because it is non-destructive (Thygesen et al., 2003).

In this study, antioxidant capacity was measured using trolox equivalent antioxidant capacity (TEAC) assay and the Folin-Ciocalteu assay was used to determine phenolic contents in colored rice varieties. To date, no studies have been reported in about the use of FT-IR in determination of antioxidant capacity and total phenolic contents in rice. The objectives of this study were to compare the chemical analysis of antioxidant capacity and phenolic contents to a Fourier Transform Infrared (FT-IR) Spectroscopy method, between uncooked rice and cooked rice.

1.2 Research objectives

The objectives of this thesis are:

1.2.1 To study variations in total antioxidant capacity of Thai glutinous rice varieties (forty-eight colored rice and two white rice)

1.2.2 To compare total antioxidant capacity between uncooked rice and cooked rice.

1.2.3 To compare the competence of the chemical analysis methods to Fourier Transform Infrared (FT-IR) Spectroscopy method in determinations of antioxidant capacity and phenolic contents.

1.3 Research hypothesis

Although they are generally called black rice, color variation of the so-called black rice does exist. Some are brown and some are black. Since anthocyanin pigments are known to be responsible for the red, purple and blue colors of many plants species including cereals (Araceli et al., 2009; Awika et al., 2005), different Thai black rice varieties with different color should have different amount of anthocyanins which reflects different antioxidant capacity, different amounts of total phenolics, flavonoids and proanthocyanidins. Anthocyanins have been reported to be highly instable and susceptible to degradation (Isabel Louro et al., 2011; Melissa et al., 2011). Because rice is cooked prior to consumption, the question of whether antioxidant capacity of rice is reduced after cooking was raised.

FT-IR spectroscopy has been applied as the method to determine phenolic content and total antioxidant capacity in some plants and some cereals (Augustin et al., 2011; Xiaonan et al., 2011). The advantages of FT-IR spectroscopy over

traditional chemical method are simple sample preparation, less time required, no chemicals reagents needed while satisfactory precision and sensitivity are still retained. Due to these advantages of FT-IR spectroscopy, it was used in parallel with traditional chemical method. The comparison of the results from these two methods was expected to provide information about the feasibility of the more convenient FT-IR spectroscopy method to quantitate antioxidant capacity and antioxidants contents in rice.

1.4 Scope and limitation of the study

Forty-eight colored rice and two white rice varieties were the subjects in the study. The rice grains were provided by Rice Grains Research Center, Nong Khai province. In order to answer the two questions, 1) Are black rice with different colors have different amount of anthocyanin, total phenolics, flavonoid and proanthocyanidins as well as different antioxidant capacity, and 2) Is the antioxidant capacity of rice decreased by the cooking process, both uncooked and cooked rice grains of all varieties were subjected to five experiments to determine the total antioxidant capacity, total phenolic compounds, total flavonoids, total proanthocyanidins and anthocyanin contents. FT-IR spectroscopy was also used to determine these values.

1.5 Expected results

Antioxidant capacity of each rice variety under the study was expected to be reported as well as the total content of antioxidants: phenolics, flavonoids, proanthocyanidins and anthocyanins. Based on the results, candidate varieties of rice for the introduction into nutritionally enhanced rice breeding program would be suggested. Data on antioxidant capacity and antioxidants content in cooked rice would also be useful fact for rice consumers who expect to gain extra nutritional value from black rice. Application of FT-IR spectroscopy for determinations of antioxidant capacity and antioxidant contents were also tested. The results from the use of FT-IR spectroscopy should serve as the technical guideline for those who are interested in the measurement of plant antioxidants.

CHAPTER II

LITERATURE REVIEW

2.1 Rice

Rice is the seed of the monocot plants *Oryza sativa* (Asian rice) or *Oryza glaberrima* (African rice) shown in Figure 2.1. Rice is one of the major cereal grains that serves as the important staple food for the world's human population, especially in Asia and the West Indies. It is the grain with the third-highest worldwide production, after maize (corn) and wheat (Cohen et al., 1994). Since a large portion of maize crops is grown for purposes other than human consumption, rice is the most important grain with regard to human nutrition and caloric intake, providing more than one fifth of the calories consumed worldwide by human. There are many varieties of rice and culinary preferences tend to vary regionally. For example in India, non-sticky rice is preferred as implied in a saying "grains of rice should be like two brothers, close but not stuck together", while in the Far East there is a preference for softer, stickier varieties. Not only it is important as a staple food, but rice also has considerable cultural importance. For example, rice is first mentioned in the Yajur Veda and it is frequently referred to in Sanskrit texts. Rice is often seen associated with prosperity and fertility, therefore there is the custom of throwing rice at weddings in French culture (Crawford and Shen, 1998).

Rice is normally grown as an annual plant, although in tropical areas it can survive as a perennial and can produce a ratoon crop for up to 30 years (Douangboupha et al., 2006). Rice plant can grow to 1-1.8 m (3.3-5.9 ft) tall depending on the variety and soil fertility. It has long, slender leaves of 50-100 cm (20-39 inches) long and 2-2.5 cm (0.79-0.98 inches) broad. The small wind-pollinated flowers are produced in a branched arching to 30-50 cm (12-20 inches) long pendulous inflorescence. The edible seed is a grain (caryopsis) of 5-12 mm (0.20-0.47 inches) long and 2-3 mm (0.079-0.12 inches) thick. Rice cultivation is well-suited to countries and regions with low labor costs and high rainfall, as it is labor-intensive to cultivate and it requires ample water. Rice can be grown practically anywhere, even on a steep hill or mountain. Although its parent species are native to South Asia and certain parts of Africa, centuries of trade and exportation have made it commonplace in many cultures worldwide.

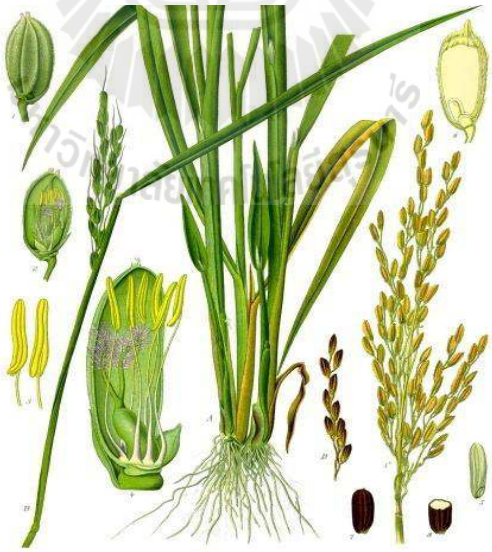


Figure 2.1 *Oryza sativa*, commonly known as Asian rice.

(Source: http://www.en.wikipedia.org/wiki/File:Oryza_sativa)

2.1.1 Morphology of the rice plant

Rice is generally considered a semiaquatic annual grass that can survive in tropical perennials and produce new shoots from nodes after harvest (ratooning). At maturity, rice plants are composed of the main stems and shoots. Each bears the flowering or crop yield, plant height, panicle terminal to a variety of different environmental ranging from about 0.4 meters to over 5 meters. The stages in which rice grows from seed into plants include seed germination, seeding and tillering stages, and the reproductive stage. The reproductive stage is composed of the panicle initiation and heading stages.

Rice grains (Figure 2.2) are firmly attached to the wall or membrane of the ovary (pericarp). Grain consists of two major parts 1) the chamber called the husk or hull. The husk includes lemma, palea, awn, rachilla and sterile lemmas 2) the edible part called caryopsis including pericarp, tegmen or seed coat, aleurone, starch endosperm and embryo (Te-Tzu Chang and Eliseo, 1965).

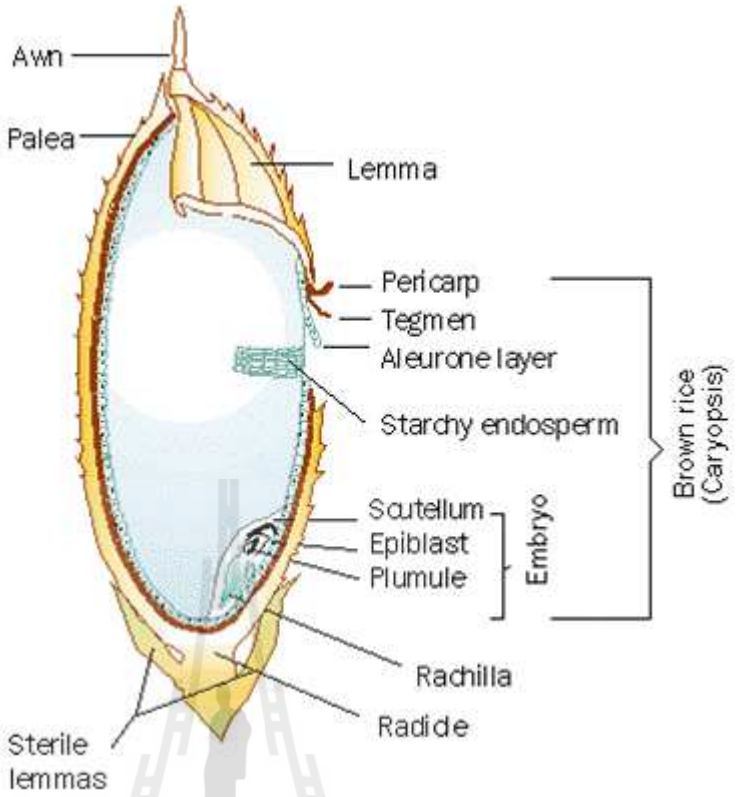


Figure 2.2 Morphology of seed rice grain.

(Source: http://www.teksengricemill.com/knowled/structure.htm)

2.1.2 Glutinous rice

Glutinous rice (*Oryza sativa* var. *glutinosa* or *O. sativa*; also called sticky rice, sweet rice, waxy rice, botan rice, biroin rice, mochi rice, and pearl rice) (Kenneth and Michael, 2002) is a type of short-grained Asian rice that is especially sticky when cooked. It is called glutinous in the sense of being glue-like or sticky and not in the sense of containing gluten. While called "sticky," it is not to be confused with the other varieties of Asian rice, which becomes sticky to one degree or another when cooked.

In Thailand glutinous rice is known as *khao niao* in Central Thailand and Isan; and as *khao nueng* in Northern Thailand (Figure 2.3). Northern Thais (Lanna people) and northeastern Thais, traditionally eat glutinous rice as their staple food, whereas non-sticky white rice or *Khao chao* is favored by people in the South and the central of the country as well as some northeastern Thais who were influenced by the Khmer-Thai. Such Thais are from Surin province and neighboring areas (Kenneth and Michael, 2002).

Glutinous rice does not contain dietary gluten (i.e. it does not contain glutenin and gliadin), and should be safe for gluten-free diets. The two major components of starch are amylose and amylopectin. Glutinous rice is distinguished from non-glutinous type of rice by having no (or negligible amounts of) amylose, and high amounts of amylopectin. Amylopectin is responsible for the sticky quality of glutinous rice. The difference has been traced to a single mutation that was selected for by farmers (Kenneth and Michael, 2002). Glutinous rice can be used either milled or unmilled (that is, with the bran removed or not removed). Milled glutinous rice is white in color and fully opaque (unlike non-glutinous rice varieties, which are somewhat translucent when raw), whereas the bran can give unmilled glutinous rice a purple or black color. Black and purple glutinous rice are distinct strains from white glutinous rice. In developing Asia, there is little regulation to prevent toxic dyes being added to color adulterated rice. Both black and white glutinous rice can be cooked as grains or ground into flour and cooked as paste or gel.

Figure 2.3 Thai traditional glutinous rice.

(Source: http://www.asiarecipe.com/stickyrice.html)

Glutinous rice is one of the main ingredients in *yam naem khao thot* or *naem khluk* (Figure 2.4), an Isan snack made of crumbled crisp-fried glutinous rice balls, minced pork, ginger, green chillies, peanuts and onion.

Figure 2.4 Yam-naem or name-khluk.

(Source: http://www.dishaday.blogspot.com/2007/11/yam-naem.html)

Famous sweets and desserts among tourists in Thailand is *khao niao mamuang* (Figure 2.5). *Khao lam* is sticky rice with sugar and coconut cream cooked in specially-prepared bamboo sections of different diameters and lengths. It can be prepared with white or dark purple (*khao niao dam*) varieties of glutinous rice. Sometimes a few beans or nuts are added and mixed in. Thick *khao lam* containers may have a custard-like filling in the center made with coconut cream, egg and sugar (Figure 2.6). *Khao tom mat* is, steamed sticky rice mixed with banana and wrapped in banana leaf (Figure 2.7).

Figure 2.5 Khao-niao-mamuang.

(Source: http://www.joysthaifood.com/mango/thai-food-recipe-kao-niao-mamuang/)

Figure 2.6 Khao-lam.

(Source: http://www.thai-blogs.com/2008/sticky-rice-in-bamboo/)

Figure 2.7 Khao-tom-mat.

(Source: http://www. bangkrod.blogspot.com/2010/khao-tom-mat)

2.1.3 Cultivation of glutinous rice

Glutinous rice is a type of rice grown in Laos, Myanmar, Vietnam, Bangladesh, China, Japan, Korea, Philippines, Thailand, Taiwan, Cambodia and Indonesia. An estimated 85% of Lao rice production is of this type. The rice has been recorded in the region for at least 1,100 years. Non-glutinous varieties with better yield were improved and introduced into many Asian countries during the Green Revolution. While adopted by many, Lao farmers rejected such varieties due to their preference of traditional sticky varieties (Kenneth and Michael, 2002). Over time, higher-yield varieties of glutinous rice have become available from the Lao National Rice Research Program. By 1999, more than 70% of the area along the Mekong River Valley was used to grow these newer varieties. In China, glutinous rice has been grown for at least 2,000 years. According to the legend, it was used to make the mortar in the construction of the Great Wall of China. Chemical tests have confirmed that this is true for the city walls of Xian (Kenneth and Michael, 2002).

Thailand is an agricultural country, most of the farmers cultivate crops such as rice, corn and soybean. The rice planting area takes approximately 11.3% of the agriculture area in Thailand. The Central and North-East of the country are the highest rice production areas, followed by the North and the South. Thailand has been one of the world's largest rice exporters for more than 20 years. In the year 2550, 9.20 million tons of rice exported was worth 119,304 million baht.

2.1.4 Colored glutinous rice

Glutinous or sticky rice has obvious features. The cooked grains stick like glue together. Grains of glutinous rice are opaque. They may be long or short depending on the variety. The variations in the color of glutinous rice grains are shown in Figure 2.8. They can be white, red, brown, purple or black. The grain color is the outcome of how the pigments are mixed. Glutinous rice is widely consumed in Thailand, Laos, and is the staple food of people in the Northeast and the North of Thailand. In addition to direct consumption, colored glutinous rice varieties are also used to produce rice flour and desserts such as steamed custard and in some other recipes.

Figure 2.8 Variation of color of glutinous rice grains.

(Source: http://www.all-creatures.org/recipes/i-rice-blacksticky.html)

Pigments of rice are mainly found in the trunk, leaves, and almost all parts of the inflorescence (Figure 2.9). Brown red and purple colors are determined by the substance called procyanidin, peonidin and cyaniding respectively (Min et al., 2011). In the seeds these substances are found in the pericarp layer (Figure 2.10). Anthocyanin was found at high levels at pericarp in black rice. There are studies that identified the type anthocyanin by using matrix-assisted laser desorption/ionization mass spectrometry (MALD-MS) analysis. The results showed that different anthocyanins were found in pericarp at different localization of the grain. They may be pentose moiety like including cyaniding-3-*O*-pentoside and petunidin-3-*O*-pentoside or hexose moiety such as cyaniding-3-*O*-hexoside and petunidin-3-*O*-hexoside (Yukihiro et al., 2012). The color appearing on grains is the combined quality of the three pigment substances. Cyaniding and peonidin belong to the class of anthocyanin flavonoid compounds, whereas procyanidin is classified into proanthocyanidin class of flavonoid compounds. All these pigments are antioxidants (Ryu et al., 1998). The anthocyanins present in black rice are chrisanthemin, keracyanin, and peonidin 3-glucoside, while those found in red rice are catechin and catechatannin (Kim et al., 2008). There have been a few previous reports on phenolic compounds (flavonoids, anthocyanins and proanthocyanidins) contents in the rice grains as shown in Table 2.1.

Table 2.1 Phenolic compounds reported in rice grains.

Colored rice	Phenolics	Anthocyanins	Proanthocyanidins	Reference
Black (Artemide)	10.3 ± 1.7 g/kg	20.5 ± 0.01 g/kg	153.07 ± 6.08 mg/kg	Finocchiaro et al., 2010.
Red (RNC1)	4.8 ± 0.3 g/kg		118.56 ± 3.28 mg/kg	Finocchiaro et al., 2010.
Red (RNC2)	5.0 ± 0.5 g/kg		115.42 ± 2.89 mg/kg	Finocchiaro et al., 2010.
Red (RNC5)	3.8 ± 0.3 g/kg		110.12 ± 3.59 mg/kg	Finocchiaro et al., 2010.
Red (Perla Rosso)	3.0 ± 0.3 g/kg		99.18 ± 1.66 mg/kg	Finocchiaro et al., 2010.
Red (Ermes)	3.5 ± 0.3 g/kg		93.90 ± 4.94 mg/kg	Finocchiaro et al., 2010.
White (Perla)	1.8 ± 0.1 g/kg			Finocchiaro et al., 2010.
White (Gladio)	1.8 ± 0.2 g/kg			Finocchiaro et al., 2010.
White (Augusto)	1.9 ± 0.0 g/kg			Finocchiaro et al., 2010.
Light brown	151.8 mg GEA			Shen et al., 2009
Red rice	470.1 mg GEA			Shen et al., 2009
Black rice	1055.7 mg GEA			Shen et al., 2009
Light brown	69 mg GEA			Goffman and Bergman, 2004
Red rice	214 mg GEA			Goffman and Bergman, 2004
Black rice	274 mg GEA			Goffman and Bergman, 2004

GEA = gallic acid.

Anthocyanin is a type of secondary metabolite in plants, in the form of glucoside in the vacuole of plant cell that endows the nice color. Anthocyanin plays an important role in UV protection and phytopathogens during plant development. It is well known as strong scavenger of free radicals. It has numerous benefits for health such as, promoting visual acuity, inhibiting lipid peroxidation and protecting from certain cancers and mutation diseases (Madhuri and Reddy, 1999). Bong et al. (2007) reported the composition of anthocynins in two rice cultivars including Ilpum and Heugjinju. The result showed that Heugjinju contained three types of anthocyanins, while Ilpum showed no detectable level of the substance. Moreover, they also assessed the expression of five anthocynin biosynthetic genes, including phenylalanine ammonia lyase (*PAL*), chalcone synthase (*CHS*), flavonone 3β-hydroxylase (*F3H*), dihydroflavonol reduvtase (*DFR*) and anthocyanin synthase (*ANS*) in different tissues of those cultivars. It was revealed that the expression was more in the leaves and seeds of Heugjinju than in llpum. Furthermore, two genes, *DFR* and *ANS* were highly expressed (Bong et al., 2007).

Figure 2.9 Rice panicle.

(Source: http://arbeericecenter.blogspot.com.asian-sunrice-harvest.html)

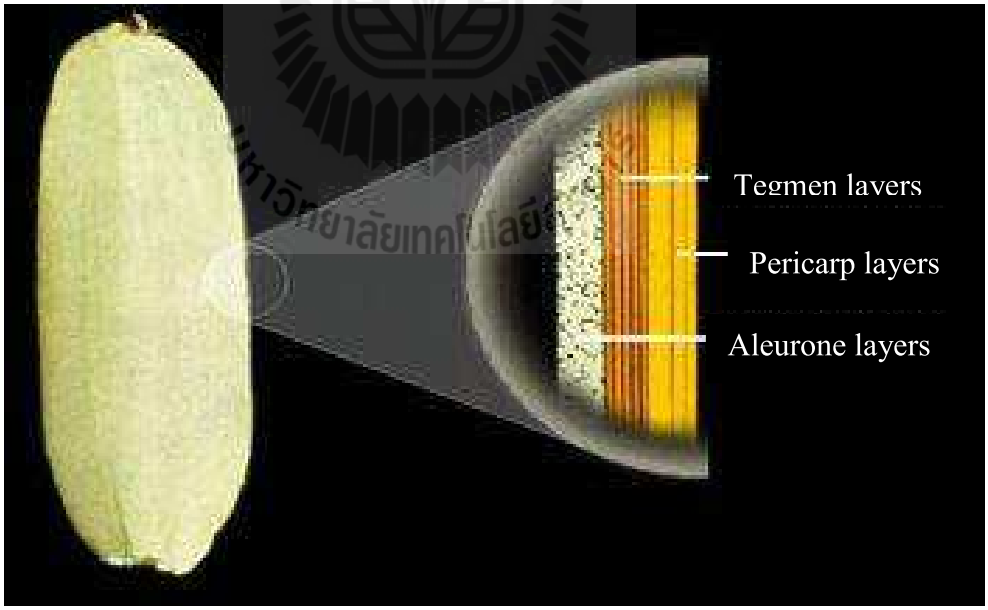


Figure 2.10 Pericarp layers.

