CHAPTER II

LITERATURE REVIEW

2.1 γ-Oryzanol and Rice Bran

γ-Oryzanol was first extracted from rice (*Oryza sativa* L.) bran oil in 1954 (Kaneko and Tsuchaya, 1954) and named it also contained a hydroxyl group. γ-Oryzanol, hereafter referred to as oryzanol, was originally assumed to be a single component but later was shown to be a mixture of ferulic acid (4-hydroxy-3-methoxycinnamic acid) esters of triterpene alcohols and plant sterols. Cycloartenyl ferulate, 24-methylelnecycloartanyl ferulate and campesteryl ferulate are the three major components (**Figure 2.1, b**) and account for approximately 80% of oryzanol in rice bran (Akihisa *et al.*, 2000; Fang *et al.*, 2003; Xu and Godber, 1999 and 2001). The content of oryzanol in rice bran oil can range from 1-3% (Seetharamaiah and Prabhaker, 1986; Rao *et al.*, 2002) depending on the rice varieties and analytical methods.

Oryzanol is a white or slightly yellowish white, odorless crystalline substance or powder, which is stable in air, and is insoluble in water, moderately soluble in methanol, petroleum ether, diethyl ether and *n*-heptane, more in isopropyl alcohol and practically soluble in acetone and chloroform (Bucci *et al.*, 2003).

Rice bran is a relatively abundant byproduct of rice milling which contain 15-20% oil by weight. The potential for extracting high value products from rice bran for the food and pharmaceutical industries is well recognized (Hargrove, 1994). Rice bran is a rich natural source of oryzanol containing up to 3g/kg rice bran (Xu *et al.*, 2001), whereas some components of oryzanol, mainly sitostanyl and campestanyl ferulate and lesser amounts of sitosteryl and campesteryl ferulate, can be also found mostly in the inner pericarp of corn, wheat, rye and other grains, as well as, various fruits, vegetables, and herbs (Seitz, 1989).

A rice kernel consists of 20% hull, 72% endosperm, 6% bran and 2% germ, structure of which is illustrated in **Figure 2.2** (Juliano and Bechtel, 1985; Lu and Luh, 1991). Rice bran is the soft germ and several soft layers, pericarp, seed coat, nucellus and aleurone layer, surrounding the hard starchy endosperm. Additional constituents of the bran, include 12-16% proteins, 34-52% available carbohydrates, 7-11% crude fiber, and 7-10% ash. The typical crude rice bran oil is composed of 68-71% triacylglycerols, 2-3% diacylglycerols, 5-6% monoacylglycerols, 2-3% free fatty acids, 5-7% glycolipids, 3-4% phospholipids (mainly phosphatidylcholine, phosphatidylethanolamine and phosphatidylinositol), 2-3% waxes, and 4.2% unsaponifiable fraction (McCaskill and Zhang, 1999; Sayre and Saunders, 1990).

In comparison with other vegetable oils, crude rice bran oil tents to contain higher levels of non-triglyceride components, most of which are removed during further refining process.

2.2 Rice

Rice (genus *Oryza*) is tolerant to desert, hot, humid, flooded, dry and cool conditions, and grows in saline, alkaline and acidic soils with cycle of 3-7 months. The span of one cycle varies depending on its type and the growing environment. Growing rice requires an extensive irrigation system and properly leveled fields. A uniformity leveled field enables each rice kernel to absorb the same amount of moisture from the field. This uniform moisture level runs too high, the rice may spool faster.

Of 23 *Oryza* species, two are cultivated: *Oryza sativa*, which originated in the humid tropics of Asia, and *O. glaberrima*, from West Africa. Asian cultivated rice has evolved into three eco-geographic races - *indica*, *japonica* and *javanica*. In West Africa, *O. glaberrima* Steud. adds to the diversity of rice. In the limited number of varieties studied, iron and zinc can range between 1-6 mg and protein between 5-14 g per 100g of rice. If better utilized, these varieties with higher nutritional value could contribute to reducing the global burden

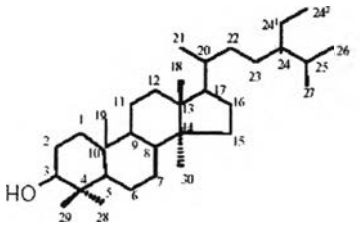
of malnutrition. Moreover, *O. rufipogon*, possibly a third form, has distinctive partitions into South Asian, Chinese, New Guinean, Australian, and American forms (Adair, 1972).

The origins of rice have been debated for some time. The first written records of rice trace back to a rice planting decree issued by a Chinese Emperor around 2800 BC. Later rice consumption spread around the world and was quickly assimilated into the diets of the people of Central Asia, the Mediterranean, and eventually the USA and South America.

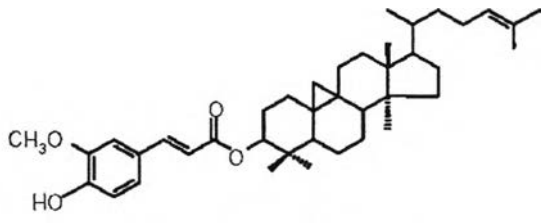
There are about 83,000 varieties of rice cultivated in the world today, most of them in Asia, and more than 17,000 are found in Thailand. They are collected and conserved at the National Rice Seeds Storage Laboratory for Genetic Resources. The varieties of Thai rice were first spread to the world market when Thai rice named "*Pin Kaew*" and other local varieties won the first, second, third and eight other prizes at the World Grain Contest held in Canada in 1933. At present, the well-known Thai jasmine rice has become very famous for its premium quality and aroma rice products. Other excellent varieties from breeding are: RD6 (glutinous rice), RD15, RD21, RD23, Phitsanulok 60-1, Suphanburi 60, Chainat 1, Luengpateaw 123 and many more.

First use of rice in Thailand can be traced back to some 5,500 years ago (about 3,500 BC.). This was the result of the research of three Japanese scientists, Tayada Watabe, Tomoya Akihama and Osamu Kinoshita, who found the husks of Thai rice in the bricks at 108 archeological sites in 39 provinces all over Thailand. The studies indicated that during the 5th-14th century, there were three sizes of rice grain: the large type, the round type (short grain) and the slender type (long grain). Only two types of rice grain now remain in Thailand, i.e., the round type in the North and the Northeast; the slender type in the Central Plain and the South. Unfortunately, the large type is extinct (Huke and Huke, 1990).

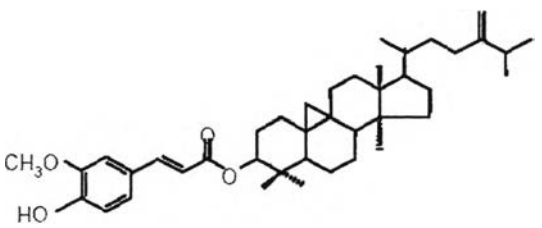
(a) a sterol with carbon numbering



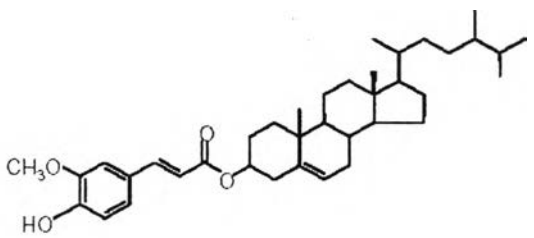
(b1) cycloartenyl ferulate



(b2) 24-methylenecycloartanyl ferulate



(b3) campesteryl ferulate



(c) cholesterol

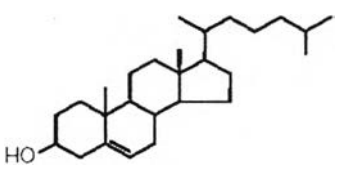


Figure 2.1 Chemical structures of (a) a sterol with carbon numbering, (b1)-(b3) oryzanol components and (c) cholesterol.

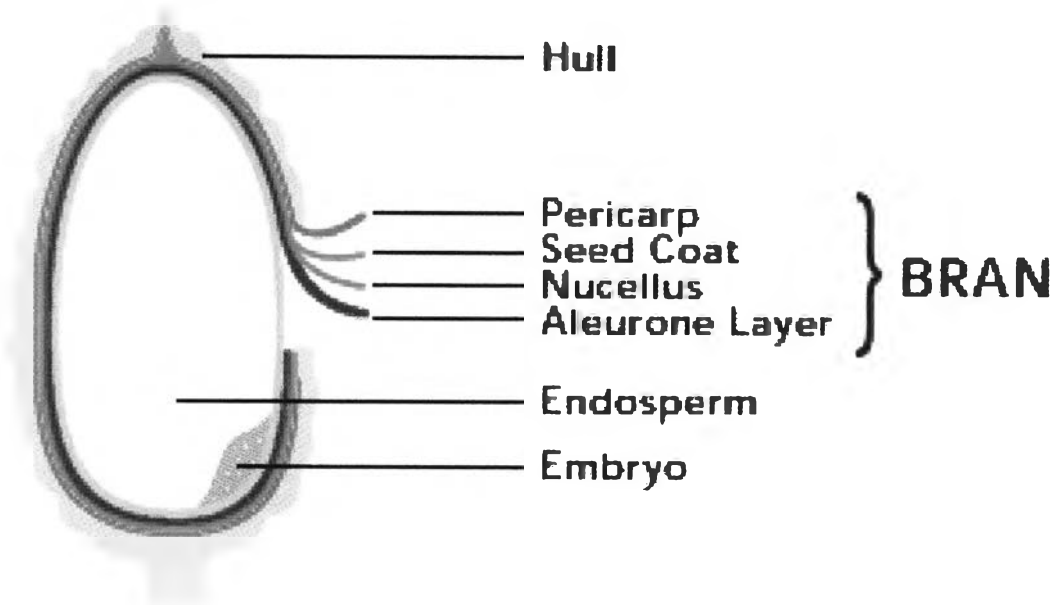


Figure 2.2 Rice kernel structure adapted from Orthoefer, 1996.

2.2.1 Classification of Rice

In general, the rice family could be classified into three main categories depending on how we classified them:

2.2.1.1 By Size: Short, Medium and Long Grain

(a) *Long grain rice*, such as jasmine and basmati, is more than three times as long as wide. Once cooked, grains stay separate, light and fluffy.

(b) *Medium grain rice* is between two and three times as long as wide. When cooked, the grain retains more moisture than long grains with a bit of cling; and

(c) *Short grain rice* (red rice and glutinous rice) is less than twice as long as wide, it is creamier in texture and sticks together once cooked and is used for cooking Thai glutinous rice or the Vietnamese ping-pong rice balls.

2.2.1.2 By Waxiness: Waxy or Nonwaxy

(a) *Waxy rice* is low in amylose, usually absorbs less water and is slightly sweet when cooked. It is suitable for steaming. Waxy rice is also known as sweet, sticky, or glutinous rice; and

(b) *Nonwaxy rice* is high in amylose and usually cooks into dry separate grains.

2.2.1.3 By Rice Characteristics

(a) *White rice* has been milled to remove its bran. Often, it is also polished to remove even the last traces of bran. Its color is white or cream-colored;

(b) *Aromatic rice*, such as jasmine and basmati, produces a subtle seductive scent as it cooks. When the rice is served hot, it is still aromatic. Some of the aromatic rice are part of the Thai family, with soft, slightly clingy grains when cooked, while others are Della-type rice that cook to a drier, fluffier texture;

(c) *Jasmine rice*, known as "*khao chao*", is a low-amylose long grain aromatic rice. Thai jasmine rice is produced from *Kao Dok Mali 105* that was originally developed in Thailand. It has clear, translucent, crystalline long grains. It is usually absorption-cooked to make the rice soft and slightly clinging;

(d) *Glutinous rice*, known as "*khao neeo*" in Thai and Lao, and as sweet rice or sticky rice in the world market. Thai sticky rice has a medium to long grain and is slightly aromatic. When raw, the grains are opaque white - unlike the translucent grains of nonsticky Thai rice;

(e) *Parboiled rice* has been steamed in order to dissolve the nutrients from the bran and then pressurized to deposit the nutrients in the kernel. The result is more nutritious and evenly textured white rice;

(f) *Brown rice* has been taken from its husk but not yet milled and polished. Some "brown rice" is not brown but red or black, because the bran is naturally colored;

(g) *Thai black rice* is a black variety of Thai long-grain sticky rice, which does not stick together because the black outer bran separates the grains when cooked; and

(h) *Wild rice*, the North American wild rice is not actually rice but the seed of a water grass (*Zizania aquatica*). Recently, wild rice has been successfully cultivated in California and elsewhere.

