CHAPTER 1

Introduction

1.1 Rice

Rice, *Oryza sativa* L., is a major cereal mainly crop in the developing world. It is an important staple food source for over half of the world's population and approximately 95% of its production is in Asia [1]. In the Asia-Pacific region, rice is a daily food and it is believed to provide more health benefits than other carbohydrate-based foods because it contains several nutrients and antioxidative compounds such as vitamin B complex, vitamin E complex (tocopherols and tocotrienols), phytosterols, phytic acid, oryzanols and phenolic compounds [2−6].

1.1.1 Morphology of rice grain

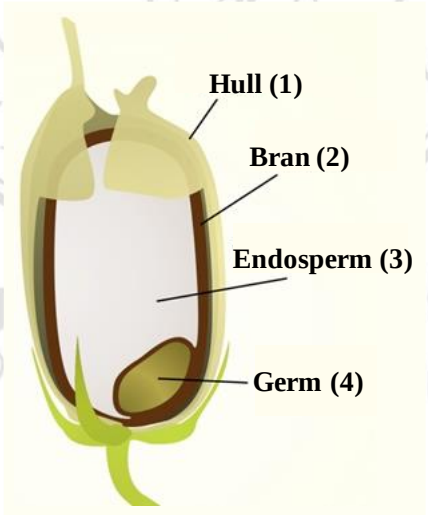


Figure 1.1 Morphology of rice grain

The parts of the rice grain **(Figure 1.1)** are divided into rice hull **(1)**, bran part **(2)** containing pericarp, seed coat nucellus and aleurone layer, endosperm **(3)** and germ **(4)**. The percentages of these components are shown in **Table 1.1.**

Table 1.1 Components of rice grain

Component	Percent
Hull	20%
Bran & Germ	10%
Starchy endosperm	70%

1.1.2. Rice bran

Rice bran, a part of the rice kernel that contains pericarp, aleurone, and subaleurone fractions, is a byproduct of rice milling and weighs 10% of the total rice grain weight. Rice brans, oils, and hulls contain a large number of bioactive compounds, with pigmented brans containing many more bioactive compounds than white brans.

Rice bran oil is concentrated in the germ and bran layers in rough rice grain. Rice bran oil contains ~96% of saponifiable fractions and ~ 4% unsaponifiable fractions which include phytosterols, sterol esters, triterpene alcohols, hydrocarbons and tocopherols **(Table 1.2)** [7].

Table 1.2 Rice bran oil compositions

Lipid Type			Percent
Saponifiable lipids			90-96
	Neutral lipids		88-89
		Triacylglycerols	83-86
		Diacylglycerols	3-4
		Monoacylglycerols	6-7
		Free fatty acids	2-4
		Waxes	6-7
	Glycolipids		6-7
	Phospholipids		4-5
Unsaponifiable lipids			2-4
	Phytosterols		43
	Sterol esters		10
	Triterpene alcohols		28
	Hydrocarbons		18
	Tocopherols		1

1.1.3 Pigmented rice

Although rice are widely consumed as white rice, there are many special rice cultivars that contain pigments such as black, purple and red. Pigmented rices

shown in **Figure 1.2** have a color on the pelea, lemma and other inside parts, such as pericarp, tegment and aleurone layer [8].

Figure 1.2 Pigmented rice

A great deal of interest is given to the association between the consumption of pigmented rice and the improvement of human health due to the great antioxidant potency of phenolic compounds they contain. Phenolic compounds or polyphenols are important antioxidants because of their high redox potentials. They act as reducing agents, hydrogen donors, singlet oxygen quenchers and as metal chelating agents. Health-related effects of phenolic compounds such as antibacterial, antimutagenic, anticarcinogenic, antithrombotic and vasodilatory activities have been reported [9-14].

Pigmented rice has been reported to contain acetylated procyanidin, anthocyanins, and other phenolics with significant free radical scavenging activity. Pigments in black rice are located in the aleurone layer as a mixture of anthocyanins. Those colorings are naturally occurring compounds that belong to the family of flavonoids, in which pelargonidin, cyanidin, delphinidin, peonidin, petunidin and malvidin represent the most commonly occurring anthocyanins [15]. It has been reported that feeding black rice to rabbits in place of white rice had resulted in increased high-density lipoprotein concentrations in hypercholesterolemic rabbits which corresponded to a reduction in the size of atherosclerotic lesions in these animals [16].

1.1.4 Riceberry rice

Riceberry, a black purple rice variety (*Oryza Sativa* L.), is a new breeding line developed by the Rice Research Center, Kasetsart University, Kamphaeng Saen, Nakhon Pathom, Thailand **(Figure 1.3)**. It is a cross-bred strain from Khao Hom Nin Rice variety which is well known as containing high antioxidant properties and Khao Dawk Mali (KDML) 105 which is well known as a fragrant rice. Riceberry oil extracted from the bran part of Riceberry grain by a cold pressure technique had been reported to contain high antioxidant contents such as γ-oryzanol, α-tocopherol, γ-tocotrienol, β-carotene, lutein, co-enzyme Q10 phenolic compounds, quercetin, isorhamnetin and other active compounds [17-18].

Figure 1.3 Riceberry rice

Physical properties of Riceberry rice

Height	: 145 cm
Harvest stage	: 127 dates
Product	: 600-700 kg/farm
% Brown rice	: 78%
% Head rice	: 50%
Length of seed	: paddy 11 mm
Brown rice	: 7.8 mm

Nutrition properties of brown rice, Riceberry

Amylose	: 18.3	%
Vitamin E	: 577	μg/100g

β-carotene	: 21.61 μg/100g
Antioxidant Activity (FRAP)	: 30.91 μmole TE/g
Antioxidant Activity (ORAC)	: 134.29 μmole TE/g
Phytate	: 659.24 mg/100g
Polyphenol	: 202.5 mg/100g
Tannin	: 66.78 mg/100g

1.2 Effective components in rice bran

1.2.1 Phenolic Compounds

Phenolic compounds or polyphenols are substances containing a phenolic ring bearing one or more hydroxyl substituents. Hydroxybenzoic and hydroxycinnamic acids have a single-ring structure with absorption maxima at 280 nm for the C6-C1 skeleton of hydroxybenzoic acid derivatives (gallic acid, protocatechuic acid, *p*-hydroxybenzoic acid, vanillic acid and syringic acid) and at 320 nm for the C_6-C_3 skeleton of hydroxycinnamic acid derivatives (*p*-coumaric acid, ferulic acid, caffeic acid, sinapic acid, chlorogenic acid and cinnamic acid) [19]. However, flavanoids comprise three ring structures and can be further classified into anthocyanins, flavan 3-ols, flavones, flavanones and flavonols. Chemical structures of some phenolic compounds were shown in **Figure 1.4**.

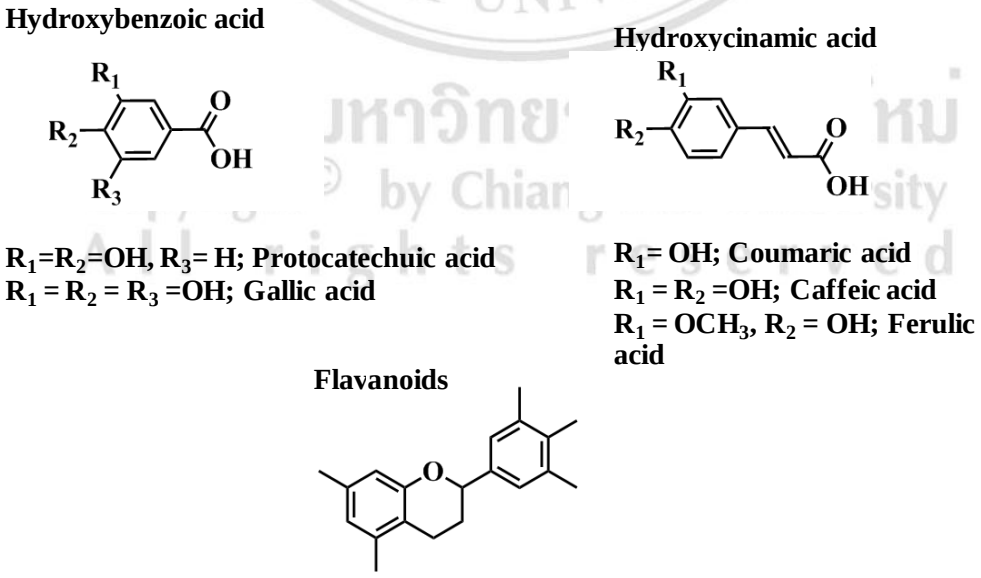


Figure 1.4 Chemical structures of some phenolic compounds

Since, phenolics act as reducing agents, hydrogen donors, singlet oxygen quenchers and as metal chelating agents. Their important bioactivities are antioxidants. Moreover, health-related effects of phenolic compounds such as antibacterial, antimutagenic, anticarcinogenic, antithrombotic and vasodilatory activities have been reported [20-25].

Cocoa, potato, yam, tomato, kale, Brussels sprouts, broccoli and others dark green leafy and brightly-colored vegetables as well as legumes and cereals also rices, in addition to spices and fruits such as cherries and citrus, are particularly rich in phenolic compounds.

