1999

The Potential of High Oryzanol Rice Bran Oil as an Antioxidant in Whole Milk Powder.

Jackin Njagi Nanua
Louisiana State University and Agricultural & Mechanical College

INFORMATION TO USERS

This manuscript has been reproduced from the microfilm master. UMI films the text directly from the original or copy submitted. Thus, some thesis and dissertation copies are in typewriter face, while others may be from any type of computer printer.

The quality of this reproduction is dependent upon the quality of the copy submitted. Broken or indistinct print, colored or poor quality illustrations and photographs, print bleedthrough, substandard margins, and improper alignment can adversely affect reproduction.

In the unlikely event that the author did not send UMI a complete manuscript and there are missing pages, these will be noted. Also, if unauthorized copyright material had to be removed, a note will indicate the deletion.

Oversize materials (e.g., maps, drawings, charts) are reproduced by sectioning the original, beginning at the upper left-hand corner and continuing from left to right in equal sections with small overlaps.

Photographs included in the original manuscript have been reproduced xerographically in this copy. Higher quality 6" x 9" black and white photographic prints are available for any photographs or illustrations appearing in this copy for an additional charge. Contact UMI directly to order.

Bell & Howell Information and Learning
300 North Zeeb Road, Ann Arbor, MI 48106-1346 USA
800-521-0600

THE POTENTIAL OF HIGH ORYZANOL RICE BRAN OIL AS AN ANTIOXIDANT IN WHOLE MILK POWDER

A Dissertation

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
Doctor of Philosophy

in

The Department of Dairy Science

by
Jackin Njagi Nanua
BSc. University of Nairobi, 1979
MSc. University of Reading, 1989
December, 1999

ACKNOWLEDGEMENTS

The author would like to acknowledge the many wonderful people whose assistance has made this work possible. He would like to thank Dr. Charles White and all the staff of the Department of Food Science, Mississippi State University for the assistance and use of the departmental facilities at MSU. He would like to extend special thanks to Dr. Steve Grace of Biodynamics Institute for guidance and use of the Electron Spin Resonance Spectroscopy equipment, Dr. Zhimin Xu of Food Science Department, Ms Sandra Traylor and Chuck Boeneke for their invaluable assistance and encouragement during laboratory work. He would also like to extend his thanks to Dr. Isaiah Warner of Chemistry Department for allowing him to use his laboratory and Dr. Fulbert Namwamba for his editorial assistance. Much gratitude goes to the author's major professor Dr. John U. McGregor for his guidance, funding, and encouragement and to his committee members Drs. R. H. Gough, R. W. Adkinson, D. Hwang and S. Godber for their advice. He would like to extend his gratitude to Dr. William Patrick, The Louisiana Methodist World Hunger Council Scholarship Program and the LSU College of Agriculture for financial support.

The author would like to extend very special thanks to his dear wife Mrs. Omisda Kageni Njagi, his beloved children Miriam, Ruth, John and Mercy, his parents Evangeline and Frederick Nanua for their love patience and sacrifice.

Finally, he is thankful to God, who gives 'the Spirit of a Sound mind' and whose Grace is always sufficient.

All these have made the research work to be great fun.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

ABSTRACT

Lipid autoxidation of whole milk powder (WMP) during storage lowers its organoleptic quality and causes accumulation of oxidation products. This study evaluated the effectiveness of high-oryzanol (2.5%) rice bran oil (RBO) as an antioxidant in WMP. The powder was fortified with 0.1% and 0.2% RBO and its oxidation studied during accelerated storage at 45° C and 0.31 water activity for 40 days. Free radicals were determined by electron spin resonance spectroscopy. Oxidation was estimated by determining thiobarbituric acid reactive substances (TBARS) and cholesterol oxidation products using gas chromatography. The effect of RBO on flavor and consumer preference of freshly manufactured milk powder was also evaluated. The oryzanol content of the WMP was 0.00 μg/g, 49.16 μg/g and 180.11 μg/g for 0.0%, 0.1% and 0.2% RBO fortified samples respectively. The α-tocopherol content was 0.00, 3.72 and 7.54 μg/g for 0.0%, 0.1% and 0.2% RBO fortified samples respectively. Addition of RBO significantly affected the color of the powder. The fortified powders had darker, more yellow color than control samples. Consumer tests showed that 0.2% RBO caused a detectable flavor change in milk powder, but at 0.1%, it had no significant effect on flavor. The RBO at 0.1% and 0.2% caused a significant reduction in free radicals during manufacture and after 10 days storage. High-heat powder had higher concentrations of free radicals than low-heat powder. Lipid oxidation was not significantly affected by RBO immediately after manufacture, but was significantly reduced by 0.1% and 0.2% RBO after storage for 10 or more days. The TBARS for all powder samples increased up to 30 days storage but reduced on further storage, probably due to their reaction with proteins. Rice bran oil did not have

a significant effect on the accumulation of COP. The data on COP however was not conclusive, due to wide variability that may have resulted from thermal degradation of COP in the GC column during analysis. Results indicated that RBO reduced oxidation of WMP. Further work is necessary to determine the optimum application rate and method for process optimization.

CHAPTER ONE: INTRODUCTION

Review of Literature

Milk Powder Production

Whole milk powder (WMP) is obtained by the evaporation of water from pasteurized milk, to give a powdery product containing not more than 5% moisture and between 26% and 40% milk fat by weight on an 'as is' basis. Vitamins A and D may be added. Other optional safe and suitable ingredients that may be added include emulsifiers, antioxidants, stabilizers, anti-caking agents and characterizing flavors (FDA, 1996). Transportation costs and storage space are reduced by the reduction in mass and bulk. The resulting low water activity reduces the rate of chemical reactions and inhibits development of microorganisms, leading to improved shelf life. During processing, the objective is to obtain a long shelf life product that resembles the original product upon reconstitution at minimum cost (Cari'c, 1994). This objective calls for water removal with minimum damage to the milk components. Water can be removed by several methods such as spray drying, drum drying, evaporation, freeze-drying, foam-drying, ultrafiltration and reverse osmosis. Whereas freeze-drying can be used to produce a powder with minimum heat damage, it is expensive and might not be suitable for large-scale production. Drum drying is lower in cost but causes excessive heating of the milk, which leads to heat damage. The method that gives the most effective compromise in terms of cost, throughput and product quality, and that is widely used commercially, is evaporation under vacuum followed by spray drying.

The average gross composition of WMP and skim milk powder (SMP) is given in Table 1.

Table 1. Average composition of whole and skim milk powders[1]

Constituent	Grams in 100grams.	
	WMP	SMP
Water	3.5	4.3
Protein	25.2	35.0
Lipids	26.2	1.0
Carbohydrates	38.1	51.9
Ash	7.0	7.8

[1]Source: Cari'c (1994).

World Production of Milk Powder

Due to their long shelf life and nutritional value, milk powders are often used in nutritional intervention programs as emergency food aid. Situations that result in mass food shortages are often caused by factors such as civil strife, war, earthquakes, epidemics and drought. Dried milks are also used in the formulation of infant formula and specialty products such as beverages, bakery products, puddings, soups, meat and meat substitute products.

The world annual milk powder production for the years 1992 – 1997 stood at over 5.5 million tons. About half this amount was exchanged in international commerce. During that period, SMP production was greater than WMP (Table 2). The

leading producers of WMP in descending order are EC, New Zealand, Brazil, Argentina and Australia, while those for skim milk powder are EC, USA, Australia, New Zealand and Japan. The leading world exporters of milk powders are EC, New Zealand, and Australia. The combined value of WMP and SMP for 1996 was about eleven trillion dollars (FAO, 1998).

Table 2. World production and exports (in parenthesis) of some dairy products [2]

Product	1992-94	1995	1996	1997 (est.)
	(,000 metric tons)			
WMP	2315 (1076)	2396 (1257)	2424 (1116)	2549 (1161)
SMP	3366 (1031)	3436 (1221)	3368 (1246)	3510 (1288)
Butter & Ghee	6836 (721)	6615 (678)	6644 (675)	6764 (760)
Cheese	14334 (903)	14634 (1048)	15023 (943)	15384 (998)

[2]Source: FAO, 1998.

Quality Problems

A major problem in handling WMP is its susceptibility to oxidative rancidity in the presence of oxygen, which, apart from giving objectionable odors, can also result in potentially toxic products (O'Connor and O'Brien, 1995). Commercially, WMP is packaged under controlled atmosphere in gas-impermeable, moisture-proof and opaque packaging materials. Oxygen level is reduced to a minimum and replaced with an inert gas, usually nitrogen. Reduced oxygen concentration slows the rate of oxidation and other undesirable reactions such as Maillard reactions. Packaging WMP under nitrogen has also been shown to reduce the rate of lysine loss (Cari'c, 1994). High

moisture content can lead to lactose crystallization, caking and reduced solubility of the product (Jouppila *et al.* 1997). The protection offered by the packaging material is lost once the package is opened.

When WMP is used for nutritional intervention relief efforts, it is normally transported in bulk packages. Once the package is opened for distribution, the product is exposed to environmental conditions and the milk fat can undergo oxidative reactions if not used immediately. The problem is compounded if the ambient conditions are hot and humid, as these reactions are faster at elevated temperatures and water activities (Tuohy *et al.*, 1981; Stapelfeldt *et al.*, 1997a).

At high humidity, milk powder can absorb moisture leading to lactose crystallization and decreased solubility. Lactose in freshly spray dried milk occurs as amorphous lactose, which is unstable and hygroscopic and thus absorbs moisture when exposed to high relative humidity (RH), and then converts irreversibly to the crystalline state. This leads to reduction in reconstitution properties, lumping and caking of the powder (Vuataz, 1988; Lai and Schmidt, 1990). Crystallization of lactose causes a release of adsorbed water thus increasing the amount of moisture in the remaining amorphous matrix (Roos and Karel, 1992). The rate of crystallization increases with increase in storage relative humidity up to a maximum. The maximum extent of crystallization has a parabolic relationship to the RH, with a maximum at a RH of 70% (Jouppila *et al.*, 1997). The types of crystals formed depend on the RH and temperature during storage. Jouppila *et al.* (1997) reported that at 55% RH, anhydrous mixture of α- and β-lactose crystals were formed with a molar ratio of 5:3, but at 76.4% and 85.8% RH, they detected other crystal forms such as anhydrous α- and β-lactose and α-lactose

monohydrate. Other investigators have reported formation of α-lactose monohydrate at 77% RH, 21°C (Aguilar and Zieglar, 1994), 57.2% RH and 25 °C (Vuataz, 1988) and as anhydrous β -lactose at 39.1%, 49.3% RH and 25 °C (Vuataz, 1988). The critical RH for milk powder lactose crystallization has been reported to be 37% at 24 °C (Jouppila *et al.*, 1997). Milk powder should therefore be stored at RH lower than 37% to avoid lactose crystallization with the subsequent loss of reconstitution properties and other quality characteristics of the powder.

Oxidation of Milk Lipids

Lipid oxidation reactions occur more or less spontaneously under mild conditions and are therefore referred to as autoxidation reactions. Oxidative deterioration of milk lipids during the storage of milk powder is a major concern for the dairy industry due to its negative effects on milk flavor and health of consumers (Emanuel *et al.*, 1991; O'Connor and O'Brien, 1995; Peng *et al.*, 1987; Weihrauch, 1988). Milk lipids contain unsaturated fatty acids, phospholipids and cholesterol that can undergo oxidative deterioration in the presence of oxygen. Autoxidation reactions are catalyzed by light and transition metal ions such as iron and copper (Caric', 1994). Phospholipids are usually found in the fat globule membrane and are oxidized faster than fatty acids, due to catalysis by copper, which is a natural constituent of the fat globule membrane (Walstra and Jenness, 1984). Lipid oxidation is initiated by the formation of free radicals that react with unsaturated fatty acids to give odorless and tasteless but highly unstable hydroperoxides as the primary oxidation products. Formation of free radicals may be triggered by factors such as metal catalysts, irradiation, or active oxygen species, and occur when a hydrogen atom is abstracted

from the α-methylene group adjacent to the double bond (Badings, 1960; O'Connor and O'Brien, 1995; Schaich, 1980). The types of free radicals formed depend on the fatty acid composition. Fatty acids with conjugated double bonds can be attacked directly at the double bond by oxygen without the formation of free radicals (Badings, 1960). Transition metals, such as copper, and heme proteins also catalyze the decomposition of hydroperoxides, resulting in more free radicals, which in turn initiate new chain reactions. Copper and cytochromes are found in the fat globule membrane and are thought to be responsible for autoxidation reaction initiation. Active oxygen species involved in milk fat oxidation include singlet oxygen ($^1O_2$), hydroxyl radicals ($HO^{\cdot}$), ozone (O_3), hydrogen peroxide (H_2O_2) and superoxide radical ($O^{\bullet}_2$), some of which are the products of other biochemical reactions and photoreactions (Korycka-Dahl and Richardson, 1980). Normal triplet oxygen can be transformed to singlet oxygen by radiation (Walstra and Jenness, 1984). Singlet oxygen is also formed in addition to free radicals, when iron catalyzes the decomposition of hydroperoxides (O'Connor and O'Brien, 1995). The hydroperoxides undergo further oxidative reactions, resulting in the production of secondary oxidative products such as aldehydes, ketones, alcohols, and acids, many of which have strong odors and affect the sensory and functional properties of milk (Caric',1994; McCluskey *et al.*, 1997; O'Connor and O'Brien, 1995; Stapelfeldt *et al.*, 1997a; Stapelfeldt *et al.*, 1997b). These chain reactions are terminated when the free radicals react with each other to form stable non-radical molecules. Antioxidants also react with the free radicals, hence stopping the reaction.

The main hydroperoxide decomposition products responsible for rancid flavors include saturated and unsaturated aldehydes, ketones, hydrocarbons and alcohols, in

addition to many minor compounds. Some of the principal carbonyl compounds are n-hexanal, 2-octenal, 2-nonenal, 2-4-heptadienal, 2-4-nonedienal, 1-octen-3-one, 4-cis-heptenal, 6-transnonenal, 2-4-decadienal and many others. Some of these compounds have very low sensory threshold levels and affect milk flavor even at very low concentrations. The relative concentrations of these compounds give rise to the various off-flavors in oxidized milk products (O'Connor and O'Brien, 1995).

Health Aspects of Lipid Oxidation

Apart from the fatty acids, cholesterol can also be oxidized resulting in the formation of numerous products generally referred to as cholesterol oxidation products (COP). Cholesterol oxidation products in food have been associated with development of arteriosclerosis in test animals (Emanuel *et al.*, 1991). Peng *et al.* (1985) reported that rabbits given 2.5 mg/kg of 25-hydroxycholesterol and cholestane-3β,5α,6β–triol intravenously developed balloon-like protrusions and lesions on the luminal surface of the aortas after 24 hours. They reported that cholestane-3β,5α,6β–triol caused more endothelial damage than 25-hydroxycholesterol. Endothelial edema was also evident. Steinberg (1988) discussed the pathogenesis of atheriosclerosis and concluded that, one of the causes of fatty streaks was the intake of oxidized Low Density Lipoproteins-Cholesterol (LDL). Steinberg (1988) hypothesized that fatty streaks in arteries occur when monocytes and macrophages penetrate the endothelium, inhabit the sub-endothelial space and accumulate lipids becoming foam cells. The foam cells secrete active oxygen species or lytic enzymes that either attack the intracellular matrix, hence loosening the epithelial cells, or attack and kill the endothelial cells directly. It was also shown that the monocytes do not take in LDL but take in oxidized LDL. In the

presence of pro-oxidants such as copper, LDL is taken in by monocytes forming foam cells. Antioxidants such as α-tocopherol prevent formation of fatty streaks by preventing LDL oxidation in the tissues. It can therefore be concluded that LDL is oxidized in the tissues in the presence of pro-oxidants. Oxidized lipids are able to covalently bind to proteins, making them chemostatic for monocytes through acetyl-LDL receptor, while native LDL does not bind to protein. When these lipoproteins are established in the intima, the monocytes are able to attach themselves to, and penetrate the endothelial layer through acetyl-LDL receptor. Oxidized LDL are also able to enter the monocytes, converting them to foam cells, and are also directly toxic to endothelial cells and this can cause lesions that finally develop into arteriosclerosis. Taylor *et al.* (1979) reported that pure cholesterol fed to rabbits was not arteriogenic whereas COP were toxic to aortic endothelial and smooth muscle cells. They found that 25-hydroxycholesterol was the most potent angiotoxic agent followed by Cholestane-3β,5α,6β–triol. This was in contrast to the findings of Peng, *et al.* (1985), who reported that Cholestane-3β,5α,6β–triol caused more damage to arterial endothelium in test animals than 25-hydroxycholesterol. Emanuel *et al.* (1991) found that human subjects who were fed powdered eggs containing high levels of COP increased their plasma COP, which were then cleared from the bloodstream. It is possible that some of the COP were absorbed by monocytes while others were incorporated into the arterial walls. Consumption of COP could increase the risk of arterial endothelial damage and arteriosclerosis. As noted earlier, oxidized lipids can covalently bind to proteins and this could affect protein bioavailability.

A number of investigators have reported the occurrence of COP in various milk products including milk powder. McCluskey *et al.* (1997) found the main COP in milk powder to be 7-hydroxy derivatives, 7-keto-derivatives, cholestane-triol α-epoxides and β-epoxides. They reported that COP levels were less for milk from cows fed a vitamin E supplement than for cows on restricted feed. Nourooz-Zadeh and Appelqvist (1988) reported variable amounts of α-epoxycholesterols, β-epoxycholesterols, cholestene-diol, cholestane-triol and hydroxycholesterols in stored commercial milk powder samples. They observed that COP increased during storage periods up to 22 months but that there was no significant difference between 22 and 31 month-old samples. They reported higher levels of COP in small pack milk samples than in bulk packaged samples. Whole milk powder had mean cholesterol content of 2.0 mg/g fat while skim milk powder had 189mg/g fat. Sanders *et al.* (1989) found COP in some cheese powders, infant formula and milk powders with epoxides being the main COP. Cholesterol oxides have also been found in other foods such as, egg powder, freeze dried chicken, beef and turkey (Sander *et al.*, 1988); processed turkey meat products (King *et al.*, 1998); pancake mix, French fries, some food concentrates (Won Park and Addis, 1985) and tallow (Won Park and Addis, 1986). Dried egg products have the highest concentration of COP compared to other food products.