(Source: http://www.knowledgebank.irri.org/morph/Rice_grain.htm)

2.2 Free radicals and antioxidants

2.2.1 Free radicals

Free radicals are chemical species that possess an unpaired electron in the outer (valence) shell of the molecule. They are highly reactive and they have low chemical specificity. Therefore they can react with most molecules in its vicinity. This includes lipids, proteins, carbohydrates and DNA. It tries to gain stability by capturing the needed electron. They do not survive in their original state for very long and quickly react with their surrounding. Therefore, free radicals attack the nearest stable molecule. When the attacked molecule loses its electron, it becomes a free radical itself, beginning a chain reaction (Figure 2.11). Once the process is started, it can cascade, finally resulting in the disruption of a living cell (Cheeseman and Slater, 1999).

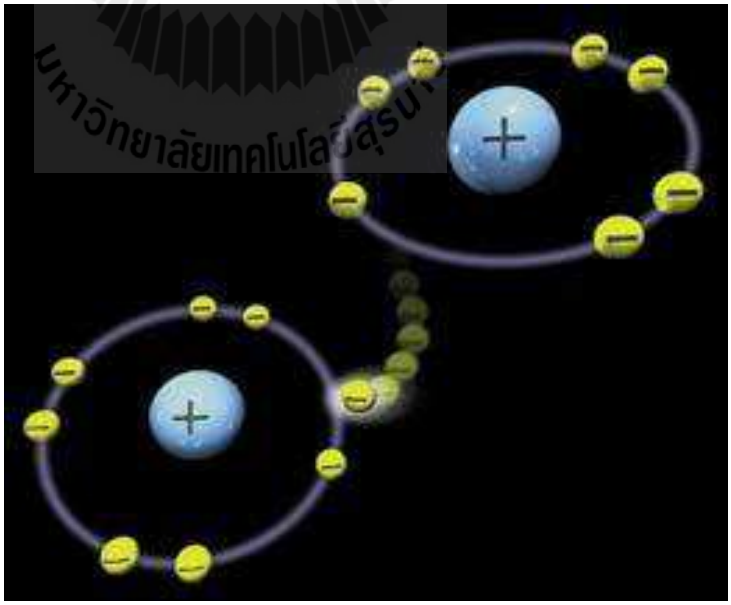


Figure 2.11 Free radical attacking the nearest stable molecule.

(Source: http://www.bestofbothworldsaz.com/morph/Free radical.htm)

2.2.2 Reactive oxygen species (ROS)

Reactive oxygen species (ROS) are derived from the metabolism of molecular oxygen. Most reactive ROS are endogenously generated as by-products during mitochondrial electron transport, phagocytes, exercise, and inflammation and some ROS are externally generated by environmental pollutants, cigarette smoke, stress, ultraviolet light, ozone and certain drugs (Buechter, 1988; Halliwell, 1999). In addition ROS are formed as necessary intermediates of metal catalyzed oxidation reactions. Atomic oxygen has two unpaired electrons in separate orbits in its outer electron shell. This structure makes oxygen susceptible to radical formation. The sequential reduction of oxygen through the addition of electrons leads to the formation of a number of ROS including: superoxide, hydrogen peroxide, hydroxyl radical, hydroxyl ion, and nitric oxide (Figure 2.12).

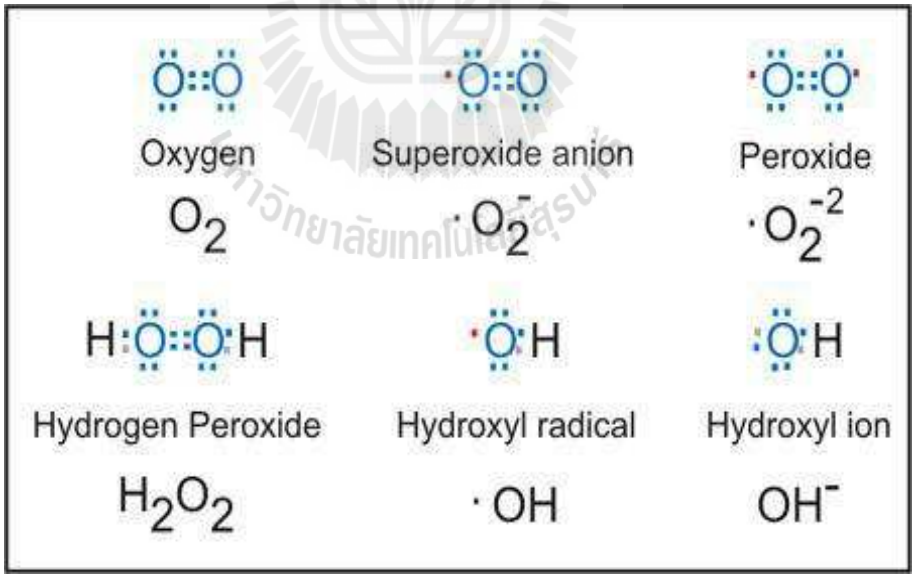


Figure 2.12 The common ROS.

(Paul Held, 2010)

2.2.3 Cellular defense against ROS

The human body has several mechanisms to counteract damage by free radicals and other ROS. These act on different oxidants in different cellular compartments. The protection of cells from ROS has two systems. First is a system of enzymes including superoxide dismutases, glutathione peroxidases and catalase, which decrease concentrations of most harmful oxidants in the tissue (Lea, 1966). Several essential minerals including copper, zinc, selenium and manganese are necessary for the formation or activity of these enzymes. The second system of defence against free radical damage is the presence of antioxidants. Antioxidants are molecule stable enough to donate an electron to a rampaging free radical and neutralize it, thus reducing its capacity to damage. Some such antioxidants including uric acid, glutathione, ubiquinol are produced during normal metabolism in the body. Although about 4000 antioxidants have been identified, the best known are vitamin C, vitamin E and carotenoids. Many other non-nutrient food substances, generally phenolic or polyphenolic compounds, display antioxidant properties and, thus, may be important for health (Harman, 1992; Lea, 1966).

2.2.4 Antioxidants

Antioxidants are substances or enzyme, or other substances that can prevent or slow oxidation of the starting material or the substrate. The substrates can react in the cell. These include the substances of nearly all types in the body such as proteins, lipids, carbohydrates, and DNA (Aboul-Enein et al., 2005; Adom et al., 2005). Occasionally, free radicals are produced at high amount and outpace the antioxidation reactions by antioxidants in the body. Oxidative stress has effects on the cell, such as

the oxidation of carbohydrates, proteins and DNA. In addition, it breaks molecules having (S-H) bonds and membrane (Adom et al., 2005). The effect on cells and cell damage lead to the severity of coronary artery, cancer and immune disease. Normally, antioxidant substance is divided into two categories: the antioxidants found in the body and the antioxidants found in food. The antioxidants found in the body are enzyme and non-enzyme types superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPX), glutathione reductase (GR), and glutathione transferase (GST). Non-enzymes antioxidants in the body are the substances such as glutathione, lipoic acid, ceruloplasmin, albumin, transferrin, haptoglobin, hemopexin, uric acid, bilirubin, and cysteine. The antioxidants found in food, include vitamin E, carotenoids, ascorbic acid, ubiquinone, thiols, imosins, pyruvate, gallic acid, phenolics, and flavonoids (Benzie, 2003; Brand-Williams et al., 1995; Caill et al., 2007; Calliste et al., 2001).

Antioxidants destroy free radicals by binding with free radicals to reduce the reaction at the start or stop the chain reaction, the reaction does not occur. When there are fewer free radicals the initial reaction was less and the effect to cell membrane or the DNA is less, so that the potential risk of these diseases is reduced too.

There are many studies on the antioxidant effects on normal subjects or patients with cancer or other diseases. Blot et al. (1993) studied about the antioxidants and the risk of cancer, in Chinese patients with cancer, and women who were at high risk for cancer. The result showed that beta carotene, vitamin E and C could reduce the incidence of stomach cancer and other cancer significantly, while Anonymous (1994) reported about the prevention of cancer by the use of vitamin E and beta-

carotene. The result found that beta-carotene to the rate of lung cancer is to quit smoking increased significantly. However, vitamin E had no effect either.

2.3 Antioxidants from plants

Although oxidation reactions are crucial for life, they can also be damaging. Hence, plants and animals maintain complex systems of multiple types of antioxidants, such as glutathione, vitamin C, and vitamin E as well as enzymes such as catalase, superoxide dismutase and various peroxidases (Sies, 1993). Low levels of antioxidants, or inhibition of the antioxidant enzymes, cause oxidative stress and may damage or kill cells.

Antioxidants in fruits and vegetables provide protection against harmful free radicals and have been strongly associated with reduced risk of chronic diseases, such as diabetes, cancer, cardiovascular disease, and Alzheimer's disease (Cao et al., 1996; Chen et al., 2006; Lindley, 1998; Liu, 2004; Velioglu et al., 1998; Vinson et al., 1998). These antioxidants include phenoic compounds, flavonoids, vitamins, carotenoids and endogenous metabolites (Finocchiaro et al., 2010). Phenolic compounds are found in a variety of foods such as fruits, vegetables and grains. The concentration and types of compounds obtained from these plants are different due to the environmental factors, genetic factors and processing condition (Kris-Etherton et al., 2002).

Plants are a major source of antioxidants. Antioxidants are found abundantly in beans, grain products, fruits and vegetables. Fruits with different color provide different beneficial compounds-lutein in some of the yellow pigments found in corn; beta-carotene in cantaloupe and mango; lycopene in tomatoes and watermelon, and

anthocyanins in berries. It is best to obtain these antioxidants from foods instead of supplements. Many studies have demonstrated the relationship between antioxidants capacity of consumed food and the reduction of health problems. For examples, Abdel-Aal et al. (2006); MacGhie et al. (2007); and Ryu et al. (1998) reported antioxidant capacity of the pigmented rice/or its extract in both *in vitro* and *in vivo* models, as well as other biological effects of the extracts including antimutagenic and anticarcinogenic activities (Nam et al., 2005). A recent study found that five servings of fruits and vegetables reduce the risk of stroke by 25%. Antioxidants may also enhance immune defense and therefore lower the risk of cancer and infection. In addition, they also minimize the oxidative stress caused by smoking and sunburn. (www.healthcastle.com/antioxidant, 2012). Polyphenol and flavonoids are antioxidants. There is general belief that the phenolics present in plant food contribute to the prevention of oxidative damage that is implicated in a range of diseases, including aging, cardiovascular diseases and cancer (Lin et al., 2006; Scabert et al., 2000).

A number of antioxidants from plants and their benefits to health have been reported. Black tea flavonoids (such as catechins, theaflavins and thearubigins) possess strong antioxidant properties. They protect the body from damage caused by free radical-induced oxidative stress. Grape seeds contain compounds called procyanidolic oligomers, known as PCOs or pycnogenols. They have long been seen as the most powerful phyto-antioxidants. As antioxidants, they are around 50 times stronger than vitamin E (www.ezineArticles.com/1936915, 2012). The dark blue/purple pigments of the bilberry contain flavonoids that have been shown to improve micro-circulation and act as very effective antioxidants. Bilberry is most well

known for its benefits on eye health (www.ezineArticles.com/1936915, 2012). Carrots get their bright carrot color from carotenoids, pigment antioxidants that are powerful quenchers of damaging free radicals. The most well known carotenoid in carrots is the antioxidant beta-carotene, which converts in the body to vitamin A. Carrots also contain the antioxidant vitamin E (Ichikawa et al., 2001). These are some examples from many reports which suggest that plants are rich in antioxidants.

2.4 Antioxidants in rice

Rice is an important agricultural commodity and the main staple in the diet for many Asian countries. With growing concerns regarding national health and expanding markets of functional products worldwide, some special rice cultivars, including giant embryonic rice, black rice, and red rice are being developed (www.charpa.co.th., 2010). Recently, research on the health benefits of bioactive compounds from diverse rice crops has increased extensively. Among these rice cultivars, pigmented rice was reported to have the capability of preventing atherosclerosis in mouse model and human study. These results may in part be attributable to the presence of natural antioxidants (Lu and Foo, 2001; Oki et al., 2002; Xia et al., 2003). Moreover, pigmented rice was reported to have a greater antioxidant capacity than white rice (Choi et al., 2007; Lin and Weng, 2006; Ryu et al., 1998; Sompong et al., 2010).

Black rice is an economically important rice species and derives its name from its richness in natural anthocyanin compounds (Figure 2.13), such as cyanidin 3-glucoside and peonidin 3-glucoside, which possess anti-oxidative and anti-inflammatory activities (Hu et al., 2003). Previous investigations have shown that

dietary supplementation of black rice pigment significantly inhibits atherosclerotic plaque formation in rabbits (Ling et al., 2001). In addition, black rice contains many beneficial components, including polyphenolics, flavonoids, vitamin E, phytic acid, and c-oryzanol. These antioxidant compounds eliminate reactive oxygen species (ROS) such as lipid peroxide and superoxide anion radicals and lower cholesterol content (Ichikawa et al., 2001; Nam et al., 2005).

Flavonoids are one group of phenolics, which consists of two aromatic rings linked by 3 carbons that are usually in an oxygenated heterocycle ring (Liu, 2004). Anthocyanins are a group of reddish to purple water-soluble flavonoids that are the primary pigments in the red and black grains, and have been widely identified and characterized in cereal grains (Abdel-Aal et al., 2006). The major components of anthocyanins in coloured rice are cyaniding-3-O-β-glucoside and peonidin-3-O-β-glucoside (Abded-Aal et al., 2006; Yawadio et al., 2007). In addition to this, there have been a few reports on characterization of other flavonoids such as flavonols, flavanols, flavones, and flavanones. Proanthocyanidin (PA or PAC), also known as procyanidin, oligomeric proanthocyanidin (OPC), is a class of flavanols. Proanthocyanidins are essentially polymer chains of flavonoids such as catechins (www.herbalchem.net, 2011). Proanthocyanidins represent a group of condensed flavan-3-ols, such as procyanidins, prodelphinidins and propelargonidins that can be found in many plants, most notably apples, maritime pine bark, cinnamon, cocoa, grape seed, grape skin, and black grains (Shen et al., 2009). However, bilberry, cranberry, black currant, green tea, black tea, and other plants also contain these flavonoids. Proanthocyanidins have strong antioxidant properties. Foods rich in proanthocyanidins have high free radical absorbance capacity which has been linked

to numerous health benefits such as weight management, cell health, and cardiovascular health (Dykes and Rooney, 2007). Scientists continue to research the relevance of proanthocyanidins' strong anti-oxidant properties *in vivo* for such applications as cancer prevention and cardiovascular health (Dykes et al., 2007). United States Department of Agriculture (USDA) does maintain a database of proanthocyanidin content and structure for many foods, but dietary supplements proanthocyanidin content has not been well documented (Cai et al., 2004; Dykes et al., 2007).

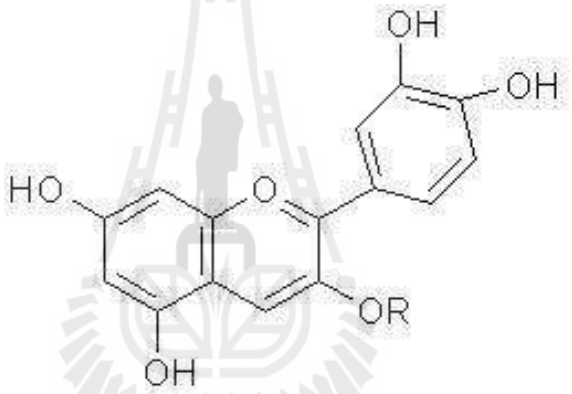


Figure 2.13 Chemical structure of anthocyanin.
(Rodrigo et al., 2011)

2.5 Antioxidant assays

After several studies on the importance of antioxidant in biological systems by counteracting of oxidative stress that causes several human diseases including chronic inflammation, diabetes mellitus, arthrosclerosis, neurodegenerative disorders, and certain types of cancer have been conducted, there is a great interest of quantification of antioxidants and determination of antioxidant capacities of a number of specific

and food compound. In June 2004, Orlando have the first was convened on antioxidant methods by International Congress to the purpose of dealing with analytical issues relative to assessing antioxidant capacity (AOC) in food, nutraceuticals, botanicals, and other dietary supplements. Keyword from this Congress, summarize will be to dealing with chemistry of antioxidant analytical methods (Halliwell et al., 1995; Huang et al., 2005).

In the biological systems, the factor that provides challenge in the assay of antioxidant capacity is have at least four normally sources of antioxidants 1) enzymes such as catalase, superoxide dismutase, and glutathione peroxidase 2) large molecules, for example, proteins, albumin, and ferritin 3) small molecules including glutathione, ascorbic acid, uric acid, carotenoids, polyphenols and 4) hormones (melatonin, estrogen, etc.). In addition, there have the source multiple of free radical and oxidant including nitric oxide ($NO^{\cdot}$), superoxide anion ($O_2^{\cdot -}$), hydroxyl ($HO^{\cdot}$), etc. In the some cases of antioxidants may act by a different single mechanism depending on the reaction system and by multiple mechanisms in a single system.

The literature, to have many method to developed and tested, these methods have still been discussed for advantages and limitations. The standard method for claiming antioxidant capacity, it does not seem to have a consensus for concluding the most convenient method, such as, carrying out the analysis in the physiological irrelevance pH, the limitations for determination of hydrophilic antioxidants, the concern on light sensitivity of initiators or probes, the problems occurring in determination of reaction endpoint, possible interference from certain food components, the use of different standards for expressing results that causes the difficulties in comparison.

2.5.1 Antioxidant mechanisms

In the literature for the reaction mechanisms have to the confusion foe the need to provide a protocol involving measurement of more than one property multiple activities of polyphenols is outlined and the dominant activity depends on the medium and type of antioxidants. The source of antioxidant have the response may be different to different radical or oxidation. For example, carotenoids are exceptional singlet-oxygen scavengers but are not particularly good quenchers of peroxyl radicals relative to phenolics. Therefore, the source of all free radical or all antioxidants in a complex system, there are not a single assay accurately reflecting the mechanism of reaction (Prior et al., 2005).

On the basis, antioxidants can inactivate free radicals by two major mechanisms: 1) hydrogen atom transfer (HAT) reaction and 2) single electron transfer (SET) reaction. Both the based methods to determine the mechanism and the efficiency of antioxidants. In the all sample, almost always occur of HAT and SET mechanisms and can be made difficult to their difference. Huang et al. (2005) and MacDonald-Wicks et al. (2006), they were study to Trolox equivalent antioxidant capacity (TEAC), Total phenol assay by using the Folin-Ciocalteu Reagent (FCR), and and 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging capacity assays was considered under the methods utilizing ET mechanism, whereas Prior et al. (2005), study to classified under the methods utilizing both SET and HAT mechanisms, because difficulty in the interpretation of inhibition mechanisms of these radicals.

2.5.1.1 Hydrogen atom transfer (HAT) reaction

In Prior et al. (2005), report, HAT-based methods measure the classical ability of an antioxidant to quench free radicals by hydrogen donation to form stable compounds. HAT-based methods are more relevant to the radical chain-breaking antioxidant capacity.

$$X^{\cdot} + AH \longrightarrow XH + A^{\cdot}$$

The peroxyl radicals play a key role in the unwanted lipid oxidation in food and biological systems measured by HAT-based method. The sacrificial antioxidants, represented by vitamin E, are critical in protecting polyunsaturated fatty acid esters in foods and in cell membranes from autoxidation. Vitamin E functions through a HAT mechanism. A HAT-based method, represented by the oxygen radical absorbance capacity (ORAC), total radical-trapping antioxidant parameter (TRAP) assays, involves peroxyl radicals as the oxidant and provides a useful information on radical chain-breaking capacity (Dejian et al., 2005). Moreover, it can be translated to its inhibition capacity of fatty acid autoxidation in an actual food system.

ORAC assay: Glazer (1990) and Ghiselli et al. (1995) they are is the based upon the early work of ORA assay, Cao et al. (1993) developed further. ORAC measures antioxidant inhibition of peroxyl radical induced oxidations and thus reflects classical radical chain breaking antioxidant activity by H atom transfer (Ou et al., 2001). In the general assay, the peroxyl radical reacts with a fluorescent probe to form a nonfluorescent product, which can be quantitated easily by fluorescence. ORAC values are usually reported as Trolox equivalents. A standard curve is generated using the AUC for five standard concentrations of Trolox, and the Trolox equivalents of the samples are calculated using the following linear or quadratic relationships (Y = a +

bX, linear; or $Y = a + bX + cX^2$) quadratic) between Trolox concentration (Y)(μM) and the net area under the FL decay curve (X) ($AUC_{sample} - AUC_{blank}$). Data are expressed as micromoles of Trolox equivalents (TE) per liter or per gram of sample (μmol of TE/g or μmol of TE/L) (Ou et al., 2001; Prior et al., 2003). The ORAC assay has been used to study the AOC of many compounds and food samples (Davalos et al., 2004; Prior et al., 2003; Wada and Ou, 2002; Wang et al., 1996). Industry has accepted the method to the point that some nutraceutical manufacturers are beginning to include ORAC values on product labels (www. nw.com, 2004).