1.2.2 Sterols and triterpenoids

Sterols and triterpenoids are a group of naturally occurring substances derived from hydroxylated polycyclic isopentenoids having a 1,2-cyclopentanophenanthrene structure **(Figure 1.4)**. These compounds contain a total of 27-30 carbon atoms (the number of carbon atoms in the biosynthetic precursor squalene oxide) in which a side chain with carbon atoms 2-7 is attached at the carbon 17 position (C-17). Their structures are closely related and varied depending on the extent of modifications of the ring system and side chain variations [20]. Thus, the number and position of double bonds in both the polycyclic and side chain systems of sterols can be different. In addition, the side chains can also bebroadened, lengthened, or shortened at certain carbon positions beyond C-22. Chemical structures of some sterols and triterpenoids in plant are shown in **Figure 1.5.**

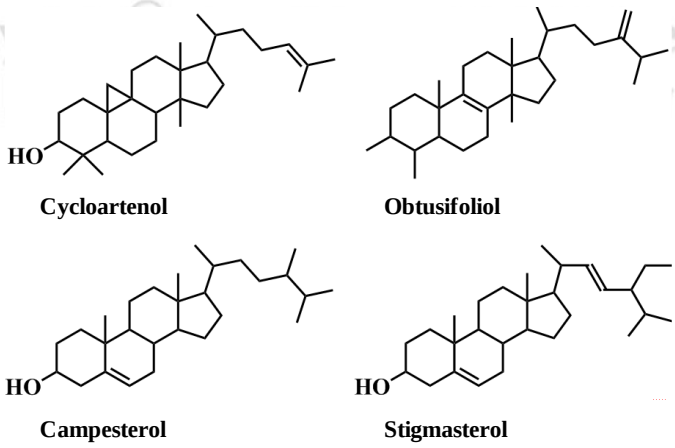
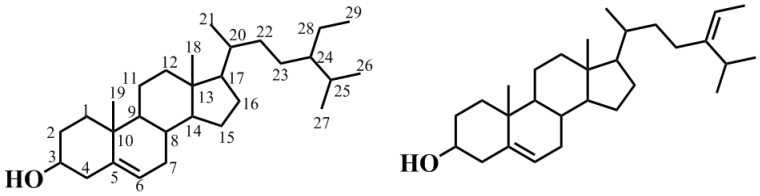


Figure1.5 Chemical structures of some plant sterols and triterpenoids



***β*-Sitosterol** **△5-avenasterol**

Figure1.5 (*Cont.*) Chemical structures of some plant sterols and triterpenoids

Sterols and triterpenoids are known to have a wide range of biological activities and physical properties. Plantsterols (i.e. phytosterols), in particular, are important agricultural products for health and nutrition industries. They are useful emulsifiers for cosmetic manufacturers and supply the majority of steroidal intermediates and precursors for the production of hormone pharmaceuticals [21]. A number of plant sterol with specific structures are known to inhibit oxidative deterioration of oils serving as potential antipolymerization agents for frying oils. Hypocholesterolemic activities of some phytosterols (e.g. soysterols, vegetable oil components and sitosterol) have been documented. The saturated analogues of phytosterols and their esters have been suggested as effective cholesterol-lowering agents offering cardiologic health benefits [22]. Commercial margarines formulated with certain levels of phytosterols are currently available in several countries. Reported phytosterol data [23] for some plant foods and vegetable oils have shown that nuts and oils contain higher levels (2: 1%) of sterols than fruits and vegetables <<0.05% **(Table1.3)**. It is noteworthy that the compositional distributions of phytosterols in certain vegetable oils have been used for their identifications despite their presence in the lipids as minor constituents [24]. Hence, phytosterols and other non-saponifiable compounds in oils are often used as markers for the assessment of adulterated oils [25].

Table 1.3 Some reported phytosterols and triterpenoids concentrations in foods and vegetable oils (mg/100 g) [23]

Food	Phytosterols and triterpenoids
Potato	5
Tomato	7
Pear	8
Lettuce	10

Table 1.3 *cont*.

Food	Phytosterols and triterpenoids
Carrot	12
Apple	12
Onion	15
Banana	16
Fig	31
Garbanzo bean	35
Kidney bean	127
Soybean	161
Pecan	108
Almond	143
Cashew nut	158
Peanut	220
Sesame seed	714
Peanut oil	207
Olive oil	221
Soybean oil	250
Cotton seed oil	324
Safflower oil	444
Sesame oil	865
Corn oil	968
Rice bran oil	1190

1.2.3 γ-Oryzanols

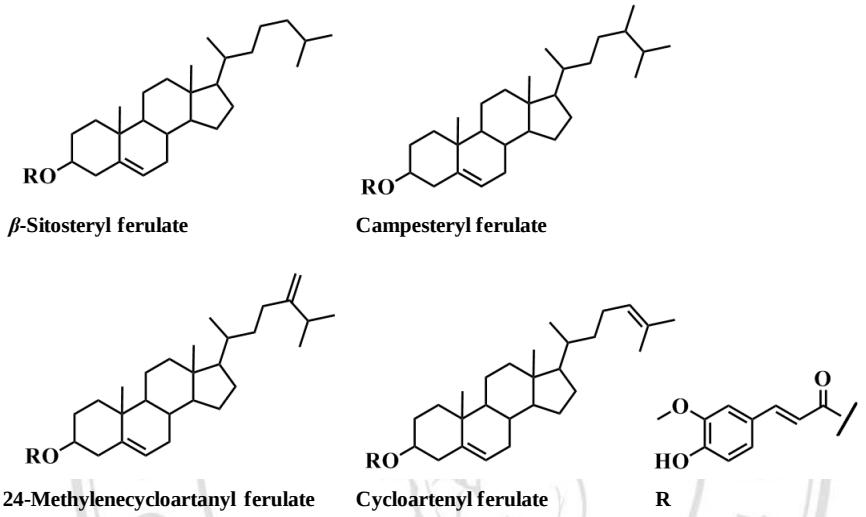
γ-Oryzanol has the chemical structure that of two molecules in one, first a plant sterol and second ferulic acid. The sterol (triterpenyl alcohol) is the largest part of the molecule. Ferulic acid is one of the hydroxycinnamic acids, a subgroup of plant phenolic acids.

γ-Oryzanol component of rice bran oil was first presumed to be a single component. But later it was determined to be a mixture of 10 ferulate esters of triterpene alcohol [26] the fraction of which contains ferulate (4-hydroxy-3 metoxy cinnamic acid) esters of triterpene alcohols and plant sterols. Cycloartenyl ferulate, campesteryl ferulate, 24-methylenecycloartanyl ferrulate and *β*-sitosteryl ferulate are the major components accounted for 80 percent of total *γ*-oryzanols. Their structures are shown in **Figure 1.6**.

γ-Oryzanol inhibits tumor promotion, reduces serum cholesterol levels, used to reduce blood cholesterol levels, treat nerve imbalance, as well as being an antioxidant

and preservative, and can also be used to treat nerve imbalance and disorders of menopause [27-29].

Rice bran oil remains the best natural source for γ-oryzanols. There is no conclusive evidence of the presence of this compound in any other plants than rice. However, this does not mean rice is the only source of γ-oryzanols in nature.



β-Sitosteryl ferulate **Campesteryl ferulate**

24-Methylenecycloartanyl ferulate **Cycloartenyl ferulate** **R**

Figure1.6 Chemical structures of the four main components of γ-oryzanols

1.2.4 Tocopherols and tocotrienols

An essential nutrient for the body, vitamin E is made up of four tocopherols and four tocotrienols. Both of the tocopherols and tocotrienols occur in alpha, beta, gamma and delta forms, determined by the number of methyl groups on the chromanol ring [30]. The alpha form has three methyl groups, the beta and gamma forms have two methyl groups and the delta has only one methyl group. The slight difference between tocotrienols and tocopherols lie in the unsaturated side chain having three double bonds in its farnesyl isoprenoid tail [31-32]. Each form, alpha, beta, gamma and delta, has slightly different biological activity [33]. The structures of them are shown in **Figure 1.7**.

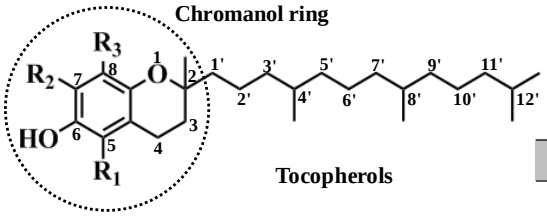
All of the tocotrienol and tocopherol isomers have antioxidant activity due to the ability to donate a hydrogen atom (a proton plus electron) from the hydroxyl group on the chromanol ring, to a free radical in the body. This process inactivates

("quenches") the free radical by effectively donating a single unpaired electron (which comes with the hydrogen atom) to the radical.

As natural antioxidants, tocopherols protect food from oxidation by protecting the stability of oils and fats. The bioavailability of tocopherols for humans depends on adequate amounts of lipids and fats in the diet and on the consumption of plant foods [34].

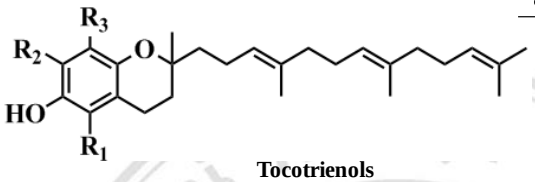
Tocopherols are also known as four different chemical variations of vitamin E. α-Tocopherol is traditionally recognized as the most highly active form of vitamin E in humans [35]. It is a major fat-soluble antioxidant that acts in the cellular membrane [36]. While α- tocopherol is mainly important for its vitamin E efficacy, γ-tocopherol is the superior antioxidant for oxidation-sensitive oil [37]. As an antioxidant, vitamin E acts to protect human cells against the effects of free radicals which are potentially damaging byproducts of energy metabolism. Free radicals can harm cells and tissues through damage to proteins, DNA and lipids and may contribute to the development of cardiovascular disease and cancer [38-39]. High dietary levels of vitamin E are known to protect against abnormal blood clotting, heart attacks, strokes and cancer which arise from the abnormal oxidation of cholesterol and fatty acids [35,40]. Specifically, symptoms caused by α-tocopherol deficiency can be alleviated by tocotrienols. Thus, tocotrienols may be viewed as being members of the natural vitamin E family not only structurally but also functionally. Thus, one model for the function of vitamin E in the body is that it protects cell membranes, active enzyme sites and DNA from free radical damage.

