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Fecal steroid excretion of rats fed rice bran oil and oryzanol

Zhaoli Dai

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FECAL STEROID EXCRETION OF RATS
FED RICE BRAN OIL AND ORYZANOL

A Thesis

Submitted to the Graduate Faculty of the
Louisiana State University and
Agricultural and Mechanical College
in partial fulfillment of the
requirements for the degree of
Master of Science

in

The Department of Food Science

by
Zhaoli Dai
B. S., Jinan University, Guangzhou, China, 1999
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ABSTRACT

Rice bran oil (RBO) has been considered healthy cooking oil with potent effects to reduce the risks of atherosclerosis and coronary heart disease. The relatively high level of unsaponifiable components contributes to the healthy benefits of RBO. This study investigated the possibility that the cholesterol-lowering effects of oryzanol are through increased fecal steroid excretion. The objective was to compare fecal cholesterol and bile acid excretion as well as bioavailability among different forms of oryzanol. The correlation between bioactivity and bioavailability of oryzanol was investigated as well.

Forty-seven female breeder rats were used as animal models. Oryzanol was added to an AIN-93 M maintenance diet at 2.8g/kg as the treatments. Treatments were either oryzanol in RBO, dissolved oil form of oryzanol in corn oil, or crystalline form of oryzanol added directly to the diet. Control was corn oil. Fecal samples were collected at week 6 and 10. Fecal cholesterol, oryzanol, and bile acids were analyzed with GC-FID.

Results showed no significant differences between the two collection periods ($P > 0.05$). Oryzanol in RBO produced the greatest fecal cholesterol excretion among the diet groups ($P < 0.05$). Both fecal cholesterol and total bile acid excretion were significantly higher in rats fed RBO oryzanol than the average of the other two treatment groups ($P < 0.001$). It may indicate that the hypocholesterolemic activity of RBO with oryzanol was more effective than oryzanol in corn oil, through synergistic effects of the components in the unsaponifiable contents. The possible mechanism for RBO and oryzanol to lower cholesterol levels appeared to be through inhibiting cholesterol absorption and bile acid reabsorption to enhance fecal cholesterol and bile acid excretion.

The effect of treatment compared to control was greater for bile acid excretion than for cholesterol excretion, which may suggest the primary mechanism of the hypocholesterolemic effect of oryzanol may be through the interference of bile acid reabsorption in the enterohepatic circulation.

The correlation among different forms of oryzanol to the bioactivity was unclear, although the results showed fecal recovery of oryzanol was positively related to fecal steroid excretion. It might indicate the absorbability of oryzanol was negatively related to fecal steroid excretion.

CHAPTER 1

INTRODUCTION

Lowering total plasma cholesterol concentration has been shown to reduce coronary heart disease (CHD). Consuming natural dietary components may reduce plasma cholesterol (Lin et al., 2004; Graaf et al., 2002; Kerckhoffs et al., 2002.). FDA (2000) endorsed health claims show that food products containing adequate amounts of plant sterols/stanols may reduce CHD. Natural compounds such as phytosterol and oryzanol have been shown to have potent cholesterol-lowering effects in various animal and human studies (Graff et al., 2002; Hakala et al., 2002; Trautwein et al., 2002; Vissers et al., 2000; Rong et al., 1997; Kahlon et al., 1996.). Due to the minimal toxic side effects of these compounds in natural plant seeds, they may provide an alternative dietary supplement in place of existing pharmaceutical agents to improve serum cholesterol levels and thus reduce the risks of atherosclerosis and heart disease.

Rice bran oil (RBO) has been used in some Asian countries as cooking oil (Orthofer, 1996; Rukmini and Raghuram, 1991). The oil is concentrated in the germ and bran layers, which are dispersed during rice milling (Orthofer, 1996). Oil content in rice bran is about 20% (Orthofer, 1996), containing ~ 43.8% oleic acid and ~ 39.3% linoleic acid, which is similar to the fatty acid profile of peanut oil (Edwards et al., 1994).

Rice bran oil contains relatively high unsaponifiable fractions (UF), such as plant sterols and stanols, γ -oryzanol, tocopherols and tocotrienols, compared to other common vegetable oils. Rice bran contains ~ 3000mg/kg γ -oryzanol and up to 300mg/kg vitamin E. These two compounds have been considered as potent antioxidants and may be bioactive in prevention of certain chronic diseases (Xu et al., 2001). Studies showing rice

bran oil lowering plasma cholesterol more effectively than other commonly used vegetable oils, suggest plant sterols, γ -oryzanol and tocotrienols may be the major active compounds responsible for this significant effect (Hakala et al., 2002; Qureshi et al., 2001; Sugano and Tsuji, 1997; Rogers et al., 1993; Kahlon et al., 1992; Rukmini and Raghuram, 1991).

γ -Oryzanol, was first extracted from rice bran oil as ferulate esters of plant sterols (Rogers et al., 1993). It has been purified and identified as having 10 components: cycloartenyl, 24-methylenecycloartanyl, campesteryl, Δ^7 -campestenyl, sitosteryl, Δ^7 -sitostenyl, stigmasteryl, Δ^7 -stigmasteryl, and sitostanyl and campestanyl ferulates (Xu and Godber, 1999). γ -Oryzanol was used to decrease plasma cholesterol, platelet aggregation, cholesterol absorption from cholesterol-enriched diets and aortic fatty streaks (Hakala et al., 2002). Other than the promising cholesterol-lowering effects, γ -oryzanol may have other pharmacological effects such as regulation of the estrous cycle, growth-accelerating action, and the ability to promote skin capillary circulation (Rong et al., 1997). Results from a number of animal and human studies (Vissers et al., 2000; Rong et al., 1997; Sugano and Tsuji, 1997; Hegsted et al., 1993; Yoshino et al., 1989.) have demonstrated the cholesterol-lowering benefits of RBO and/or γ -oryzanol.

γ -Oryzanol is also a potential antioxidant as its chemical structure contains phenolic moiety (Hakala et al., 2002, Xu et al., 2001). In Xu et al. (2001)'s study, they demonstrated that γ -oryzanol might be a more important antioxidant in the reduction of cholesterol oxidation than vitamin E in rice bran. Using a cholesterol oxidation system, they found a 10-fold higher level of γ -oryzanol than vitamin E in rice bran and all the

components in γ -oryzanol exhibited significant antioxidant activity, with 24-methylenecycloartanyl ferulate having the highest antioxidant activity.

The similar structures of plant sterols and γ -oryzanol to that of cholesterol suggested that the similarity might be responsible for the hypocholesterolemic effects of RBO. These components might compete with the binding sites of cholesterol and inhibit cholesterol absorption in the intestine, causing greater amounts of cholesterol to be excreted and metabolized to bile acids in the feces (Vissers et al., 2000; Sugano and Tsuji, 1997; Rogers et al., 1993; Rukmini and Raghuram, 1991).

Cholesterol is synthesized in virtually all cells in the body from acetyl-CoA. The capacity is greatest in the liver and the intestinal wall. These tissues supply over 90% of the endogenous plasma cholesterol, and the endogenous synthesis accounts for more than two thirds of the total cholesterol store. Cholesterol is delivered to the liver in the form of chylomicron remnants, as well as in the form of LDL-C and HDL-C. The disposal of cholesterol comes from reabsorbed cholesterol from the gut in the enterohepatic circulation and HDL. The liver disposes of cholesterol by excretion in bile as free cholesterol and after conversion to bile salts (Devlin, 1997). It is estimated that neutral sterol, most of which is cholesterol, represents about 55%, and bile acids 45% of total steroid excretion in the feces (Groff and Gropper, 1995). When the amount of dietary cholesterol is reduced, *de novo* biosynthesis of cholesterol in the liver and the intestine is increased to satisfy the needs of other tissues and organs, as well as to replace bile salts and cholesterol lost from the enterohepatic circulation in the feces (Devlin, 1997).

The bile acids are the end products of cholesterol metabolism. Chemical analyses have identified two primary bile acids, cholic acid and chenodeoxycholic acid, which are

synthesized in the liver and derived from cholesterol (Uchida et al., 1984). The primary bile acids are composed of 24 carbon atoms, contain two or three OH groups, and have a side chain that ends in a carboxyl group (Devlin, 1997). The primary bile acids would be conjugated with taurine or glycine in the liver to form conjugated bile acids, which are excreted into the intestine where they are partially deconjugated and transformed into a series of secondary bile acids by bacterial fermentation (Grundy et al., 1965). Other minor amounts of bile acids include 17 secondary bile acids, such as deoxycholic acid and 3 α , 12 α -dihydroxy-7-oxo-5- β -cholanic acid, etc., which are also synthesized from the actions of the gut flora on primary bile acids (Hawkins et al., 2002). Since bile acids can return to the liver during enterohepatic circulation, prevention of their returning to the liver would stimulate the conversion of cholesterol to bile acids and therefore its excretion. This might be the main hypothetical mechanism for the hypocholesterolemic effect (Rong et al., 1997; Sugano and Tsuji, 1997; Kahlon et al., 1996; Rukmini and Raghuram, 1991) of oryzanol in RBO. However, the complex structures of bile acids make it very difficult to determine and identify each component (Grundy et al., 1965).

This study investigated cholesterol-lowering effects of RBO by analysis of fecal cholesterol and bile acids, using a gas chromatography with flame ionization detector (GC-FID). However, some studies have suggested that various plant sterols might have more hypocholesterolemic effects than γ -oryzanol (Trautwein et al., 2002; Vissers et al., 2000; Weststrate and Meijer, 1998). The control diet in this study contained corn oil, which has higher levels of linoleic acid than RBO. One of the objectives in this study was to investigate if γ -oryzanol is responsible, at least partially, for the cholesterol-lowering effects of RBO. The other objective was to compare bioactivity of three different forms

of γ -oryzanol associated with its effects on fecal steroid excretion, compared with γ -oryzanol recovery in the feces. It was hypothesized that RBO γ -oryzanol would have greater bioactivity than γ -oryzanol dissolved in corn oil, followed by crystalline γ -oryzanol.

This study was a continuation of the experiment of Gillespie (2003). The result showed that bioavailability of crystalline γ -oryzanol was lower relative to crystalline γ -oryzanol dissolved in corn oil.

Since the lipid profile of rice bran oil and peanut oil are similar (Rukmini and Raghuram, 1991), it might have been more appropriate to use peanut oil than corn oil, in order to minimize the differences of the component profile between oils in the control and the treatment groups. Also, a cholesterol-enriched diet would have been more likely to manifest potential hypocholesterolemic effects of rice bran oil, as γ -oryzanol might affect cholesterol metabolism by altering dietary cholesterol absorption (Rong et al., 1997).

CHAPTER 2

LITERATURE REVIEW

2.1 NUTRITIONAL PROFILE OF RBO

Rice bran oil is concentrated in the germ and bran layers in rough rice grain. Crude rice bran oil contains ~96% of saponifiable fractions and ~ 4% unsaponifiable fractions, which include phytosterols, sterolesters, triterpene alcohols, hydrocarbons, and tocopherols (Table 1). Oryzanol content is about 2% in crude rice bran oil. These unsaponifiable fractions were reported to be bioactive and to have positive nutritional and health benefits (Orthoefer, 1996).

The hypolipidemic effect of RBO was investigated in animal and human studies. Generally, these studies showed a positive cholesterol-lowering effect of RBO, although the results may vary due to factors such as subject models, endogenous cholesterol level in the body, concentration of active components in the diets, and duration of the diet treatment. These studies appeared to indicate the bioactive components of RBO act synergistically to induce hypocholesterolemic effects (Visser et al., 2000; Sugano and Tsuji, 1997; Kahlon et al., 1992; Rukmini and Raghuram, 1991).

Some researchers reported that supplements of rice bran were effective in lowering total cholesterol and LDL levels in human subjects with moderate hypercholesterolemia (Hundemer et al., 1991). However, serum cholesterol was found to be lowered when patients with mild hypercholesterolemia consumed 300 g/d of unpolished rice, or 100 g/d of stabilized rice bran, while consuming either 30g/d of rice bran for 6 weeks or 60 g/d of rice bran for 4 weeks were shown to be ineffective (Hakala et al., 2002).

Table 1. Composition of Crude Rice Bran Oil

Component	%
Saponifiable lipids	90-96
Neutral Lipids	88-89
Triglycerides	83-86
Diglycerides	3-4
Monoglycerides	6-7
Free fatty acids	2-4
Waxes	3-4
Glycolipids	6-7
Phospholipids	4-5
Unsaponifiable lipids	4.2
Phytosterols	43
Sterolesters	10
Triterpene alcohols	28
Hydrocarbons	18
Tocopherols	1

2.2 RICE BRAN OIL AND ITS EFFECTIVE COMPONENTS

2.2.1 Fatty Acids

RBO has a nonspecific fatty acid profile among the vegetable oils. The amount of linoleic acid is rather moderate, and RBO also contains a relatively high proportion of oleic acid. The fatty acid composition of RBO is similar to that of peanut oil, thus many studies used peanut oil as a control (Table 2). One study with rats fed RBO diet demonstrated a significant reduction in total serum cholesterol, LDL-cholesterol, and an increase in fecal steroid excretion compared with the same concentration of peanut oil diet (Rukmini and Raghuram, 1991). Another study by Seetharamaiah and Chandrasekhara (1988) found that RBO lowered serum cholesterol levels in rats more than those fed with peanut oil, when cholesterol and cholic acid were incorporated into the experimental diets. These results suggested that some minor components in RBO such as the unsaponifiable fraction may play a role in the hypocholesterolemic action (Rukmini and Raghuram, 1991). When a small portion of unsaponifiables from RBO was added to peanut oil diets of the rats, the cholesterol-lowering action was magnified (Ikeda et al., 1985).