Factors Affecting Milk Powder Oxidation

Lipid oxidation in milk powder during storage is influenced by the nature of the milk, heat pretreatment, storage temperature, water activity and oxygen level. It has been found that milk from some cows undergoes oxidative rancidity faster than milk from others. This type of milk is referred to as spontaneous milk. In terms of

susceptibility to autoxidation, milk can be grouped into spontaneous, susceptible and non-susceptible milk. Spontaneous milk will undergo autoxidation in the absence of pro-oxidants such as iron or copper; susceptible milk will develop oxidized flavor in the presence of copper or iron; while non-susceptible milk does not oxidize even in the presence of iron or copper (Dunkley and Franke, 1967). This phenomenon has been attributed to hereditary factors, stage of lactation and feeding regime of the dairy cows. King (1958) found that spontaneous milk had a higher copper concentration in the fat globule membrane than non-susceptible milk. O'Connor and O'Brien (1995) suggested that spontaneous oxidation is a function of the concentrations of pro-oxidants and antioxidants in milk. Spontaneous oxidation of milk has not been fully understood although investigators have suggested that enzymes such as xanthine oxidase and cytochromes react with copper to generate singlet oxygen (O'Connor and O'Brien, 1995). Granelli (1996) reported that oxidative stability was higher in milk with higher levels of antioxidants such as tocopherol and carotene, and lower levels of superoxide dismutase and polyunsaturated fatty acids. Milk powder made from spontaneous milk was more prone to autoxidation than powder made from normal milk. In that study, it was found that epoxides and hydrocholesterols increased with storage time while there was no significant increase in 7-ketocholesterol. Granelli (1996) concluded that 7-ketocholesterol is not a good indicator of cholesterol oxidation. This is in contrast to the findings of McCluskey *et al.* (1997), and Nourooz-Zadeh and Appelqvist (1988) who found 7-ketocholesterol to be a common cholesterol oxide in milk powder. Copper and iron, which are powerful pro-oxidants, are normal constituents of fat globule membranes. Antioxidants such as tocopherols and ascorbic acid are also found in milk.

Increasing the amount of tocopherols in the diet of lactating cows has been shown to increase vitamin E content in milk and to reduce fat oxidation in dried milk (McCluskey *et al.*, 1997). Milk from pasture fed cows is generally less prone to spontaneous oxidation than cows fed on dry feed due to a higher content of tocopherols in the pasture (Bruhn and Franke, 1971; Urbach, 1990). It is therefore safe to predict that increasing the level of antioxidants in milk will retard lipid oxidation during storage.

Heat treatment of milk has long been known to affect the oxidative stability of milk. Heat denatures some of the whey proteins especially β-lactoglobulin leading to activation of thiol (sulfhydryl) groups, which have antioxidant properties (O'Connor and O'Brien, 1995). It has been shown that the rate of development of thiobarbituric acid reactive substances (TBARS) during storage is lower for high-heat than for low-heat milk powder (Stapelfeldt *et al.*, 1997a). McCluskey *et al.* (1997) reported that peroxide value (PV), TBARS and COP in freshly dried milk were higher for high than for low-heat milk but this reversed during storage. Nourooz-Zadeh and Appelqvist (1988) also reported the presence of COP in fresh high-heat milk powder but were absent in fresh low and medium-heat milk powders. The higher initial oxidation could be attributed to the increased rate of oxidation during heat treatment as a result of higher temperature and longer holding times. The lower rate of autoxidation during storage of high heat powder could be attributed to the higher amounts of sulfhydryl groups produced during heat treatment (O'Connor and O'Brien, 1995). The sulfhydryl groups react with free radicals to form stable molecules hence terminating the chain reaction. Reactive thiols are also thought to bind metal ions such as copper, hence decreasing its pro-oxidant capacity (Walstra and Jenness, 1984). Severe heat treatment of milk can

initiate non-enzymatic browning reactions such as Maillard reactions, which lead to production of some reducing compounds of the enediol type (Walstra and Jenness, 1984). Once started, Maillard reactions continue during storage, even at low temperature, resulting in off-colors, off-odors and loss of some amino acids such as lysine. Heat damage can also lead to a decrease in powder solubility. This tends to limit the use of heat treatment as an antioxidative tool in milk powder production.

Water activity (a_w) has been reported to affect the rate of lipid autoxidation in milk powder by a number of investigators. Tuohy *et al.* (1981) reported an increase in lipid oxidation of milk powder during storage, with increased water activity. Stapelfeldt *et al.* (1997a) reported a positive correlation between water activity and the rate of TBARS development for milk powder during storage at 45 °C, but not for powder stored at 25 °C. They found no significant difference in TBARS or sensory characteristics between milk powder samples stored at a_w 0.11 and 0.23 at 25°C, but there was a significant increase in TBARS for those stored at a_w of 0.31 at 45°C. They attributed this to a combination of increased autoxidation and Maillard reaction at the higher water activity and temperature. High a_w and temperature can also result in protein polymerization and reduced solubility of the powder. Wewala (1990) reported that increasing a_w in the range of 0.09-0.28 improved oxidative stability of milk powder with no adverse effects on flavor, color, flowability or lumpiness when stored at 30 °C but not at 40 °C; solubility was decreased for a_w above 0.26. A water activity range of 0.21-0.24 was proposed for optimum stability of whole milk powder. Development of color is faster at elevated temperature because of the reaction of lipid oxidation products with proteins and Maillard reactions. These reactions could also lead to protein

polymerization, decreased solubility and loss of lysine (Kieseker and Clarke, 1984; Kikugawa, 1986; Stapelfeldt *et al.*, 1997a; O'Connor and O'Brien, 1995). From these studies, it is therefore necessary to store whole milk powder in cool dry environments so as to increase its shelf life.

Use of Anti-oxidants in Milk Powder

A number of compounds have been studied as possible antioxidants in milk powder. Busch *et al.* (1952) reported that nordihydroguaiaretic acid (NDGA) decreased fat oxidation in milk powder when applied at the rate of 0.05% of the fat. They reported that ascorbic acid at 50 mg/L was synergistic to NDGA but did not show antioxidative properties on its own. Abbot and Waite (1962) reported that butylated hydroxyanisole (BHA) applied at a rate of 0.01%, decreased lipid oxidation and that dodecyl-gallate was a more effective antioxidant than propyl-gallate and NDGA. They also reported that a flavone, 6-dodecyl-3-7-2'-5'-tetrahydroxy flavone, was as effective as dodecyl-gallate as an antioxidant. Tamsma *et al.* (1963) listed antioxidants in decreasing order of effectiveness as: lauryl-gallate, propyl-gallate, NDGA, ascorbyl palmitate, BHA, ascorbic acid, dihydroquercetin, sodium diethyldithiocarbamate and thiodipropionic acid. They reported that heating milk at 74°C improved oxidative stability more effectively than all the antioxidants tested. Hill *et al.* (1969) reported that thiodipropionic acid, propyl-gallate and butylated hydroxytoluene (BHT) significantly reduced lipid oxidation in milk and that they were superior to NDGA, and carboxymethylmercaptosuccinic acid. In a review on antioxidants for use in milk powder, Hammond (1970) classified antioxidants as primary antioxidants and synergists. Primary antioxidants react with intermediate products terminating the chain

reactions while synergists were defined as the ones that combine with metal ions preventing them from catalyzing the reactions. The review reported that the most effective antioxidants for use in milk were, BHT, Propyl-gallate and thiodipropionic acid. Thiodipropionic acid gave a grape-like flavor at concentrations above 0.01% (w/w) while propyl-gallate gave off-color to baked products. Min *et al.* (1990) observed that foam dried milk was more stable to oxidation than spray dried milk. They further observed that propyl gallate at concentration of 100ppm of dry powder reduced oxidation of milk fat more than BHA or tertiary butylhydroquinone at the same concentration. These reported studies indicate that antioxidants can improve the oxidative stability of WMP during storage.

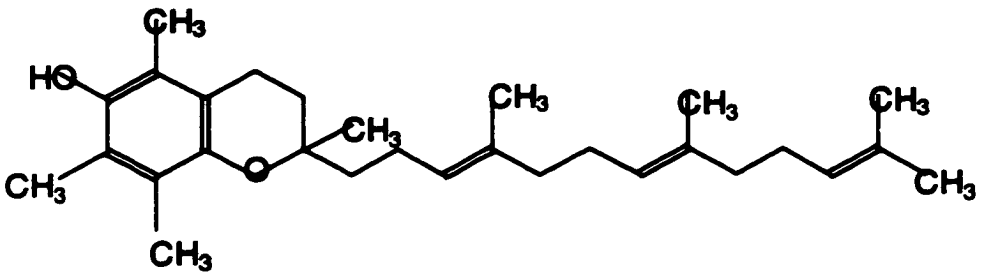
Current FDA legislation allows the use of "safe and suitable" antioxidants in whole milk powder. However, no specific antioxidants are given and ferulic acid is not one of the approved antioxidants for use in foods (FDA, 1996). Some of the approved antioxidant plant extracts however, have high levels of ferulic acid as one of the active ingredients (Graf, 1992).

Anti-oxidative Properties of Rice Bran Oil

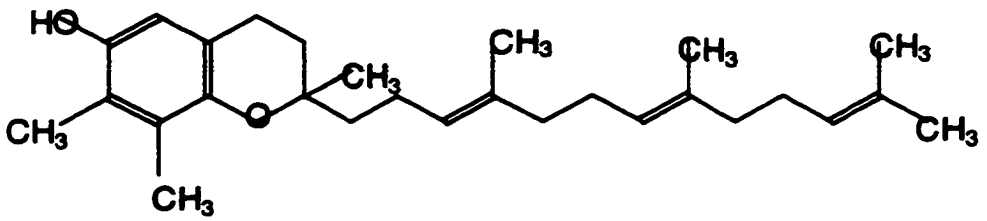
Several researchers have reported that rice bran oil (RBO) has anti-oxidative properties which are attributed to the presence of oryzanol, tocotrienols and tocopherols (Graf, 1992). Oryzanol is a common collective term referring to esters of ferulic acid with cyclic dimethyl and desmethyl sterols and other compounds (Norton, 1995). Other names for ferulic acid are 3-(4-hydroxy-3-methxyphenyl)-2-propenoic acid, 3-methoxy-4-hydroxycinnamic acid, caffeic acid 3-methyl ether and coniferic acid. It usually occurs as the *trans*-isomer in plants but equilibrates to 23% *cis* and 77% *trans* form

upon storage at room temperature (Fenton *et al.*, 1978). Oryzanol was first isolated from rice bran oil (*Orysae sativa S*), and identified as a family of ferulic acid esters of unsaturated triterpenoid alcohols and given the name γ-oryzanol (Diack and Saska, 1994). It is not a single compound but a group of compounds referred to as α-, β-, and γ-oryzanol (Norton, 1995). Figure 1 gives the chemical structure of some common tocopherols and tocotrienols while Fig. 2 gives the chemical structure of some oryzanols.

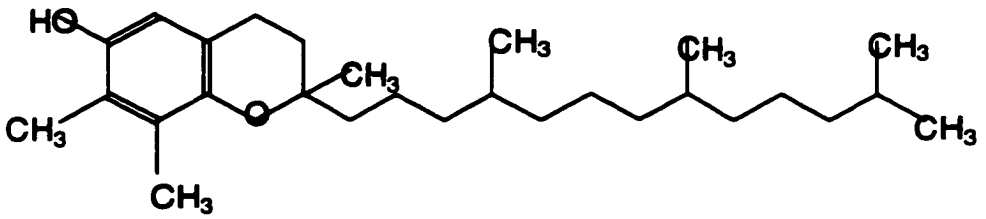
Snacks fried in rice bran oil have been shown to be more stable to oxidative deterioration than those fried in other vegetable oils. Snacks fried in RBO and stored at 40 °C under light intensity of 10,000 lux developed detectable rancid odors after 45 hours compared to 37 hours for those fried in corn oil, 19 hours for those fried in canola oil, 17 hours for soy bean oil and 12 hours for cotton seed oil. RBO was also found to be the most thermostable of the oils tested (Anonymous, 1991). Oryzanol has been reported to reduce the oxidation rate of linoleic acid (Yagi and Ohishi, 1979). The antioxidant properties of oryzanol are attributed to the ferulic acid moiety. Ferulic acid terminates free radical chain reactions by giving a hydrogen atom, which neutralizes the radical, while its unpaired electron is resonance stabilized by relocation in the entire molecule. This stable radical does not participate in chain reactions thereby terminating the reaction. Ferulic acid is also a strong ultra-violet radiation absorber, and therefore protects sensitive compounds from the UV autoxidative degradation (Graf, 1992). Direct addition of oryzanol in food, as an antioxidant has been approved in some countries such as Japan, but not in USA.



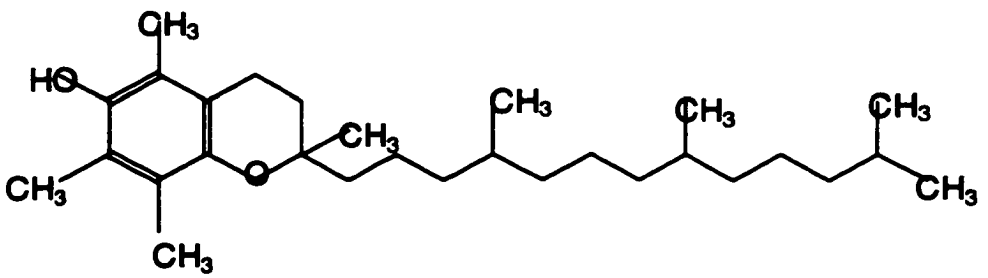
Alpha-tocotrienol



Gamma-tocotrienol



Gamma-tocopherol



Alpha-tocopherol

Figure 1. Tocopherol and tocotrienol molecules

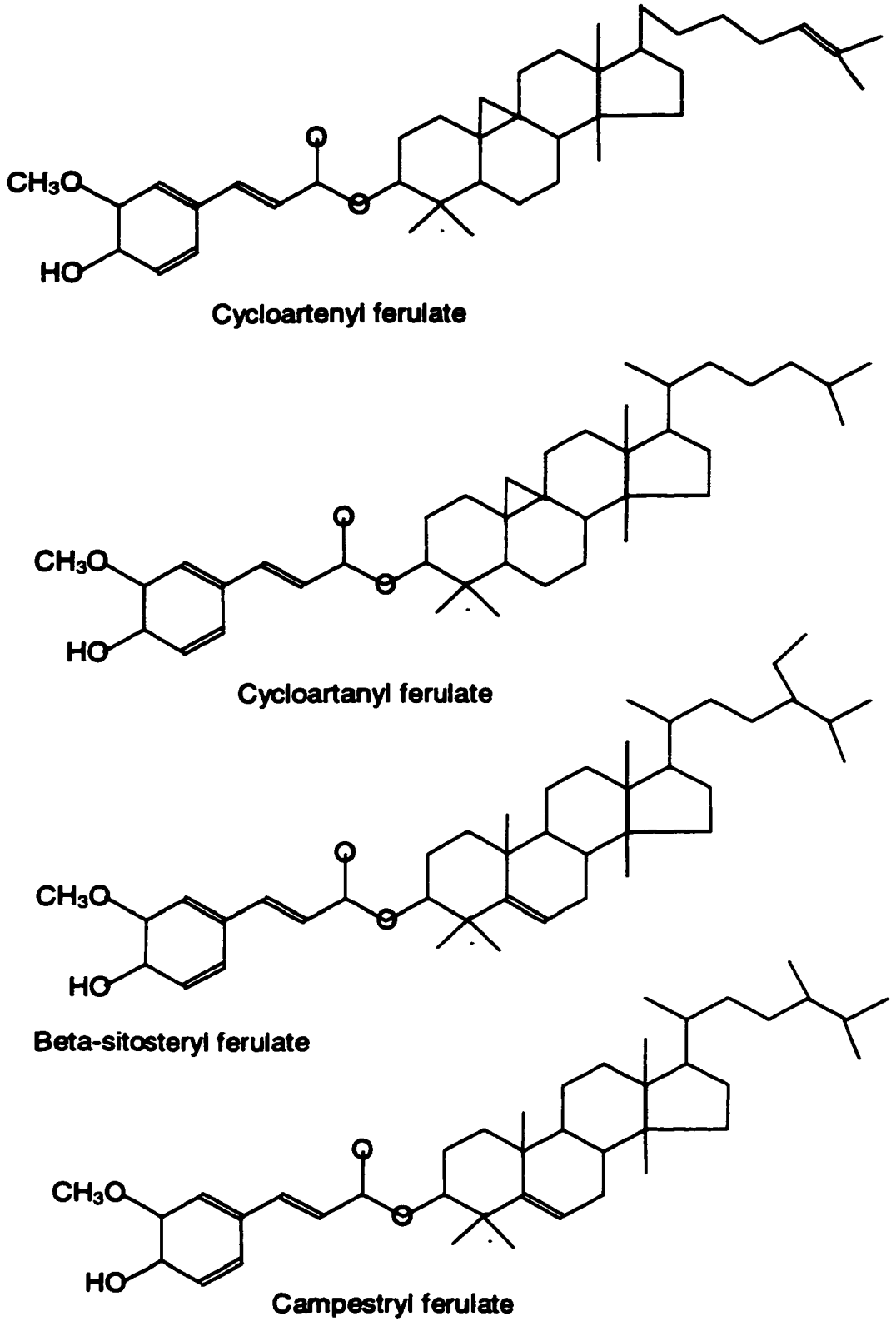


Figure 2. Chemical structure of some common oryzanols

However, some high ferulic acid natural plant extracts have been approved as antioxidant concoctions (Graf, 1992).

Gamma-oryzanol is part of the unsaponifiable matter in RBO and it is possible to retain it in the oil by partially refining the oil. Yoon and Kim (1994) reported no significant difference between the γ-oryzanol and tocopherol content of crude and degummed rice bran oil. They also reported a slight increase in tocopherol and γ-oryzanol content of bleached oil after deodorizing. This could be due to the relative increase in concentration when the flavor compounds are removed. Alkaline refining significantly reduced tocopherol and oryzanol content thus lowering the oxidative stability of the oil. There was also no significant difference in stability between crude and degummed oil. This indicates that it might be possible to de-gum and deodorize the oil without significantly reducing its anti-oxidative properties as long as alkaline refining is omitted.

Tocotrienols differ from tocopherols (vitamin E) in that tocotrienols have three double bonds on the hydrocarbon side chain while it is saturated in tocopherols. There are two common vitamin E molecules; α-tocopherol has three methyl groups attached to the chromane ring while γ-tocopherol has two methyl groups (Fig. 1). Tocopherols and tocotrienols have antioxidative properties with the latter being more potent (Yamaoka *et al.*, 1991).

Increasing vitamin E content improves the oxidative stability of fatty food products. Kingston *et al.* (1998) reported improved oxidative stability of cooked pork from pigs that had been fed a high vitamin E ration compared to those fed a control ration. The vitamin E content in the muscle of test pigs was 4.24 μg/g compared to

0.97μg/g muscle for control pigs. Other researchers have reported similar improvements in oxidative stability of meats from animals fed high vitamin E rations (Cannon *et al.*, 1998; Engeseth *et al.*, 1993; Silva *et al.*, 1994; Wulf *et al.*, 1995). These findings suggest that tocopherols and tocotrienols have antioxidant properties. McCluskey, *et al.* (1997) found that feed supplemented with vitamin E resulted in an increase in vitamin E content of milk. The oxidative stability of the milk lipids during storage of milk powder made from the high vitamin E milk was improved and the heat stability of whey proteins of the powder was also improved. Chen *et al.* (1998) found that increasing the dietary α-tocopherol content for laying hens increased the α-tocopherol content of the egg-yolk. The oxidative stability of the egg yolk improved with increase in α-tocopherol content up to 50 μg/g, but α-tocopherol had a pro-oxidative effect on the egg yolks with 75 μg/g.