2.2.2 Jasmine Rice

Jasmine rice is the world's best quality rice which originates in Thailand. This rice is soft, easy to cook and very fragrant and used with every Thai and oriental dishes. Jasmine rice is commonly known as **fragrant rice, scented rice** or **aromatic rice**.

Jasmine rice has world famous reputation in appearance, cooking texture and aroma. This special aromatic fragrance gives its famous name- "Jasmine Rice". One reason that makes Jasmine rice special is the ability to adapt to different dishes for different tastes. Jasmine Rice is the world favorable rice because of its soft and sticky qualification as well as natural scent and is exclusively grown in Thailand.

Jasmine rice is grown in Thailand's northeastern region producing the best quality rice. Currently there are 4 sub-varieties of jasmine rice: Kao Dok Mali, Go Ko 15, Klongluang and Suphan. All 4 sub-varieties offer similar cooking rice.

In this study, Pathumthani 1 and Kao Dok Mali 105 were used for identification of oryzanol and comparison with Sunpatong 1 (glutinous rice) and Go Ko Chai Nat 1 (ordinary white rice), see **Figure 2.3**.

2.3 Biological Aspects of Oryzanol

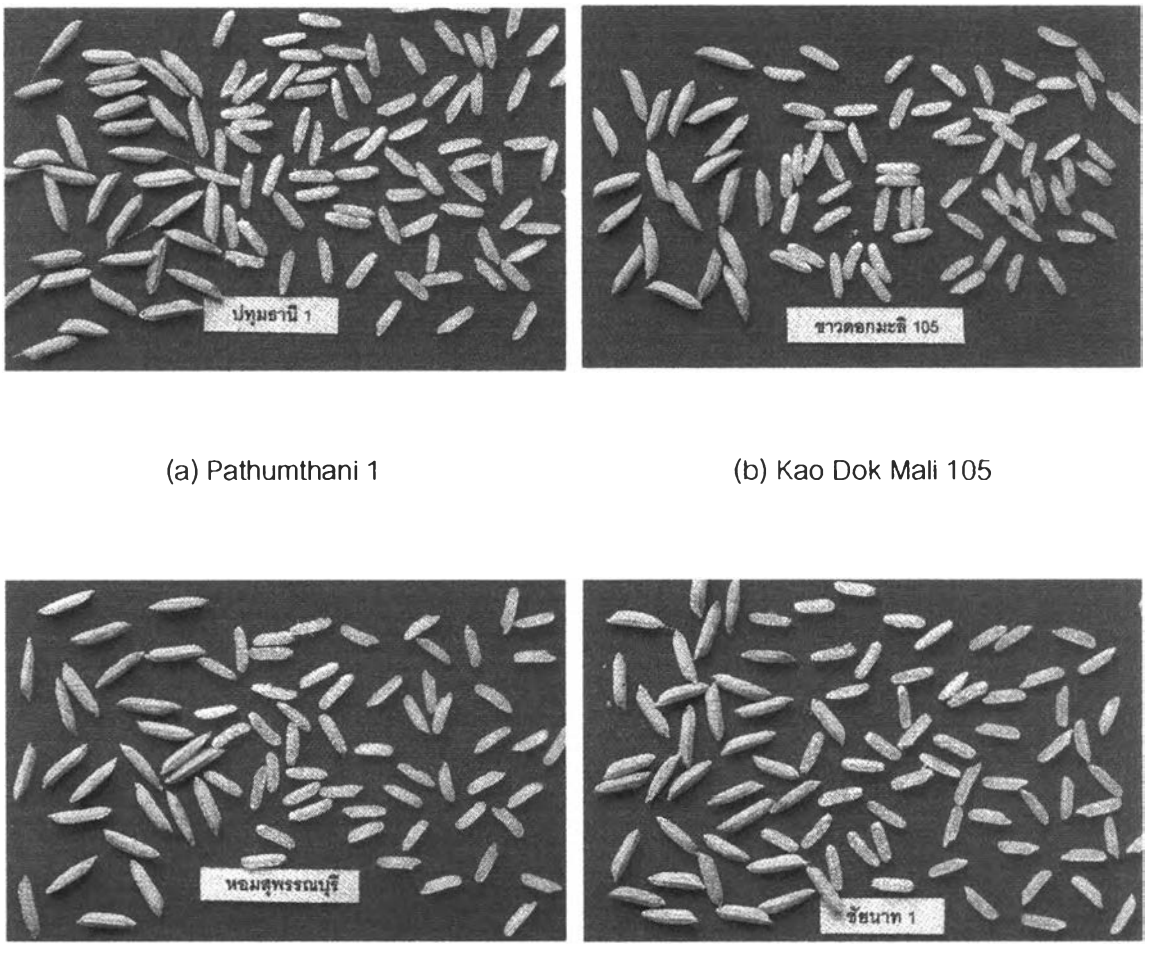
The biological function of oryzanol in the plant has not been clearly described. Since oryzanol protects rice bran oil from oxidation, inhibits peroxidation of lipids mediated by iron or UV radiation (Jariwalla, 2001), it seemed plausible; therefore, that oryzanol could be candidates for kernel pathogen resistance compounds for both fungi and insects (Norton, 1994; Seitz, 1989). Reasons for this include: (i) the localization of the compounds within the pericarp; (ii) the specialized character of the sterol portion of the ester in corn and related cereals (they are primarily saturated forms of normal membrane sterols); and (iii) preliminary data revealed that there were perhaps a dozen or more cinnamic acid derivative esters of sterols, most at low level, present in corn and rice bran (Moreau *et al.*, 1999; Ramarathnam *et. al.*, 1988).

As mention before, oryzanol is a mixture of sterol ferulate, one may view it from two standpoints, triterpenes of sterols and ferulic acid. Triterpenoids can be categorized under the class synonymously named terpenoids, terpenes or isoprenoids, which are various,

widespread and numerous natural products derived from a common biosynthetic pathway based on mevalonate as the parent compound, with the important subclass of steroids (Banthorpe, 1991). The general pathway of biosynthesis (**Figure 2.4**) is enzymatically controlled and starts from acetyl-CoA, to mevalonate, to 2*E*,6*E*-farnesyl pyrophophate, to squalene, and then to triterpenoids, with a final chair-boat-chair-boat configuration (Bramley, 1997). 24-Methyl cholesterol is present in higher plants as a mixture of its (24-*R*)- and (24- *S*)-epimers campesterol and dihydrobrassicasterol (Rubinstein *et al.*, 1976; Rendell *et al.*, 1986) and is a precursor for the synthesis of brassinosteroids (**Figure 2.5**).

Sterols are isoprenoid-derived compounds that play essential roles in the development of all eukaryotic organisms. Bulk sterols, such as cholesterol in animals, ergosterol in yeast and sitosterol and campesterol in plants, are integral membrane components which serve to regulate the fluidity and permeability of membranes and directly affect the activity of membrane associated proteins, including enzymes and signal transduction components (Hartmann, 1998). These same sterols also serve as biosynthetic precursors of steroid hormones, including mammalian androgens, estrogens and glucocorticoids; insect ecdysteroids; fungal antheridiol and oogoniol; and plant brassinosteroids (Clouse, 2000).

Plant sterols, also called phytosterols, are further formed from triterpenoids or cycloartenol (Vissers *et al.*, 2000), but the sequence of the modifications of the sterol ring system and side chain can differ from species or even in various tissues or during different developmental stages in a plant. Plant sterols, supposedly produce in the cytosolic and microsomal compartments of the plant cells, occur in plants as free sterols and conjugated forms, that is, steryl ester of fatty acids or phenolic acids, steryl glycosides, and acylated steryl glycosides (Piironen *et al.*, 2000).



(a) Pathumthani 1 (b) Kao Dok Mali 105

(c) Hom Suphan Buri (d) Go Ko Chai Nat 1

Figure 2.3 Physical appearances of Jasmine rice kernel: (a) Pathumthani 1, (b) Kao Dok Mali 105, (c) Hom Suphan Buri and ordinary white rice kernel: (d) Go Ko Chai Nat 1.

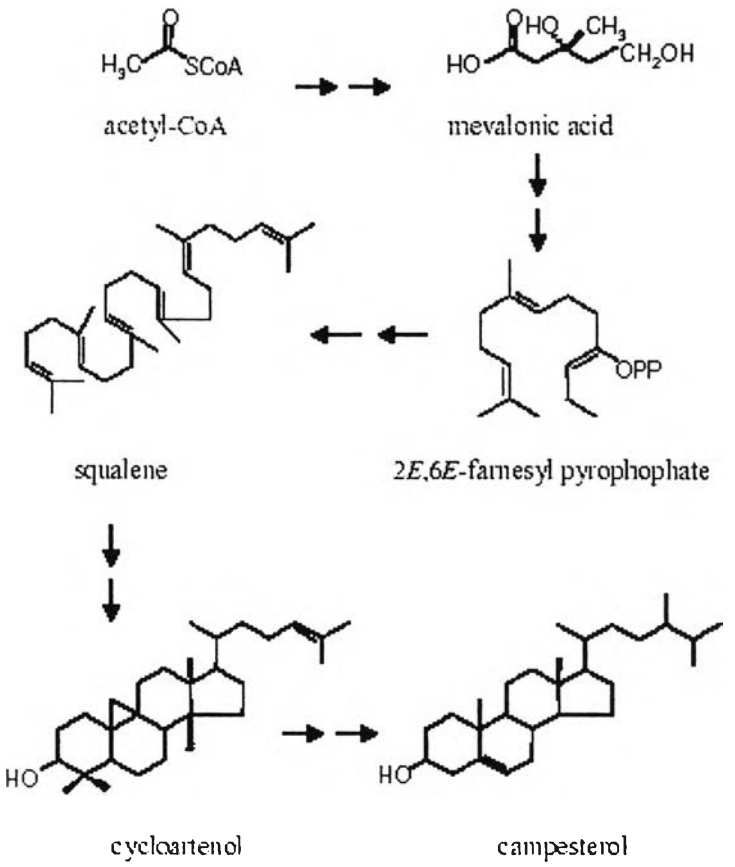


Figure 2.4 Brief pathway for biosynthesis of terpenoids in plants.

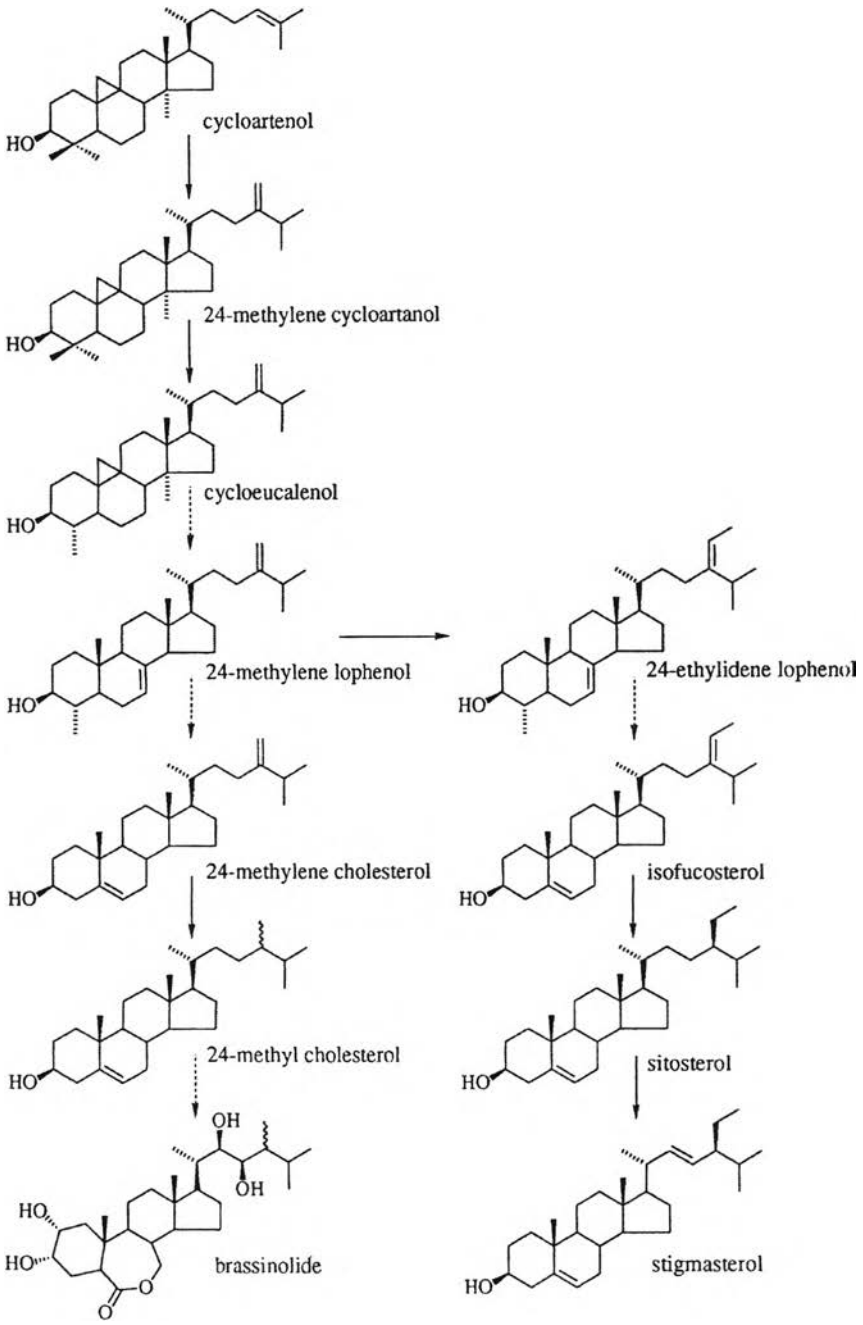


Figure 2.5 Simplified biosynthetic pathway of sterols in higher plants. The dashed arrows indicate more than one biosynthetic step not shown here. (Schaller *et al.*, 1998)