Many research claims of tocotrienols' health benefits for human beings have been made. Tocotrienol is more effective antioxidant than tocopherol because its unsaturated side chain facilitates better penetration into saturated fatty layers of the brain and liver [41-42]. Tocotrienols can lower tumor formation, DNA damage and cell damage [43-44].



Tocopherols

	R_1	R_2	R_3
α	CH_3	CH_3	CH_3
β	CH_3	H	CH_3
γ	H	CH_3	CH_3
δ	H	H	CH_3



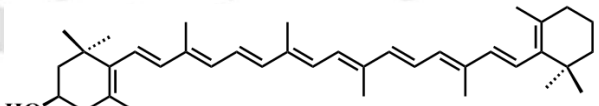
Tocotrienols

Figure 1.7 Structures of tocopherols and tocotrienols

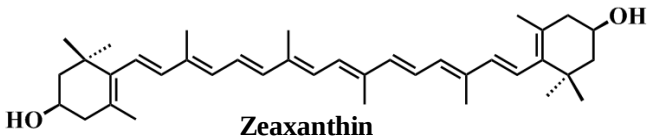
The tocopherols and tocotrienols are found in selected vegetable oils, including rice bran oil and palm oil, wheat germ, barley, saw palmetto, anatto and certain other types of seeds, nuts, grains and the oils derived from them [45]. This variant of vitamin E typically only occurs at very low levels in nature [46].

1.2.5 Carotenoids

Carotenoids are a class of natural fat-soluble pigments that are associated with the lipidic fractions. Carotenoids are polyisoprenoid compounds and can be divided into two main groups; (a) carotenes or hydrocarbon carotenoids, which composed of only carbon and hydrogen atoms and (b) xanthophylls that are oxygenated hydrocarbon derivatives. They contain at least one oxygen function such as hydroxy, keto, epoxy and methoxy or carboxylic acid groups **(Figure 1.8)**.

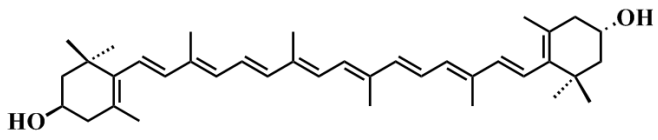


Cryptoxanthin

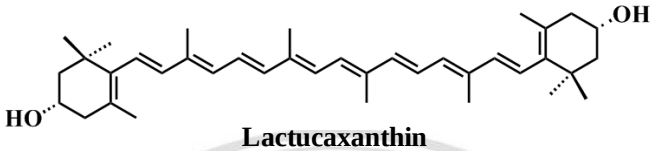


Zeaxanthin

Figure 1.8 Structure of selected carotenoids



Lutein



Lactucaxanthin

Figure 1.8 (*Cont.*) Structure of selected carotenoids

In humans, carotenoids play two primary roles. Most of them give antioxidant activity and some of them are converted into vitamin A. Carotenoids that the body converts to vitamin A are referred to as "provitamin A" carotenoids. The most well known of this group are *β*-carotene and *α*-carotene. Some of the better known carotenoids without provitamin A activity but with very high antioxidant activity are lutein, lycopene and zeaxanthin. Vitamin A which has many vital systemic functions in humans can be produced within the body from certain carotenoids, notably *β*-carotene [47]. Dietary *β*-carotene is obtained from a number of fruits and vegetables such as carrots, spinach, peaches, apricots and sweet potatoes [48].

As carotenoids are among the active components of fruits and vegetables with potential health effects, an enhancement of carotenoid levels might thus be desirable. *α*-Carotene was found in carrots, pumpkin and red and yellow peppers and cryptoxanthin was obtained from oranges, tangerines, peaches, nectarines and papayas. Lycopene, the hydrocarbon carotenoid that gives tomatoes their red color, is particularly effective at quenching the destructive potential of singlet oxygen [49]. Lutein and zeaxanthin xanthophylls, found in corn and in leafy greens such as kale and spinach, are believed to function as protective antioxidants in the macular region of the human retina [50]. Astaxanthin, a xanthophyll found in salmon, shrimp and other sea foods, is another naturally occurring xanthophyll with potent antioxidant properties [51].

1.3 Health benefit of rice

1.3.1 Antioxidant activity

Rice contains good quality protein, high contents of fiber and vitamins. Pigmented rice varieties have the potential to promote human health because they contain antioxidative compounds that have the ability to inhibit the formation or to reduce the concentrations of reactive cell-damaging free radicals [52]. These compounds include anthocyanins (glycosides), cyanidin-3-*O*-*β*-D-glucoside and peonidin-3-*O*-*β*-glucoside [53] anthocyanidins (aglycones), cyanidin and malvidin [54], polymeric procyanidins [55] the phenolic compounds anisole, 4-hydroxycinnamic acid (*p*-coumaric), 4,7-dihydroxyvanillic acid, protocatechuic acid methyl ester, syringaldehyde and vanillin [56] the phenolic compounds ferulic, sinapinic acids, the sucrose esters 6'-*O*-(E)-feruoylsucrose and 6'-*O*-(E)- sinapoylsucrose, [57] the ferulic acid sterol ester (*γ*-oryzanol) [58] and the alkaloid 4-carbomethoxy-6-hydroxy-2-quinolone [59].

1.3.2 1,1-Diphenyl-2-picrylhydrasyl (DPPH) free radical-scavenging assay

DPPH assay is one of the most widely used methods for screening antioxidant activity of plant extracts. DPPH is a stable, nitrogen-centered free radical which produces violet color in ethanol solution. It was reduced to a yellow colored product, diphenylpicryl hydrazine, with the addition of the fractions in a concentration-dependent manner that can be easily monitored using a spectrophotometer since DPPH radical has an intense absorption maximum around 520 nm. The reduction in the number of DPPH radical, which correlated with the number of available hydroxyl groups, has been used to assess the efficiency of antioxidants in plants [60].

1.3.3 Anticancer activity

Vitamin E inhibits lipid peroxidation in biological membranes and reduces oxidative stress which are causes of carcinogenesis. Vitamin E can suppress cell cycle progression and inhibits cell proliferation in prostate carcinoma, colon adenocarcinoma and osteosarcoma cells [61]. Also, vitamin E induces apoptosis in human gastric adenocarcinoma cells [62]. The *in vitro* effect of phytic acid has been demonstrated in human colon and breast cancer [63], whereas the *in vivo* effect of tricin has been

demonstrated in cancers of breast and colon [64-65]. The other compounds in rice bran that show anti-proliferation and apoptosis induction of cancers are phytosterols, *β*-sitosterol, campesterol and stigmasterol [66].

Phytochemicals contained in pigmented rice bran can suppress tumor progression or carcinogenesis [67]. It has been found that ferulic acid, a strong membrane anti-oxidative agent found in pigmented rice bran, is an effective constituent that prevents carcinogen- induced oral and colon carcinogenesis in rats [68]. Ferulic acid also plays a role in inhibition of cell growth and induction of apoptosis in HepG2 cells [69]. However, more significant constituents in rice bran that protect against cancer are waiting to be discovered.

1.4 Extraction methods

1.4.1 Solvent extraction and saponification

In a typical procedure for vegetable oilseeds, a sample is homogenized by a commercially available grain mill and extracted with absolute ethanol in a soxhlet apparatus overnight in a steam bath. Water and petroleum ether are added to the cooled extract and shaken in a separatory funnel. Evaporation of the top organic layer under water aspirator pressure leaves the total lipid extract. For saponification of the lipid extract or crude/refined vegetable oils [70-72], an aliquot of the oil sample is stirred overnight at room temperature with 1 M ethanolic potassium hydroxide. The mixture is diluted with water and extracted with three portions of diethyl ether. The combined ether extract is saponified again with ethanolic potassium hydroxide, washed with several batches of distilled water until neutral to pH paper and then dried sequentially with short columns of anhydrous sodium sulfate, deactivated alumina and anhydrous sodium sulfate. Removal of solvent yields an unsaponifiable residue suitable for chromatographic quantification of sterols.

1.4.2 Supercritical fluid extraction

A supercritical fluid (SF) is a material that can be either liquid or gas, used in a state about the critical temperature (T_c) and critical pressure (P_c) where gases and liquids can coexist. It shows unique properties that are different from those of either gases or liquids under standard conditions. A gas, when compressed isothermally to

pressure more than its critical pressure, exhibits enhanced solvent power in the vicinity of its critical temperature. Such fluids are SF.

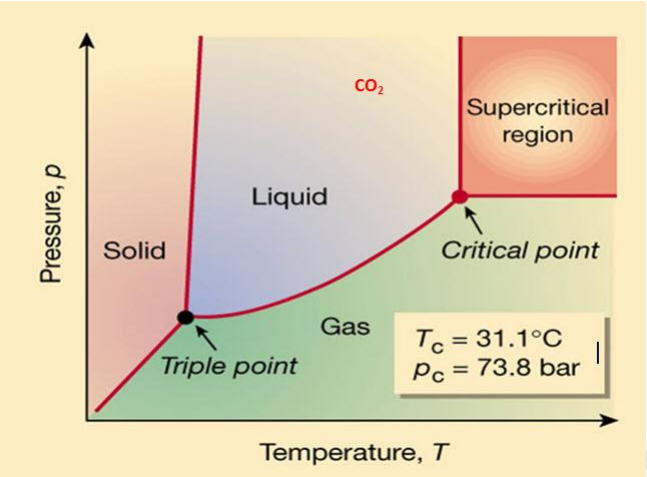


Figure 1.9 Carbon dioxide pressure-temperature phase diagram

A typical phase diagram for a pure substance **(Figure 1.9)** shows temperature and pressure regions when the substance occurs as a single phase (solid, liquid, and gas). Such regions are bounded by curves indicating the coexistence of two phases (solid-gas, solid-liquid and liquid-gas) which are involved in sublimation, melting and vaporization equilibia, respectively. The three curves intersect at the so-called triplepoint (T_P), where the solid, liquid and gas phases coexist in equilibrium. The properties of SF showed as followings:

(i) SF has highly compressed gases which combine properties of gases and liquids in an intriguing manner.