Health Aspects of Rice Bran Oil

Apart from antioxidant properties, RBO has been reported to possess some potential health benefits. Rong *et al.* (1997), reported that hamsters fed a hypercholestrolemic diet containing 1% oryzanol showed a significant decrease in plasma total cholesterol, (LDL, intermediate density lipoproteins ⟨IDL⟩ and very low density lipoproteins ⟨VLDL⟩) compared to control hamsters fed the same diet but without added oryzanol. Oryzanol also caused a reduction in cholesterol absorption and reduced the formation of aortic fatty streaks. Seetharamaiah and Chandrasekhara (1993) compared the effect of oryzanol, curcumin and ferulic acid on rat serum cholesterol and lipoproteins. They reported that feeding rats on diets containing 0.5% oryzanol, 0.15% curcumin and 75mg% ferulic acid lowered serum total cholesterol and

serum lipoproteins (LDL & VLDL). Rats fed a diet containing 0.5% oryzanol, 0.15% curcumin and 75mg/g ferulic acid had a decrease in total serum cholesterol of 20%, 20% and 25% respectively. These supplements also lowered the ratio of LDL to HDL cholesterol. Oryzanol and curcumin decreased liver cholesterol but ferulic acid did not. These researchers reported that oryzanol was a superior hypocholesterolemic agent compared with ferulic acid or curcumin. The cholesterol lowering effect of rice bran oil in elderly humans has been reported to be similar to corn and canola oil but superior to olive oil (Lichtenstein *et al.*, 1994). In that study, the level of oryzanol and tocotrienol in the RBO was 0.51 and 0.49 μg/g respectively and, at this level, oryzanol may not be sufficient to have a significant effect under the conditions of the study. These researchers reported that tocotrienols block cholesterol synthesis, thus lowering its concentration in the blood.

Rice bran oil is also a good source of vitamin E. Godber (unpublished data) found 243 μg/g and 354 μg/g α-tocopherol in degummed and crude oil, respectively. The oil was also rich in α-tocotrienols with 212 μg/g and 313 μg/g for refined and crude oil respectively. Rogers *et al.* (1993) reported tocopherol and tocotrienol contents ranging from 88 to 1609 ppm and 72 to 1157 ppm respectively for commercial RBO samples. The wide range could be due to differences in rice varieties and manufacturing methods. Gamma-oryzanol content in RBO depends on the processing method. Yoon and Kim (1994) reported that γ-oryzanol content decreased with degree of refining of RBO from 1.6% in crude oil to 0.77% in refined oil. Rice bran oil is widely consumed in Japan (80,000 tons per annum) and is gaining acceptance in other countries such as India (Sugano and Tsuji, 1997). No adverse health effects have been associated with

RBO consumption in these countries. Being a naturally occurring food constituent, use of RBO is likely to gain widespread acceptance if it is proved to be an effective antioxidant in milk powder.

Gamma-oryzanol use in Human Nutrition

A number of firms such as Vitamin Power® and AC Humco (Houston,TX) are promoting γ-oryzanol as a nutrition supplement. They offer it as a natural steroid for lean muscle development. The author is not aware of any published work in support of the claim that γ-oryzanol assists lean muscle development. Benecol, a margarine developed in Europe, is being promoted for its cholesterol lowering properties by McNeil Consumer Healthcare (Fort Washington, PA). This margarine is fortified with plant extracts containing sitostanyl ferulate, which is one of the γ-oryzanol fractions found in rice bran oil, as the active ingredient. In a review, Sugano and Tsuji (1996) reported that RBO consumption is being promoted in Japan and India, due to its plasma cholesterol lowering properties. They attributed the cholesterol lowering properties of RBO to its γ-oryzanol, tocopherol and tocotrienol content. Currently, γ-oryzanol is manufactured in Japan as a by-product of rice oil refining. Rice bran oil production in USA started in 1994 (McCaskill and Zhang, 1999) and thus is in its infancy. Rice bran accounts for about 8% of the paddy but contains about 75% of the oil found in rice (McCaskill and Zhang, 1999). In most cases, it is used as animal feed or discarded as waste. Riceland Foods (Stuttgart, AR) are involved in the production and development of high-oryzanol RBO, which could improve the oxidative stability of fatty foods and could have therapeutic effects.

Statement of the Problem

Many of the developing countries lie within the hot and humid tropics and experience wide seasonal variations in milk production. Whole milk powder production could serve as a market outlet for farmers during glut and later be reconstituted by the consumers during the periods of scarcity. In addition, these countries have often been recipients of food aid that include milk powder. Although milk powder serves as a highly nutritious food ingredient, it develops oxidative rancidity rapidly once it is removed from the package. This can lead to economic loss, and health problems associated with consumption of oxidized lipids. Controlled atmosphere (CA) storage has been used commercially to protect the powder from these changes but this protection is lost once the package is opened. Antioxidants have been shown to delay autoxidation of milk powder but the FDA has not approved any specific antioxidant for milk. A number of antioxidants have however been approved for use in other foods.

Addition of RBO to milk powder might not be considered an additive for purposes of regulation since RBO is an acceptable food product. Rice bran oil has antioxidative properties, which have been attributed to its γ-oryzanol, tocotrienol and tocopherol content. There is a need to develop safe, effective and acceptable antioxidants for whole milk powder to improve its oxidative stability. Since RBO is rich in oryzanol, tocopherol and tocotrienols, which are powerful antioxidants, there is a need to investigate its application as an antioxidant in food systems and particularly in milk powder. Rice bran oil is a natural vegetable oil and no health problems have been associated with its consumption. The author is not aware of any published work, on the

use of oryzanol as an antioxidant in milk. An investigation on the effect of high oryzanol RBO on the oxidative stability and sensory properties of whole milk powder could lead to improvements in WMP quality. This could also provide an additional use for the RBO unsaponifiable matter, which is a byproduct of oil refining, thus increasing profitability.

This dissertation work is an evaluation of the potential of RBO as an antioxidative additive in whole milk powder. The study investigated the effect of the oil on lipid oxidation, cholesterol oxidation, free radical formation and consumer preference of milk powder.

CHAPTER TWO: FORTIFICATION OF WHOLE MILK POWDER WITH HIGH ORYZANOL RICE BRAN OIL: EFFECTS ON CHEMICAL AND SENSORY CHARACTERISTICS

Introduction

The most widely used method for the commercial production of milk powder is evaporation of milk under vacuum followed by spray drying. Spray drying involves the introduction of milk in droplet form into a hot air chamber. Due to the large surface area to volume ratio created by the droplets, evaporation of water is very rapid causing almost instantaneous drying. This has a cooling effect and hence avoids excessive temperatures that could otherwise lead to heat damage of the powder particals. The basic operations in milk powder production are clarification, standardization, heat treatment, homogenization, evaporation, drying and packaging (CariNc, 1994). Processing conditions can affect physical and chemical properties of the powder but can be varied to give a product with desired characteristics. The degree of concentration affects particle size, bulk density, and solubility of powder (Morr and Richter, 1988). Heat treatment destroys all vegetative pathogens, lowers the microbial load, inactivates some milk enzymes, especially lipase, and activates sulfhydryl groups of β-lactoglobulin. Sulfhydryl groups can increase resistance to autoxidation during storage. An increase in cholesterol oxides has been reported during this pre-concentration heating process in the manufacture of high heat milk powder (Nourooz-Zadeh and Appelqvist, 1988). Excessive heating can lead to Maillard and other reactions, which might lower the powder quality.

Addition of other ingredients to milk is done for various reasons. Milk is oftentimes fortified with vitamin D (Walstra and Jennes, 1984). The FDA allows the addition of antioxidants in whole milk powder (WMP) but no specific antioxidants are given (FDA, 1996). Dietary intake of antioxidants is reported to have beneficial health effects. Lichtenstein *et al.* (1994) reported that, rice bran oil (RBO) reduced plasma low-density lipoproteins (LDL) in moderately hypercholesterolemic humans. Nicolosi *et al.* (1991) reported that rice bran oil (RBO) reduced total cholesterol and LDL in non-human primates. Other investigators have also reported similar cholesterol lowering effects due to consumption of RBO (Qureshi *et al.*, 1997; Rong *et al.* 1997; Sugano and Tsuji, 1997; Graf, 1992). These properties are thought to be due to the oryzanol, tocopherol and tocotrienol content of RBO. Consumption of dietary antioxidants has been associated with reduced incidence of coronary heart disease (CHD). In a 25-year collaborative study in seven countries, Verschuren *et al.* (1995) reported that there were significant differences in CHD incidence for similar cholesterol intake levels between different cultures. As part of the same study, Kromhout *et al.* (1989) reported that there were dietary differences among the groups studied, and those whose diet was rich in antioxidants had a reduced risk of CHD mortality. In the same study, Ocke *et al.* (1995) reported that intake of vitamin C was inversely related to stomach-cancer mortality.

Since RBO has been shown to have cholesterol lowering and antioxidative properties, addition of RBO in milk powder might improve its oxidative stability and affect the cholesterol metabolism of consumers. Addition of RBO to milk powder might however affect some chemical and organoleptic properties of the milk. This

study was carried out to investigate the effect of RBO on some of the chemical and sensory characteristics of the milk powder.

Materials and Methods

Manufacture of Low Heat Milk Powder

Milk powder was manufactured as described by Stapelfeldt *et al.* (1997a) with some modifications. Whole milk, with 3.6% fat, which had been pasteurized at 74 °C for 20 seconds, was collected from the Mississippi State University Dairy Plant. The milk was then evaporated using a rising film, APV laboratory vacuum evaporator (APV Pasllac Anhydro AS, Copenhagen, Denmark) under vacuum at 45-50 °C to 40 % total solids. Three 3-kg samples of the concentrated milk were weighed into stainless steel buckets. High Oryzanol RBO (Riceland Foods Inc., Stuttgart, Arkansas) was added to each sample so as to have 0.00, 0.10 and 0.20% RBO (w/w) in the original milk. The oil and milk were mixed using a hand stirrer and homogenized at 14,000 kPa. The milk was then spray dried in an APV Laboratory Model Spray Drier (APV Anhydro, Φstomarken, Soborg, Denmark), fitted with a peristaltic pump and a rotary atomizer. The air inlet temperature was 200 °C and the outlet temperature 100°C. The treatments were replicated three times. The dried milk was then put into resealable Ziploc® bags (Johnson Home Storage Inc., Racine WI) shipped to Louisiana State University and stored at –40° C until further procedures were applied.

Manufacture of High Heat Powder

A second batch of whole milk, 3.6% fat, which had been pasteurized at 74°C for 20 seconds was further heat treated at 88°C for 15 minutes in a steam jacketed vat and cooled to 7°C. It was concentrated to 40% solids using the same procedure as described

previously. Three 3-kg samples of the concentrated milk were taken and treated as described for the low-heat powder. The milk was then spray dried with an air inlet drying chamber temperature of 200°C and an outlet temperature of 105°C. The treatments were replicated three times and all the samples handled as for the low-heat powder.

Chemical and Sensory Analysis

Samples were then analyzed by methods described in the following paragraphs. Samples that were not analyzed immediately were stored in plastic bags at –40° C until needed.

Moisture Content Determination

Moisture content was determined using the oven-drying method at 102 ± 2 °C for 3 hours as described by Bradley *et al.* (1993).

Fat Content Determination

Fat content was analyzed using Rose-Gottrieb method as described by Bradley *et al.* (1993). One gram of milk powder was placed in a 50-ml beaker, one milliliter water was added and rubbed into a paste using a stainless steel spatula . Nine milliliters more water and 1.1 ml ammonium hydroxide (0.9 s. g.) was added and mixed to dissolve all the milk. The sample was warmed over a boiling water bath and quantitatively transferred to an extraction flask with 10 ml denatured ethanol and allowed to cool. The sample was extracted by mixing with 25 ml ethyl ether for 1 minute and 25 ml petroleum ether for the same length of time. The ether layer was decanted into a pre-weighed fat dish and the extraction repeated three times with 15 ml of the ethers each time. The ether extracts were mixed and the solvents evaporated

gently on a hot plate, followed by drying at 102 ± 2 °C for 3 hours. The dish was cooled in a dessicator, weighed and the weight of the fat was determined by subtracting the weight of the dish. Percentage fat was then calculated.

Color Determination

Color was determined using a Minolta Spectrophotometer Model CM508d camera (Minolta Co., Japan). Milk samples were placed in clear transparent plastic bags and the camera was pressed against the bag. The camera was set to take three photographs of the powder and record the mean L, a, and b values.

Oryzanol and Vitamin E Analysis

Saponification and solid phase extraction (SPE) was done following a modification of the procedure described by Shin *et al.* (1997). One gram WMP, 5 ml ethanol and 0.1 g ascorbic acid were placed in a 15-ml test tube. The contents were mixed by vortexing, incubated at 80 °C for 10 minutes, 0.15 ml of 80% KOH was added and allowed to saponify for 15 minutes. The contents were allowed to cool to room temperature. Two ml of 15% acetic acid was added, mixed and centrifuged for 5 minutes using a Babcock centrifuge. The supernatant was siphoned using a Pasteur pipette and placed in a test tube. The residue was extracted two more times by adding 5 ml ethanol plus 2 ml 15% acetic acid, mixing and centrifuging as described above and the supernatants were pooled. The 3-ml Supelclean LC-18 SPE tubes (Supelco, Bellfonte, PA) were washed with 2 ml methanol followed by 2 ml1% acetic acid. The extract was passed through the SPE tube at less than 2ml per minute and the eluent discarded. The SPE tube was washed sequentially with 2 ml water, 2 ml of 1% acetic acid and 0.5 ml methanol/water (50:50 v/v) to remove the unretained material. The SPE

tube packing was dried under helium using the Visidry™ attachment to the manifold (Supelco, Bellfonte, PA). Oryzanol and the vitamin E vitamers were eluted thrice with 0.5 ml ethyl acetate/hexane (20:80 v/v). The solvent was dried under a stream of nitrogen and diluted with 1 ml mobile phase.

The high performance column chromatography (HPLC) system consisted of a Waters model 501 pump (Waters, Milford, MA), Waters model 717 plus autosampler (Waters, Milford, MA), and a Waters 486 tunable absorbance UV/VIS detector (Waters, Milford, MA). The HPLC Analysis method described by Xu and Godber (Unpublished) was modified for the analysis of the extract. The γ-oryzanol was analyzed isocratically using a Waters Nova Pak® 3.9x150 mm C-18 column (Waters, Milford, MA). The mobile phase was ethanol/acetonitrile/dichloromethane/acetic acid (50:44:3:3 v/v) at a flow rate of 1.4 ml/min at ambient temperature. The detector was set at 330 nm wavelength. Data was acquired and processed using Waters Millenium[32] Software (Waters, Milford, MA). Vitamin E was analyzed using the same procedures as γ-oryzanol but the detector wavelength was set at 290 nm.

A γ-oryzanol standard curve was generated using RBO with known oryzanol concentration, which was dissolved in 10 ml diethyl ether to obtain a 500 μg/ml γ–oryzanol solution. Predetermined quantities of the solution were transferred to test tubes so as to have 0, 100, 200, 300, 400 and 500 μg γ–oryzanol. The solvent was dried under a stream of helium. Saponification and solid phase extraction was performed in the same manner as the WMP samples. The extracts were subjected to HPLC analysis as described for the WMP samples and the area of three prominent peaks regressed against the amount of γ-oryzanol. The three peaks whose area was to be used for

quantification of oryzanol were identified by their retention times. The area of the peaks was then added. The regression equation obtained from the standards was applied to the peaks area to quantify the oryzanol content of WMP samples.

To quantify vitamin E, a standard solution containing 400 μg/g tocopherol (Sigma Chemical Co., St. Louis MO) in the mobile phase was prepared. Predetermined volumes of the standard solution were made up to one ml with the mobile phase so as to have 0, 4, 8, 12, 16, 20, and 40 μg/g tocopherol. The samples were then analyzed by HPLC following the procedures described for the WMP samples. The peak areas were used to make a standard curve and a regression equation generated using Microsoft Excel™ (Microsoft Corporation, Seattle, WA). The regression equation was used to calculate the amount of vitamin E in the samples and expressed as μg/g of the WMP.

Sensory Evaluation

Milk powder was mixed with distilled water at the ratio of 125 g powder to one liter of water in a warring blender at room temperature. After all the lumps dissolved, the milk was pasteurized at 72 °C for 15 seconds using a laboratory scale plate pasteurizer and cooled to 4° C.

The Triangle test, as described by Lawless and Heymann (1998), was used to evaluate the flavor differences for milk reconstituted from 0.1% and 0.2% RBO milk powder with that reconstituted from milk powder without added RBO. Milk samples were presented to the panelists under red light to mask any differences in color. Preference test, as described by Lawless and Heymann (1998), was also carried out to determine the effect of 0.1% and 0.2% RBO addition to milk powder on the consumer preference of the reconstituted milk as compared with control sample.

The sensory panel was drawn from students and workers from Louisiana State University. Samples were coded with three digit random numbers, which were generated using the Microsoft Excel® software (Microsoft Corporation, Seattle WA).

Statistical analysis

The general linear model (GLM) procedure (SAS® software for Windows Ver. 6.12 SAS Institute, Cary, NC) was used for data analysis. Sample means were compared using the Tukey procedure. Rice bran oil content was treated as the fixed independent variable while heat treatment was treated as a block effect in a completely randomized block design. A confidence level of 95% was as the criteria for declaring difference in means. Sensory data was analyzed using the X2 probability table for triangle and paired preference tests (Lawless and Heyman, 1998).

Results and Discussion

Moisture, Fat Content and Antioxidants

The milk powders had mean moisture content of 1.03 ± 0.54% (Table 3), which is lower than the expected value of 3 to 4%. The low moisture content of the powder could have been due to the high exit temperature during the drying process. Added RBO had no significant effect on the moisture content, but moisture was significantly lower for high heat powder than low heat powder. The ideal outlet temperature should be 95°C or lower (Cari'c, 1994). Fortifying milk with 0.2% RBO significantly increased the fat content ($P < 0.05$), but there was no significant increase at the 0.1% RBO level (Table 3). Addition of RBO increased the γ-oryzanol and vitamin E content of the WMP (Table 3). Low heat WMP had higher vitamin E and γ-oryzanol content than high heat powder. During processing, some milk powder samples developed

electrostatic properties and adhered to the drier wall. This problem was more prevalent for high-heat milk powder. This could have the effect of increasing the resident time of the powder in the drying chamber thereby lowering the moisture content. This could have also caused thermal degradation of some milk components.

Table 3. Effects of rice bran oil supplementation on the mean composition of whole milk powder*

RBO %	Moisture %	Fat %	γ-Oryzanol (ppm)	Vitamin E (ppm)
0.00	0.90	33.26[a]	0.00[a]	0.00
0.10	1.21	34.12[ab]	49.16[a]	3.72
0.20	0.97	34.61[b]	180.11[b]	7.54

* Numbers in the same column followed by the same letter are not significantly different ($P < 0.05$).