TRAP assay: This method monitors the ability of antioxidant compounds to interfere with the reaction between peroxyl radicals generated by AAPH or ABAP [2,2′-azobis(2-amidinopropane) dihydrochloride] and a target probe (Ghiselli et al., 1995). Different variations of the method have used oxygen uptake (Wayner et al., 1985), fluorescence of R-phycoerythrin Ghiselli et al., 1995), or absorbance of 2, 2′-azinobis (3-ethylbenzothiazoline-6-sulfonic acid (ABTS) (Bartosz et al., 1998) as the reaction probe. The basic reactions of the assay are similar to those of ORAC. Requirements for the assay are that the probe must be reactive with $ROO^{\cdot}$ at low concentrations, there must be a dramatic spectroscopic change between the native and oxidized probe (to maximize sensitivity), and no radical chain reaction beyond probe oxidation should occur. Typically, oxidation of the probe is followed optically (Bartosz et al., 1998) or by fluorescence (Ghiselli et al., 1995). Antioxidant activity has been determined as time to consume all of the antioxidant, by extension of the lag time for appearance of the oxidized probe when antioxidants are present, and by percent reduction of a reaction. TRAP values are usually expressed as a lag time or reaction time of the sample compared to corresponding times for Trolox. The

TRAP assay is designed and is most often used for measurements of in vivo AOC in serum or plasma because it measures nonenzymatic antioxidants, such as glutathione, ascorbic acid, α-tocopherol, and β-carotene (Wayner et al., 1985).

2.5.1.2 Single electron transfer (SET) reaction

SET-based methods detect the ability of a potential antioxidant to transfer one electron to reduce any compounds including metals, carbonyls, and radicals.

$$M\ (III) + AH \longrightarrow AH^{\bullet} + \mathbf{M\ (II)}$$

In SET methods, relative reactivity is based on deprotonation and ionization potential of the reactive functional group, so SET reactions are pH dependent. In general, ionization potential values decrease with increasing pH, reflecting increased electron-donating capacity with deprotonation (Prior et al., 2005). The pH values have an important effect on the reducing capacity of antioxidants. At acidic conditions, the reducing capacity may be restrained due to prorogation on antioxidant compounds, whereas, in basic conditions, proton dissociation of phenolic compounds would increase sample's reducing capacity (Huang et al. 2005). SET reactions can be relatively slow and need long times to reach completion so traditionally measure relative percent decrease in product rather than kinetics or total antioxidant capacity (Ozgen et al., 2006). Compared to HAT, the SET mechanism is strongly solvent dependent due to solvent stabilization of the charged species (Ou et al., 2002).

Ferric Reducing Antioxidant Power (FRAP) is general chemistry to AOC methods utilizing SET reaction mechanisms. The FRAP assay was originally

developed by Benzie (1996) to measure reducing power in plasma, but the assay subsequently has also been adapted and used for the assay of antioxidants in botanicals (Benzie and Szeto, 1999; Gil, 2000). The reaction measures reduction of ferric 2, 4, 6-tripyridyl- s-triazine (TPTZ) to a colored product (Benzie, 1996). The reaction detects compounds with redox potentials of < 0.7 V (the redox potential of Fe^{3+}-TPTZ), so FRAP is a reasonable screen for the ability to maintain redox status in cells or tissues. Reducing power appears to be related to the degree of hydroxylation and extent of conjugation in polyphenols (Pulido et al., 2000). However, FRAP cannot detect compounds that act by radical quenching (H transfer), particularly thiols and proteins (Benzie and Szeto, 1999). This causes a serious underestimation in serum.

The FRAP assay does not measure thiol antioxidants, such as glutathione. FRAP actually measures only the reducing capability based upon the ferric ion, which is not relevant to antioxidant activity mechanistically and physiologically. However, in contrast to other tests of total antioxidant power, the FRAP assay is simple, speedy, inexpensive, and robust and does not require specialized equipment. The FRAP assay can be performed using automated, semiautomatic, or manual methods.

Although the TEAC and DPPH assays are usually classified as SET reactions, these two indicator radicals in fact may be neutralized either by direct reduction via electron transfers or by radical quenching via H atom transfer (Jimenez et al., 2004). Reactivity patterns and mechanisms are thus difficult to interpret without detailed information about the composition and structures of antioxidants being

tested. Interpretation is particularly difficult when small molecule reducing agents such as ascorbic acid are present in extracts of phenols.

TEAC or ABTS assay was first reported by Miller et al. (1993), which is based on the scavenging ability of antioxidants to the long-life radical anion $ABTS^{\bullet+}$. In this assay, ABTS is oxidized by peroxyl radicals or other oxidants to its radical cation, $ABTS^{\bullet+}$, which is intensely colored, and AOC is measured as the ability of test compounds to decrease the color reacting directly with the $ABTS^{\bullet+}$ radical. Results of test compounds are expressed relative to Trolox.

TEAC assay is operationally simple and has been used in many research laboratories. Absorption maxima were shown to be at wavelengths of 414, 752, and 842 nm in aqueous media and 414, 730, and 873 nm in ethanolic media (Arnao, 2000). Another advantage of TEAC assay is t hat it permits study over a wide pH range. Although frequently used at pH 7.4, the stability of ABTS radical at this pH has been reported to be problematic. For standard antioxidants such as Trolox or ascorbic acid, ABTS radical at pH 7.4 provided reliable endpoint values after 10 min. However, with standard phenolics, the results at 10 min are estimates only and do not represent equilibrium endpoint values based on oxidation. Also, with ABTS radical at pH 7.4, values for the antioxidant capacity of the standard phenolics were 5– 20 % greater than the values determined at pH 4.5 (Ozgen et al. 2006).

2, 2-Diphenyl-1-picrylhydrazyl (DPPH) assay. The radical is one of the few stable organic nitrogen radicals, which bears a deep purple color. It is commercially available and does not have to be generated before assay like $ABTS^{\bullet+}$. This assay is based on the measurement of the reducing ability of antioxidants toward $DPPH^{\bullet}$. The ability can be evaluated by electron spin resonance (EPR) or by

measuring the decrease of its absorbance. The widely used decoloration assay was first reported by Brand-Williams et al. (1995). Antioxidant assays are based on measurement of the loss of DPPH color at 515 nm after reaction with test compounds (Bonder et al., 1997), and the reaction is monitored by a spectrometer. The percentage of the DPPH remaining is calculated as

$$\% \text{ DPPH}^{\bullet}_{REM} = 100 \text{ x } [\text{DPPH}^{\bullet}_{REM}]/[\text{DPPH}^{\bullet}]_{T=0}$$

The percentage of remaining DPPH$^{\bullet}$ (DPPH$^{\bullet}_{REM}$) is proportional to the antioxidant concentration, and the concentration that causes a decrease in the initial DPPH$^{\bullet}$ concentration by 50% is defined as EC_{50}. The time needed to reach the steady state with EC_{50} is defined as T_{EC50}. Sanchez-Moreno et al. (1998) further introduced another parameter to express antioxidant capacity, called “antiradical efficiency (AE)”. It was defined as

$$AE = 1/EC_{50}T_{EC50}$$

The DPPH assay is considered to be mainly based on an SET reaction, and hydrogen-atom abstraction is a marginal reaction pathway (Ou et al., 2005).

DPPH assay, the test is simple and rapid and needs only a UV -vis spectrophotometer to perform, which probably explains its widespread use in antioxidant screening. However, interpretation is complicated when the test compounds have spectra that overlap DPPH at 515 nm. Carotenoids, in particular, interfere (Noruma et al., 1997). Use of DPPH to measure AOC is plagued by many drawbacks. The assay is not a competitive reaction because DPPH is both radical probe and oxidant. DPPH color can be lost via either radical reaction (HAT) or reduction (SET) as well as unrelated reactions, and steric accessibility is a major determinant of the reaction. Thus, small molecules that have better access to the

radical site have higher apparent AOC with this test. DPPH has a relatively small linear reaction range of only 2 -3-fold. DPPH is stable nitrogen radical that bears no similarity to the highly reactive and transient peroxyl radicals involved in lipid peroxidation. Many antioxidants that react quickly with peroxyl radicals may react slowly or may even be inert to DPPH due to steric inaccessibility. DPPH also is decolorized by reducing agents as well as H transfer, which also contributes to inaccurate interpretations of AOC. Thus, AOC is not fairly rated by the ability of antioxidants to react with DPPH.

2.6 Fourier transform infrared spectroscopy (FT-IR)

2.6.1 Definition

FT-IR stands for Fourier Transform Infrared, the preferred method of infrared spectroscopy. In infrared spectroscopy, IR radiation is passed through a sample. Some of the infrared radiation is absorbed by the sample and some of it is passed through (transmitted). The resulting spectrum represents the molecular absorption and transmission, creating a molecular fingerprint of the sample. Like a fingerprint no two unique molecular structures produce the same infrared spectrum (Griffiths and De Hasseth, 2007). This makes infrared spectroscopy useful for several types of analysis, including, it can identify unknown materials; can determine quality or amount of components in mixture of sample (Figure 2.14). Therefore, FT-IR is preferred over dispersive of fuller methods in IR analysis for several reasons, such as it can increase speed or collecting a scan every second, provides a precise measurement method which requires no external calibration, increase sensitivity-one second can be co-

added together to ratio out random noise, and mechanically simple with only one moving part etc.

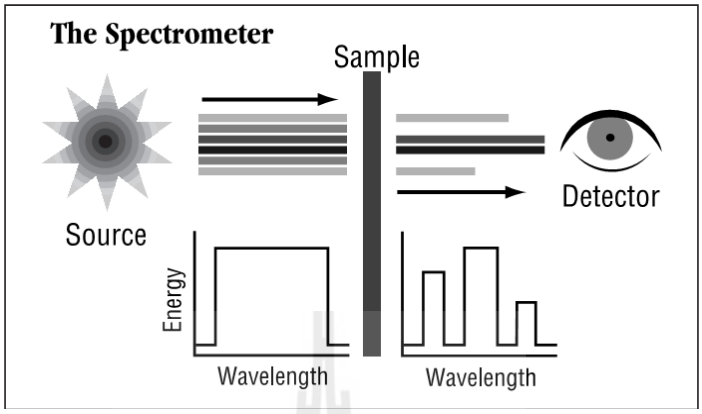


Figure 2.14 The principle of spectrometer.

(Griffiths and De Hasseth, 2007)

2.6.2 Infrared spectroscopy

Infrared (IR) spectroscopy is one of the most common spectroscopic techniques used by organic and inorganic chemists. Simply, it is the absorption measurement of different IR frequencies by a sample positioned in the path of an IR beam. The main goal of IR spectroscopic analysis is to determine the chemical functional groups in the sample. Different functional groups absorb characteristic frequencies of IR radiation. Using various sampling accessories, IR spectrometers can accept a wide range of sample types such as gases, liquids, and solids. Thus, IR spectroscopy is an important and popular tool for structural elucidation and compound identification.

Infrared radiation spans a section of the electromagnetic spectrum having wavenumbers from roughly 13,000 to 10 cm^{-1} or wavelengths from 0.78 to 1000 μm. It is bound by the red end of the visible region at high frequencies and the microwave region at low frequencies. IR absorption positions are generally presented as either

wavenumbers or wavelengths (ƛ). Wavenumber defines the number of waves per unit length. Thus, wavenumbers are directly proportional to frequency, as well as the energy of the IR absorption. The wavenumber unit (cm^{-1}, reciprocal centimeter) is more commonly used in modern IR instruments that are linear in the cm^{-1} scale. In the contrast, wavelengths are inversely proportional to frequencies and their associated energy. IR absorption information is generally presented in the form of a spectrum with wavwnumber (Figure 2.15).

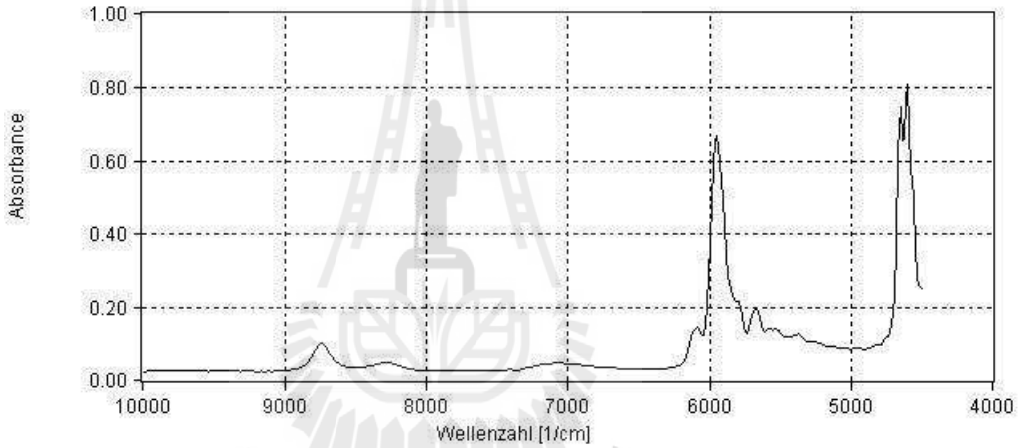


Figure 2.15 The infrared spectrum of polystyrene.
(Shabaka, 1992)

The IR region is commonly divided into three small areas: near IR, mid IR, and far IR (Figures 2.16 and 2.17). This in study focuses on the most frequently used mid IR region, between 4,000 and 400 cm^{-1} (2.5 to 25 μm), because the majority of FT-IRs operate in this wavenumber range.

	Near IR	**Mid IR**	**Far IR**
Wavenumber	**13,000-4,000 $^{cm-1}$**	**4,000-200 $^{cm-1}$**	**200-10 $^{cm-1}$**
Wavenumber	**0.78-2.5 μm**	**2.5-50 μm**	**50-1,000 μm**

Figure 2.16 The three areas wavenumber of infrared spectrum.

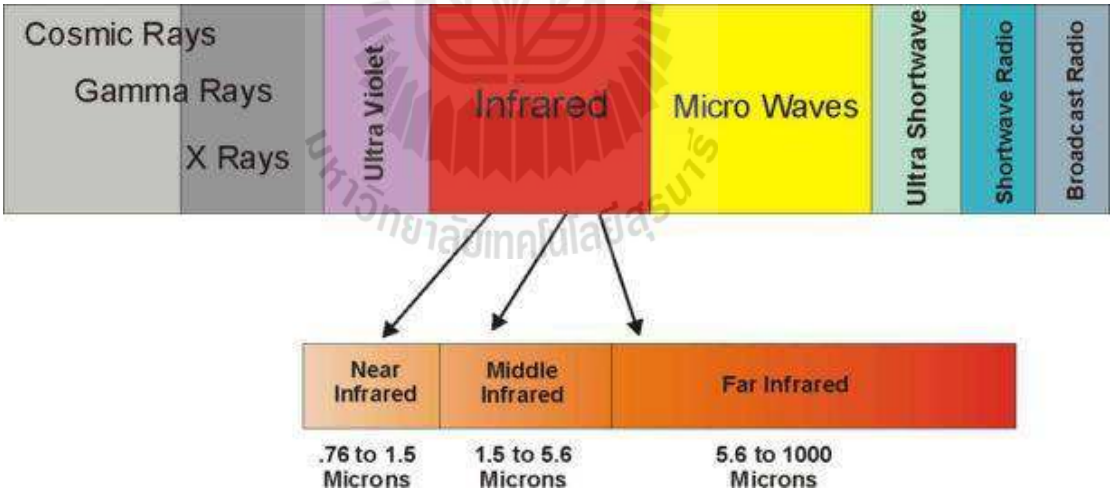


Figure 2.17 Infrared spectrum.

(Shanshan et al., 2005)

2.6.3 Infrared absorption

At temperatures above absolute zero, all the atoms in molecules are in continuous vibration with respect to each other. When the frequency of a specific vibration is equal to the frequency of the IR radiation directed on the molecule, the molecule absorbs the radiation. Each atom has three degrees of freedom, corresponding to motions along any of the three Cartesian coordinate axes (x, y, z). A polyatomic molecule of n atoms has 3n total degrees of freedom. However, 3 degrees of freedom are required to describe translation, the motion of the entire molecule through space. Additionally, 3 degrees of freedom correspond to rotation of the entire molecule. Therefore, the remaining 3n – 6 degrees of freedom are true, fundamental vibrations for nonlinear molecules. Linear molecules possess 3n – 5 fundamental vibrational modes because only 2 degrees of freedom are sufficient to describe rotation.

The total number of observed absorption bands is generally different from the total number of fundamental vibrations. It is reduced because some modes are not IR active and a single frequency can cause more than one mode of motion to occur. Conversely, additional bands are generated by the appearance of overtones (integral multiples of the fundamental absorption frequencies), combinations of fundamental frequencies, differences of fundamental frequencies, coupling interactions of two fundamental absorption frequencies, and coupling interactions between fundamental vibrations and overtones or combination bands (Fermi resonance). The intensities of overtone, combination, and difference bands are less than those of the fundamental bands. The combination and blending of all the factors thus create a unique IR spectrum for each compound. The major types of molecular vibrations are stretching

and bending. The various types of vibrations are illustrated in Figure. 2.18. Infrared radiation is absorbed and the associated energy is converted into these type of motions. The absorption involves discrete, quantized energy levels. However, the individual vibrational motion is usually accompanied by other rotational motions. These combinations lead to the absorption bands, not the discrete lines, commonly observed in the mid IR region.

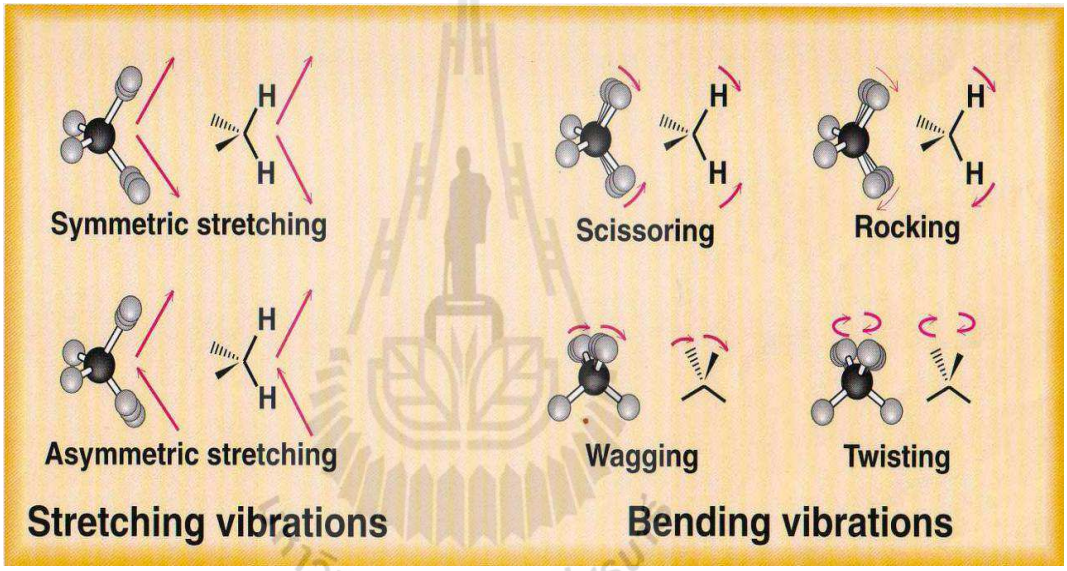


Figure 2.18 Molecular vibration: molecular absorb energy when the frequency of electromagnetic wave, is equal to the frequency of oscillation (the infrared).

(Yue and Hongwei, 2012)

2.6.4 Simplified optical layout of a typical FT-IR spectrometer

In simple terms, IR spectra are obtained by detecting changes in transmittance (or absorption) intensity as a function of frequency. Most commercial instruments separate and measure IR radiation using dispersive spectrometers or Fourier transform spectrometers. Fourier transform spectrometers have recently replaced dispersive

instruments for most applications due to their superior speed and sensitivity. They have greatly extended the capabilities of infrared spectroscopy and have been applied to many areas that are very difficult or nearly impossible to analyze by dispersive instruments. Instead of viewing each component frequency sequentially, as in a dispersive IR spectrometer, all frequencies are examined simultaneously in Fourier transform infrared (FT-IR) spectroscopy.

The normal instrumental process (Figure 2.19) begins when Infrared energy is emitted from a glowing black-body source. This beam passes through an aperture which controls the amount of energy presented to the sample (and, ultimately, to the detector). Of them the beam enters the interferometer where the “spectral encoding” takes place. The resulting interferogram signal then exits the interferometer. After that the beam enters the sample compartment where it is transmitted through or reflected off of the surface of the sample, depending on the type of analysis being accomplished. This is where specific frequencies of energy, which are uniquely characteristic of the sample, are absorbed. Finally of the beam passes to the detector for final measurement. The detectors used are specially designed to measure the special interferogram signal. Then the measured signal is digitized and sent to the computer where the Fourier transformation takes place. The final infrared spectrum is then presented to the user for interpretation and any further manipulation.

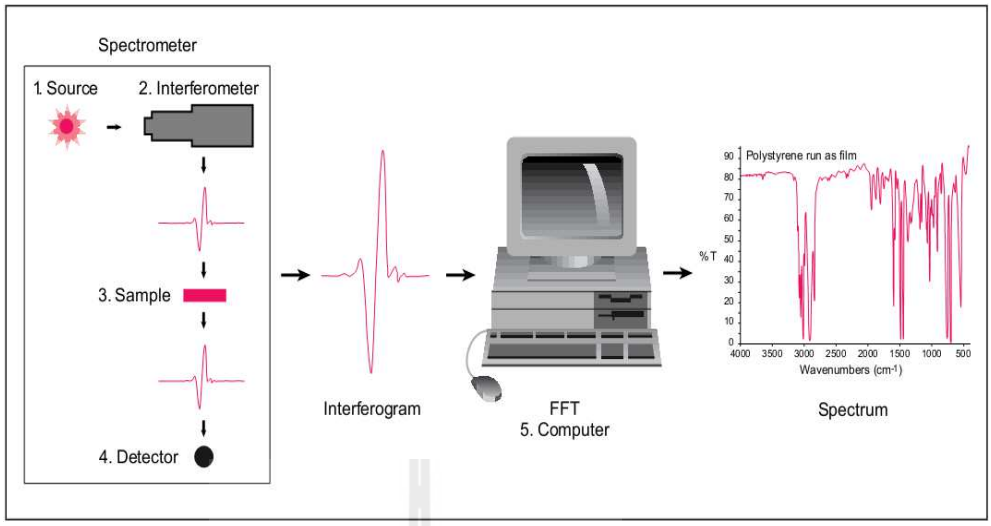


Figure 2.19 The sample analysis process of FT-IR spectroscopy.