Plant sterols fall into one of three categories: 4-desmethylsterols (no methyl groups); 4-monomethylsterols (one methyl group) and 4,4-dimethylsterols (two methyl groups) (**Table 2.1-2.2**). The most common plant sterols found in vegetable oils are campesterol (2.6–38.6%), β-sitosterol (40.2–92.3%) and stigmasterol (0.0–31.0%), and structurally related to cholesterol, classified as 4-desmethylsterols of the cholestane series (Abidi, 2001; Kochhar, 1983), but they have a different side-chain configuration. Saturation of the sterols with hydrogen leads to the formation of plant stanols, such as campestanol and sitostanol. Because the human body can not synthesize plant sterols, these components are derived only from the diet. They are usually present in vegetable oils, nuts, cereals and beans (Kerckhoffs *et al.*, 2002). Plant sterols are assumed to be hydrolysed in the gut by sterolester hydrolase, and therefore the different fractions should have the same effects on the cholesterol absorption in the human small bowel (Normén *et al.*, 1999; Swell *et al.* 1954).

Plant stanols are less abundant in these dietary sources than plant sterols. Compared with cholesterol, plant sterols and stanols are absorbed less and their biliary excretion is faster. Consequently, their serum levels are very low. The different plant sterols and stanols are not equally absorbed. This was demonstrated in a study by Heinemann and colleagues (1993) in which the intestinal absorptions of cholesterol and different plant sterols and stanols were compared in healthy men, using an intestinal perfusion technique over a 50 cm segment of the upper jejunum. Absorptions of cholesterol, campestanol, campesterol, stigmasterol and sitosterol were 33, 13, 10, 5 and 4%, respectively. Absorption of sitostanol is minimal. Total plasma plant sterol concentrations in healthy adults range from 7 mmol/L to 24 mmol/L, which accounts for less than 1% of total plasma sterol concentrations (Salen *et al.*, 1985).

Several mechanisms responsible for the different rates of absorption of plant sterols have been suggested: (i) micellar solubility is a major factor which affects the absorption rate (Slota *et al.*, 1983; Ikeda *et al.*, 1988a). Discrimination between absorbable and non-absorbable sterols may occur during the process of their uptake into intestinal mucosa

(Borgstrom, 1968; Child and Kuksis, 1983); (ii) slower rate of transfer of sitosterol from the cell surface to intracellular site, compared to that of cholesterol, may also contribute to lower absorption rate for the plant sterol (Ikeda *et al.*, 1990); and (iii) other studies have indicated that mucosal esterification could be a possible site of discrimination in sterol absorption (Ikeda *et al.*, 1988b). Thus, it appears that limited absorption rate for plant sterols is due to a combination of several factors.

On the other hand, ferulic acid has the biosynthetic origin from the aromatic amino acid L-phenylalanin, itself synthesized from chorismate, the final product in the Shikimate pathway (**Figure 2.6**). The introduction of a second hydroxyl group into *p*-coumaric to give caffeic acid is catalyzed by monophenol mono-oxygenased, a well-known group of plant enzymes (Macheix *et al.*, 1990; Strack, 1997). Methylation of caffeic acid leads to the formation of ferulic acid, which together with *p*-coumaric acid, are the precursors of lignins.

Much of the ferulic acid occurs as ester, ether, or acetal bonds either to structural components of the plants (cellulose, protein, lignin), or larger polyphenol (flavonoids), or smaller organic molecules (e.g. glucose, tartaric acid) or other nature products, e.g. terpenes (Klick and Herrmann, 1987; Robbins, 2003; Winter and Herrmann, 1986).

The biological role of ferulic acid has been connected with diverse functions, including nutrient uptake, protein synthesis, enzyme activity, photosynthesis, structural components and thus inhibits germination (Einhellig, 1986; Wu *et al.*, 1999, 2000). Moreover, some studies imply a defensive role of ferulic acid with repellent property and a physiological function in plant mineral metabolism.

Table 2.1 Some natural occurring 4,4'-desmethylsterols

Desmethylsterol	
5-Avenasterol	7-Avenasterol
Brassicasterol	22,23-Dihydro-BR
Campesterol	Chondrillasterol
25-Dehydro-CD	Campestanol
7-Campesterol	7-Campestenol
Cholestanol	Cholesterol
Clerosterol	Clionasterol
Crinosterol	22,24-Dihydrobrassicasterol
Desmosterol	Ergostanol
Ergosterol	Ergostatetraenol
Fucosterol	Fungisterol
Isofucosterol	24-Methylencholesterol
Poriferasterol	22,23-Dihydro-PO
Poriferastenol	Schotenol
Sitosterol	Sitostanol
α-Spinasterol	25-Dehydro-SP
5,23-Stigmastadienol	5,24-Stigmastadienol
Stigmastanol	7-Stigmastenol
22,7-Stigmastenol	Stigmasterol
7,22,25-Stigmastatrienol	7,25-Stigmastadienol
Vernosterol	Zymostenol
Zymosterol	

Table 2.2 Some natural occurring 4-methylsterols and 4,4'-dimethylsterols

4-Methylsterol	4,4'-Dimethylsterol
Citrostadienol	α-Amyrin
Cycloeucalenol	β-Amyrin
Gramisterol	Butyrospermol
Lophenol	Cycloartanol
4α-Methylzymostenol	Cycloartenol
4α-Methylzymosterol	Cyclobranol
31-Norcycloartenol	Cyclolaudenol
31-Norlanostenol	Lanostenol
Obtusifoliol	Lupeol
Methylvernosterol	24-Methylencycloartanol
Ethyllophenol	24-Methylenlanostenol
31-Norlanosterol	Parkeol
	4,4',14-Timethyl-24-methylencholesterol
	Tirucalladienol
	Dihydrolanosterol
	Erythrodiol
	Uvaol
	Euphol

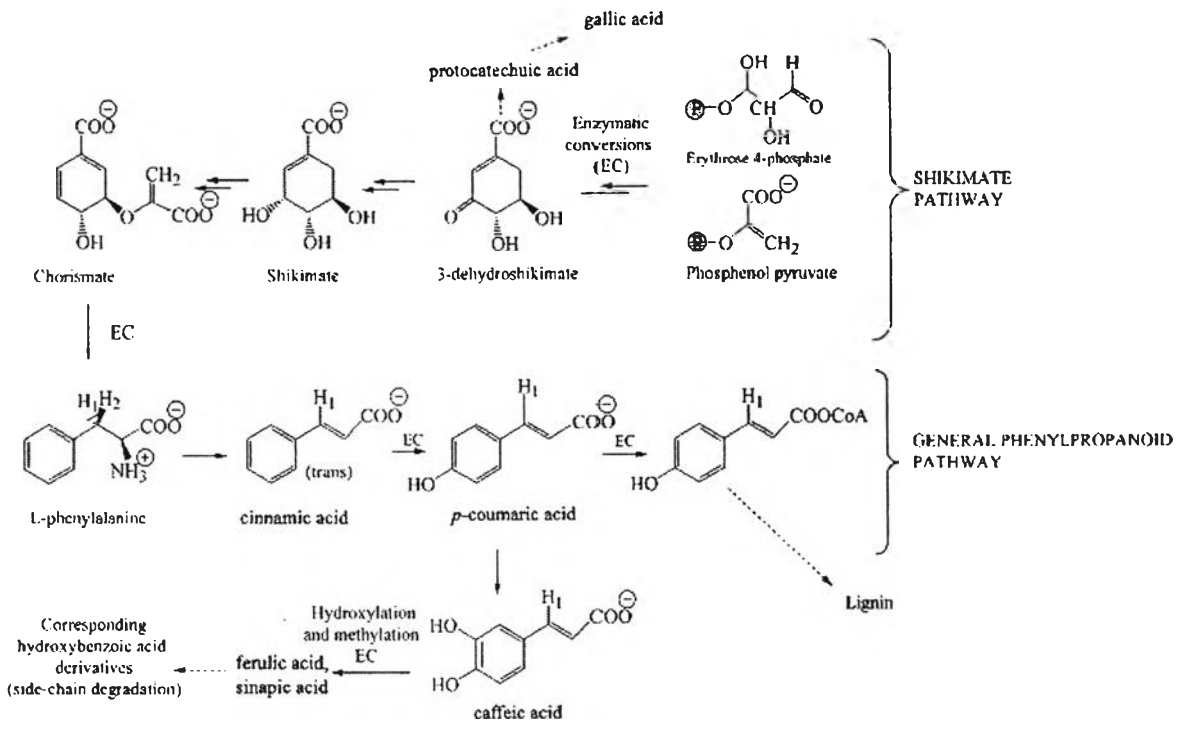


Figure 2.6 Biosynthetic pathways to hydroxybenzoate and hydroxycinnamate derivatives. The dashed arrows indicate more than one biosynthetic step not shown here. (Robbins, 2003)

2.4 Analytical Studies of Oryzanol

It has been established that oryzanol is a mixture and the number of individual components found in oryzanol depends on the rice cultivar and chromatographic approaches. The most commonly used techniques for the determination of oryzanol in rice bran oil are HPLC and UV spectrophotometry. The former is generally applied to investigate the chemical composition of the mixture, for example, in 1957 Shimizu *et al.* separated three major components of oryzanol (designated A, B, and C; Ohta and Shimizu, 1957); subsequently, Endo and his coworkers were able to separate well seven components (Endo *et al.*, 1968, 1969). Normal-phase High Performance Liquid Chromatography (HPLC), a column packed with spherical silica used by Diack and Saska (1994), could only separate oryzanol into two fractions and each fraction contained at least two or more constituents. Identification and quantification of each individual component of oryzanol would be difficult due to incomplete separation.

Reverse-phase HPLC, in contrast, could separate mixture of oryzanol used one- or two-part mobile phase mixtures of methanol, methanol and water, or methanol and acetronitrile. These methods could identify five (Norton, 1995; Rogers *et al.*, 1993) or six (Evershed *et al.*, 1988) individual components of oryzanol. In addition, Xu and Godber (1999) separated and identified ten components of oryzanol from rice bran oil using preparative normal-phase HPLC to concentrate oryzanol and to reduce interfering substances and reverse-phase HPLC to isolate and collect each component. These ten components were identified as Δ^7-stigmastenyl ferulate, stigmasteryl ferulate, cycloartenyl ferulate, 24-methylenecycloartanyl ferulate, Δ^7-campestenyl ferulate, campesteryl ferulate, Δ^7-sitostenyl ferulate, sitosteryl ferulate, campestanyl ferulate and sitostanyl ferulate (**Figure** 2.7). The three major components among these are cycloartenyl ferulate, 24-methylenecycloartanyl ferulate and campesteryl ferulate. Six novel feruloyl esters of triterpene alcohols and sterols, viz., cycloeucalenyl and 24-methylenecholesteryl *trans*-ferulates, and cycloartenyl, 24-methylenecycloartanyl, 24-methylcholesteryl, and sitosteryl *cis*-ferulates, besides five known *trans*-ferulates, cycloartenyl, 24-methylenecycloartanyl,

24-methylcholesteryl, sitosteryl, and stigmastanyl *trans*-ferulates, and one known *cis*-ferulate, stigmastanyl c*is*-ferulate, were isolated from the methanol extract of edible rice bran by Akihisa *et al.* (2000). This is the first instance for the characterization of naturally occuring ferulate of 4α-methylsterol, i.e., cycloeucalenyl *trans*-ferulate. Furthermore, Fang and his colleague (2003) successfully separated and identified twenty-four components of oryzanol by using liquid chromatography-mass spectrometry/ mass spectrometry (LC-MS/MS) technique. This is the first evidence for the presence of hydroxylated feruiate esters and caffeate esters as part of oryzanol in rice bran (see **Table 2.3** for summary).