(ii) SF can lead to reactions, which are difficult or even impossible to achieve in conventional solvents.

(iii) SF has solvent power similar to light hydrocarbons for most of the solutes.

(iv) Solubility increases with increasing pressure. Rapid expansion of supercritical solutions leads to precipitation of a finely divided solid. This is a key feature of flow reactors.

(v) The fluids are commonly miscible with permanent gases (e.g. N_2 or H_2) and this leads to much higher concentrations of dissolved gases than can be achieved in conventional solvents [73].

The biggest interest for the last decade has been the applications of supercritical carbon dioxide because it has a near ambient critical temperature (31°C), thus biological materials can be processed at temperatures around 35°C. The density of the supercritical carbon dioxide (SC-CO_2) at around 200 bar pressure is close to that of hexane, and the solvation characteristics are also similar to hexane, thus, it acts as a non-polar solvent. The major advantage is that a small reduction in temperature or a slightly larger reduction in pressure, will result in almost the entire solute precipitating out as the supercritical conditions are changed or made sub-critical. SF can produce a product with no solvent residues. Examples of pilot and production scale products include decaffeinated coffee, cholesterol-free butter, low-fat meat, evening primrose oil and squalene from shark liver oil, etc. The solvation characteristics of SC-CO_2 can be modified by the addition of an entrainer such as ethanol, however, some entrainer remains as a solvent residue in the product, negating some of the advantages of the "residue-free" extraction.

Supercritical fluid extraction (SFE) is the process of separating one component (the extractant) from another (the matrix) using SF as the extracting solvent. Extraction is usually from a solid matrix but it can also be from liquids. SFE can be used as a sample preparation step for analytical purposes, or on a larger scale to either strip unwanted material from a product (e.g. decaffeination) or collect a desired product (e.g. essential oils). CO_2 is the most used SF, sometimes modified by co-solvents such as ethanol or methanol. Extraction conditions for SC-CO_2 are above the critical temperature of 31°C and critical pressure of 74 bar. Addition of modifiers may slightly alter this. SFE mostly uses CO_2 at high pressure to extract the high value products from natural materials.

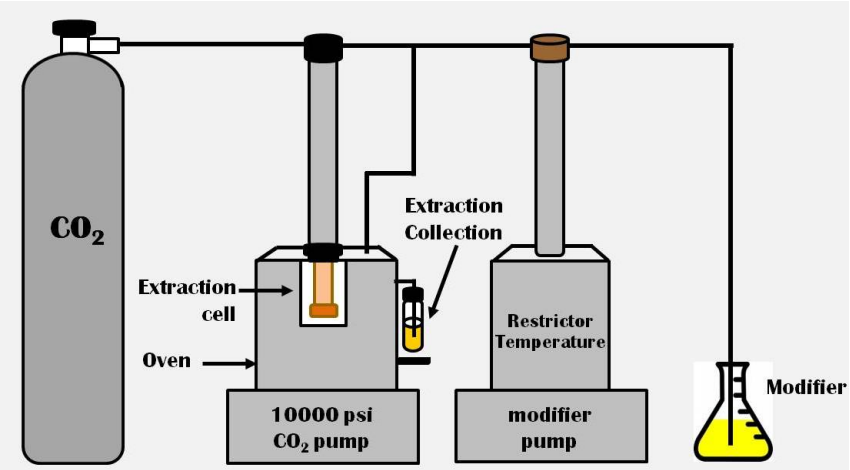


Figure 1.10 Schematic diagram of SFE apparatus

A schematic diagram of the SFE apparatus is shown in **Figure 1.10.** SF extractor consists of a tank of the mobile phase, usually CO_2, a pump to pressurize the gas, an oven containing the extraction vessel, a restrictor to maintain a high pressure in the extraction line, and a trapping vessel. The liquid is pumped to a heating zone where it is heated to supercritical conditions. It then passes into the extraction vessel where it rapidly diffuses into the solid matrix and dissolves the material to be extracted. The dissolved material is swept from the extraction cell into a separator at lower pressure and the extracted material settles out. The CO_2 can then be cooled, recompressed and recycled or discharged to atmosphere.

SFE is an alternative to liquid extraction using solvents such as hexane or dichloromethane. There will always be some residual solvent left in the extract and matrix. Thus, there is always some level of environmental contamination from their uses. In contrast, carbon dioxide is easy to remove simply by reducing the pressure, leaving almost no trace and it is also environmentally benign. Moreover, the CO_2 is non-toxic, nonflammable, odorless, tasteless, inert, and inexpensive. Due to its low critical temperature 31°C, carbon dioxide is known to be perfectly adapted in food, aromas, essential oils and nutraceutical industries.

1.5 Gas chromatography (GC)

A gas chromatograph (GC) is a chemical analysis instrument for separating chemicals in a complex sample. **Figure 1.11** illustrates the major components of a

general GC system which are carrier gas, injector, column, detector and data system. The injector provides the means to introduce a sample into a continuous flow of carrier gas. A gas chromatograph uses a flow-through narrow tube known as the column, through which different chemical constituents of a sample pass in a carrier gas at different rates depending on their various chemical and physical properties and their interaction with a specific column filling, called the stationary phase. As the chemicals exit the end of the column, they are detected and identified electronically. The function of the stationary phase in the column is to separate different components, causing each one to exit the column at a different time (retention time, t_R). Other parameters that can be used to alter the order or time of retention are the carrier gas flow rate, and the temperature of oven.

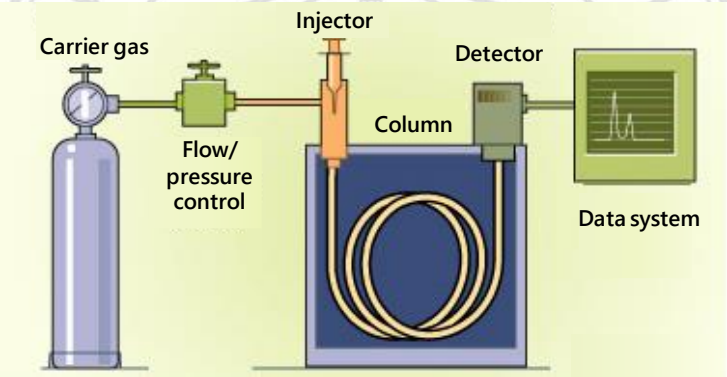


Figure 1.11 Schematic diagram of GC

The capillary column is held in an oven that can be programmed to increase the temperature gradually (or ramped, in GC terms). This helps our separation. As the temperature increases, those compounds that have low boiling points elute from the column sooner than those that have higher boiling points. Therefore, there are actually two distinct separating forces, temperature and stationary phase interactions mentioned previously.

As the compounds are separated, they elute from the column and enter a detector. The detector is capable of creating an electronic signal whenever the presence of a compound is detected. The greater the concentration in the sample, the bigger the signal. The signal is then processed by a computer. The time from when the injection is made (time zero) to when elution occurs is referred to as the t_R.

While the instrument runs, the computer generates a graph from the signal. This graph is called a chromatogram. Each of the peaks in the chromatogram represents the signal created when a compound elutes from the GC column into the detector. The x-axis shows the t_R, and the y-axis shows the intensity (abundance) of the signal. If the GC conditions (oven temperature ramp, column type, etc.) are the same, a given compound will always exit (elute) from the column at nearly the same t_R. By knowing the t_R for a given compound, we can make some assumptions about the identity of the compound. However, compounds that have similar properties often have the same retention times. Therefore, more information is usually required before making an identification of a compound in a sample containing unknown components [74].

1.6 Gas chromatography-mass spectrometry (GC-MS)

Gas chromatography-mass spectrometry (GC-MS) is an instrumental technique, comprising a GC coupled to a mass spectrometer (MS), by which complex mixtures of chemicals may be separated, identified and quantified. This makes it ideal for the analysis of the hundreds of relatively low molecular weight compounds found in environmental materials. In order for a compound to be analysed by GC-MS, it must be sufficiently volatile and thermally stable. In addition, functionalised compounds may require chemical derivatization, prior to analysis, to eliminate undesirable adsorption effects that would otherwise affect the quality of the data obtained. Samples are usually analyzed as organic solutions, consequently materials of interest (e.g. soils, sediments, tissues, etc.) need to be solvent extracted and the extract subjected to various "wet chemical" techniques before GC-MS analysis is possible.