(Griffiths and De Hasseth, 2007)

FT-IR instruments have distinct advantages over dispersive spectrometers: Better speed and sensitivity. A complete spectrum can be obtained during a single scan of the moving mirror, while the detector observes all frequencies simultaneously. There is only one moving part, the moving mirror, resulting in less wear and better reliability. Sensitivity is dramatically improved with FT-IR for many reasons. The detectors employed are much more sensitive, the optical throughput is much higher which results in much lower noise levels, and the fast scans enable the coaddition of several scans in order to reduce the random measurement noise to any desired level (referred to as signal averaging). Although the spectra of many samples can be satisfactorily run on either FT-IR or dispersive instruments, FT-IR spectrometers are the preferred choice for samples that are energy-limited or when in-creased sensitivity is desired. A wide range of sampling accessories is available to take advantage of the capabilities of FT-IR instruments.

2.6.5 Application of FT-IR in biology

Recently, spectroscopy has been used as a major tool for the identification of antioxidants in medical research, food science and industrial development. Advance research has been conducted to improve the evaluation by developing FTIR technique in which the results confer more accurate and rapid than conventional spectroscopy method. First, FRIT is used extensively in medicine to diagnostic some cancers. For example, prostate cancer cell can be differentiated from normal cell by determining the quantity of peptide in human tissue. In addition, Dimitrova and colleagues showed that breast cancer tumors contained the significantly change in the secondary peptide structure than that of normal cell as identified by FTIR. Second, the use in FTIR has been shown in food science to identify some ingredients containing in the products. For example, Mercela and colleagues characterized the component in food additive polysaccharides by FTIR at the 1,200-800 cm^{-1} wavenumber region and showing that those polysaccharides were glucomannan and carrageenan. Finally, FRIT has been used widely for the identification of antioxidant activities in fruit and vegetables. Recently, Xiaonan and colleagues have shown that total phenolic compound in garlics and elephant garlic from California, Oregon, Washington, and New York contain antioxidant activity by FTIR. Fruit extracts such as blueberry, grape and blackberry were found to contain antioxidant activity (Henry et al., 2005). Moreover, Augustin and colleague (2011) have shown that propolis extracts have the antioxidant activity by FTIR and UV-vis spectroscopic.

CHAPTER III

MATERIALS AND METHODS

3.1 Rice grain samples and preparation

Rice grains of forty-eight glutinous rice varieties including nineteen dark rice, twenty-seven red rice and two brown rice were obtained from Rice Research Center, Nong Khai province. They were grains from rice that were sown in June, transplanted on late July, and harvested in November in the year 2010 (Table 3.1). In this study, the color of the rice has been classified by the intensity of the color of the grain after dehusked. Two popular white rice e varieties: Khao Dawk Mali 105 (KDML 105) and Kor Kho 6 (RD 6) were included in the study. After harvest, the grains were dried to 13% ± 1% of moisture and kept at 4 ^{o}C.

Table 3.1 The varieties of glutinous color rice from Nong Khai province (N=48).

GS.NO	Rice varieties	The shell
00621	Niao-Dam	Black with brown spots
01708	Niao-Dam	Dam
02723	Niao-Dam	Dam
03359	Niao-Dam	Dam
03437	Niao-Dam	Dam
05563	Niao-Dam	Dam
05760	Niao-Dam	Dam
06008	Niao-Dam	Black with brown spots
06257	Niao-Dam	Black with brown spots
06722	Niao-Dam	Dam
06827	Niao-Dam-Noi	Yellow-Green
07278	Niao-Dam Tub Moo	Dam
07279	Niao-Dam Makleua	Dam
09379	Niao-Dam Tub Moo	Dam
09406	Niao-Dam	Dam
09474	Niao-Dam	Dam
09475	Niao-Dam	Black with brown spots
09476	Niao-Dam	Dam
14378	Niao-Dam Puang	Dam
15110	Niao-Dam	Dam
18014	Niao-Dam	Black with brown spots
19057	Niao-Dam	Yellow-Green
19845	Niao-Dam	Dam
21427	Niao-Dam	Black with brown spots
21626	Niao-Dam	Yellow-Green
21629	Niao-Dam	Black with brown spots
21831	Niao-Dam	Black with brown spots
21972	Niao-Dam Rai	Black with brown spots
91124	Krasaen Niao Daeng	Brown
7677	Kkao Klam	Dam
89038	Klam	Black with brown spots
91151	Niao-Dam	Black with brown spots
87061	Dor Khao Dam	Black with brown spots
88073	Kkao Klam	Black with brown spots
88083	Niao-Dam	Black with brown spots
9284	Kkao Klam	Black with brown spots
87009	Dor Eklam	Black with brown spots
91130	Kkao Klam	Black with brown spots
87046	Kkao Klam	Yellow-Green
89057	Niao-Dam	Black with brown spots
88013	Kkao Klam	Yellow-Green
88028	Niao-Dam	Yellow-Green
88168	Kkao Klam	Black with brown spots
88069	Kkao Klam	Black with brown spots

Table 3.1 (continued)**.**

GS.NO	Rice varieties	The shell
88063	Kkao Klam	Black with brown spots
88138	Kkao Klam	Black with brown spots
91195	Kkao Klam	Dam
87090	Kkao Klam	Dam
106971	Kkao Klam	Dam
9284	Kkao Klam	Dam

Grains of fifty rice varieties were dehusked using rice dehusking machine at Rice research center, Nakhon Ratchasima province. In order to prepare the cooked sample of rice grains, rice grains were soaked in water using 1:1.5 grain mass: water volume ratio and cooked over boiling water. Properly cooked grains were achieved if there was no transparent bit left in the grains and the grains turned soft. Both uncooked and cooked rice were freezed at -20 ^{o}C for one night. They were then freeze-dried for three days. The grains were grounded into flour in Satake Rice Machine (Stake Co., Japan). The resulted rice flour was passed through a 100-mesh sieve on a Cyclone Sample Mill (UDY Corporation, USA). Both raw and cooked samples were kept at 4 ^{o}C until use for the analysis in screw-capped plastic containers.

3.2 Antioxidant extraction from rice

Thirty ml of methanol containing 1% HCl was used for the extraction of 1 g each rice flour sample by mixing the flour into the solution and let stand at room temperature for 24 hr. The solution was collected and the extraction is repeated for one more time using the same rice flour sample. The methanolic extracts from the

first and the second round of extraction were pooled and centrifuged at 4,000 g for 15 min. The supernatant was collected and stored at 4 ^{o}C until used (Shen et al., 2009).

3.3 Chemicals and reagents

The Folin–Ciocalteu reagent, phenolic acid standards (gallic acid), 2, 2-Azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS), rutin, and potassium blue were purchased from Fluka-Merck (Austria). 6-hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid (Trolox), methanol, hydrochloric acid (HCL), and ammonium iron (III) sulfate dodecahydrate were purchased from Aldrich-Qrec (Austria).

3.4 Chemical assays

3.4.1 Total antioxidant capacity (TAC) assay

Total antioxidant capacity assays of rice extracts were carried out using a spectrophotometer by the improved 2, 2-azino-bis-(3-ehylbenzothiazoline-6-sulphonic acid) diammonium salt (ABTS) radical cation method as described in Bao et al. (2005) and Cai et al. (2004). The TAC was evaluated according to the Trolox equivalent antioxidant capacity (TEAC) method based on ABTS radical cation decoloration in the presence of antioxidants (Pellegrini et al., 1999). A spectrophotometer was used to monitor the decelerate of radical cation.

The method used for the ABTS radical cation preparation was based on the methods described in Bao et al. (2005) and Cai et al. (2004). The ABTS radical cation was prepared by reacting a 7 mM aqueous solution of ABTS with 2.45 mM (final

concentration) potassium persulfate (Appendix B), then allowing the mixture to stand in the dark at room temperature for 12-16 hr before use. Three point nine ml of the obtained $ABTS^{+}$ solution was taken for the absorbance measurement at 734 nm. The optical density (OD) read was adjusted to 0.70 ± 0.02 with methanol. The OD 0.7 reflects suitable amount of the ABTS radical cation of which the reaction with antioxidants can be easily followed. To perform the antioxidation reaction, 0.1 ml of rice extract was added and mixed thoroughly with the prepared $ABTS^{+}$ solution. The reaction mixture was kept at room temperature for 6 min, and after that the absorbance at 734 nm was recorded. Since the antioxidant capacity would be expressed as Trolox equivalent antioxidant capacity (mM TEAC), the graph representing the relationship between the amount of Trolox used in the antioxidation reaction and the OD read at 734 nm was produced and used as the standard (Figure 3.1). Trolox standard solution in 80% methanol at the concentrations of 0, 0.2, 0.4, 0.6, 0.8, 1.0, 1.2 and 1.4 mM (Appendix A) were prepared and assayed under the same condition: the reaction at room temperature for 6 min. Results were expressed in terms of Trolox equivalent antioxidant capacity (TEAC, mM Trolox equivalents per 1 g of rice dry weight).

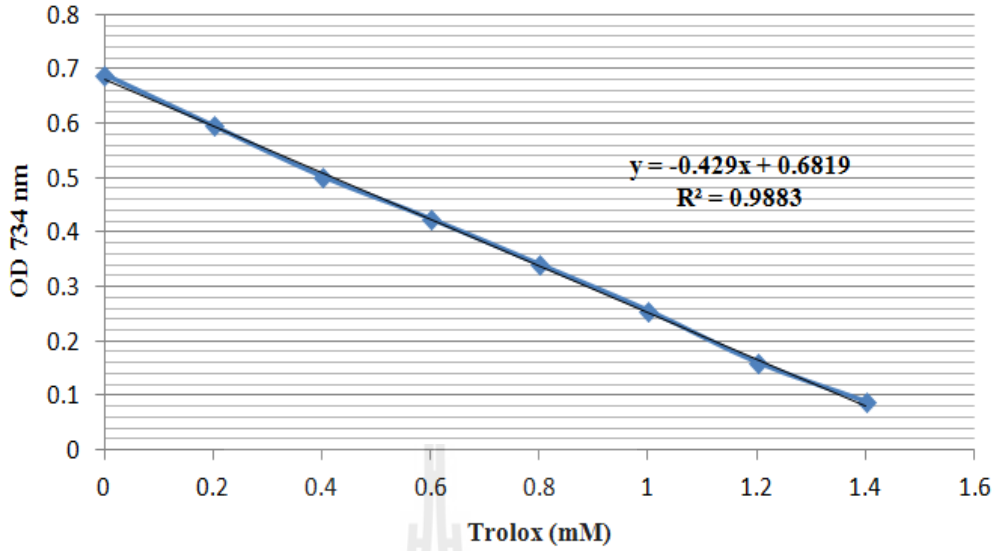


Figure 3.1 Calibration curve for Trolox equivalent antioxidant capacity values determination.

3.4.2 Determination of total phenolic compounds

Total phenolic content was assayed by the Folin-Ciocalteu colorimetric with slight modification (Bao et al., 2005; Cai et al., 2004; Shen et al., 2009). The aliquots (1.0 mL) of diluted rice extract solution were mixed with 0.5 ml of 0.5 N Folin-Ciocalteu reagent. The reaction was then neutralized to pH 7, with saturated carbonate solution (7.5 g/L) (Appendix B). After incubation for 2 h at room temperature, the resulted blue solution was, then taken to measure the absorbance at 760 nm using a spectrophotometer. The optical density read was used to determine the total phenolic compound in each reaction based on the calibration curve. The calibration curve was prepared using gallic acid solution, at concentrations of 0, 200, 400, 600, 800 and 1,000 mg/L as standard solutions (Appendix A) (Figure 3.2). The total phenolic

contents were expressed as mg/L of gallic acid equivalent (mg GAE) per 1 g of rice dry weight.

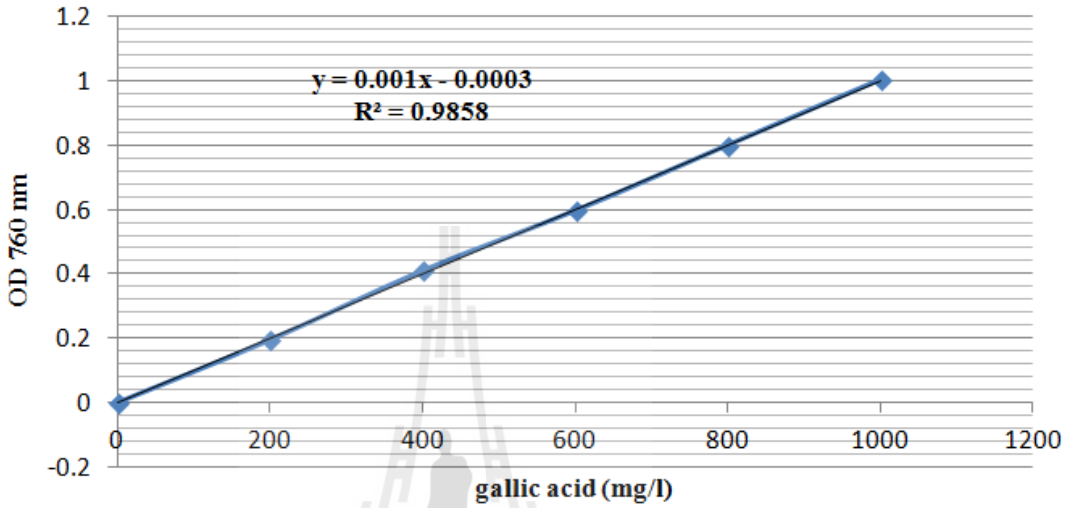


Figure 3.2 Calibration curve of gallic acid for total phenolic compound determination.

3.4.3 Determination of total flavonoid contents

Total flavonoid content was demonstrated by a colorimetric method (Bao et al., 2005). Aliquots of 0.5 mL of diluted rice extracts solution was pipetted into 15 mL polypropylene conical tubes containing 2 mL double distilled H_2O and then mixed with 0.15 mL of 5% $NaNO_2$ (Appendix B). The sample had been let to stand for 5 min before 0.15 mL of 10% $AlCl_3.6H_2O$ (Appendix B) was added to it. The mixture was allowed to stand for another 5 min, and then 1 mL 1 M NaOH (Appendix B) was added. The reaction solution was well mixed and kept for 15 min at room temperature. After that the absorbance was determined at 415 nm. Total flavonoid content was calculated using the standard rutin curve at concentrations of 0, 20, 40,

60, 80 and 100 µg/mL (Appendix A), and expressed as µg/ml rutin equivalent (µg RE) per 1 g of rice dry weight (Figure 3.3).

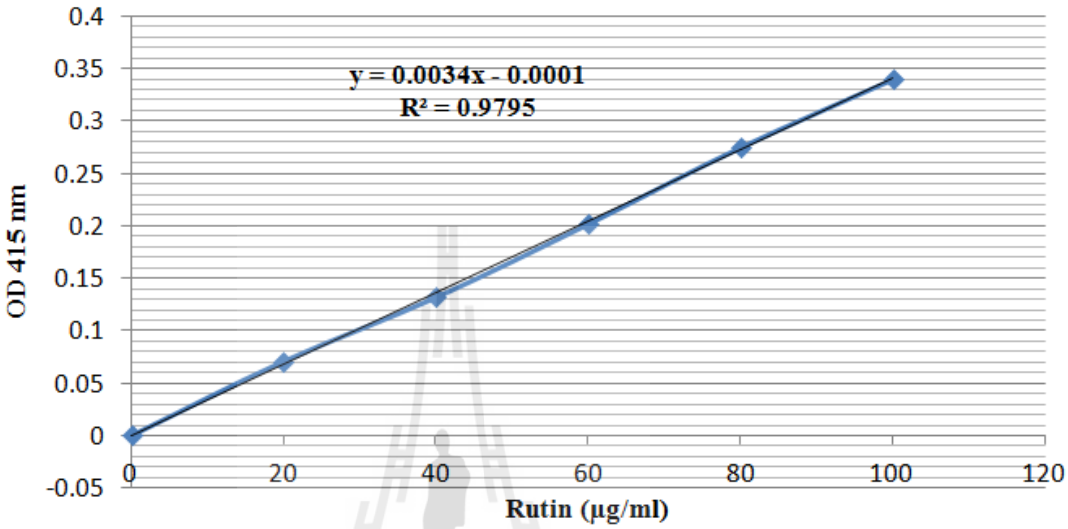


Figure 3.3 Calibration curve of rutin for total flavonoid content determination.

3.4.4 Determination of proanthocyanidins

Proanthocyanidins were quantified by butanol/HCl depolymerization on the TAC extracts (Porter et al., 1986). Into a glass tube sealed with Teflon-lined screw cap, 6 mL of butanol/concentrated HCl (95:5, v/v) and 100 µL of 2% (w/v) solution of $NH_4Fe(SO_4)_2.12H_2O$ in 2 M HCl (Appendix B) were added to 1 mL of methanol rice extracts. The solution was thoroughly mixed and warmed in a water bath run at 95.0 ± 0.2 ℃ for 50 min. Absorbance of the reaction mixture was measured at 540 nm using a spectrophotometer. The quantification was performed based on calibration curve of cyaniding chloride at concentrations of 0, 20, 40, 60, 80 and 100 µg/mL

(Appendix A). The proanthocyanidin contents in rice extract were expressed as µg/mL of cyaniding equivalents (Figure 3.4) per 1 g of rice dry weight

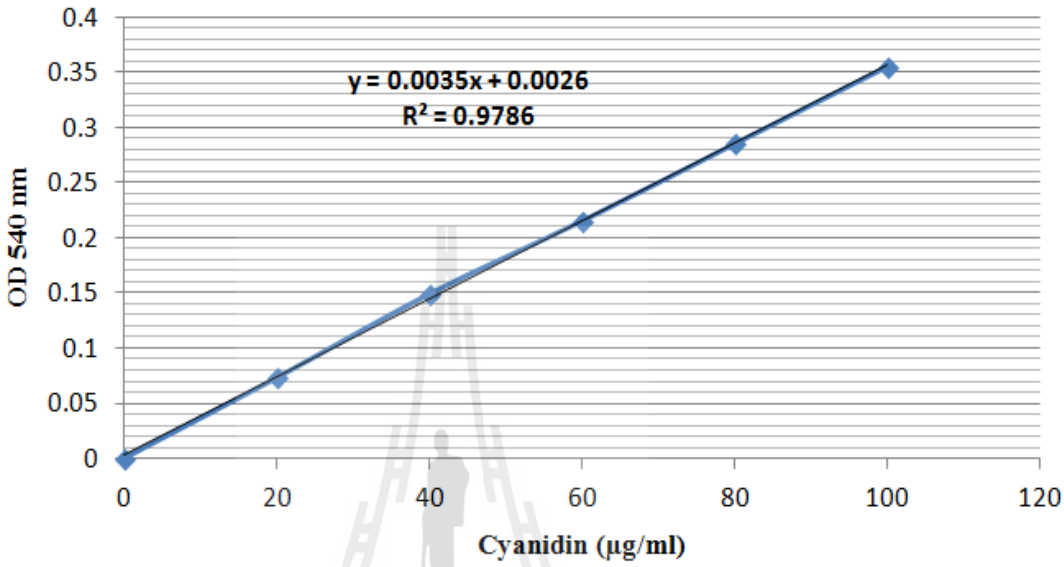


Figure 3.4 Calibration curve of cyanidin for proanthocyanidin determination.

3.4.5 Determination of monomeric anthocyanin contents

Determination of the total amount of anthocyanin contents was done using the reported spectrophotometric method (Abdl-Aal and Hucl, 1999). Anthocyanins were extracted with acidified methanol (85:15 (v/v) methanol: 1 M HCl) with a solvent to sample ratio of 1:10. The reaction solution was well mixed for 5 min at room temperature. Absorbance of the reaction was measured at 540 nm using a spectrophotometer. The major anthocyanin in the extract was likely to be cyaniding-3-glucoside (Giusti and Wrolstad, 2005). Therefore, the total monomeric anthocyanin contents of crude extract was calculated in terms of cyaniding-3-glucoside. The

concentration of monomeric anthocyanin pigments was calculated by the following equation:

Monomeric anthocyanin pigment (mg/g)

$$C = (A/\mathscr{E}) \times (vol/1{,}000) \times MW \times (1/\text{ weight of sample}) \times 10^6$$

C is the concentration of anthocyanins (mg/g), where MW represents molecular weight of cyaniding-3-glucoside (449), vol is the total volume of the extract, $\mathscr{E}$ is molar absorptivity of cyaniding-3-glucoside (25,965 $cm^{-1}m^{-1}$) and A is the absorbance measured at 535 nm (Giusti and Wrolstad, 2005).

