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Metabolic aspects of oryzanol in rats

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METABOLIC ASPECTS OF ORYZANOL IN RATS

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 School of Human Ecology

by
Michelle Smith Gillespie
B.S., Louisiana State University, 1991
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DEDICATION

This manual is dedicated with love to my dearest friend and husband, Jeffrey

Morris Gillespie, who has selflessly supported me through my graduate studies.

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ABSTRACT

Using a rat model, this study compared the bioavailability of three forms of oryzanol, a compound extracted from rice bran oil that has been associated with improved cholesterol levels. Various methods of extracting lipids from serum and liver were explored to determine oryzanol absorption. Cholesterol levels were obtained via enzymatic colorimetric assay and GCMS.

Sprague Dawley retired female breeder rats were sustained for 11 weeks on a cholesterol-free diet to which either no oryzanol was added (n = 19) or 2.8 g/kg of oryzanol was added as: 7% oryzanol rice bran oil (RO, n = 8), crystalline oryzanol (CO, n = 8), or crystalline oryzanol dissolved in tocopherol-stripped corn oil (DO, n = 9). The percentage of dietary oryzanol recovered in the feces of rats fed the CO diet (41.9 ± 2.21, mean $\pm$ SEM) was significantly higher compared to the RO (28.3 ± 3.54) and DO (27.8 ± 2.63) groups ($P < 0.05$), suggesting that bioavailability of crystalline oryzanol is lower relative to the oil forms.

Oryzanol was not detected during GCMS analysis of serum following hexane extraction. Similarly, hexane extractions of liver analyzed via HPLC and GCMS did not reveal oryzanol. Hexane may not be an appropriate solvent for extracting oryzanol, or perhaps the compound is metabolized prior to its entry into the liver or bloodstream.

Although serum cholesterol levels determined by colorimetric enzymatic assay did not differ significantly among the groups, levels were highest in the RO group, followed in sequence by the DO, CO, and oryzanol-free controls. It is likely that a cholesterol-enriched diet is required for the hypolipidemic effect of oryzanol to be realized. Additionally, it is plausible that the cholesterol values from the oryzanol-fed

animals were inflated by phytosterols in the serum.   Hexane extractions of serum

analyzed via GCMS resulted in unintelligible data, suggesting that hexane is not a

suitable solvent for the extraction of cholesterol from serum. Although a 2:1

chloroform:methanol mixture (Folch et al., 1956) resulted in more uniform values, it

appeared that the extraction of total cholesterol from lipoproteins was incomplete.

CHAPTER 1

INTRODUCTION

Cardiovascular disease is the leading cause of death in the United States and currently costs Americans an estimated $351.8 billion per year (American Heart Association, 2002). It is well established that an association exists between cholesterol levels and incidence of heart attack. Low-density lipoprotein (LDL) cholesterol levels of 130 mg/dL or higher and high-density lipoprotein (HDL) cholesterol levels of 40 mg/dL or lower are both considered risk factors for cardiovascular disease in humans. Of Americans aged 20 and older, 48.6% of males and 43.3% of females have LDL levels high enough to be considered at risk. In this same group, 39.0% of males and 14.9% of females have HDL levels low enough to be considered at risk. (American Heart Association, 2002. Data from NHANES III, 1988-94, CDC/NCHS.)

Much research has been done to identify components of food that positively alter cholesterol levels and reduce risk of cardiovascular disease. Cereal brans, including that of rice, have shown promising results. Consumption of rice bran has significantly reduced plasma and liver cholesterol levels in hamsters (Kahlon, Saunders, Chow, Chiu, & Betschart, 1990; Kahlon, Chow, Sayre, & Betschart, 1992; Kahlon, Edwards, & Chow, 1998). LDL levels have decreased while HDL levels have remained stable in humans fed diets containing rice bran (Hegsted, Windhauser, Morris, & Lester, 1993). In particular, the oil extracted from rice bran has shown positive effects on cholesterol levels in both animals (Sharma & Rukmini, 1986) and humans (Raghuram, Rao, & Rukmini, 1989).

Rice bran oil is extracted from the germ and bran layers of brown rice and contains both saponifiable and unsaponifiable compounds. The saponifiable fraction

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consists primarily of triglycerides. Smaller amounts of diglycerides, monoglycerides, free fatty acids, waxes, glycolipids, and phospholipids are also present (Orthoefer, 1996). The most prevalent fatty acids are palmitic, oleic, and linoleic (Wilson, Ausman, Lawton, Hegsted, & Nicolosi, 2000). Compared to other edible oils, rice bran oil has a relatively high unsaponifiable fraction of 4.2% (Itoh, Tamura, & Matsumoto, 1973). The unsaponifiable components include phytosterols, tocopherols, tocotrienols, and oryzanol. Oryzanol was originally identified as a single compound, however it is now known that oryzanol is a group of various triterpene alcohols esterified to ferulic acid (Xu & Godber, 1999).

The component of rice bran oil that is responsible for the cholesterol-lowering effect is still uncertain. High levels of serum cholesterol-raising fatty acids in rice bran oil compared to other vegetable oils have led researchers to explore the unsaponifiable fraction (Wilson et al., 2000). Despite the apparent inferior fatty acid content of rice bran oil, this group reported similar reductions in total cholesterol and LDL levels in monkeys consuming diets containing rice bran, canola, and corn oil. In another study, LDL levels dropped and HDL levels were unchanged or increased in rats on diets supplemented with the unsaponifiable matter from rice bran oil (Sharma & Rukmini, 1987). More specifically, studies have suggested that oryzanol is primarily responsible for the hypolipidemic effects of rice bran oil. Addition of oryzanol to rat diets containing rice bran oil was associated with lower cholesterol levels compared to rat diets containing rice bran oil alone (Seetharamaiah & Chandrasekhara, 1989). Oryzanol administered to hyperlipidemic persons resulted in improved cholesterol levels (Yoshino et al., 1989).

Medicines currently available for treatment of hypercholesterolemia include bile acid sequestrants and HMG-CoA reductase inhibitors, also known as statins. Studies supporting the idea that oryzanol is an active component of rice bran oil with hypolipidemic qualities justify the exploration of pharmaceutical possibilities for this compound as well. Comparing the metabolic aspects of various forms of dietary oryzanol in rats may be helpful in elucidating the mechanism by which oryzanol is able to lower cholesterol levels, as well as providing insight into an optimal way to package it for use as a cholesterol-lowering agent.

The primary objectives of this study were (1) to determine if crystalline oryzanol is as bioavailable as oryzanol in rice bran oil or that dissolved in corn oil, and (2) to investigate the relative cholesterol-lowering capabilities of the three forms. It was hypothesized that oryzanol within rice bran oil would be more efficiently metabolized and/or absorbed than the crystalline form, and therefore more effective at improving cholesterol levels. It was surmised that the bioavailability of crystalline oryzanol would be enhanced by dissolving the compound in corn oil, although perhaps not extensively enough to make its hypolipidemic efficacy comparable to that of the rice bran oil form. The secondary objective was to develop methodology for determining the presence of oryzanol in serum and liver.

This study compared amounts of oryzanol in the feces of rats fed one of the following forms: 7% oryzanol rice bran oil, crystalline oryzanol, or crystalline oryzanol dissolved in corn oil. Dietary oryzanol recovered from the feces represented that not absorbed or metabolized in the gastrointestinal tract. Various procedures were explored to effectively extract oryzanol from serum and liver for quantification by HPLC and

GCMS methods. Serum was analyzed to determine whether or not any oryzanol entered the bloodstream without being metabolized. Livers were analyzed to detect any absorbed oryzanol that had not been metabolized. Total cholesterol levels were assessed using both an enzymatic colorimetric method and GCMS.

Many studies that reported an association between consumption of rice bran oil and lower cholesterol levels in rats used high-cholesterol diets. The rat diets in this study were cholesterol-free. It is likely that a diet containing cholesterol is necessary for the hypolipidemic effect of oryzanol to be realized. Also, if absorption of oryzanol is enhanced by cholesterol in the gut, the cholesterol-free diets in this study may have resulted in higher fecal oryzanol and lower liver oryzanol than would have occurred with cholesterol-fed rats. Another limitation of this experiment stems from the fact that the rats were not fasted prior to sacrifice, preventing meaningful analysis of serum for LDL values.

CHAPTER 2

REVIEW OF LITERATURE

Rice Bran Improves Cholesterol Levels

Animal Studies

Various sources of dietary fiber have been shown to lower cholesterol in animal models. Rice bran, in particular, has shown an exemplary capability for improving cholesterol status in mice, rats, and hamsters. This effect appears to be enhanced in diets rich in cholesterol. Hundemer, Nabar, Shriver, and Forman (1991) constructed an experiment to compare the cholesterol-lowering effects in mice of rice bran, soybean fiber, oat bran, and barley bran when added to a 0.06% cholesterol diet. Rice bran was the most effective bran supplement at lowering liver cholesterol concentrations and significantly lowered total plasma cholesterol with respect to the control diet. In addition, the HDL cholesterol levels were maintained in mice consuming the rice bran diet, resulting in higher HDL to total cholesterol ratios.

In another study, rats showed significantly lower levels of liver triglycerides and liver cholesterol when they were fed a rice bran versus a wheat bran diet (Topping, Illman, Roach, Trimble, Kambouris, & Nestel, 1990). When fish oil was added to test diets, rice bran was more effective than wheat bran at preventing the fish oil's effect of decreasing LDL receptor activity and served to enhance the fish oil's ability to lower triglycerides. The rice bran diet also increased LDL receptor activity in the liver more than the wheat bran diet, effectively lowering plasma cholesterol levels. Again, HDL levels were unaffected by the rice bran diets, while a decrease in this "good" cholesterol was associated with the wheat bran diets.

Hamsters were used to compare the cholesterol-lowering effects of stabilized rice bran, parboiled rice bran, rice bran and wheat bran combined, the full-fat and defatted versions of each, and oat bran in a 0.5% cholesterol diet (Kahlon et al., 1990). Liver weights were significantly reduced by both full-fat rice bran diets, as were liver cholesterol concentrations and total liver cholesterol amounts when compared to the control. The liver cholesterol concentrations in particular were significantly lower in animals consuming full-fat stabilized rice bran than all other groups.

Again, hamsters were used as a model to examine potential cholesterol-lowering benefits of varying levels of full-fat rice bran in both 0.3% cholesterol and cholesterol-free diets (Kahlon et al., 1992). In animals fed test diets containing dietary cholesterol, all groups consuming rice bran diets had significantly lower liver weights than controls consuming a diet with cellulose in place of rice bran. Plasma cholesterol was significantly reduced in hamsters consuming the highest level, 43.7%, of rice bran in the diet, regardless of dietary cholesterol when compared to the bran-less controls. Interestingly, in test groups with cholesterol added to the diet, a significant correlation was found between the levels of rice bran in the diet and reductions in plasma cholesterol levels.

Another hamster study, in which dietary cholesterol was only 0.25%, compared rice bran diets to diets containing oat bran, whole barley, and various levels of beta-glucan-enriched barley (Kahlon, Chow, Knuckles, & Chiu, 1993). No significant improvement was seen in either total plasma cholesterol levels or HDL to total cholesterol levels. This appears to contradict other published findings; however, these results may be explained by the lower cholesterol content of the test diets used in this

experiment. In contrast, all bran diets showed a significant decrease in total liver cholesterol compared to the control.

A fourth hamster study published by the same primary author was used to compare the benefits of adding bran from rice, oats, corn, and wheat to a 0.3% cholesterol diet (Kahlon et al., 1998). In this study, animals fed rice bran had the lowest liver weights and liver cholesterol levels. Liver cholesterol concentrations were significantly lower for the rice bran diet than for either the corn or wheat bran diets. Although total plasma cholesterol was not significantly lower in any of the treatment groups when compared to the control, the levels associated with rice bran were the lowest of all bran test diets. Also, hamsters in the rice bran group had significantly lower VLDL levels and the highest HDL to total cholesterol ratios when compared to animals consuming all other bran diets. Finally, the rice bran group showed the lowest digestibility rate and the highest lipid excretion rate of all groups.

Fukushima, Fujii, Yoshimura, Endo, and Nakano (1999) designed an experiment to determine if the benefit of rice bran treatment decreased in rats when their cecums were removed. In rats, the cecum is the main site of dietary fiber fermentation which produces short chain fatty acids. The authors theorized that increased levels of short chain fatty acids would alter lipid metabolism, perhaps by hindering cholesterol absorption due to a change in intestinal fluid viscosity, or by directly inhibiting *de novo* cholesterol synthesis in the liver. A test diet of rice bran was fed to both cecectomized and sham-operated animals. A third group that did not undergo surgery consumed a bran-less diet and served as the control. Both test diets contained 0.5% cholesterol. Rice bran diets appeared to be responsible for significant reductions in serum total cholesterol,

serum triglycerides, and liver cholesterol concentrations. Although the serum HDL levels were also lower in the rice bran groups, HDL to total cholesterol ratios in these groups were higher than that of the control group. The sham-operated group fed rice bran showed the lowest liver cholesterol concentrations as well as the lowest total plasma cholesterol levels, leading the authors to conclude that rice bran might lower cholesterol by increasing short chain fatty acid production in the cecum. However, Topping et al. (1990) found evidence to the contrary. Although increased short chain fatty acid concentration was associated with a rice bran diet relative to a wheat bran diet, no correlation between short chain fatty acids and cholesterol was observed. These researchers therefore concluded that the beneficial effect of rice bran with respect to cholesterol levels was not due to short chain fatty acid production.

Human Studies

Rice bran appears to be effective at improving cholesterol levels in humans, as well. Hegsted et al. (1993) found rice bran to be equivalent to oat bran in lowering cholesterol in 11 mildly hypercholesterolemic subjects. Treatment diets each contained 300 mg/day of cholesterol and were supplemented with either 100 g/day of stabilized rice bran or an equal amount of oat bran. All subjects consumed both diets during two 3-week periods that were separated by a 1-week bran-less control diet. Total cholesterol levels were significantly reduced in both bran diets when compared to the control. Although HDL levels dropped initially during the bran consumption, the overall effect was an improvement in HDL to total cholesterol ratios.

Changes in plasma lipid levels were studied when 24 men with slightly above normal cholesterol levels consumed test diets containing 35 g/day of wheat bran, 60

g/day of rice bran, or 95 g/day of oat bran (Kestin, Moss, Clifton, & Nestel, 1990). The varying amounts of the different brans provided a constant amount of total dietary fiber of 11.8 g/day. All of the subjects consumed each of the three bran diets in random order for 1-week durations. Only oat bran was effective at reducing plasma cholesterol when compared to baseline levels. However, the highest rise in HDL was associated with the rice bran diet, resulting in an improved HDL to total cholesterol ratio. This ratio was significantly higher than that seen in the wheat bran group. Also, plasma triglycerides were lower in the rice bran versus the wheat bran diet, which was again statistically significant. Comparing this study to Hegsted et al. (1993), less dramatic results from the rice bran diet may be due to the use of unequal amounts of the various cereal brans.

In comparing the impact of rice bran on postprandial lipemia with that of oat bran, wheat fiber, and wheat germ in males with normal cholesterol, Cara et al. (1992) found marginally positive effects. Test diets were consumed in random order by all subjects and were supplemented with 39.4 g of rice bran, 40.0 g of oat bran, 12.5 g of wheat fiber, or 40.0 g of wheat germ. Postprandial serum triglyceride rises were attenuated by all cereal additions, including rice bran. Rice bran also significantly reduced the overall chylomicron response to eating, although not as effectively as the other test diets. Unlike the other cereals, rice bran was not associated with significantly lower cholesterol levels seven hours after the meal. The weaker impact of rice bran reported here compared to other published findings may be explained due to the following: (1) this study did not analyze long-term effects of dietary changes, and (2) subjects had normal levels of cholesterol.

Oil Fraction of Rice Bran Is Responsible for
Improving Cholesterol Levels

Animal Studies

Multiple researchers have reported results suggesting that the active component of rice bran that is capable of lowering LDL levels while stabilizing or increasing HDL levels is in the oil that can be extracted from the bran. In an effort to promote domestically produced edible oil in India, Sharma and Rukmini (1986) explored possible health benefits of rice bran oil. They compared cholesterol and triglyceride levels in rats maintained for two months on a diet containing 10% rice bran oil to those of animals fed a control diet with an equal amount of groundnut oil. Half of the animals in each group had 0.1% cholesterol and 0.05% cholic acid added in place of a portion of the starch. In cholesterol-fed animals, the rise in serum cholesterol due to the high-cholesterol diet was more effectively moderated by rice bran oil than groundnut oil. Also, HDL levels rose significantly in the cholesterol-fed rice bran oil group compared to the cholesterol-fed controls. In addition, serum and liver triglycerides, as well as liver cholesterol, tended to be lower in this group. Similar trends occurred in the rats that did not have added dietary cholesterol.

A later study by Kahlon et al. (1990) found that the ability of rice bran to improve cholesterol levels in hamsters diminished when the oil content was removed, leading the authors to hypothesize that the oil was the component responsible for the positive effect. Edwards and Radcliffe (1994) compared effects of rice bran oil on lipid status to the effects of corn oil, which had previously been shown to have a beneficial effect on cholesterol levels. In an evaluation of rats fed cholesterol-free diets containing 10% of either rice bran oil or corn oil, no difference was found in serum triglycerides,

cholesterol, or phospholipids between the two groups. Therefore, although the study did not utilize a true control group, the researchers concluded that rice bran oil was as effective as corn oil in improving cholesterol levels in rats fed cholesterol-free diets. An earlier study comparing the effects of rice bran oil to groundnut oil found superior cholesterol-improving effects in rice bran oil. Significantly lower total cholesterol in serum, with a tendency toward higher HDL-cholesterol levels were found in rats consuming cholesterol-free, 10% rice bran oil versus groundnut oil diets (Seetharamaiah & Chandrasekhara, 1989). A similar comparison was made in a more recent rat study between rice bran oil and high-linoleic acid safflower oil, again using feeds not enriched with cholesterol. Serum cholesterol levels did not differ significantly between the two test groups, implying comparable beneficial qualities with respect to cholesterol among the two oils (Radcliffe, Imrhan, & Hsueh, 1997).