All of these methods are time consuming because they require purification of oryzanol by liquid-liquid fractionation followed by low-pressure column chromatography (Norton, 1995) or low-pressure column chromatography followed by preparative normal-phase HPLC (Xu and Godber, 1999). A simpler method proposed would involve purification of oryzanol from rice bran by liquid-liquid extraction using a solvent mixture which can be used as mobile phase in the subsequent reverse-phase HPLC step (Norton *et al.*, 1995). The on-line coupling of liquid chromatographic pre-separation with capillary gas chromatography (on-line LC-GC) is also an elegant and efficient approach for analysis of oryzanol (Kamm *et. al.*, 2001; Miller *et al.*, 2003).

Identification of individual compounds usually performed by gas chromatography-electron impact mass spectrometry (GC-EI-MS), positive chemical ionization- mass spectrometry (CI-MS), and nuclear magnetic resonance (NMR) (Akihisa *et al.*, 2000; Diack and Saska, 1994; Rogers *et al.*, 1993; Xu and Godber, 1999; Yasukawa *et al.*,1998).

UV spectrophotometry (De and Bhattacharyya, 2000; Gong and Yao, 2001; Qiu and Tang, 1998; Tsuchiya *et al.*, 1957) is preferred to determine its total content, the absorption spectra of the individual components of oryzanol are almost identical (Xu and Godber, 1999), because it is more simple, rapid and inexpensive than HPLC; even so, these

methods, measuring the absorbance at a single wavelength where the contribution from the oil matrix could be significant, may produce wrong results, particularly for samples with low concentrations of the analyte. Besides, they do not furnish evidence the presence of unexpected interferents, as the accuracy of the results cannot be verified. Bucci *et al.* (2003) using second derivative spectrophotometry found that it is possible to get rid of the interference of the oil matrix.

2.5 Influence of Rice Bran Oil Processing on Oryzanol

After milling, hydrolytic rancidity by enzymes such as lipase in the bran occurs rapidly. Methods developed to accomplish stabilization are heating with an extruder or microwave oven, freezing and treating with chemicals.

The stability and storability of oryzanol in rice bran after extrusion has been studied by Shin *et al.*(1997). The loss of oryzanol and total vitamin E increased when the extrusion temperature increased, which were in a temperature-dependent manner, but the degree of total vitamin E loss was greater than that of oryzanol loss.

After rice bran stabilization, crude rice bran oil is extracted and then refined before human consumption (Scheme 2.1). The process of rice bran oil refining usually involves degumming, deacidification (refining), bleaching, deodorization, and winterization. It has been reported that, in general, the total sterol loss after oil refining may be between 10 to 70%, depending on the type of oil and the processing conditions employed, and the sterol content gradually decreases in each step of the refining process (Kochhar, 1983).

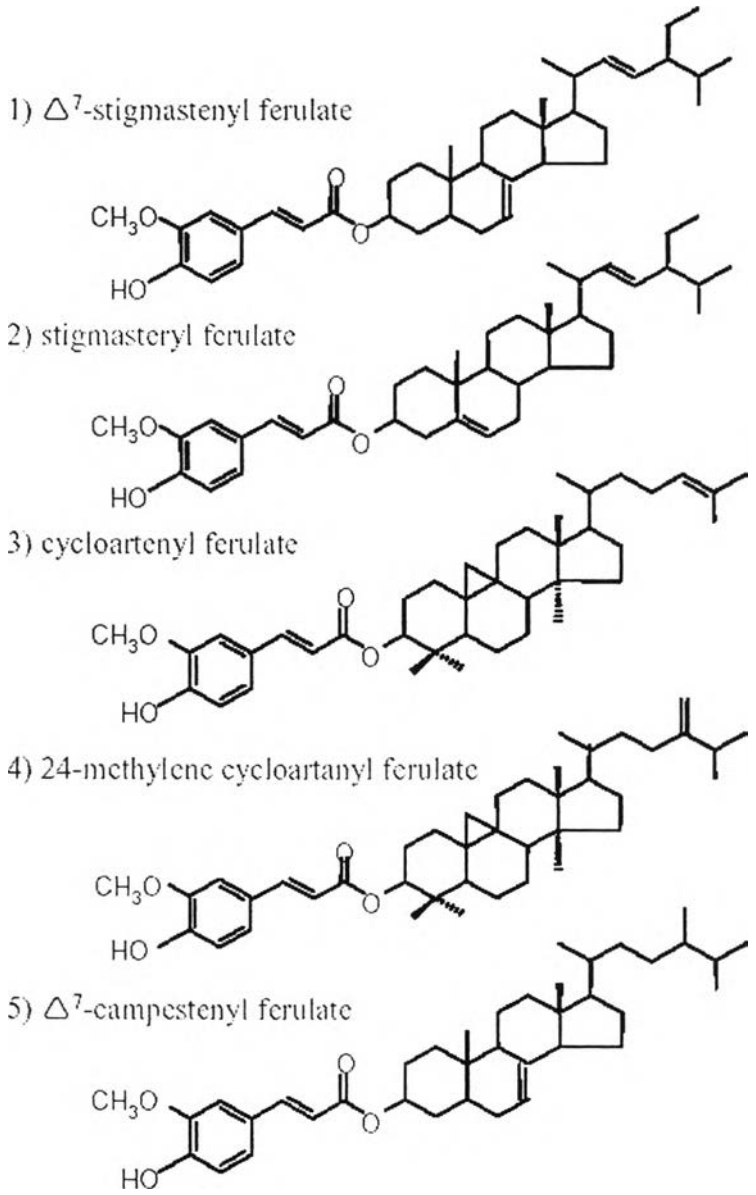


Figure 2.7 Chemical structures of oryzanol constituents isolated from rice bran oil. (Xu and Godber, 1999)

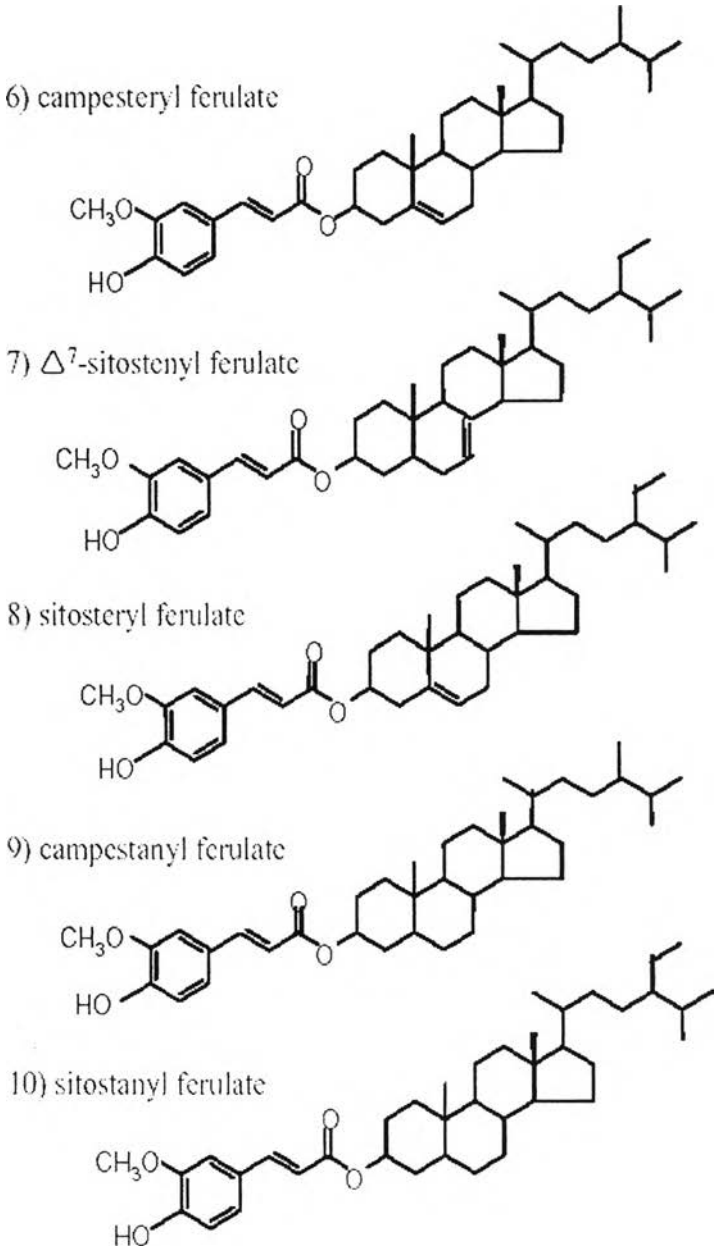


Figure 2.7 Chemical structures of oryzanol constituents isolated from rice bran oil. (Xu and Godber, 1999)

Table 2.3 Chemical structures of triterpene alcohol and sterol ferulates previously reported in rice bran

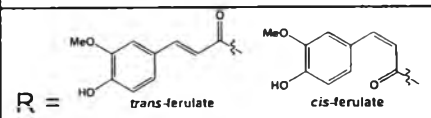
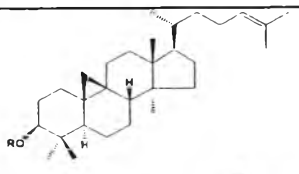
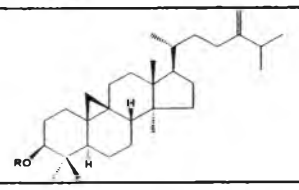
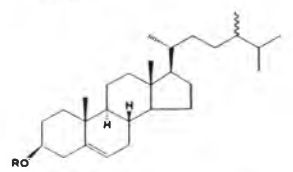
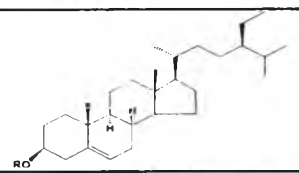
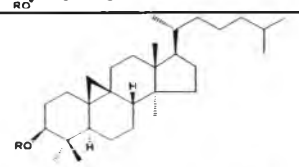
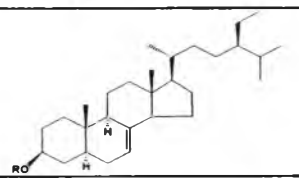
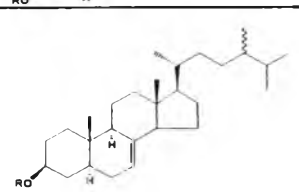
Oryzanol	Structure	Literature report
STEROL FERULATES		
Cycloartenyl (*cis*-, *trans*-) ferulate		Akihisa *et al.*, 2000 Roger *et al.*,1993 Xu and Godber,1999
24-Methylenecycloartanyl (*cis*-, *trans*-) ferulate		Akihisa *et al.*, 2000 Roger *et al.*,1993 Xu and Godber,1999
Campesteryl or 22-Dihydrobrassicasteryl *trans*-ferulate		Yasugawa *et al.*,1998 Xu and Godber,1999
Sitosteryl (*cis*-, *trans*-) ferulate		Akihisa *et al.*, 2000 Roger *et al.*,1993 Xu and Godber,1999
Cycloartanyl *trans*-ferulate		Roger *et al.*,1993
Δ^7-Sitosteryl or Schottenyl *trans*-ferulate		Fang *et al.*, 2003 Iwatsuki *et al.*, 2003 Xu and Godber,1999
Δ^7-Campesteryl or 24-Methyllathosteryl *trans*-ferulate		Fang *et al.*, 2003 Iwatsuki *et al.*, 2003 Xu and Godber,1999

Table 2.3 (continued) Chemical structures of triterpene alcohol and sterol ferulates previously reported in rice bran