Edwards and Radcliffe (1994) compared the effects on serum lipids in rats with RBO and corn oil (Table 3), which has higher linoleic acid than RBO. Their results suggested that these two vegetable oils had a similar effect on serum total cholesterol level, at least at the concentration of the oils they used; however, the effects on serum cholesterol level may vary when incorporated into atherogenic diets fed to rats for a period longer than 5 weeks. In Wilson et al. (2000)'s study, they fed RBO and corn oils as 20% of energy, respectively, to monkeys for four weeks. RBO produced similar

Table 2. Fatty Acid Composition of Rice bran Oil and Peanut Oil

Fatty acid (%)	Rice bran oil	Peanut oil
C14:0	0.6	-
C16:0	21.5	14.4
C18:0	2.9	3.1
C18:1	38.4	42.6
C18:2	34.4	35.9
C18:3	2.2	-
C20:0	-	2.7
C22:0	-	1

Table 3. Fatty Acid Composition of Rice bran Oil and Corn Oil

Fatty acid (%)	Rice bran oil	Corn oil
C16:0	12.7	9.65
C18:0	2.0	2.14
C18:1	43.8	28.1
C18:2	39.3	58.9
C18:3	1.1	0.79
C20:0	0.59	0.37
C20:1	0.52	-

reductions in serum total cholesterol and LDL-C compared with corn oil, which has higher polyunsaturated fats and lower saturated fats. These researchers concluded that unsaponifiable fractions in RBO could compensate for its high saturated fats and thus UF played a predominant role in decreasing cholesterol levels without reducing serum HDL-C compared with other vegetable oils. However, Nicolosi et al. (1991) found that RBO and corn oil were equally effective in reducing serum cholesterol level in monkeys.

2.2.2 Unsaponifiable Fractions (UF)

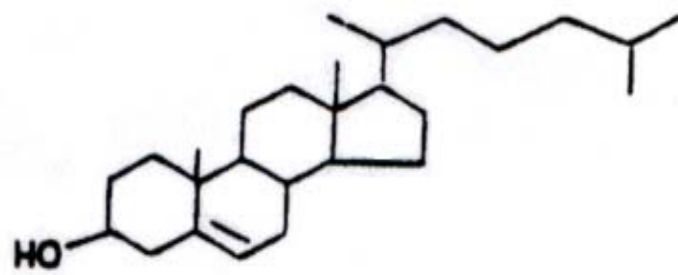
Rice bran oil (RBO) has been observed and confirmed to have hypocholesterolemic effect in a variety of human and animal studies. It has a propensity for improving serum cholesterol levels and lipoprotein profiles compared with more commonly used vegetable oils. This may be due to its relatively high contents of unsaponifiable fractions (UF) than most common vegetable oils. Refined RBO may contain 1.5-2.6% of UF and crude RBO may contain up to 5% of UF, while most refined vegetable oils contain 0.3-0.9% of UF (Rong et al., 1997). UF is composed of various forms of plant sterols, triterpene alcohols, 4-methyl sterols, oryzanol, tocopherols and tocotrienols, and some less polar components. In a study by Nagao et al. (2001), the hypocholesterolemic effect of RBO was compared with a pure extract of UF and found to be similar in both. Thus, studies have suggested that UF in RBO may play a very important role in cholesterol-lowering activity (Nagao et al., 2001; Rong et al., 1997). As mentioned earlier, the lipid profiles are similar in RBO and peanut oil. A study suggested that unsaponifiable fractions might play a role in the cholesterol-lowering effects in RBO compared to the peanut oil control diet (Seetharamaiah and Chandrasekhara, 1988).

In Kahlon et al. (1996)'s study, they reported that unsaponifiables from RBO added to cellulose control diet tended to lower total plasma cholesterol levels, but the effect was not significant compared with the control diet. They suggested that intact rice bran might have synergistic cholesterol-lowering effects as other components act together with the unsaponifiable fractions (UF). They also reported that liver cholesterol decreased up to 3-fold with rice bran diets compared with the cellulose diets with equivalent levels of UF. However, rice bran diet with added UF had significantly higher fat excretion and significantly greater plasma and liver cholesterol reduction than other rice bran (without adding UF) diets, which may indicate UF plays a main role in lowering cholesterol levels. In addition, significant excretion of neutral sterol in feces was observed with intake of neutral sterol. Furthermore, fecal excretion of fats and neutral sterol were significantly negatively correlated with liver cholesterol, which suggested the possible mechanisms of hypocholesterolemic effects by UF through diminished absorption-reabsorption of cholesterol and lipid from the digestive tract and interference with cholesterol metabolism by sterols from UF in RBO.

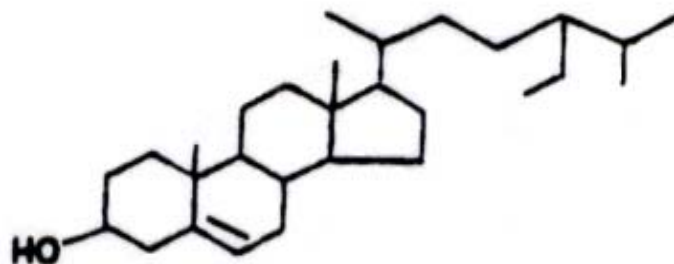
Nagao et al. (2001) concluded from their study that UF in RBO leads to a decreased serum cholesterol concentration by interrupting the absorption of intestinal cholesterol rather than by modifying VLDL-C, LDL-C and HDL-C in the liver, as an elevation of cholesterol excretion into feces was reported. However, a dietary effect on the fecal bile acid excretion was not found, which may be because bile salts were not added to the diet in their study.

2.2.2.1 Plant Sterols (Phytosterol)

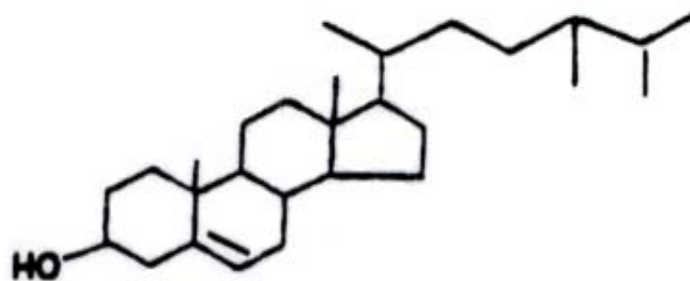
Campesterol, β -sitosterol and stigmasterol are the most abundant plant sterols found in edible vegetable oils (Figure1). Among these plant sterols, β -sitosterol has been recognized as the predominant component in the cholesterol-lowering action (Trautwein et al., 2002). It is generally assumed that plant sterols inhibited intestinal absorption of dietary and biliary cholesterol, because of the similarity between the structures of plant sterol and that of cholesterol. Some studies indicated that plant sterols contributed more hypocholesterolemic effects than unsaponifiable fractions. In addition, some plant sterols may be more active than others (Wilson et al., 2000). Large doses of plant sterols may inhibit cholesterol absorption in humans by lowering serum cholesterol level (Vissers et al., 2000). In the study of Vissers et al. (2000), they compared the cholesterol-lowering actions of plant sterols from RBO with triterpene alcohols from peanut oil in normolipidemic volunteers. The concentrates were added to margarine that had the same fatty acid composition, and the subjects consumed the sunflower oil as a control, peanut oil, and RBO margarines for 3 weeks each. Their results showed that RBO margarine decreased serum total cholesterol and LDL-C compared with the control margarine, but it did not affect HDL-C level. Whereas the peanut oil margarine did not affect serum total, LDL-, or HDL-cholesterol compared with the control margarine. These researchers suggested that the cholesterol-lowering effects may be due to the 4-desmethylsterols especially β -sitosterol, but not 4,4'-dimethylsterols such as cycloartenol. Another study of Weststrate and Meijer (1999) found no effects of concentrated RBO sterols in healthy normocholesterolaemic and mildly hypercholesterolaemic human subjects,



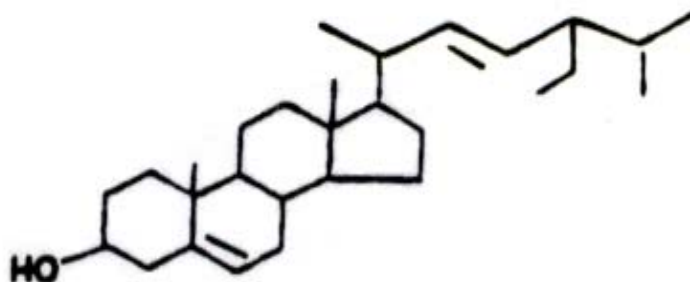
Cholesterol



Sitosterol



Campesterol



Stigmasterol

Figure 1. Chemical Structures of Cholesterol and Major Plant Sterols

which was in contrast to Vissers et al. (2000). Weststrate and Meijer (1999) found that sterol-esters mainly sitosterol, campesterol and stigmasterol from soybean oil was effective in lowering plasma total cholesterol and LDL-cholesterol levels without affecting HDL-cholesterol concentration. They suggested that the more similar structures of plant sterols to cholesterol may contribute more effective competition with cholesterol in the micelles.

However, other researchers fed rats a combination of the 4,4'-dimethylsterol cycloartenol and the 4-desmethylsterol β -sitosterol, and found that the effect on cholesterol levels was similar to β -sitosterol alone. β -sitosterol was less absorbed and thus more effective in inhibiting cholesterol absorption (Ikeda et al., 1985). The lack of the effect of 4,4'-dimethylsterols could be that they may not displace intestinal cholesterol from the micelles due to the less comparable molecular structure of cholesterol (Kerckhoffs et al., 2002).

On the other hand, Trautwein et al. (2002) suggested that the cholesterol-lowering effects of RBO might due to higher concentration of plant sterol (mostly in the form of β -sitosterol) rather than dimethylsterols (found primarily in the oryzanol fraction). They also suggested that esterified sterols and stanols were equivalent in lowering total plasma cholesterol and LDL cholesterol by inhibiting cholesterol absorption and stimulating fecal cholesterol excretion, but did not affect bile acid excretion as cholesterol 7 α -hydroxylase activity did not differ. They fed to hamsters with a fat- or cholesterol-enriched diet, combined with esterified 4-desmethylsterols from soybean oil, or esterified hydrogenated 4-desmethylsterols from the same source, or esterified 4,4'-dimethylsterols from oryzanol in RBO for 5 weeks. The results showed that both 4-desmethylsterols and

-stanols with 0.25% dose had equal effects and significantly ($P < 0.05$) lowered serum total cholesterol (TC) by 15%. However, the effect of 4,4'-dimethylsterols at the optimal dose, which was 0.24%, lowered serum TC by 10%. The possible explanation might be the chemical structure similarity of these sterols to cholesterol, since 4,4'-dimethylsterols such as cycloartenol have two additional methyl groups at the site adjacent to the hydroxyl group (Trautwein et al., 2002). The daily fecal excretion of neutral sterols was significantly higher in sterol- and stanol- supplemented diets, but not in those fed with 4,4'-dimethylsterols. Thus, Trautwein et al. (2002) suggested diminished intestinal cholesterol absorption may not be the primary mechanism of 4,4'-dimethylsterols in lowering serum TC in rats, but of sterols and stanols (Ikeda et al, 1985; Miettinen and Vanhanen, 1994; Vanhnen et al., 1994; Weststrate and Meijer, 1999). For fecal bile acid excretion, the hamsters fed 0.48% 4,4'-dimethylsterols had a significantly higher fecal output than the control diet group, but the results were not significant with either the sterol or the stanol group. This might indicate bile acid excretion as one of the primary mechanisms of cholesterol-lowering effects of 4,4'-dimethylsterols in RBO.

Another study of Trautwein et al. (1997) compared different dietary fats on cholesterol profiles in hamsters. They found that rapeseed oil affected cholesterol metabolism by reducing cholesterol absorption, due to higher fecal output of cholesterol and bile acids in hamsters. Because the fatty acid profile is similar between rapeseed oil and olive oil, while rapeseed oil has more palmitic acid and 2.5 fold higher plant sterols, they attributed the greater hypocholesterolemic effects of rapeseed oil to its plant sterol concentration and/or the impact of saturated fats in rapeseed oil.

2.2.2.2 γ -Oryzanol

The most characteristic component of RBO is γ -oryzanol, which is a mixture of ferulic acid esters of triterpene alcohols and sterols in rice bran oil (Figure 2). Cycloartenol ferulate, 24-methylene cycloartanyl ferulate and campesterol ferulate are the major components of γ -oryzanol (Xu and Godber, 1999). About 20% of unsaponifiable fraction in RBO is oryzanol (Rong et al., 1997). γ -Oryzanol may be used to inhibit tumor promotion, reduce serum cholesterol levels, and to treat nerve imbalance and disorders of menopause (Fang et al., 2003).

The study by Seetharamaiah and Chandrasekhara (1989) was done to determine if oryzanol alone in RBO had hypocholesterolemic activity. The refined RBO, which contained traces of oryzanol, was used as one of the treatment diets, 0.5% oryzanol added to the same refined RBO was the other treatment, and peanut oil as a control diet. Their results in a cholesterol-enriched diet in rats showed that serum TC decreased 38% on the RBO diet and was further lowered by 20% with additional oryzanol when compared with the control. They concluded that the cholesterol-lowering effect of RBO was attributable to oryzanol as well as to other components in the RBO.