In another rat study comparing rice bran and safflower oil, half of the animals were randomly assigned to cholesterol-free test diets in which one of these two oils, or a varying combination of the two, contributed 10% of the weight (Koba, Liu, Bobik, Sugano, & Huang, 2000). The other half were assigned to similar diets with 0.5% cholesterol. Among the animals not fed dietary cholesterol, total cholesterol levels were similar between the rice bran oil and safflower oil groups, but HDL levels were significantly higher in the rice bran oil group. This resulted in a higher HDL:total cholesterol ratio for this test group, although the difference was non-significant. A 7:3 rice bran oil:safflower oil ratio in the diet appeared optimal with respect to cholesterol levels. The average HDL:total cholesterol ratio was significantly higher for this group

than all other groups. However, the superior effects of both the rice bran oil only group and the 7:3 combination group disappeared in the cholesterol-fed animals.

Human Studies

In a human study, hyperlipidemic patients who were willing to change their dietary habits to reduce risk of heart disease agreed to replace their usual cooking oils with rice bran oil (Raghuram et al., 1989). A control group that continued habitual use of palm or groundnut oils in cooking was used for comparison. Significant reductions in serum cholesterol and triglycerides were seen after 15 days in the experimental group. Blood analyses after 30 days revealed even greater variation between the experimental and control subjects. The most responsive patients to the change in dietary oils were those with the highest initial cholesterol and triglyceride levels.

A later study compared the hypolipidemic impact of rice bran oil with canola, corn, and olive oils, which are more commonly used in Western diets, again using human subjects with moderately elevated blood cholesterol levels (Lichtenstein et al., 1994). All diets contained 80 mg of cholesterol with one of the four oils constituting two-thirds of the fat in the different diets and were all consumed for one month by each subject in a random order. Total and LDL cholesterol levels were similar during the rice bran, canola and corn oil-enriched diet consumption periods. These oils resulted in total and LDL cholesterol levels that were significantly lower than when subjects were maintained on the diet containing olive oil. No significant variation in HDL levels among the four groups meant that inferior cholesterol ratios were associated with the olive oil diet in relation to the others. All diets showed similar results with respect to plasma triglycerides. The outcome of this study suggests that rice bran oil can be considered as

beneficial as the more commonly consumed canola and corn oils with respect to heart

disease risk factors in hyperlipidemic subjects.

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**<u>Unsaponifiable Fraction of Rice Bran Oil Is
Capable of Hypolipidemic Action</u>**

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Animal Studies

Early research by Sharma and Rukmini (1987) explored the possibility that the

unsaponifiable portion of rice bran oil alone was responsible for hypocholesterolemic

effects. A control group of rats was maintained for eight weeks on a 1% cholesterol diet

with 10% groundnut oil and was compared to three experimental groups. In these

groups, either 0.2% or 0.4% unsaponifiable matter of rice bran oil was added or rice bran

oil replaced groundnut oil. Although the 0.2% unsaponifiable addition was ineffective at

significantly altering cholesterol levels compared to the control group, both the rice bran

oil substitution and the 0.4% addition of unsaponifiables significantly lowered liver

triglycerides, liver cholesterol, and total serum cholesterol while maintaining HDL levels.

Cholesterol levels were statistically indistinguishable between the diet containing

groundnut oil plus 0.4% unsaponifiables from rice bran oil and the diet in which the

groundnut oil was replaced with rice bran oil, showing that the active component is likely

contained in the unsaponifiable fraction.

Nagao, Sato, Takenaka, Ando, Iwamoto, and Imaizumi (2001) recently reported

similar benefits of unsaponifiables prepared from rice bran oil in a strain of exogenously

hypercholesterolemic rats. Animals were maintained for two weeks on a 0.5%

cholesterol diet with a 10% fat content from: (1) rice bran oil, (2) a mixture of palm and

safflower oils with a similar fatty acid content to that of rice bran oil, or (3) the oil

mixture described in (2) plus 0.25% of unsaponifiable content prepared from rice bran

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oil. Serum and liver total cholesterol concentrations were significantly lower and HDL levels significantly higher in both groups of rats consuming the unsaponifiables (within rice bran oil or added to the simulated rice bran oil) versus rats assigned to the simulated rice bran oil without added unsaponifiables. Higher fecal excretion of cholesterol was noted in the two unsaponifiable groups as well, although bile acid and coprostanol excretion was similar in all three groups. Analysis of liver mRNA's revealed no differences in prevalence of HMG-CoA reductase, LDL receptors, or cholesterol 7a-hydroxylase among the three experimental groups. This information led the authors to conclude that the unsaponifiable fraction of rice bran oil acts to lower cholesterol by interrupting cholesterol absorption in the gut, and not by altering hepatic cholesterol metabolism.

In a recent primate study, monkeys were fed in random order a control diet representative of the typical American diet and three experimental diets in which 20% of the energy content was comprised of either rice bran oil, canola oil, or corn oil (Wilson et al., 2000). Based on the comparative fatty acid contents of the three oils, both canola and corn oil diets were expected to outperform the diet containing rice bran oil. However, HDL levels were maintained on the rice bran oil diet and fell during consumption of the other two. No significant variation among the diets was noted with respect to total cholesterol levels. Given the higher saturated fat content and lower unsaturated fat content of the rice bran oil, these results suggest the unsaponifiable fraction is critical for this oil's ability to decrease risk of cardiovascular disease.

Human Studies

Lichtenstein et al. (1994), whose research was referred to previously, found hypolipidemic effects of rice bran oil to be comparable to that of canola, corn, and olive

oils in human subjects with moderately elevated cholesterol levels. Since results from the rice bran oil-enriched diet exceeded predictions using calculations based on fatty acid content, the authors surmised that a fraction of the oil other than the fatty acid portion, likely the unsaponifiables, must be acting to lower total cholesterol.

An experiment designed to assess the impact of plant sterols present in the unsaponifiable fraction from rice bran oil, used healthy volunteers with normal cholesterol levels (Vissers, Zock, Meijer, & Katan, 2000). The subjects were instructed to continue usual dietary and physical activity habits while supplementing their diets with a control margarine containing only trace amounts of plant sterols or one enriched with 2.1 g/day of the sterols from rice bran oil for three weeks each. The enriched margarine significantly lowered total and LDL cholesterol versus the control. HDL levels were unaffected by consumption of the enriched margarine.

Oryzanol in Unsaponifiable Fraction of Rice Bran Oil Is an Active Hypolipidemic Ingredient

Animal Studies

It has been hypothesized that oryzanol is the primary active ingredient in rice bran oil that is responsible for its ability to improve cholesterol levels. Seetharamaiah and Chandrasekhara (1989) attempted to answer the question of whether or not oryzanol was the sole ingredient in rice bran oil inducing this positive effect on cardiovascular health status. They examined the impact of the addition of 0.5% oryzanol to an experimental diet enriched with 1% cholesterol and 10% refined rice bran oil that contained only trace amounts of oryzanol. Liver weights of rats fed oryzanol were lower than those maintained on diets consisting of refined rice bran oil with no added oryzanol. In addition, the oryzanol-supplemented diet was associated with lower total cholesterol

levels. However, since improved cholesterol status was also observed in rats fed the diet with refined rice bran oil when compared to those maintained on a control diet containing 10% groundnut oil, the authors concluded that oryzanol, although critical, could not be the only active component.

Assuming oryzanol to be an important element in improving cholesterol levels, the optimal dosage and length of treatment time necessary to realize benefits was studied (Seetharamaiah & Chandrasekhara, 1988). Oryzanol was extracted from rice bran as detailed by Seetharamaiah and Prabhakar (1986), and was added in a crystalline form to test diets in varying amounts. Rats were randomly assigned to diets that were enriched with 1% cholesterol and 0.15% bile salts, and either devoid of oryzanol or oryzanol-enriched at levels of 0.2, 0.5, 1.0 and 2.0%. A control group was fed a diet free from both cholesterol and oryzanol. Liver weights of cholesterol-fed rats were significantly lower when oryzanol of any percentage was consumed. Significant variations in blood cholesterol levels were present by week five, at which time oryzanol-fed animals had lower levels compared to all other cholesterol-fed rats. Oryzanol also appeared to prevent HDL levels from dropping due to dietary cholesterol. A significant decrease in plasma triglyceride levels was seen in the 0.5% oryzanol group. Due to this apparent advantage, and the fact that liver cholesterol, triglycerides, and phospholipids in this group were significantly lower than in rats fed diets not enriched with oryzanol, the authors concluded that 0.5% oryzanol was an optimal dosage.

Research in this same laboratory was conducted to compare the cholesterol-lowering ability of oryzanol to that of two known hypolipidemic compounds, curcumin (yellow pigment in turmeric) and ferulic acid (Seetharamaiah & Chandrasekhara, 1993).

Rats fed a high cholesterol diet had similar HDL levels, but significantly higher serum cholesterol levels than animals maintained on a similar diet supplemented with any of the three compounds being tested. The most preferred HDL:LDL ratio was seen in the oryzanol-fed group. Both oryzanol and curcumin were effective at lowering total liver cholesterol, as well as esterified cholesterol and triglycerides. The authors reported oryzanol as a superior hypolipidemic agent to curcumin and ferulic acid.

Rong, Ausman, and Nicolosi (1997) published findings touting the ability of oryzanol to reduce cholesterol absorption in hamsters. Animals were sustained for 7 weeks on a hypercholesterolemic diet of 5% coconut oil and 0.1% cholesterol to which either no or 1% oryzanol had been added. Both oryzanol and cholesterol were dissolved in the coconut oil prior to being added to the diet. The oryzanol supplemented diet was associated with a significant decrease in total and non-HDL cholesterol levels in plasma. Compared to the control group, the oryzanol-fed animals experienced a 25% reduction in percent cholesterol absorption. In contrast, liver and intestinal HMG-CoA reductase activities were similar between the groups suggesting no differences in endogenous cholesterol synthesis.

A second experiment from which results were published in the same paper was a modification of the first. The oryzanol level in the experimental diet was reduced from 1.0% to 0.5% and the length of the study was increased to 10 weeks. Again, hamsters ingesting oryzanol showed an improved cholesterol status. Compared to the controls, this group had reduced plasma total cholesterol, reduced triglycerides, and more optimal HDL:non-HDL ratios. The authors concluded that oryzanol was a critical component of rice bran oil's hypolipidemic capacity and that its ability to reduce dietary cholesterol

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absorption was at least partially responsible for the improved cholesterol status seen in oryzanol-fed hamsters.

Human Studies

Sixty-seven patients with elevated cholesterol levels volunteered to take 300 mg/day of gamma-oryzanol so that its effect on cholesterol in humans could be examined (Yoshino et al., 1989). Although there was no control group, the patients were stratified into three groups according to the World Health Organization hyperlipidemia classification and encouraged not to change dietary or lifestyle habits for the 3-month duration of the study. All groups experienced a decline in total cholesterol levels, and to a significant degree in type IIb (high cholesterol and high triglycerides) and IIa (high cholesterol only) patients. At the conclusion of the experiment, mean values for total triglyceride levels were significantly lower and the type IIb subjects showed a significant increase in HDL levels. It should be noted that, although significant, these were small changes in cholesterol status. Importantly, however, no adverse effects of oryzanol supplementation were observed.

Absorption of Oryzanol

To my knowledge, only one publication to date attempts to quantify the absorption of oryzanol. Fujiwara, Sakurai, Sugimoto, and Awata (1983) investigated the absorption and metabolism of gamma-oryzanol in rats by (1) administering an oral dose of a triolein solution of radioactively labeled gamma-oryzanol at the C-3 position of ferulic acid, and (2) injecting the radioactively labeled compound into the mid-ileal lumen of the small intestine. In the oral dose portion of the experiment, radioactive urinary and fecal excretion lasted for 72 hours. The fecal radioactive excretion

represented 84.5% of the total dose. While 9.8% of the radioactivity was recovered in the

urine, the fraction of urine expected to have contained oryzanol did not show any

radiation, leading the authors to believe that oryzanol absorbed in the intestine was

completely metabolized in the body. Radioactivity was also detected in the blood,

reaching the highest level of radioactivity four hours after oral administration. When

radioactive-labeled gamma-oryzanol was injected into the lumen, the majority of it

remained in the luminal fluid, suggesting that oryzanol is poorly absorbed in the gut.

Measurement of Absorption of Oryzanol in Liver

To my knowledge, no studies have measured the absorption of oryzanol in liver

without using radioactive tracers. However, a plethora of research exists in which

cholesterol in this organ is quantified. Oryzanol from rice bran oil is a mixture of at least

ten ferulate esters of triterpene alchohol (Xu & Godber, 1999). The chemical structures

of the triterpene alcohols are similar to that of cholesterol (Figure 1, Figure 2).

Therefore, it is logical to attempt to measure oryzanol using an accepted method for
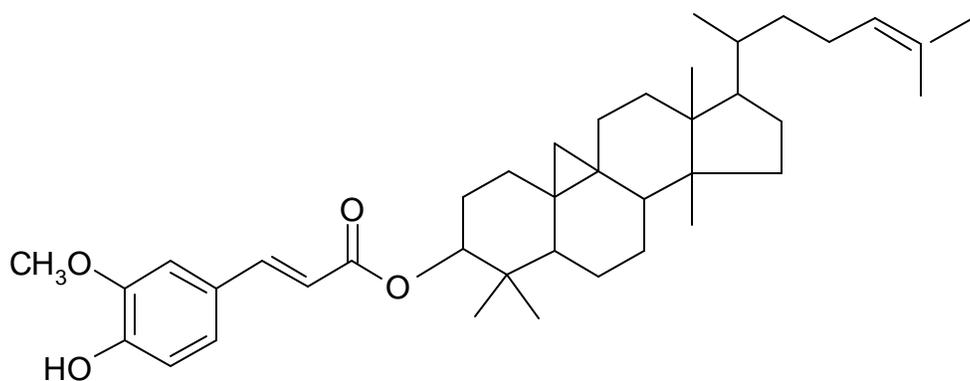
measuring cholesterol.

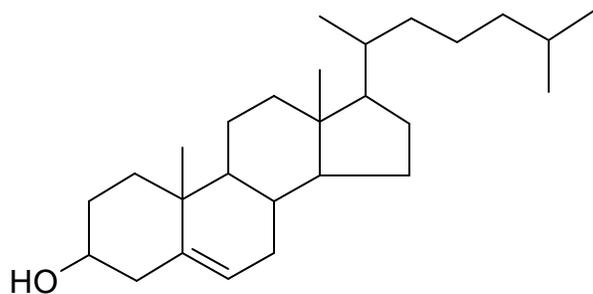**FIGURE 1** Cycloartenyl ferulate, a common form of oryzanol.

FIGURE 2 Cholesterol.

A review of the literature disclosed that scientists frequently chose the extraction

method of Folch, Lees, and Stanley (1957) to extract lipids from tissues (Koba et al.,

2000; Fukushima et al., 1999; Hundemer et al., 1991). Others used modified versions of

this technique (Lee, Akoh, Flatt, & Lee, 2000; Wilson et al., 2000). After extracting

lipids from liver or adipose tissue, various methods such as enzymatic kits (Kahlon et al.,

1992, 1993, 1998; Hundemer et al.; Wilson et al.), gas-liquid chromatography

(Fukushima et al., 1999) and high performance liquid chromatography (HPLC) (Koba et

al.; Lee et al.) were used to quantify the cholesterol. Xu and Godber (1999) have

demonstrated that oryzanol in rice bran oil can be accurately quantified using an HPLC

method.

Summary

Varying and sometimes conflicting results have been reported regarding the

effects of rice bran, rice bran oil, the unsaponifiable content of rice bran oil, and oryzanol

on serum and liver cholesterol. Many variables were at work including the species used,

the duration of the studies, the specifications for the diets, and the analytical methods

utilized. Nevertheless, the body of research suggests that rice bran or a component of

rice bran, such as oryzanol, is likely capable of lowering risk of heart disease by

improving cholesterol levels.

CHAPTER 3

METHODS

Animals and Diets

Sprague Dawley, nine month old, retired breeder rats were purchased from Harlan Sprague Dawley, Inc. (Indianapolis, IN) to serve as the animal models. Upon arrival, laboratory animals were individually housed in suspended wire-mesh-bottomed, stainless steel cages in a temperature controlled room with a 12-hour light/dark cycle. Animals were allowed free access to feed and water during the entire study. A total of 47 rats were blocked by weight and then randomly assigned to a treatment group. Twenty rats were used as controls and the remaining 27 were equally divided among three experimental diet groups. All rats assigned to experimental diets and half of the rats serving as controls were ovariectomized two weeks into the study, while the remaining control animals underwent a sham surgery.

All diets (Table 1) were a modification of the AIN-93M rodent diet formulated by Reeves, Nielsen, and Fahey (1993) for nutritional maintenance of adult rats. Experimental diets were a replication of the control (C) diet with the exception of the substitution of high-oryzanol rice bran oil for corn oil in the rice bran oil (RO) diet, and the addition of 2.8 g/kg oryzanol in the crystalline oryzanol (CO) and dissolved oryzanol (DO) diets. The 2.8 g/kg of supplemental oryzanol was calculated based on the 7% oryzanol content of the high-oryzanol rice bran oil to allow for an equal amount of oryzanol in all three test diets. Rice bran oil used in the RO diet was donated by Riceland, Inc. (Stuttgart, AR). Crystalline oryzanol, purchased from Samlong Chemical Co., LTD. (Changxin, Jiangsu, China), and corn oil stripped of tocopherols, purchased

from Dyets, Inc. (Bethlehem, PA), were used in the CO and DO diets. Crystalline

oryzanol and corn oil were simply added as separate ingredients in the CO feed mixture.