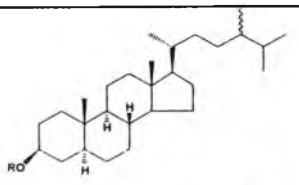
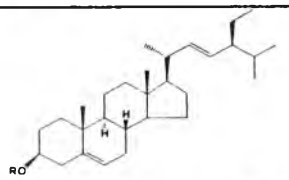
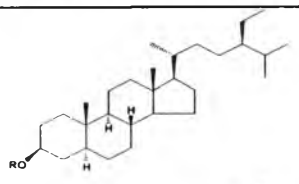
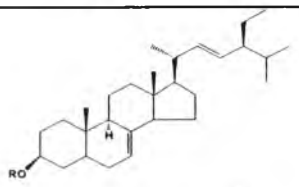
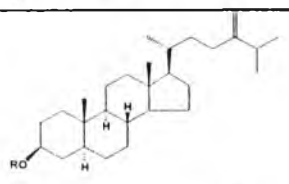
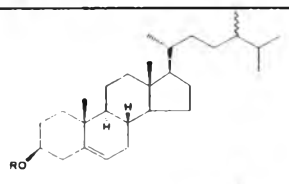
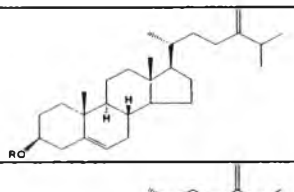
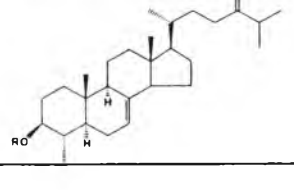
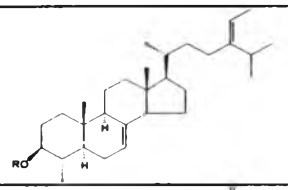
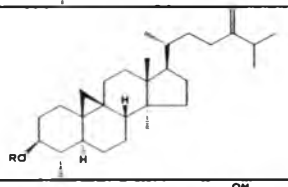
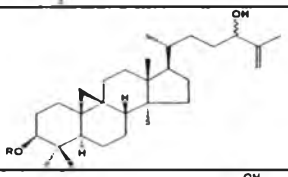
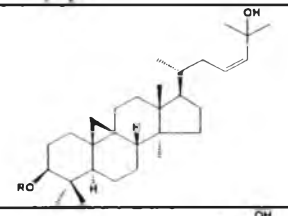
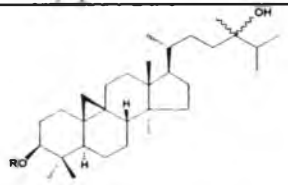
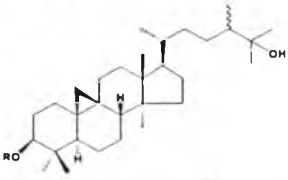
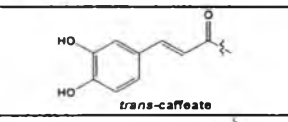
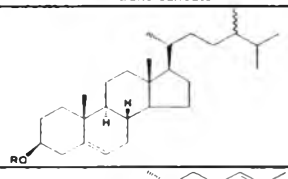
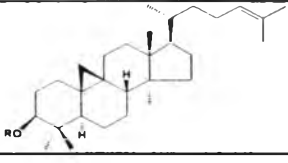
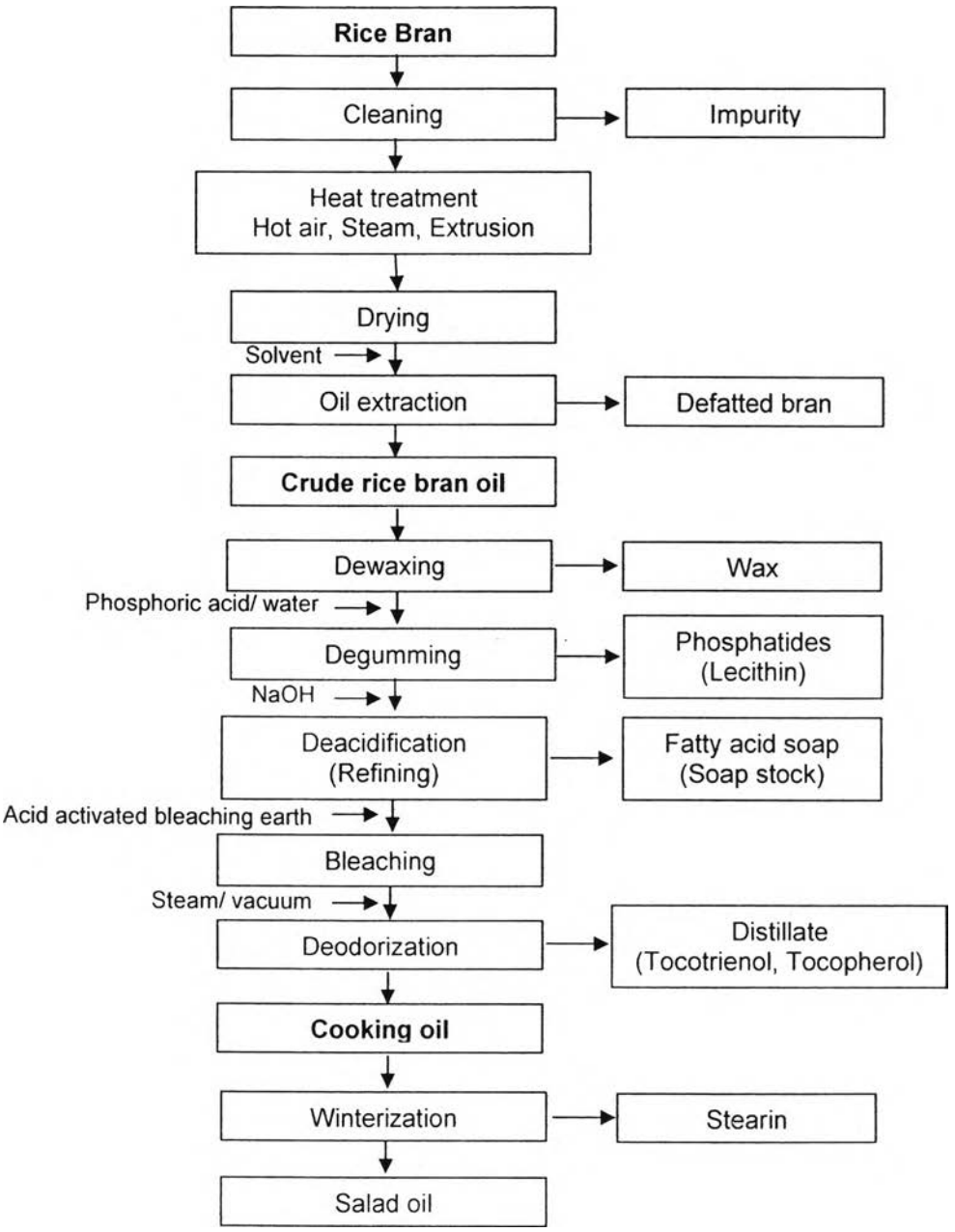
Oryzanol	Structure	Literature report
Campestanyl or 24-Methylcholestanyl *trans*-ferulate		Fang *et al.*, 2003 Iwatsuki *et al.*, 2003 Xu and Godber,1999
Stigmasteryl (*cis*-, *trans*-) ferulate		Akihisa *et al.*, 2000
Stigmastanyl or Sitostanyl (*cis*-, *trans*-) ferulate		Akihisa *et al.*, 2000 Xu and Godber,1999
Δ^7-Stigmastenyl *trans*-ferulate		Xu and Godber,1999
24-Methylenecholestanyl *trans*-ferulate		Iwatsuki *et al.*, 2003
24-Methylcholesteryl *cis*-ferulate		Akihisa *et al.*, 2000
24-Methylenecholesteryl (*cis*-, *trans*-) ferulate		Akihisa *et al.*, 2000
Gramisteryl (*cis*-, *trans*-) ferulate		Akihisa *et al.*, 2000

Table 2.3 (continued) Chemical structures of triterpene alcohol and sterol ferulates previously reported in rice bran

Oryzanol	Structure	Literature report
Citrostadienol (*cis*-, *trans*-) ferulate		Akihisa *et al.*, 2000
Cycloeucalenyl *trans*-ferulate		Akihisa *et al.*, 2000 Fang *et al.*, 2003
(24*R*,*S*)-cycloart-25-ene-3 β,24-diol-3β-trans-ferulate		Fang *et al.*, 2004
cycloart-23*Z*-ene-3β,25-diol-3β-*trans*-ferulate		Fang *et al.*, 2004
24-hydroxy-24-methylcycloartanyl *trans*-ferulates		Fang *et al.*, 2003
25-hydroxy-24-methylcycloartanyl *trans*-ferulate		Fang *et al.*, 2003
CAFFEATE ESTERS		
campesteryl *trans*-caffeate		Fang *et al.*, 2003
cycloartenyl *trans*-caffeate		Fang *et al.*, 2003

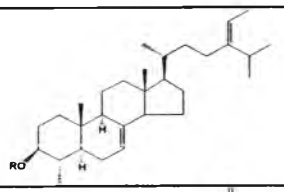
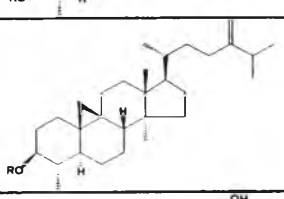
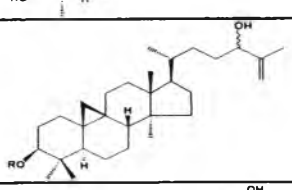
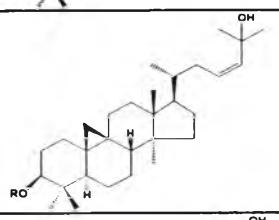
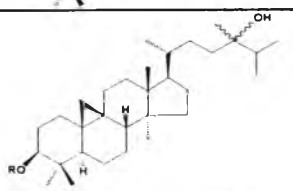
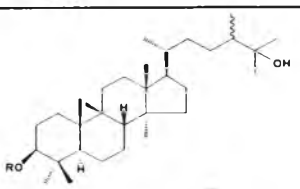
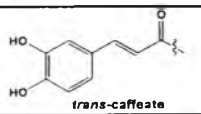
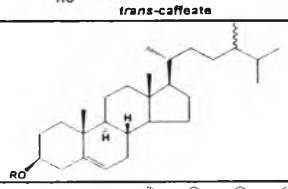
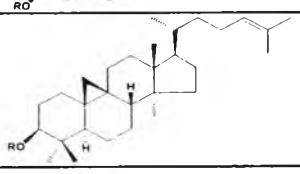
In the deacidification step, the use of alkali such as caustic soda (NaOH) to remove free fatty acids forms soapstock with a significant amount of oryzanol, which is later separated by settling or centrifugation. The soapstock can be reacidified to yield dark acid oil, in which the free fatty acids can be distilled under high vacuum and leaves oryzanol in the residue called pitch. It has been proposed to recover oryzanol from the inexpensive pitch as a valuable byproduct (Das *et al.*, 1998). In the bleaching step, the purpose is to remove color materials by heating to 85°C and treating with adsorbents such as activated carbon and activated bleaching earth. It has been recorded that partial modification of sterols, i.e. dehydration and oxidation, and deacylation of sterol esters may occur during bleaching. In the deodorization step to remove volatile compounds with undesirable flavors, steam distillation under reduced pressure is usually used with the expectation that nonvolatile off-flavor substances will be thermally degraded, become volatile and be distilled away.

Finally, the hydrogenation process, which is important in the oil industry, allows the liquid oils to be converted into semisolid or solid fats for special applications such as margarine. The process is to add hydrogen to double bonds in the fatty acid chains, which are susceptible to oxidation, and thus also improves the oxidative stability of the oil. The effect of bleaching, deodorization and hydrogenation on oryzanol content in refined oil has not been clearly studied, which may be related to the fact that oryzanol is mainly lost after neutralization. Since it has been noted that oryzanol has several double bonds and possesses antioxidant activity, if it survives neutralization, oryzanol content may further decrease during these steps where oxidation may occur.