3.5 FT-IR Instrumentation and Spectral Collection

FT-IR spectra of rice flour samples were recorded at room temperature (ca. 20 °C) using the Attenuated Total Reflectance (ATR)-FTIR Spectroscopy (Bruker Optics Ltd, Ettlingen, Germany) with single reflection ATR sampling module coupled with MCT detector cooled with liquid nitrogen over the measurement range from 4000-400 cm^{-1}. The measurements were performed with a spectral resolution of 4 cm^{-1} with 64 scans co-added.

The rice flour samples were left in the desiccators for 3 hr to remove all the moisture and then allowed to equilibrate to room temperature (ca. 20 °C) before scanning. The HART crystal cell was cleaned with 20 µl of methanol before spectra collection. The background setting was carried out by the measurement without sample on the cell prior to the spectra collection from the samples. Small amount of rice flour sample was taken with a spatula tip and carefully onto the clean HATR crystal cell. The beam was emitted from the source and the spectra were collected.

The spectral collection was repeated five times for each rice flour sample. The number of rice flour samples was 50 (n=50). Since all the samples were completely dry, there was no possible interference from methanol or water with the spectral collection. As a result, the intensity of spectral bands was increased (Lu et al., 2010). The crystal cell was cleaned with methanol between each spectral collection from each sample.

3.6 Chemometric Analysis

Data preprocessing was conducted before chemometric model development. The spectra were processed using 2nd derivative and vector normalized by the Savitzky-Golay method (using 3rd polynomial and, 9 smoothing points setting) and then normalized using Extended Multiplicative Signal Correction in the spectral regions to enhance the resolution of superimposed bands and to minimize problems from unavoidable baseline shifts.

Chemometric models were established based on processed spectra, including dendrogram analysis (using discriminant function analysis, DFA), cluster analysis (using principal component analysis, PCA), partial least-squares regression (PLSR), and loading plot analysis. DFA is one forms of chemometrics which can be used to construct branched dendrogram structures (Jarvis and Goodacre, 2004). PCA is used to reduce the dimensionality of multivariate data while preserving most of the variances (Lu et al., 2010). PLSR is a bilinear regressed analytical method that establishes the relationship between spectral features and reference values (i.e. concentration of analyzes). PLSR models were evaluated in terms of latent variables, correlation coefficient (r value), standard error and outlier diagnostics. The calibration

PLSR model was created, following by cross validation (either leave-one-out or from other batches of data). The validated calibration model could be performed to do prediction of samples outside. Loading plots were derived from chemometric analyses and used for explaining linear segregation of chemometric models based on various functional groups. The wavenumbers between 1800 and 700 cm^{-1} were selected for all chemometric models (DFA, PCA and PLSR) in the current study.

3.7 Statistical analysis

Analysis of variance (ANOVA), correlation analysis and principal component analysis of the results were performed in SAS (Software Version 13. SAS Institute Inc.). Within each variable, significant differences among the means were assessed with the *Duncan's* test for comparisons ($p < 0.05$).

CHAPTER IV

RESULTS AND DISCUSSION

4.1 Chemicals analysis

4.1.1 Total antioxidant capacity (TAC) of rice with different colors

There are a number of methods used to analyze the amount of antioxidant capacity, for example, Trolox equivalent antioxidant capacity (TEAC), 2,2-diphenyl-1-picrylhydrazyl (DPPH), oxygen radical absorbance capacity (ORAC), ferric reducing antioxidant power (FRAP) assays . In this study, total antioxidant capacity of rice was measured using the TEAC assay which also known as 2, 2-azinobis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) assay. Although TEAC assay is usually classified as Single Electron Transfer (SET) based method, the free radicals may be quenched either by hydrogen donating or by direct reduction via electron transfers from the antioxidant (Jimenez et al., 2004). The assay is based on the scavenging ability of antioxidant for the long-life radical anion $ABTS^{+}$ (Miller et al., 1993). In this assay, the total antioxidant capacity was evaluated according to the method based on ABTS radical cation discoloration in the presence of antioxidant (Pellegrini et al., 1999). The pre-formed radical monocation of 2, 2-azinobis (3-ethylbenzothiazoline-6-sulfonic acid) ($ABTS^{\bullet+}$) is generated by oxidation of ABTS with potassium persulfate and is reduced in the presence of hydrogen-donating antioxidants. Inhibition of the

radical $ABTS^{\bullet+}$ in the presence of rice samples was indicated by the discoloration of the solution as measured by optical density read at 734 nm. The absorbance at 734 nm was converted of the amount of Trolox in the reacting having the same absorbance by using the standard curve: a plot of absorbance at 734 nm versus concentration of trolox used in standard antioxidation reaction. Therefore, the antioxidant capacity was calculated as Trolox equivalents (mM TEAC). In the method, the rice extract was added after generation and quantification of $ABTS^{\bullet+}$ to prevent the possible overestimation and to reduce the interference of compounds with oxidants during radical formation. If rice extract was added into the reaction medium before the radical $ABTS^{\bullet+}$ had been formed, antioxidant in the rice extract could have reacted with oxidizing agents instead of the radical. This would have led to the overestimation of antioxidant capacity of the sample (Re et al., 1999; Van den Berg et al., 1999). The TAC of all rice varieties were measured in both uncooked and cooked forms. The average TAC values of each rice varieties from three measurements are presented in Table 4.1. The results showed that TAC in raw rice was higher than in cooked rice, and pigmented rice had higher TAC than white rice. The values of TAC of each color category of rice are summarized in (Table 4.2). The uncooked dark rice showed highest TAC, followed by red, brown and white rice, respectively. White rice varieties had mean TAC values of 0.081 mM TEAC, ranging from 0.073 to 0.088 mM TEAC. Within the category of brown rice, the mean TAC was 0.207 mM TEAC, ranging from 0.190 to 0.223 mM TEAC, whereas the red rice had the mean value of 0.323 mM TEAC, ranging from 0.160 to 0.435 mM TEAC. The dark rice samples had mean TAC values of 0.458 mM TEAC, ranging from 0.347 to 0.608 mM TEAC, around five times of that of the white rice. Similarly, in cooked rice, the pigmented rice

genotypes showed higher TAC than the white rice. White rice varieties had mean TAC values of 0.067 mM TEAC, ranging from 0.057 to 0.076 mM TEAC. Within the category of brown rice, the mean TAC was 0.131 mM TEAC, ranging from 0.123 to 0.139 mM TEAC, whereas the red rice had the mean value of 0.173 mM TEAC, ranging from 0.086 to 0.309 mM TEAC. The nineteen dark varieties had mean TAC of 0.220 mM TEAC, which was double and quadruple that of brown and white rice, respectively. It was found that, when cooked, the TAC of dark rice, red rice, brown rice and white rice decreased (Figure 4.1), by 51.97, 46.44, 36.71 and 17.28 percent, respectively. The greatest reduction of TAC was observed in cooked dark rice, while white rice showed the least TAC reduction when cooked. Nonetheless, TAC of cooked colored rice was still higher than cooked white rice, as shown in Table 4.2. Even after cooking, dark rice remained to be the rice with highest TAC, respectively followed by red, brown and white rices (Figure 4.1). Analysis of variance (ANOVA) showed a significant correlation between color of rice and total antioxidant capacity ($p \leq 0.05$). These results are in agreement with the work previously reported. Higher antioxidant activity for rice grains with black and red pericarp color was also reported by Goffman and Bergman (2004) using DPPH assay, and using 2, 2-azinobis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) assay reported by Shen et al. (2009). That higher antioxidant activity was observed for the rice grains with black and red pericarp colores, which was 8 to 14 times higher than the antioxidant activity observed for the rice grains with a light brown pericarp color, indicates a difference in the antioxidants activity of the grains depending on pericarp color (Melissa et al., 2011).

Cao et al. (1996) and Wang et al. (1996) reports to antioxidant activity of grains, dry beans, fresh vegetables and fruits were analyzed using the DPPH methods, by use Trolox is reference standard and the antioxidant activity of the sample is expressed in micromoles of Trolox equivalents (TE) per 100 gm of sample (Table 4.3).

Table 4.1 The data on average TAC of all rice varieties in uncooked and cooked forms.

Rice varieties	GS NO.	TAC (mM Trolox equivalents)	
		Uncooked	Cooked
Dark rice			
	00621	0.424	0.204
	01708	0.435	0.186
	02723	0.442	0.221
	03359	0.417	0.125
	03437	0.524	0.260
	05563	0.347	0.197
	06722	0.608	0.417
	09406	0.389	0.183
	14378	0.407	0.244
	21427	0.400	0.174
	91151	0.486	0.200
	87061	0.468	0.309
	88083	0.444	0.251
	87009	0.563	0.288
	91130	0.440	0.151
	88138	0.514	0.151
	91195	0.531	0.232
	87090	0.370	0.160
	9284	0.486	0.218
Red rice			
	05760	0.435	0.214
	06008	0.328	0.144
	06257	0.379	0.251
	06827	0.223	0.113
	07278	0.291	0.151
	07279	0.368	0.139
	09379	0.291	0.137
	09474	0.421	0.214
	09475	0.417	0.230
	15110	0.340	0.148
	18014	0.435	0.195

Table 4.1 (continued).

Rice varieties	GS NO.	TAC (mM trolox equivalents)	
		Uncooked	Cooked
Red rice			
	19057	0.277	0.197
	21626	0.384	0.139
	21629	0.398	0.309
	21831	0.160	0.086
	21972	0.204	0.113
	7677	0.347	0.216
	89038	0.386	0.172
	88073	0.260	0.160
	87046	0.256	0.134
	89057	0.365	0.160
	88013	0.326	0.235
	88028	0.187	0.111
	8168	0.307	0.204
	88069	0.295	0.139
	88063	0.356	0.211
	106971	0.298	0.137
Brown rice			
	91124	0.190	0.123
	9284	0.223	0.139
White rice			
	KDML 105	0.088	0.076
	RD 6	0.073	0.057

Table 4.2 Total antioxidant capacity of uncooked and cooked rice in each color category: dark (n=19), red (n=27), brown (n=2) and white (n=2)**.**

	Total antioxidant capacity expressed as mM TEAC*			
	Dark rice	Red rice	Brown rice	White rice
Uncooked				
Mean ± SD	0.458 ± 0.067[a]**	0.323 ± 0.123[b]**	0.207 ± 0.206[c]**	0.081 ± 0.010[d]**
Range	0.347 - 0.608	0.160 - 0.435	0.190 - 0.223	0.073 - 0.088
Cooked				
Mean ± SD	0.220 ± 0.067[a]**	0.173 ± 0.051[a]**	0.131 ± 0.113a[b]**	0.067 ± 0.013[b]**
Range	0.125 - 0.417	0.086 - 0.309	0.123 - 0.139	0.057 - 0.076

*Within the total number of 50 rice samples analyzed, there are 19 dark rice, 27 red rice, 2 brown rice and 2 white rice.

**Values with the same letter are not significantly different *Duncan's* test for comparisons ($p < 0.05$).

The comparison was performed between rice with different colors within the same category, i.e. uncooked or cooked.

Table 4.3 Antioxidant activity from foods (Cao et al., 1996; Wang et al., 1996)**.**

Foods	**Antioxcidant activity (TE/100 grams)**
Blueberries	3300
Red grapes	1350
Lima beans	1055
Red cabbage	1000
Wheat flour (refined)	600
Broccoli flowers	500
Spinach	500
Green grapes	400
Tomato	300
Green beans	175
Green cabbage	150

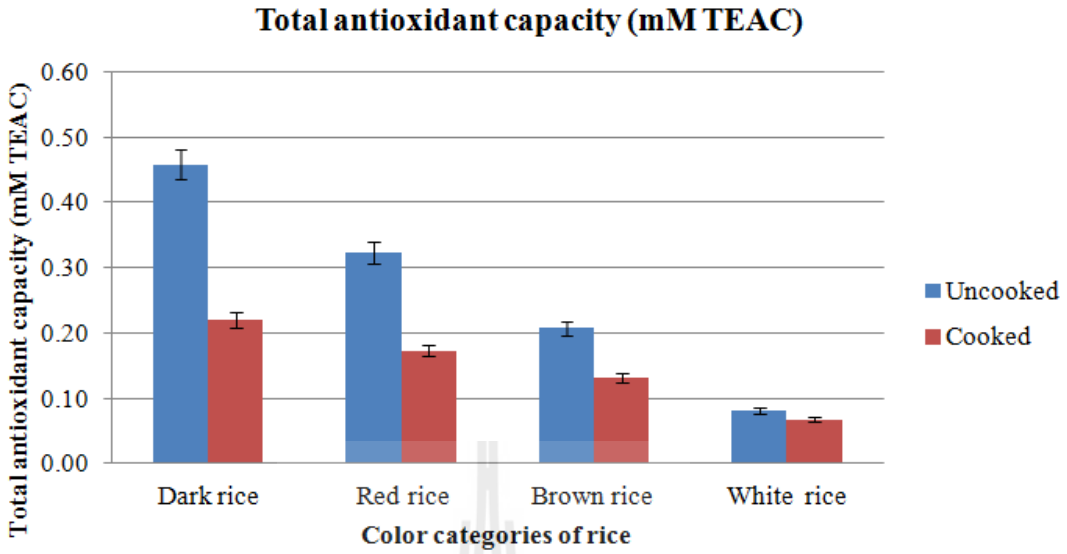


Figure 4.1 Comparison of TAC between uncooked and cooked rice.

4.1.2 Phytochemicals: phenolic compound, flavonoid, anthocyanin and proanthocyanidin contents in colored and white rice

Fruits and vegetables are good sources of natural phytochemicals. Researchers have demonstrated the phenotypic diversity of some phytochemicals in rice bran layer (Dykes and Rooney, 2007; Liu, 2007). For example, the environmental and genotypic effects on tocotrienol, tocopherol, γ-ory-zanol contents of rice (Bergman and Xu, 2003), and the minerals contents and their correlation with other qualitatiative traits of rice (Jiang et al., 2007). In rice, it was found that bran color was highly statistically significant for bran phenolic contents. Flavonoids are one group of phenolics. Anthocyanins and proanthocyanidins are in the group of reddish to purple water soluble favonoids that are primary pigments in the black and red grains, and have been widely identified and characterized in cereal grains (Abdel-Aal et al., 2006). Similar to other cereal grains, the phenolic compounds in rice exist in the soluble and

insoluble forms. The total polyphenols content in light brown rice grains has been shown to be constituted of the soluble form for 38% (Adom and Liu, 2002) to 60% (Mira et al., 2009), and around 81% in red and black pericarp colored grains (Mira et al., 2009). Isabel et al. (2011) found that the average content of phenolics in pigmented rice (409.7 ± 62.9 mg FA eq/100 g) was about 4 times higher than that of the non-pigmented rice (99.4 ± 19.1 mg FA eq./100 g). The type and concentration of polyphenols in rice grain vary among genotypes and are related mainly to the pericarp color. Normally, grains with a light brown pericarp color have a lower concentration of phenolic compounds compared to those with red and black pericarp colours (Tian et al., 2004; Zhou et al., 2004).

In this study, the phytochemicals of rice were determined to reveal the additional value of whole-grain rice consumption apart from carbohydrate it provides. Furthermore the colored rice has drawn great interest because of both its high contents in phenolics and flavonoids such as anthocyanins and proanthocyanidins derivatives. Since, the concentration of these compounds is also affected by processing the measurement was also performed in cooked rice. In rice, phenolic contents are mainly associatied with the pericarp. Hu et al. (2003) and Zhou et al. (2004) reported the reduction of phenoic compounds in the grain as a result of processing to obtain polished grains, which was the method used to prepare rice for consumption in Brazil. Additionally, rice has to be fully cooked by hydrothermal treatment prior to consumption. There is little information about the effect of such procedure on the phenolic contents in the rice grain. The possible reductions of phytochemicals, including phenolics, flavonoids, anthocyanins and proanthocyanidins due to cooking process, have not received enough attention in rice grains.

The here presented work evaluated and compared concentrations of phytochemicals including total phenolic compounds, total flavonoid, proanthocyanidins and anthocyanin contents of colored rice and white rice varieties as well as the effect of processing on the concentration of phytochemicals in the rice grain. The data obtained from all rice varieties are presented in Table 4.4. The average values and rang of phytochemicals of the uncooked and cooked rice in each color category are reported in Table 4.5 and Table 4.6, respectively. Among all the rice accessions both uncooked and cooked, the pigmented rice genotypes showed higher phenoic compound, total flavonoid, proanthocyanidin and anthocyanin contents than the white rice. Phenolic compounds of uncooked and cooked rice ranged from 92.30 mg GAE/L to 1199.30 mg GAE/L and 48.30 mg GAE/L to 418.30 mg GAE/L, respectively (Table 4.5 and 4.6). Dark rice, both uncooked and cooked showed high average level of phenolic compounds of 780.87 mg GAE/L and 277.24 mg GAE/L, respectively. Cooking process decreased phenolic compound content by of dark rice 70.90% (Figure 4.2). Among the red rice, the average phenolic compound content of uncooked was 572.93 mg GAE/L and that of cooked rice was 248.67 mg GAE/L. The value decreased by 56.60%. The average phenolic compound contents of uncooked and cooked brown rice were 218.30 mg GAE/L and 135.30 mg GAE/L, respectively. The most reduction percentage by 38.03% was revealed in brown rice (Table 4.5 and 4.6; Figure 4.2). White rice contained the lowest level of phenolic compound compared to all colored rice varieties. Uncooked white rice had 114.80 mg GAE/L while cooked rice had 46.30 mg GAE/L. The value decreased by 59.67% (Table 4.5 and 4.6; Figure 4.2). Similar results were obtained by other research groups. Goffman and Bergman (2004) reported average concentrations of 274, 213 and 69 mg GAE

100 g^{-1} for black, red and light brown pericarp color rice grains, respectively, while in another study the average concentrations of 1055.7, 470.1 and 151.8 mg GAE 100 g^{-1} for black, red and light brown pericarp color rice grains were noted, respectively (Shen et al., 2009). Likewise, the average concentration of 409.7 mg GAE 100 g^{-1} in the pigmented group of rice, which was 4 times higher than the value of 99.4 mg GAE 100 g^{-1} in the non- pigmented group were recorded (Isable et al., 2011). Correspondingly, analysis of variance (ANOVA) showed a significant effect of genotype on phenolic compound, total flavonoid, proanthocyanidin and anthocyanin contents ($p < 0.05$) from this study in both uncooked and cooked rice.

Table 4.4 Total phenolic compounds, flavonoid, anthocyanin contents and proanthocyanidins of colored rice and white rice varieties in the study.

Rice varieties		Phytochemical contents							
		Total phenolic compounds (mg/L)		Flavonoid (mg/g)		Anthocyanin contents (µg/L)		Proanthocyanidins (µg/L)	
		Uncooked	Cooked	Uncooked	Cooked	Uncooked	Cooked	Uncooked	Cooked
Dark rice	**NO**								
	00621	920.30	418.30	342.79	210.02	1018.33	417.09	362.97	114.10
	01708	768.30	274.30	360.02	192.38	1042.38	356.22	372.97	88.40
	02723	941.30	322.30	385.91	209.44	1269.26	356.91	465.54	89.25
	03359	602.30	138.30	292.67	103.26	949.70	403.95	334.97	108.97
	03437	1199.30	412.30	357.08	188.55	1094.95	328.55	394.11	76.68
	05563	842.30	342.30	345.61	198.55	959.38	287.05	337.54	59.25
	06722	702.30	315.30	315.02	187.08	834.18	340.31	307.82	82.11
	09406	567.30	235.30	309.73	175.61	951.77	292.58	334.11	62.68
	14378	612.30	286.30	330.32	227.97	771.24	343.77	263.25	82.97
	21427	617.30	306.30	293.26	209.73	633.59	336.16	202.97	79.25
	91151	833.30	277.30	360.02	179.14	1043.08	323.71	372.11	75.25
	87061	715.30	281.30	301.50	191.50	731.81	296.04	234.97	63.54
	88083	541.30	245.30	296.20	153.85	627.36	246.24	202.11	42.97
	87009	876.30	248.30	395.61	178.26	735.96	245.55	244.97	43.25
	91130	843.30	242.30	363.26	85.02	798.21	233.10	272.11	36.68
	88138	865.30	152.30	397.67	189.73	1097.72	273.22	394.11	54.40
	91195	1115.30	258.30	359.73	161.79	1111.56	322.32	400.11	75.82
	87090	616.30	179.30	301.50	160.91	771.93	267.68	259.82	51.82
	9284	657.30	332.30	339.14	219.14	973.91	336.85	343.25	80.68

Table 4.4 (continued).