For the DO group, crystalline oryzanol was completely dissolved in the corn oil portion

by stirring constantly with a stirbar for approximately one hour on a slightly warmed hot

plate before adding the solution to the diet mix. The diets were stored in air-tight

containers at -20 °C.

TABLE 1

Diets in grams per kilogram.

Ingredients	C	RO	CO	DO
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	0	40	40
Rice bran oil	0	40	0	0
Cellulose	50	50	50	50
Mineral Mix AIN-93M	35	35	35	35
Vitamin Mix AIN-93M	10	10	10	10
Choline bitartrate	2.5	2.5	2.5	2.5
L-cystine	1.8	1.8	1.8	1.8
Oryzanol	0	0*	2.8	2.8**

*Oryzanol (2.8 g) was contained in the high-oryzanol rice bran oil; no additional amount
was added.
**Dissolved in the stripped corn oil.

Diet samples were analyzed using a reverse-phase high-performance liquid

chromatographic (HPLC) method in order to verify the oryzanol content of each test diet.

To prepare each sample for analysis, a 0.25 g portion was weighed in a 10-mL glass test

tube. Five mL of hexane was then added and the contents were vortexed for 15 seconds.

This was followed by incubation in a 60 °C water bath for 30 minutes. The test tube was

removed and vortexed for 15 seconds, twice, at equal intervals during the water bath and finally upon removal. The sample was then centrifuged using a Beckman model J-6B centrifuge (Palo Alto, CA) at 4,000 xg for 10 minutes and a portion of the upper layer of hexane solution was siphoned off and transferred into a vial for HPLC analysis.

The HPLC system (Waters, Milford, MA) and method of analysis was as described by Xu and Godber (1999) in their separation of gamma-oryzanol components. Fifty µL of prepared extract was injected using a Waters 715 Ultra Wisp autosampler. A mobile phase of methanol:acetonitrile:dichloromethane:acetic acid (50:44:3:3) was used at a flow rate of 1.4 mL/min (Waters 510 pump) with a C18 reverse phase column from Supelco (4.6 mm x 25 cm, Bellefonte, PA). The Waters 486 tunable absorbance detector was set at a wavelength of 330 nm. Analysis was performed in triplicate for each of the three oryzanol-containing diets and the values within each group were averaged. A formula based on a standard curve for oryzanol was used to convert peak areas to mg/g units (Appendix A).

Data Collection

Food was replenished on Monday, Wednesday, and Friday of each week and food intake was calculated as weight of food offered less that not eaten or spilled. Body weights were recorded once per week and notes were made regarding any health problems. At weeks 6 and 10, each animal was placed in an individual metabolism cage for 24 hours according to a random schedule to collect feces. Samples were collected in plastic containers and frozen until analyzed.

Daily oryzanol intake for each rat was calculated as the product of the percent oryzanol in each diet as determined by the HPLC analysis, and the average daily amount

of food consumed during and one to four days prior to its 24-hour stay in the metabolism cage. The number of days averaged to obtain the daily intake value varied due to the Monday, Wednesday, Friday food intake record and the random metabolism cage schedule. The decision to use averaged data over these time periods was based primarily on findings from two recent rat studies. Dvir et al. (2000) reported 15.06 hours were required for the first detection of excreted intake from a 2% cellulose diet. In a separate study, 19.5 to 24.7 hours were required for 80% of intake from a 5% fiber diet to be excreted (Ferguson & Harris, 1998). The diets used in the current study contained 5% fiber from cellulose. Additionally, Fujiwara et al. (1983) noted that radioactive fecal excretion ceased within 72 hours of an oral dose of radioactively labeled gamma-oryzanol administered to rats.

Beginning on Monday of week 12, approximately 10 rats were sacrificed each morning in random order. Animals were anesthetized with isoflurane and terminated via cardiac puncture and cervical dislocation. Whole blood drawn from cardiac puncture in the morning was collected in a glass vacutainer, placed on ice and allowed to clot. Samples were centrifuged each afternoon at 1,000 xg for 10 minutes at room temperature using a Dynac™ centrifuge (Clay Adams, Parsippany, NJ). The resulting serum was placed in eppendorf tubes and frozen at -20 °C. Livers were excised, rinsed, blotted, weighed, divided into three aliquots, wrapped in aluminum foil, and placed immediately in liquid nitrogen. Livers were transferred later the same day to a freezer maintained at -20 °C for storage.

Fecal Analysis

Feces were analyzed for oryzanol content by HPLC so that the percentage of dietary oryzanol that was neither absorbed nor metabolized could be compared among the

25

three diet groups. In preparation for analysis, frozen fecal specimens were allowed to thaw at room temperature. The specimens were then transferred to individual glass jars and dried in a VWR 1410 vacuum oven (West Chester, PA) at 60 °C and –15 inHg of pressure. Samples were weighed periodically during the drying process to verify that moisture had been eliminated. Once dehydrated, each sample was ground with a mortar and pestle, returned to the glass container, capped, and placed in a Dry-Keeper desiccator cabinet purchased from Bel-Art Products (Pequannock, NJ) for storage. A 0.25 g portion of each of the dried fecal samples was analyzed using HPLC by an identical method as detailed for the diet analysis above. Again, peak areas were converted to mg/g units according to a formula based on a standard curve for oryzanol (Appendix A). The daily fecal excretion of oryzanol was extrapolated from the amount of oryzanol detected in each 0.25 g fecal sample. Daily fecal excretion of oryzanol was divided by daily oryzanol intake to provide the percentage of dietary oryzanol recovered in the feces.

<div align="center"><u>**Serum Analysis**</u></div>

Cholesterol Quantification Using Enzymatic Colorimetric Assay

To examine the effect of the various diets on cholesterol levels, serum samples from all five groups were analyzed for total cholesterol using an enzymatic colorimetric method (Infinity™ cholesterol reagent, procedure no. 401, Sigma Diagnostics, Inc., St. Louis, MO). Serum was allowed to thaw at room temperature prior to lipid analysis. Ten µL of serum sample was added to 1.0 mL of reagent in a glass test tube. The contents were vortexed and allowed to incubate in a 37 °C water bath for five minutes before a portion was transferred to a cuvette. Colorimetric readings were done using a Hitachi U-2000 UV/Vis spectrophotometer (Weston, MA) set at 500 nm. Assays were performed in

duplicate. When the reproducibility of assay values for a particular sample was at least 90%, the two values were averaged. Otherwise, a third assay was performed and an average of all three was used. To reduce variation caused by possible hemolysis of the sample, the absorption value was reduced by the colorimetric reading obtained from analyzing 10 µL of serum added to 1.0 mL of water. The total cholesterol value was then calculated by dividing the adjusted absorption value of the prepared serum sample by the absorption value of the calibrator, and multiplying the result by the concentration of the calibrator, 200 mg/dL (Appendix A).

Oryzanol Detection Using GCMS

Unexpected results of higher cholesterol levels in oryzanol-fed rats compared to the controls led to the question of whether or not the assay was measuring phytosterols as well as cholesterol. Therefore, a serum sample from the RO group was prepared for analysis by gas chromatography and mass spectrophotometry (GCMS) to determine the presence of oryzanol. The serum sample was allowed to thaw at room temperature and 0.5 mL was measured into an 8-mL glass vial. An extraction was done by adding 1.0 mL of hexane, vortexing for 15 seconds, centrifuging for 10 minutes at approximately 500 xg, and transferring the supernatant to a clean 10-mL test tube. The hexane extraction was repeated twice and the resulting extract was divided between two test tubes. A Zymark TurboVap LV evaporator (Hopkinton, MA) with a nitrogen flow at 10 psi and 45 °C for 20 minutes was used to evaporate the solvent. One test tube was stored for possible future analysis, while contents in the other test tube were derivatized by adding 0.1 mL of pyridine and 0.1 mL of N,O-bis(trimethylsilyl) acetamide (BSA) + trimethylchlorosilane (TMCS) from Supelco (Bellefonte, PA). This was vortexed and

placed in a 30 °C water bath for 30 minutes. GCMS was performed as described by Xu and Godber (1999). One μL of derivatized sample was injected into a Saturn 2000 GCMS (Varian, Walnut, Creek, CA) with an injection port temperature of 300 °C. The initial oven temperature was 100 °C and was increased 3 °C per minute to a maximum of 280 °C. Helium was used as the carrier gas at a flow rate of 1.0 mL/min.

The resulting chromatograph of the RO sample revealed small peaks with greater retention times than the cholesterol peak, suggesting the possible presence of phytosterols. To determine whether these peaks included oryzanol, the procedure was repeated with a sample from the C group for comparison. When similar peaks resulted from GCMS analysis of the C serum, the possibility of the peaks being representative of oryzanol was eliminated.

Cholesterol Quantification Using GCMS

Hexane Extraction. To validate the cholesterol values from the enzymatic colorimetric assay, the serum sample set was reanalyzed for total cholesterol via GCMS. An 8.7 ppm stock solution of a-cholestane dissolved in hexane served as a standard. After allowing serum samples to thaw at room temperature, 0.25 mL of serum and 1.0 mL of the internal standard stock solution was measured into a 7-mL glass vial. (Only 0.25 mL of stock solution was added to the first five samples. Following analysis of these samples, the amount of stock solution was adjusted from 0.25 to 1.0 mL to obtain internal standard and cholesterol peak areas of similar size). Next, one mL of hexane was added. The contents were vortexed, shaken vigorously, and then centrifuged at 1,000 xg for 10 minutes. The supernatant was transferred to a clean test tube. This extraction process was performed in triplicate, each time transferring the supernatant to the same

test tube. The cumulative solvent was evaporated using a CentriVap Console vacuum centrifuge (Labconco, Kansas City, MO) at 45 °C for 20 minutes. The samples were then derivatized by adding 0.1 mL pyridine and 0.1 mL BSA + TMCS, capping, vortexing, and incubating in a 30 °C water bath for 30 minutes. One µL of sample was injected into the GCMS for quantification of cholesterol under the same conditions as outlined above.

Chloroform:Methanol Extraction Results from GCMS analysis of serum following extraction with hexane varied greatly from sample to sample. Due to these unintelligible values, the method was abandoned prior to completing analysis of the entire sample set. In a second attempt, the methodology was adjusted to extract lipids according to Folch, Lees, and Sloane Stanley (1956). A 2:1 solution of chloroform:methanol was used as the reagent. The internal standard a-cholestane (0.7 mg) was added directly to the reagent (250 mL), creating a 2.8 ppm concentration. Per Folch et al., a rinsing solution was created by mixing an 8:4:3 ratio of chloroform: methanol:water, allowing layers to form, and retaining the upper phase. The amount of rat serum remaining from the study constrained the following analysis to three samples per diet group. One-half mL of serum and 7.0 mL of chloroform:methanol reagent was added to a 15-mL test tube. The contents were vortexed for 30 seconds and then filtered by pouring into a small glass funnel lined with Whatman 7.0 cm No.1 qualitative filter paper (Springfield Mill, Maidstone, Kent., England). An additional 3.0 mL of reagent was used to rinse the filter paper. The filtered solution was collected into a clean 15-mL test tube and 2.0 mL of distilled water was added. Contents were vortexed for 30 seconds and centrifuged at 2,000 xg for five minutes in a Hermle Z383K centrifuge from Labnet International, Inc. (Woodbridge, NJ). The supernatant was removed using a Pasteur

pipette and discarded. Twice, approximately 1.5 mL of the rinsing solution was used to rinse the tube, and again the supernatant was discarded. Next, 2.0 mL of methanol was added, the contents were vortexed 30 seconds, and the solvents were completely evaporated in the CentriVap Console at 45 °C for 2 ½ hours. Derivitization followed using 0.1 mL each of pyridine and BSA + TMCS as described previously. The dervatized contents were diluted with 1.8 mL of hexane prior to being injected into the GCMS instrument. The settings for the GCMS were identical to those detailed previously.

Standard Curve. A standard curve was produced in order to translate GCMS peak areas obtained for cholesterol into actual serum cholesterol concentrations. Fifty mL of hexane was added to 2.0 mg of cholesterol standard. One mL of this solution was added to each of three 15-mL test tubes. The solvent was evaporated in the CentriVap Console set at 45 °C for 20 minutes. Next, 10.0 mL of the 2.8 ppm solution of a-cholestane in chloroform:methanol reagent was added to each test tube. Again, the solvent was evaporated using the CentriVap Console set at 45 °C for 2 ½ hours. To create varying degrees of dilution, the following amounts of hexane were added to the three test tubes containing the cholesterol and a-cholestane standards: 1.3, 1.8, and 2.3 mL. One µL from each dilution was injected for GCMS analysis. Again, GCMS specifications remained the same as mentioned earlier. (See Appendix A for resulting formula).

<u>**Liver Analysis**</u>

Developing Methodology for Oryzanol Quantification

Detection of oryzanol in the liver would suggest that at least some oryzanol is absorbed intact. Several procedures were tested to formulate an acceptable methodology

for preparing liver samples for quantification of oryzanol content using HPLC or GCMS analysis. A 0.25 g portion of liver from a rat not included in the feeding study was weighed and placed in a glass tube with a conical shaped bottom. Two mL of hexane were added and the contents were homogenized using a Duall® PTFE pestle (Kimble/Kontes, Vineland, NJ) suitable for soft tissues. The pestle was driven by a Black & Decker ¼ inch, 120 volt drill (Townsend, MD) with electrical power supply regulated at 60 volts by a variable transformer. Aluminum foil was wrapped around the top of the conical tube during homogenizing to limit evaporation of the solvent. Although portions of blended liver adhered to the pestle and tube, it was apparent that the tube could easily handle a larger liver sample. Therefore, in a second attempt, the liver size was increased to 0.5 g and hexane was added incrementally (0.5 mL and 1.0 mL) during homogenization and a final 1.0 mL was used to flush the pestle and tube. The benefit of flushing was questionable as the majority of the liver tissue appeared to remain on the inside of the conical tube. The products of both of these methods were discarded.

Two samples of liver excised from a rat not included in the feeding regimen for this project, along with two samples of liver from a rat in the RO group, were prepared for analysis next. A 0.5 g portion of each was weighed and placed in a conical tube. The duplicate sample from the rat not in the study was spiked with 0.005 g of crystalline oryzanol. Again, hexane was added incrementally in amounts of 0.5 mL and 1.0 mL with homogenization after each addition. Finally, 0.5 mL of hexane was used to flush the pestle. However, flushing did not appear to be advantageous and was abandoned in future procedures. Homogenized mixtures were transferred to clean test tubes and each was vortexed for 15 seconds and centrifuged for 10 minutes at 4,000 xg. Prior to

centrifuging, the duplicate sample from the RO rat was incubated in a 60 °C water bath for 30 minutes, and vortexed twice at 10 minute intervals during incubation and upon removal. The supernatants of each of the four samples were transferred to HPLC vials. The same method used to determine oryzanol content in the feces and diet via HPLC analysis was followed.

Curves produced by the chromatograph for the two samples representing the RO group did not differ substantially, suggesting that the incubation period was superfluous (Appendix B). Although the areas beneath the curves of these RO samples were greater than that of the first sample from the rat not fed oryzanol, all three curves were similar in shape and did not resemble the curve of the duplicate sample to which crystalline oryzanol had been added. Since the peak did not represent oryzanol, the compound being detected was unknown.

HPLC Analysis

The method was adjusted slightly a fourth and final time and one liver from each of the five experimental groups was analyzed. Additionally, a duplicate sample of liver from group C was spiked with 0.01 g of cholesterol in an attempt to identify the visible peak. Again, approximately 0.5 g of liver was weighed and placed in a conical glass tube. One mL of hexane was added and the contents were homogenized for 60 seconds. Another 1.0 mL of hexane was added and the contents were homogenized an additional 30 seconds. The homogenate was poured into a clean test tube and centrifuged at 4,000 xg for 10 minutes. The supernatant was transferred into a vial and HPLC analysis as detailed previously was performed. Peaks resulting from HPLC analysis of liver did not

appear to be representative of oryzanol or cholesterol. Therefore, GCMS technology was utilized next to identify the compound being detected.

GCMS Analysis

During a repeated HPLC analysis of a liver extract from the RO group, the most prominent peak was collected into a clean test tube. Solvents were evaporated under nitrogen flow at 45 °C for 30 minutes at a pressure of 10 psi. The sample was derivatized by adding 0.1 mL of hexane, 0.2 mL of BCl_3-methanol, and 0.1 mL of 2,2-dimethoxypropane. The mixture was incubated for 10 minutes at 60 °C. Upon removal, 0.5 mL of hexane and 0.1 mL of water were added and the tube was shaken. Layers were allowed to form and the upper organic layer was transferred using a Pasteur pipette into a clean vial containing a small amount of anhydrous sodium sulfate. Again, the tube was shaken, contents were allowed to settle, and the upper layer was transferred into a clean vial. GCMS analysis was performed using specifications as detailed above for serum samples. A search of probable compounds associated with the resulting GCMS peaks revealed that the collected sample most likely consisted primarily of C:16 fatty acids.

Oil Analysis

HPLC analysis of the tocopherol-stripped corn oil used in the CO and DO diets was conducted to verify that any phytosterols present in the oil would not be measured as oryzanol. The high-oryzanol rice bran oil used in the RO diet was analyzed for comparison. Approximately 0.5 g each of oil were weighed into glass test tubes. An extraction was carried out by adding 2.0 mL of hexane, vortexing for 15 seconds, and centrifuging for 10 minutes at 4,000 xg. The supernatant was transferred into an HPLC vial for analysis as previously described.