A human study conducted by Yoshino et al. (1989) showed the long-term safety and significant hypolipidemic effects of oryzanol. The subjects with hyperlipidemia were treated with 300mg of γ -oryzanol for three months. The results showed that total triglycerides and LDL decreased significantly by the third month ($P < 0.05$). The plausible explanation might be the inhibition of cholesterol absorption in the intestine. Also HDL was significantly elevated at the third month in all of the subjects, although the mechanism was not clear. The researchers suggested that γ -oryzanol would be a safe and

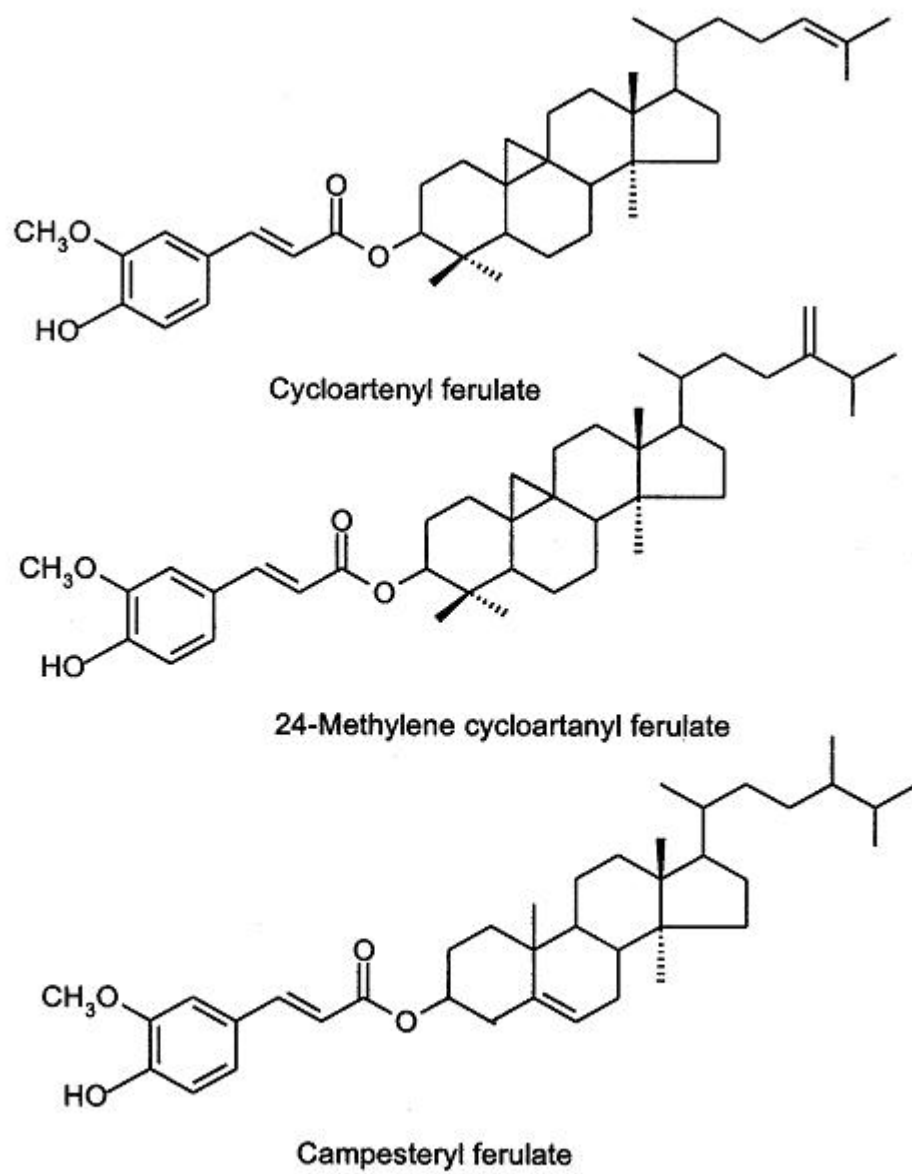


Figure 2. Three Major Components of γ -Oryzanol.

effective long-term agent to control plasma cholesterol levels in patients with mild hypercholesterolemia, although its effect proved relatively weaker than other hypolipidemic drugs.

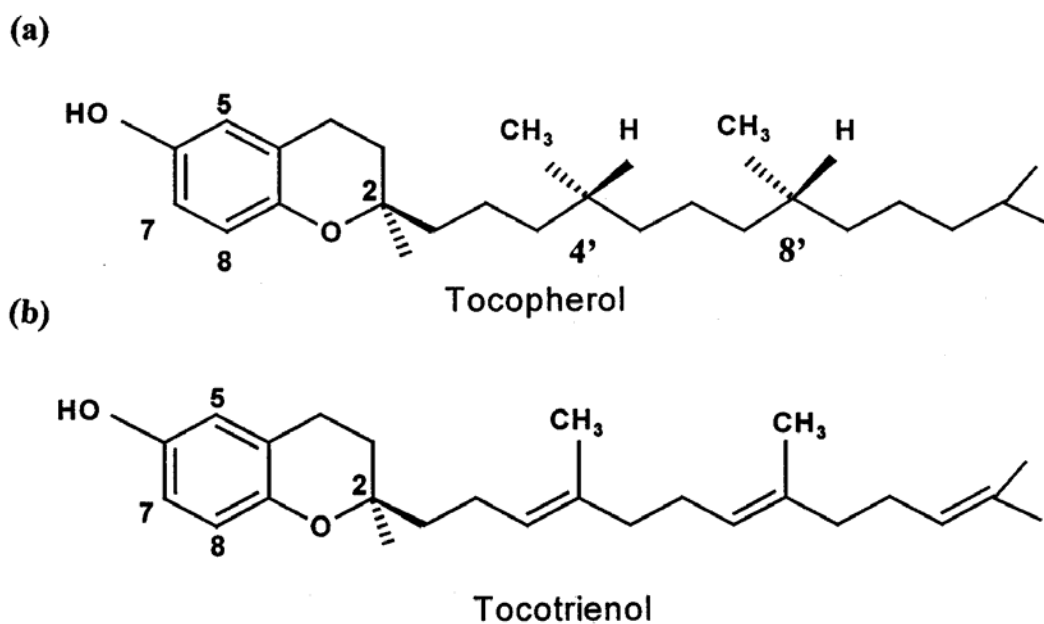
Rong et al. (1997) reported that oryzanol was an active ingredient responsible, at least partially, for the hypocholesterolemic activity of RBO. They found that non-HDL-C by 57% was reduced when adding 0.5% oryzanol to a diet containing coconut oil to hamsters for 10 weeks; although, increasing oryzanol to 1% and feeding hamsters with the diet for 7 weeks resulted in a 34% reduction in non-HDL-C. Further, aortic fatty streak formation was reduced 67% in both of the oryzanol-treated hamsters. However, both of the oryzanol treatments did not change *de novo* cholesterol synthesis as measured by HMG-CoA reductase activity. Also Rong et al. (1997) pointed out that the concentration of oryzanol in the first and second treatments were 8-fold and 4-fold higher, respectively, than the concentration in physically refined RBO. On the other hand, the dietary cholesterol levels may modify the influence of oryzanol, as no significant effects on plasma lipids or lipoproteins were observed. It may suggest that oryzanol affected cholesterol metabolism by altering dietary cholesterol absorption. The study by Sharma and Rukmini (1987) reported that an increased excretion of fecal total sterol and total bile acids occurred when oryzanol was added to the diet fed to the mice. However, they could not elucidate the mechanisms of its hypocholesterolemic effects. In Sugano and Tsuji (1997)'s study, when rats were fed either cholesterol-enriched or cholesterol-free diets, the rats fed with RBO or RBO with additional oryzanol led to a significant decrease in cholesterol levels.

The similar structures of oryzanol to cholesterol suggests that oryzanol might compete for the binding sites with cholesterol, leading to more cholesterol and bile salt excretions in the feces. Also cycloartenol is a precursor of phytosterols and it may be absorbed and stored in the liver so it might compete with the binding sites of cholesterol in the liver, causing greater amounts of cholesterol and bile acid excretion (Rukmini and Raghuram, 1991).

Another study reported by Kiribuchi et al. (1983) found that cycloartenol magnified the hypocholesterolemic effects of soy sterols in rats, probably through increasing fecal steroid excretion. Other researchers suggested that the structure of the sterols in γ -oryzanol might be less effective in lowering cholesterol absorption, as γ -oryzanol was less effective than the fatty acid esters of plant sterols and stanols in lowering total cholesterol levels in some human studies (Weststrate and Meijer, 1998; Hakala et al., 2002).

2.2.2.3 Tocopherols and Tocotrienols

RBO is also a rich source of tocotrienols, and the content and biological activities are higher than those of tocopherols (Sugano and Tsuji, 1997; Qureshi et al., 2001). Tocotrienols differ from tocopherols in having three double bonds in the isoprene side chain. The subtle structural differences among tocotrienols are relative to the number and location of methyl groups on the chroman rings (Figure 3). The structural differences may influence their biological activities. δ -Tocotrienol is the most potent cholesterol inhibitor among the known tocotrienols, followed by γ -tocotrienol and α -tocotrienol (Qureshi et al., 1996a). Also, tocotrienols differ substantially in their capacity to suppress tumor cell proliferation (Quershi et al., 2000). Their multitherapeutic properties,



Position of methyl group	Tocopherols	Tocotrienols
5,7,8-Trimethyl	α -T	α -T3
5,8-Dimethyl	β -T	β -T3
7,8-Dimethyl	γ -T	γ -T3
8-Monomethyl	δ -T	δ -T3

Figure 3. Molecular Formulas of Tocopherols and Tocotrienols.

such as hypocholesterolemic, antioxidant, antithrombotic, anticancer and anti-inflammatory have been reported in various animal and human studies (Quershí et al., 2001). Qureshi et al. (2000) isolated and identified two novel tocotrienols, d-P₂₁-T3 (desmethyl tocotrienol) and d-P₂₅-T3 (didesmethyl tocotrienol) from stabilized rice bran. Male chickens (6 week old) were fed a tocols-containing diet (except α -tocopherol and β -tocotrienol) for 24 days, and the results showed that the serum total and LDL-C were significantly ($P < 0.05$) decreased by 13-31% and 17-78%, respectively, when compared to the control group. Among the tocotrienol-containing diets, the total and LDL-C levels were lowered in the following order: d-P₂₅-T3 > d-P₂₁-T3 > δ -T3 > γ -T3 > α -T3. Similar effects were also observed in which these tocotrienols inhibited HMG-CoA reductase activity. The mechanism was considered to be by suppression of HMG-CoA reductase activity through a post-transcriptional mechanism (Parker et al., 1993). Among the known tocols, d-P₂₅-T3 exhibited the greatest cholesterol lowering activity in chickens, which may be due to its maximum inhibition of HMG-CoA reductase activity.

Another study by Qureshi et al. (2001), looked at the effects of tocols, including two novel tocotrienols, d-P₂₅-T3 and d-P₂₁-T3, from rice bran on inhibition of atherosclerotic lesions in C57BL/6 ApoE-deficient mice. Their results showed that the mice fed for 14 weeks with a low fat diet supplemented with TRF₂₅ (a mixture of tocols with high ratio of tocotrienol to tocopherol purified from rice bran) or d-P₂₅-T3 decreased atherosclerotic lesion size by 42% and 47% ($P < 0.01$), respectively, whereas the α -tocopherol supplemented diet had only 11% ($P = 0.62$) reduction. It was suggested that tocotrienols had greater antioxidant activity to suppress cholesterol oxidation and melanoma cell proliferation than those of tocopherols.

2.3 POSSIBLE MECHANISMS OF CHOLESTEROL-LOWERING EFFECTS OF RBO

The cholesterol-lowering effects of RBO are probably attributable to its relatively high UF, which play a more important role than its fatty acids. Three major groups of UF are physiologically bioactive in improving cholesterol levels in animals and human subjects. These include plant sterol/stanol, γ -oryzanol and tocopherols (tocopherol and tocotrienol). These compounds work synergistically to exhibit hypocholesterolemic effects. The possible mechanisms for each group of compounds are summarized as follows.

Plant sterol-stanol: Several mechanisms of plant sterols effect on cholesterol concentration in the body may include: 1) formation of a nonabsorbable complex with cholesterol; 2) altering the size and/or stability of the micelles; 3) interferences with cholesterol esterification in the mucosal cell; 4) interacting with protein receptors which are required in cholesterol absorption (Rong et al., 1997).

γ -Oryzanol: In a review (Rukmini and Raghuram, 1991) of biochemical aspects of the hypolipidemic action of RBO, the hypothesized mechanisms by which oryzanol lowers cholesterol levels may include: 1) Oryzanol inhibits cholesterol absorption by interfering with its absorption, at least by the mode of luminal action, as well as, or instead of intracellular events that reflect long-term effects of a feeding regiment. 2) Oryzanol exercises its effects on cholesterol metabolism at sites other than the intestine. Studies suggested its hypocholesterolemic effects by reducing non-HDL lipid profiles but no such effect on HDL-C.

Tocopherols: Higher contents of tocotrienol than tocopherol in rice bran (Qureshi et al., 2001; Qureshi et al., 2000; Rukmini and Raghuram, 1991) in which RBO has more

significant antioxidant properties. All the tocopherols share three features in an antioxidant system (Qureshi et al., 2000): 1) a hydrogen donor group, 2) an atom having at least one lone pair of electrons, and 3) a side chain comprising one or more isoprenoid-like units. Tocopherols exhibited significant antioxidant activity that inhibits cholesterol oxidation (Xu et al., 2001). The cholesterol-lowering activities by tocopherols include reducing serum TC and LDL-C by suppressing HMG-CoA reductase activity through a novel post-transcriptional mechanism (Qureshi et al., 2000). Tocotrienols may also reduce atherosclerotic lesion size while the mechanism was unknown (Qureshi et al., 2001).