Rice varieties		Phytochemical contents							
		Total phenolic compounds (mg/L)		Flavonoid (mg/g)		Anthocyanin contents (µg/L)		Proanthocyanidins (µg/L)	
		Uncooked	Cooked	Uncooked	Cooked	Uncooked	Cooked	Uncooked	Cooked
Red rice	**NO**								
	05760	508.30	213.30	310.02	163.26	688.93	277.36	224.97	57.54
	06008	882.30	444.30	345.61	187.97	742.19	209.58	248.68	28.11
	06257	642.30	343.30	282.97	199.14	700.69	269.75	228.97	50.68
	06827	677.30	224.30	277.97	175.30	723.51	239.30	240.68	41.25
	07278	636.30	246.30	292.67	186.79	651.57	260.07	212.40	47.82
	07279	602.30	242.30	325.91	198.85	731.81	306.42	248.40	64.11
	09379	522.30	212.30	274.44	160.32	708.98	264.22	233.82	50.40
	09474	805.30	586.30	346.50	236.50	729.74	280.13	243.54	58.68
	09475	546.30	208.30	277.97	200.61	666.10	237.94	216.97	39.82
	15110	616.30	247.30	297.97	218.26	695.15	253.85	229.25	46.68
	18014	662.30	232.30	163.26	65.32	680.63	501.47	223.54	150.40
	19057	637.30	243.30	360.02	138.55	618.37	241.44	197.82	41.25
	21626	887.30	250.30	352.08	172.67	746.34	255.92	246.68	47.25
	21629	475.30	207.30	239.44	147.67	419.16	48.41	113.82	12.68
	21831	312.30	185.30	224.44	110.91	337.54	40.11	79.25	8.68
	21972	408.30	148.30	260.91	190.32	391.50	213.04	102.97	29.25
	7677	486.30	323.30	292.67	191.40	632.90	266.30	204.40	52.11
	89038	563.30	229.30	315.61	200.91	515.31	226.87	154.68	35.25
	88073	613.30	197.30	280.91	175.61	462.05	224.84	131.25	35.25
	87046	447.30	197.30	280.02	185.02	351.38	44.26	87.54	10.40
	89057	684.30	225.30	362.67	143.55	702.07	56.02	231.82	21.54

Table 4.4 (continued).

Rice varieties		Phytochemical contents							
		Total phenolic compounds (mg/L)		Flavonoid (mg/g)		Anthocyanin contents (µg/L)		Proanthocyanidins (µg/L)	
		Uncooked	Cooked	Uncooked	Cooked	Uncooked	Cooked	Uncooked	Cooked
Red rice	**NO**								
	88013	265.30	173.30	165.91	174.73	273.22	60.86	54.97	17.54
	88028	437.30	245.30	260.91	162.38	486.95	221.34	142.11	32.68
	8168	505.30	224.30	281.50	187.38	467.58	83.69	134.68	26.97
	88069	519.30	175.30	277.67	177.97	431.61	65.01	119.82	19.54
	88063	593.30	247.30	362.20	170.91	695.15	232.40	228.40	36.68
	106971	532.30	241.30	295.61	159.73	599.01	214.42	189.82	30.11
Brown rice									
	91124	249.30	180.30	175.02	97.38	473.12	240.01	124.97	41.25
	9284	187.30	90.30	201.20	165.32	314.72	213.73	72.11	29.25
White rice									
	KDML 105	137.30	48.30	41.37	29.44	77.46	24.89	25.54	2.40
	RD 6	92.30	44.30	42.97	26.79	74.70	26.28	25.25	2.68

Table 4.5 Average values and ranges of phenolic compound, flavonoid, proanthocyanidin and anthocyanin contents of uncooked dark, red, brown and white rice.

Rice varieties	Phenolic compound (mg GAE/L)	Flavonoid (µg RE/L)	Proanthocyanidin (µg/L)	Anthocyanin (mg/g)
Dark rice				
Mean ± SD	780.87 ± 182.84[a]	339.31 ± 34.57[a]	321.04 ± 73.38[a]	919.64 ± 177.95[a]
Range	541.30 - 1199.30	292.67 - 397.67	202.11 - 465.54	627.36 - 1269.26
Red rice				
Mean ± SD	572.93 ± 146.11[a]	289.18 ± 51.50[a]	184.12 ± 60.75[b]	587.01 ± 146.55[b]
Range	265.30 - 887.30	163.26 - 362.67	54.97 - 248.68	273.22 - 746.34
Brown rice				
Mean ± SD	218.30 ± 43.84[b]	188.11 ± 18.51[b]	98.54 ± 37.37[bc]	393.92 ± 112.00[b]
Range	187.30 - 249.30	175.02 - 201.20	72.11 - 124.97	314.72 - 473.12
White rice				
Mean ± SD	114.80 ± 31.81[b]	42.97 ± 0.00[c]	25.39 ± 0.20[c]	76.08 ± 1.95[c]
Range	92.30 - 137.30	42.97 – 42.97	25.25 - 25.54	74.70 - 77.46

Mean values within a column superscripted by the same letter are not significantly different at *Duncan's* test for comparisons ($p < 0.05$).

The comparison was performed between rice with different colors within the same category, i.e. uncooked or cooked.

Table 4.6 Average values and ranges of phenolic compound, total flavonoid, proanthocyanidin and anthocyanin contents of cooked dark, red, brown and white rice.

Rice varieties	Phenolic compound (mg GAE/L)	Total flavonoid (µg RE/L)	Proanthocyanidin (µg/L)	Anthocyanin (mg/g)
Dark rice				
Mean ± SD	277.24 ± 74.36[a]	180.10 ± 36.24[a]	72.00 ± 21.15[a]	316.17 ± 50.74[a]
Range	138.30 - 418.30	85.02 - 227.97	36.68 - 114.10	233.10 - 417.09
Red rice				
Mean ± SD	248.67 ± 88.93[ab]	173.40 ± 33.49[a]	40.46 ± 26.64[a]	207.22 ± 105.40[a]
Range	148.30 - 586.30	65.32 - 236.50	8.68 - 150.40	40.11 - 501.47
Brown rice				
Mean ± SD	135.30 ± 63.63[bc]	131.35 ± 48.04[a]	35.25 ± 8.48[ab]	226.87 ± 18.58[a]
Range	90.30 - 180.30	97.38 - 165.32	29.25 - 41.25	213.73 - 240.01
White rice				
Mean ± SD	46.30 ± 2.82[c]	28.11 ± 1.87[b]	2.54 ± 0.19[b]	25.58 ± 0.98[b]
Range	44.30 - 48.30	26.79 - 29.44	2.40 - 2.68	24.89 - 26.28

Mean values within a column superscripted by the same letter are not significantly different at *Duncan's* test for comparisons ($p < 0.05$).

The comparison was performed between rice with different colors within the same category, i.e. uncooked or cooked.

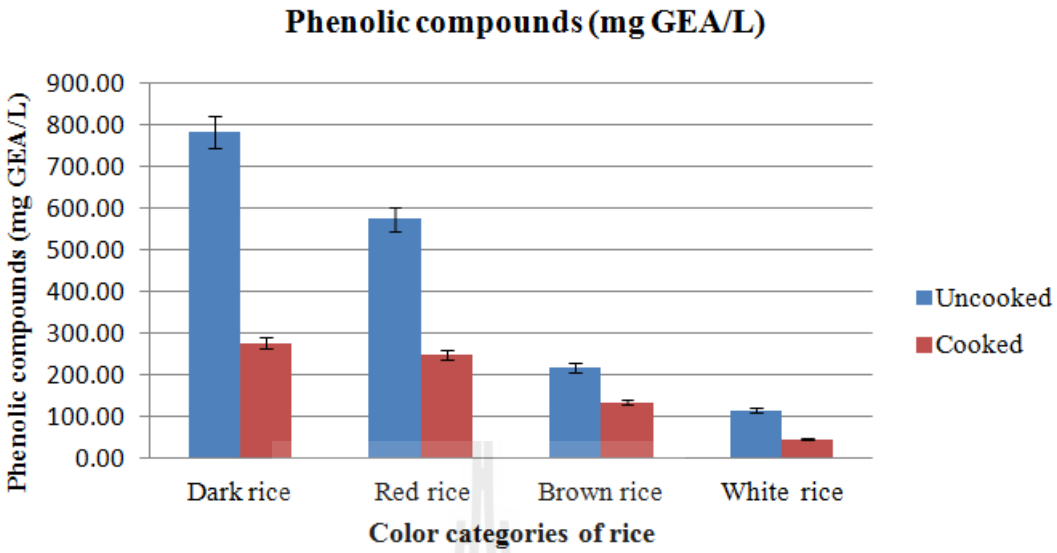


Figure 4.2 Comparison of phenolic compounds between uncooked and cooked rice.

Flavonoids have been reported to have antioxidant and anticancer activities (Adom and Liu, 2002; Dykes and Rooney, 2007; Hu et al., 2003). Flavonoids are one group of phenolics and are compounds of two aromatic rings joined by a 3 carbon link; they include flavonols, flavones, flavonones, flavanols, anthocyanins and proanthocyanidins. Flavonoids are located in the pericarp of all cereals. Thus, in this study we evaluated of values of flavonoid contents in uncooked and cooked colored rice as well as in white rice for the comparison. Flavonoid contents in uncooked and cooked forms of all rice varieties ranged from 42.97 µg RE/L to 397.67 µg RE/L and 29.44 µg RE/L to 227.97 µg RE/L, respectively. The dada from each variety is shown in Table 4.4, while the average and range of flavonoid contents in each rice color category are presented in Table 4.5 and Table 4.6. In uncooked form, the average flavonoid contents among the white, brown, red and dark rice were 42.97 µg RE/L, 188.11 µg RE/L, 289.18 µg RE/L and 339.31 µg RE/L, respectively (Table 4.5). And average flavonoid contents among the white, brown, red and dark rice in cooked form

were 28.11 µg RE/L, 131.35 µg RE/L, 173.40 µg RE/L, and 180.10 µg RE/L, respectively (Table 4.6). Cooking has been shown to decrease the amount of flavonoid contents in dark, red, brown and white rice by 46.93, 40.04, 30.18 and 34.58 percent, respectively (Figure 4.3). Even though dark rice had the highest average level of flavonoid content than the red rice, some dark rice varieties were revealed to have lower flavonoid contents than red rice. Similarly some red rice varieties had lower contents than brown rice, whereas all of white rice had lower contents than brown, red and dark rice. Such results were also similar by Yun et al. (2009). The white rice had mean flavonoid content (131.6 mg RE/100g), lower than those of red rice (147.2 mg RE/100g) and black rice (240 mg RE/100g). It was explained that, instead of the mainly found antioxidants and proanthocyanidins, such red and brown rice varieties may have other flavonoid compositions, e.g. flavonols, flavanols, flavones and flavonones, at high levels which made the total flavonoid content higher than that of the varieties supposed to have higher values.

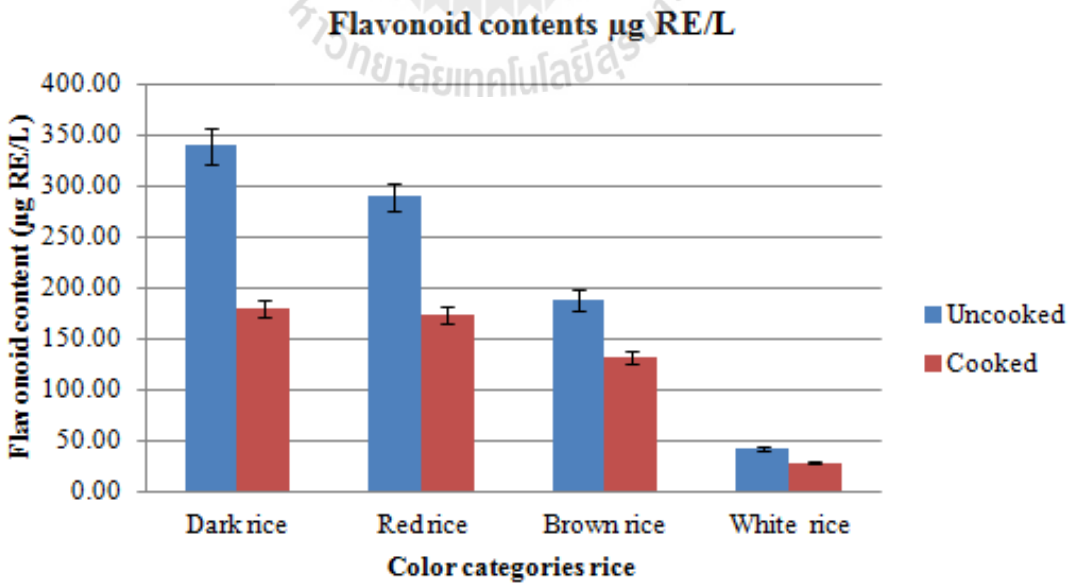


Figure 4.3 Comparison of flavonoid contents between uncooked and cooked rice.

Anthocyanins are water-soluble pigments that contribute to black, purples, blues and red in plant foods (i.e. blackberries, strawberries, blueberries and rice) and are the major flavonoids studied in pericarp of pigmented cereal varieties of barley, rice, rye, maize and wheat. Anthocyanins are the six common antioxidant in the nature, including cyaniding, peonidin, petunidin, pelargonidin, delphinidin and malvinidin. A molecule of proanthocyanidins or procyanidins is consisted of polymerized flavonol units. They are compounds found in sorghum with a pigmented testa layer, barley and red finger millets (Abdel-Aal et al., 2006; Choi et al., 1994). In this study anthocyanins and proanthocyanidins in colored rice grain were measured the obtained data from uncooked and cooked rice were compared. The average values of anthocyanin and proanthocyanidin content of rice in all categories were compared. It was shown that the dark rice had the highest value followed by red, brown and white rice respectively. This was true for both uncooked and cooked form of rice (Table 4.5 and 4.6). Uncooked, dark varieties had an average anthocyanin content of 919.64 mg/g, ranging from 627.36 mg/g to 1269.26 mg/g and an average proanthocyanidins of 321.04 µg/L, ranging from 202.11 µg/L to 465.54 µg/L. In red rice, the average of anthocyanin contents of 587.10 mg/g, ranging from 273.22 mg/g to 746.34 mg/g and an average proanthocyanidins of 184.12 µg/L, ranging from 54.97 µg/L to 248.68 µg/L, respectively, whereas among the brown rice an average anthocyanin contents of 393.92 mg/g, ranging from 314.72 mg/g to 473.12 mg/g and an average proanthocyanidins of 98.54 µg/L, ranging from 72.11 µg/L to 124.97 µg/L. The two white rice varieties had of anthocyanin contents and proanthocyanidins were 76.08 mg/g and 25.39 µg/L, ranging from 74.70 mg/g to 77.46 mg/g and from 25.25 µg/L to 25.54 µg/L, respectively (Table 4.5). In cooked form rice, white rice

had average anthocyanin contents of 25.58 mg/g, ranging from 24.89 mg/g to 26.28 mg/g, and an average proanthocyanidins of 2.54 µg/L, ranging from 2.40 µg/L to 2.68 µg/L (Table 4.6). Among the brown rice, the average of anthocyanin contents of 226.87 mg/g, ranging from 213.73 mg/g to 240.01 mg/g, and an average proanthocyanidins of 35.25 µg/L, ranging from 29.25 µg/L to 41.25 µg/L, respectively, whereas among the red rice, it average anthocyanin contents of 207.22 mg/g, ranging from 40.11 mg/g to 501.47 mg/g and an average proanthocyanidins of 40.46 µg/L, ranging from 8.68 µg/L to 150.40 µg/L were recorded. The nineteen dark rice varieties had average anthocyanin contents of 316.17 mg/g, ranging from 233.10 mg/g to 417.09 mg/g, and an average proanthocyanidins of 72.00 µg/L (around double amount of the brown rice), ranging from 36.68 µg/L to 114.14 µg/L (Table 4.6). It was found, when cooked the amount of anthocyanin contents and proanthocyanidins in dark, red, brown and white rice reduced by 65.63, 64.70, 42.41 and 66.38 percentages for anthocyanin contents, respectively, and 77.58, 78.03, 64.23 and 90.00 percentages for proanthocyanidins, respectively (Figure 4.4 and 4.5). Even though dark rice varieties had the highest average level of anthocyanin and proanthocyanidin contents, some red rice accessions were revealed to have higher contents than the dark rice. Likewise, the red rice had higher average level of contents than the brown rice, but some red rice varieties had lower contents of these compounds than the brown rice. However, all varieties of white rice had the lowest contents of anthocyanin and proanthocyanidins.

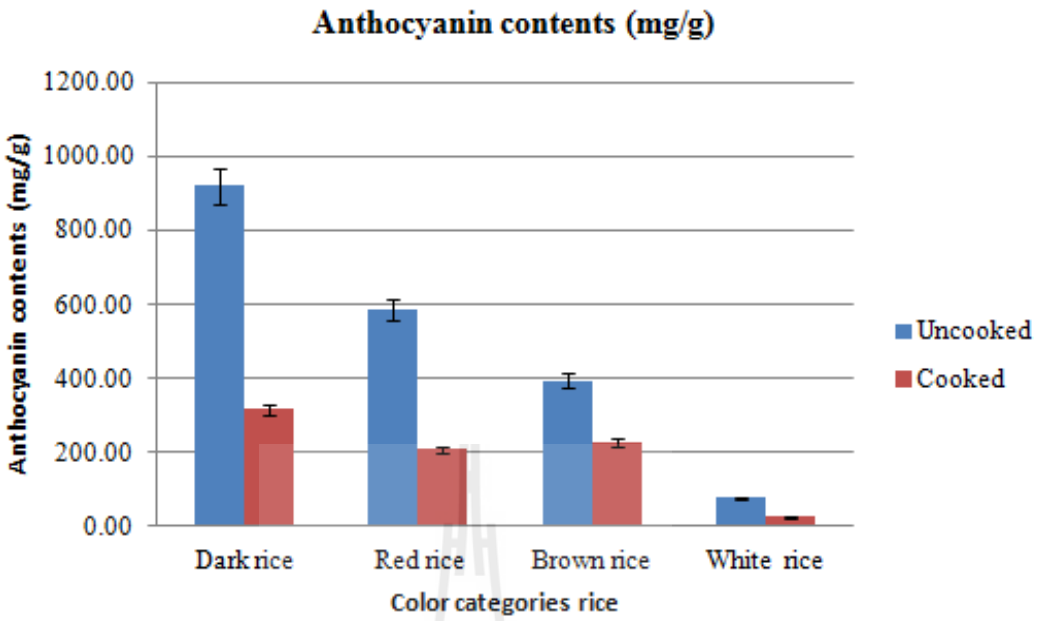


Figure 4.4 Comparison of anthocyanin contents between uncooked and cooked rice.

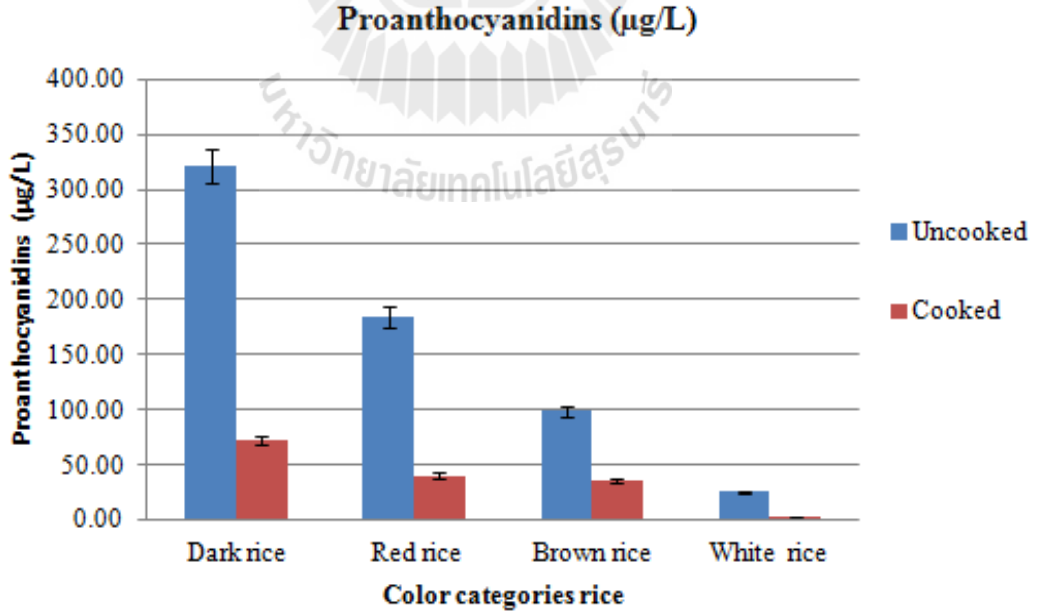


Figure 4.5 Comparison of proanthocyanidins between uncooked and cooked rice**.**

The comparison of the reduction percentages of total antioxidant capacity and phytochemicals including phenolic compounds, total flavonoids, anthocyanins, and proanthocyanidins in colored rice and white rice before and after cooking is shown in Table 4.6. The results showed that, when cooked, total antioxidant capacity, phenolic compounds, flavonoids, anthocyanin and proanthocyanidins reduced at least 30 percent. Dark rice, had the highest percentage reduction after having been cooked, followed by white rice, red rice and brown rice. According to the data shown in Table 4.7 flavonoids seemed to be most resistant to cooking process. Its reduction percentage was the lowest compared to other compounds in other rice categories. In contrast, the most sensitive compound to cooking process appeared to be proanthocyanidins as indicated by its highest reduction.

There are report from Japan and Korea about the survey of 10 pigmented *Oryza sativa* L. indica varieties which demonstrated that cyanidin-3-glucoside (0-470 mg/100 g) and peonidin-3-glucoside (0-40 mg/100 g) were the predominant anthocyanins. A similar result was shown by Abel-Aal et al. (2006). However, the rice variety was not specified. Abel-Aal et al. (2002) also noted the presence of several cyanidin diglucosides and a cyaniding rutinoside in these samples. When the main kind of flavonoids associated with kernel pigmentation in rice and other cereals was investigated, proanthocyanidin was typically observed in the red kernels and not in black ones, whilst high levels of anthocyanins were found only in the black kernels (Abdel-Aal et al., 2008; Finocchiaro et al., 2007; Hosseinian et al., 2008; Hu et al., 2007; Jang and Xu, 2009; Knievel et al., 2009; McCallum and Walker, 1990; Miyamoto and Everson, 1958; Nagao et al., 1957; Nam et al., 2006; Oki et al., 2002). This fact was confirmed for the black rice cv. Venere, which did not contain detectable

amounts of proanthocyanidins. And for the five red rices studied here, which, on the other hand, contained no detectable amounts of anthocyanins; although, tiny amounts of these latter compounds can be found in red rice with LC-MS (Finocchiaro et al., 2007), as can be expected because of the role of anthocyanins as intermediates in proanthocyanidin synthesis (Lepiniec et al., 2006). Abdel-Aal et al. (2006) found that the red kernel contains about 35 times less anthocyanins than black rice.