Statistical Analysis

The SAS statistical software package version 8.2 (SAS Institute, Cary, NC, USA) was used to analyze the data from this study. Analysis of variance (ANOVA) was performed using PROC MIXED to compare the percentage of dietary oryzanol recovered in the feces among the three experimental groups fed oryzanol. Comparisons were also made between the two fecal collection periods, as well as among diet group and collection period combinations. Tukey's test was used to determine specific differences among the data. Total cholesterol levels of all five test groups were also compared using ANOVA obtained via PROC GLM. Results were considered significant when $p \leq 0.05$.

Human Pilot Study

A pilot study was designed to determine if any oryzanol from the high-oryzanol rice bran oil might be absorbed in the human gut and present in a sufficient amount in the blood to be detected. Also, using human serum to investigate the possible presence of oryzanol allowed for conservation of rat serum needed for cholesterol analysis. The average daily intake of rice bran oil for the RO group was used to calculate a comparable daily intake for a 70 kg person based on body weight. This resulted in an amount that was unfeasible to consume. Therefore, a second calculation was done based on the percentage of calories in the rat diet attributable to the rice bran oil (Appendix C). It was estimated that the RO test diet contained 9.6% of calories as rice bran oil. For a 2,000 kcal diet, 21.33 g of rice bran oil would provide 9.6% of the calories. A muffin recipe (Appendix D) was modified to contain double this amount in a yield of six muffins, so that three muffins per day could be consumed by a human volunteer during a 48-hour period.

A non-fasting blood sample was drawn at 1:00PM by the Student Health Center at Louisiana State University. The lab provided serum prepared by allowing the whole blood to clot and then centrifuging and transferring to a clean glass vacutainer. The serum was divided into three eppendorf tubes and stored at -20 °C. The human volunteer consumed six muffins according to the following schedule:

Day 1
1:30PM – muffin 1
7:30PM – muffin 2

Day 2
9:00AM – muffin 3
2:00PM – muffin 4
8:00PM – muffin 5

Day 3
8:00AM – muffin 6

A second non-fasting blood sample was drawn at 1:00PM on day three by the LSU Student Health Center and the resulting serum was provided. The same protocol was followed as for the first blood drawn.

HPLC Analysis of Serum for Oryzanol Detection

The before and after serum samples were analyzed for oryzanol content via HPLC. A 0.3 g portion of each sample was weighed in a test tube in duplicate. Two mL of hexane was added to each test tube and the contents were vortexed for 15 seconds. Samples were then centrifuged at 4,000 xg for 10 minutes and the supernatant was transferred to an HPLC vial. The HPLC analysis was performed as described previously for rat serum. Results from this procedure did not reveal any oryzanol, and graphs from the HPLC analysis of the before and after serum were indistinguishable. To produce a more concentrated extract, 1.0 g of serum was mixed with 2.0 mL of hexane, vortexed for

15 seconds, centrifuged at 4,000 xg for 10 minutes, and the supernatant was then

transferred to a clean test tube. An additional 3.0 mL of hexane was added to the serum,

the contents were vortexed and centrifuged, and the supernatant was transferred to the

test tube containing the previous extraction. Nitrogen gas at 45 °C and 10 psi for 30

minutes was used to evaporate the solvent from the serum extracts. A final 0.5 mL of

hexane was added, the contents were vortexed, and the HPLC analysis was repeated. In

spite of the concentrated sample, no oryzanol was detected.

Serum Cholesterol

In addition, a cholesterol assay was performed on the before and after serum

samples. The same enzymatic colorimetric method was followed as for the rat serum

detailed in the serum analysis section previously.

CHAPTER 4

RESULTS AND DISCUSSION

Weight Gain, Final Weight, and Liver Weight

Final weight was not significantly different (P=0.2088) among the three oryzanol (RO, CO, DO) and two control diet groups (C, SH) (Figure 3, Table 2). However, weight gain approached significance (P=0.0597) with the greatest variation between the SH and RO rats. In general, all five groups showed a gradual increase in weight over the 11-week feeding period with the exception of a dip at week 4 and a leveling off in the C, SH, and DO groups beginning at week 10. Feed efficiency was significantly higher in animals fed rice bran oil (RO) versus the sham-operated (SH) control group. Differences in feed efficiency among the remaining groups were not significant.
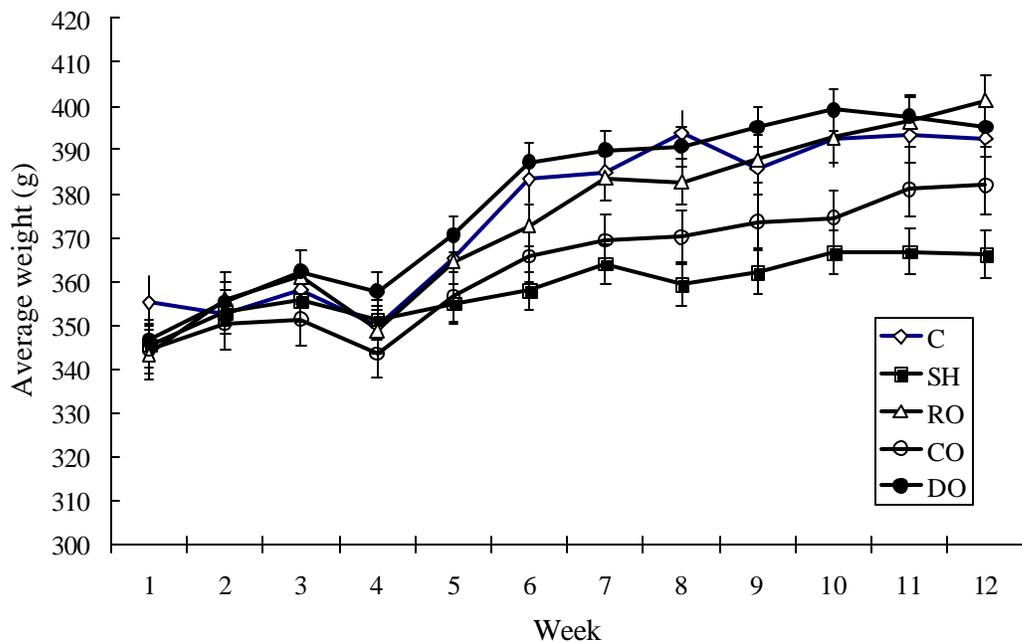

FIGURE 3 Average weekly weight by diet group. Data points are means ± SEM.

These results are consistent with those reported by Edwards and Radcliffe (1994), where rats fed diets of either rice bran oil or corn oil for 28 days did not differ significantly with respect to final weight, weight gain, or feed intake. Likewise, rat studies comparing rice bran oil to groundnut oil revealed similar consumption and weight gain between test groups (Sharma & Rukmini, 1986), even when rice bran oil feeds were supplemented with additional amounts of the unsaponifiable fraction (Sharma & Rukmini, 1987) or oryzanol (Seetharamaiah & Chandrasekhara, 1989). Previous comparisons of feed intake and growth between groups of rats assigned to feeding regimens of rice bran oil versus safflower oil were also statistically indistinguishable (Radcliffe et al., 1997; Koba et al., 2000).

In this research, liver weights were not statistically different among the experimental groups (Table 2). Some studies have shown significant reduction in liver weights in animals consuming oryzanol (Seetharamaiah & Chandrasekhara, 1989; 1988). However, an experiment comparing rice bran oil to other edible oils (Sharma & Rukmini, 1986), and another investigating the effects of unsaponifiables in rice bran oil (Sharma & Rukmini, 1987) did not find significant differences. In summary, the weight gain, final weight, and liver weight results reported here were not surprising, as it was not expected that oryzanol would necessarily affect these outcomes.

<p align="center">**Balance Study**</p>

Feed Intake

Although feed intake did not differ significantly among the three oryzanol-fed groups (P = 0.1451), animals assigned to the rice bran oil (RO) diet consumed more on

average than other diet groups (Table 3, Table 5). Perhaps the rats found the flavor or texture of this feed to be more palatable.

TABLE 2

Effect of test diets on total weight gain, final body weight, feed efficiency, and liver weight.[1,2]

Diet Group	Weight Gain (g/81 days)	Final Weight (g)	Feed Efficiency[3]	Liver Weight (g)
C (9)	39 ± 6.2	393 ± 13.7	2.9 ± 0.43a,b	9.941 ± 0.2978
SH (10)	21 ± 9.7	366 ± 9.2	1.5 ± 0.68a	10.332 ± 0.3008
RO (8)	60 ± 11.4	401 ± 11.8	4.2 ± 0.70b	10.226 ± 0.3243
CO (8)	37 ± 11.8	382 ± 14.8	2.8 ± 0.81a,b	9.689 ± 0.5469
DO (9)	50 ± 7.8	395 ± 7.2	3.5 ± 0.45a,b	9.865 ± 0.2985

[1]Values are means ± SEM. The number of animals in each group follows the diet group name in parentheses.
[2]Different letters within a column represent significant differences ($P < 0.05$).
[3]Feed Efficiency is calculated as g of weight gained / 100 g of feed intake.

Feed intake during the second stay in the metabolism cages (Period B) was significantly less for all diet groups both independently and combined when compared to the first stay (Period A) (Table 3, Table 5). It was initially hypothesized that the larger sizes of the rats at the latter stage inhibited their access to feed in the confined metabolism cages. However, a review of records for the entire study revealed that feed intake averaged across all groups declined steadily after week 5 of the feeding regimen. Similarly, between week 7 and the completion of the study, a decreasing trend occurred within each diet group. The gradual increase in average weight of the rats over this same period, at least through the second collection period, leads to the conclusion that the drop in feed consumption for Period B as compared to Period A was due to increased feed efficiency in the maturing animals.

Oryzanol Intake

Oryzanol intake values were calculated based on actual oryzanol concentrations of the diet mixtures. HPLC analysis of diet samples revealed a slightly lower percentage of oryzanol in the RO diet mixture (2.3 g/kg) versus the crystalline oryzanol (CO) and crystalline oryzanol dissolved in corn oil (DO) diets, which were both equivalent to the predicted concentration of 2.8 g/kg. The HPLC analysis also revealed differences in chromatograph curve shapes between the crystalline form and the rice bran oil version (Appendix E). Obviously, the decrease in feed intake associated with Period B translated to a decrease in the average oryzanol intake, as well (Table 3, Table 5). However, significant differences were not found in oryzanol intake among the three oryzanol-fed groups.

TABLE 3

Feed and oryzanol intake associated with fecal collection periods of oryzanol-fed rats.[1,2]

Diet Group	Batch	Feed Intake (g/day)	Oryzanol Intake (mg/day)
RO (8)	A	20 ± 0.9	45.387 ± 2.0353
	B	17 ± 1.0	39.137 ± 2.3416
	Both	18 ± 0.7	42.262 ± 1.7021
CO (8)	A	18 ± 0.4	51.862 ± 1.0890
	B	15 ± 0.7	42.233 ± 1.9515
	Both	17 ± 0.6	47.047 ± 1.5933
DO (9)	A	18 ± 0.8	50.379 ± 2.3361
	B	15 ± 0.6	43.484 ± 1.5680
	Both	17 ± 0.6	46.931 ± 1.6005

[1]Values are means ± SEM. The number of animals in each group follows the diet group name in parentheses.
[2]Significant differences are noted in Table 5.

Oryzanol Recovery

Combining data from both fecal collection periods revealed that a significantly higher percentage of dietary oryzanol was recovered in the feces of animals fed the CO (41.9 ± 2.21) diet compared to both the RO (28.3 ± 3.54) and DO (27.8 ± 2.63) diets (Table 4, Table 5). Analysis of data collected solely from Period A also resulted in significantly higher recovery percentages of dietary oryzanol in the fecal matter of the CO (44.6 ± 3.33) versus the RO (26.2 ± 5.26) and DO (26.0 ± 2.88) groups. The trend was in the same direction for Period B data, but numbers were not significant. No oryzanol was detected in the feces of C or SH rats which consumed oryzanol-free diets. Significant differences in data related to the oryzanol balance study are summarized in Table 5. See Appendix F for examples of HPLC curves from each diet group.

TABLE 4

Total weight of dry feces, amount of oryzanol detected in feces, and percentage of dietary oryzanol recovered in the feces.[1]

Diet Group	Batch	Feces (g)	Fecal Oryzanol (mg)	% Oryzanol Recovered In Feces
RO (8)	A	1.220 ± 0.0940	11.818 ± 2.3186	26.2 ± 5.26
	B	1.071 ± 0.1479	11.643 ± 1.6122	30.4 ± 4.96
	Both	1.146 ± 0.0868	11.731 ± 1.3643	28.3 ± 3.54
CO (8)	A	1.365 ± 0.0593	23.116 ± 1.7483	44.6 ± 3.33
	B	0.996 ± 0.0718	16.536 ± 1.4123	39.2 ± 2.82
	Both	1.180 ± 0.0636	19.826 ± 1.3510	41.9 ± 2.21
DO (9)	A	0.956 ± 0.1773	13.383 ± 1.8682	26.0 ± 2.88
	B	0.784 ± 0.1410	12.617 ± 1.7500	29.6 ± 4.50
	Both	0.870 ± 0.1118	13.000 ± 1.2452	27.8 ± 2.63

[1]Values are means ± SEM. The number of animals in each group follows the diet group name in parentheses.

TABLE 5

Significant effects of test diets on feed intake, oryzanol intake, feces, fecal oryzanol and percentage of dietary oryzanol recovered in the feces.[1]

Diet Group	Batch	Feed Intake (g/day)	Oryzanol Intake (mg/day)	Feces (g)	Fecal Oryzanol (mg)	% Oryzanol Recovered In Feces
	A vs B	P < 0.05	P < 0.05	P < 0.05	P < 0.05	NS
CO vs RO		NS	NS	NS	P < 0.05	P < 0.05
CO vs DO		NS	NS	NS	P < 0.05	P < 0.05
CO	A vs B	NS	NS	NS	P < 0.05	NS
CO vs RO	A	NS	NS	NS	P < 0.05	P < 0.05
CO vs DO	A	NS	NS	NS	P < 0.05	P < 0.05

[1]NS implies a non-significant result. P < 0.05 implies significance.

Only one previously published experiment measuring fecal recovery of dietary oryzanol was located. In 1983, Fujiwara et al. found oryzanol to be poorly absorbed. After injecting an oral dose of radioactively labeled crystalline oryzanol (at the C-3 position of the ferulic acid), 84.5% of the radioactivity was recovered in the feces. In a second experiment published in the same article, only 10 to 20% of oryzanol appeared to be metabolized in the intestine. This would suggest that at least 64.5% of intact oryzanol was excreted in the feces. This is much higher than the findings of this study. Although the highest recovery rates were seen in the CO group collected during Period A, the average recovery rate was only 44.6%. It is possible that a portion of oryzanol in the feces was bound up in lipid complexes rendering hexane incapable of complete extraction. This occurrence would have resulted in average recovery values lower than the true mean. Or perhaps, the percentage of oryzanol metabolized in the study by Fujiwara et al. was underestimated due to the small sample size of three.

An alternative way to view the results from the oryzanol balance study is to consider the percentage of dietary oryzanol not recovered in the feces. This portion is assumed to have been either metabolized or absorbed, or both. The percentage of dietary oryzanol not recovered in the feces was significantly higher for the RO and DO groups compared to the CO group (Figure 4). This data supports our hypothesis that dissolving crystalline oryzanol in oil enhances its bioavailability.

Again, we must consider the likelihood that hexane extraction of oryzanol in the feces was not complete. It is possible that oryzanol in rice bran oil or dissolved in corn oil is less likely to be bound up in lipid complexes in the intestinal lumen than crystalline oryzanol. This explanation could be tested by repeating the extraction with a chloroform:methanol mixture (Folch et al., 1957) and comparing the results to those from the hexane extraction.

Rice Bran Oil and Corn Oil Analysis

Although both the tocopherol-stripped corn oil and the high-oryzanol rice bran oil generated HPLC peaks with retention times between four and ten minutes, the pinnacle of the rice bran oil peak appeared at 7.354 minutes, while the highest point on the corn oil graph was not reached until 9.382 minutes. Moreover, the area under the curve for a similar sample size was nearly 70-fold for the rice bran oil versus the corn oil. Results from this qualitative test suggested that while some compounds other than oryzanol might have been quantified as oryzanol, the impact on recovery values would have been minimal. Oryzanol was quantified during HPLC analysis of the feces, serum, and liver using peaks with retention times between approximately 5.5 and 11.5 minutes.
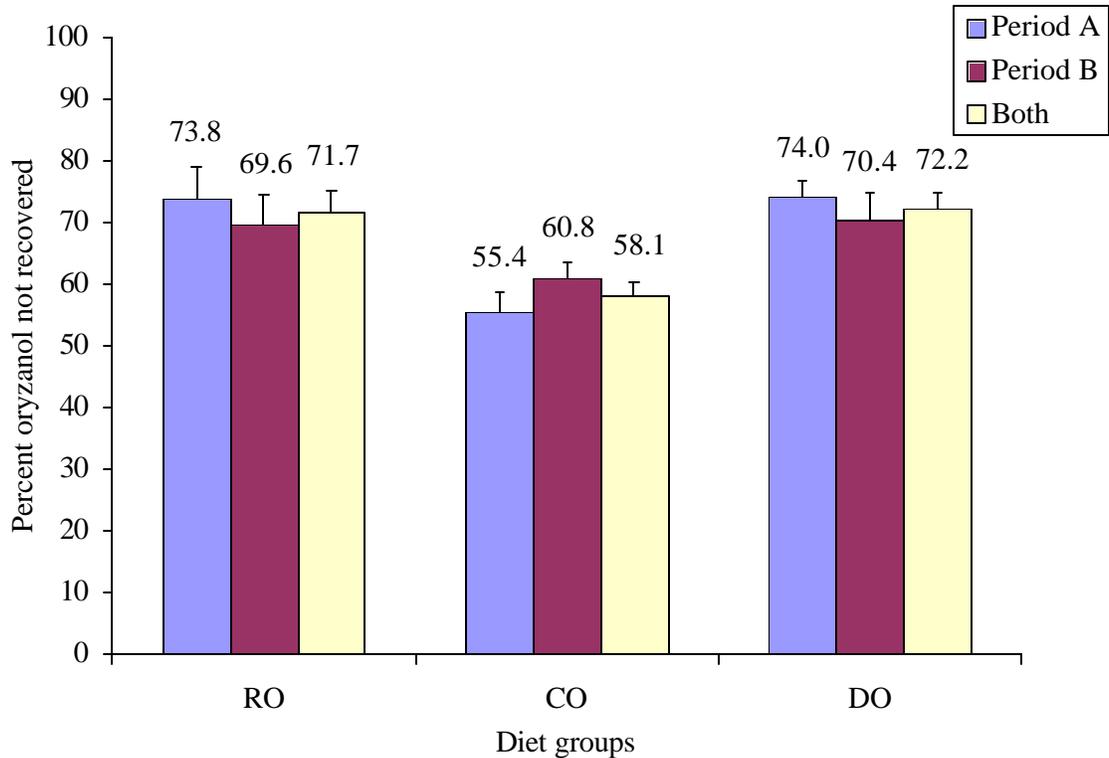
FIGURE 4 Percentage of dietary oryzanol not recovered in the feces by diet group and batch. Values reflect means. Error bars represent SEM.