Scheme 2.1 Flowchart for conventional rice bran oil production. (Kao and Luh, 1980; Nicolosi *et al.*, 1994)

Table 2.3 (continued) Chemical structures of triterpene alcohol and sterol ferulates previously reported in rice bran

Oryzanol	Structure	Literature report
Citrostadienol (*cis-*, *trans-*) ferulate		Akihisa *et al.*, 2000
Cycloeucalenyl *trans*-ferulate		Akihisa *et al.*, 2000 Fang *et al.*, 2003
(24*R*,*S*)-cycloart-25-ene-3 β,24-diol-3β-trans-ferulate		Fang *et al.*, 2004
cycloart-23*Z*-ene-3β,25-diol-3β -*trans*-ferulate		Fang *et al.*, 2004
24-hydroxy-24-methylcycloartanyl *trans*-ferulates		Fang *et al.*, 2003
25-hydroxy-24-methylcycloartanyl *trans*-ferulate		Fang *et al.*, 2003
CAFFEATE ESTERS		
campesteryl *trans*-caffeate		Fang *et al.*, 2003
cycloartenyl *trans*-caffeate		Fang *et al.*, 2003

In the deacidification step, the use of alkali such as caustic soda (NaOH) to remove free fatty acids forms soapstock with a significant amount of oryzanol, which is later separated by settling or centrifugation. The soapstock can be reacidified to yield dark acid oil, in which the free fatty acids can be distilled under high vacuum and leaves oryzanol in the residue called pitch. It has been proposed to recover oryzanol from the inexpensive pitch as a valuable byproduct (Das *et al.*, 1998). In the bleaching step, the purpose is to remove color materials by heating to 85°C and treating with adsorbents such as activated carbon and activated bleaching earth. It has been recorded that partial modification of sterols, i.e. dehydration and oxidation, and deacylation of sterol esters may occur during bleaching. In the deodorization step to remove volatile compounds with undesirable flavors, steam distillation under reduced pressure is usually used with the expectation that nonvolatile off-flavor substances will be thermally degraded, become volatile and be distilled away.

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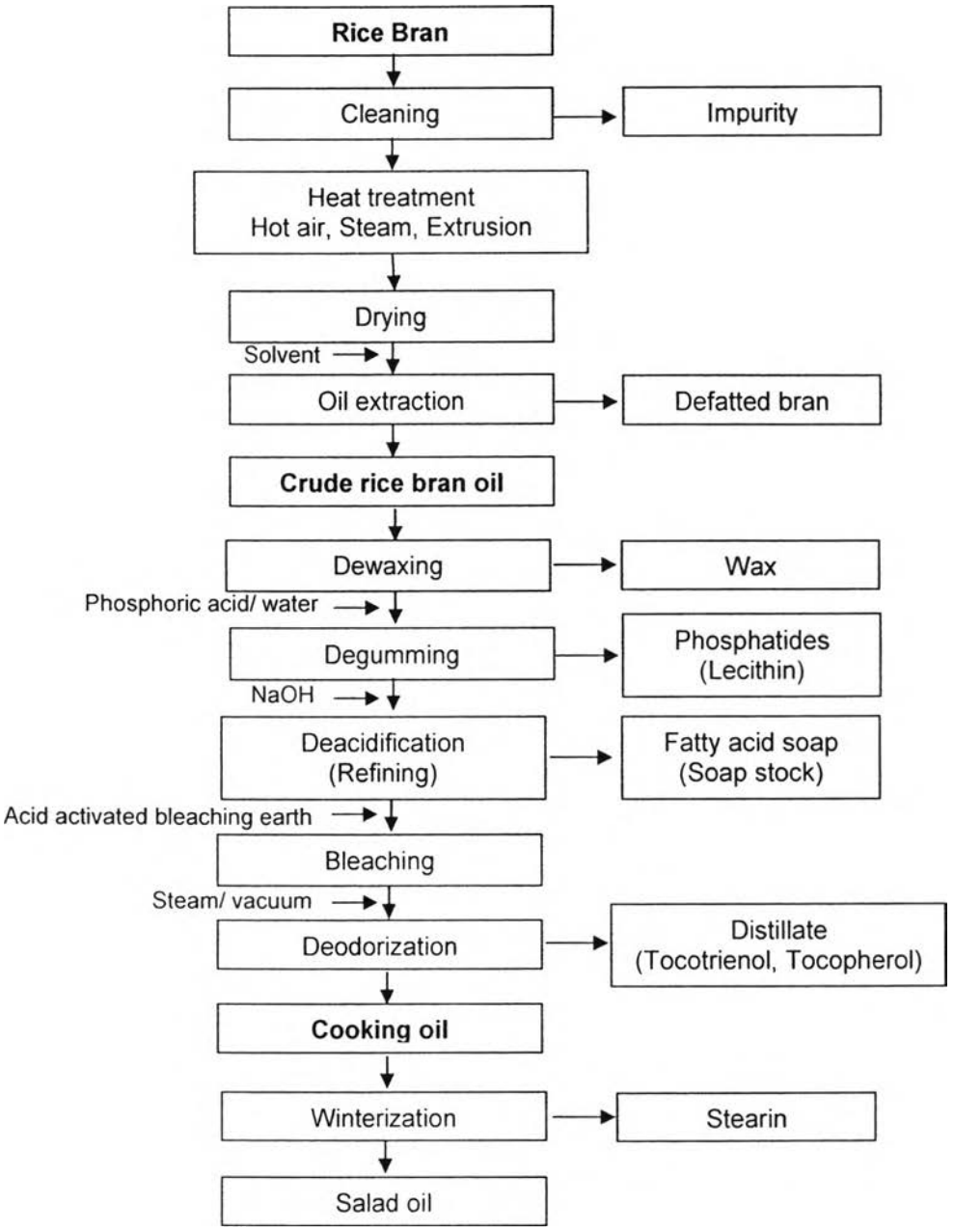

Scheme 2.1 Flowchart for conventional rice bran oil production. (Kao and Luh, 1980; Nicolosi *et al.*, 1994)

Not only oryzanol but also tocotrienols are lost during each step of rice bran oil refining process, and after processing, up to 90% can be lost. As regards preventing the loss of minor constituents of oryzanol, rice bran oil used in this study was not obtained from commercial rice bran oil. This implies that new refining methods for rice bran oil must be developed and the recoveries of these beneficial ingredients must be well optimized in order to increase the value of rice bran oil.

2.6 Toxicology and Carcinogenicity Studies of Oryzanol

The safety of oryzanol for human consumption was tested by toxicological studies on rats fed a diet containing 10% rice bran oil compared to groundnut oil for three generations (Rukmini, 1988). The chemical composition between these two oils was similar, except the unsaponifiable matter of rice bran oil (4.1%) was higher. The rice bran oil effect on the growth performance, including weight gain and feed efficiency, was not significantly different and the effect on fat absorption and the retention of nitrogen, phosphorus and calcium was not significantly different. Moreover, the toxicological effect of rice bran oil on reproductive performance was evaluated for two matings and three generations. The results indicated that no abnormalities were found, which were comparable to groundnut oil groups on the percentage of conception, birth weight, litter size, weaning weight, preweaning mortality and the number of days taken to deliver from the date of introduction for mating. The mutagenic potential of rice bran oil for deep frying and repeated heating was also found to be negative, which demonstrated remarkable oxidative stability.

Furthermore, the short-term safety of oryzanol was assessed using the Rec assay (bacterial DNA repair test), the Ames test (bacterial reverse mutation test), the rat bone marrow chromosome aberration test, and the metabolic cooperation inhibition test using Chinese hamster V79 cells (Tsushimoto *et al.*, 1991). Oryzanol has been shown to be non-genotoxic and non-inhibitory of cellular communication.

Additionally, potential carcinogenesis of oryzanol was studied by feeding mice a diet containing oryzanol up to 2g/kg body weight/day for 78 weeks (Tamagawa *et al.*, 1992b) and feeding rats for 2 years (Tamagawa *et al.*, 1992a). No treatment-related change was found in general condition, food consumption, mortality, organ weight and hematology. Histopathological examination showed that the tumor incidents were not significantly different between the treated groups and the control groups.

Modifying effects of oryzanol were also studied in a rat wide-spectrum organ carcinogenesis model. The three combined carcinogens used were 2,2'-dihydroxy-di-n-propylnitrosamine (1g/kg body weight ×2 intraperitoneal injections, i.p.), *N*-etyhl-*N*-hydroxyethylnitrosamine (1.5g/kg body weight ×2 intragastric administration) and 3,2'-dimethyl-4-aminobiphenyl (75mg/kg body weight ×3 subcutaneous injections) treatment at intervals of 3 to 4 days. The results showed that the oryzanol treatment tended to lower the incidences of pancreatic eosinophilic foci and liver hyperplastic nodule and hepatocellular carcinoma, but increased the incidences of lung adenoma and carcinoma. However, the 1% oryzanol dose given in their study was 100 to 150 times higher than what oryzanol is usually given for medical use (>300mg/person/day) (Hirose *et al.*, 1991). Also, the correlation between oryzanol ingestion and human lung cancer and the interaction with other ingredients in the diet have not been reported in any epidemiological data.

Examination of the modifying potential of oryzanol on mammary carcinogenesis in female Sprague-Dawley rats pretreated with a single intragastric dose of 7,12-dimethylbenz(a)anthracene revealed no significant differences in the final incidences and multiplicities of mammary tumours, even if oryzanol tended to (nonsignificantly) decrease the size of the tumour (Hirose *et al.*, 1999). The lung tumour promotion potential may not be due to oryzanol's major metabolite, ferulic acid, since ferulic acid at a dietary dose of 2% did not show such enhancing effect in Sprague-Dawley female rats pretreated with 1,2-dimethylhydrazine and N-methylnitrosourea (Hirose *et al.*, 1994).

On the other hand, another study of modifying effects on carcinogenesis was demonstrated using a similar animal model (Imaida *et al.*, 1990) but with two different combined carcinogens, 1,2-dimethylhydrazine (40mg/kg body weight ×3 subcutaneous injections within one week) and 1-methyl-1-nitrosourea (20mg/kg body weight ×2 i.p. administrations, twice a week for 2 weeks). The results showed that 1% ferulic acid had weak inhibitory effect and did not promote lung adenoma and carcinoma as oryzanol did in the study by Hirose *et al.* (1991).

Research suggests that oryzanol taken in moderately high amounts (up to 600 mg per day) for prolonged periods of time (6 months) may cause a variety of adverse side-effects, including dry mouth, sleepiness, hot flushes, irritability, and light-headedness in some individuals (Takemoto *et al.*, 1977).

At the time of writing, there were no well-known drug interactions with oryzanol. To the auther's knowledge, there was the only one study about properties of oryzanol on cytochrome P450 (CYP) enzymes. Most oxidative metabolism of drugs is catalyzed by CYP enzymes, which comprise a large family of hemoproteins (Gonzalez, 1989; Guenoerich, 1992). Researchers found that oryzanol had little inhibitory effects on CYP activities, indicating that this compound would not be expected to cause clinically significant interactions with other CYP-metabolized drugs at expected therapeutic concentrations (Umehara *et al.*, 2004).

In summary, oryzanol has been approved and commercially available in many countries such as Japan but government and industry in the United States of America are comparably more conservative. Studies suggest that oryzanol is not carcinogenic to animals and rice bran oil was safe for human consumption. There have been no reported acute or chronic side effects of oryzanol and the evidence of its beneficial effects in humans is increasing and the market is growing. Thus, extensive and well-designed clinical trials

with regard to the potential of oryzanol and ferulic acid as chemopreventors or promoters and the mechanisms for carcinogenic or anticarcinogenic effects are needed.

2.7 Phamacokinetic of Oryzanol

While oryzanol has been utilized in many countries as food additives and pharmaceuticals, there are only a few studies in English exploring the digestibility, absorption, and metabolism of oryzanol in the human gastrointestinal tract. Many of the studies used animal models such as rabbits and rats and they were also done using thin layer chromatography in the period when the analytical method to identify oryzanol and its metabolites was not advanced.

Oryzanol is mainly metabolized to ferulic acid in animals and humans (Fujiwara *et al.*, 1982, 1983; Umehara *et al.*, 2004). Animal studies have shown that less than 10% of oryzanol taken in via the diet is actually absorbed. The small amounts that are absorbed travel to the liver where the compound is separated into the sterol and ferulic acid. In addition, seventy-two hours after oral administration of ^{14}C labeled oryzanol (C-3 position of ferulic acid) to rats, 10% of the radioactivity was excreted by urine and 85% by feces. Intact oryzanol could not be detected in the urine but ferulic acid, dihydroferulic acid, *m*-hydroxyphenylpropionic acid, *m*-coumaric acid, hippuric acid, and *m*-hydroxyhippuric acid in free and conjugated forms (glucuronides, sulfates) were identified as urinary metabolites of oryzanol, see **Figure 2.8** for chemical structures (Fujiwara *et al.*, 1983). Ferulic acid, vanillic acid, acetovanillone, hippuric acid, and vanilloylglycine but not intact oryzanol have been detected as urinary metabolites in rabbits (Fujiwara *et al.*, 1980).