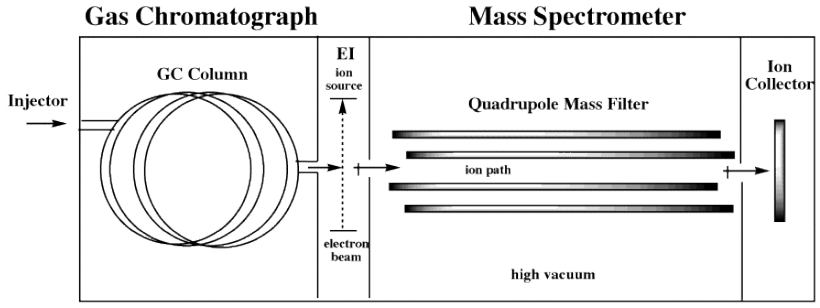


Figure 1.12 Schematic diagram of a GC-MS

Schematic diagram of a GC-MS shown in **Figure 1.12** comprises GC and MS parts. The MS part included ion source, mass analyzer and detector regions. The sample solution is injected into the GC inlet where it is vaporized and swept onto a chromatographic column by the carrier gas. The sample flows through the column and the compounds comprising the mixture of interest are separated by virtue of their relative interaction with the coating of the column and the carrier gas. The latter part of the column passes through a heated transfer line and ends at the entrance to ion source where compounds eluting from the column are converted to ions.

Traditionally, two potential methods exist for ion production. The most frequently used method is electron ionization (EI) and the occasionally used alternative is chemical ionization (CI). For EI a beam of electrons ionize the sample molecules resulting in the loss of one electron. A molecule with one electron missing is called the "molecular ion" and represented by $M^{+\cdot}$ (a radical cation). When the resulting peak from this ion is seen in a mass spectrum, it gives the molecular weight of the compound. Due to the large amount of energy imparted to the molecular ion it usually fragments producing further smaller ions with characteristic relative abundances that provide a "fingerprint" for that molecular structure. This information may be then used to identify compounds of interest and help elucidate the structure of unknown components of mixtures. CI begins with the ionisation of methane (or another suitable gas), creating a radical which in turn will ionise the sample molecule to produce $[M+H]^+$ molecular ions. CI is a less energetic way of ionising a molecule hence less fragmentation occurs with CI than with EI. CI yields less information about the detailed structure of the molecule but yields the molecular ion. Sometimes the molecular ion cannot be detected using EI, hence the two methods complement one another. Once ionised a small positive is used to repel the ions out of the ionisation chamber.

The next component is a mass analyzer which separates the positively charged ions according to various mass related properties depending upon the analyser used. Several types of analyser exist: quadrupoles, ion traps, magnetic sector, time-of-flight, radio frequency, cyclotron resonance and focusing to name a few. The most common of mass analyzers are quadrupoles and ion traps. After the ions are separated, they enter a detector the output from which is amplified to boost the signal. The detector sends information to a computer that records all of the data produced, converts the electrical

impulses into visual displays and hard copy displays. In addition, the computer also controls the operation of the mass spectrometer [75].

1.7 High performance liquid chromatography (HPLC)

High performance liquid chromatography (HPLC) is unquestionably the most widely used of all of the analytical separations. The reasons for the popularity of the method are sensitivity, ready adaptability to accurate quantitative determination, suitability for separating nonvolatile species or thermally fragile ones and widespreaded applicability to substances that are of prime interest to industry.

HPLC instruments consist of a reservoir of mobile phase, a pump, an injector, a separation column and a detector as shown in **Figure 1.13**. Compounds are separated by injecting a plug of the sample mixture onto the column. The different components in the mixture pass through the column at different rates due to differences in their partitioning behavior between the mobile liquid phase and the stationary phase. Solvents must be degassed to eliminate formation of bubbles. The pumps provide a steady high pressure with no pulsating, and can be programmed to vary the composition of the solvent during the course of the separation. Detectors rely on a change in UV-Visible absorption or fluorescence after excitation with a suitable wavelength.

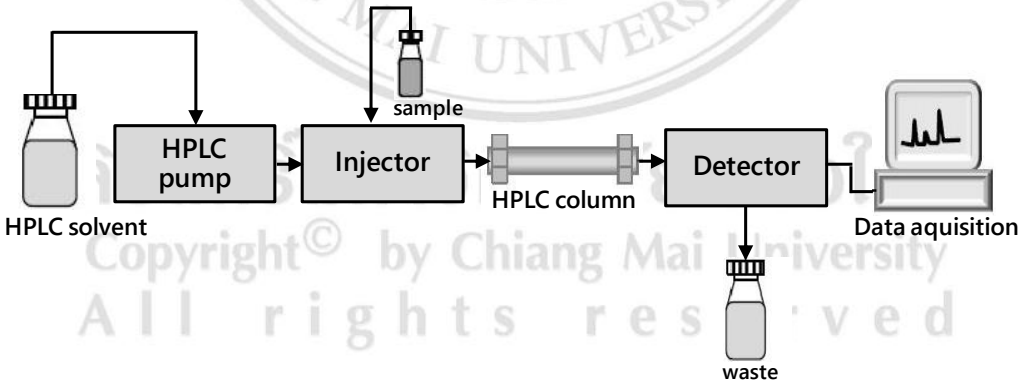


Figure 1.13 Schematic diagram of HPLC

1.7.1 Normal phase chromatography

Normal phase (NP)-HPLC was the first kind of HPLC setup used and retains analyte based on polarity. This method uses a polar stationary phase and a non-polar mobile phase and is used when the analyte of interest has a polar nature. The polar analyte associates with and is retained by the polar stationary phase. Adsorption

strengths increase with increased analyte polarity. The interaction between the polar analyte and the polar stationary phase (relative to the mobile phase) increases the elution time. The interaction strength not only depends on the functional groups in the analyte molecule, but also on steric factors. The affect of sterics on interaction strength allows this method to resolve or separate structural isomers. Use of more polar solvents in the mobile phase will decrease the retention time of the analytes while more hydrophobic solvents tend to increase retention times. Very polar solvents in a mixture tend to deactivate the column by occupying the stationary phase surface. This is somewhat particular to normal phase because it is most purely an adsorptive mechanism as the interactions are with a hard surface rather than a soft layer on a surface.

NP-HPLC had fallen out of favor in the 1970's with the development of reversed phase (RP)-HPLC because of a lack of reproducibility of retention times as water or protic organic solvents changed the hydration state of the silica or alumina chromatographic media. Recently it has become useful again with the development of hydrophillic interaction liquid chromatography bonded phases which utilize a partition mechanism providing reproducibility.

1.7.2 Reversed phase chromatography

The reversed phase (RP)-HPLC consists of a nonpolar stationary phase and a polar mobile phase. It was developed due to the increasing interest in large nonpolar biomolecules. One common stationary phase is a silica which has been treated with RMe_2SiCl, where R is a straight chain alkyl group such as $C_{18}H_{37}$ or C_8H_{17}. The retention time is therefore longer for molecules which are more non-polar in nature, allowing polar molecules to elute more readily. Increasing of retention time can be done by adding a polar solvent to the mobile phase or decrease retention time by adding a more hydrophobic solvent. RP chromatography is so commonly used that it is not uncommon for it to be incorrectly referred to as "HPLC" without further specification. Structural properties of the analyte molecule play an important role in its retention characteristics. In general, an analyte with a larger hydrophobic surface area (C-H, C-C and generally non-polar atomic bonds, such as S-S and others) results in a longer retention time because it increases the molecule's non-polar surface area which is non-interacting with the water structure. On the other hand, polar groups such as -OH, $-NH_2$,

COO^- or $-NH^{3+}$ reduce retention as they are well integrated into water. Very large molecules, however, can result in an incomplete interaction between the large analyte surface and the ligands alkyl chains and have problems entering the pores of the stationary phase. Presently, RP-HPLC is the most popular mode of liquid chromatography for determining phenolic compounds and other natural products in plant extracts [76].

1.8 Liquid chromatography-mass spectrometry (LC-MS)

Liquid chromatography-mass spectrometry (LC-MS) is an analytical chemistry technique that combines the physical separation capabilities of liquid chromatography (LC) with the mass analysis capabilities of MS. Schematic diagram of LC-MS are shown in **Figure 1.14**. LC is used to introduce thermally labile to MS. The combination of the electrospray (ES) ion source with HPLC becomes the LC-MS interface in recent year. It is a particularly powerful combination since this ionization technique covers a wide range of samples that are commonly separated by HPLC. Electrospray ionization (ESI) takes place at atmospheric, but the technique differs significantly from atmospheric pressure chemical ionization (APCI) in that nebulization and ionization of the mobile phase is effected by an electric field applied to the end of a restricted inlet nozzle. In two variations on this interface, nebulization is assisted by a stream of nitrogen introduced coaxially with the mobile phase (commonly called ion spray) or by ultrasonication. A major advantage of ES is its ability to form multiply charged ions that have high masses but low mass-to-charge ratio (*m/z*) values and can be detected using inexpensive quadrupole (low-mass-range) mass analyzers.

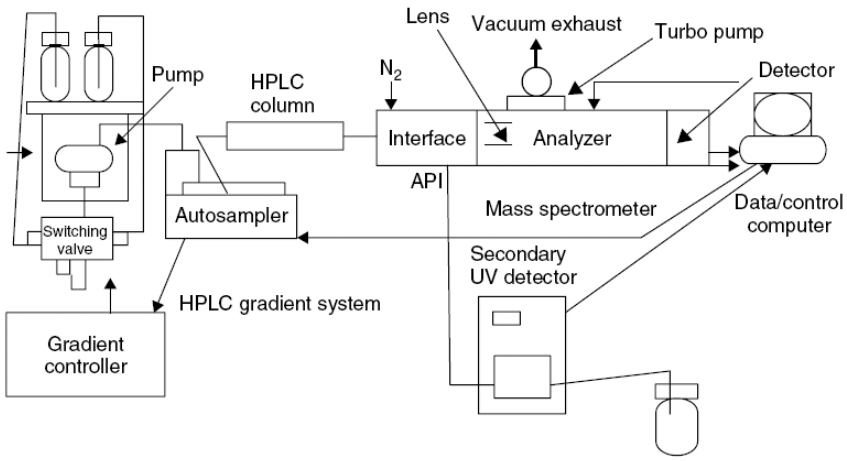


Figure 1.14 Schematic diagram of LC-MS

All ions in an ionization chamber are to be analyzed according to *m/z*. Ions have an electrical charge that permits them to be controlled by various electrical fields. They are separated by their *m/z* values in a mass analyzer. There are several types of mass analyzer including magnetic, transmission quadrupole, quadrupole ion trap, magnetic ion trap or time-of-flight analyzer. Ions are analyzed according to their abundance along an *m/z* scale [77].