Serum Cholesterol

Enzymatic Colorimetric Assay

Serum from the five experimental groups were analyzed for total cholesterol by enzymatic colorimetric assay to compare the effects of the test diets. Total cholesterol levels were highest in the RO group followed in decreasing order by the DO, CO, C and SH groups (Table 5). However, differences between the test groups were not statistically significant.

Since the sham-operated (SH) rats did not have their ovaries removed, it was expected that the cholesterol levels in this group would be lower than the ovariectomized controls (C). The removal of ovaries and cessation of menses have both been associated

TABLE 6

Comparison of total cholesterol values by diet group obtained from enzymatic colorimetric assay and GCMS using hexane or chloroform:methanol extraction.[1]

| | | | GCMS | | | |
| | Enzymatic Colorimetric Assay | | Hexane | | Folch Method | |
Diet Group	n	Cholesterol (mg/dL)	n	Cholesterol (mg/dL)	n	Cholesterol (mg/dL)
C	9	119.5 ± 5.02	3	16.4 ± 14.5	3	22.9 ± 3.44
SH	10	98.1 ± 5.79	2	30.8 ± 5.7	3	15.8 ± 6.75
RO	8	135.0 ± 16.57	4	14.0 ± 7.6	3	22.8 ± 3.23
CO	8	123.8 ± 10.24	3	6.0 ± 5.8	3	20.4 ± 2.40
DO	9	128.1 ± 13.75	5	18.5 ± 7.6	3	34.9 ± 11.5

[1]Values are means ± SEM.

with higher total cholesterol levels due to the drop in estrogen production (Davidson, Maki, Karp, & Ingram, 2002). No previous studies were found that specifically examined differences in cholesterol between ovariectomized rats fed oryzanol and non-ovariectomized rats sustained on oryzanol-free diets. Therefore, no prediction was made as to where the SH group would fall with respect to the ovariectomized oryzanol-fed groups.

However, the trend among the ovariectomized control and oryzanol diet groups was the exact reverse of what was hypothesized based on the review of literature. Researchers reported lower total cholesterol levels in rats fed oryzanol compared to controls (Seetharamaiah & Chandrasekhara, 1988, 1989, 1993). Likewise, Yoshino et al. (1989) reported reduced total serum cholesterol in hyperlipidemic patients consuming 300 mg/day of gamma-oryzanol.

In each of these studies, however, diets were enriched with cholesterol. Previous research has shown a substantial increase in total serum cholesterol of rats sustained on a

cholesterol-enriched diet when compared to cholesterol-free diet groups. Sharma and Rukmini (1986) found an increase of over 100 mg/dL in rats fed a diet supplemented with 0.1% cholesterol and 0.05% cholic acid. These researchers also found that a cholesterol-enriched diet was required to see significant decreases in serum lipid levels of animals sustained on a diet containing rice bran oil versus groundnut oil. In a study using cholesterol-free diets for all test groups, serum cholesterol levels between rats fed rice bran oil versus corn oil did not differ (Edwards & Radcliffe, 1994). Therefore, it was theorized *post hoc* in our study, that a diet containing cholesterol might be required to detect a cholesterol-lowering effect of oryzanol in rats. Similarly, use of oryzanol to lower cholesterol in humans may prove to be more effective for those not able to comply with a very low-cholesterol diet on a long-term basis.

A second plausible explanation for this reversed trend is that the Infinity cholesterol reagent used in the colorimetric assays was not specific for cholesterol. In other words, the oxidase that reacts with cholesterol to form hydrogen peroxide may also be capable of a similar reaction with a phytosterol such as oryzanol. It is the hydrogen peroxide product that is ultimately quantified and used to calculate the total cholesterol concentration in the serum sample.

To test this theory, a small amount of high-oryzanol rice bran oil was dropped into a glass test tube containing the cholesterol reagent. Upon vortexing, a cloudy, pink mixture formed. This procedure was repeated using each of the following: stripped corn oil, a vortexed mixture of stripped corn oil and crystalline oryzanol, and crystalline oryzanol alone. All three turned a pale shade of pink. Therefore, the hypothesis that the

cholesterol levels in the oryzanol-fed rats were erroneously inflated due to higher levels of phytosterols in the serum was not rejected.

GCMS

Hexane Extraction. Results from GCMS analysis of serum extracts prepared by hexane extraction were highly variable. This outcome suggested that hexane was not useful as an extraction solvent for this medium. The procedure was abandoned prior to analysis of the entire data set. It is not likely that the values obtained by this method in any way reflect even true relative cholesterol levels. However, the relationship was as follows: CO < RO < C < DO < SH.

Chloroform:Methanol Extraction. As with results from the enzymatic colorimetric assays, lowest total cholesterol values were associated with the SH group following the chloroform:methanol extraction of Folch et al. (1957). Serum availability limited analysis to only three samples from each of the five test groups. Although small sample size and variability prevented statistical significance, values from the remaining groups had the following relation: CO < RO < C < DO.

Results from this GCMS analysis were substantially lower than those obtained using the enzymatic colorimetric assay. Cholesterol values obtained by GCMS ranged from only 16% of the cholesterol values resulting from the enzymatic assay in the SH and CO groups to 27% for the DO group. Averaging all cholesterol values from both sets of analyses resulted in GCMS values at only 19% of the enzymatic assay. This suggests that although the Folch method is advantageous over extraction with hexane, further perfection of the extraction and/or subsequent GCMS procedure is required.

Oryzanol in Serum

GCMS analysis following hexane extraction of RO serum compared to that of a control rat did not reveal the presence of oryzanol. Likewise, no oryzanol was detected in the HPLC analysis of serum from the human pilot study (Appendix G). If oryzanol is present in the serum, it is likely that it is transported through the blood like cholesterol as a component of lipoproteins. Therefore, in light of the results from the cholesterol analysis, it is possible that hexane was not an optimal extraction reagent for serum oryzanol.

It would not be surprising, however, if oryzanol is in fact poorly absorbed. As mentioned above, Fujiwara et al. (1983) reported minimal absorption of oryzanol. They noted an 84.5% fecal recovery rate of the radioactivity from an oral dose of labeled oryzanol. In addition, 9.8% of the radioactivity was recovered in the urine, but no oryzanol was detected in the radioactive fraction implying that the compound being quantified was likely only its ferulate metabolite.

These researchers also reported radioactivity in the blood, but it was not determined whether the detected compound was intact oryzanol or ferulic acid. Although it is not likely that oryzanol is present in the blood, further analysis following a superior extraction procedure is necessary for an unequivocal conclusion. Additionally, it would be worthwhile to investigate whether or not oryzanol metabolites are present in serum. It is possible that metabolites were not detected in this experiment due to GCMS retention times of these compounds exceeding the studied range of zero to 65 minutes.

Oryzanol in Liver

As with analysis of the serum, HPLC and GCMS analysis of hexane extracts of homogenized liver did not reveal oryzanol (Appendix H). Again, it is probable that

hexane was not effective at extracting lipids from the tissue. However, it seems likely that significant uptake of intact oryzanol into the liver would have been detected. Considering these results and previous studies that have suggested poor absorption of oryzanol and phytosterols, it is likely that the oryzanol content of liver is either none or very small.

Fate of Dietary Oryzanol

Averaged over both collection periods, the percentage of dietary oryzanol not recovered in the feces ranged from 58.1% in the CO group to 72.2% in the DO group. The explanation for the disappearance of oryzanol is uncertain. One possible fate of dietary oryzanol is absorption into the epithelial cells of the small intestine as an intact compound. Due to its insolubility in an aqueous solution, it is predicted that oryzanol would be incorporated into micelles in the intestinal lumen with the aid of bile. The micelle formation allows non-polar substances to be transported to the brush border of the mucosal cells for intestinal absorption (Mayes, 2000). Once inside the intestinal cells, it is reasonable that oryzanol would be incorporated into chylomicrons for transport through the lymph in a similar manner to that of dietary cholesterol. In refute of this theory, Fujiwara et al. (1983) reported that oral injections of gamma-oryzanol resulted in entry of the intact compound into the systemic system via the portal vein.

Although oryzanol may be absorbed as an intact compound, it is probable that at least some percentage of dietary oryzanol is metabolized prior to entering the systemic system. Esterases present in the mucosal cells of the small intestine and in the luminal microflora of the large intestine are likely capable of cleaving the ester bond connecting the ferulic acid to the sterol portion of the compound (Andreason, Kroon, Williamson,

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& Garcia-Conesa, 2001). The expected metabolites of oryzanol are ferulic acid and the various triterpene alcohols. The three most abundant triterpene alcohols in oryzanol are cycloartenol, 24-methylenecycloartanol, and campesterol (Xu & Godber, 1999).

Adam et al. (2002) investigated the absorption of ferulic acid in rats using an intestinal perfusion model. Absorption rates of perfused ferulic acid were directly proportional to the concentration of the perfusion. The authors suggested, therefore, that ferulic acid is absorbed by passive diffusion or by facilitated transport. In a feeding study published in the same work, Adam et al. reported a 50% absorption rate in rats of ferulic acid added to a semi-purified diet. High recovery of ferulic acid in urine and undetectable plasma concentrations within 18 hours of a meal was interpreted to mean that ferulic acid is readily and completely eliminated in urine.

There is some evidence that cycloartenol is absorbed and stored in the liver (Rukmini & Raghuram, 1991). Ikeda et al. (1985) reported cycloartenol absorption four times that of ß-sitosterol in rats. A literature search utilizing Web of Knowledge on February 13, 2003, did not produce any references concerning intestinal absorption of 24-methylenecycloartenol. The absorption rate of campesterol has been estimated near 10% in humans (Heinemann, Axtmann, & Von Bergmann, 1993). In rats fed a diet containing unsaponifiables from rice bran oil, Nagao et al. (2001) reported double concentrations of serum campesterol versus levels in those not consuming the unsaponifiables. Triterpene alcohols are similar to cholesterol in structure and may be incorporated into the chylomicron surface as is free cholesterol. Evaluation of serum and liver for ferulic acid and these triterpene alcohols in future studies would provide additional insight into oryzanol metabolism.

Yet another explanation for dietary oryzanol not detected in the feces is that neither oryzanol nor its metabolites are absorbed. Rather, oryzanol is metabolized in the gut and the products are then excreted. It is well accepted that the absorption of phytosterols is limited relative to cholesterol (Moghadasian, 2000). Therefore, although partial absorption of these metabolites is possible, a high excretion rate is likely. Further research could explore the extent to which oryzanol metabolites are excreted.

Mode of Action of Oryzanol as Hypolipidemic Agent

Serum cholesterol concentrations did not differ significantly between experimental groups in this study. However, a substantial number of previous studies have attributed decreases in serum and liver cholesterol to intake of rice bran and its unsaponifiable fraction, particularly oryzanol. Several theories exist regarding modes of action of cholesterol-lowering agents, including: (1) Interrupting a step in the biochemical synthesis of *de novo* cholesterol. Statins, the most effective cholesterol-lowering group of pharmaceuticals currently on the market, work in this manner by inhibiting the HMG-CoA reductase enzyme (Mayes, 2000). (2) Attenuating absorption of dietary cholesterol. Due to their cholesterol-like structure, some phytosterols, especially saturated compounds such as sitostanol, are thought to work this way via competitive absorption (Jones & Kubow, 1999). (3) Inhibiting enterohepatic circulation of bile acids by limiting reabsorption in the ileum. Bile acid excreted in the feces necessitates liver production of new bile which requires cholesterol as a substrate (Grundy, 1986). In turn, LDL receptors are up-regulated as a result of diminished hepatic cholesterol stores. Cholesterol-lowering drugs known as bile acid sequestrants utilize this mode of action.

If it is true that a cholesterol-enriched diet is required to see the hypolipidemic effects of oryzanol, then one would conclude that oryzanol works to lower cholesterol levels by inhibiting absorption of dietary cholesterol rather than by controlling the synthesis of de novo cholesterol. This theory is in agreement with Nagao et al. (2001) whose research team reported that neither rice bran oil nor its unsaponifiable fraction alone significantly altered messenger RNA for HMG-CoA reductase, LDL receptor, or cholesterol 7a-hydroxylase. They concluded that unsaponifiables from rice bran oil inhibited intestinal absorption of dietary cholesterol. The same conclusion was reached by Rong et al. (1997) who studied the effects of oryzanol on cholesterol levels in hamsters. Kahlon et al. (1996) inferred that the unsaponifiable content of rice bran oil exerted its hypolipidemic action by increasing both fecal fat and neutral sterol excretion. Sharma and Rukmini (1986) previously hypothesized that increased bile acid excretion in rats consuming rice bran oil was due to unsaponifiables creating an unsuitable environment for micelle formation or inhibiting reabsorption of bile acids.

It is possible that intact oryzanol is responsible for inhibition of dietary cholesterol absorption and/or increased excretion of bile acids. However, due to the likelihood that esterases in the gut are capable of cleaving the ester bond, it is possible that the cholesterol-lowering effect of oryzanol can be attributed in part to its metabolites. Ferulic acid alone has been shown to lower serum LDL in rats fed a high-cholesterol diet (Seetharamaiah & Chandrasekhara, 1993). Kiribuchi, Miura, & Tokuda (1983) reported a synergistic cholesterol-lowering effect of cycloartenol and 24-methylenecycloartanol in combination with soysterol fed to rats. Mildly hypocholesterolemic men consuming a

phytosterol mixture that was 27% campesterol for four weeks experienced a significant

drop in serum cholesterol levels (Matvienko et al., 2002).

Conclusions

This study suggests that more than 50% of dietary oryzanol is either absorbed or

metabolized in the gut. Some metabolism of oryzanol is probable due to the presence of

esterases in the intestinal lumen and mucosal cells. It remains uncertain what percentage

of oryzanol or its metabolites are absorbed. Previous research suggests that oryzanol,

ferulic acid, and at least some of the plant sterol components of oryzanol are absorbed to

a limited degree. Regardless, dissolving crystalline oryzanol in oil appears to enhance

bioavailability to the extent that it is comparable to high-oryzanol rice bran oil.

Assuming that oryzanol does possess hypolipidemic qualities, its mechanism of

action is of interest. Oryzanol did not significantly affect serum cholesterol levels in this

study. Therefore, it is likely that dietary cholesterol is necessary for the hypolipidemic

benefits of oryzanol to be realized in rats. This suggests that oryzanol and/or its

metabolites work to lower cholesterol levels by inhibiting absorption of dietary

cholesterol. In humans, oryzanol as a pharmaceutical may not be capable of improving

cholesterol levels beyond the degree that would be obtainable by adhering to a very low

cholesterol diet. However, given the high cholesterol content of the typical Western diet,

further research regarding oryzanol as a cholesterol-lowering agent is certainly justified.

The chloroform:methanol method modified from Folch et al. (1957) appeared to

be superior to hexane; however, the discrepancy between cholesterol values obtained via

enzymatic colorimetric assay and GCMS indicates a shortcoming. Although the

extraction methods used in this study appear to have some degree of fallibility, it is

reasonable to conclude that the presence of intact oryzanol is minimal or non-existent in serum and liver.

Future Directions

Hexane may have been a poor extraction reagent in this balance study due to the possibility that some fecal oryzanol is bound up in lipid structures. Therefore, it is suggested that remaining fecal samples be extracted using a chloroform:methanol method (Folch et al., 1957). Oryzanol recovery quantification via HPLC could be repeated in order to validate or dispute the results reported here.

Although oryzanol's mode of action remains uncertain, growing evidence supports the theory that oryzanol lowers cholesterol levels by (1) inhibiting dietary cholesterol absorption and (2) increasing excretion of bile acids. Quantifying bile acids and neutral sterols would indicate whether excretion rates are higher for the more bioavailable oil forms of oryzanol. Measuring the expected metabolites of oryzanol in feces and urine, such as ferulic acid, cycloartenol, 24-methylenecycloartanol, and campesterol from rats fed the three oryzanol diets would provide information concerning the rate at which oryzanol is metabolized in the gut and whether the rate varies between oil and crystalline forms. Results would be more meaningful if the experiment was repeated using a high cholesterol diet and significant differences in cholesterol levels were discovered.