2.3.1 Antioxidant Activity of Oryzanol and Tocotrienols in RBO

Cholesterol oxidation products have been suggested as a major cause of heart disease. Reduction of cholesterol and LDL-C oxidation may contribute to the potential cholesterol-lowering property of RBO (Xu et al., 2001). Similar to lipid oxidation, cholesterol oxidation is initiated by free radicals to produce hydroperoxide, peroxide, and other oxidized products. In the study of Xu et al. (2001), using a cholesterol-oxidation system, three major γ -oryzanol components, which are 24-methylene cycloartenyl ferulate, cycloartenyl ferulate and campesterol ferulate, showed significant antioxidant activity after 24 hours of oxidation, whereas oxidation products were not significantly lower in each treatment of tocopherols after 24 hours of oxidation. Also, the oxidation products in the treatment of γ -oryzanol were significantly lower than those of tocopherols. It was assumed that the greater antioxidant activity of γ -oryzanol than tocopherols might be due to the similarity between the structure of γ -oryzanol and that of cholesterol. Overall, the antioxidant activity of both γ -oryzanol and tocopherols contribute to the potential anti-atherogenic effects of RBO.

2.4 FECAL STEROID EXCRETION RELATED TO CHOLESTEROL LEVEL

The effect of RBO through fecal steroid excretion was considered as the mode of action of UF, specifically, the effect of plant sterols may be through fecal neutral sterol excretion. However, there have not been many investigations on cholesterol-lowering effects of RBO related to fecal steroid excretion (Figure 4), especially on human subjects. Kahlon et al. (1996) suggested that the cholesterol-lowering activity in RBO was probably through increasing excretion of fat and neutral sterol as they found that the hamsters fed UF diet or UF added to rice bran oil had significantly higher fat excretion. However, their results for fecal neutral sterol excretion did not negatively correlate with plasma cholesterol, while the fecal fat excretion did. Sharma and Rukmini (1986) reported increased fecal excretion of both neutral sterols and bile acids in rats when fed RBO compared with those fed peanut oil.

2.4.1 Studies of Functional Foods Related to Fecal Steroid Excretion and Cholesterol-lowering Effects

An increased excretion of fecal neutral sterol and bile acids increases the conversion of excess cholesterol to bile acids in the enterohepatic circulation. Preliminary studies showed that the effects of certain components in plant foods (such as pectin, oat bran, etc.) on lowering serum cholesterol levels could be due to secretion of bile acids and neutral sterol in the feces (Garcia-diez et al., 1996).

Neutral sterol includes cholesterol, coprostanol, cholestanol and coprostanone (Lin et al., 2004). In fecal neutral sterol (Figure 5), coprostanol is a metabolite of cholesterol, formed by the action of gut microflora through fermentation. Bile acids converted from cholesterol in the enterohepatic circulation exist as 24 carbon units and

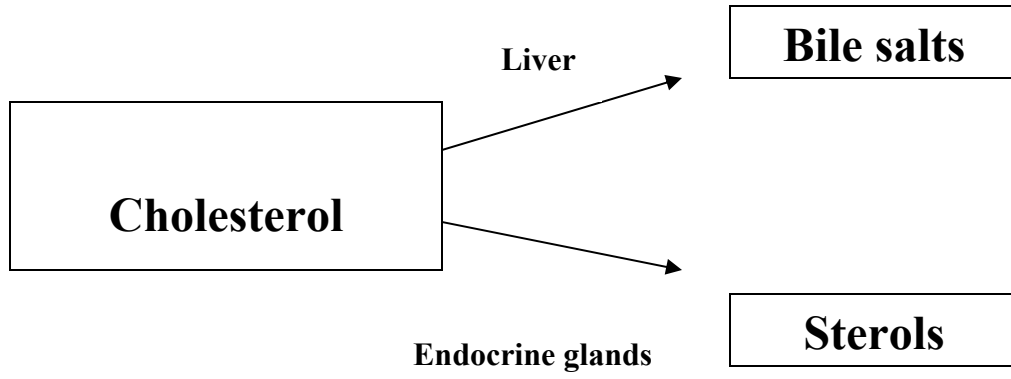
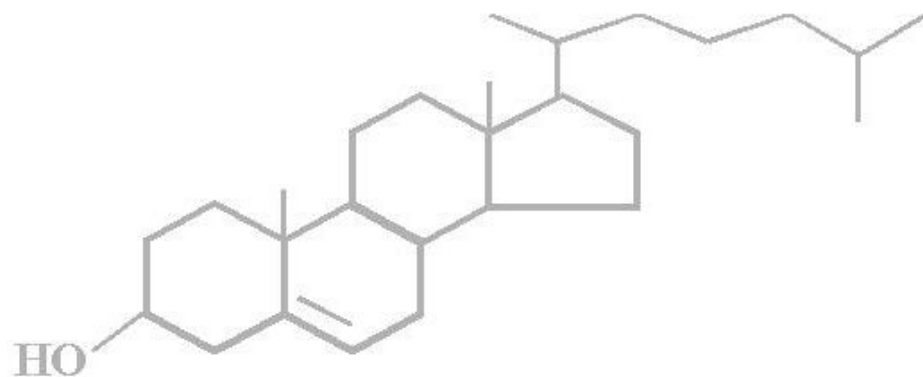
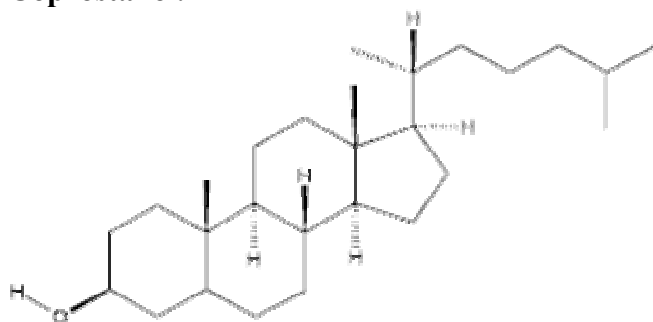


Figure 4. Brief Biochemical Pathway of Conversion of Cholesterol to Bile Salts and Sterols.

a) Cholesterol.



b) Coprostanol.



c) Cholestanol.

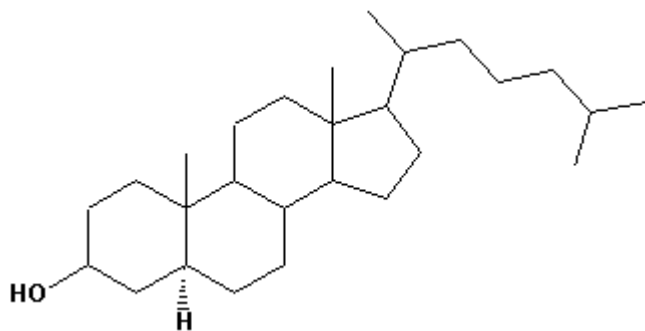


Figure 5. Chemical Structures of Major Neutral Sterols.

include cholic acid, chenodeoxycholic acid, and their bacterial metabolites, deoxycholic acid and lithocholic acid (Batta et al., 1984).

Differences in cholesterol absorption were confirmed by measurement of neutral sterol levels in feces, and then the decrease in absorption of cholesterol was accompanied by the increase in the synthesis of cholesterol by the liver. In the study of Hawkins et al. (2002), they reported that neutral sterol accounted for about 2% of fecal lipids.

Cholesterol may represent about 70% of the total fecal neutral sterols, followed by coprostanol, but cholestanone would account for just 1-2% of total neutral sterol excreted (Trautwein et al., 1997, 1999).

The synthesis of bile acids provides a direct means of converting cholesterol into bile acid (Figure 6) that facilitates the direct secretion of endogenous and exogenous cholesterol from the liver. The proportion of primary and secondary bile acids may depend on 7 α -hydroxylase activity and/or primary bile acid availability (Cheung, 1998).

The study of Hawkins (2002) suggested that cholic acid was an *in vivo* mediator of negative feedback regulation of bile acid biosynthesis by the recruitment of different co-regulators to a target gene following activation by a ligand. The study of Vahouny et al. (1987) was concerned with effects of fiber supplements on fecal bile acids and neutral sterols. They concluded that consistent results were observed in all of the insoluble fiber sources with increased fecal neutral sterols and more fecal cholic acids and a marked drop in hydrodeoxycholate levels. Their results suggested that primary bile acids represent a greater percentage of fecal bile acids, indicating reduced metabolism by colonic bacteria. However, their data on fecal excretions of sterol outputs did not appear to correlate directly with the observed effects of the fiber supplements on plasma

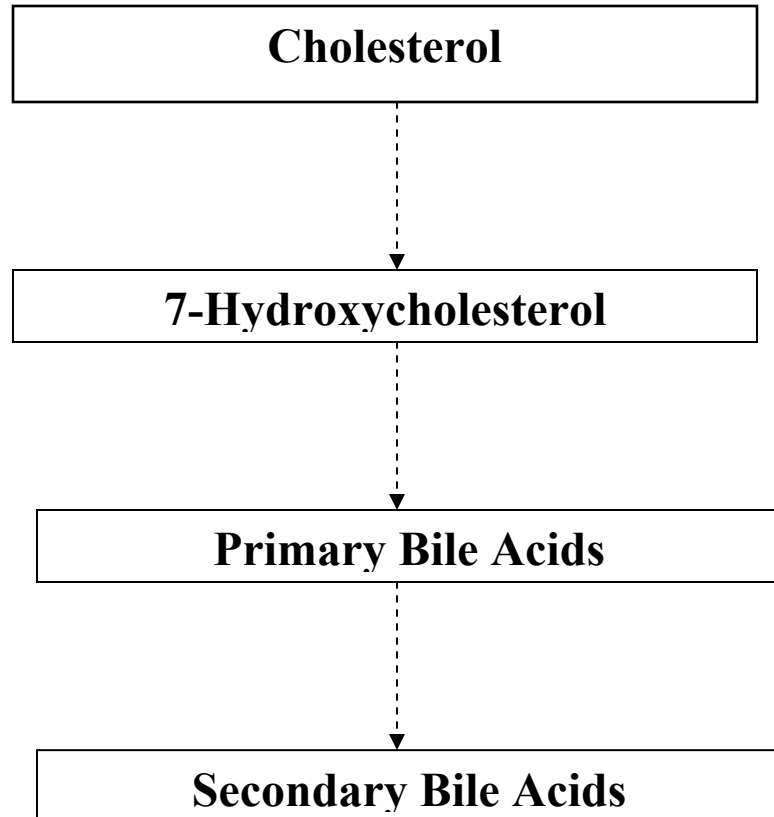


Figure 6. Brief Pathway of Synthesis of Primary and Secondary Bile Acids.

cholesterol levels in the rats. They concluded that the fiber-induced changes in fecal bile acid content, concentration or composition, might not be the sole mechanism involved in lowering of serum cholesterol levels.

In the study of Uchida (1984), they found that dietary cholesterol was preferentially converted to chenodeoxycholic acid, although the precise mechanism is not fully understood. It is widely accepted that the phytosterols form non-absorbable complexes with cholesterol, and bile acids play an essential role in cholesterol absorption. In addition, they also suggested that bile acids derived from chenodeoxycholic acid decreased more than that derived from cholic acid.

The study of Nakamura et al. (2000) on the effects of rutin on hyperlipidemic rats suggested that fecal bile acid excretion increased significantly only in the rats administered with a certain amount of rutin, while there were no significant changes in cholic acid and chenodeoxycholic acid or in the ratio of primary bile acids. Another study on fecal steroid excretion in rat by oral administration of gymnemic acids found similar results as cholesterol-lowering effects were dose dependent and they found that fecal excretion of cholesterol and cholic acid derived bile acids had a positive correlation with gymnemic acids (Nakamura et al., 1999). However, some studies showed that deoxycholic acid was the most abundant bile acid excreted in the stool of the mice (Schwarz et al., 1996).

2.5 LIPID METABOLISM IN OVARIECTOMIZED RATS

Ovariectomized (OVX) rats have been found to have higher cholesterol levels than normal rats due to the deficiency of estrogen (Junqueira et al., 2002). OVX increased body energy store and susceptibility of non-HDL-C to oxidation (El-Swety et al., 2002). The deficiency of estrogen appears to be responsible for the effects since estrogen replacement prevents the extra energy gain. Thus, the energy flux is accompanied by adaptation of lipid metabolism, such as increased hepatic lipid production and elevated circulation level of lipoproteins. However, OVX did not alter liver cholesterol content (Picard et al., 2000). Picard et al. (2000) tested the ability of EM-652.HCl, an estrogen antagonist to prevent obesity and abnormality of lipid metabolism. The study showed that EM-652.HCl could prevent most of the abnormalities of energy and lipid metabolism in the OVX rats treated with the EM-652.HCl. agent. Nogowski et al. (1998) studied the effect of genistein on lipid metabolism in OVX rats. They concluded genistein strongly inhibited lipogenesis in the liver and adipocytes of OVX rats, while cholesterol metabolism seemed to have unexpected results.

However, some bioactive components in vegetable oils such as RBO and corn oil have hypolipidemic effects and also RBO was used to treat disorders of menopause (Fang et al., 2003). Thus, it is of interest to determine the differences in fecal steroid excretion between OVX rats and sham operated rats, although they were treated with corn oil in the control groups.