1.9 Nuclear magnetic resonance spectroscopy (NMR)

Nuclear magnetic resonance spectroscopy (NMR) is a technique used to determine a compound's unique structure. It identifies the carbon-hydrogen framework of an organic compound. Using this method and other spectroscopic methods including infrared and mass spectrometry, scientists are able to determine the entire structure of a molecule. In this discussion, we will focus on ^{1}H-NMR or proton nuclear magnetic resonance. Even though there are many other spectrometers including ^{13}C-NMR (carbon nuclear magnetic resonance) ^{15}N-NMR (Nitrogen nuclear magnetic resonance) and ^{1}H-NMR was the first and is the most common atom used in nuclear magnetic resonance spectroscopy.

The atomic nucleus is a spinning charged particle and it generates a magnetic field. Without an external applied magnetic field, the nuclear spins are random and spin

in random directions. But when an external magnetic field is present, the nuclei align themselves either with or against the field of the external magnet.

Each group of chemically equivalent protons gives rise to a signal. Chemically equivalent protons are protons that are in the same environment. The same magnetic force will create overlapping signals on the spectrum. Therefore, we can determine how many sets of equivalent protons that are in a molecule by looking at the number of signals in its ^{1}H-NMR spectrum. The positions of the signals in an NMR spectrum are based on how far they are from the signal of the reference compound. This information tells us the kind of proton or protons that are responsible for the signal.

One method of solving this problem is to report the location of an NMR signal in a spectrum relative to a reference signal from a standard compound added to the sample. Such a reference standard should be chemically unreactive and easily removed from the sample after the measurement. Also, it should give a single sharp NMR signal that does not interfere with the resonances normally observed for organic compounds. Tetramethylsilane, $(CH_3)_4Si$, usually referred to as TMS, meets all these characteristics and has become the reference compound of choice for proton and carbon NMR.

Since the separation (or dispersion) of NMR signals is magnetic field dependent, one additional step must be taken in order to provide an unambiguous location unit. This is illustrated for the acetone, methylene chloride and benzene signals by clicking on the previous diagram. To correct these frequency differences for their field dependence, they are divided by the spectrometer frequency (100 or 500 MHz in the example), as shown in a new display by again clicking on the diagram. The resulting number would be very small, since we are dividing Hz by MHz, so it is multiplied by a million, as shown by the formula in the blue shaded box. Note that ν_{ref} is the resonant frequency of the reference signal and ν_{samp} is the frequency of the sample signal. This operation gives a locator number called the “chemical shift”, having units of parts-per-million (ppm) and designated by the symbol δ. Proton chemical shift ranges are shown in **Figure 1.15**.

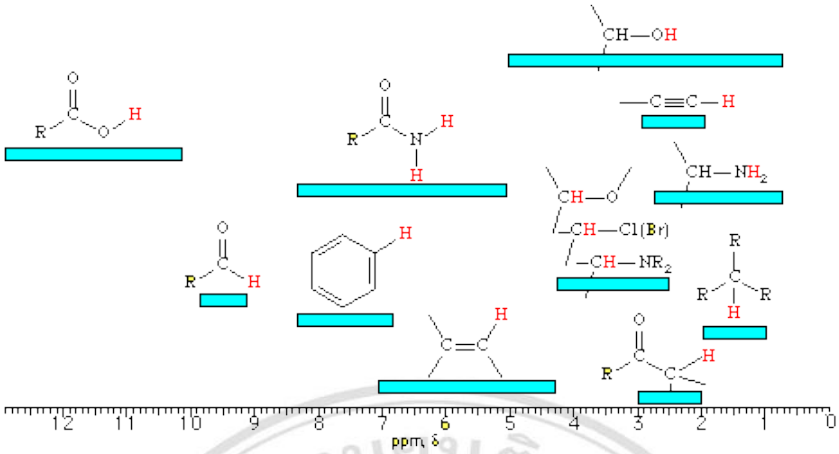


Figure 1.15 Proton chemical shift ranges

Integration is the area measurement that tells us the relative number of protons that give rise to each signal. Beer's Law says that the amount of energy absorbed or transmitted is proportional to a certain number of moles present. The area under each signal is proportional to the amount of radio energy and number of equivalent protons that give rise to that signal. The numbers do not always correspond to the exact or absolute number of protons. Instead, it tells us the relative number or ratio of the amount of equivalent protons.

Using ^{1}H-NMR, the structure of a molecule can be determined using the four components shown on the spectra, signals, chemical shift, integration and splitting patterns. ^{13}C-NMR can also be used and this eliminates some structures that would be possible when only the ^{1}H-NMR is utilized [78].

1.10 Literature review

1.10.1 Phytochemicals in some rice varieties and their analyses

Rice bran is the most nutritious part of rice and a good source of bioactive phytochemicals. A number of researches have reported the phytochemical profiles in rice such as phenolic compounds, sterols, triterpenoids, tocopherols, tocotrienols and carotenoids. Most of these phytochemicals are recognized as bioactive compounds that can improve human health and well-being.

Several phenolic compounds have already been identified in rice. Grains with light brown pericarp color present mainly low molecular weight phenolics

(approximately 85%). In those with red and black pericarp color prevail the compounds with higher molecular weight [56]. The main phenolics in rice grains with light brown pericarp color are the phenolic acids, mainly ferulic acid (255-362 mg/kg grain) and *p*-coumaric acids (70-152 mg/ kg grain). These two phenolic compounds were identified by Zhou and co-workers [79]. Other eight potentially chemopreventive phenols, protocatechuic acid, *p*-coumaric acid, caffeic acid, ferulic acid, sinapic acid, vanillic acid, methoxycinnamic acid and tricin, in the extracts of bran and intact brown rice were also identified by Hudson and co-workers [80].

Tian and coworker [57] developed the method for the determination of phenolic compounds, 6′-O-feruloylsucrose, 6′-O-sinapoylsucrose, ferulic acid, sinapinic acid, *p*-coumaric acid, chlorogenic (3-caffeoylquinic) acid, caffeic acid, protocatechuic acid, hydroxybenzoic acid, vanillic acid and syringic acid in brown and germinated rice. The rice samples were extracted with 70% ethanol, filtered and defatted. The defatted aqueous solution was subjected to solid-phase extraction using a C_{18} silica gel cartridge. The 70% acidic methanol elution was analyzed directly by HPLC and high-performance liquid chromatography-electrospray ionization-mass spectrometry (HPLC-ESI-MS). Phenolic compounds were separated with a C_{18} RP column by gradient elution using 0.025% trifluoroacetic acid in purified water (A) and acetonitrile (B), 0 min, 5% B; 5 min, 9% B; 15 min, 9% B; 22 min, 11% B; and 38 min, 18% B) as the mobile phase at a flow rate of 0.8 ml/min. Detection limits ranged from 0.10 to 0.35 ng per injection (5:l). Relative standard deviations of 0.22–3.95% and recoveries of 99-108% were obtained for simultaneous determination of these phenolic compounds.

Usually, grains with red and black pericarp presented higher antioxidant activity than those with light brown pericarp color [81] due to high amount of higher molecular weight polyphenols such as bioactive flavonoids.

Kitsada and coworkers [82] identified and quantified some anthocyanins in glutinous and nonglutinous Thai black rice varieties by HPLC-ESI-MS. The major anthocyanins, cyanidin-3-*O*-glucoside and peonidin-3-*O*-glucoside, were found in the ranges of 16.01-34.40 and 2.43-7.36 μg/mL, respectively. The comparative study in terms of quantity of these phytochemicals and antioxidant capacity of the black rice

bran extracts suggested the contribution of overall phenolic components rather than that of these particular anthocyanin pigments.

Sriseadka and coworkers [83] identified and characterized structures of flavonoids and their glycosides in bran extracts of seven Thai black rice varieties, KDML 105, BT No.3, 132-1-1-4-1-1-1-3, 132-1-1-3-3-1-4-4, 49-6-6-6-1-0, Riceberry, 1000-0-0-1 and 16815, by sequential uses of RP- HPLC with a photodiode array (PDA) detector and a combined ESI-MS. Eleven flavonoids were detected, and six of these were found for the first time in rice bran. These were taxifolin-7-*O*-glucoside, myricetin-7-*O*-glucoside, isorhamnetin-3-*O*-acetylglucoside, isorhamnetin-7-*O*-rutinoside, 5,6,3′,4′,5′-pentahydroxyflavone-7-*O*-glucoside, and 5,3′,4′,5′-tetrahydroxyflavanone-7-*O*-glucoside. The quantitative results revealed that different rice varieties possessed flavonoids in different concentrations. The most abundant glycoside derivatives of flavonoids widely distributed among the rice varieties was monoglucoside such as quercetin-3-*O*-glucoside, isorhamnetin-3-*O*-glucoside and isorhamnetin-3-*O*-glucoside.

Non-polar fraction of rice bran is rich in unsaturated linoleic and oleic fatty acids and bioactive compounds such as *γ*-oryzanols, phytosterols, tocopherols and tocotrienols. The following observations are designed to stimulate further use of rice bran oil and some of its bioactive components as ingredients in health-promoting functional food.