A review of the literature suggests that the chloroform:methanol solvent (Folch et al., 1957) is an appropriate reagent for extracting lipids from serum and liver. However, results obtained in this study were highly variable. It would be worthwhile to perfect the extraction methodology in order to analyze liver and serum cholesterol via GCMS in

future studies. Rats should be fasted prior to blood draw so that total, LDL, and HDL cholesterol values can be obtained. Oryzanol concentrations in liver, adipose tissue, and urine could be quantified using the perfected extraction procedure as well.

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APPENDIX A

FORMULAS

Calculation for oryzanol concentration in test diets and feces

$$X = \frac{PA}{4107260 * SS}$$, where X is the concentration value in mg/g units, PA is the

area under the HPLC curve for the oryzanol peak, and SS is the sample size in g.

Calculation for serum cholesterol levels obtained by enzymatic colorimetric assay

$$TC = \frac{AAV}{CAL} \times 200 \text{ mg/dL}$$, where TC is the total cholesterol value in mg/dL, AAV

is average of the colorimetric absorption values for the prepared serum sample, and CAL

is the colorimetric absorption value of the calibrator.

Calculation for a-cholestane standard in serum samples analyzed by GCMS

$$X = \frac{PA + 1221.8}{30,000,000}$$, where X is the concentration value in µg/µL units and PA is

the area under the GCMS curve for the a-cholestane peak.

Calculation for serum cholesterol analyzed by GCMS

$$X = \frac{PA + 2012}{4,000,000}$$, where X is the concentration value in µg/µL units and PA is the

area under the GCMS curve for the cholesterol peak.

Standard curves for a-cholestane and cholesterol

Cholestane

$y = 3E+07x - 1221.8$
$R^2 = 0.8952$

CHOL

$y = 4E+06x - 2012$
$R^2 = 0.9778$
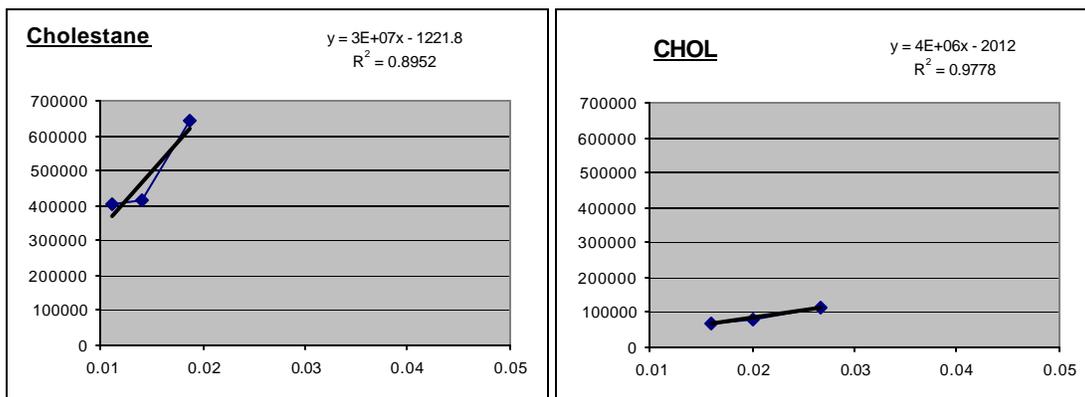
APPENDIX B

HPLC OF LIVER SAMPLES PERFORMED DURING METHODOLOGY DEVELOPMENT
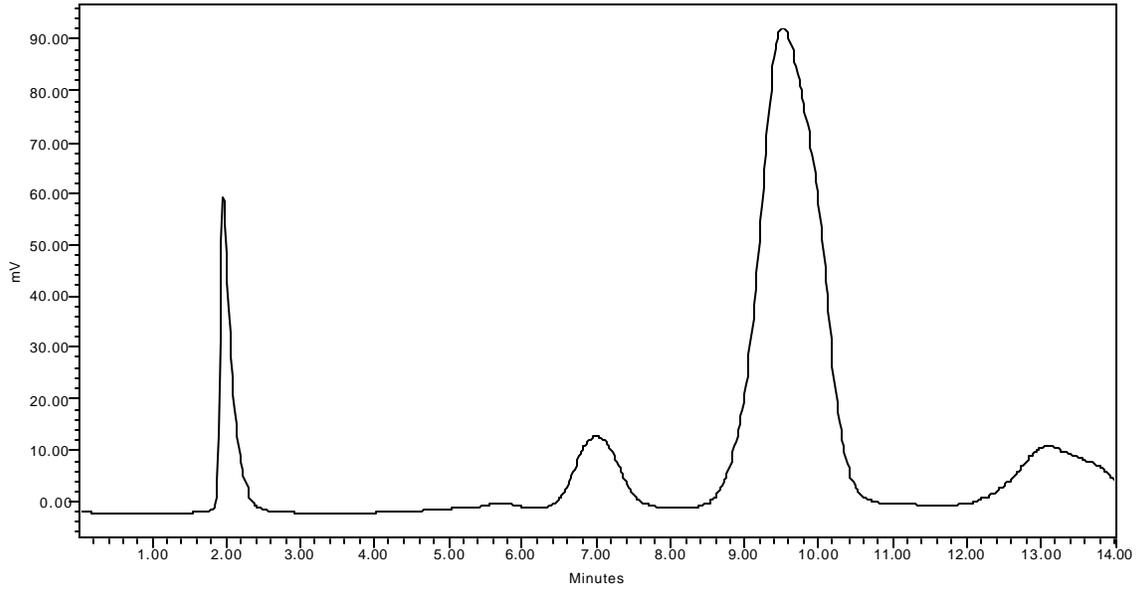
Test Liver 1
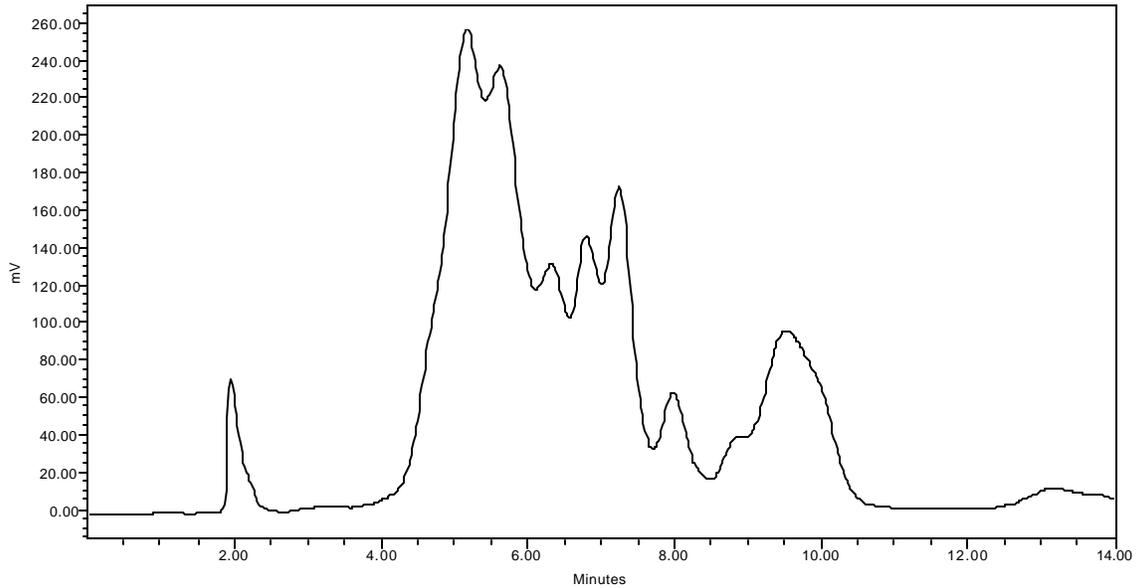
Test Liver 2 – Spiked with cholesterol
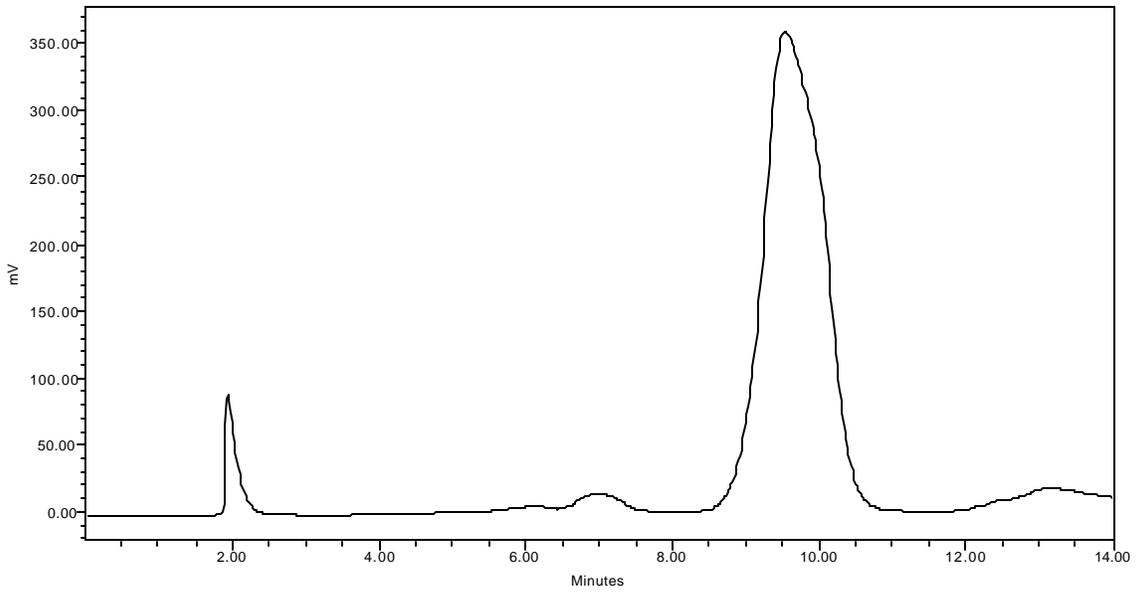
RO 8 – No water bath

RO 8 – Water bath

64
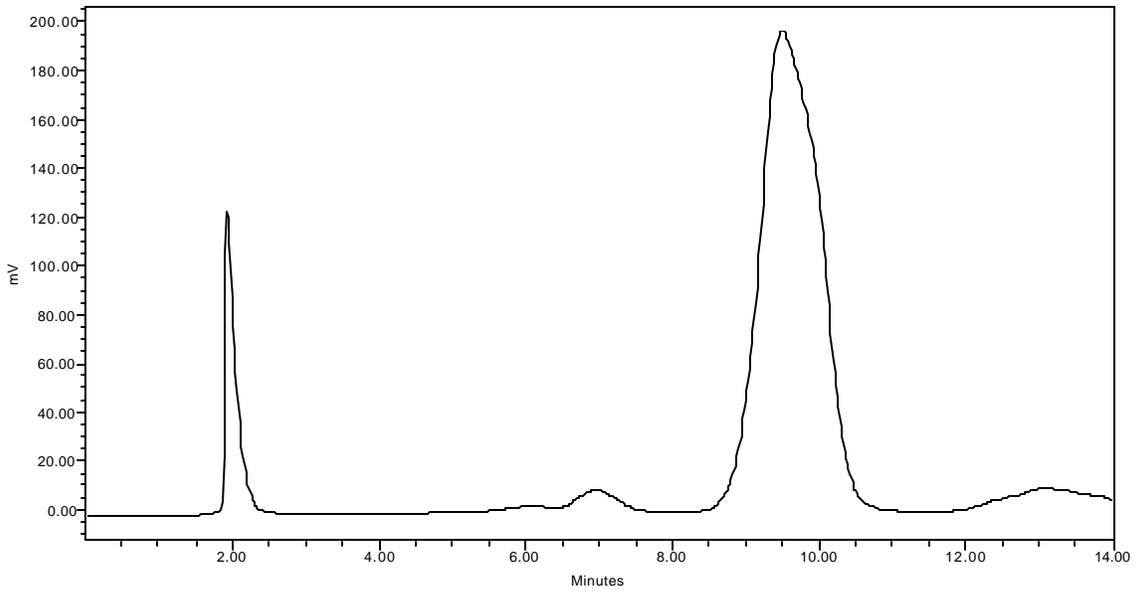
APPENDIX C

DAILY RICE BRAN OIL INTAKE FOR HUMAN PILOT STUDY

The following calculations are used to estimate a daily intake amount of rice bran oil for a 70 kg person that is comparable to the RO diet based on the percentage of total calories that are contributed by high-oryzanol rice bran oil.

RO Diet

Rice bran oil content of RO test diet: 40 g/kg

Approximate calorie content of 1 g of rice bran oil: 9 kcal

Caloric content of high-oryzanol rice bran oil in RO diet: $40 \times 9 = 360$ kcal/kg

Total caloric content of RO diet: 3742 kcal/kg diet

Percent calories of rice bran oil in RO diet: $(360 / 3742) \times 100 = 9.6\%$

Estimate for Human Diet

Caloric amount for 70 kg person on 2,000 kcal diet: $2000 \times 0.096 = 192$ kcal

Quantity for 70 kg person on 2,000 kcal diet: $192 / 9 = \textbf{\textit{21.33 g/day}}$

APPENDIX D

MUFFIN RECIPE FOR HUMAN PILOT STUDY

Combine and allow to cool:
    3/8 c. 10-grain cereal
    ¾ c. boiling water
    ½ tsp. instant decaffeinated coffee

In medium bowl, mix well:
    1 egg white
    1/8 c. light brown sugar, packed
    ¼ c. high-oryzanol rice bran oil (7% oryzanol)
    10-grain cereal mixture from above
    ½ tsp. vanilla extract
    1/3 c. chopped dates
    1 Tbl. sunflower seeds
    2 Tbl. semi-sweet chocolate chips

In small bow, combine well:
    ¾ c. cake flour, unsifted
    1 tsp. baking powder
    ¼ tsp. baking soda
    ¼ tsp. salt

Fold wet and dry mixtures together until just moistened. Line six-cup muffin tin with paper cups that have been coated with non-stick cooking spray. Divide batter evenly among muffin cups. Bake at 400°F for 20-25 minutes.

Yield: 6 muffins.

Adapted from Twelve Grain Muffins,
www.bicyclesource.com/body/nutrition/muffins/twelve-grain-muffin.html.

APPENDIX E

HPLC OF DIET SAMPLES
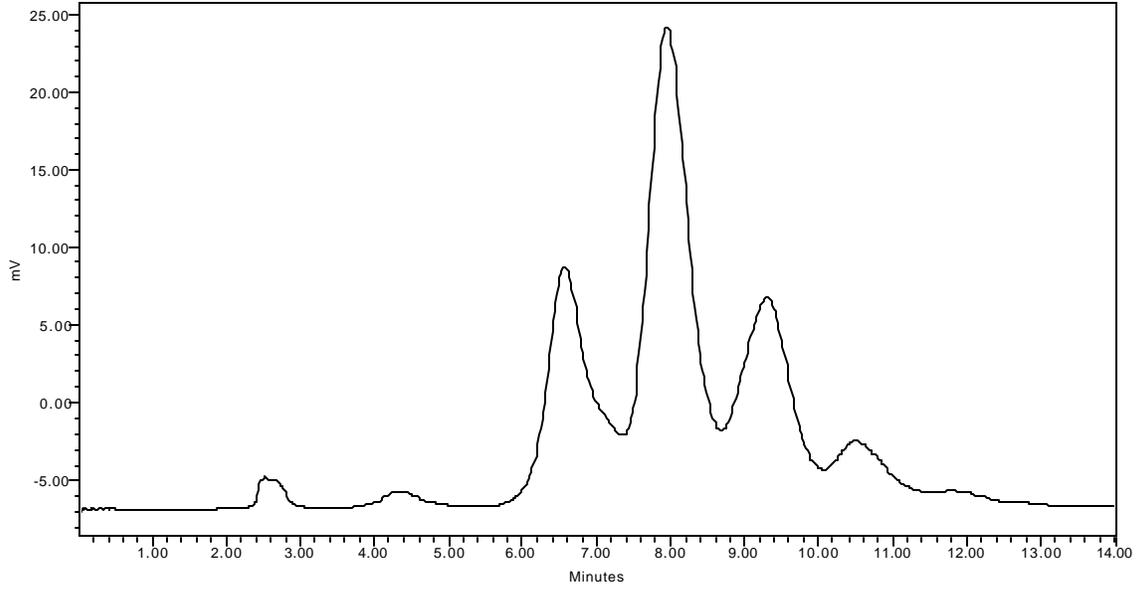
RO Diet Sample
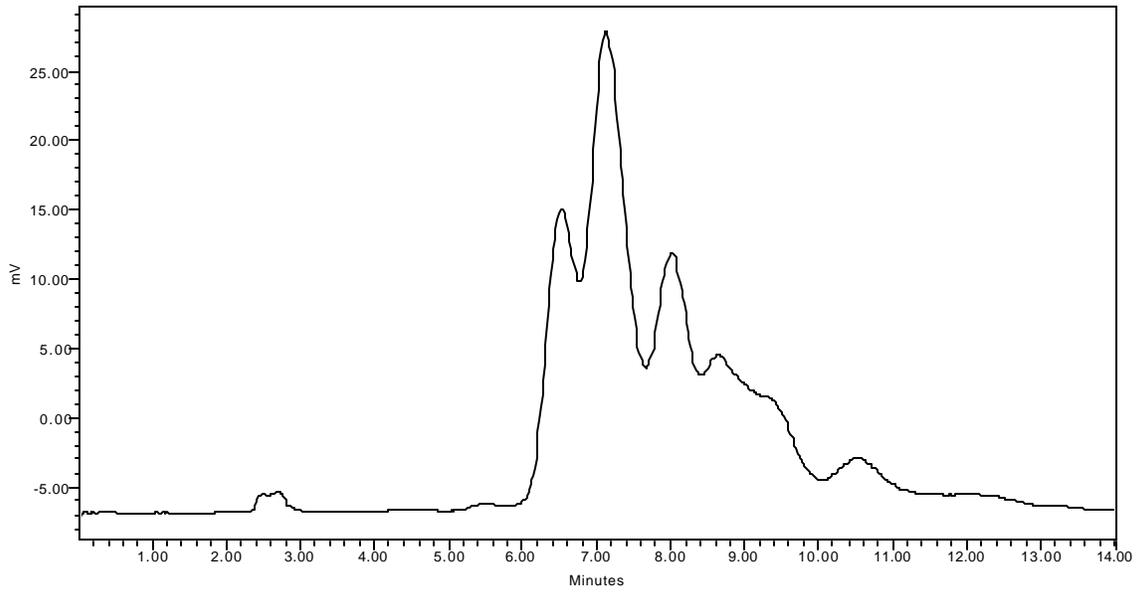
CO Diet Sample
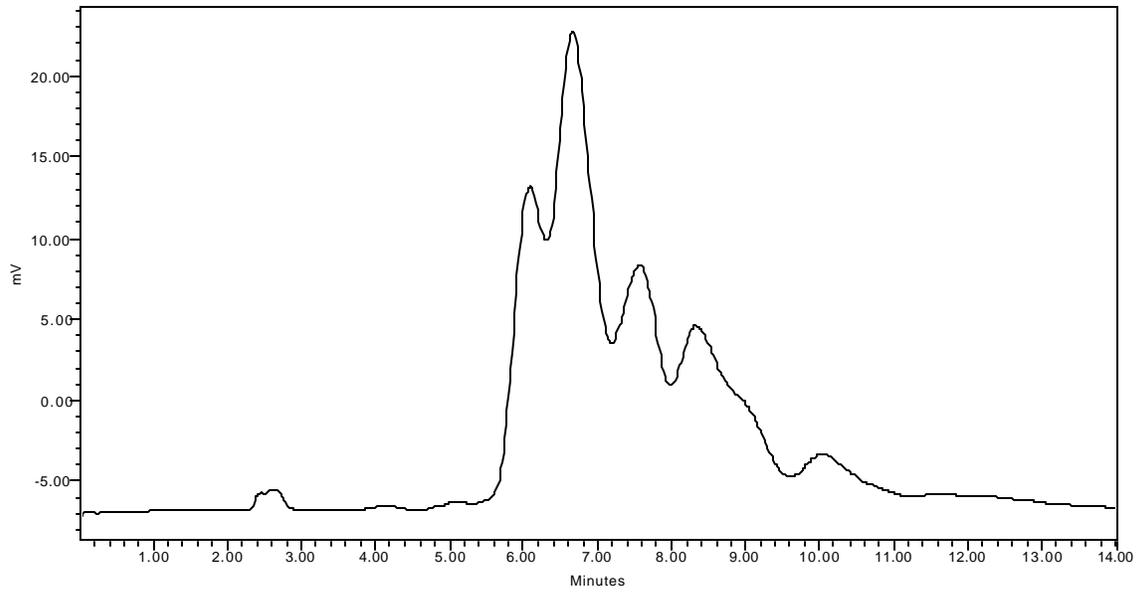
DO Diet Sample