Color

Rice bran oil caused a significant reduction in the 'L' value (white), and increased the 'b' value (yellow) but had no effect on the 'a' value (red) ($p < 0.05$) (Table 4). High heat powder had a significantly lower 'L' (lightness) value than low heat powder and higher 'b' value (yellow) but no effect on the 'a' value (red) (Table. 4). High oryzanol rice bran oil has a pale yellow color and this was reflected by an increase of the powder 'b' and 'L' values. Heat treatment of milk led to a darkening of the powder due to browning reactions. Heat can cause the carbonyl groups and amino groups of proteins to react giving brown pigments (Koga *et al.*, 1997). Milk has both proteins and reducing sugars, mainly lactose, and therefore heating can cause Maillard reactions, which result in darkening of the powder (Cari'c 1994). This agrees with the findings of

Nielsen *et al.* (1997), who reported that whole milk powder darkened and became more yellow during accelerated storage but had no effect on redness. Quinones *et al.* (1998) reported that the protein content of milk had an effect on the color of liquid milk. Lowering protein content reduced the 'L' and 'a' values but increased the 'b' value of 2% and 3.3% fat milk. Other factors that have been shown to affect the color of market milk are: fat content that causes an increase in 'L' value, homogenization that increases the 'L' value, and UHT pasteurization that caused an increase of 'L' but a decrease in 'a' and 'b' values (Bergann and Schick, 1997). Koga *et al.* (1997) reported that cooking oil increased in 'redness' after frying model papers, which had been soaked in amino acid solutions. Heat did not have any significant effect on the redness (a) value.

Table 4. Effect of rice bran oil on whole milk powder color*

RBO level	'L' (Lightness)	'b' (Yellow)	'a' (Red)
0.0	95.96[a]	9.56[a]	3.05[a]
0.1	95.23[b]	12.28[b]	-0.91[a]
0.2	95.06[b]	13.31[b]	-0.95[a]

*Values in the same column followed by the same letter are not significantly different ($P < 0.05$).

Sensory Evaluation

Fortification of milk with 0.1% RBO had no significant effect on sensory characteristics of the milk powder (Table 5). However at 0.2% RBO a detectable effect ($P < 0.05$) on the sensory characteristics of reconstituted milk were observed (Table 5). The difference could have been due to the effect of RBO on the flavor of milk. This could

have been a direct contribution of the rice oil to flavor or could have been indirect due to the effect of RBO on oxidation.

Table 5. Triangle test results for 0.1% and 0.2% rice bran oil fortified whole milk powder against control

	N	Correct responses	Critical correct responses	
Sample			α=0.01	α=0.05
0.1% RBO	17	5	11	9
0.2% RBO	21	12*	13	12

* Significant difference ($P < 0.05$)

Fortification of milk powder with 0.1% RBO had no significant effect on consumer preference when compared with control milk (Table 6). Milk powder without RBO was preferred ($P < 0.01$) to milk with 0.2% RBO (Table 6). This decrease in preference could be due to the detectable flavor change caused by the addition of rice bran oil. Rice flavor in milk is not a familiar characteristic and this is likely to be unacceptable when tasted for the first time. Consumers who are familiar with rice flavors might not find the product objectionable and as such, products containing RBO could be developed for the export market. This however would have to be investigated further.

Addition of RBO to milk powder at the rate of 0.1% did not have a detectable effect on the sensory properties of the powder except on color, which was more yellow than control. At 0.2% RBO, there was a detectable effect on sensory properties and fat content of the milk powder. This suggests that RBO can be added to milk powder at 0.1% without a significant effect on the acceptability of the powder.

Table 6. Paired preference test results for 0.1% and 0.2% rice bran oil fortified whole milk powder against control

	N	Preference for:		Critical value	
Sample		Control	With RBO	α=0.01	α=0.05
0.1% RBO	19	13	6	16	15
0.2% RBO	21	17*	4	17	16

*Significant preference ($P < 0.05$)

CHAPTER THREE: INFLUENCE OF HIGH ORYZANOL RICE BRAN OIL ON FREE RADICAL FORMATION IN WHOLE MILK POWDER

Introduction

Lipid oxidation is initiated by the formation of free radicals that react with unsaturated fatty acids to give odorless and tasteless, but highly unstable hydroperoxides, as the primary oxidation products. Free radicals are formed when a hydrogen atom is abstracted from the methylene group adjacent to the double bond, the α-methylene group (Bading, 1960). Free radicals are very reactive and react with unsaturated monocarboxylic acids leading to the production of more free radicals among other products. Apart from initiating autoxidation reactions in fat, some free radicals have been shown to cause DNA damage, which can lead to mutations and development of tumors (Pryor *et al.*, 1998; Elliott, 1999). Hydroxyl radicals are produced during autoxidation of unsaturated fatty acids and are more toxic than other free radical species (Zangh *et al.*, 1996). Formation of free radicals from lipids may be triggered by factors such as metal catalysts, irradiation, or active oxygen species, and occur when a hydrogen atom is abstracted from the α-methylene group adjacent to the double bond (Badings, 1960; O'Connor and O'Brien, 1995; Schaich, 1980). The free radicals react with unsaturated fatty acid forming more free radicals in a chain reaction. This propagates the autoxidation reactions. The reaction is terminated when the free radicals react with each other to give stable molecules, or when a hydrogen atom is donated to the free radical making it stable.

Rice bran oil (RBO) has been reported to have anti-oxidative properties (Gaf, 1992; Yagi and Ohishi, 1979). This antioxidative property is attributed to the high contents of γ-oryzanol, tocopherol and tocotrienol in RBO (Yamaoka *et al.*, 1991; Yoon & Kim, 1994). One of the anti-oxidative mechanisms of γ-oryzanol is through neutralization by donating a hydrogen atom to the free radical, thus stopping the chain reaction (Graf, 1992). Gamma-oryzanol also absorbs UV radiation and can slow down electromagnetic radiation-induced formation of free radicals. Zangh *et al.* (1996) reported that an extract from ginseng plant scavenged free radicals. Ginseng extract is used in China for the treatment of several conditions including antiaging, immuno-enhancer, antistress and anti-tumor (Zangh *et al.*, 1996). The therapeutic effects of this extract have been linked to its antioxidative properties. Gamma-oryzanol, which has antioxidative properties, could therefore have therapeutic effects since it might inhibit development of free radicals in foods and oxidative cell damage in humans.

Free radicals have an unpaired electron that has a magnetic moment and can resonate in a changing magnetic field under the right conditions. The free radicals can thus give an electron spin resonance (ESR) signal while lipids do not normally give one (Chapman and GoNi, 1986). Free radicals have been detected in milk powder by ESR spectroscopy (Stapelfeldt *et al.*, 1997b). These researchers reported that free radical concentrations during storage of whole milk powder as determined by ESR correlated well with sensory characteristics and thiobarbituric acid reactive substances (TBARS). The author is not aware of any published work on the use of RBO as an anti-oxidant in dried milk products. Addition of high oryzanol RBO to milk powder is likely to slow the formation of free radicals and hence reduce the rate of autoxidation during storage.

This research was carried out to study the effect of high-oryzanol rice bran oil on the formation of free radicals in whole milk powder. Milk powder was fortified with high oryzanol RBO and analyzed for free radical using ESR spectroscopy. Lipid oxidation was determined by measuring thiobarbituric acid reactive substances (TBARS).

Materials and Methods

Manufacture of Low Heat Milk Powder

Milk powder was manufactured following the procedures described by Stapelfeldt *et al.* (1997a) with some modifications. Whole milk, 3.6% fat, which had been pasteurized at 74 °C for 20 seconds, was collected from The Mississippi State University Dairy Plant. It was then evaporated under vacuum at 45-50° C to 40 % total solids using a rising film, APV laboratory Vacuum evaporator (APV Pasllac Anhydro AS, Copenhagen, Denmark). Three 3-kg samples of the concentrated milk were taken and high oryzanol rice bran oil (Riceland Foods Inc. Stuttgart Arkansas) was added to each, giving 0.00, 0.10 and 0.20% RBO in the original milk. The oil and milk were mixed using a hand stirrer and homogenized at 14000 kPa. The milk was then spray dried in an APV Laboratory Model Spray Drier (APV Anhydro, Φstomarken, Soborg, Denmark), fitted with a peristaltic pump and a rotary atomizer. The air inlet temperature was 200 °C and the outlet temperature 100°C. The treatments were replicated three times.

Manufacture of High Heat Powder

A second batch of whole milk, 3.6% fat, which had been pasteurized at 74°C for 20 seconds, was collected from Mississippi State University Dairy Plant and heat treated at

88°C for 15 minutes in a steam jacketed vat and cooled to 7°C. It was concentrated to 40% solids, RBO added as for low-heat powder and dried using the same procedure as described previously except that the outlet temperature was 105°C. The treatments were replicated three times.

Storage of milk powder

Duplicate 25-g samples of the powders were stored in open petri dishes at 45° C, over a saturated aqueous solution of magnesium chloride of a_w 0.31 (Nielsen *et al.* 1997) for 10 days. Storage space in the incubator was randomly allocated for each sample. Free radicals were analyzed using ESR spectroscopy at the beginning and end of the storage time. Fat oxidation was estimated by determining thiobarbituric acid reactive substances at the beginning and end of storage time. Samples that were not analyzed immediately were stored at –40° C until analyzed.

Electron Spin Resonance Spectroscopy

Electron spin resonance spectroscopy was carried out using a modification of the procedure described by Stapelfeldt *et al.* (1997b). Milk powder was placed into cylindrical ESR tubes with a 3 mm internal diameter and compacted using a flexible wire to two centimeters from the bottom of the tubes. Electron spin resonance spectra were recorded at room temperature using a Varian 109 ESR spectrometer (Varian Associates, Palo Alto, California) operating at the "X" band and employing 100 kHz field modulation. 1,1-Diphenyl-2-picrylhydrazyl (DPPH) (Sigma Chemical Co. St. Louis, MO), which is an organic stable free radical, was used as a concentration and g-value standard. Instrument settings were: sweep width 100 gauss (3310-3410 gauss), microwave power 2.0 mW, modulation frequency 9.365 ± 0.005 GHz, modulation

amplitude 4.0 gauss, receiver gain $8x10^3$, time constant 0.5 sec, scan time 2 minutes and sampling frequency 15/sec. Data was acquired with GRAMS/386 for Chromatography data acquisition software (Galactic Industries Corp. Salem, NH).

Quantification of Free Radicals

To determine the amount of powder in the ESR tubes, some of the tubes were cut so as to fit in the analytical balance. Milk powder was placed into the tubes, compacted in the same way as for the ESR and weighed. The depth of the powder in the tubes was determined and used to calculate the mean weight per cm, which was 0.041g/cm. This value was used to determine the weight of the milk powder in the ESR tubes. The signal peaks were analyzed using National Institute of Environmental Health EPR software (National Institute of Environmental Health, Research Triangle Park, NC) to give the difference between maximum and minimum points. The double integration method is typically employed to calculate the free radical concentration. However, in this investigation, there was variation in the sample ESR baseline, a factor that introduced errors in the calculation of peak area and hence could not be used to quantify the free radicals. The difference between maximum and minimum peak height was however not affected by the baseline variation. It was found to be proportional to the number of spins and thus was used to calculate the free radical concentration in the powder. To make a standard curve, known quantities of DPPH were analyzed for the ESR signal under similar conditions as for the samples, but with receiver gain set at 10.0. By taking the difference between the peak maximum and minimum and plotting it against the amount (moles) of DPPH, a standard curve was made. A regression curve was drawn using Microsoft EXCEL™ software (Microsoft Corporation, Seattle, WA)

and the regression equation from the standard curve used to calculate the number of spins per gram of fat in the milk samples.

Thiobarbituric Acid Reactive Substances

Thiobarbituric acid reactive substances were determined following the procedure described by Farkas *et al.* (1997). Five gram samples of milk powder were weighed in a 50-ml beaker and quantitatively transferred into a 500-ml Kjeldahl flask with 93 ml distilled water. Into the flask was added, 1.4 ml 4N HCl, 400 µl Tween 80 (Amresco®, Solon, OH) to emulsify the fats, 200 µl antifoam A (Sigma Chemical Co. St. Louis, MO). A 200 µl BHT (Sigma Chemical Co. St. Louis, MO) solution was also added to minimize any further oxidation, plus a few boiling glass beads. The contents of the flasks were thoroughly mixed by shaking and connected to Kjeldahl distillation units. The heating element was set at position 6.5 and about 50 ml distillate collected. The volume of the distillate was recorded. Five milliliters of the distillate was placed in a 20-ml test tube with a Teflon screw cap and 5 ml 0.02M TBA solution in glacial acetic acid (Sigma Chemical Co. St. Louis, MO) was added. After mixing, the test tubes were placed in a boiling water bath for 35 minutes and cooled in a tap water bath at room temperature for 10 minutes. A blank was prepared by using 5 ml of distilled water instead of distillate. Absorbance was read at 538nm using a Shimadzu UV-VIS-NIR Scanning Spectrophotometer model UV-3101PC (Shimadzu Corporation, Kyoto, Japan). A standard curve was made by reacting 5 ml of 1 - 10 $x10^{-7}$ M 1,1,3,3,-tetraethoxypropane (TEP) with TBA as described above. Absorbance values for the standard concentrations were used to create a linear regression equation using Microsoft Excel software (Microsoft Corporation, Seattle, WA). A linear curve was drawn with

absorbance as the abscissa axis and the molar concentration of TEP as the ordinate. All calculations were done using the Microsoft Excel® software. Sample absorbance data was converted into milligrams malonaldehyde using the linear regression equation obtained from the standard curve.

Percent recovery was determined by diluting 50 µl of 10^{-3} M TEP stock solution, in duplicate, with 93 ml distilled water in a 500 ml Kjeldahl flask and distilling as for the samples. The distillate was made to 50 ml with distilled water, a 5-ml portion of the distillate was reacted with 5 ml TBA as described above and absorbance read at 538 nm. Another 50 µl of the TEP stock solution was diluted to 50 ml with distilled water, a 5 ml portion taken and treated as for the distillate and the absorbance at 538 nm read.

Percent recovery = $A_{538,distilled}/A_{538,nondistilled} \times 100\%$ ..... Equation 1.

Results and Discussion

Quantification of Free Radicals from the ESR Signal

Electron spin resonance spectroscopy is a technique that can be used to detect free radicals and other paramagnetic species unambiguously. In this study, an ESR signal was detected in the milk samples and based on its g-value, it could be assigned to a carbon or oxygen centered organic free radical. Paramagnetic metals such as copper or manganese can also produce ESR signals, but signals from their salts were recorded to the eliminated the possibility that the signal arose from such metals. The ESR signals from these metal salts were different from the signals from the milk powder and from DPPH. Typical spectrum for milk powder samples and DPPH are shown (fig.3)

and they have similar shapes and comparable g-values. The g-value for the milk powder was calculated from the equation given by Weil *et al.* (1994).

$g_x - g_{std} = - g_{std}(B_x - B_{std})/B_{std}$ .....Equation 2

(g_x = g-value of unknown, g_{std} = g-value of standard (DPPH), B_x = center field position of unknown EPR spectrum, B_{std} = center field position of EPR spectrum of the standard). The g-value for WMP was 2.0041.

The concentration of free radicals in milk powder was estimated using DPPH as a spin standard. The standard curve of peak height difference against moles of DPPH was linear with, $R^2 = 0.997$ and the regression equation was:

$y = 0.0003x + 0.05$ ..... Equation 3

(where x = peak height difference, y = Moles DPPH

To calculate the moles of radicals for milk powder, a correction factor of 0.9 was applied to the regression equation to correct for the purity of the standard.

Radicals/g milk powder = $y/m \times N \times g_d/g_s \times 0.9$ ..... Equation 4

(m = g milk powder, N= Avogadro's number $\{6.0228 \times 10^{23}\}$, g_d = receiver gain for DPPH and g_s = receiver gain for milk sample)

Equation 4 was divided by the fat fraction of the milk powder to convert it into radicals per gram of milk fat.

Effects of RBO on Free Radicals

Rice bran oil and heat treatment did not have significant effect on the free radical content of WMP after manufacture. When data from both blocks were considered, RBO had no significant effect on free radical formation but there was a slight decrease in free radical concentration with RBO content after 10 days storage.

There was a significant block interaction ($P < 0.05$). When data was analyzed for each block separately, the free radicals in milk fortified with 0.2% RBO were significantly lower ($P < 0.05$) than in powder without RBO after 10 days of storage (Table 7 & Figure 5). There was an increase in the free radical concentration during storage for ten days for all samples but those fortified with RBO had lower concentrations than the ones without RBO. Rice bran oil had no significant effect on high-heat milk free radicals after 10 days of storage.

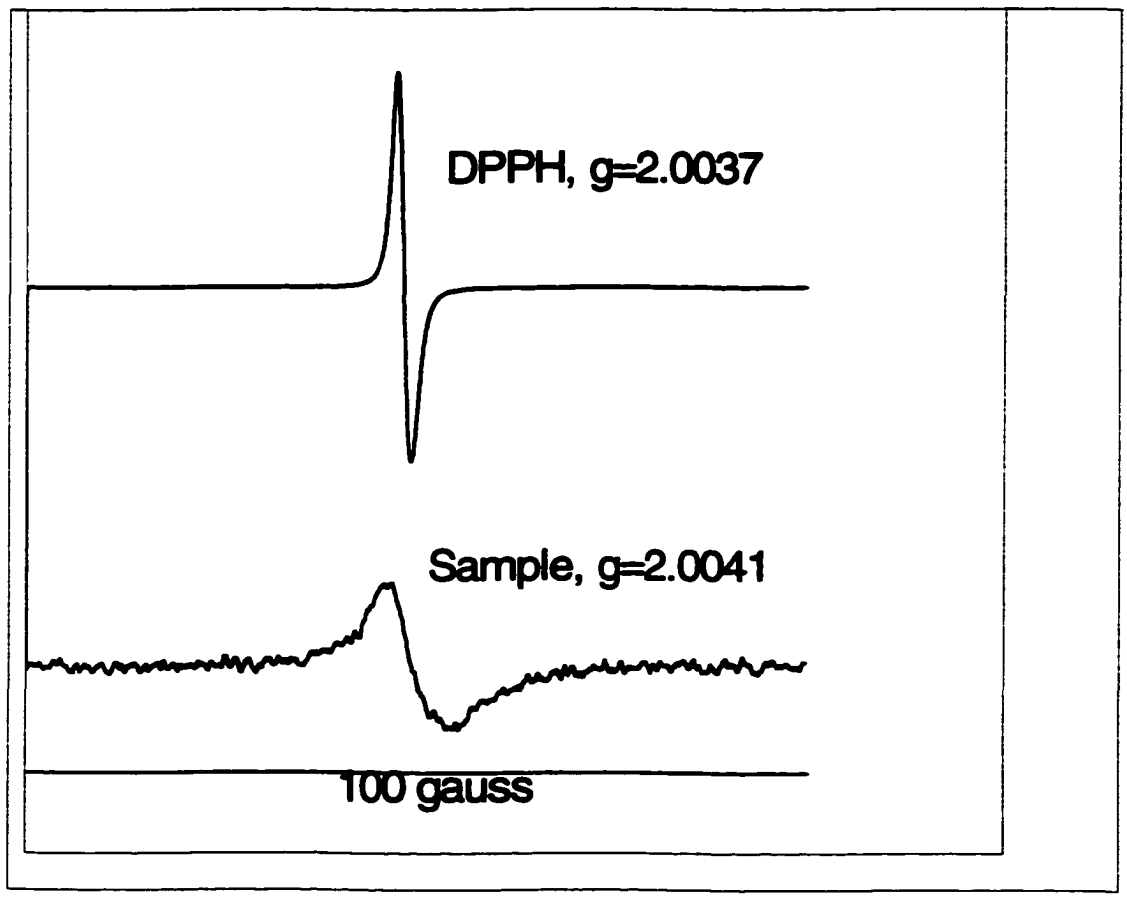


Figure 3. Typical electron spin resonance spectrum for 1,1-diphennyl-2-picrylhydrazyl and milk powder sample

High heat WMP had significantly higher free radicals (3.15×10^{15} radicals/g) than low heat WMP (2.99×10^{15} radicals/g) after 10 d storage ($P < 0.05$).