Fuleki and Ricardo (2003) reported that thermal processing caused degradation of proanthocyanidins, depolymerization of higher oligomeric and polymeric structures into dimers and trimers and also concomitant polymerization reaction. Several reports have demonstrated that phenolic compounds from different foods suffered decomposition under high temperatures, and this effect depended on the temperature, type of compounds in the sample, time of processing and other conditions (Larrauri et al., 1997; Piga et al., 2003).

The thermal processing and cooking affect anthocyanin stability distribution in foods, as most foods undergo some type of cooking or thermal processing prior to consumption. The stability of anthocyanins in foods is affected by the pH, temperature, glycosidic linkages, and food matrix interactions that occur during processing (Delgado et al., 2000). At present, little is known about the effects of various thermal cooking methods on the composition and retention of anthocyanins in black rice. Black rice is generally cooked for longer periods than white rice by boiling and/or steaming it. It is commonly cooked in a pot using just enough water to be absorbed into the rice during cooking (absorption method) or using an electric rice cooker as these are increasingly available worldwide and simplify the rice cooking process. Alternatively, pressure cooking can be used to speed the cooking process. In

many countries, rice is soaked prior to cooking to soften its texture and help decrease cooking times. Miki et al. (2009) studied the effects of anthocyanins in black rice. The results showed that the soaking had no significant impact on anthocyanin stability or retention. It was also found that the content of cyanidin-3-glucoside decreased significantly across all cooking methods by pressure cooking, in the greatest decreases (79.8%), followed by the rice cooker (74.2%) and gas range (65.4%). A similar result was shown by Abdel-Aal and Hucl. (2003) and by Cabrita et al. (2000). The loss of anthocyanins in black rice may be attributed to the degradation or decomposition of anthocyanins arising from thermal processing. Many reports suggested that cyaniding-3-glucoside in black rice was degraded predominantly into protocatechuic acid during cooking.

Table 4.7 The percentage reduction of TAC and phytochemicals including phenolic compounds, total flavonoids, anthocyanins, and proanthocyanidins due to cooking process in rice.

Rice varieties	Total antioxidant capacity %	Phenolic compounds %	Total flavonoids %	Anthocyanins %	Proanthocyanidins %
Dark rice	57.97	70.90	46.93	65.63	77.58
Red rice	46.44	56.60	40.04	64.70	78.03
Brown rice	36.71	38.03	30.18	42.41	64.23
White rice	12.78	59.67	34.58	66.38	90.00

4.2 FT-IR analysis

4.2.1 FT-IR spectral features of colored rice

FT-IR spectral features of uncooked colored rice and cooked colored rice are shown in Figure 4.6 and 4.7, respectively. FT-IR was operated in the wavenumber rang of 4000 - 400 cm^{-1}. Chemicals bonds in the sample vibrated when interacting with infrared, the condition in which infrared absorbance occurred. Since the sample absorbed infrared radiation in the wavenumber ranges according to the functional groups in its structure and the wavenumber absorbed was characteristic to each type of functional group, the correlation between the infrared spectrum and the sample structure could be made. The bands between the wavenumbers of 1800 - 750 cm^{-1} (fingerprint regions) especially reflected the chemical compositions of proteins, lipid, carbohydrate, polysaccharides and polyphenols in plant extracts. The distinctive peak at the wavenumber of 813 cm^{-1} was caused by ring CH deformation (Movasaghi et al., 2008), which may reflect information about the structure of polyphenols which constituted of CH ring in the molecules (Schulz and Baranska, 2007). The peak at 868 cm^{-1} was assigned to the left-handed helix DNA (Dovbeshko et al., 2002). The bands in the wavenumbers of 985-1028 cm^{-1} were caused by the vibrational frequency of $–CH_2OH$ groups of carbohydrates: the C–O stretching vibration coupled with C–O bending of the C–OH groups, C–O, C–C stretching and C–O–H deformation motions (Andrus, 2006; Mordechai et al., 2001; Schulz and Baranska, 2007). The band at 1339 cm^{-1} was assigned to the stretching in-plane C−O vibration combined with the ring structure of phenyl while the wavenumber of 1370 cm^{-1} was assigned to the stretching C−H and deformation C−O (Mordechai et al., 2001; Schulz and Baranska, 2007). The distinctive band at 1635 cm^{-1} is assigned to the ring C−C stretch of phenyl. This

distinctive absorption peak was also observed in the qualitative study of polyphenolic components in garlic (Schulz and Baranska, 2007). For high wavenumber, the peak at 2928 cm^{-1} was due to CH_2 antisymmetric stretch of methyl groups mainly from lipids (Lu and Rasco, 2010; Naumann, 2001). The distinctive band at 3260 cm^{-1} was assigned to O−H stretching of carbohydrates (Lu et al., 2010) as well as the vibrational frequency in aromatic ring of alcohol and phenol (Zanyar et al., 2008) (Table 4.8) (Figure 4.6 and 4.7).

The attention was paid on the absorption peak at 1635 cm^{-1} which resulted from the ring CC stretch of phenyl. In this work, infrared spectrum of Trolox was generated to serve as standard for comparison and confirmation for the presence of phenolic compounds in rice samples (Figure 4.8). Distinctive peak at 1635 cm^{-1} was clearly observed in spectrum of Trolox. The absorbance difference of different samples suggested the difference in phenolic content in the samples. At 1635 cm-1 wavenumber, the infrared spectra of both uncooked and cooked rice corresponded with the results from chemical assays. Dark rice contained the highest level of phenolic compound followed by red brown and white rice.

Table 4.8 The absorption wavenumbers found in rice of all color categories.

Peak (cm^{-1})	**Assignment**
813	Ring CH deformation Polyphenols which constituted of CH ring
868	Left-handed helix DNA
985-1028	Vibrational frequency of $–CH_2OH$ groups of carbohydrates: the C–O stretching vibration coupled with C–O bending of the C–OH groups, C–O, C–C stretching and C–O–H deformation
1339	C−O vibration Ring structure of phenyl
1370	C−H and deformation C−O
1635	Ring C−C stretch of phenyl
1738	C=O stretching (lipids)
2928	CH_2 antisymmetric stretch of methyl groups mainly from lipids
3260	O−H stretching of carbohydrates Aromatic ring of alcohol and phenol

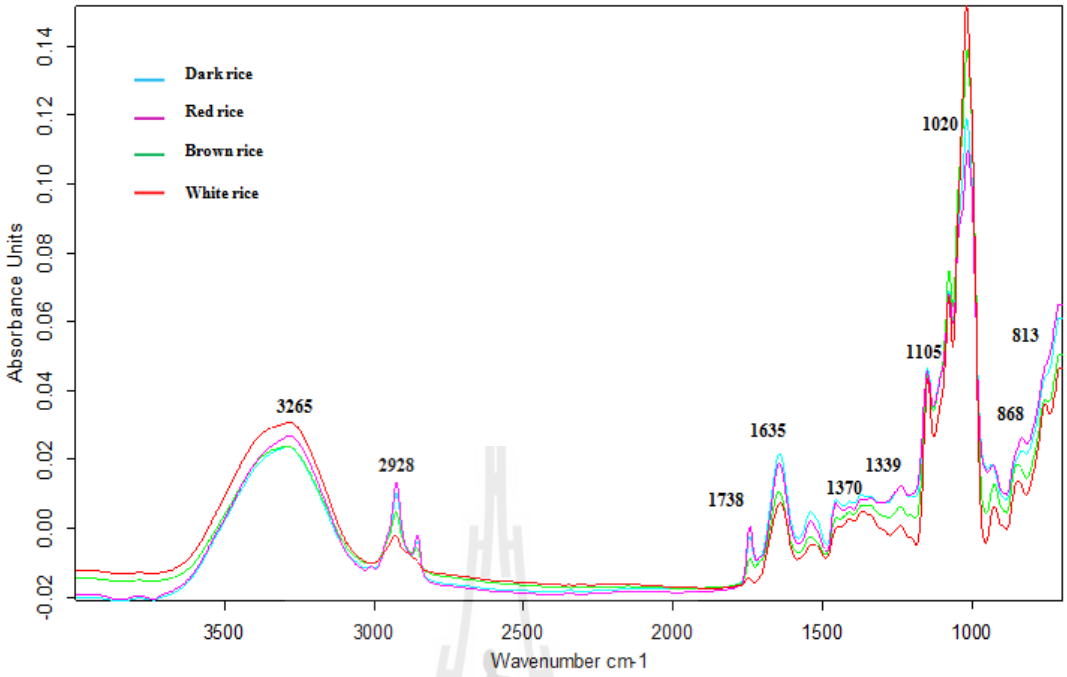


Figure 4.6 Representative FT-IR average spectra of uncooked rice.

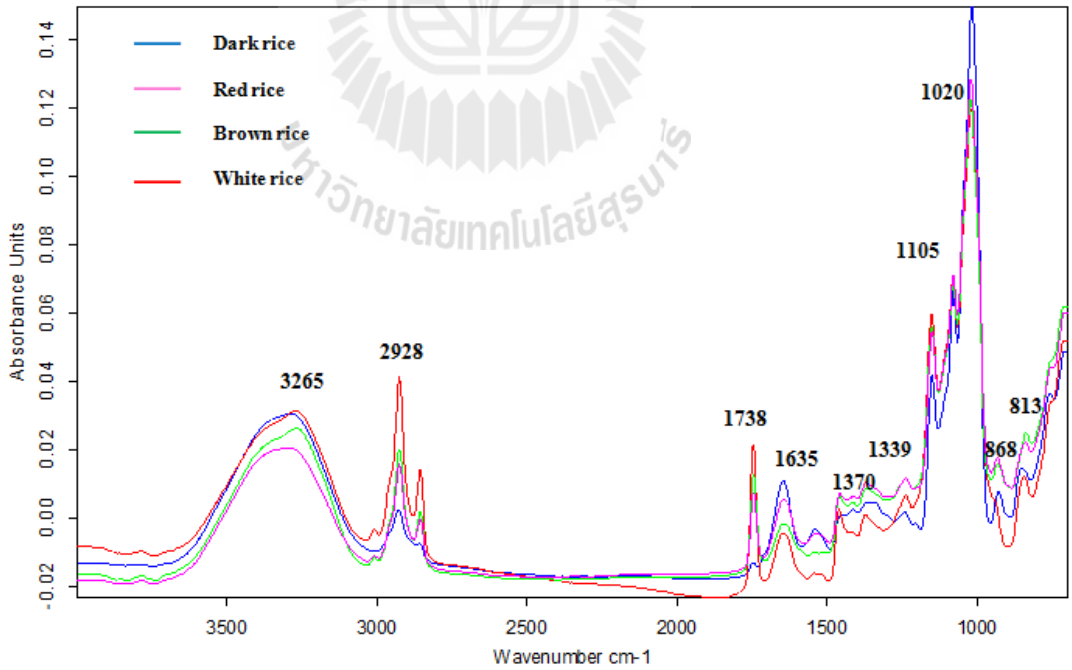


Figure 4.7 Representative FT-IR average spectra of cooked rice.

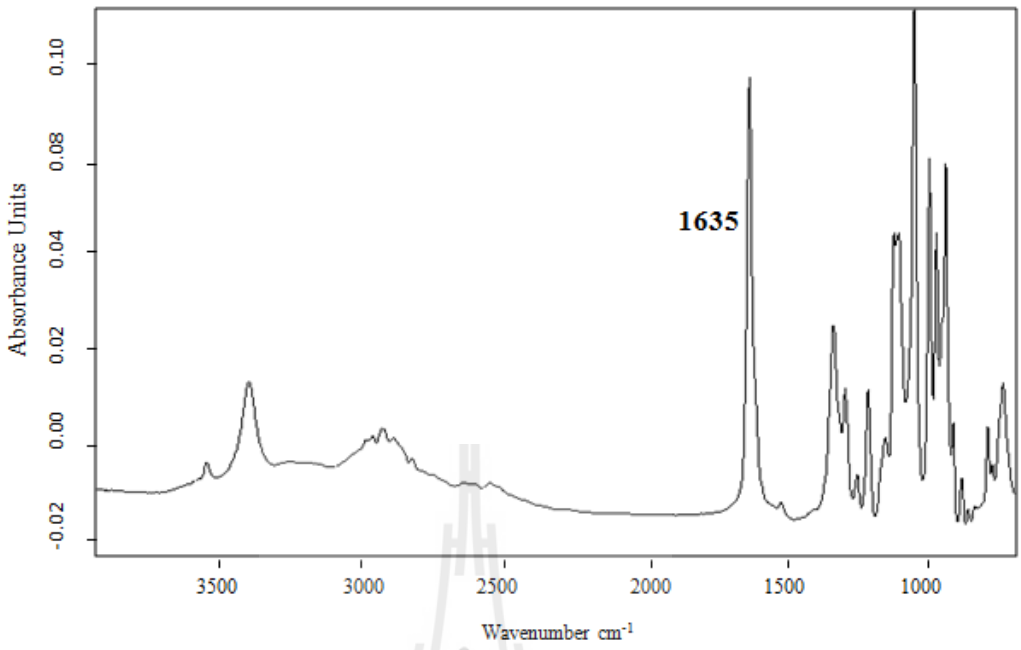


Figure 4.8 Representative FT-IR spectra of Trolox to serve as standard.

4.2.2 Classification of colored rice varieties by PCA and DFA analysis

Discriminant function analysis (DFA) was performed to clarify rice varieties according to FT-IR spectral features. A dendrogram–based chemometric model was used for hierarchical cluster analysis based on selected PCs from PCA model to produce dendrograms shown in Figure 4.9. Grouping pattern of rice varieties are not different in uncooked and cooked rice (Figure 4.9 A and 4.9 B). All dark rice varieties were clustered together and were separated from other varieties, two white rice varieties were grouped together, whereas classification of red and brown rice into different groups cannot be resolved.

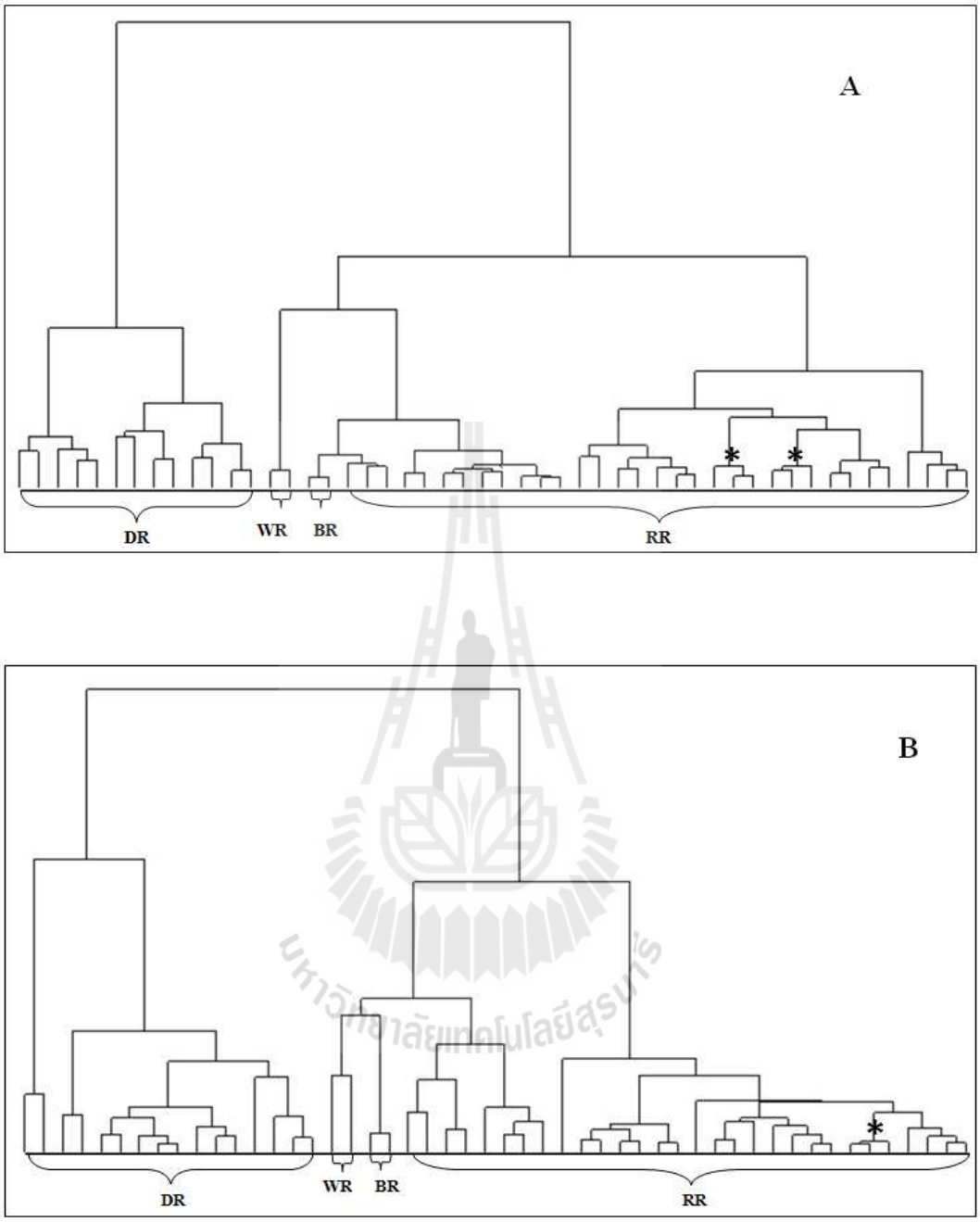


Figure 4.9 DFA results of uncooked (A) and cooked (B) rice of all varieties, including dark rice (DR), red rice (RR), brown rice (BR) and white rice (WR).

*Dark rice varieties

The PCA loading plots identified wavenumber associated with the high contribution in the linear regression model. In other words, the peak at specific wavenumber resulted from vibration properties that discriminate the samples (Lu and Rasco, 2010; Lu et al., 2010). The PCA loading plots of rice varieties that explained the segregation shown in Figure 4.10. The peaks positively associated with the most discrimination among different varieties of rice were located around wavenumber of 1200 to 800 cm^{-1}, wavenumber of 1400 cm^{-1} and between wavenumber of 1800 and 1500 cm^{-1}. The wavenumber at 1736 cm^{-1} is due to the C=O stretching of lipids (Dovbeshko et al., 1997) and the wavenumber at 1679 cm^{-1} is due to C=O stretching vibration that are H−bonded (Dovbeshko et al., 1997). Most of these distinctive peaks are associated with phenolic ring structures. For example, the wavenumber at 1630 and 1589 cm^{-1} are assigned to ring C−C stretch of phenyl (Schulz and Baranska, 2007); the wavenumber at 1559 cm^{-1} is assigned to the ring base (Dovbeshko et al., 1997); the wavenumber at 1510 cm^{-1} is due to in-plane CH bending vibration from the phenyl ring (Schulz and Baranska, 2007); the wavenumber at 1489 cm^{-1} is due to in-planed CH bending vibration. The other wavenumbers are due to the presence of carbohydrate groups including, the wavenumber at 1050 cm^{-1} is assigned to C−O stretching coupled with C−O bending of the C−OH and the wavenumber at 1025 cm^{-1} due to vibrational frequency of –CH2OH groups (Schulz and Baranska, 2007). The loading plots of PCA provided reasonable explanations that concentration of phenolic compounds constitute the greatest vibration and contribute to different antioxidant capacity of different rice colored varieties.