HPLC OF FECAL SAMPLE FROM EACH DIET GROUP
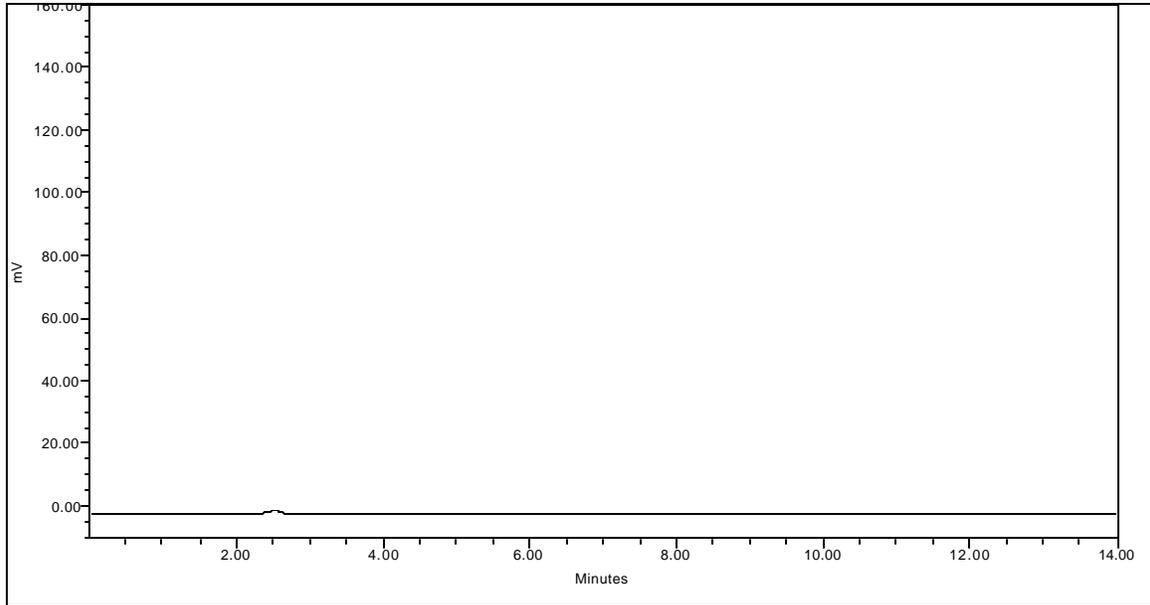
C 10 A

SH 5 A

RO 3 A

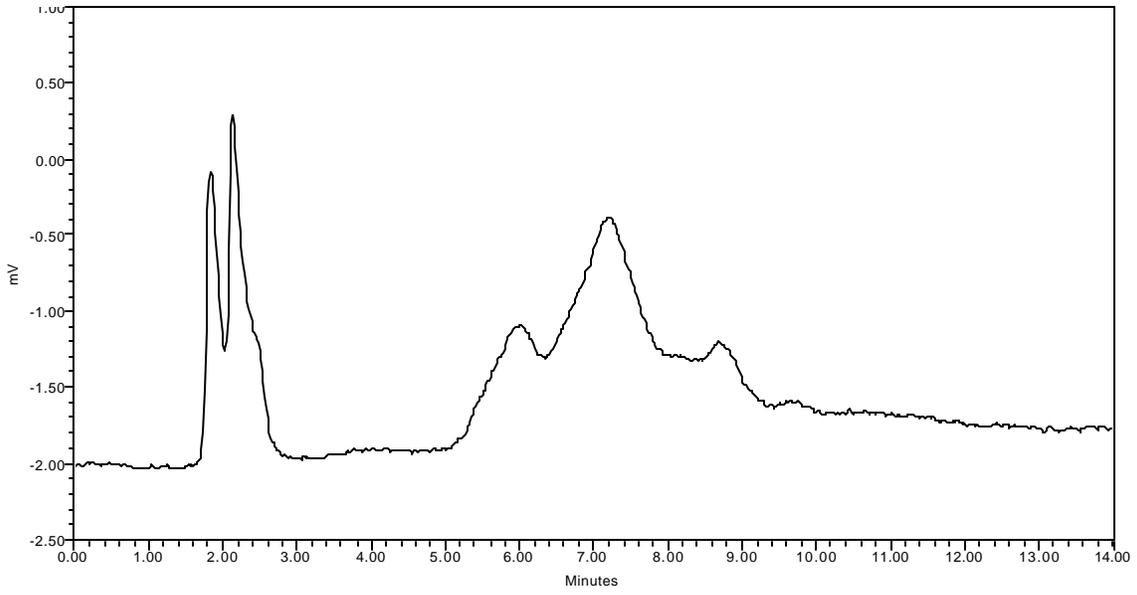**CO 7 A**
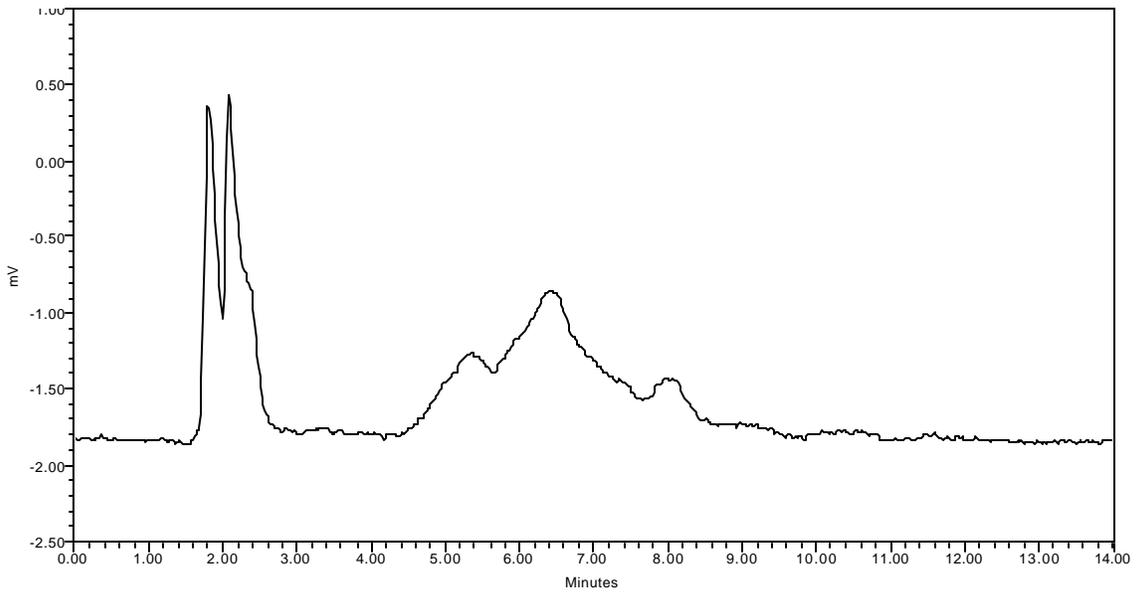
70
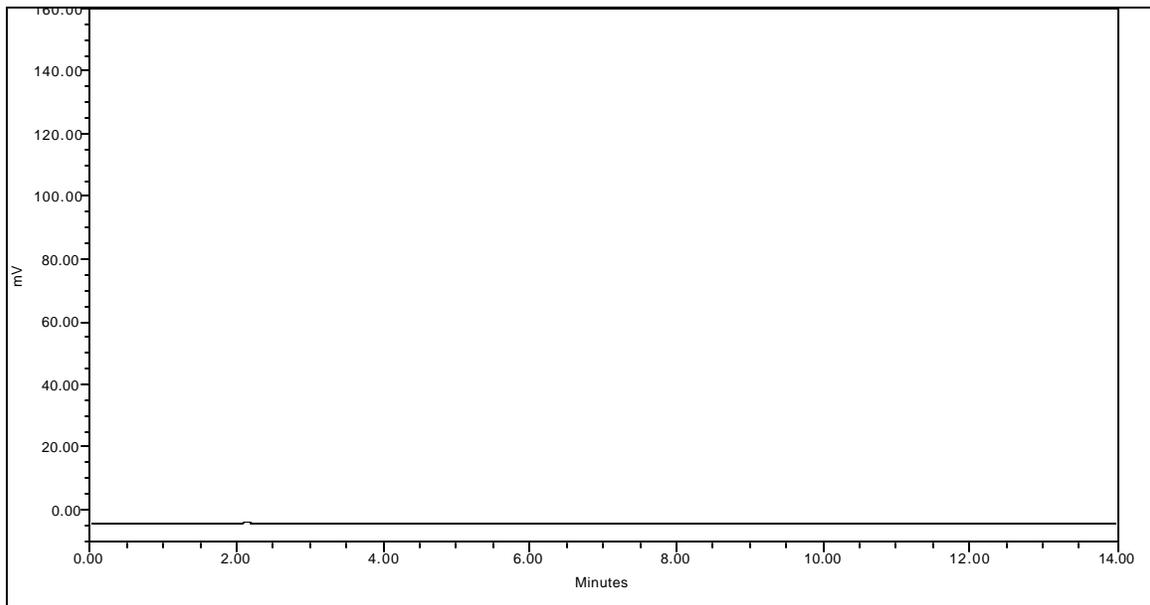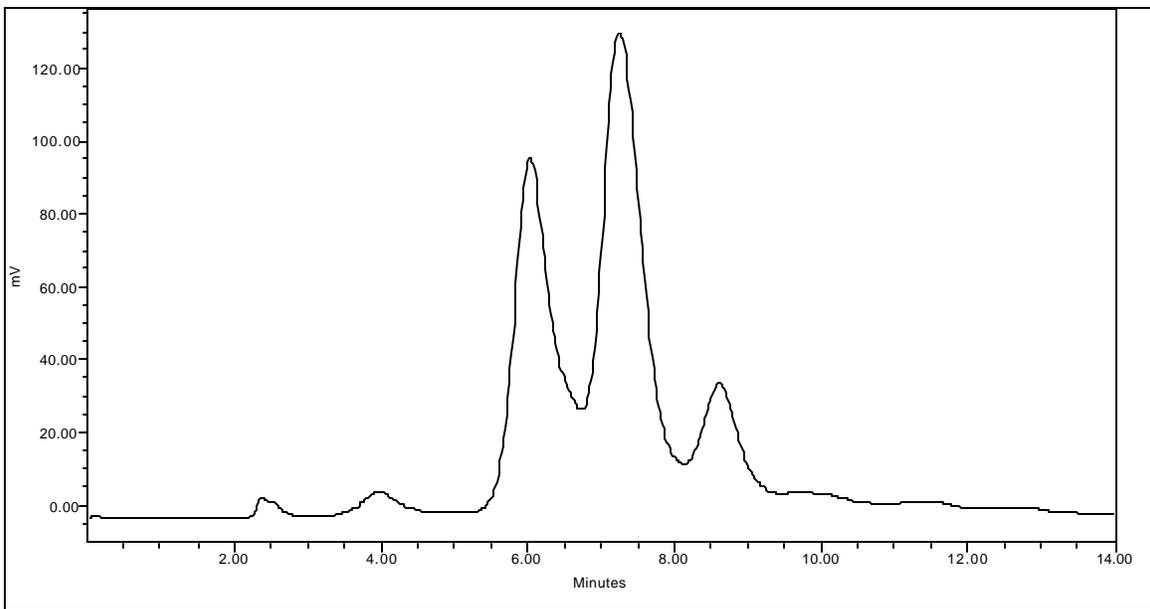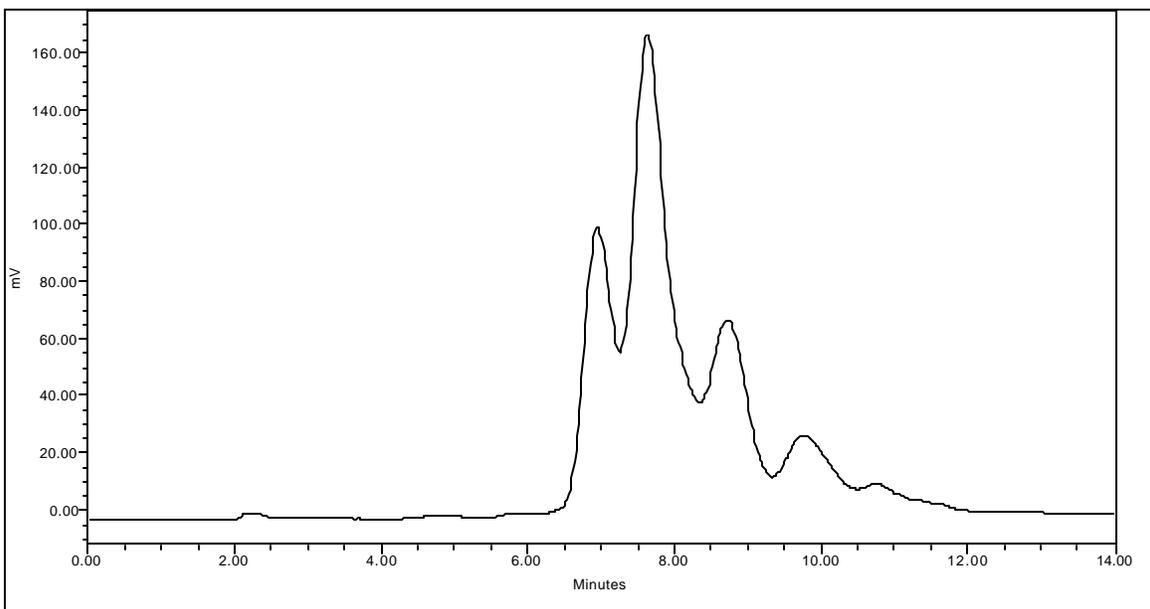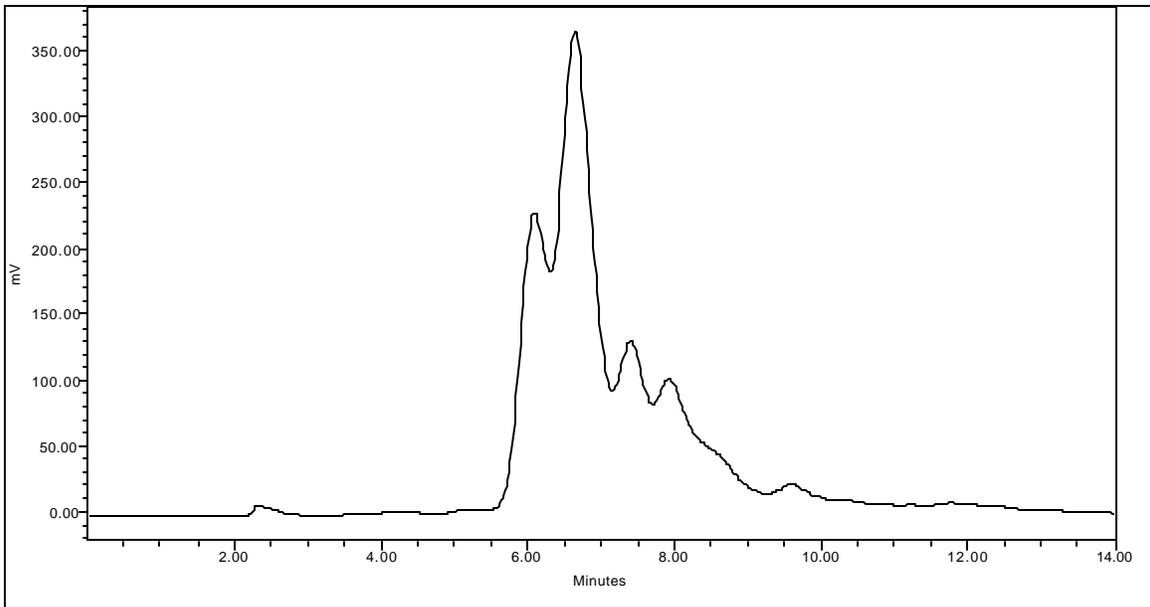
DO 5 A