Table 7. Effects of rice bran oil addition on free radical concentration in low-heat whole milk powder

RBO%	Day One	Day Ten
	radicals (x 10^{15}) per g milk fat	
0.0	2.55[a]	3.22[a]
0.1	2.22[b]	3.00[a b]
0.2	2.29[ab]	2.76[b]

[a,b] Numbers in the same column followed by same letter are not significantly different ($P < 0.05$)

These results are in contrast to the generally accepted view that heat treatment improves the oxidative stability of milk. Apart from increasing the anti-oxidative substances in the milk powder, the RBO also increased the amount of polyunsaturated fatty acids (PUFA). Rice bran oil, with an iodine value range of 99 - 108 g I_2/100g (Gunstone, 1997), is more unsaturated than milk fat, with iodine value 25 - 45 g I_2/100g (Mulder and Walstra, 1974). These PUFA are more prone to autoxidation than saturated fatty acids. This could increase the rate of free radical formation under severe conditions to a level where the beneficial effects of RBO are upset. During the drying of high heat milk powder, there was an increase in the resident time in the drier and an increase in the exit temperature. This increased heat treatment could have increased the rate of free radical formation for the milk powder, especially at higher levels of RBO. This phenomenon might account for the higher rate of oxidation for the high-heat milk powder, but there is need for further investigation. There was a positive correlation

between the free radical concentration in the powder and the TBARS after ten days of storage (figure 4), with a correlation coefficient, $R^2 = 0.28$.

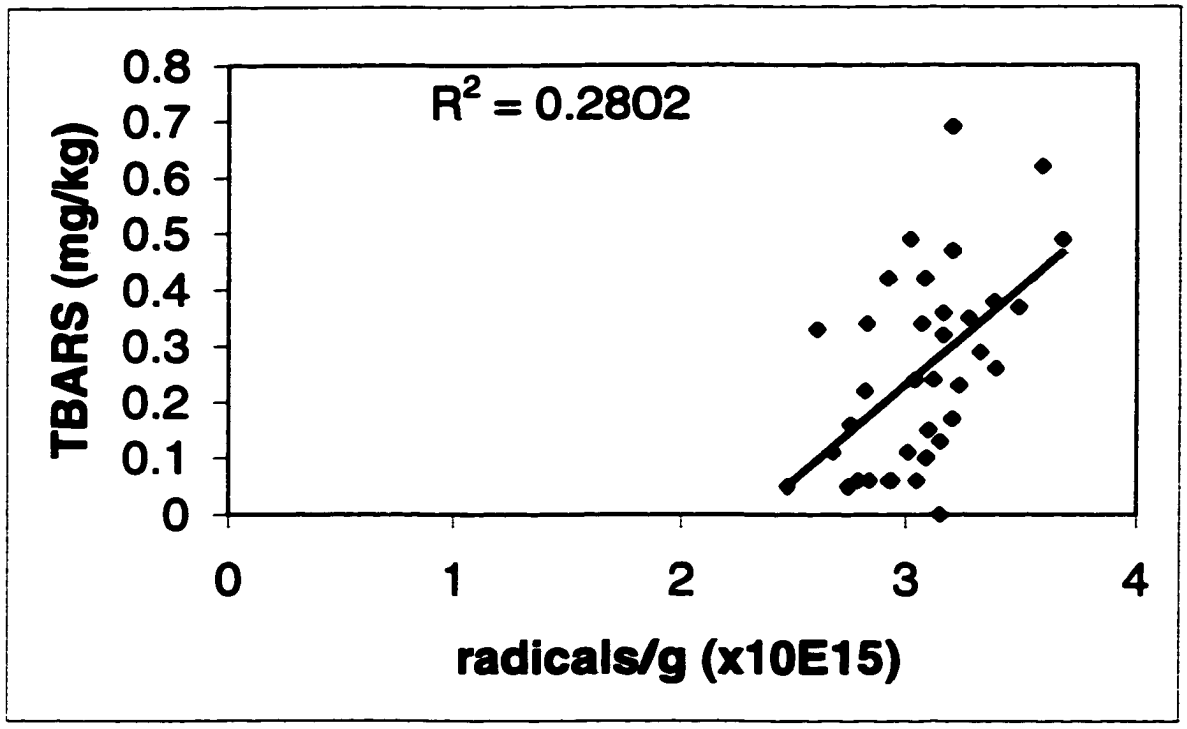


Figure 4. Relationship between free radicals and thiobarbituric acid reactive substances of milk powder after storage for10 d

The low correlation coefficient is because free radicals are the first intermediate products of lipid autoxidation, which react to give TBARS at a later stage (Badings, 1960; O'Connor and O'Brien, 1995). Stapelfeldt *et al.* (1997b) reported that the concentration of free radicals showed a linear correlation with TBARS detected at 450 nm but not at 532 nm. They attributed this discrepancy to the fact that the products formed during the oxidation of milk lipids are different from the products of other lipids, due to differences in fatty acid composition. Milk fat has low concentrations of

PUFA and higher concentrations of monounsaturated fatty acids than most other fats. Oxidation of monounsaturated fatty acids gives TBARS that produce a yellow pigment instead of the pink pigment observed in PUFA oxidation (Kosugi *et al.*, 1987). Although they are not a true measure of the extent of oxidation, free radicals are highly reactive, and can give an indication of the oxidative stability of the dried milk during storage. The milk powder that has a higher level of free radicals is likely to undergo oxidative deterioration faster than milk powder with a low concentration of free radicals. The RBO had a significant effect on the development of TBARS ($P < 0.05$) after ten days of storage, but had no significant effect on the initial TBARS (Table 8).

Table 8. Effect of rice bran oil on oxidation level of whole milk powder after manufacture and 10 d storage

RBO (%)	Day 0	Day 10
	Mean TBARS (mg malonaldehyde/kg fat)	
0.0	0.10[a]	0.23[a]
0.1	0.07[a]	0.07[b]
0.2	0.06[a]	0.08[b]

*Numbers in the same column, followed by the same letter are not significantly different ($P < 0.05$).

Stapelfeldt *et al.* (1997b) reported an increase in free radical concentration during storage of milk powder to a peak followed by decrease with further storage. Free radicals are intermediate compounds in the autoxidation of lipids hence their concentration decreases as they are used up. Stapelfeldt *et al.* (1997b) reported that at the initial stages, free radical concentration correlated well with sensory characteristics

of the powder. However, as storage time increased, sensory quality decreased although free radicals decreased. This was attributed to free radicals being used up as carbonyl compounds are formed. Electron spin resonance spectroscopy could therefore be developed as a quick method of determining initial stages of milk powder oxidation.

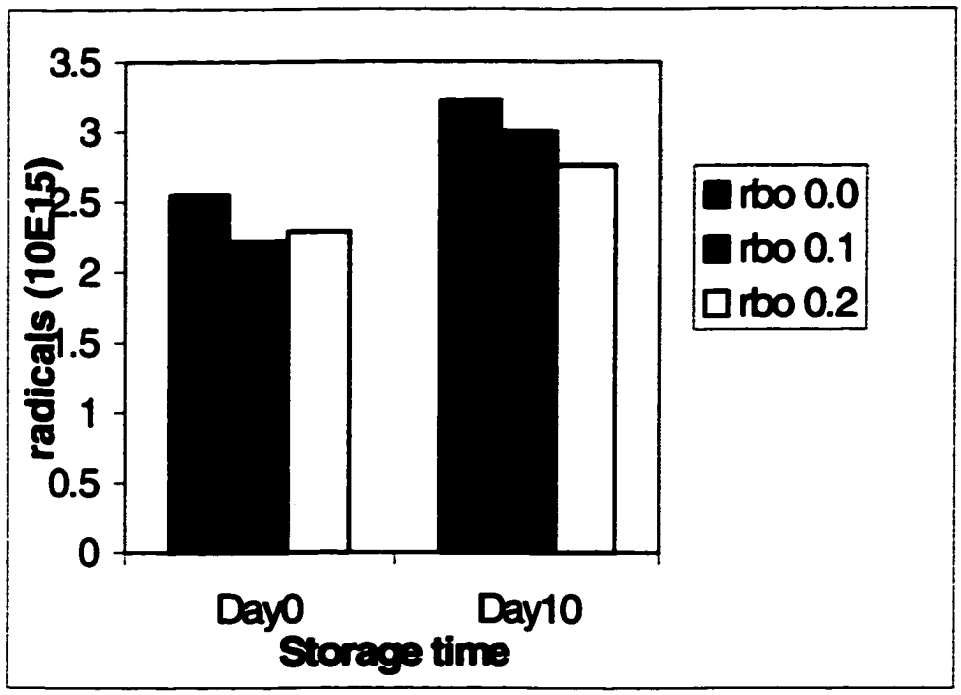


Figure 5. Mean free radicals for milk powder with different rice bran oil contents

In conclusion, results indicated that high oryzanol RBO was effective in reducing the formation of free radicals during manufacture and storage of milk powder. Free radicals are the first products of lipid autoxidation and they propagate the reaction, therefore, their reduction could slow the oxidative deterioration of whole milk powder during storage. Free radicals have been implicated in DNA damage in test animals and can lead to development of tumors (Pryor *et al.*, 1998). Reduction of free radicals thus gives added health benefits to milk powder. Rice oil is widely consumed in many

countries especially in Japan and South-East Asia with no reported adverse effect. Its incorporation in milk powder is therefore not likely to cause any health hazards to the consumers.

CHAPTER FOUR: INFLUENCE OF HIGH ORYZANOL RICE BRAN OIL ON OXIDATIVE STABILITY OF WHOLE MILK POWDER

Introduction

The quality of whole milk powder during storage is affected by a number of physical and chemical reactions, especially moisture uptake and autoxidation. Whole milk powder contains milk fat that contains some polyunsaturated monocarboxylic acids (Christie, 1995; Swaisgood, 1996) and is therefore prone to autoxidation during storage. Antioxidants are defined as substances which, when present at low concentrations in relation to the oxidizable substrate, will delay the onset and slow down or stop the oxidation process (Nawar, 1996; Halliwell, 1995). Milk lipid oxidation occurs through a free radical mediated chain reaction. The main fat-soluble antioxidants allowed for use in food are monohydric or polyhydric phenols with various ring substitutions (Nawar, 1996). Tocopherols (vit. E) and tocotrienols also have anti-oxidative properties (O'Connor and O'Brien, 1995). Primary antioxidants react with intermediate products, especially free radicals, and give stable products, thus terminating the chain reactions. Milk has some natural antioxidants, especially vitamin E and C. The concentrations of these antioxidants in milk vary according to their abundance in the feed. Attempts have been made to increase the vitamin E content of milk through its supplementation in the feed (Dunkley *et al.*, 1968; Tamsma *et al.*,1963). These researchers reported that vitamin E supplementation in the feed increased its concentration in milk, but only about 2% of the ingested vitamin was transferred to milk, thus making it economically untenable. Antioxidants have been

used to improve milk flavor during storage of the powder but some of them adversely affect the flavor (Tamsma *et al.*,1963). A number of vitamins such as vitamin E, C, and β-carotene have anti-oxidative properties (Elliot, 1999) and this is responsible for their bioactivity. These vitamins could be useful as antioxidants in food systems (Elliot, 1999). A number of phytosterols such as γ–oryzanol have been reported to have antioxidative properties (Graf, 1992). Rice bran is a good source of γ–oryzanol and is a constituent of the unsaponifiable matter in rice bran oil refining.

Rice bran oil has substantial amounts of vitamin E, tocotrienols and γ-oryzanol, all of which have anti-oxidative properties. Addition of RBO to milk is therefore likely to improve the oxidative stability of the dried milk. In addition, the RBO is likely to increase the vitamin E content of the powder thus improving it nutritional quality. Peroxide value is often used to monitor oxidation of fats. However, the peroxides are unstable and do not persist long in milk. Thiobarbituric acid reactive substances, which are more stable than peroxides, were used in this study to estimate the level of milk oxidation. This study was conducted to determine the effect of high-oryzanol rice bran oil on the oxidation of milk powder during storage.

Materials and Methods

Manufacture of Low Heat Milk Powder

Milk powder was manufactured as described in chapter two. Whole milk, 3.6% fat, which had been pasteurized at 74° C for 20 seconds, was evaporated under vacuum at 45-50° C to 40 % total solids using a rising film, APV laboratory Vacuum evaporator (APV Pasilac Anhydro AS, Copenhagen, Denmark). Three 3-kg samples of the concentrated milk were taken and high-oryzanol rice bran oil added to each to obtain

0.00, 0.10 and 0.20% RBO in the original milk. The oil and milk were mixed using a hand stirrer and homogenized at 14000 kPa. The milk was then spray dried in an APV Laboratory Model Spray Drier (APV Anhydro, Φstomarken, Soborg, Denmark), fitted with a peristaltic pump and a rotary atomizer. The air inlet temperature was 200 °C and the outlet temperature 100°C. The treatments were replicated three times.

Manufacture of High Heat Powder

A second batch of whole milk, 3.6% fat, which had been pasteurized at 74°C for 20 seconds, was further pasteurized at 88°C for 15 minutes in a steam jacketed vat and cooled to 7°C. It was then concentrated to 40% solids and RBO was added as described for low-heat powder and dried using the same procedure as described previously except that the outlet temperature was 105°C. The treatments were replicated three times.

Storage Conditions

Duplicate 25-g samples of the powders were stored in open petri dishes at 45° C, over saturated aqueous solution of magnesium chloride of a_w 0.31 (Nielsen *et al.*, 1997) for 40 days. Storage space in the incubator was randomly allocated for each sample. They were then analyzed for TBARS after 0, 10, 20, 30 and 40 days of storage. Samples that were not analyzed immediately were stored at –40° C until analyzed. The moisture content at the end of the storage period was also determined.

Thiobarbituric Acid Reactive Substances

Thiobarbituric acid reactive substances (TBARS) were determined following the procedure described by Farkas *et al.* (1997). Five grams milk powder was weighed in a 50-ml beaker and transferred into a 500-ml Kjeldahl flask with 93 ml distilled water and mixed. To this were added 1.4 ml 4N HCl, 400 µl Tween 80 (Amresco®, Solon, OH),

200 µl antifoam A (Sigma Chemical Co. St. Louis, MO), 200 µl butyrated hydroxytoluene (Sigma chemical co. St. Louis, MO) and a few boiling glass beads. The contents of the flasks were thoroughly mixed by shaking and connected to the Kjeldahl distillation units. The heating element was set at position 6.5 and about 50 ml distillate collected. The volume of the distillate was recorded. Five ml of the distillate was placed in a 20-ml test tube with a Teflon screw cap and 5 ml 0.02M TBA solution in glacial acetic acid (Sigma Chemical Co. St. Louis, MO) was added. After mixing, the test tubes were placed in a boiling water bath for 35 minutes and cooled in a tap water bath at room temperature for 10 minutes. Absorbance was read at 538nm using a Shimadzu UV-VIS-NIR Scanning Spectrophotometer model UV-3101PC (Shimadzu Corporation, Kyoto, Japan). A blank determination was made by using 5 ml of distilled water instead of the distillate.

A standard curve was made by reacting 5 ml of 1.0 to 10.0 x10^{-7}M solutions of 1,1,3,3,-tetraethoxypropane (TEP) with TBA as described above. Absorbance values for the standard concentrations were used to create a linear regression equation using Microsoft Excel™ software (Microsoft Corporation, Seattle, WA). A linear curve was drawn with absorbance as the abscissa and the molar concentration of TEP as the ordinate. All calculations were done using the Microsoft Excel™ software. The linear regression equation obtained from the standard curve was used to convert the sample absorbance units into mg malonaldehyde.

Recovery Studies

To determine percent recovery, 50 µl of 10^{-3} M TEP stock solution in duplicate was placed in a 500 ml Kjeldahl flask and diluted with 93 ml distilled water. The flask

was connected to the distillation unit and distilled until almost 50 ml distillate was collected. The distillate was made to 50 ml with distilled water and a 5-ml portion was reacted with 5 ml TBA as described earlier. The absorbance was read at 538 nm wavelength. Another 50 µl of the TEP stock solution in duplicate was diluted to 50 ml with distilled water, a 5 ml portion taken and treated as for the distillate and the absorbance at 538 nm read.

Percent recovery = $A_{538,distilled}/A_{538,nondistilled}$ x100% ..... Equation 4.

Statistical analysis

Analysis of variance (ANOVA) was carried out using the Mixed and the General Linear Model (GLM) procedure in SAS® software for Windows Version 6.12 (SAS Institute, Cary, NC). Treatment sample means were compared for significance of difference using the Tukey procedure. Pasteurization method was treated as a block effect in a completely randomized block design while RBO concentration was taken as the fixed treatment effect. A type 1 error probability ($P < 0.05$) was as the criteria for declaring differences.

Results and Discussion

Thiobarbituric acid (TBA) reactive substances are a group of lipid oxidation products that react with TBA to form colored products. Although malonaldehyde is used as the standard, there are other compounds that also react with TBA. The standard curve and regression equation obtained from TEP solutions gave a linear correlation as shown in the Fig. 6 with R^2=0.99 and a regression equation:

$y = 170.97x - 0.1602$ .....Equation 5.

(Where, y = concentration of TEP, x = Absorbance). The percent recovery was 83.3%.