Following a single oral administration of oryzanol at 300 mg to humans, the peak plasma concentrations of oryzanol and ferulic acid were 37.6 and 36.6 ng/ml, respectively. Ferulic acid was excreted in urine at 2.4-2.8% of the dose/day but oryzanol was not detected (Odomi *et al.*, 1983). *In situ* intestinal experiments using ^{14}C-labeled oryzanol

showed that the ester linkage of oryzanol was partly hydrolyzed in the intestine during absorption (Fujiwara *et al.*, 1983).

The enzymes responsible for the hydrolysis of oryzanol during metabolization have not been fully studies; however, a previous *in vitro* study showed that oryzanol was hydrolyzed to ferulic acid in rat liver, intestine, pancreas and kidney preparations, with possible involvement of carboxylesterase (Kudo and Akiyama, 1982). Two recent studies have shown that steryl ferulates are hydrolyzed by mammalian digestive enzymes. Moreau and Hicks (2004) demonstrated that synthetic sitostanyl ferulate is hydrolyzed by cholesteryl esterase and pancreatin, whereas steryl ferulates of oryzanol were only hydrolyzed by cholesteryl esterase. Furthermore, Miller and his colleague (2004) screened commercially available enzyme preparations for their suitability to hydrolyze oryzanol *in vitro* and indicated that pancreatic cholesteryl esterase plays an essential role in the metabolization of oryzanol *in vivo*.

2.8 The Potential Functionality of Oryzanol to Human Health

A number of studies in human and animals have shown that rice bran oil is as effective as other vegetable oils in lowering plasma cholesterol levels (Lichtenstein *et al.*, 1994; Rukmini and Raghuram, 1991). In some cases, rice bran oil lowered plasma cholesterol more effectively than other commonly used vegetable oils rich in linoleic acid (Rukmini and Raghuram, 1991); this effect can be attributed to the occurrence of specific components in rice bran oil, oryzanol (and its constituents, triterpene alcohols), tocopherols, and perhaps tocotrienols (Nicolosi *et al.*, 1994; Rohrer and Siebenmorgen, 2004; Rukmini and Raghuram, 1991; Sugano and Tsuji, 1997).

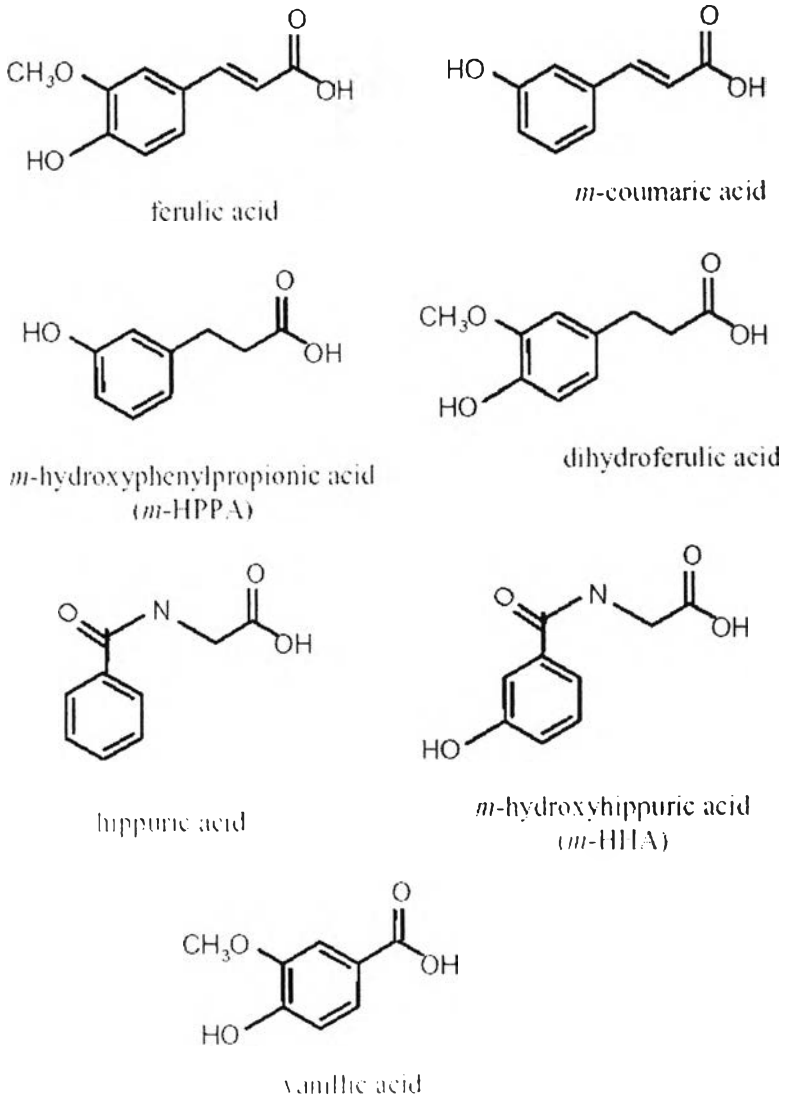

Figure 2.8 Chemical structures of ferulic acid and its urinary metabolites. (Fujiwara *et al.*, 1983, and Booth *et al.*, 1957)

2.8.1 Antioxidant Activity

In order to understand the function of antioxidants, first of all, the oxidative stress caused by the generation of reactive oxygen species (ROS) associated with the presence of oxygen even under physiological conditions and the antioxidant defense system in human organisms must be discussed. ROS are either radicals, atoms or a group of atoms that contain at least one unpaired electron, such as peroxyl radicals (ROO$^{\cdot}$), hydroxyl radicals ($OH^{\cdot}$), the nitric oxide radicals (NO$^{\cdot}$) and the superoxide anion radicals ($O_2^{\cdot-}$), or non-radical compounds, such as hydrogen peroxide (H_2O_2) and singlet oxygen ($^1O_2$), which are capable of oxidizing molecules. ROS may have positive, negative or zero charge and sometimes are also called oxidants or prooxidants.

The antioxidant defense system *in vivo* includes enzymatic and non-enzymatic antioxidants. The major enzymatic antioxidants are superoxide dismutase ($O_2^{\cdot-} \rightarrow H_2O_2$), catalase ($H_2O_2 \rightarrow H_2O$), glutathione peroxidase (organic hydroperoxides ROOH$\rightarrow H_2O$). The non-enzymatic antioxidants are dietary antioxidants among which the most prominent are ascorbic acid (vitamin C), tocopherols and tocotrienols (vitamin E), carotenoids and flavonoids.

2.8.1.1 Antioxidant Activity of Oryzanol against Cholesterol Oxidation

Oxidation products of cholesterol are considered to be mutagenic and carcinogenic compounds (Ansari *et al.*, 1982; Gerhardt and Gallo, 1998; Paniangvait *et al.*, 1995; Watanabe *et al.*, 1988; Woods and O'Brien, 1999). These products also are harmful to many cells in blood vessels, such as macrophage and endothelial cells, and contribute to plaque formation, which results in a variety of cardiovascular and pulmonary diseases (Kumar and Singhao, 1991; Lyon and Brown, 1999; Morel and Lin, 1996; Wilson *et al.*, 1997). Cholesterol oxidation is initiated by free radicals to produce hydroperoxides, peroxides, and other degradation products.

The antioxidant activity of oryzanol has been evaluated in a few models using chemicals to induce lipid oxidation and measuring the generation of oxidized products. One of them is a cholesterol oxidation model accelerated by 2,2'-azobis(2-methylpropionamidine) dihydrochloride (AAPH), which was established to compare the inhibitory capability of three major components of oryzanol including cycloartenyl ferulate, 24-methylenecycloartanyl ferulate and campesteryl ferulate and vitamin E on production of oxidized cholesterol (Xu and Godber, 2001). The results suggested that 24-methylene cycloartanyl ferulate had the highest antioxidant activity and all three major components of oryzanol had higher antioxidant activity than vitamin E components. Due to oryzanol structure, they may have greater ability to associate with cholesterol in the small droplets of the emulsion and become more efficient in protecting cholesterol against free radical attack. And it was proposed that the antioxidant mechanism of oryzanol might be due to its intramolecular hydrogen-bonded methoxyphenols, where hydrogen donation would occur more readily (Huang *et al.*, 2002; Xu and Godber, 2001).

The antioxidant activity of the three major components of oryzanol was also investigated and compared with that of α-tocopherol and ferulic acid using linoleic acid model (Xu and Godber, 2001). Researchers found that free fatty acid and α-tocopherol had greater activity than did the three ferulic acid esters at a lower concentration due to relatively larger molecular structure of sterol or triterpene portion than free ferulic acid. Moreover, lipid peroxidation has been shown to be prevented in the retina by oryzanol because of its antioxidant property (Hiramitsu and Armstrong, 1991). As for antioxidant activity of oryzanol, Seetharamaiah and colleagues (1990) found that oryzanol significantly inhibited platelet aggregation.

2.8.1.2 Anti-carcinogenesis of Oryzanol

Since various triterpene alcohols and sterols and their oxygenated derivatives exhibits activity on *in vivo* primary screening assay for antitumor-promoters by 12-*O*-tetradecanoylphorbol-13-acetate (TPA) in mice and on tumor promotion in two-stage

carcinogenesis in mouse skin initiated by 7,12-dimethylbenz[*a*]anthracene (DMBA) and promoted by TPA, oryzanol from rice bran extract also has inhibitory effect against TPA-induced inflammation in mice (Akihisa *et al.*, 1997 and 2000; Yasukawa *et al.*, 1991 and 1997). Moreover, using wheat, rye, and corn bran oils comprising oryzanol and sterols as food ingredients might be advantageous because their sterol and sterol ferulate constituents have, as shown in this study, possible cancer chemopreventive properties (Hakala *et al.*, 2002; Iwatsuki *et al.*, 2003). Moreover, it might be worth noting that plant sterols (phytosterols), especially sitosterol (Sietz, 1989), are suggested to have protective effect against the most common cancers in the developed countries (Awad and Fink, 2000) including colon (Raicht *et al.*, 1980), prostate (Awad *et al.*, 2000a), and breast (Awad *et al.*, 2000b). Thus, oryzanol are suggested to be valuable antitumor promoters or potential cancer chemopreventive agents.

The theory has also been approached in a review of the antioxidant chemistry of ferulic acid (Graf, 1992). It was explained that the antioxidant activity of ferulic acid is due to its phenolic hydroxyl group that has hydrogen donating property to scavenge a reactive radical and form a phenoxy radical. This radical is then stabilized resonantly because the unpaired electron can be delocalized across the entire molecule (Figure 2.9). The second phenolic hydroxyl group can enhance the radical-scavenging property by providing additional resonance stabilization and form quinone. The methoxyl group of ferulic acid partially destabilizes the phenoxy radical and impairs its antioxidant activity.

2.8.2 Hypocholesterolemic Capacity

The composition of the human diet plays an important role in the management of lipid and lipoprotein concentrations the blood. Reduction in saturated fat and cholesterol intake has traditionally been the first goal of dietary therapy lowering the risk for cardiovascular disease (Kerckhoffs *et al.*, 2002).

A hypocholesterolemic effect of different vegetable oils blended with rice bran oil was found in healthy young women fed for 7 days (Suzuki and Osima, 1970). This effect was also reported in rats fed a normal diet containing 7% rice bran oil or a high cholesterol diet containing 1% cholesterol and 7% rice bran oil for 4-7weeks and there was also significantly decreased levels of serum and liver total cholesterol and low-density lipoprotein (LDL) cholesterol and a slightly increased level of high-density lipoprotein (HDL) cholesterol in both the normal diet and the high cholesterol diet (Sunitha *et al.*, 1997).

Assuming oryzanol to be an important element in improving cholesterol levels, the optimal dosage and length of treatment time necessary to realize benefits was studied (Hakala *et al.*, 2002; Seetharamaiah and Chandrasekhara, 1988). Later, oryzanol was found to be responsible for the effect (Seetharamaiah and Chandrasekhara, 1989). The rice bran oil (10%) diet showed markedly lower serum and liver total cholesterol levels in rats and rice bran oil spiked with oryzanol (0.5%) reduced it even more significantly. This was also confirmed by Rong *et al.* (1997) in hamsters fed a diet containing 0.1% cholesterol with or without 1% oryzanol for 7 weeks. The oryzanol-fed animals had 28% reduction in plasma total cholesterol and 34% reduction of combined LDL and very low density lipoprotein (VLDL) cholesterol, as well as 25% decrease in cholesterol absorption. Moreover, oryzanol showed no effect on endogenous cholesterol synthesis, as measured by the liver and intestinal HMG-CoA reductase activities, which is an important indication that the decreased cholesterol level in plasma may have resulted from lower cholesterol absorption and that cholesterol synthesis by HMG-CoA reductase may not increase to adjust the cholesterol level in response to the decreased cholesterol uptake.