CHAPTER 3

METHODOLOGY

3.1 ANIMAL MODELS

A total of 47 nine-month old Sprague Dawley retired female breeder rats were purchased from Harlan Sprangue Dawley, Inc. (Indianapolis, IN) and served as the animal models in this study. They were individually housed in suspended wire-mesh-bottomed cages in a controlled environment (temperature controlled, 12-hour light/dark cycle). The rats were blocked by weight and then randomly assigned to a diet group. Twenty rats were used as controls with half ovariectomized (OVX) two weeks prior to the experiment while the others underwent a sham surgery; the remaining 27 rats were ovariectomized (Colona, 2002) as well, and equally divided among three dietary treatments (Gillespie, 2003).

3.2 DIETS

The diets (Table 4) (Gillespie, 2003) were a modification of the formula based on the AIN-93 M maintenance diet, which may have a better balance of essential nutrients for adult rodents (Reeves et al., 1993). All of the animals were sustained for 11 weeks on a cholesterol-free diet that was either a control group diet or treatment diets with 2.8g/kg of oryzanol. Tocopherol stripped corn oil was used in the control group and two of the treatment groups, and RBO was used in the other treatment group. They were grouped as follows:

(C & SH) - Control diets (n=20): Corn oil;

(CO) - 2.8 g/kg of crystalline oryzanol (n=9): Corn oil;

(DO) – 2.8 g/kg of crystalline oryzanol dissolved in corn oil (n=9): Corn oil;

(RO) - 7% oryzanol rice bran oil (n=9): RBO.

Table 4. Diet Formula.

Ingredient (g/kg diet)	C & SH	CO	DO	RO
Casein	150	150	150	150
Sucrose	100	100	100	100
Cornstarch	455.7	455.7	455.7	455.7
Dextrinized cornstarch	155	155	155	155
Corn oil, stripped	40	40	40	0
Rice bran oil	0	0	0	40
Cellulose ^c	50	50	50	50
Mineral Mix AIN-93 M	35	35	35	35
Vitamin Mix AIN-93M	10	10	10	10
Choline bitartrate	15	15	15	15
L-cystine	1.8	1.8	1.8	1.8
Oryzanol	0	2.8	2.8 ^b	2.8 ^a

- a. 2.8 g of Oryzanol was originally contained in the high- oryzanol rice bran oil.
- b. Oryzanol added and dissolved in the tocopherols stripped corn oil.
- c. 50g cellulose contained 5% of fiber.

The 2.8g/kg of oryzanol supplementation was to allow for an equal amount of oryzanol in all the treatment diets, based on the 7% oryzanol concentration in the high oryzanol rice bran oil. The RBO used in the study was donated by Riceland, Inc. (Stuttgart, AR). Corn oil stripped of tocopherols was purchased from Dyets, Inc. (Bethlehem, PA), and crystalline oryzanol was purchased from Samlong Chemical Co., LTD. (Changxin, Jiangsu, China). For the DO group, crystalline oryzanol was completely dissolved in the corn oil portion before adding the solution to the diet mix. Whereas for

CO group, the crystalline oryzanol was added directly to the diet mix. Diets were stored in airtight containers at -20°C.

3.3 COLLECTION AND PREPARATION OF FECES

Foods were replenished three times each week. At weeks 6 and 10, referred to as period A and B, respectively, each animal was placed in an individual metabolism cage for 24 hours according to a random schedule to collect feces. Fecal samples were collected in plastic containers and frozen until analyzed.

Frozen fecal specimens were allowed to thaw at room temperature. Feces from each animal was transferred to individual glass jars and dried in a VWR 1410 vacuum oven (West Chester, PA) at 60°C under -15 in. Hg pressure. After dehydration, each fecal sample was ground and kept in a capped glass container in a Dry-Keeper desiccator cabinet by Bel-Art Products (Pequannock, NJ) for storage. Steroid extracts from a 0.25 g portion of each fecal sample were analyzed using Gas Chromatography Flame Ionization Detector (GC-FID) as detailed in a later section.

3.4 EXTRACTION OF FECAL STEROIDS

The extraction procedures were patterned after Cheung (1998), Vahouny et al. (1987) and Evrard and Janssen (1968) with some modifications. An aliquot of 0.25g of each fecal sample was weighed into a clean 15-mL test tube, then 0.2mL of 50ppm stock solution of α -cholestane (ICN Biomedicals Inc. Aurora, OH) dissolved in hexane was added as an internal standard for cholesterol, along with 0.1mL of 100ppm stock solution of β -cholanic acid (ICN Biomedicals Inc. Aurora, OH) in hexane as an internal standard for bile acids. After vigorous vortexing for 30 seconds, the mixture was transferred to a 50-mL 24/40 stoppered test tube, which connected to a circulating condenser system.

After acid-digesting with 4mL of acetic acid at 110°C for 60 minutes and cooling, 5mL of toluene (Mallinckrodt, Paris, KN) and 1mL of distilled water were added to the sample. As toluene and acetic acid are miscible, 1 mL of distilled water was added to separate the organic solvent extract. The liquid mixture then was transferred to a plastic 40-mL centrifuge tube, capped and vortexed using a Type 37600 Mixer (Thermolyne Corporation, Dubuque, IW). After centrifuging at 1,500xg for 4 minutes at 22°C in a Hermle Z383K centrifuge (Labnet International, In. Woodbride, NJ), the supernatant of acetic acid-toluene extract was transferred using a Pasteur pipette to a clean 40-mL test tube. This procedure was repeated three times. The toluene-lipid extract was then completely evaporated using a CentriVap Console vacuum (Labconco, Kansas City, MO) at 98°C for 60 minutes, without coldtrap. The lipids were then subjected to enzymatic hydrolysis to the bile acid conjugates with 12 units of stock solution choloylglycine hydrolase (Sigma Chemical, St. Louis, MO) by adjusting the mixture to the optimized pH at 5.6, using a 410 A pH Meter (Orion Research Inc. Boston, MA). Two mL of 0.2N pH5.6 sodium acetate buffer solution (Appendix A) and 1 mL of 1.85% EDTA (Sigma Chemical, St. Louis, MO) solution were added to the dried lipids. After vortexing and measuring the pH, 1 mL of choloylglycine hydrolase solution was added and the pH was measured again. The mixture was adjusted to pH5.6 by adding ~3-4 mL of the sodium acetate buffer and then incubated in a water bath at 37°C for 5 minutes.

3.4.1. Fecal Neutral Sterol Extraction and Analysis

The deconjugated lipids were saponified with alcoholic potassium hydroxide (AOCS, 1988) by adding a drop of 80% KOH (w/v) with a Pasteur pipette, and then

adding 37.5 μ L of absolute ethanol USP. The mixture then was vortexed for 30 seconds and incubated in a water bath at 45°C for 30 minutes.

After cooling, cholesterol (including oryzanol) was extracted with 5mL of Petroleum ether (Fisher, Fairlawn, NJ) and the extraction was repeated three times. After the mixture was transferred to a 40mL centrifuge tube, capped, vortexed, and centrifuged in the Hermle Z383K centrifuge, using the same condition as mentioned earlier. After that, the organic solvent was allowed to separate into two layers. The petroleum supernatant was transferred with a Pasteur pipette to a clean 15-mL test tube and then all extracts were combined and dried completely using the CentriVap Console vacuum at 65°C for 55 minutes.

The neutral sterol, after evaporation, was light-yellow color. After cooling, 0.2mL of hexane was added and vigorously vortexed for one minute. The liquid was carefully transferred to a 2mL vial (Supelco, Rena, Nevada) for GC and capped. One μ L of the hexane-sterol extract was injected with a syringe (Varian, Walnut, Creek, CA) into a CP-3800 Gas Chromatography Flame Ionization Detector with a SACTM-5 Capillary Column (Supelco). This type of capillary column does not require TMS derivatization and is able to separate sterols (SupelcoTM Column Test Report). The operating condition used was as follows: an injection port temperature of 300°C with 100:1 split ratio; the column oven temperature was 270°C that was held for 18 minutes; the detector temperature was 200°C. Helium was used as a carrier gas at a flow rate of 2 mL/min.

3.4.2. Fecal Bile Acid Extraction and Analysis

The residue in the 40mL centrifuge tube after extraction of sterols contained most

of the bile acids. The residue was acidified with concentrated HCl by adding ~ 9 drops with a Pasteur pipette then was adjusted to pH 1 using the pH measuring tip. After vigorous vortexing, the color of the content was dark yellow. Anhydrous diethyl ether (Fisher, Fairland Malinckrodt, Paris, KN) was used as the solvent to extract total bile acids. Again, the mixture was capped and vigorously vortexed before centrifugation in the Hermle Z383K centrifuge. The operating condition of the centrifuge was as described previously. Extraction was repeated three times and the supernatant of the diethyl ether-bile acid extracts were combined and evaporated using the CentriVap Console vacuum at 60°C for 45 minutes. After cooling, the bile acid extract was methylated (Appendix B) with 2mL of BCl₃-methanol (Supelco, Bellefonte, PA) and 1mL of 2, 2-dimethoxypropane (Sigma, St. Louis, MO). The mixture was vortexed for 2 minutes and incubated in a 65°C water bath for 10 minutes. Two mL of hexane and 1mL of distilled water was added after cooling. The mixture was vigorously vortexed and centrifuged for 2 minutes using a Physicians Compact Centrifuge (Clay Adams, Parsippany, NJ) to allow the two layers to separate upon standing. The upper hexane layer was very carefully transferred with a Pasteur pipette to a clean 15-mL test tube containing anhydrous Na₂SO₄ to absorb the excess water from the organic layer. After vortexing and centrifugation with the Physicians Compact Centrifuge, the clear hexane-bile acid extract was transferred to a 2-mL GC vial (5896A, Hewlett Packard), capped and put into the autosampler. The operating conditions were: an injection port temperature of 250°C; initial oven temperature was 42°C and maintained for 2 minutes, then increased at 4.0 degrees per minute to 222°C and maintained for 6 minutes. The column was a SupelcoTM2380 Capillary Column (Supelco). Helium was used as a carrier gas at a flow rate of 2 mL/min.

3.5 OPTIMIZATION OF ANALYTICAL CONDITIONS

To determine the major components for neutral sterol and bile acids, we used some standards to do the trials before developing the method for analysis. For cholesterol and oryzanol, 100ppm stock solution of α -cholestane dissolved in hexane and 100ppm stock solution of cholesterol standard (ICN Biomedicals Inc. Aurora, OH) were first analyzed to determine the retention time. One μ L of 100ppm α -cholestane, or 100ppm cholesterol standard, or mixture of both, was injected into the CP-3800 GC-FID with the SACTM-5 Capillary Column (Supelco). The operating conditions were set as described in section 3.4.1. The peaks of α -cholestane and cholesterol appeared at the retention time of \sim 5.77 min. and \sim 9.45 min., respectively.

For bile acids, 100ppm stock solution with cholic acid standard (ICN Biomedicals Inc. Aurora, OH), or chenodeoxycholic acid standard (ICN Biomedicals Inc. Aurora, OH), or deoxycholic acid standard (ICN Biomedicals Inc. Aurora, OH), or total bile acid standard (ICN Biomedicals Inc. Aurora, OH) were performed using the same procedure as that described for bile acid extraction. The peaks of β -cholanic acid appeared at 2.83 min., cholic acid at 6.7 min., chenodeoxycholic acid at 11.53 min., deoxycholic acid at 34.0 min., and lithocholic at 37.0 min. Primary bile acids represented the total of cholic acid and chenodeoxycholic acid. Secondary bile acids represented the total of deoxycholic acid and lithocholic acid. Total bile acids represented the total of primary bile acids and secondary bile acids.

3.6 FECAL ORYZANOL RECOVERY

Since the chemical structure of oryzanol (esters of ferulic acid and sterols) is similar to that of cholesterol, oryzanol was extracted into sterols during cholesterol extraction. To determine this, we used purified crystalline oryzanol with the same extraction procedure as was used for cholesterol. Oryzanol was separated into three major compounds, which were most likely cycloartenol ferulate, 24-methylene cycloartanyl ferulate and campesterol ferulate, as they are the major components of γ -oryzanol (Xu and Godber, 1999). The peaks appeared in the chromatogram at 11.7 min., 14.3 min., and 17.0 min. The results of oryzanol were depicted as these three major components.

3.7 GEL FORMING

During fecal lipid or steroid extraction, the solution must be separated immediately upon centrifuging. If the solution does not separate immediately upon centrifuging, it forms a gel, which makes it difficult for extraction. Because of the large amount of soaps in the aqueous phase, gel formation occurred, in which case gentle warming or addition of 20% NaCl solution was used to solve the problem (Evrard and Janssen, 1968).

3.8 STATISTICAL ANALYSIS

Statistical Analysis System (SAS) version 9.0 (SAS Institute, Cary, NC, USA) was utilized for all statistical analysis. One-way analysis of variance (ANOVA) was used to compare the differences of data among the group means. Paired t-test was used to compare the differences of the two fecal collecting periods (A & B) in cholesterol, oryzanol and bile acid excretion within the diet group means. LSD test was used to

determine specific differences among the means. All data represent MEANS \pm SEM; $p \leq 0.05$ was considered significantly different.

CHAPTER 4

RESULTS AND DISCUSSION

4.1 COMPARISON OF DIFFERENCES BETWEEN TWO FECAL COLLECTION PERIODS

A paired t-test was used to compare feces collected in periods A and B of each animal for cholesterol excretion, fecal oryzanol excretion, cholic acid (CA), chenodeoxycholic acid (CDCA), primary bile acids (PBA), deoxycholic acid (DCA), lithocholic acid (LCA), secondary bile acids (SBA), and bile acids total (BAT). Total sample sizes were ninety-four with forty-seven for each period.