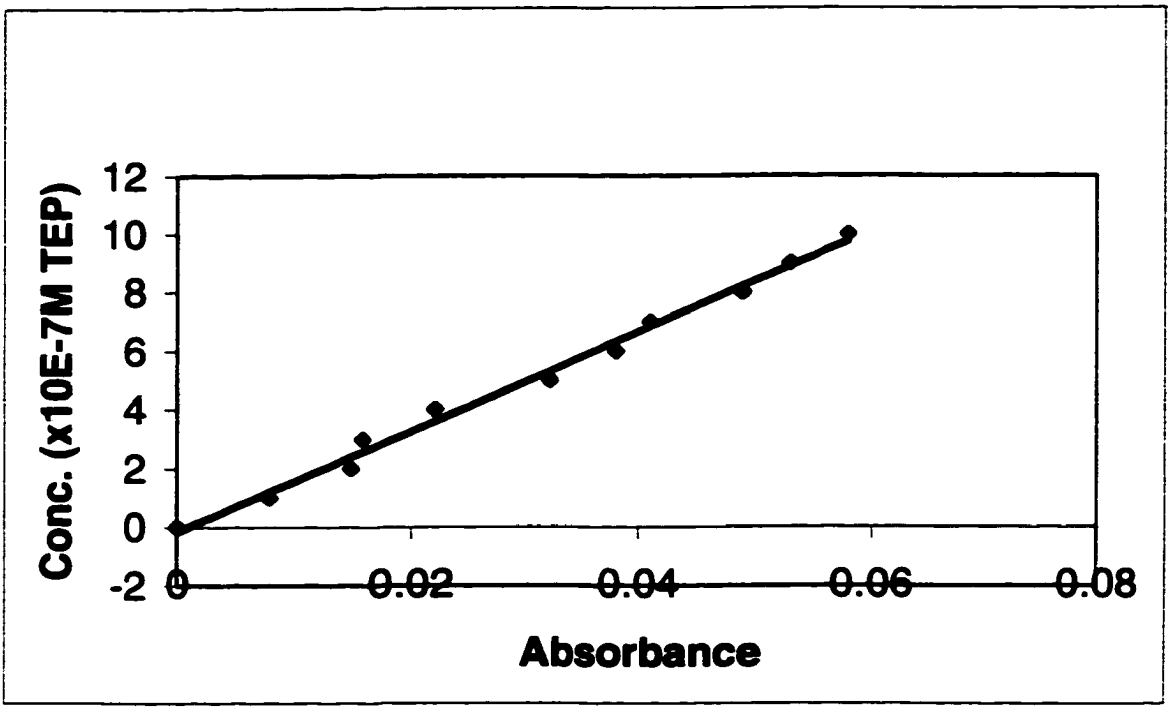


Figure 6. Thiobarbituric acid reactive substances standard curve

Milk powder oxidation, as measured by TBARS, increased with storage time from a mean of 0.11 mg malonaldehyde/kg after manufacture, to 1.35 mg malonaldehyde/kg after 30 days storage, but the TBARS decreased with further storage (Figure 7). The lowering of TBARS after 40 days of storage could have been due to decomposition of malonaldehyde and other compounds that react with TBA to give colored products. Malonaldehyde can react with protein in an oxidizing system resulting in abnormally low TBARS values (Nawar, 1996). Milk powder has a high protein content and thus the reaction between malonaldehyde and protein could account for the decrease in TBARS value after storage for 40 days. Rice bran oil at 0.1% significantly reduced milk powder lipid oxidation ($P<0.05$) during storage for low heat powder (Table. 8 and Fig. 7), but had no significant effect on the oxidation of high heat

milk powder under the conditions of this study. Increasing the RBO content to 0.20% did not improve the oxidation stability of WMP further. The high-heat powder had a significantly higher oxidation level than low-heat powder (Table 9) ($P<0.05$). It was expected that high heat treatment would increase the initial level of oxidation, but reduce the oxidation level of the milk powder during subsequent storage due to production of sulfhydryl groups, which have anti-oxidative properties (McCluskey *et al.*, 1997; Tamsma *et al.*, 1963). However, this was not observed in this work. On the contrary, high heat powder had significantly higher initial oxidation level at manufacture and after subsequent storage than the low heat powder (Table. 9) ($P < 0.05$).

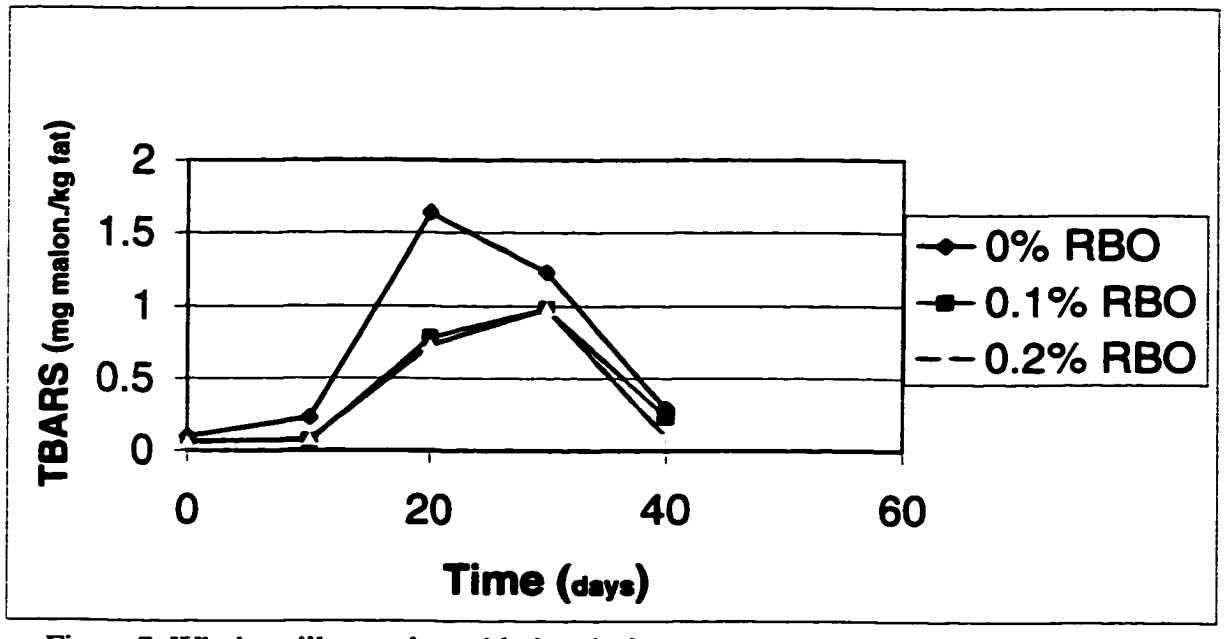


Figure 7. Whole milk powder oxidation during storage

This might be due to the reversal of the beneficial effect of heat by the excessive heat treatment. The exit temperature during spray drying of high heat powder was 105° C

instead of the desired 80 to 90° C. This high exit temperature was observed during the drying of the high heat milk while it was 100° C during the drying of low heat milk.

Table 9. Effects of rice bran oil on the mean TBARS values for low-heat and high-heat (in parenthesis) whole milk powder

RBO (%)	Storage time (days)				
	0	10	20	30	40
	TBARS (mg malonaldehyde/kg)				
0.0	0.10[a] (0.11)	0.23[a] (0.32)	1.64[a] (1.79)	1.23[a] (1.79)	0.29[a] (0.62)
0.1	0.07[a] (0.11)	0.07[b] (0.42)	0.78[b] (1.98)	0.98[b] (1.53)	0.24[a] (0.59)
0.2	0.06[a] (0.14)	0.08[b] (0.35)	0.73[b] (2.03)	0.98[b] (1.59)	0.10[b] (0.95)

*Numbers in the same column, followed by the same letter are not significantly different ($P < 0.05$).

The milk powder sometimes developed electrostatic characteristics and tended to stick to the sides of the drier. This increased the residence time of the powder in the hot drier thus increasing oxidation. This phenomenon was more prevalent during the drying of high-heat than during low-heat milk powder production.

The reasons for this behavior are not clear. However heat treatment increases the viscosity of milk (Walstra and Jennes, 1984), a phenomenon that could affect the flow properties of the concentrated milk during the drying process, especially the atomizing stage. This, in addition to the higher temperature and longer holding time during pasteurization may have increased the rate of oxidation hence contributing to the higher TBARS values for high heat milk. Fresh high heat powder had a significantly higher TBARS ($P < 0.05$) than low heat powder. Pasteurization was carried out before the addition of the RBO and thus the high heat milk did not have the protection of the

antioxidants present in the RBO during this severe heat treatment. It is possible that the milk fat became oxidized during this heat treatment and continued during the drying process to such a level that the antioxidants from the RBO were dexhausted, and thus were rendered ineffective. The RBO also has a higher amount of polyunsaturated fatty acids (PUFA) than milk. The average amount of PUFA in milk fat is about 3.6% (Walstra and Jennes, 1984), while the PUFA content of RBO is over 42% (Sugano and Tsuji, 1996). Addition of RBO to milk powder therefore increased the PUFA content, thus giving more oxidation substrate. This might partly account for the higher oxidation observed in high-heat powder than in low-heat powder. As observed in chapter two, the color of the high-heat powder was darker than low-heat powder (Lower L* value). This shows that Maillard reactions occurred more for high-heat powder and this could also accelerate the rate of lipid oxidation in the milk powder. Maillard reactions are complex and are initiated by the reaction between amines and carbonyl compounds (Damoradan, 1996). They involve condensation reactions between amino acids and reducing sugars, thus leading to loss of both the amino acid and lactose, which is the main sugar in milk. Some of the products of carbonyl compounds react readily with free amino acids to give aldehydes, ammonia and carbon dioxide. This results in loss of amino acids in what is referred to as the Strecker Degradation (Damoradan, 1996), flavor changes and reduced solubility of the powder. High heat treatment during drying of milk can thus lead to undesirable changes in the milk.

These results indicated that RBO, when added at 0.1% of the original milk, was effective in reducing the oxidation of milk lipids. As reported previously, RBO added to milk powder at 0.1%, did not impart a detectable flavor to the milk powder. It also

did not influence consumer preference of the product when compared to milk with no added RBO. The results from this study indicated that RBO is an effective antioxidant when added to milk powder. Rice bran oil addition to milk powder is likely to gain widespread acceptance since rice is a common food product in many cultures and rice oil is widely consumed in some Asian countries.

CHAPTER FIVE: INFLUENCE OF HIGH ORYZANOL RICE BRAN OIL ON CHOLESTEROL OXIDATION DURING STORAGE OF WHOLE MILK POWDER

Introduction

Cholesterol is a major sterol in human and animal tissues (Schmarr *et al.*, 1996). It is an unsaturated lipid that can undergo free radical mediated autoxidation reactions leading to the formation of oxides generally referred to as cholesterol oxidation products (COPs) (Li *et al.*, 1996; Nourooz-Zadeh and Appelqvist, 1988; Rose-Sallin *et al.*, 1995). Cholesterol accounts for about 0.31% of bovine milk lipids (Christie, 1995). Some COP that have been isolated from milk powder include 7β-hydroxycholesterol, 7-ketocholesterol, 5α,6α-epoxycholestanol, 5β,6β -epoxycholestanol, and 25-hydroxycholesterol (McCluskey *et al.*, 1997). Consumption of cholesterol oxides has been associated with arteriosclerosis and coronary heart diseases hence their presence in food is a serious health concern. Although milk is not a major source of dietary cholesterol, it can contribute to the total cholesterol intake. The development of COP during storage of whole milk powder should raise legitimate health concerns.

Rice bran oil (RBO) has been reported to slow lipid oxidation (Yagi and Ohishi, 1979; Graf, 1992). It has also been shown to reduce cholesterol uptake and slow the formation of fatty streaks in test animals (Rong *et al.*, 1997). The antioxidative properties of RBO are thought to be due to the presence of γ–oryzanol, tocopherol and tocotrienol in the oil (Graf, 1992). Addition of RBO to milk powder is therefore likely to inhibit cholesterol oxidation during storage of whole milk powder.

This study was conducted to investigate the effect of high-oryzanol rice bran oil on the oxidation of cholesterol during storage of whole milk powder.

Materials and Methods

Manufacture of Low Heat Milk Powder

Milk powder was manufactured following the procedures described earlier in chapter two. Whole milk, with 3.6% fat, which had been pasteurized at 74° C for 20 seconds, was evaporated under vacuum at 45-50° C to 40 % total solids. Three 3-kg samples of the concentrated milk were taken and high-oryzanol rice bran oil was added to each to obtain 0.00, 0.10 and 0.20% RBO in the original milk. Milk was then homogenized at 14000 kPa. The milk was spray dried in an APV Laboratory Model Spray Drier that was fitted with a peristaltic pump and a rotary atomizer. The air inlet temperature was 200° C and the outlet temperature 100° C. The treatments were replicated three times.

Manufacture of High Heat Powder

A second batch of whole milk with 3.6% fat, which had been pasteurized at 74°C for 20 seconds, was heat-treated at 88°C for 15 minutes. It was then concentrated to 40% solids, RBO was added and it was homogenized and dried using the same procedure as described for low-heat powder although the outlet temperature was 105°C. The treatments were replicated three times.

Milk Powder Storage

Duplicate samples of 25 g of the milk powder samples were stored in open petri dishes at 45° C, over a saturated aqueous magnesium chloride solution of a_w 0.31 (Nielsen *et al.*, 1997) for 40 days. Storage space in the incubator was randomly allocated for each sample. Cholesterol oxidation products (COP) were determined after 0, 20, and 40 days of storage.

Direct Saponification and Extraction of Cholesterol Oxidation Products

Cholesterol oxidation products were extracted following the direct saponification method as described by Dionisi *et al.* (1998), with minor modifications. One gram of milk powder was placed in a 40-ml test tube with a Teflon screw cap and 200 µL (100 µL for day 40) of a 100 µg per ml 5α-cholestane solution in ethyl ether (Sigma Chemical Co. St. Louis, MO) was added as an internal standard. Ten ml 1N-KOH in methanol was added and shaken to suspend the milk powder sample and dissolve all the fat. The samples were left at 20°C for 18 to 20 hours with occasional shaking to allow milk-fat to saponify. Ten milliliters of distilled water was added and mixed. To extract non-saponifiable matter, 10 ml ethyl ether was added, mixed and left standing until the two layers separated. The upper organic layer was siphoned with a Pasteur pipette. Extraction was repeated thrice and the pooled extract was transferred into a 125-ml separatory flask and washed once with 15 ml0.5N KOH and twice with 15 ml 0.47M sodium sulfate. The extract was then dried by shaking with anhydrous sodium sulfate (Fisher Scientific, Fair Lane, NJ) and filtered through Whatman no. 1 filter paper. The flask was rinsed with 10 ml diethyl ether that was also passed through the filter paper. The filtrate was evaporated at room temperature using a nitrogen flash evaporator to about 2 ml, transferred to a 2-ml vial and the solvent was evaporated under a stream of nitrogen. The extract was stored at –40° C until GC analysis was carried out.

Purification of Cholesterol Oxidation Products

Cholesterol oxidation products were separated from unsaponifiable matter using a modification of the solid phase extraction (SPE) procedure described by Dionisi *et al.*

(1998) and McCluskey *et al.* (1997). The unsaponifiable extracts were dissolved in 2 ml of hexane and applied to a 3-ml aminopropyl- SPE cartridge (Supelco, Bellfonte, PA), that had been activated with 3 ml hexane. The SPE cartridge was first eluted with 6 ml hexane/ethyl acetate (95/5, v/v) followed by 10 ml hexane/ethyl acetate (90/10, v/v). The COP were then eluted with 7.5 ml acetone and the acetone dried under a stream of nitrogen.

Trimethylsilyl Ester Derivatization

Cholesterol oxidation products were converted to trimethylsilyl esters as described by Dionisi *et al.* (1998) and quantified by GC as described by Won Park and Addis (1985) with some modifications. To the extract, 100 µl of N,O-bis(trimethysilyl)trifluoroacetamide (BSTFA) (Sigma Chemical Co. St. Louis, MO), 20 µl of dry pyridine, and 2 µl of trimethylchlorosilane (TMCS) (Sigma Chemical Co. St. Louis, MO) were added. The reaction was allowed to proceed overnight at room temperature and then dried under a stream of nitrogen. The extract was then re-dissolved in 100 µl of GC grade hexane/N,O-bis(trimethylsilyl)acetamide (9/1 v/v) for injection into the GC column.

Gas Chromatography Analysis

All GC work was performed using a Hewlett Packard GC model 5890 (Hewlett Packard Co., Avondale, PA), which was fitted with a 30-m fused silica bonded phase capillary column (Supelco, Bellfonte, PA), with an internal diameter of 0.25 mm, and an automatic sample injector. A flame ionization detector (FID), set at 300° C, was used to record the retention time. The carrier gas was helium set at 70 kPa column head pressure. Sample volume was 3 µl, and injector temperature 270°C. Signals from the

FID were recorded using a Hewlett Packard integrator model 3396A (Hewlett Packard Co., Avondale, PA), with chart speed of 0.5 cm/min. Attenuation was set at –2 and threshold at –2. The cholesterol oxides used as standards were; 7β-hydroxycholesterol, 7-ketocholesterol, 5 α,6α -epoxycholestanol, 5 β,6 β -epoxycholestanol, and 25-hydroxycholesterol (Sigma Chemical Co., St. Louis, MO).

Comparison of retention time of peaks due to the standards with the retention times of sample peaks was used to identify cholesterol oxides in milk powders. The peak areas were used to quantify the COP fractions detected in the milk powder samples.

Recovery Studies

Recovery studies were not done due to instrument failure. To quantify the COP, 100% recovery was assumed.

Identification of COP Peaks and Standard Curves

For each COP, a 100 µg/ml solution was prepared by dissolving one milligram of the pure chemical in 10 ml diethyl ether. For each COP, 300 µl, 500 µl, 700 µl and 900 µl of the 100 µg/ml the COP solution placed into 4 sample vials and the solvent dried under a stream of nitrogen. Trimethylsilyl ester derivatization and GC analysis were carried out following the same procedures as described for the samples. The area of peaks obtained from the GC spectrum for each COP was compared to determine peaks whose area increased with an increase in the COP concentration. For each COP, a regression analysis for the peak area against COP concentration was carried out using Microsoft Excel® software (Microsoft Corporation, Seattle, WA). The regression equation and correlation coefficient (R^2) values were recorded. The peak with the

highest R^2 was assumed to be due to the added COP and its retention time used to identify the compound in the milk sample. For quantification of COP, the regression equations were modified by setting the intercept to zero. This lowered the R^2 but gave a more estimate of the concentration of COP in the samples. This modified regression equation was used to estimate the concentration of the particular COP in milk powder samples.

Statistical analysis

Analysis of variance (ANOVA) was carried out by the General Linear Model (GLM) procedures using the SAS® software for Windows Version 6.12 (SAS Institute, Cary, NC). Treatment sample means were compared for significance of difference using the Tukey procedure. Pasteurization method was treated as a random block effect in a completely randomized block design while RBO level was taken as the fixed treatment effect. An alpha level of 0.05 was used to determine significance of treatment differences.

Results and Discussion

Identification of COP Peaks and Standard Curves

The linear regression equations obtained for the cholesterol oxides are given in Table 9. The chromatograms of the pure cholesterol oxides showed more peaks than would be expected for pure compounds. The origins of these peaks could not be ascertained but other investigators have reported that some COP undergo extensive thermal degradation during GC analysis (Park and Addis, 1985; Van Lier and Smith, 1968). However, Park and Addis (1985) did report that trimethylsilyl derivatives of COP did not decompose during capillary gas-liquid chromatography. The large number

of peaks that were observed from pure cholesterol oxides may be explained by thermal decomposition of COP. This is possible if there were some underivatized COP present in the samples. A typical chromatogram of one of the COP is shown in Figure 8. The peak areas of the GC chromatograms had abnormally large variability. This was more prevalent during the analysis of the standards and was traced to problems with the signal integrator, which at times failed to integrate some signals while overestimating others. The high variability in peak area resulted in a coefficient of variation (cv) of 155%. Dionisi *et al.* (1998) reported a cv of between 1.3 for 7-ketocholesterol and 141.4 for 7-hydroxycholesterol. This large cv might have affected the statistical significance of the data. Another possible cause of the large variability could be the nature of degradation of the COP. It has not been established whether the fragmentation is random or systematic. Random fragmentation of the COP can result in different artifacts, thus making quantification of the compound difficult.