There are only a few oryzanol studies on humans. A mild but clear hypocholesterolemic effect was observed when 300 mg of oryzanol was administered daily to each of 67 subjects for 3 months (Yoshino *et al.*, 1989). Although there was no control group, the patients were stratified into three groups according to the World Health Organization hyperlipidemia classification and encouraged not to change dietary or lifestyle

habits for the 3-month duration of the study. All groups experienced a decline in total cholesterol levels, and to a significant degree in type IIb (high cholesterol and high triglycerides) and IIa (high cholesterol only) patients. Sasaki *et al.* (1990) studied the effects of oryzanol on serum lipids of twenty chronic schizophrenic patients with dyslipidemia. Each patient was given 100mg of oryzanol three times daily for 16 weeks. The results showed that total cholesterol and LDL levels decreased significantly with no side effects recorded, suggesting that oryzanol is safe and effective in the treatment of dyslipidemia.

In a recent human study, in which the subjects received 30 g/day of margarine enriched with various plant sterols for 3.5 weeks, it was found that oryzanol was less effective than the fatty acid esters of the common 4-desmethyl sterols and stanols (i.e., sitosterol, campesterol, and the respective stanols) in lowering total and LDL-cholesterol (Weststrate and Meijer, 1998). The authors suggested that the difference may be partly due to the lower intake of sterols from the oryzanol-enriched margarine (1.5 g/day) than from the margarines enriched with other plant sterols (3.3 g/day) and partly due to the structure of the sterols in oryzanol that may be less effective in lowering cholesterol absorption.

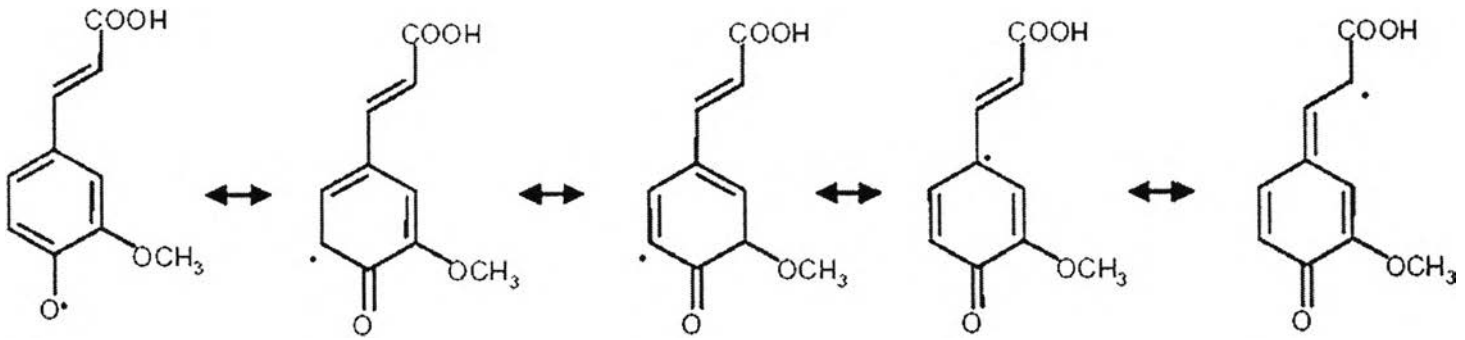

Figure 2.9 Resonance stabilization of ferulic acid radical. (Graf, 1992)

In the process of digestion, oryzanol was partly hydrolyzed in gut to be plant sterols and ferulic acid, and hence plant sterol portion of oryzanol may play an important role in reducing plasma cholesterol. The consumption of plant sterols and plant stanols lowers blood cholesterol levels by inhibiting the absorption of dietary and endogenously produced cholesterol from the small intestine and the plant sterols are only very poorly absorbed themselves. This inhibition is related to the similarity in physicochemical properties of plant sterols, stanols and cholesterol, and it is generally accepted that there are three possible mechanisms by which this inhibition occurs:

(a) **The co-precipitation of cholesterol and plant sterols:** In the intestinal lumen, cholesterol is found in solution with other fats; however, as monoglycerides and fatty acids are absorbed from the gut, concentration of the less well absorbed substances, e.g. sterols increases. When their concentration reaches a critical level, similar substances can precipitate from the solution. This may happen with cholesterol and plant sterols, because of their similarity in structure. Both cholesterol and plant sterols in the free state are poorly soluble in micelles, and in fact mutually limit each others solubility. Hence the greater the amount of plant sterols, the lower the solubility and perhaps the greater the amount of cholesterol precipitated. Cholesterol in the crystalline form cannot be absorbed.

(b) **The competition for space in mixed micelles:** Micelle or mixed micelles are very efficient detergent-structures that solubilize the lipids secreted in the small intestine. Mixed micelles are composed of bile salts, phospholipids, tri-, di- and monoglycerides, fatty acids, free cholesterol and fat soluble micronutrients. Under normal circumstances, absorption of cholesterol into intestinal mucosal cells requires cholesterol to be incorporated into bile acid micelles for solubilization. As there is only limited capacity in the micelles for carrying cholesterol, compounds with similar structures to cholesterol such as plant sterols and stanols can compete with cholesterol for this space. Therefore increasing the amounts of plant sterols and stanols results in less and less cholesterol in mixed micelles and thereby decreased absorption of cholesterol from the gut. *In vitro* and *in vivo* studies suggest that plant sterol have a greater affinity for micelles and can therefore

displace cholesterol from the micelles (Ikeda and Sugano, 1983; Heinemann *et al.*, 1991; Ikeda *et al.*, 1988). Armstrong and Carey (1987) reported that the solubilization of plant sterol in micelles was more energetically favored than cholesterol, thus providing a thermodynamic explanation for decreased cholesterol solubility in the presence of plant sterol.

(c) **The increase in cholesterol excretion:** The obligatory increase in cholesterol excretion that accompanies reduced cholesterol absorption was also observed (Carr *et al.*, 2002; Sugano *et al.*, 1977; Grundy *et al.*, 1969; Lees *et al.*, 1977; Becker *et al.*, 1993; Kudchodkar *et al.*, 1976). The current results indicate that the majority of cholesterol excreted in the feces was of hepatic origin, which provides the most likely explanation for the reduced plasma and liver cholesterol concentration observed with plant sterol feeding. A novel finding by Carr and his coworker (Carr *et al.*, 2002) was that plant sterol feeding caused an alteration in the gallbladder bile acid profile, thus reducing the hydrophobicity index of the bile. The hydrophobicity index (Heuman, 1989) is an overall estimation of the cholesterol-solubilizing capacity of bile and is based on the relative hydrophobicity of individual bile acids. Studies with individual bile acids indicate that more hydrophobic bile acids have a greater capacity to solubilize cholesterol (Armstrong and Carey, 1987), suggesting that a lower hydrophobicity index may be an indicator of reduced cholesterol solubilization in the small intestine.

In summary, oryzanol has shown promising hypocholesterolemic capacity. To postulate the hypocholesterolemic mechanism of oryzanol, the cholesterol absorption and transport in humans must be discussed. The cholesterol in the human body comes from two sources. One is endogenous cholesterol, which is synthesized in the liver. Some may go into VLDL and be secreted into the blood and some are converted to bile salts, stored in the gallbladder, released into the duodenum as biological detergents to form micelles with dietary lipids, reabsorbed at the ileum and return to the liver through the hepatic portal vein. The other source is exogenous dietary cholesterol, which is incorporated into micelles,

absorbed at the jejunum and travels to the liver through the lymphatic system and bloodstream. During any one day, the cholesterol in the intestinal lumen is typically two-thirds from endogenous sources and one-third from dietary sources. Cholesterol absorption in humans can vary widely (15 to 75%; Grundy, 1983). It has been demonstrated that decreased serum cholesterol, specifically LDL, can result from the inhibition of cholesterol absorption (Gylling and Miettinen, 1995).

2.8.3 Other Pharmaceutical Properties

The theory behind oryzanol supplementation is the effect it has on the body's hormonal system - specifically the ability to increase testosterone levels. Oryzanol is also thought to stimulate the hypothalamus to secrete Growth Hormone Releasing Hormone (GHRH), which stimulates the release of Human Growth Hormone (HGH). Both testosterone and Human Growth Hormone have muscle-building effects. Oryzanol may also have an effect on endorphins. Endorphins are released during exercise and help reduce the feeling of fatigue. Athletes who feel less fatigue are able to train longer (Wheeler and Garleb, 1991).

A 1997 study tested the effect of oryzanol supplements during resistance (strength) training in 22 college-aged males who had been weight training for more than one year. The subjects were divided into two groups. Both groups completed the same weight-training program. The researchers measured body composition, muscle strength, power, heart rate, blood pressure, hormones such as testosterone, and blood lipids such as cholesterol. A double-blind, placebo-controlled study found that 9 weeks consumption of oryzanol at a dose of 500 mg daily affected neither anabolic hormone levels nor performance (Fry *et al.*, 1997).

In contrast, two studies published in 1990s suggest the ferulic acid supplements may be beneficial. In the first study, six trained weight lifters received 30 mg per day of ferulic acid for eight weeks while four lifters received a placebo. Body weight and strength (measured by one maximum lift) increased in the group receiving the supplements. The

authors concluded that some aspects of weight training might be helped by ferulic acid supplementation. The second study measured hormone levels in six trained male endurance runners who took 50 mg per day of ferulic acid for three days. Levels of endorphins were greater when the athletes were taking the supplements than when they took the placebo (Grunewald and Bailey, 1993; Wheeler and Garleb, 1991).

The authors of the 1990 studies thought the results were promising but in need of more research. The 1997 study measured more aspects in a larger number of people and is considered the strongest study. The results of that study would not support the use of oryzanol as a performance enhancer. It is interesting to note that in rat studies where oryzanol was injected, growth hormone manufacture and release was decreased – not increased as many supplements claim. The reason for the decrease is not known (Fry *et al.*, 1997).

In addition, oryzanol is known to have antibiotic and bactericidal properties and has been shown to be highly effective against lipogenic liver cirrhosis in spontaneously hypertensive rats, an animal having natural abnormalism in lipid embolism (Suzuki *et al.*, 2002). Evidence from animal studies suggests that oryzanol may help prevent ulcers, but meaningful human trials are lacking (Cicero and Gaddi, 2001).

Oryzanol has also been advocated as a treatment for menopausal symptoms, but the basis of this potential use consists of evidence far too weak to be relied upon at all. In one study, oryzanol injected into rats altered levels of circulation luteinizing hormone (LH) (Cicero and Gaddi, 2001).

In summary, some of the biological effects of oryzanol and rice bran oil are: (i) reduction of cholesterol in the blood of experimental rats and human volunteers (Raghuram *et al.*, 1989; Seetharamaiah and Chandashekara, 1989), (ii) improvement of capillary action of blood vessels (Kamimura *et al.*, 1964), (iii) anti-aging effect similar to tocopherols

(Noboru and Yusho, 1970), (iv) anti-dandruff and anti-itching properties (Shugo, 1979). Many product patents based on oryzanol are available in the literature (Horst and Andreas, 2004; Imai *et al.*, 1994; Kaimal, 1999; Krishna *et al.*, 2001; Noboru and Yusho, 1970; Shugo, 1979).

2.9 Applications of Oryzanol

Since the functionality of oryzanol has been found to be promising, oryzanol may have great market potential and be applied to a wide range of products as an ingredient for druds, nutraceuticals, functional foods, and feeds, as well as cosmetics (Parrado *et al.*, 2003). It has been approved in Japan for several conditions, including menopausal symptoms, mild anxiety, stomach upset, and high cholesterol. In the US, it is widely used as a sports supplement, as well as for reducing cholesterol; still, there is no more than preliminary evidence that oryzanol is effective for any purpose.

Oryzanol can be enriched in food products such as cereal and margarine for its cholesterol-lowering and antioxidant effects, put into frying oil for its stability and into food coating or packaging material for antioxidant potential to extend shelf life and prevent color changes in the products. It can also be supplemented into non-food products such as sun protection skin lotion due to UV absorption, skin-care products for repairing dry and sensitive skin, and nail lacquers for preventing discoloration of nails.