Results (Table 5) showed that there was no significant difference ($P>0.05$) in the data between period A and period B within each diet group; as such the following results were presented as the combined data from two periods. Feed intake in period B was significantly less in all diet groups when compared to that in period A, but weight gain was more efficient in period B (Gillespie 2003). Gillespie's (2003) results showed that significant differences of fecal oryzanol excretion existed in the individual data between period A and B in the CO group, but no statistically significant differences were found in individual data in the DO and RO groups between the two periods in her study. However, the methods of analysis used in the two studies were different, which may have affected the results.

4.2 CHOLESTEROL EXCRETION

RO group showed significantly greater cholesterol excretion than other diet groups (Table 6). Specific comparison of cholesterol excretion among the treatment groups also showed that RO group had significantly higher cholesterol excretion than the average of the other treatment groups ($P<0.001$).

Table 5. Comparison of Fecal Steroids between Collection Periods of A and B^{1, 2, 3}

Group	CHOL ⁴	ORY ⁵	CA ⁶	CDCA ⁷	PBA ⁸	DCA ⁹	LCA ¹⁰	SBA ¹¹	BAT ¹²
C (A)									
Mean	1.349	-	0.109	0.110	0.219	0.410	0.188	0.598	0.817
SEM	0.237	-	0.024	0.032	0.047	0.187	0.031	0.240	0.247
C (B)									
Mean	1.173	-	0.101	0.050	0.151	0.206	0.322	0.528	0.679
SEM	0.122	-	0.049	0.037	0.088	0.095	0.102	0.300	0.313
A vs. B	NS	NS	NS	NS	NS	NS	NS	NS	NS
SH (A)									
Mean	1.640	-	0.087	0.042	0.129	0.195	0.095	0.290	0.419
SEM	0.276	-	0.039	0.011	0.035	0.127	0.044	0.142	0.184
SH (B)									
Mean	1.447	-	0.045	0.032	0.077	0.107	0.087	0.194	0.271
SEM	0.233	-	0.012	0.009	0.033	0.005	0.007	0.013	0.031
A vs. B	NS	NS	NS	NS	NS	NS	NS	NS	NS
CO (A)									
Mean	1.565	3.958	5.117	3.196	9.513	12.688	7.724	20.412	29.925
SEM	0.218	0.337	0.952	0.741	1.985	4.159	2.132	5.880	3.196
CO (B)									
Mean	2.303	3.530	5.563	3.336	7.701	14.376	6.422	20.798	30.595
SEM	0.166	0.857	0.802	0.524	1.755	3.529	1.737	3.756	3.191
A vs. B	NS	NS	NS	NS	NS	NS	NS	NS	NS
DO (A)									
Mean	1.599	3.043	2.546	1.719	4.265	9.358	10.215	19.573	23.838
SEM	0.201	0.619	0.855	0.455	1.013	3.413	3.212	2.954	3.234
DO (B)									
Mean	1.631	3.077	1.488	1.471	2.949	7.712	9.165	16.877	19.826
SEM	0.287	0.543	0.211	0.575	0.274	2.070	1.049	4.450	5.564
A vs. B	NS	NS	NS	NS	NS	NS	NS	NS	NS
NO (A)									
Mean	2.459	7.251	8.247	7.572	15.819	15.291	12.819	28.110	43.929
SEM	0.471	0.305	2.405	4.420	4.033	2.750	1.480	4.587	7.767
NO (B)									
Mean	3.162	8.670	10.229	4.910	15.139	15.721	8.087	23.808	38.947
SEM	0.431	0.473	1.594	1.016	2.371	1.926	1.733	4.820	5.673
A vs. B	NS	NS	NS	NS	NS	NS	NS	NS	NS

¹Values are presented as peak area (PA) ratios to two internal standards accordingly.

²Letter A or B follows the diet group name in parentheses represents collection period A or B, respectively.

³NS implies a non-significant result (P>0.05).

⁴Cholesterol.

⁵Oryzanol.

⁶Cholic acid.

⁷Chenodeoxycholic acid.

⁸Primary bile acids.

⁹Chenodeoxycholic acid.

¹⁰Lithocholic acid.

¹¹Secondary bile acids.

¹²Bile acids (total).

Table 6. Effects of the Diets on Fecal Cholesterol Excretion^{1, 2, 3}

Diet Group	C (10)	SH (10)	CO (8)	DO (9)	RO (8)
Mean	1.261 ^C	1.544 ^{B,C}	1.934 ^B	1.615 ^{B,C}	2.811 ^A
SEM	0.101	0.145	0.164	0.222	0.118
RO vs. AVE (CO & DO)	P<0.001				

¹Values are presented as peak area (PA) ratio of cholesterol to α -cholestane (internal standard).

²Sample size used in each group follows the diet group name in parentheses.

³Values within a row with the same letter of superscript are not significantly different (P>0.05).

The effect may be caused by oryzanol or possibly other components of UF in RBO (Seetharamaiah and Chandrasekhara, 1989). In Seetharamaiah and Chandrasekhara (1989)'s study, additional oryzanol was added to RBO and the results showed that serum total cholesterol level was lowered. However, they concluded that the hypocholesterolemic effect of RBO was not due to oryzanol alone. Furthermore, the differences among CO, DO, and SH groups were not significant (P>0.05), and there were no statistical significant differences among DO, SH and C groups either.

The lack of a significant difference between the sham and the CO and DO diets may be due to hypocholesterolemic effects of the corn oil. Typical corn oil contains 1.18% β -sitosterol (Rukmini and Raghuram 1991), which has been recognized as the predominant component in the cholesterol lowering action (Trautwein et al., 2002), although the corn oil used in this study was stripped of tocopherol that may have also impacted phytosterol levels. Ikeda et al. (1985) reported that combining the 4,4'-dimethylsterol cycloartenol (one of the major components in oryzanol) and 4-desmethylsterol (β -sitosterol) in diets fed to rats showed that the effects on decreased cholesterol levels were similar to β -sitosterol by itself. If this was the case in this study, it

might explain why there was no significant difference in fecal cholesterol excretion among those corn oil diet groups.

Although it was hypothesized that oryzanol in oil form would be more bioactive and thus induce more fecal steroid excretion than the crystalline form, the CO group showed significantly higher cholesterol excretion than the C group. Thus, the results found in this study did not support this hypothesis. Interestingly, Vissers et al. (2000) suggested that the effect of sterols in rice bran oil on serum lipoprotein in humans might be due to plant sterols, but not oryzanol. If plant sterol did play a major role, it might also explain the similar effects on fecal cholesterol among the corn oil diet groups. However, CO and DO treatment groups contained extremely high concentration of oryzanol (2.8g in 40 g in corn oil), thus it was assumed that DO would show more significant cholesterol lowering effects as well. No published studies compared the crystalline form and dissolved oil form of oryzanol. Thus, this comparison requires more study to determine relative bioactivities of different forms of oryzanol.

4.3 FECAL ORYZANOL EXCRETION AMONG THE TREATMENT GROUPS

RO produced significantly higher oryzanol excretion ($P < 0.05$) than the other treatment groups (Table 7), followed by CO and DO groups. The oryzanol that is not recovered from the feces would indirectly reflect that which was absorbed plus that which was changed by the digestive process into a form that was not detectable. It is known that plant sterols and oryzanol are poorly absorbed in the small intestine (Ling et al., 1995; Fujiwara et al., 1983). Gillespie (2003) found that the CO group had significantly higher oryzanol excretion than RO and DO groups, and similar levels of oryzanol was excreted between RO and DO groups. Oryzanol absorption was not

measured in this study, so the difference in absorption among the treatment groups could not be determined directly, but it appears that in all treatment diets, a fairly substantial portion of the ingested oryzanol is changed into a form that is not detectable.

The fact that there was no difference between CO and DO groups indicated that the change in oryzanol during digestion was not significantly different between the dissolved oil form of oryzanol and the crystalline form.

Table 7. Fecal Oryzanol Excretion among Three Treatment Groups^{1,2,3}

Diet Group	CO (8)	DO (9)	RO (8)
Mean	3.744 ^B	2.914 ^B	7.956 ^A
SEM	0.439	0.484	0.777
RO vs. AVE (CO & DO)*	P=0.004		

¹Values are arbitrary and presented as peak area (PA) ratio of oryzanol to α -cholestane (internal standard).

²Sample size used in each group follows the diet group name in parentheses.

³Values within a row with the same letter of superscript are not significantly different (P>0.05).

* Significant effects of test treatment diets on different bile acid excretion. NS implies a non-significant result (P>0.05).

However, Gillespie (2003) found that the dissolved oil form of oryzanol had lower fecal recovery than the crystalline form. A possible explanation could be the analytical methods were very different in the two studies. The HPLC analysis of oryzanol used by Gillespie (2003) may more accurately measure total oryzanol than using GC-FID. Ten components of oryzanol have been identified using HPLC (Xu and Godber, 1999), but only three components were detected using the GC-FID method. Certain components might be more predominantly detected in HPLC. Also, differences in extraction and

sample preparation between the two studies may have contributed to the divergent results.

It is logical that bioavailability of oryzanol in RBO would be higher than the dissolved form of crystalline oryzanol in corn oil, since compatibility of oryzanol in RBO should be higher than in corn oil. However, this study showed a significantly higher fecal oryzanol excretion in the RO group than the other treatment groups. Perhaps, more intact forms of oryzanol were excreted in the feces in the RO group that were more readily detected by the GC-FID method, and thus it appeared that more oryzanol was excreted in the feces.

The metabolism of oryzanol has not yet been elucidated very clearly. Fujiwara et al. (1983) reported that 9.8% of the ¹⁴C-labeled γ -oryzanol was excreted as major urinary metabolites such as ferulic acid, dihydroferulic acid, m-hydroxyphenylpropionic acid, m-coumaric acid, m-hydroxyhippuric acid and hippuric acid. Of the administered γ -oryzanol, 10-20% was metabolized to yield ferulic acid in the small intestine and 84.5% was excreted in the feces, thus possibly as much as 64.5% of intact γ -oryzanol would be excreted in the feces; however, an extremely low percentage (0.3%) was absorbed in the lymphatic ducts. Fujiwara et al. (1983) concluded that oral intake of oryzanol was mainly absorbed in the blood via the portal vein. This might be the explanation for why more oryzanol was detected in the feces in CO group than in DO group, because crystalline oryzanol might be excreted in much more intact forms than the dissolved oil form.

If the data for cholesterol excretion is associated with oryzanol excretion, these findings indicate that fecal cholesterol excretion might not necessarily relate negatively to fecal oryzanol excretion, at least based on the results found in this study. This could be

explained through the relatively higher level of UF in RBO and their synergistic activities. Nagao et al. (2001) suggested that fecal cholesterol excretion was significantly higher in RBO and RBO simulated oil plus UF than RBO simulated oil, which indicated that UF played an important role in fecal cholesterol excretion. An appreciable amount of UF in RBO, including oryzanol and other plant sterols, may interfere with intestinal cholesterol absorption to a greater extent than oryzanol by itself. Another concern was if the cholesterol-free diet in this study would produce fecal steroid excretion by the effects of RBO or oryzanol. Sharma and Rukmini (1986) reported a significant neutral sterol excretion was found in rats fed a cholesterol-free diet compared to the control peanut oil diet. An extremely high concentration of oryzanol used in this study might magnify cholesterol-lowering effects of oryzanol in either RBO or corn oil.

4.4 FECAL BILE ACID EXCRETION

4.4.1 Primary Bile Acids

All of the treatment groups except DO showed a significantly higher CA excretion than the control groups ($P < 0.05$). The results of CA excretion (Table 8) suggested that oryzanol may play a more important role in bile acid excretion. Among the treatment groups the highest excretion was found in the RO group, where it was significantly higher than CO and DO groups; also, a significant difference was found between CO and DO groups ($P < 0.05$). The specific comparison of the effects among the three treatment groups showed that RO had significantly greater effects on CA excretion than the average of the other two treatment groups ($P < 0.001$). The results may indicate that the RO group, which also contained high concentration of UF had a significantly higher fecal bile acid excretion than the other two treatment groups because oryzanol

might be more bioactive in RBO than in corn oil. The significant difference between CO and DO groups was unexpected, but may reflect differences in oryzanol digestibility as previously described.

Table 8. Effects of the Diets on Fecal Primary Bile Acid Excretion^{1,2,3}

Bile acids	C (7)	SH (8)	CO (7)	DO (8)	RO (7)
CA ⁴	0.105 ^C	0.066 ^C	5.34 ^B	2.012 ^C	9.238 ^A
SEM	0.052	0.020	0.873	0.532	2.145
RO vs. AVE (CO & DO) [*]	P<0.001				
CDCA ⁵	0.08 ^C	0.037 ^C	3.266 ^B	1.595 ^B	6.241 ^A
SEM	0.026	0.013	0.656	0.447	1.137
RO vs. AVE (CO & DO) [*]	P<0.0001				
PBA ⁶	0.185 ^C	0.103 ^C	8.607 ^B	3.607 ^C	15.479 ^A
SEM	0.077	0.031	1.473	0.898	3.191
RO vs. AVE (CO & DO) [*]	P=0.0001				

¹Values are arbitrary and presented as peak area (PA) ratio of bile acids to β -cholanic acid (internal standard).

²Sample size used in each group follows the diet group name in parentheses.

³Values within a row with the same letter of superscript are not significantly different (P>0.05).

⁴Cholic acid.

⁵Chenodeoxycholic acid.

⁶Primary bile acids.

^{*} Significant effects of test treatment diets on different bile acid excretion. NS implies a non-significant result (P>0.05).

Fecal CDCA excretion (Table 8) showed that all of the treatment groups had significantly higher excretion than the control groups. Again RO had the greatest CDCA excretion among the groups, which was also significantly higher than the average excretion of CO and DO groups (P<0.0001). No significant difference in CDCA excretion was noted between CO and DO groups. The significant differences (P<0.05)

between the treatment groups and the control groups supported the possibility that oryzanol may play a major role in fecal bile acid excretion.