Cholesterol Oxidation Products in Milk Powder

The cholesterol oxide, 25-hydrocholesterol (25-OH) was detected in milk samples stored for 40 days and in some of the milk samples stored for 20 days but none was detected in milk samples immediately after manufacture. Rice bran oil had no significant effect on COP content in WMP. The concentration of 25-OH was 5.90, 6.09 and 5.71 ppm, for 0%, 0.1% and 0.2% RBO fortified WMP respectively. The other COP were not detected in this study. A possible reason for this could be due to instrument sensitivity. An analysis of α–cholestane gave no signal for concentrations of 20 ppm and lower. However, concentrations of 30 ppm and above gave a definite signal. Levels of less than 20ppm COP in milk have been reported. Rose-Sallin *et al.*

(1995) reported COP levels between 0.1 and 0.9 ppm of lipids in milk powder stored open for one year. Nourooz-Zadeh *et.al.* (1988) reported COP values ranging from 1.0 to 10 ppm of lipids in whole dried milk while Dionisi *et al.* (1998) reported values ranging from 0.01 to 22 ppm.

Table 10. Mean gas chromatography retention times and the regression equations for the selected cholesterol oxidation products' standards

Compound	RT (min.)	Reg. Equation	Reg. coefficient
5α,6α-epoxycholestanol	23.15	Y = 395.81x – 4149;	R^2 = 0.907
5 β,6β-epoxycholestanol	12.12	Y = 102.37x – 2003.7;	R^2 = 0.920
7-ketocholesterol	25.45	Y = 7.178x – 138.75;	R^2 = 0.881
25-hydroxycholesterol	35.5	Y = 293.81x – 10280;	R^2 = 0.917
5α-cholestane	14.15	Y = 8.685x + 63.15;	R^2 = 0.939

These reported values are below the detection limit of 30 ppm for the particular instrument used in this study. It was possible however, to detect 25-OH. There was a decrease in the mean 25-OH content with increase in RBO content (Figure 9) but the differences were not statistically significant. The mean concentrations of 25-OH in the milk samples were 11.7 ppm, 6.1 ppm and 5.7 ppm for 0%, 0.1% and 0.2% RBO milk powder respectively. In their review of cholesterol oxides in foods, Paniangvait *et al.* (1995) reported values of 3ppm 25-OH in high-heat skim milk powder, 2,200 to 16,200 ppm in Indian ghee, 100 to 800 ppm in dried Parmesan cheese and trace to 600 ppm in butter. There were no reported values for 25-OH in whole milk powder but some other COP were detected. Addis *et al.* (1989) reported a number of COP in infant formula powders ranging from 3 to 35 ppm. Nourooz-Zadeh and Appelqvist (1988) reported

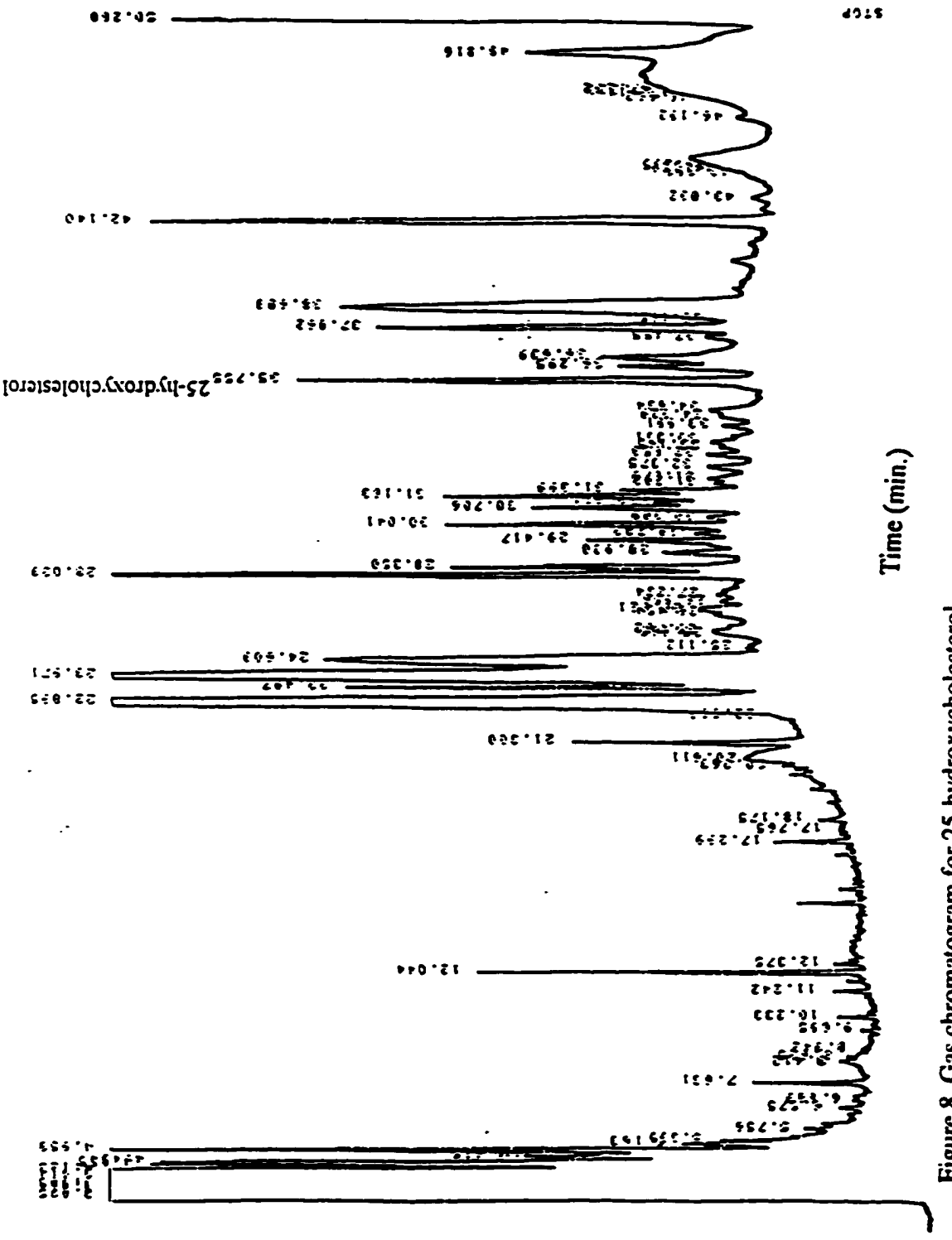


Figure 8. Gas chromatogram for 25-hydroxycholesterol

the presence of COP in whole milk powder that had been stored for 12 months but did not detect any 25-OH. They did detect 25-OH and other COP in skim milk powder, which had been stored for different times. These reports indicate that COP, among them 25-OH, develop in stored milk powder.

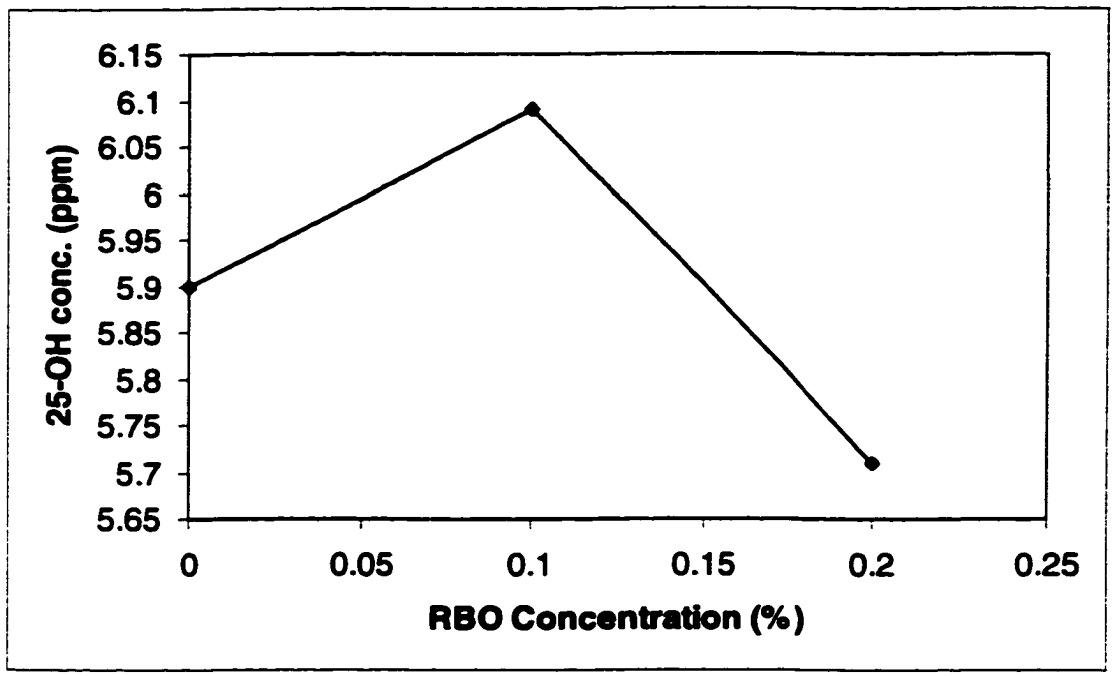


Figure 9. Changes in 25-hydroxycholesterol with rice bran oil content

Three of the GC chromatograms obtained from the analysis of milk samples are given in Figures 10, 11 and 12. There was a prominent peak with a retention time of about 16.5 min, whose area increased with storage time. The identity of the peak was not ascertained but it was most certainly a chemical reaction product, probably, an oxidation reaction. The area of this peak was independent of RBO level. Since RBO was shown to inhibit oxidation, this peak is not likely to be due to a product of lipid oxidation. There was also an increase in the number and area of peaks with storage time (Fig. 10, 11 and 12). This could be due to increased amounts of COP during

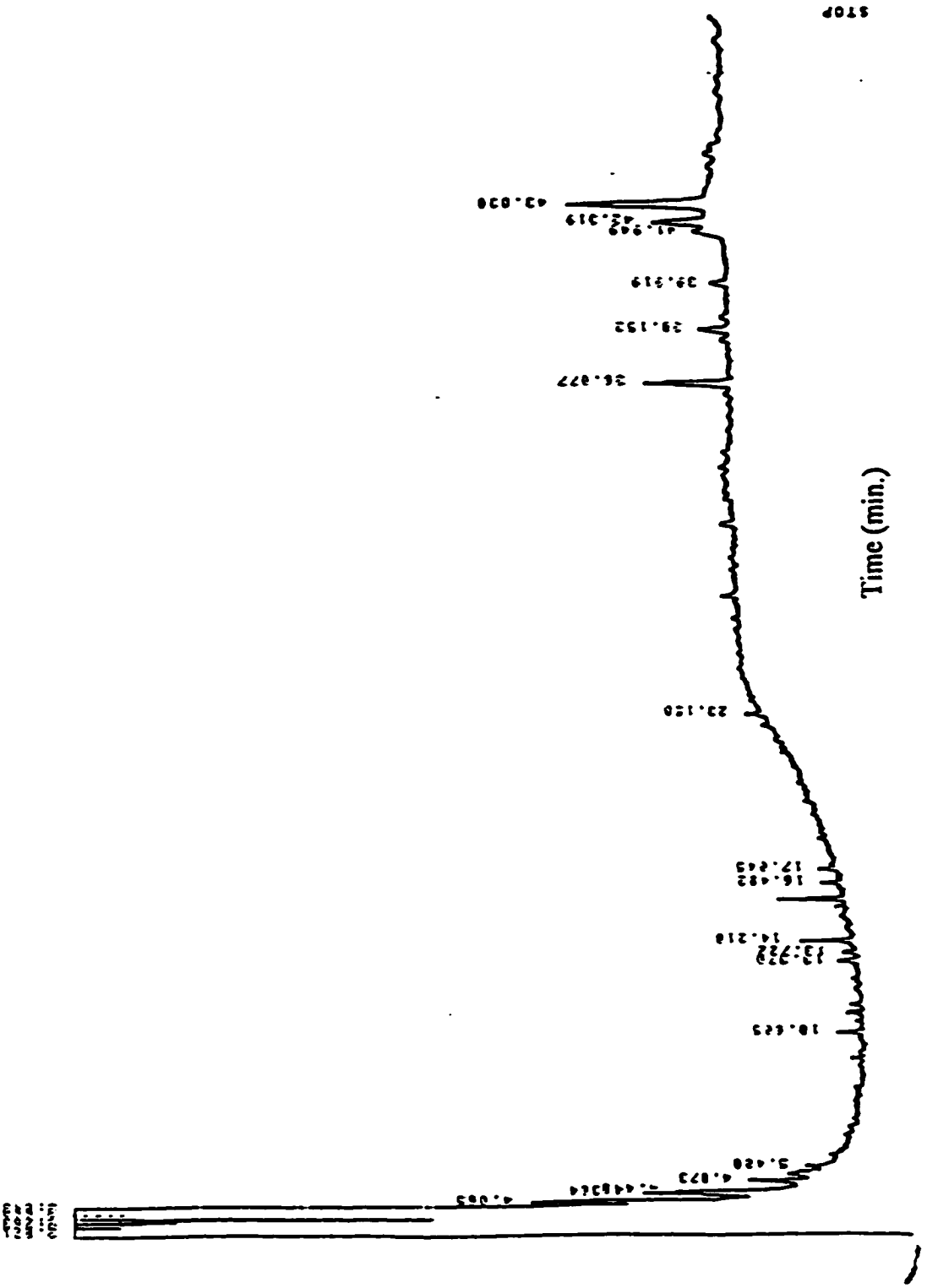


Figure 10. Gas chromatogram for control whole milk powder sample cholesterol oxidation products after manufacture

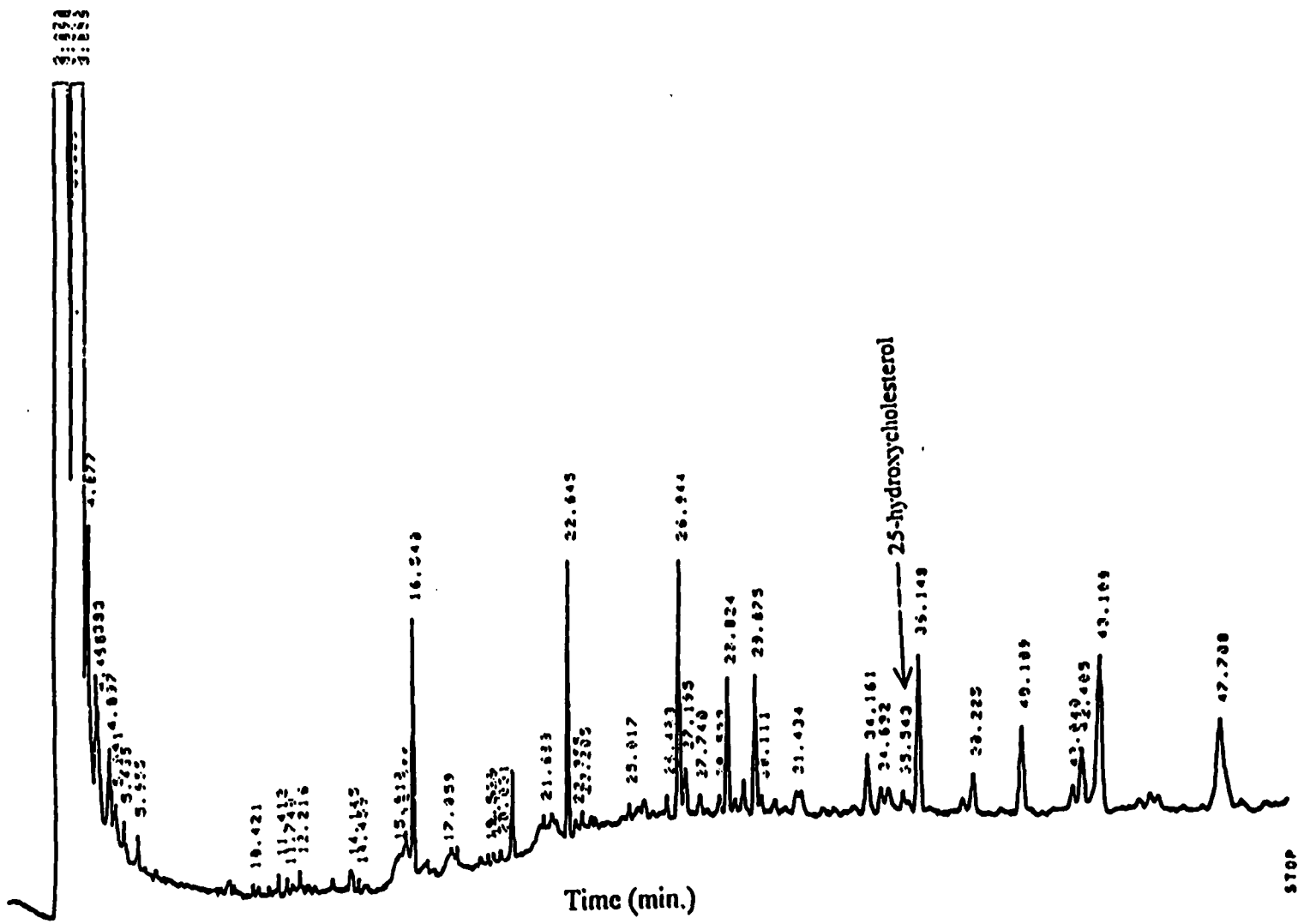


Figure 11. Gas chromatogram for control whole milk powder sample cholesterol oxidation products after 20 days' Storage

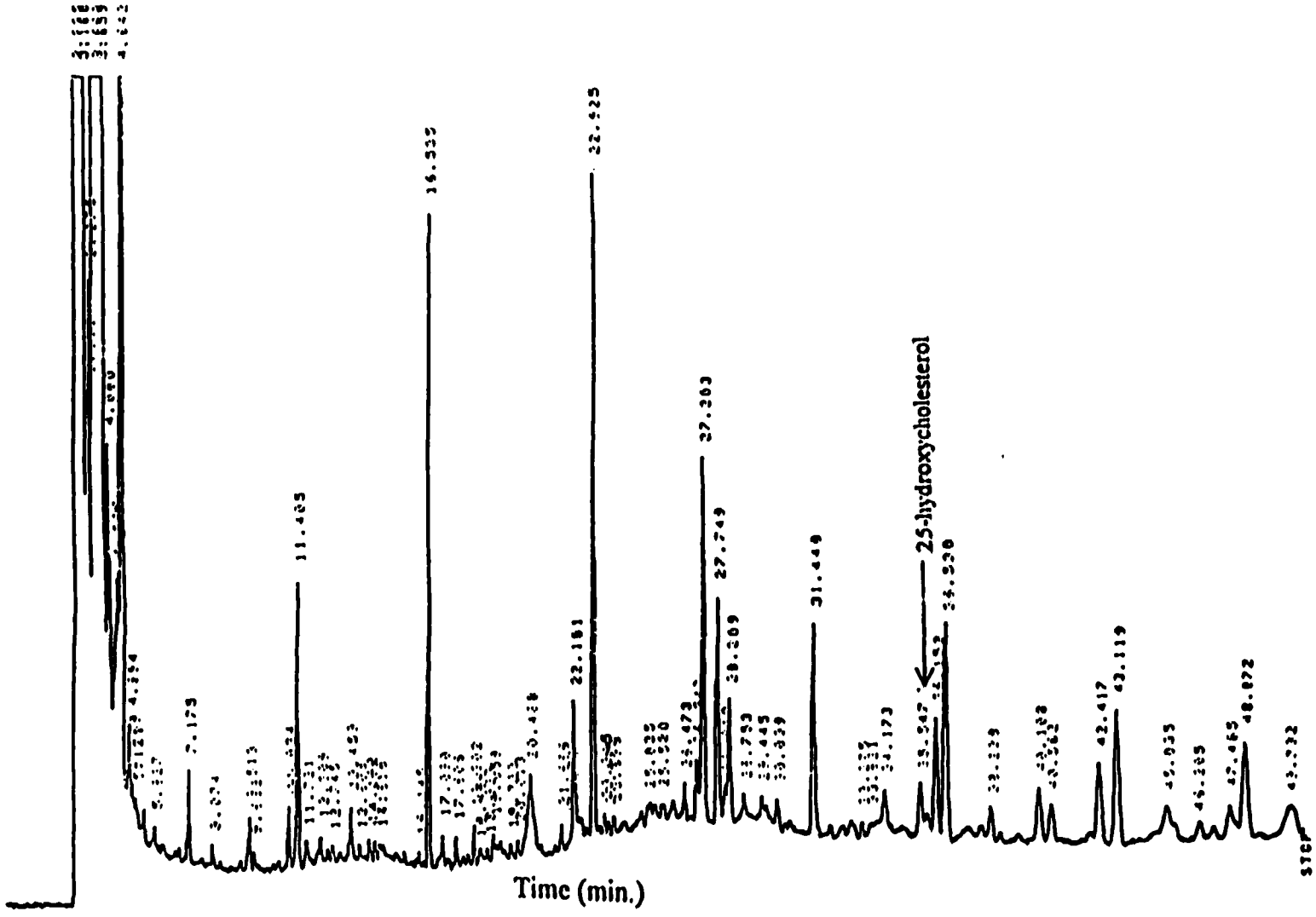


Figure 12. Gas chromatogram for control whole milk powder sample cholesterol oxidation products after 40 days' Storage

storage. As stated earlier, COP can undergo thermal degradation during GC analysis. It follows that the greater the amount of COP, the greater the number and intensity of the peaks.