APPENDIX G

HPLC OF HUMAN SERUM TO DETECT ORYZANOL
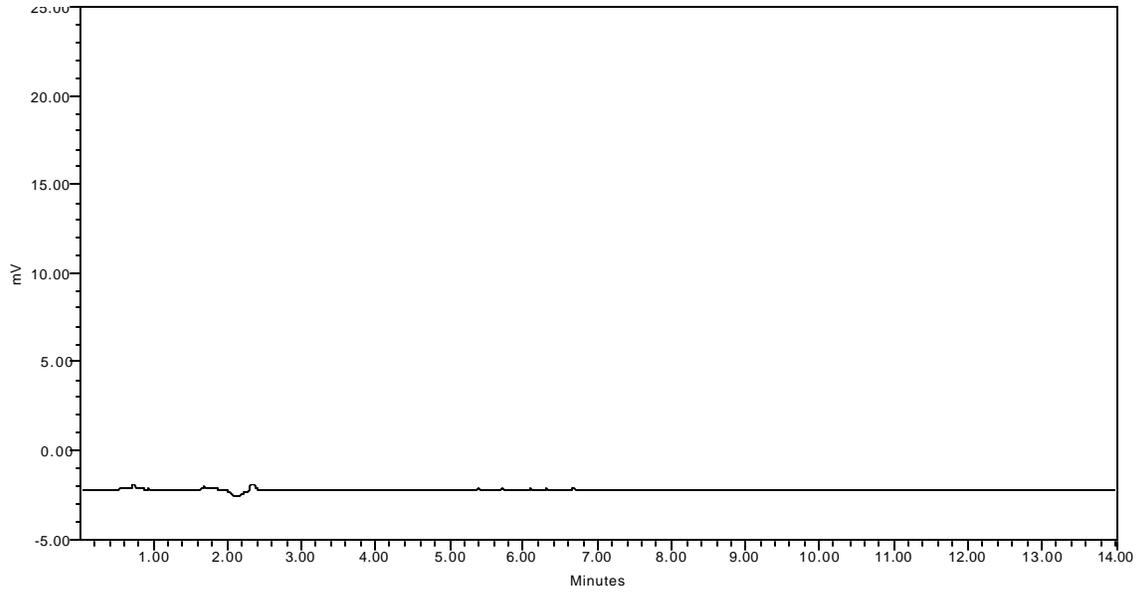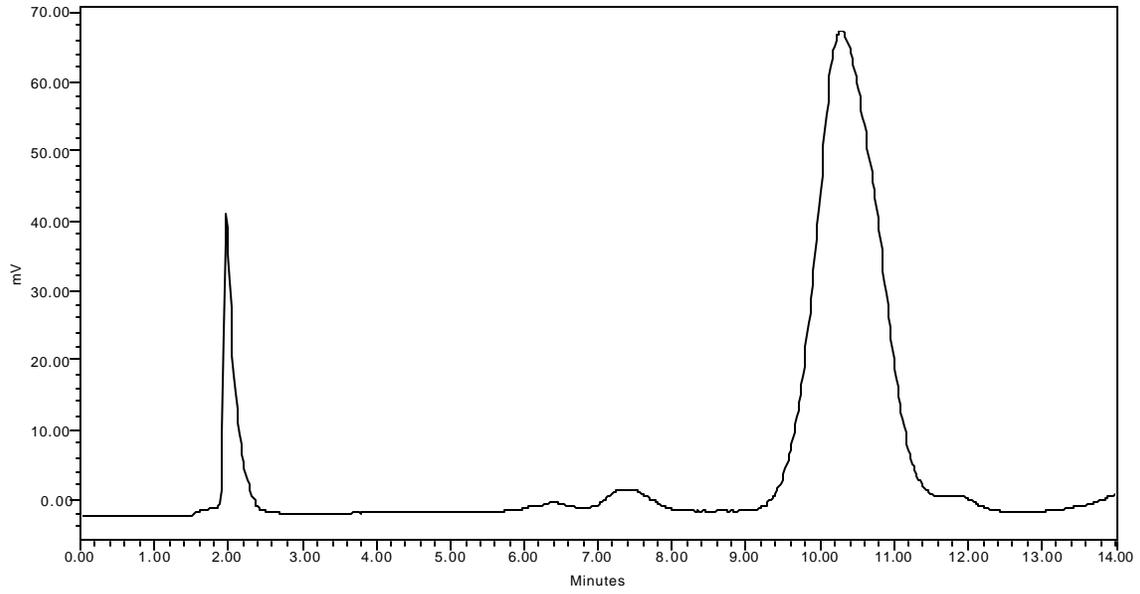

BEFORE
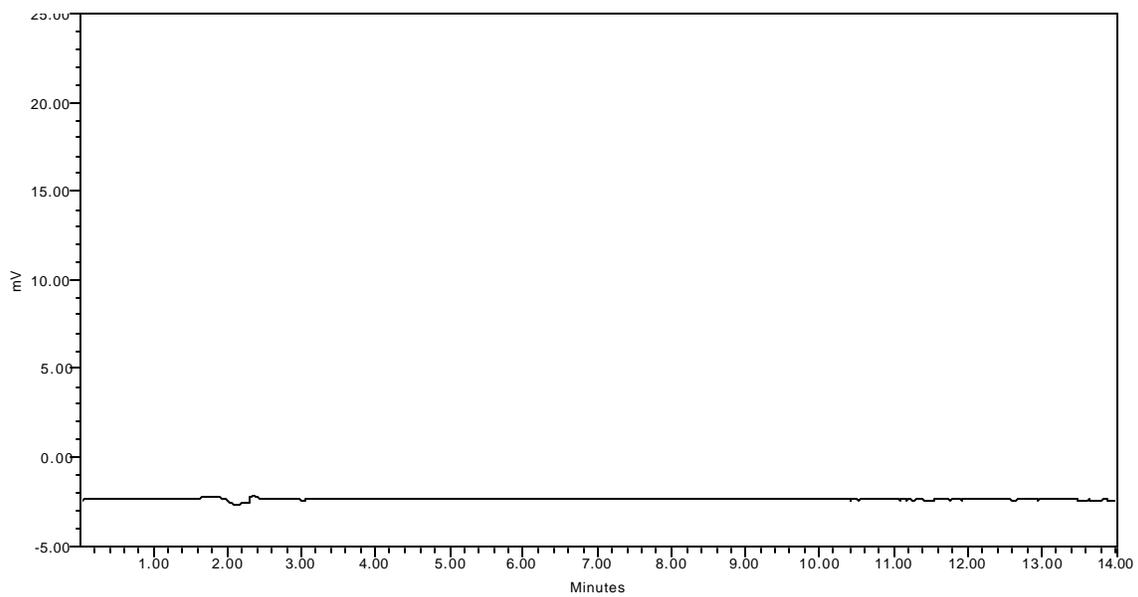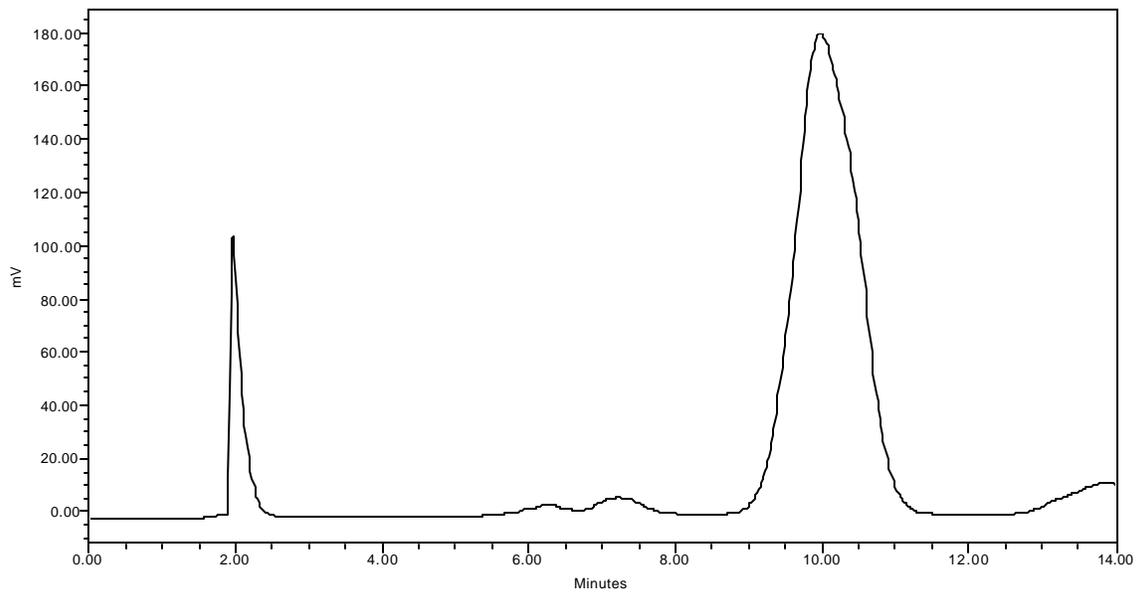

AFTER

BEFORE (CONCENTRATED)

AFTER (CONCENTRATED)

APPENDIX H

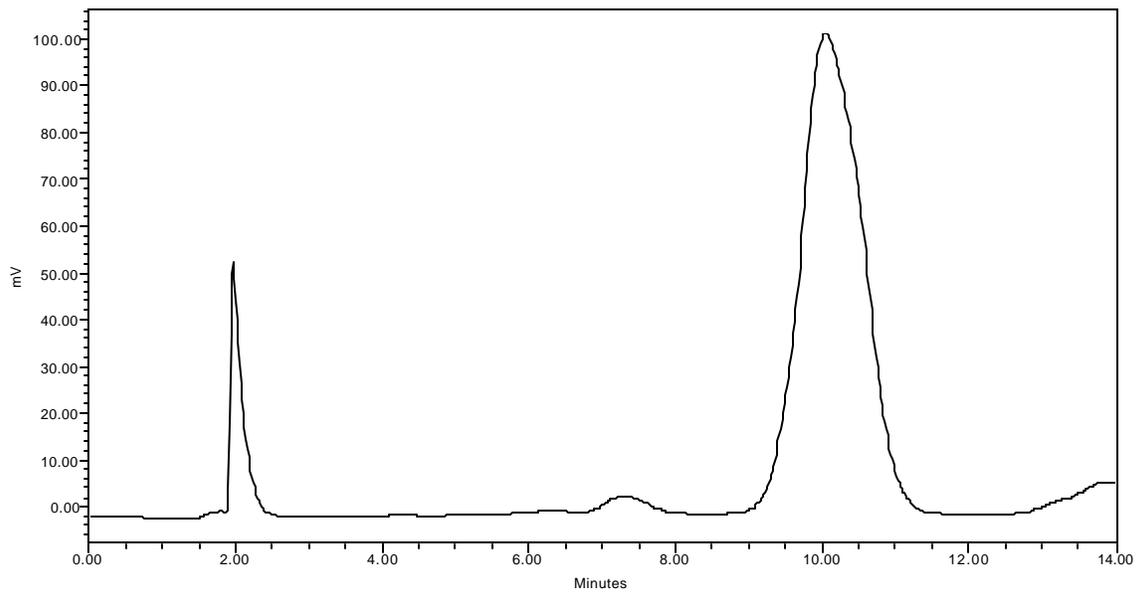## HPLC OF LIVER SAMPLE FROM EACH DIET GROUP
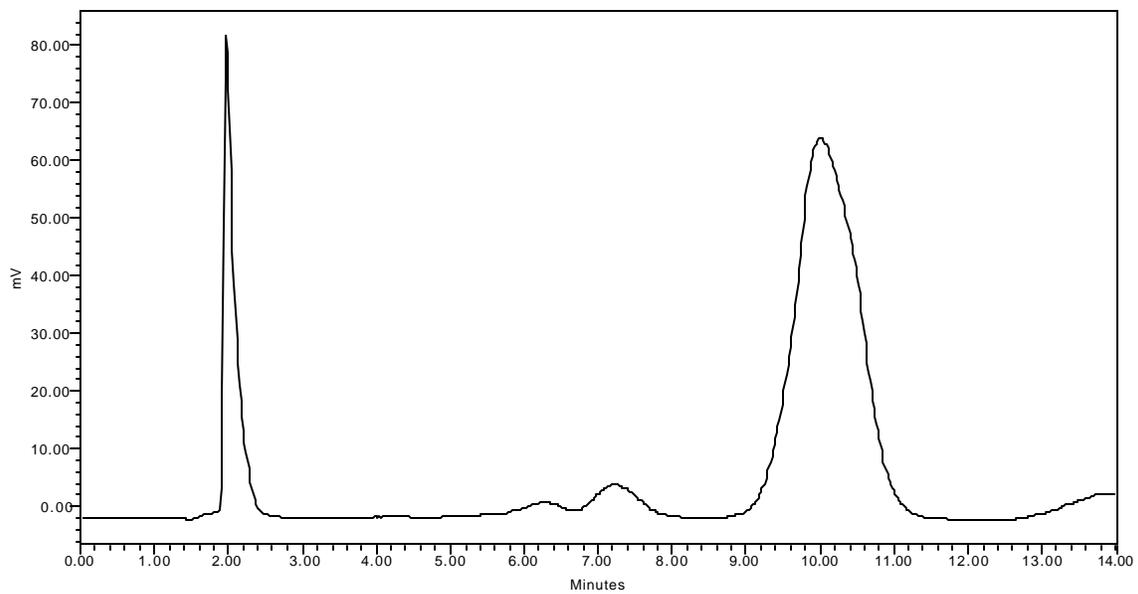

C7

SH 6
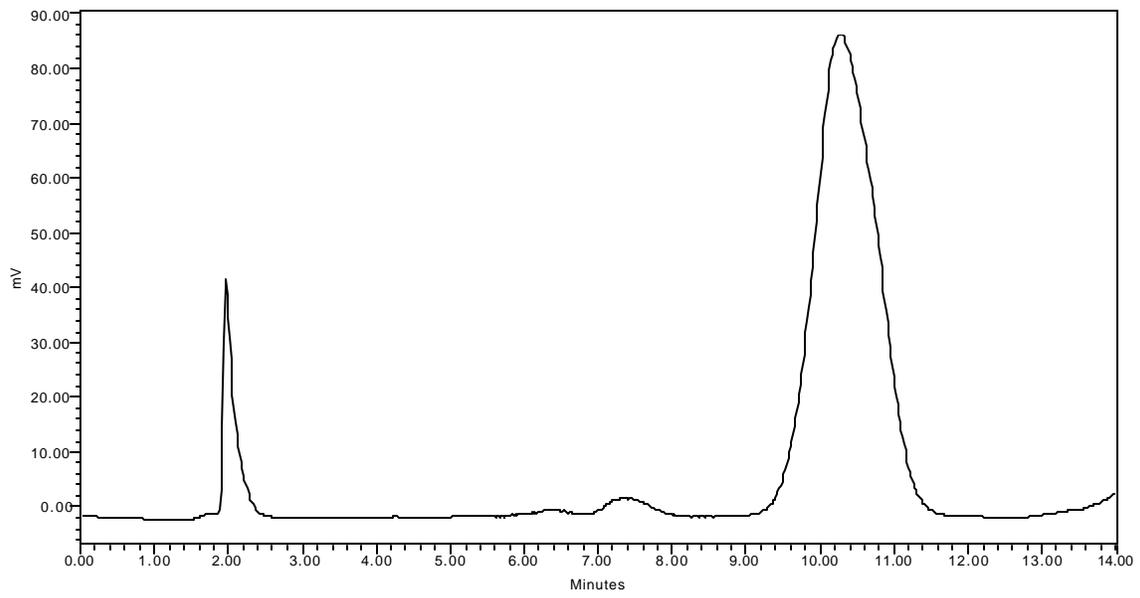
RO 2

CO 5

DO 9

VITA

Michelle Smith Gillespie was born November 16, 1969, in Zachary, Louisiana. She received her bachelor's degree in mathematics in August 1991 from Louisiana State University (LSU) in Baton Rouge, Louisiana. After graduating, she was employed for one year as a lab technician at Reichhold Chemicals in Research Triangle Park, North Carolina. In 1993, Michelle moved back to Baton Rouge and worked for four years as a systems quality control analyst and supervisor for United Companies Life Insurance Company. She was hired as an applications analyst in 1997 by LSU Computing Services, where she was responsible for maintenance and new development of programming code comprising the student financial aid and billing systems. In the fall of 2001, Michelle enrolled in the Human Nutrition and Food Division of Human Ecology at LSU to pursue a master's degree. During her studies, she worked as a graduate assistant in nutrition and food science laboratories. She is a current member of Gamma Sigma Delta and Gamma Beta Phi honor societies and a student member of American Society for Nutritional Sciences. Michelle intends to graduate in May 2003.