4.4.2 Secondary Bile Acids

DCA excretion (Table 9) was found to be the highest in the RO group among the five diet groups, and it was significantly higher in the RO group than the average of CO and DO groups ($P=0.0003$). All of the treatment groups showed a significantly greater DCA excretion than the control groups ($P<0.05$). These results also supported the possibility that oryzanol promoted fecal bile acid excretion.

Table 9. Effects of the Diets on Fecal Secondary Bile Acid Excretion^{1,2,3}

Bile acids	C (7)	SH (8)	CO (8)	DO (8)	RO (8)
DCA ⁴	0.308 ^C	0.151 ^C	13.532 ^{A,B}	8.535 ^B	15.506 ^A
SEM	0.101	0.057	3.089	3.275	2.347
RO vs. AVE (CO & DO) [*]	P=0.0003				
LCA ⁵	0.255 ^B	0.090 ^B	7.073 ^A	9.690 ^A	10.453 ^A
SEM	0.125	0.034	1.597	4.193	1.784
RO vs. AVE (CO & DO) [*]	P=0.01				
SBA ⁶	0.563 ^B	0.241 ^B	20.605 ^A	18.225 ^A	25.959 ^A
SEM	0.177	0.091	4.637	6.755	4.110
RO vs. AVE (CO & DO) [*]	P=0.0023				

¹Values are arbitrary and presented as peak area (PA) ratio of bile acids to β -cholic acid (internal standard).

²Sample size used in each group follows the diet group name in parentheses.

³Values within a row with the same letter of superscript are not significantly different ($P>0.05$).

⁴ Deoxycholic acid.

⁵ Lithocholic acid.

⁶ Secondary bile acids.

^{*}Significant effects of test treatment diets on different bile acid excretion. $P<0.05$ implies significant.

For LCA excretion (Table 9), all of the treatment groups showed significantly higher LCA excretion than the control groups. RO exhibited the highest excretion among

the diet groups, but the differences among the treatment groups were not significant ($P>0.05$); although, again, excretion for RO was significantly greater than the average of the other two treatment groups ($P=0.01$). Thus, the effect of oryzanol was exhibited in LCA excretion as well.

4.4.3 Total Bile Acids

Total bile acids (BAT) (Table 10) were the results for the combined primary bile acids (PBA) and secondary bile acids (SBA). The BAT excretion from highest to lowest was $RO>CO>DO>C>SH$. All of the treatment groups showed a significantly higher excretion of BAT than the control groups, which indicated that hypocholesterolemic effects of oryzanol might promote bile acid synthesis and/or excretion. Although RO showed the highest BAT excretion among the five diet groups, the difference was not statistically significant from that of CO. Differences of BAT excretion were not statistically significant between CO and DO groups either. However, RO exhibited statistically greater fecal bile acid excretion than the average of the other two treatment groups ($P<0.0001$).

Among the treatment groups, CA excretion was higher than CDCA excretion (Figure 7), and DCA excretion was higher than LCA excretion (except for the DO group) (Figure 8). These findings might indicate that a high dose of oryzanol increased cholic acid-derived bile acid excretion. However, DCA excretion was higher than CA excretion, and SBA excretion was higher than PBA excretion, which might indicate that oryzanol could cause fermentation in the colon, as conversion of primary bile acids to secondary bile acids generally occurs due to fermentation by the bacteria in the colon. The unexpected results for LCA excretion with DO, in which there was a higher excretion

than that of CO, could be an anomaly because the CO group showed a higher fecal steroid excretion in all the results except LCA excretion.

Table 10. Effects of the Diets on Fecal Total Bile Acid Excretion (BAT)^{1,2,3}

Bile acids	C (7)	SH (8)	CO (8)	DO (8)	RO (8)
BAT ⁴	0.748 ^C	0.344 ^C	29.212 ^{A,B}	21.832 ^B	41.438 ^A
SEM	0.203	0.115	5.256	6.496	6.089
RO vs. AVE (CO & DO) [*]	P<0.0001				

¹Values are arbitrary and presented as peak area (PA) ratio of bile acids to β -cholanolic acid (internal standard).

²Sample size used in each group follows the diet group name in parentheses.

³Values within a row with the same letter of superscript are not significantly different (P>0.05).

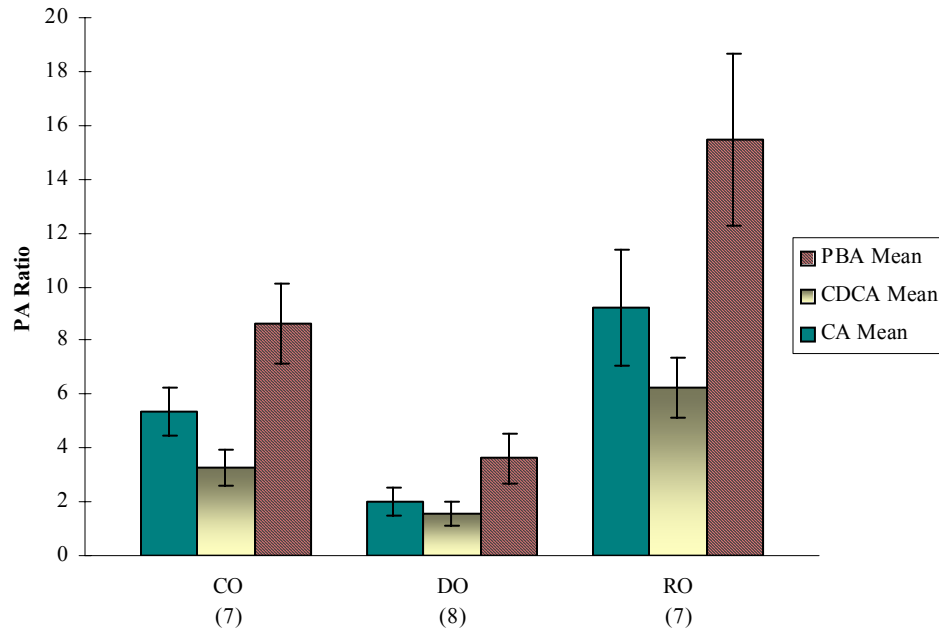
⁴ Bile acids (total).

* Significant effects of test treatment diets on different bile acid excretion. P<0.05 implies significant.

Few published papers studied cholesterol-lowering effects of oryzanol or RBO on fecal bile acid excretion. Although Sharma and Rukmini (1986) pointed out that RBO had significant effects on fecal bile acid excretion when compared with the peanut oil control diet, they did not specifically study excretion of individual neutral sterol or individual bile acids. Thus, it makes it hard to compare the results and effects of RBO or oryzanol through fecal steroid excretion in this study with other published studies.

RO and CO groups showed a significantly higher primary bile acid excretion than DO and the control groups. The bioactivity among oryzanol in three different forms appeared to be in the order of RO>CO>DO, although its bioavailability was not necessarily related to its bioactivity when considering its activity on fecal bile acid excretion. Heinemann et al. (1988) indicated that the relationship between absorbability

of plant sterols and their ability to inhibit cholesterol absorption was inverted. If that was true, it would be possible that oryzanol may have a similar effect as plant sterols because it was poorly absorbed in the body as well (Fujiwara et al., 1983).



¹Bars show mean \pm SEM.

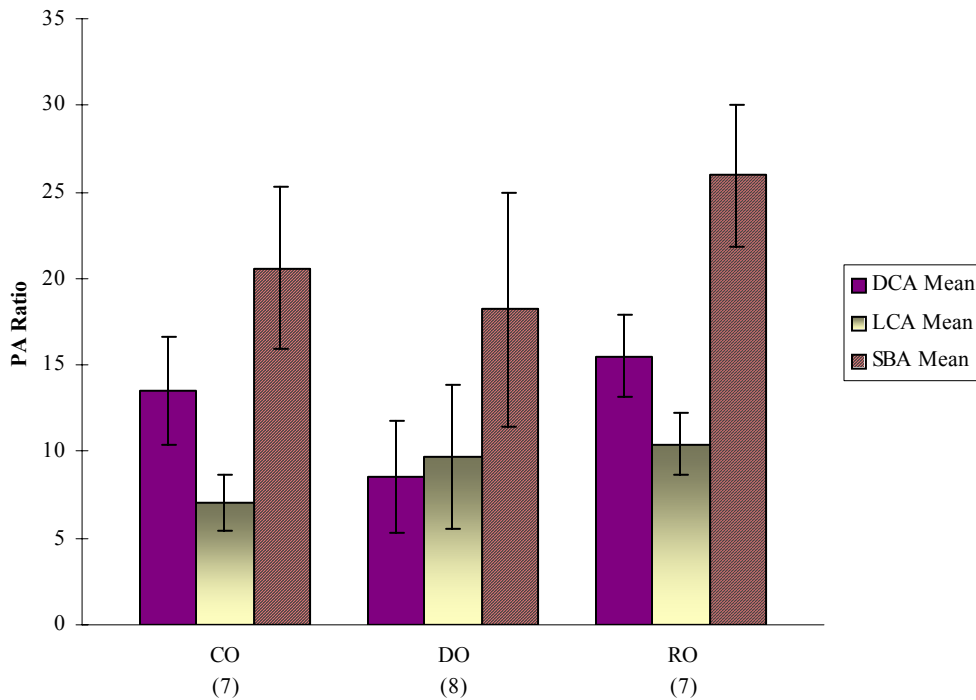
²PA Ratio represents peak area ratio of bile acids to β -cholanic acid (internal standard).

Figure 7. Comparison of Primary Bile Acid Excretion among Treatment Groups^{1, 2}.

Also it may explain partially why RO had the most γ -oryzanol recovered in the feces while it also caused the most fecal bile acid output. Similarly, CO (the crystalline form of oryzanol) had more oryzanol recovered than DO (dissolved oil form of crystalline oryzanol), and also caused more fecal bile acid output.

4.5 FECAL CHOLESTEROL VERSUS FECAL BILE ACIDS

When comparing fecal cholesterol excretion with fecal bile acids among the five diet groups, it was apparent that fecal cholesterol excretion was greater than fecal bile acid excretion in the control groups. However, among the treatment groups that contained high concentration of oryzanol, the effect was greater for bile acid excretion than



¹Bars show mean \pm SEM.

²PA Ratio represents peak area ratio of bile acids to β -cholanic acid (internal standard).

Figure 8. Comparison of Secondary Bile Acid Excretion among Treatment Groups^{1,2}.

cholesterol excretion. The explanation could be that corn oil appeared to be more responsible for inhibition of intestinal cholesterol absorption by enhancing fecal cholesterol excretion, and oryzanol may be more responsible for fecal bile acid excretion but also inhibits cholesterol absorption. Studies by Rukmini and Raghuram (1991) and

Trautwein et al. (2002) supported this point as well, as they reported that 4,4'-dimethylsterols (mostly in oryzanol) might play a major role in fecal bile acid excretion.

4.6 OVARECTOMIZED RATS AND SHAM OPERATED RATS IN THE CONTROL GROUPS

Ovarectomized (OVX) rats and intact rats (sham operated) were used in the control groups. OVX rats may have higher levels of cholesterol in the body due to the deficiency of estrogen (Junqueira et al., 2002), thus the fecal excretion was supposed to be higher than the sham rats under the same feeding condition. A general appraisal of the data for fecal steroid excretion between C and SH groups fed control diet indicates that the SH group had a higher cholesterol excretion than C group, while C group had a higher bile acid excretion than SH group; although these differences were not statistically significant ($P > 0.05$). No fecal oryzanol was detected in either of the control groups. These results suggested that the control diet used in this study had no effect on cholesterol absorption and bile acid enterohepatic circulation between the OVX and the intact rats, thus they could be regarded as similar experimental animals in this study.

4.7 FECAL BILE ACID EXTRACTION METHODS AND DETERMINATION

To simplify the results of fecal bile acids, deconjugation of bile acid conjugates was performed, thus the data in this study reflected free bile acid without the amide bonds to glycine or taurine, although a test to determine complete deconjugation was not done in this experiment. Batta et al. (1984) reported that choloylglycine hydrolase did not hydrolyze the taurine conjugate of cholic acid. As such, the inability of choloylglycine hydrolase to hydrolyze all the substrates might underestimate total bile acids present. They also suggested that choloylglycine hydrolase activity was greatly reduced when the conjugate of keto-cholic acid was the substrate. The specificity of the enzyme was toward

chenodeoxycholic acid and cholic acid conjugated with amino acids other than glycine and taurine. In addition, the enzyme activity was affected by the elongation of the side chain and hydroxyl groups in the bile acids. Their findings may indicate the prudence of using other methods to deconjugate bile acids in order to obtain a more accurate measurement of total bile acids.

Assays used to determine bile acids might affect the results differently as bile acids have extremely complex and diverse forms. Some studies reported the extraction of bile acids after deconjugation by choloylglycine hydrolase and then analysis by GC-FID after acidification and methylation, using 5β -cholanic acid or 23-nordeoxycholic acid as internal standard. Other than determination of basic bile acids, ketonic derivatives were converted by chromic acid oxidation, and standards, including different forms of conjugated and deconjugated bile acids, as well as reference ketonic esters, were used as reference standards (Nakamura, et al., 2000, Grundy et al., 1965). Some other studies used an enzymatic method to determine fecal bile acids by using 3α -hydroxy steroid dehydrogenase (Sheltawy and Josowsky, 1975). However, these methods were either too complex or too briefly described for incorporation into the analysis in this experiment.