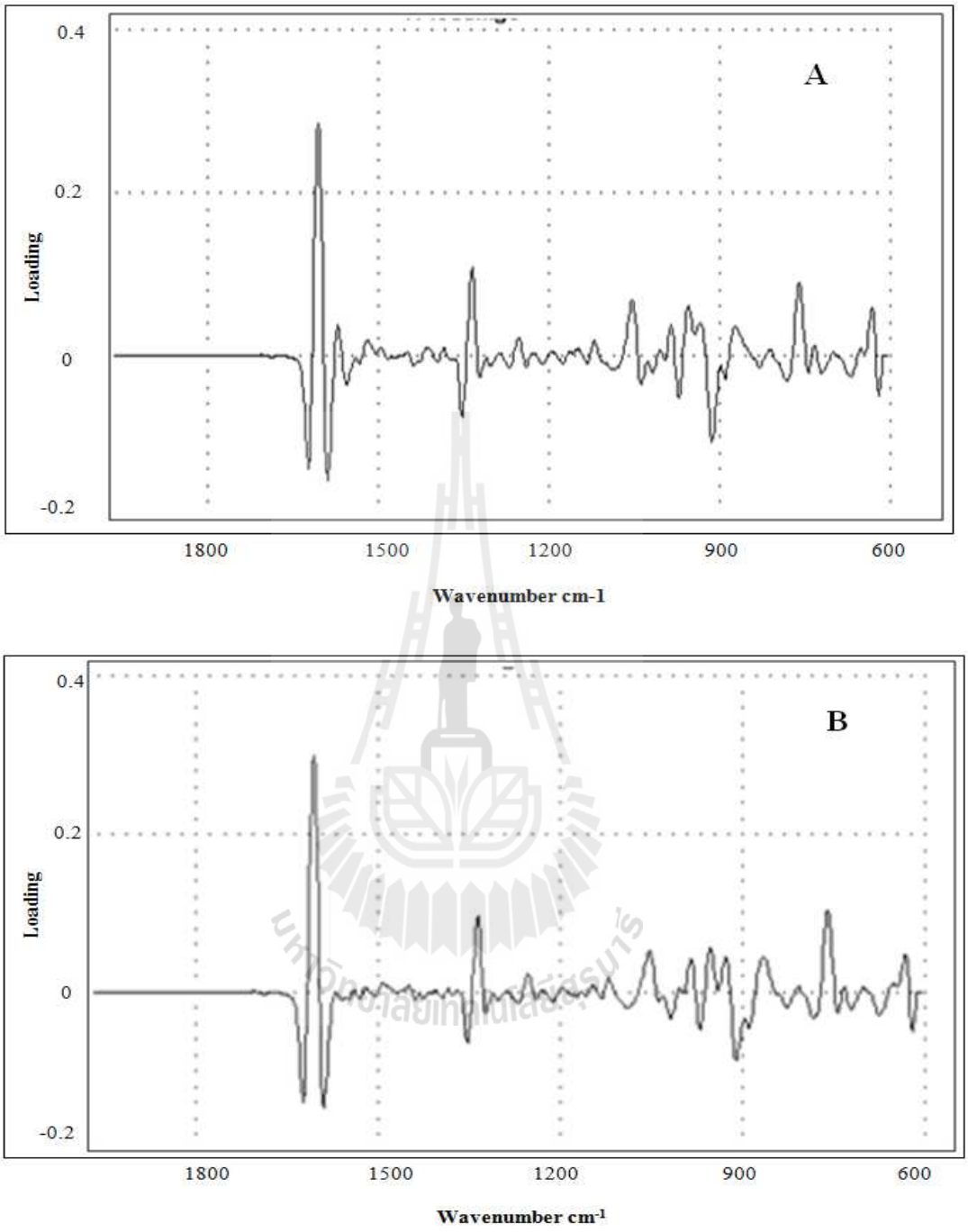


Figure 4.10 Loading plots of the PCA of rice varieties in uncooked (A) and cooked (B) forms.

4.2.3 Analysis of partial least-sequares regression (PLSR) model

PLSR models resulted parameter of cross validation (leave-one-out) for total antioxidant capacity (TAC) and total phenolic compounds of colored rice are summarized in Table 4.9. The good estimated values of TAC and total phenolic compounds by FT-IR spectroscopy were given by the regression coefficient ($R > 0.95$) with the measured reference values from TEAC and Folin-Ciocalteu assays. It was found that the standard error of calibration was low in TAC by TEAC assay and high in total phenolic compounds by Folin-Ciocalteu assay for both uncooked and cooked rice (Table 4.9). PLSR using the wavenumber from 1800 to 900 cm^{-1} was performed to establish linear regression between values measured by chemical based assays and FT-IR spectral features of uncooked (Figure 4.11) and cooked rice (Figure 4.12). These validated PLSR models were used to predict values TAC and TPC of rice varieties (n=50). Precision and errors of prediction results for the FT-IR PLSR models were comparable to those associated with the reference chemical based assays (Tables 4.10 and 4.11).

Table 4.9 PLSR models (1800-900 cm^{-1}) for determination of total antioxidant activity and phenolic compounds in rice.

Rice varieties	Total antioxidant capacity (mM TEAC)				Phenolic compounds (mg GAE/L.)			
	Mean	**Range**	**SD**	**SEC***	**Mean**	**Range**	**SD**	**SEC***
Uncooked								
Dark rice	0.443	0.379-0.551	0.047	0.010	776.93	536.10-1099.50	148.48	34.06
Red rice	0.322	0.216-0.421	0.048	0.009	567.50	280.00-882.40	140.92	27.12
Brown rice	0.200	0.189-0.213	0.016	0.011	194.80	244.10-389.60	69.72	49.30
White rice	0.078	0.051-0.106	0.039	0.027	123.30	107.30-139.30	22.62	16.00
Cooked								
Dark rice	0.219	0.154-0.399	0.054	0.012	276.25	170.90-405.50	62.50	14.33
Red rice	0.175	0.108-0.278	0.032	0.006	255.66	136.90-546.90	86.10	16.57
Brown rice	0.107	0.102-0.114	0.008	0.005	136.74	110.10-163.38	37.67	26.64
White rice	0.098	0.083-0.144	0.021	0.015	60.00	51.90-68.10	11.45	8.10

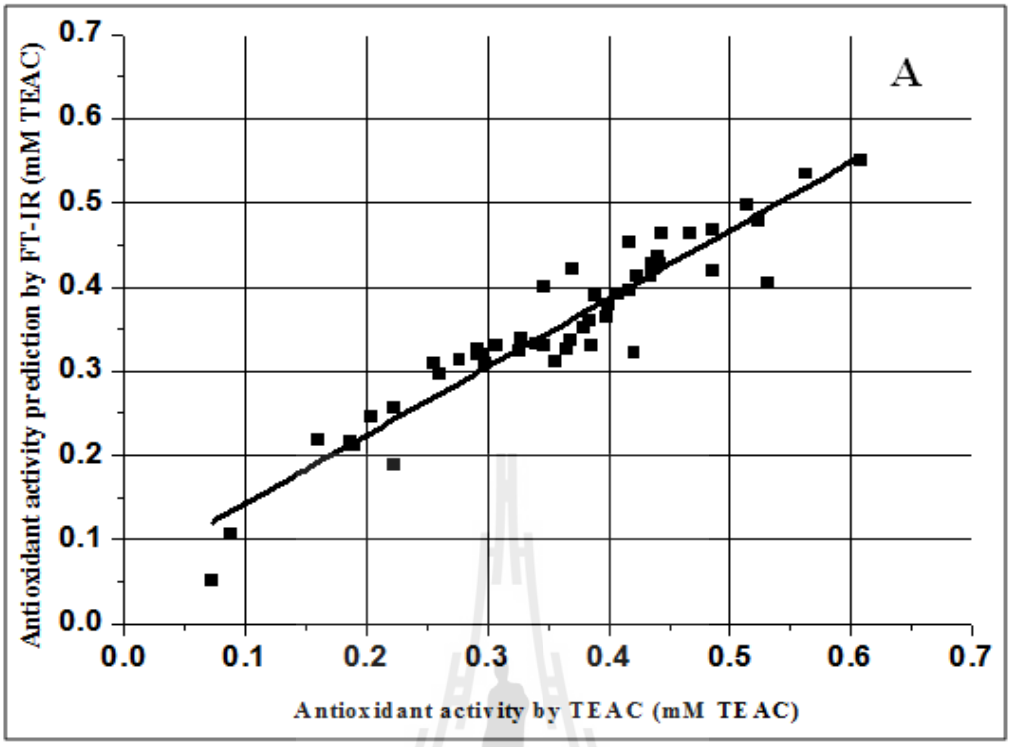
*Standard error of calibration.

Table 4.10 PLSR models for predicted total antioxidant capacity in rice varieties using FT-IR for TEAC Assay (N=50).

Rice varieties	Uncooked rice				Cooked rice			
	Reference value (mM TEAC)		IR predicted value (mM TEAC)		Reference value (mM TEAC)		IR predicted value (mM TEAC)	
	Mean	SD	Mean	SD	Mean	SD	Mean	SD
Dark rice (n=19)	0.458	0.067	0.443	0.047	0.220	0.067	0.219	0.054
Red rice (n=27)	0.323	0.323	0.322	0.048	0.173	0.051	0.175	0.032
Brown rice (n=2)	0.207	0.206	0.200	0.016	0.131	0.113	0.107	0.008
White rice (n=2)	0.081	0.010	0.078	0.039	0.067	0.013	0.098	0.021

Table 4.11 PLSR models for predicted phenoic compounds in rice varieties using FT-IR for Folin-Ciocalteu assay (N=50).

Rice varieties	Uncooked rice				Cooked rice			
	Reference value (mg GAE/l)		IR predicted value (mg GAE/l)		Reference value (mg GAE/l)		IR predicted value (mg GAE/l)	
	Mean	SD	Mean	SD	Mean	SD	Mean	SD
Dark rice (n=19)	780.87	182.84	776.93	148.48	277.24	74.36	276.25	62.50
Red rice (n=27)	572.93	146.11	567.50	140.92	248.67	88.93	255.66	86.10
Brown rice (n=2)	218.30	43.84	194.80	69.72	135.30	63.63	136.74	37.67
White rice (n=2)	114.80	31.81	123.30	22.62	46.30	2.82	60.00	11.45



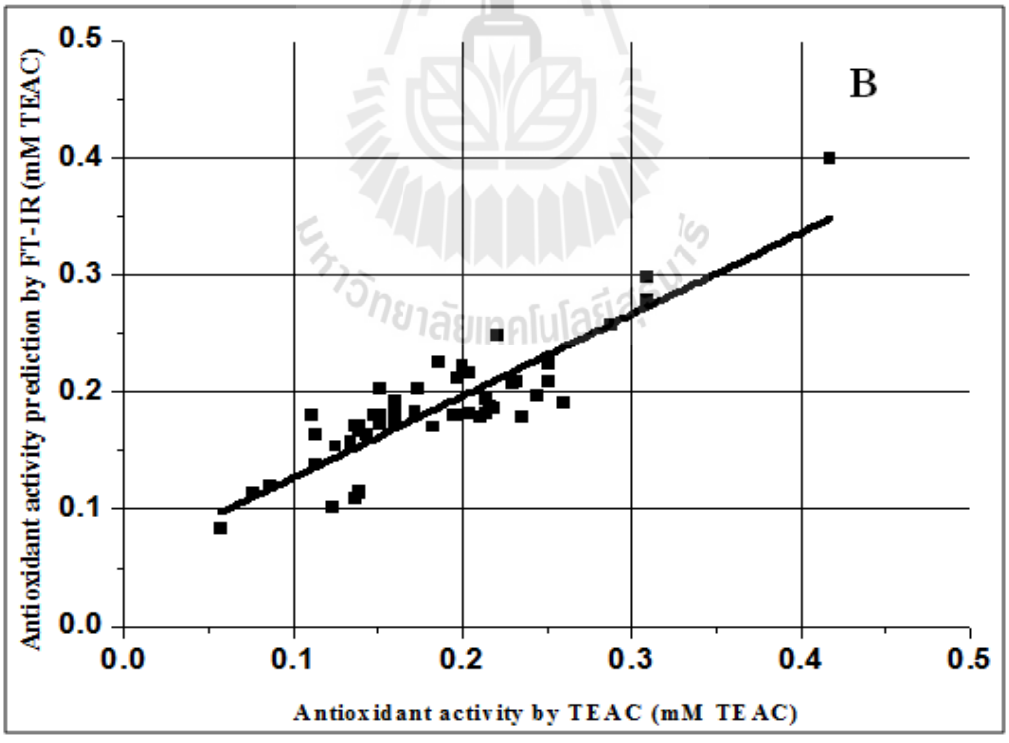


Figure 4.11 Cross-validated (leave-one-out) PLSR plots for antioxidant capacity in uncooked rice (A) and cooked rice (B) varieties (N=50) using TEAC assay.

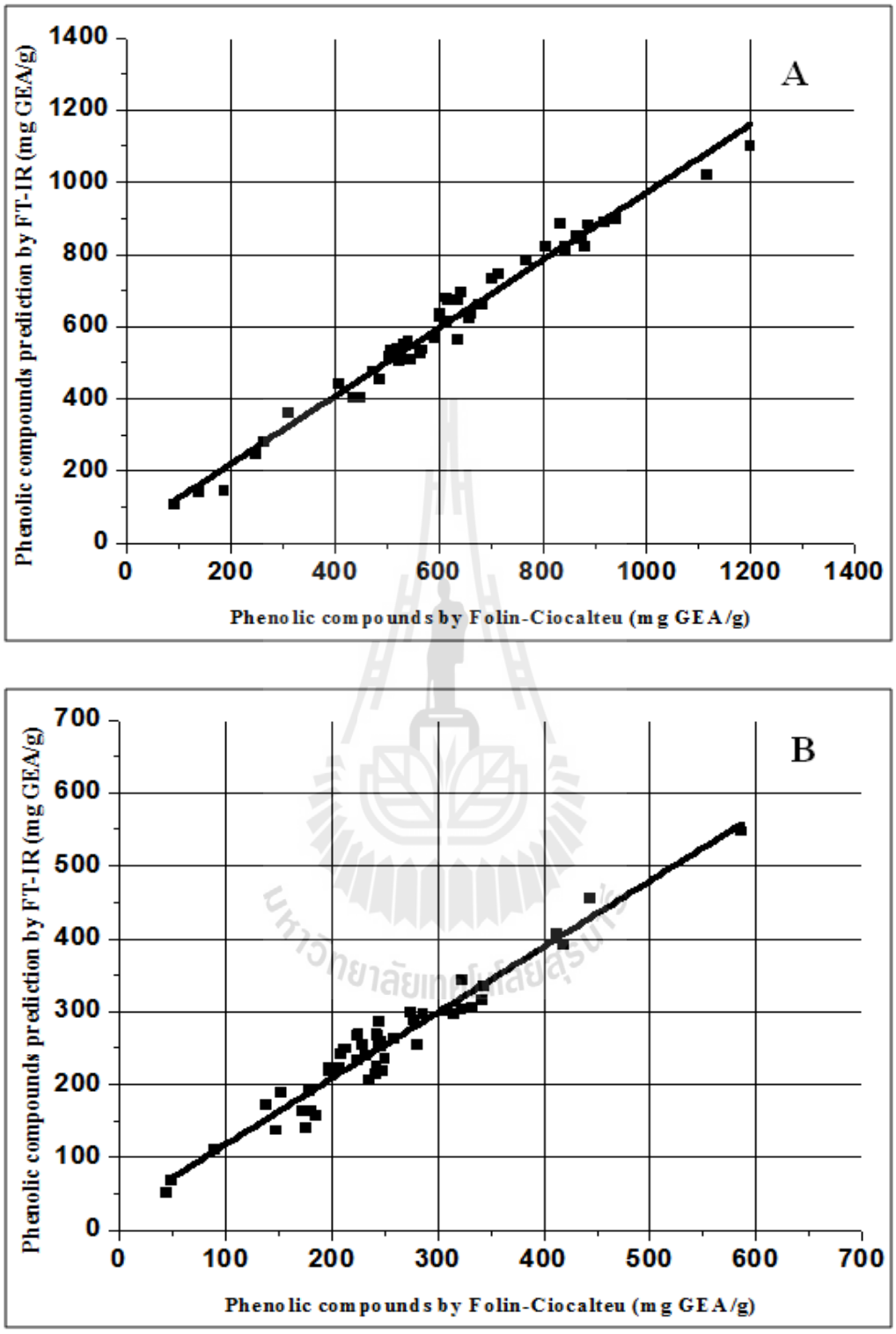


Figure 4.12 Cross-validated (leave-one-out) PLSR plots for phenolic compounds in uncooked rice (A) and cooked rice (B) varieties (N=50) using Folin-Ciocalteu assay.

In conclusion, FT-IR spectral features with chemometrics can be used to quantify and predict antioxidant capacity and phenolic compounds of colored rice varieties with a precision similar to that obtained by chemical assays. Furthermore, FT-IR requires less time for sample preparation and less cost for chemicals. In the future, this rapid detection method may be used to determine antioxidant capacity and phenolic compounds of other crops, plants, fruits and vegetable, as suggested by the results of this work and the previously published. FT-IR has been used determine antioxidant capacity and phenolic compounds in Garlic (*Allium sativum*), Elephant Garlic (*Allium ampeloprasum*), onion (*Allium cepa*), shallot (*Allium oschaninii*) (Xiaonan et al., 2011), in fruit extracts (Henry et al., 2005), and in propolis extracts (Augustin et al., 2011).

CHAPTER V

CONCLUSIONS

The aims of this study were to 1) determine and compare total antioxidant capacity of Thai glutinous rice varieties which included nineteen dark, twenty-seven red, two brown, and two white rice, 2) to compare these qualities between uncooked and cooked forms of each rice variety and 3) to compare the results from chemical analysis of antioxidant capacity and phenolic content to the results from the Fourier Transform Infrared (FT-IR) Spectroscopy method in order to draw the suggestion about the application of FT-IR in antioxidant capacity study in complex sample such as rice flour.

Significant differences in antioxidant capacity as well as in concentrations of phytochemicals contributing to antioxidant capacity were observed among rice varieties in the study. The phytochemicals of interest included phenolic compound, flavonoids, anthocyanin, and proanthocyanidin in the glutinous rice grains. The results showed that dark glutinous rice had the highest level of antioxidant capacity followed by red rice, brown rice and white rice. The results from the comparison of phytochemicals levels of all color categories of rice also revealed the same order from the highest value to the lowest value. Furthermore, a positive and significant correlation between the antioxidant capacity and levels of phytochemicals was demonstrated.

Cooking process was shown to reduced antioxidant capacity and concentration of phytochemicals in the rice grains, which should be due to the loss of the compounds in the water, thermal decomposition and, potentially, interactions with other components. The results showed that, when cooked, antioxidant capacity and phytochemicals reduced for at least 30 percent. Dark rice had the highest percentage reduction after having been cooked, followed by white rice, red rice and brown rice. The least percentage reduction of flavonoid content compared to other phenolic compounds indicated that flavonoid was most resistant to cooking process, while the most sensitive compound to cooking process appeared to be proanthocyanidin whose percentage reduction was highest.

Apart from chemical methods, FT-IR with chemometrics was performed to measure antioxidant capacity and phenolic compounds in colored glutinous rice varieties. It was found that the results from using FT-IR were comparable to those obtained from chemical methods. The precision of the two methods were concluded to be similar. Nonetheless, FT-IR has some advantages. FT-IR requires simple sample preparation, less time and reduced cost for chemicals while satisfactory precision and sensitivity are still retained. The work here presented has therefore volunteered itself as one of the reports that suggests FT-IR as the potential method to be used for determinations of antioxidant capacity and phenolic compounds of other plant extracts. Being simple, quick and convenient measuring tool, FT-IR may play an important role in the discovery of functional compounds in the new source plants. More data on absorption characteristic peaks of more compounds is crucial to push the development of the application forward.

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APPENDICES

APPENDIX A

STANDARD STOCK SOLUTIONS

A.1 Preparation of Trolox solution

1.5 mM Trolox was prepared with 80% ethanol to reach the final concentrations of 0, 0.15, 0.30, 0.60, 0.90, 1.20, and 1.50 mM. The amounts of each components are shown in Table A.1

Table A.1 Trolox solutions preparation.

Stock solution (1.5 mM Trolox) (mL)	80% Ethanol (mL)	Final conc (mM)
0	100	0
10	90	0.15
20	80	0.30
40	60	0.60
60	40	0.90
80	20	1.20
100	0	1.50

A.2 Preparation of of Rutin

5 g/L Rutin was prepared with 80% methanol to reach the final concentrations of 0, 50, 100, 200, 300, 400, 500, 700, 1000 µg/mL. The amounts of each components are shown in Table A.2

Table A.2 Rutin solutions preparation**.**

Stock solution (5 g/L Rutin) (mL)	80% Methanol (µL)	Final conc (µg/mL)
0	2,000	0
20	1,980	50
40	1,960	100
80	1,920	200
120	1,880	300
160	1,840	400
200	1,800	500
300	1,700	700
400	1,600	100

A.3 Standard solution of Gallic acid

5 g/L Gallic acid was prepared with deionized water to reach the final concentrations of 0, 50, 100, 200, 300, 400, 500, 700, 1000 mg/L. The amounts of each components are shown in Table A.3.

Table A.3 Gallic acid solutions preparation**.**

Stock solution (5 g/L Gallic acid) (mL)	Deionized water (µL)	Final conc (mg/L)
0	2,000	0
20	1,980	50
40	1,960	100
80	1,920	200
120	1,880	300
160	1,840	400
200	1,800	500
300	1,700	700
400	1,600	100

A.4 Standard solution of Cyanidin

5 g/L Cyanidin was prepared with 80% methanol to reach the final concentrations of 0, 50, 100, 200, 300, 400, 500, 700, 1000 µg/mL. The amounts of each components are shown in Table A.4

Table A.4 Cyanidin solutions preparation**.**

Stock solution (5 g/L Rutin) (mL)	80% Methanol (µL)	Final conc (µg/mL)
0	2,000	0
20	1,980	50
40	1,960	100
80	1,920	200
120	1,880	300
160	1,840	400
200	1,800	500
300	1,700	700
400	1,600	100

APPENDIX B

CHEMICAL SOLUTIONS

B.1 7 mM ABTS

ABTS 1.14 g in water 300 mL

B.2 2.54 mM Potassium persulfate

Potassium persulfate 0.198 g in water 300 mL

B.3 Carbonate 7.5 g/L

Carbonate 75 g in water 1,000 mL

B.4 5% $NaNO_2$

$NaNO_2$ 25 g in water 500 mL

B.5 10% $AlCl_3$

$AlCl_3$ 50 g in water 500 mL

B.6 1 M NaOH

NaOH 20g in water 500 mL

B.7 Butanol : HCl (95 : 5 v/v)

Butanol 950 mL : HCl 50 mL

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