Jiang and coworkers [84] reported that rice bran contained the most lipids (22.2%), followed by wheat germ, durum wheat, oat bran, wheat bran, and oat hull. Corn fine fiber contained the least amount of lipids (1.7%). *β*-Sitosterol, campesterol, and stigmasterol were the major phytosterols in these lipid extracts. Rice bran oil contained considerable amounts of cycloartenol and 24-methylenecycloartanol which were unique to these samples. Total sterol concentrations in extracted lipids were similar for rice bran, wheat bran, wheat germ, and durum wheat (21.3-15.1 mg/g). Rice bran appears to be the best source of phytosterols, with the highest oil content and high concentration of sterols.

Derakhshan-Honarparvar and coworkers [85] evaluated the quantity and quality of Iranian rice bran sterols which have antioxidant activity as well as

physiological and biological effects. Three widespread Iranian rice cultivars (Khazar, Hashemi and Alikazemi) were used for determination of their sterol contents. Rice bran samples were saponified directly after acid hydrolysis. Unsaponified materials were extracted, purified by solid phase extraction, silylated, and their sterol fractions determined by GC-MS. The sterol composition (in mg kg^{-1} bran) of these three cultivars (Khazar, Alikazemi and Hashemi) were 1,330.69, 1,279.95, 1,313.17 *β*-sitosterol; 747.52, 696.05, 756.80 campesterol; 112.80, 115.36, 114.24 $\triangle^5$-avenasterol, 38.91, 33.08, 38.24 $\triangle^7$-avenasterol; 8.05, 7.07, 7.56 cholesterol; 4.20, 3.99, 4.23 brassicasterol; and 2,722.016, 2,706.176, 2,717.68 total sterols, respectively. The highest and lowest sterol contents found in were *β*-sitosterol and campesterol, respectively.

The composition and variation of fatty acids, sterols, tocopherols and γ-oryzanol among selected varieties namely Basmati Super, Basmati 515, Basmati 198, Basmati 385, Basmati 2000, Basmati 370, Basmati Pak, KSK-139, KS-282 and Irri-6 of Pakistani rice (*Oryza sativa* L) were reported by Zubair and co-workers [86]. Oil content extracted with hexane from different varieties of brown rice seed (unpolished rice) ranged from 1.92% to 2.72%. Total fatty acid contents among rice varieties tested varied between 18240 and 25840 mg/kg brown rice seed. The rice tested mainly contained oleic (6841-10952 mg/kg) linoleic (5453–7874 mg/kg) and palmitic acid (3613-5489 mg/kg). The amounts of total phytosterols (GC and GC-MS analysis), with main contribution from *β*-sitosterol (445-656 mg/kg), campesterol (116-242 mg/kg), $\triangle^5$-avenasterol (89-178 mg/kg) and stigmasterol (75-180 mg/kg) were established to be 739.4 to 1330.4 mg/kg rice seed. The contents of *α*-, *γ*- and *δ*-tocopherols as analyzed by HPLC varied from 39.0-76.1, 21.6-28.1 and 6.5-16.5 mg/kg rice seed, respectively. The amounts of different *γ*-oryzanol components (HPLC data), identified as cycloartenyl ferulate, 24-methylenecycloartanyl ferulate, campesteryl ferulate and *β*-sitosteryl ferulate, were in the range of 65.5-103.6, 140.2-183.1, 29.8-45.5 and 8.6-10.4 mg/kg rice seed, respectively.

In addition, brans of some black rices have been reported to contain compounds in the group of carotenoids. The great interest in the study of these compounds is due to their physiological and biological functions. Some carotenoids are

involved in the cell communication and shown to be effective as free radical scavengers.

Nakornriab and coworkers [18] developed a method employing SFE followed by LC-ESI-MS for quantification of the antioxidants in groups of carotenoids and flavonoids in the bran of four Thai black rice cultivars. Trans-*β*-carotene, quercetin and isorhamnetin were identified and presented in the bran of all black rice cultivars within the range of 33.60-41.00, 1.08-2.85 and 0.05-0.83 mg/g, respectively.

Pereira-Caro and coworkers [87] analysed the secondary metabolites, anthocyanins, flovonols, carotenoids and *γ*-oryzanols, in dehulled black-purple rice cv. Asamurasaki using HPLC-PDA-MS^2. The seeds contained a high concentration of seven anthocyanins (1400 *μ*g/g fresh weight) with cyanidin-3-*O*-glucoside and peonidin-3-*O*-glucoside predominating. Five flavonol glycosides, principally quercetin-3-*O*-glucoside, quercetin-3-*O*-rutinoside and flavones were detected at a total concentration of 189 μg/g. The seeds also contained 3.9 *μ*g/g of carotenoids consisting of lutein, zeaxanthin, lycopene and *β*-carotene. γ-Oryzanol (279 *μ*g/g) was also present as a mixture of 24-methylenecycloartenol ferulate, campesterol ferulate, cycloartenol ferulate and *β*-sitosterol ferulate.

Volatile components were also identified in rice. Sukhonthara and coworkers [88] identified one hundred and twenty-nine volatile compounds in volatile oils obtained from hydrodistillation of red and black rice bran by capillary GC-MS. The result showed that myristic acid, nonanal, *(E)*-*β*-ocimene and 6,10,14-trimethyl-2-pentadecanone were the main compounds in the red rice bran, whereas myristic acid, nonanal, caproic acid, pentadecanal and pelargonic acid were the major components in the black rice bran.

Watcharapong and coworkers [89] extracted the volatiles by solid-phase microextraction (SPME) from the headspace of some black and white rice bran samples. Among all 146 volatiles, twenty-eight terpenoid odorants were acculately identified. Most of these terpenoids possess good aroma character and are varied among three groups of Thai rice, black glutinous, black non-glutinous, and white non-glutinous. Of these three groups, black non-glutinous rice contains the greater number of these monoterpenoids. However, the content of the major terpenoid odorants, which are

limonene, trans-β-ocimene, β-cymene, and linalool, is the highest in the bran of white Thai jasmine rice, Khao Dawk Mali 105 (KDML 105). An herbaceous odorant, myrcene, occurs in the bran of all black rice varieties but not in the bran of white rice. The flavor type of rice bran using these rice bran terpenoids was successfully classified using the chemometric principle component analysis method.

1.10.2 Isolation and purification of bioactive compounds in rice

Since chemical compositions that are occured in rice consist of various types of bioactive compounds or phytochemicals with different polarities, their separation still remains a big challenge for the process of identification and characterization of bioactive compounds. Practically, most of them have to be isolated or purified by the combination of several chromatographic techniques and various purification methods to obtain isolate bioactive compounds.

Miyazawa and coworkers [90] isolated the phenolic compounds, protocatechuic acid methyl ester and protocatechuic acid from black rice bran by silica gel column chromatography and identified by GC, GC-MS, infrared spectroscopy (IR) and NMR. The isolated compounds inhibited 75.4 and 60.1 % of tyrosinase activity, respectively.

Hu and coworkers [53] separated anthocyanin components, cyanidin-3-glucoside and peonidin-3-glucoside, from black rice by gel filtration and identified them using LC-MS. Proportions of cyanidin-3-glucoside and peonidin-3-glucoside exhibited antioxidant activities and free radical scavenging capacities in preventing DNA damage and LDL deterioration *in vitro*.

Ming-wei and coworkers [91] purified and identified the antioxidative composition of black rice. Four main antioxidative components, mavidin, pelargonidin-3,5-diglucoside, cyanidin-3-glucoside and cyanidin-3,5-diglucoside, were separated from the strongest antioxidative fractions by using Sephadex LH-20 resin and the structures were analyzed by UV-Vis, IR, ESI-MS, ^{1}H-NMR and ^{13}C-NMR techniques. Thus, the anthocyanin compounds are the most important substantial foundations for antioxidant property.

Chung and Shin [92] used bioactivity-guided fractionation and isolation methods to isolate five compounds, 4-carboethoxy-6-hydroxy-2-quinolone, ethyl-3,4-dihydroxy benzoic acid, 4-hydroxy-3-methoxyphenylacetic acid, 3,4-dihydroxybenzoic acid and 4-hydroxy-3-methoxycinnamic acid. These compounds showed significant antioxidant activity in a concentration-dependent manner through the scavenging of 1,1-diphenyl-2-picrylhydrazyl radicals. The structure of new compound, 4-carboethoxy-6-hydroxy-2-quinolone, was elucidated on the basis of spectroscopic evidences, particularly the results obtained *via* hetero-nuclear multiple-bond connectivity and high-resolution mass spectrometry.

1.10.3 Bioactivities of rice bran extracts

Many reports suggested that the extracts of black rice bran have beneficial effect on health. Pigmented rice and its bran extracts possess scavenging free radicals, antioxidant activity, enhancement of immune systems, and reduction of the risk of developing cancer and heart disease. Thus, several reports have emphasized on the biological activities of black rice extracts.

Kaneda and coworkers [93] examined the origin of the reactive oxygen species (ROS)-scavenging activities in the black rice extracts. Some candidates such as cyanidin-3-glucoside and cyanidin aglycone were identified. These anthocyanin compounds were found to possess both strong ROS-scavenging activities and to suppress cell-damaging effects of ultraviolet (UVB).

Nam and coworkers [67] quantitatively evaluated anti-tumor-promoting activity of rice bran extracts by measuring inhibition of Epstein-Barr virus early-antigen activation (EBV-EA) induced by the tumor promoter 12-O-tetradecanoylphorbol-13-acetate (TPA) using flow cytometry. It was found that the extracts from the pigmented varieties strongly inhibited phorbol ester-induced tumor promotion in marmoset lymphoblastoid cells B95-8 *in vitro*.