Results of different studies on fecal bile acids varied as well. Some studies reported that dihydroxy bile acids such as chenodeoxycholic acid and lithocholic acid might have increased fecal excretion under acidic pH conditions (Trautwein et al., 1998 and 1999). Vlahcevic et al. (1991) suggested that chenodeoxycholic acid might be more effective in terms of feedback inhibition of bile acid synthesis than cholic acid. It might be because less chenodeoxycholic acid and more cholic acid returned to the liver, which might stimulate cholesterol 7α -hydroxylase activity and possibly up-regulate bile acid

synthesis (Chiang and Stroup, 1994; Trautwein et al., 1999). However, other studies reported that increased cholic acid excretion was found in rats fed gymnemic acids, suggesting the compound might interfere with cholesterol absorption and bile acid reabsorption (Nakamura et al., 1999). Other studies on the effects of dietary fiber on fecal steroid excretion suggested that a higher cholic acid proportion of the fecal total bile acids might be due to the compounds being bound to cholic acid stronger than chenodeoxycholic acid (Vahouny et al., 1987; Cheung, 1998).

However, no published paper has studied whether oryzanol or RBO reflect their hypocholesterolemic effect through fecal bile acid excretion or only mention total bile acid excretion (Seetharamaiah and Chandrasekhara, 1989) as a possible mechanism. The results found in this study showed that oryzanol might increase fecal deoxycholic acid or secondary bile acids more predominately than primary bile acids, due to fermentation in the colon. Also, the results of fecal bile acid excretion supported the possibility that oryzanol may lower cholesterol levels through fecal bile acid excretion as the primary mechanism. More study may be needed to investigate the extent of oryzanol effects on specific bile acids.

4.8 CHOLESTEROL-LOWERING EFFECTS OF ORYZANOL AND PHYTOSTEROL

The main difference of the diets between the non-RBO treatment groups (CO and DO) and the control groups (SH and C) was the addition of oryzanol in CO and DO. When comparing the fecal steroid excretion between the control groups to the non-RBO treatment groups, the higher fecal steroid excretion in the two treatment groups indicated a significant hypocholesterolemic activity of oryzanol, at least at the concentration in this study. The significantly greater fecal bile acid excretion in CO and DO groups than SH

and C groups, as well as no significant differences in fecal cholesterol excretion among CO, DO and SH or DO, C, and SH groups might indicate the major mechanism of oryzanol in lowering cholesterol levels is through interference in bile acid reabsorption in the enterohepatic circulation, whereas plant sterol may interfere with cholesterol absorption, mainly. The results in this study supported that of Kiribuchi et al. (1983), who reported that cycloartenol and probably 24-methylenecycloartanol, the two major components in oryzanol, attributed cholesterol-lowering effects by increasing fecal excretion of acidic steroids. On the other hand, phytosterol has a more similar structure to cholesterol than oryzanol does. The results for fecal cholesterol excretion in this study could indicate that phytosterol has a more pronounced ability to displace cholesterol in the micelles, and thus interfere with cholesterol absorption. A sterol structure with a cyclopentane ring might be a minimum requirement for inhibiting cholesterol absorption in the intestine (Vissers et al. 2000). From Fujiwara et al. (1983)'s study, the absorption route of oryzanol appeared to be different from that of cholesterol and phytosterol, whose absorption rates were reported as ~50% and ~5%, respectively (Ling and Jones, 1995). Phytosterol along with cholesterol were incorporated into micelles via lymphatic ducts and absorbed in the small intestine. As the gut poorly absorbs phytosterol, about 95% of dietary phytosterol enters the colon and is excreted in the feces. A major mechanism of inhibition of cholesterol absorption by phytosterol is generally proposed as reduced cholesterol content in the micelles. Since phytosterol is more hydrophobic than cholesterol, they may displace cholesterol from micelles thus restricting solubility of synthesis and excretion by metabolizing enzymes in the colon. However, the influence of phytosterol on conversion of cholesterol into bile acids was not consistent in different

studies (Ling and Jones, 1995). These studies did support the possibility that both oryzanol and phytosterol were poorly absorbed in the body.

4.9 EFFECTS OF RICE BRAN OIL AND CORN OIL

Some studies reported that the cholesterol-lowering effects between RBO and corn oil were similar (de Deckere and Kover, 1996; Lichtenstein et al., 1994). In Lichtenstein et al (1994)'s study, they reported that RBO resulted in plasma lipid and apolipoprotein concentrations that were similar to other commonly used vegetable oils. However, these studies did not add other potential components to RBO to manifest the effects. Cholesterol-lowering effects might not be appreciable in conventionally used refined rice bran oil, due to the low concentration of oryzanol (51mg per 100g oil) (de Deckere and Kover, 1996). However, RBO used in this study had an extremely high concentration of oryzanol (2.8 g in 40 g oil), which might be the reason for cholesterol-lowering effects of RBO and/or oryzanol that were clearly manifested when compared to the control diets. Among the treatment groups, the fact that RO exhibited significantly higher fecal steroid excretion than CO and DO groups may suggest that the potent hypocholesterolemic effect of RBO is attributable to other components in the UF in addition to oryzanol, as RBO contains a relatively higher level of UF than corn oil. Also, a higher content of UF in RBO compared with corn oil, which has similar hypocholesterolemic effects (Kahlon et al., 1992), may be the reason for the cholesterol-lowering capabilities of RBO that were not explained by its fatty acid composition (Nicolosi et al., 1991). Another study by Kahlon et al. (1996) suggested that UF in RBO promoted fecal neutral sterol excretion probably by diminished absorption-reabsorption

of cholesterol. The results in this study also supported this point, as plant sterols and oryzanol in RBO promoted fecal cholesterol and bile acid excretion, respectively.

4.10 LIMITATIONS OF THIS STUDY

Results in this study were presented as peak area (PA) ratios of cholesterol and bile acids to the internal standards of α -cholestane and β -cholanic acid, respectively. The reason was that both neutral sterol and bile acids are complex compounds of different components, which are cholesterol metabolites with chemical structure similar to cholesterol. Cholesterol accounts for more than 70% of the total neutral sterol fraction (Trautwein et al., 1997); other neutral sterols such as coprostanol are metabolites of cholesterol, formed by the action of gut microflora (Cheung, 1998). As mentioned earlier, when the amount of dietary cholesterol is reduced, *de novo* biosynthesis of cholesterol in the liver and the intestine is increased to satisfy the needs of other tissues and organs, as well as to replace bile salts and cholesterol lost from the enterohepatic circulation in the feces (Devlin, 1997). Some studies suggested that the amount of cholesterol in fecal neutral sterol excretion might be partly due to the plant compounds added. Less cholesterol and more coprostanol would probably be excreted as increased microbial breakdown of cholesterol in the large intestine occurs due to the ingestion of plant compounds (Trautwein et al., 1999).

Bile acids are a more complex mixture of different bile acid compounds as mentioned previously. Primary bile acids are the major components, and more than a dozen different forms of secondary bile acids are derivatives of primary bile acids formed by intestinal bacteria (Hawkins et al., 2002). In the liver, both primary and secondary bile acids bind to either glycine or taurine to form conjugated bile acids. However, during the

process of bile acid metabolism, different secondary bile acid derivatives, as well as ketonic derivatives, would be formed. Because of a lack of standards for all of these components, this study focused on four major primary and secondary bile acids, to compare cholesterol-lowering effects of oryzanol and RBO reflected by PA ratio of the major compounds in fecal steroids to the internal standards. As such, the actual amount of fecal steroid excretion caused by oryzanol or rice bran oil intake was not elucidated in this study. However, this study showed that rice bran oil and its unique compound, oryzanol, promoted fecal cholesterol and bile acid excretion, which might be a major mechanism for its hypocholesterolemic effects.

Another concern was that the animals were not fed an atherogenic diet, which might affect the findings. Based on the literature review, most of the studies concerning cholesterol-lowering effects of certain bioactive components used high-fat, or high-cholesterol, or dietary neutral sterol/bile acid to magnify the effects. Rong et al. (1994) suggested that rice bran oil might exhibit its hypocholesterolemic effects by decreasing plasma LDL-C through inhibition of dietary cholesterol absorption. Sharma and Rukmini (1986) also suggested that fecal steroid excretion was greater when feeding a high cholesterol diet on a RBO study. Thus, a cholesterol or fat-enriched diet may be required to observe the specific effects of certain levels of RBO or oryzanol on serum cholesterol level changes through direct or indirect methods.

Also of concern is the lipid profile difference between RBO and corn oil. Peanut oil, which has a more similar fatty acid profile to RBO, instead of corn oil as the control diet, may have been more appropriate for the study of the hypocholesterolemic effects of RBO (Sharma and Rukmini, 1986). However, this study could serve as a preliminary

study on the cholesterol-lowering effects of RBO and oryzanol through fecal steroid excretion.

CHAPTER 5

SUMMARY AND CONCLUSION

RO group showed the greatest fecal cholesterol and bile acid excretion among the diet groups in this study and the differences were statistically significant ($P < 0.05$). In addition, fecal cholesterol and total bile acid excretion in RO was significantly higher than the average of the CO and DO groups ($P < 0.001$). On the other hand, RO group showed a significantly higher oryzanol excretion than both of the other treatment groups ($P < 0.05$). These findings indicated that the potential hypocholesterolemic effects of RBO with oryzanol were higher than corn oil with oryzanol. The cholesterol-lowering activity in the RO group may be due to synergistic effects of the components in UF, in addition to oryzanol, through the interference of cholesterol absorption and bile acid reabsorption to enhance fecal steroid excretion. However, oryzanol in RBO may be more bioactive than that in corn oil, even though the fecal recovery of oryzanol was shown to be the highest from RO in this study. Further study is needed to determine the correlation of cholesterol-lowering effects to the oryzanol recovery rate from RBO.

The results between the CO and DO group showed that fecal cholesterol and total bile acid excretion were higher in the CO group than in the DO group, which might indicate that the crystalline form of oryzanol could have a greater cholesterol-lowering effect than the dissolved oil form of oryzanol, as the crystalline form might be less absorbed. However, the differences in the data between the CO and DO groups were not statistically significant ($P > 0.05$). Further study needs to be done to elucidate whether the crystalline form or the dissolved oil form exhibits a greater potential in cholesterol-lowering effects.

Results from oryzanol in corn oil (CO and DO) and corn oil by itself (SH and C) established that significantly greater fecal bile acid excretion occurred with the oryzanol diets than the control diets, but a non-significant difference in fecal cholesterol excretion occurred between them. It might indicate the primary mechanism of oryzanol in lowering cholesterol levels may be through interference in bile acid reabsorption in the enterohepatic circulation.

It was also noticed that the effect through fecal cholesterol excretion was higher than fecal bile acid excretion in the two control groups, which may suggest corn oil appeared to be more responsible for inhibition of intestinal cholesterol absorption by enhancing fecal cholesterol excretion.

5.1 FUTURE STUDY:

It is suggested that the results of this study could be reaffirmed and/or clarified by employing the following actions in future studies:

- Using peanut oil instead of corn oil as a control diet because it is more similar to rice bran oil in fatty acid composition.
- Using high-fat or high-cholesterol diets, which may magnify the hypocholesterolemic effects of RBO.
- Using GC-MS and HPLC analysis to obtain a more accurate quantification and determination of fecal neutral sterol and bile acids.
- Evaluating the correlation between bioavailability of oryzanol and its hypocholesterolemic effects using crystalline and dissolved oryzanol forms or crystalline oryzanol added to rice bran oil instead of corn oil.

- Establish the correlation of fecal steroid excretion with serum and liver cholesterol changes.

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APPENDIX A

0.25 M SODIUM ACETATE BUFFER (PH5.6) SOLUTION.

Weigh 2.05 g anhydrous sodium acetate in a clean vial, and dissolve into 80 mL distilled water. Use a Pasteur pipette to add acetic acid to adjust pH to 5.6 exactly, measured by a 410A pH Meter. Dilute to 100 mL with distilled water (M.W. of anhydrous sodium acetate is 82g/mol. $2.05/82=0.025\text{M}$. $0.025\text{M}/100\text{mL}=0.25\text{M}$). The solution was stored at 4°C.

APPENDIX B

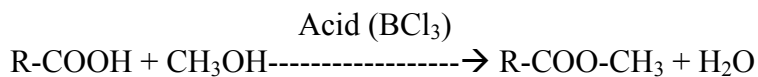
REACTION AND MECHANISM: BCL₃-METHANOL, 12% W/W.

- Procedure:

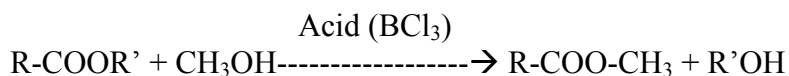
For either acid dissolve in nonpolar organic solvent, or evaporated to dryness, add 2 mL BCl₃-methanol and a water scavenger (such as 2,2-dimethoxypropane). (Water can prevent the reaction from going to completion, producing low yields). Heat at 60°C for 5-10 minutes. After cooling, add 1 mL water and 1 mL hexane. Shake the reaction vessel – it is critical to get the esters into the nonpolar solvent. Carefully remove the organic layer, and dry it over anhydrous sodium sulfate.

- Mechanism:

Esterification:



Transesterification:



VITA

Zhaoli (Joy) Dai was born and grew up in Guangzhou, China. She received her Bachelor of Science degree in food science at Jinan University, Guangzhou, in 1999.

She is independent and likes experiencing new things in life. After working three years in different global companies, her dream of studying in America came true. She was accepted into the master's program in the Department of Food Science at Louisiana State University in August 2002.

Zhaoli (Joy) is currently a master's degree candidate and will graduate in summer 2004.