Nam and coworkers [94] evaluated the antioxidative activities of extracts from the bran of rice seeds from twenty one pigmented and one non-pigmented rice cultivars using the following tests; inhibition of peroxidation of linoleic acid, inhibition of peroxidation of rabbit lipid erythrocyte membranes, reduction of potassium ferricyanide, and scavenging of superoxide anions and hydroxyl radicals. The extracts

from the pigmented rice seeds had higher antioxidative activity than those from the non-pigmented varieties.

Chen and coworkers [95] provided molecular evidence associated with the anti-metastatic effects of peonidin-3-glucoside and cyanidin-3-glucoside from black rice by showing a marked inhibition on the invasion and motility of SKHep-1 cells. This effect was associated with a reduced expression of matrix metalloproteinase (MMP)-9 and urokinase-type plasminogen activator (u-PA). Peonidin-3-glucoside and cyaniding-3-glucoside also exerted an inhibitory effect on the DNA binding activity and the nuclear translocation of AP-1. Furthermore, these compounds also exerted an inhibitory effect of cell invasion on various cancer cells (SCC-4, Huh-7 and HeLa).

In Thailand, black rice has gained increasing popularity in the rice market since their potential bioactivities were reported. Riceberry, a Thai black rice, has been developed with the aim of providing optimum nutritional benefit to general consumers, was recently reported to have anti-cancer capacity, hypoglycemic, hypolipidemic, antioxidant, and anti-inflammation properties. It is also noted to be beneficial to oxidative stress and organ histology in streptozotocin-induced diabetic rats fed with a high fat diet.

Leardkamonkarn and coworkers [96] evaluated the potential anti-cancer activity on cancer cell lines (Caco-2, MCF-7 and HL-60) by bran compounds extracted from Riceberry. Anti-proliferation and BrdU incorporation assays indicated a time–dose dependent effect of dichloromethane and methanol extracts, and that HL-60 was the most sensitive cell. DNA fragmentation assay revealed that both extracts could induce different degrees of apoptosis. The apoptotic induction pathway of each extract determined by flow cytometry and immunoblotting assays revealed various phases of cell cycle arrest with alteration of pro-apoptotic p53, caspase-3, and cyclin proteins. The bioactive compounds in each extract were chemically analysed by GC-MS and LC-ESI-MS/MS. Results revealed the presence of two major anthocyanins, cyanidin-3-glucoside and peonidin-3-glucoside, in the methanol extract, while the dichloromethane extract contained higher content of plant sterols. The latter constituents are considered the major contributors to apoptotic mechanism in the sensitive cell.

Prangthip and coworkers [97] evaluated the effects of Riceberry supplement (RB) on biochemical parameters, skeletal muscle glucose transporter 4 (GLUT4), oxidative stress and inflammation in a streptozotocin (STZ)-induced diabetes rat. The effects were due to dietary fiber supplementation and/or bioactive components, equivalent amounts of dietary fiber present in RB were also fed to STZ-induced diabetic rats. Diabetes Sprague-Dawley rats (non-FBGP 16.65 mM) were randomly divided into five groups: DM fed a high fat (HF) diet, DM-RB1 fed 5% RB, DM-RB2 fed 41% RB, DM-F1 fed 0.6% fiber and DM-F2 fed 5% fiber. After 12 weeks, significant improvement of BG, insulin, HbA1C, IPGTT and GLUT4 levels was observed in DM-RB1 and DM-RB2 groups. Hyperlipidemia was significantly improved in DM-RB2 and DM-F2 groups. Oxidative stress (TBARS), antioxidant enzymes (SOD, CAT, and GPx), oxygen radical absorbance capacity (ORAC), pro-inflammation cytokine (TNF-a and IL-6) were improved in DM-RB1 and DM-RB2 groups. Improvement of pancreas and spleen histology was found in DM-RB1 and DM-RB2 groups. These indicate the potential of RB to improve hyperglycemia and hyperlipidemia conditions as well as alleviate oxidative stressand inflammation.

Kongkachuichai and coworkers [98] determined the effects of Riceberry oil on changes in blood glucose, insulin levels, and GLUT4 transporter as well as lipid profiles in Streptozotocin (STZ)-induced hyperglycemic rats fed a high-fat diet by Riceberry rice bran oil. Seventy male Sprague-Dawley rats, aged six weeks and weighing 196.09 ± 10.46 g, were used. After two weeks, rats fed the high fat-diet were induced to hyperglycemia by two doses of STZ injections (20 and 30 mg/kg; i.p.). Normal rats were divided into two groups, one group fed with basal diet and another group fed with basal diet with the oil source replaced with 5% Riceberry oil. All rats were given free access to their diet and water for 12 weeks. After 12 weeks of supplementation, significant improvement of blood glucose, insulin, HbA1C, intraperitoneal glucose tolerance and GLUT 4 transporter level were observed in the Riceberry oil supplemented groups compared to the STZ-induced diabetic rats fed the high fat diet containing 28% corn oil group. Significant reductions in TC, LDL-cholesterol, TG and TG/HDL ratio were also shown in rats fed with Riceberry oil when compared to those of diabetic rats. Findings in the present study demonstrate that Riceberry oil, a

nutraceutical food, may be useful as an alternative food supplement for the alleviation of hyperglycemia and dyslipidemia conditions.

1.10.4 Supercritical fluid extraction

SFE has received attention as an alternative to organic solvent extraction and has been shown to be an idea method for extracting certain bioactive compounds. CO_2 is changed to its supercritical state beyond the supercritical point (73 atm, 37 °C). SC-CO_2 is nontoxic, nonflammable and simple in operation when compared with traditional extraction using solvents.

Kim and co-workers [99] studied the use of SC-CO_2 to enrich the rice bran oil in essential fatty acids including palmitic acid, linolenic acid, linoleic acid, oleic acid, stearic acid, tocopherols and squalene. The oil rich in essential fatty acids (EFA) was extracted from the domestic brown rice bran using supercritical carbondioxide (SC-CO_2) and the extracts were analyzed with GC-MS. The extracted amount of rice bran oil was dependent upon the operating pressure and temperature and the fatty acid composition of oil was varied with the reduced density of the SC-CO_2. About 70-80% of rice bran oil was extracted in 4 hours. Especially, squalene which was not found in solvent extract phase, it was identified in SFE phase only.

Xu and Godber [100] studied the advantage of SC-CO_2 extracted γ-oryzanol from rice bran in comparison with other organic extracting solvents. The experiments were carried out at 680 bar and the results showed that the amount of γ-oryzanol presented in the SFE extract was up to 80 times higher than the amount obtained through hexane extraction.

Dunford and King [101] studied the use of a fractionation column and SC-CO_2 for selective enrichment in lipid and sterol fractions of rice bran oil (RBO). They found that the phytosterol content, specially γ-oryzanol content, of deacidified RBO was about three times higher than that found in a commercially available high γ-oryzanol of RBO.

Imsanguan and co-workers [102] compared the efficiency of three extraction methods; SC-CO_2 extraction, solvent extraction and soxhlet extraction for extraction of α-tocopherol and γ-oryzanol from rice bran. The results showed that none

of the solvents could extract α-tocopherol. However, ethanol was suitable for γ-oryzanol extraction. In summary, SC-CO_2 was found to be the best solvent for extracting both α-tocopherols and γ-oryzanols from rice bran because it provided higher yields and extraction rate.

Jesus and coworkers [103] investigated the recovery of γ-oryzanol from the rice bran oil using SFE. The soxhlet technique was conducted in order to compare results with SFE. The influence of process parameters over SFE was evaluated in terms of global yield, γ-oryzanol content, γ-oryzanol recovery rate and fatty acids composition. The mathematical modeling of SFE overall extraction curve was also investigated. The condition of 30 MPa/303K presented the maximum global yield (39±1%, w/w), maximum γ-oryzanol recovery rate (31.3%, w/w), relatively high γ-oryzanol content (3.2%, w/w) and significant presence of monounsaturated and polyunsaturated fatty acids.

Tomita and coworkers [104] extracted γ-oryzanol from crushed rice bran using SC-CO_2 at various CO_2 flow rate (1-9 ml/min), temperatures (40-80°C) and pressures (20-40 MPa). The experimental extraction behavior was explained using two simple models of thermodynamic model and simple kinetic model. In addition, solubility data of rice bran oil in SC-CO_2 showed a good agreement with the values of the correlation of Chrastil model. The effects of extraction temperatures of 40, 60 and 80°C, and pressures of 20, 30, and 40 MPa on the γ-oryzanol recovery were observed. At pressure over 30 MPa, the recovery of rice bran oil was close to the recovery of oil extracted by hexane soxhlet extraction. The result implied that the yield and the recovery of rice bran oil were significantly influenced by both temperature and pressure.

1.11 The scope and aims of this research

The aims of this research work can be summarized as follows:

To screen biologically active compounds in bran of Thai black rice cv. Riceberry. The crude extracts were obtained by solvent extraction and their fractions were then isolated by column chromatography. All chemical components were characterized by GC-MS.

To identify and elucidate the structures of sterols and triterpene alcohols in the unsaponified fraction of the Riceberry extract that possessed anticancer property and to purify the active compounds using both RP-HPLC and NP-HPLC. In addition, the spectroscopic data useful for structural identification were utilized with the aid of combined chromatographic-mass spectrometric techniques such as GC-MS and LC-MS.

To quantify sterol and triterpenoid contents in some Thai white and black rice brans upon solvent extraction by solvent and then determine the contents by GC-MS.

To apply a reliable analytical method employing SFE having CO_2 as an extraction fluid as a fast green extraction technique, followed by the use of high performance liquid chromatography with fluorescence detector and diode array detector (HPLC-FLD-DAD) to quantify vitamin E, γ-oryzanols and xanthophylls in the bran of some Thai rice varieties.