In conclusion, the results obtained in this study indicate that cholesterol is oxidized during the storage of WMP. The GC method however, was not effective in detecting all the COP due to low instrument sensitivity, its low precision and thermal degradation of COP in the GC column. It was however possible to identify 25-hydroxycholesterol. Rice bran oil slightly lowered the formation of 25-hydroxycholesterol though not statistically significant. Kim (1999) reported a positive correlation between 7-ketocholesterol and TBARS in meat with a correlation coefficient (R^2) of 0.96. Since RBO reduced TBARS formation in milk powder, it can be inferred that RBO will also reduce cholesterol oxidation.

CHAPTER SIX: CONCLUSIONS AND RECOMMENDATIONS

Conclusions

Lipid autoxidation in whole milk powder (WMP) is a serious problem that needs to be controlled during long term storage. In this study, addition of high-oryzanol rice bran oil (RBO) significantly improved the oxidative stability of low-heat WMP during storage. Oxidation of high-heat WMP was slightly improved by addition of RBO but the data was not statistically significant ($P < 0.05$). The main findings of this investigation can be summarized as follows:

i) Addition of RBO to WMP at the rate of 0.1% (w/w) of the original milk affected the color of the powder, but did not have a detectable effect on the sensory properties of the WMP. When applied at 0.2% (w/w) of the original milk however, RBO had a detectable effect on sensory properties when the WMP was reconstituted and was less preferred when compared to control.

ii) High oryzanol RBO applied to milk powder at 0.1% and 0.2% (w/w) reduced the formation of free radicals during manufacture and during storage of the WMP for 10 days at 45° C and 0.31 a_w.

iii) The RBO, when added to WMP at 0.1% and 0.2% of the original milk, was effective in reducing the oxidation of milk lipids. During storage, the concentration of thiobarbituric acid reactive substances (TBARS) increased to a maximum at 30 days of storage and then decreased on further storage.

iv) **The results obtained in the study showed that cholesterol wass oxidized during the storage of WMP. Adding RBO did not have a significant effect on cholesterol oxidation. Cholesterol oxidation products (COP) were quantified using the liquid gas chromatography (GC) technique. The GC method however was not effective in detecting all the COP due to problems with instrumentation and thermal degradation of COP in the GC column.**

Lipid oxidation is initiated and propagated through free radical chain reactions. Reduction of free radicals due to RBO addition will therefore slow autoxidation reactions and provide added health benefits to the milk powder. Free radicals have been shown to be responsible for DNA damage in test animals and can lead to development of tumors (Pryor *et al.*, 1998; Elliot, 1999). Rice oil is widely consumed in many countries especially in Japan and South East Asia with no reported adverse health effects. Its incorporation in WMP is therefore not likely to cause any health hazards to the consumers. On the contrary, fortification of WMP with rice bran oil will increase the vitamin E content of the powder. Consumption of foods rich in dietary antioxidants has been associated with a reduction in the incidence of coronary heart diseases (Hertog *et al.*, 1993; Kromhout *et al.*, 1989; Verschuren *et al.*, 1995). Due to the presence of antioxidative phytosterols, tocotrienols and tocopherols, consumption of RBO is likely to reduce incidence of diseases such as arteriosclerosis, which have been associated with consumption of oxidized lipids (Lichtenstein *et al.*, 1994). At 0.1% of the original milk, RBO did not cause a detectable change in flavor, hence it is not likely to have any effect on the acceptability of the WMP when added at this level. The addition of RBO

to WMP may give the milk a competitive edge over milk imitation products such as soybean milk and non-dairy creamers.

Rice bran oil, when applied to WMP at 0.1% and 0.2% of the original milk, reduced the level of lipid oxidation during storage as determined by TBARS content of the powder. The anti-oxidative properties are most likely due to γ-oryzanol, tocopherols and tocotrienols that are present in RBO (Sugano and Tsuji, 1996). Oxidation of high-heat powder was not significantly affected by RBO addition. This was probably due to the effects of excessive heating of the powder during pasteurization and drying. It was observed that the TBARS increased to a peak and then declined upon further storage. Lipid autoxidation reactions continue during storage, as evidenced by a decrease in sensory score with storage time (Stapelfeldt *et al.*, 1997a). Reduction in TBARS with prolonged storage time indicates that they were being removed from the system probably by reacting with proteins. Nawar (1996) stated that malonaldehyde, the main compound that reacts with thiobarbituric acid to form red pigments, can react with proteins under oxidizing conditions. Dried whole milk has a high protein content. These proteins might have reacted with malonaldehyde under the conditions of this study thus reducing TBARS concentration. The suitability of TBARS as a measure of lipid oxidation in milk powders needs to be reexamined since milk proteins could react with malonaldehyde, hence reducing its concentration in the powder.

Although the reduction in cholesterol was not significantly affected by addition of RBO, the results indicated a reduction trend in COP with increased RBO content.

The results from this study indicate that RBO has potential for use as an anti-oxidant in WMP. Addition of RBO to WMP might gain widespread acceptance since rice is a common food product in many cultures, and rice oil is widely consumed in some Asian countries. The beneficial attributes of RBO are also likely to give added value to the WMP, which would be an additional asset to the marketing of the product, especially export markets.

Identification and quantification of COP was rendered difficult due to instrument failure and formation of artifacts during analysis. More accurate results might have been achieved by use of GC-MS analysis, which is reputed to be a more sensitive technique than a flame ionization detector. One of the causes of artifact formation during GC analysis is the high injection and column temperatures employed. High performance liquid chromatography (HPLC) could be used, since low temperatures are employed, in contrast to GC analysis where high temperatures are used thus avoiding thermal degradation of the oxides.

This study also determined that electron spin resonance (ESR) spectroscopy technique was able to detect and quantify free radicals in milk powder. The ESR technique is rapid and is applied directly to the WMP without further modifications, hence, it has the potential for automation.

In conclusion, it was observed that RBO reduced lipid autoxidation when applied to WMP at the 0.1% level. It did not affect consumer acceptability of the milk, and, did not significantly increase the fat content of the WMP. This suggests that RBO can be added to WMP at 0.1% of the original milk with no significant effect on the acceptability of the powder while at the same time decreasing oxidation. For consumers

concerned about the amount of fat intake, 0.1% level of application might not adversely affect preference. The health benefits from the RBO addition might offset any negative impact associated with the increased fat content. Increased fat content is not always a negative proposition. In certain parts of the world, such as parts of Sub-Sahara Africa and Asia, fat in the diet is inadequate. Low fat intake is usually coupled with inadequate intake of fat-soluble vitamins A, D, E and K and therefore an increase in fat content of the WMP may be desirable in these regions.

Recommendations

In this study, RBO was added to the milk after concentration. It would be helpful to investigate whether adding RBO to the raw milk might provide greater protection against autoxidation during pasteurization, evaporation and spray-drying operations, than adding it to the concentrated milk. There is a need to determine the minimum amount of RBO necessary to reduce lipid autoxidation in WMP.

In this study, TBARS increased to a peak and decreased upon further storage. A decrease in malonaldehyde with further storage indicated that TBARS were being used up. There is a need to investigate the fate of TBARS during oxidation of milk lipids in a protein matrix, in contrast to the oxidation of pure fat.

It was observed in this study that free radicals in milk powder could be quantified by ESR spectroscopy. This technique seems promising and needs further investigation and development.

REFERENCES

1. Abbot, J. and Waite, R. 1962. The effects of antioxidants on keeping quality of whole milk powder. I Flavones, butylhydroxyanisole, and nordihydroguaiaretic acid. Journal of Dairy Research 29 (1) 55-61.

2. Aguilar, C. A. and Zieglar, G. R. 1994. Physical and microscopic characterization of dry whole milk with altered lactose content. 2. Effect of lactose crystallization. J. Dairy Sci. 77 (5) 1198- 1204.

3. Anonymous 1991. Rice bran oil extends shelf life of snacks. Food Engineering 63 (10) 30.

4. Badings, H. T. 1960. Principles of autoxidation in lipids, with special regard to the development of autoxidation flavors. Netherlands Milk and Dairy J. 14 215-242.

5. Bergann, T. and Schick, M. 1997. Characterizing the colour of market milk using the L* a* b* colour values; relationship to fat content and methods of milk processing. Deutsche Veterinarmedizinische Gesellschaft 38 271-278.

6. Bradley, Jr., R. L., Arnold, Jr., E., Barbano, D. M., Semerad, R. G. Smith, D. E. and Vines, B. K. 1993. Chemical and physical methods. Page 433 *in* Marshall, R.T., ed. Standard methods for the examination of dairy products 16th Edition. American Public Health Association, Washington D. C.

7. Bruhn, J. C. and Franke, A. A. 1971. Influence of copper and tocopherol on susceptibility of herd milk to spontaneous oxidized flavor. J. Dairy Sci. 54 761-762.

8. Busch, A. A., Decker, C. W. and Ashworth, J. S. 1952. Keeping quality of whole milk powder. IV. Antioxidative properties of nordihydroguaiaretic acid and certain emulsifying agents. J. Dairy Sci. 35 524-532.

9. Cannon, J. E., Morgan, J. B., Schmidt, G. R., Delmore, R. J., Sofos, J. N., Smith, G. C. and Williams, S. N. 1998. Vacuum-packaged precooked pork from hogs fed supplemented vitamin E: Chemical, shelf life and sensory properties. J. Food Sci. 63 (3) 386-389.

10. Caric', M. 1994. Concentrated and dried dairy products. VCH Publishers, Inc., New York.

11. Chapman, D. and GoNi, F. M. 1986. Physical properties and spectral characteristics Page 385. *in* Gunstone, F. D., Harwood, J. L. and Padley, F. B., ed. The lipid handbook. Chapman & Hall, London, New York.

12. Chen, J. Y., Latshaw, J. D. Lee, H. O. and Min, D. B. 1998. α-Tocopherol content and oxidative stability of egg yolk as related to dietary α-tocopherol. J. Food Sci. 63 (5) 919-922.

13. Christie, W. W. 1995. Composition and structure of milk lipids. Page 1 *in* Fox, P. F., ed. Advanced dairy chemistry v.2: Lipids. Chapman & Hall London.

14. Damoradan, S. 1996. Amino acids, peptides and proteins. Page 321 *in* Fennema O. R., ed. Food chemistry 3rd Edition. Marcel Dekker Inc., New York.

15. Diack, M. and Saska, M. 1994. Separation of vitamin E and γ-oryzanols from rice bran oil by normal phase chromatography. J. American Oil Chemists Society 71 (11) 1211-1217.

16. Dionisi, F., Golay, P. A., Aeschlimann J. M. and Fay, L. B. 1998. Determination of cholesterol oxidation products in milk powders: Method comparison and validation. J. Agric. Food Chem. 46 (6) 2227-2233.

17. Dunkley, W. L., Franke, A. A. and Robb, J. 1968. Tocopherol concentration and oxidative stability of milk from cows fed supplements of d- or tocopheryl Acetate. J. Dairy Sci. 51 531-534.

18. Elliott, J. G. 1999. Application of antioxidant vitamins in foods and beverages. Food Technology 53 (2) 46-48.

19. Emanuel, H. A., Hassel, C.A., Addis, P. B., Bergmann, S. D., and Zavoral, J. H. 1991. Plasma cholesterol oxidation products (Oxyterols) in human subjects fed a meal rich in oxyterols. J. Food Sci. 56 (3) 843-847.

20. Engeseth,N. J., Gray, J. I., Booren, A. M. and Ashgar, A. 1993. Improved oxidative stability of veal lipids and cholesterol through dietary vitamin E supplementation. Meat Science 35 1-15.

21. FAO. 1997-98. Milk and milk products. Commodity market review.

22. Farkas, J. K., Floros, J. D., Lineback, D. S. and Watkins, B. A. 1997. Oxidation kinetics of menhaden oil with TBHQ. Journal of Food Science 62 (3) 505-507,547.

23. FDA. 1996. Code of Federal Regulations 21. Office of the federal register; National archives and records administration. Washington D.C.

24. Graf, E. 1992. Antioxidant potential of ferulic acid. Free Radical Biology & Medicine 13 (4) 435-448.

25. Granelli, K. 1996. The oxidative stability of lipids in milk powder in relation to some pro and antioxidants. Thesis, Swedish University of Agricultural Sciences, Upsala, Sweden.

26. Gunstone, F. D. 1997. Major sources of lipids. *in* Gunstone, F. D. and Padley, F. B. Lipid technologies and applications. Marcel Dekker, Inc. New York.

27. Halliwell, B. 1995. Antioxidant characterization: Methodology and mechanism. Biochemical Pharmacology 49 (10) 1341-1348.

28. Hammond, E. G. 1970. Stabilizing milk fat with antioxidants. American Dairy Review June 40 - 43, 76 –77.

29. Hill, L. M., Hammond, E. G., Carlin, A. F. and Seals, R. G. 1969. Organoleptic evaluation of the effectiveness of antioxidants in milk fat. J. Dairy Sci. 52 (12) 1917-1921.

30. Jenness, R. 1988. The composition of milk. Page 1 *in* Wong, N. P., Jenness, R., Keeny, M. and Marth, E. H., ed. Fundamentals of dairy chemistry, 3rd Edition Van Nostrand Reinhold Company, New York.

31. Jouppila, K., Kansikas, J. and Roos, Y. H. 1997. Glass transition, water plasticization and lactose crystallization in skim milk powder. J. Dairy Sci. 80 (12) 3152-3160.

32. Kieseker, F. G. and Clarke, D. T. 1984. The effect of storage on the properties of non-fat milk powder. Australian J. Dairy Technology 39 74-77.

33. Kikugawa, K. 1986. Fluorescent products derived from the reaction of primary amines and peroxidized lipids. Advances in Free Radical Biology and Medicine 2 389-417.

34. Kim, J. S. 1999. Increased nutritional value and antioxidative stability of restructured beef roasts with purified extracts from rice bran. Ph.D. Dissertation, Louisiana State University.

35. King, A. J., Paniangvait, P., Jones, A. D. and German, J.B. 1998. Rapid method for quantification of cholesterol in turkey meat and products. J. Food Sci. 63 (3) 382-385.

36. King, R. L. 1958. Variation and distribution of copper in milk in relation to oxidized form. Ph.D. Thesis, University of California, Davis.

37. Kingston, E. R., Monahan, F. J., Buckley, D. J. and Lynch, P.B. 1998. Lipid oxidation in cooked pork as affected by vitamin E, cooking and storage conditions. J. Food Sci. 63 (3) 386-389.

38. Koga, H.; Tone, N.; Muramoto, N.; Sakurai, H. and Katayama, O. 1997. The relation between the coloration of frying oil and the deterioration using fried model-materials. J. Japanese Society of Food Science and Technology 44 (9) 666-670.

39. Korycka-Dahl, M. and Richardson, T. 1980. Oxidative changes in milk: Initiation of oxidative changes in foods. J. Dairy Sci. 63 (7) 1181-1198.

40. Kosugi, H., Kato, T. and Kikugawa, K. 1987. Formation of yellow, orange, and red pigments in the reaction of alk-2-enals with 2-thiobarbituric acid. Analytical Biochemistry 165 456-464.

41. Kromhout, D., Keys, A., Aravis, C., Buzina, R., Fidanza, F., Giampaoli, S., Jansen, A., Menotti, A., Nedeljkkovic, S., Pekkarinen, M., Simic, B. S. and Toshima, H. 1989. Food consumption patterns in the 60s in seven countries. American Journal of Clinical Nutrition 49 889-894.

42. Lai, H. M. and Schmidt, S. J. 1990. Lactose crystallization in skim milk powders observed by hydrodynamic equilibria, scanning electron microscopy and 2H nuclear magnetic resonance. J. Food Sci. 55 (4) 994-999.

43. Lawless, H. T. and Heymann, H. 1998. Sensory evaluation of food. Chapman & Hall, New York.

44. Lichtenstein, A. H., Ausman, L. M., Carrasco, W., Gualtieri, L. J., Jenner, J. L., Ordovas, J. M., Nicolosi, R. J., Goldin, B.R. and Schaefer, E. J. 1994. Rice bran oil consumption and plasma lipid levels in moderately hypercholesterolemic humans. Arteriosclerosis and Thrombosis 14 (4) 549-556.

45. McCaskill, D. R. and Zhang, F. 1999. Use of rice bran oil in foods. Food Technology 53 (2) 50-53.

46. McCluskey, S.; J. F. Connolly, R. Devery, B. O'Brien, J. Kelly, D. Harrington and C. Stanton. 1997. Lipid and cholesterol oxidation in whole milk powder during processing and storage and processing. J. Food Sci. 62 (2) 331-337.

47. Min, D. B., Ticknor, D. B., Lee, S. H. and Reineccius, G. A. 1990. Effects of processing conditions and antioxidants on the oxidative stability and CO_2 formation in low fat dry milk. J. Food Sci. 55 (2) 401-403, 423.

48. Morr, C. V. and Richter, R. L. 1988. Chemistry of processing. Page 739 *in* Wong, N. P., Jenness, R., Keeny, M. and Marth, E. H., ed. Fundamentals of dairy chemistry, 3rd Edition Van Nostrand Reinhold Company, New York.

49. Mulder, H. and Walstra, P. 1974. The milk fat globule. Center for Agr. Publishing and Documentation. Wageningen.

50. Nawar, W. W. 1996. Lipids. Page 225 *in* Fennema O. R. ed. Food chemistry 3rd Edition. Marcel Dekker Inc., New York.

51. Nicolosi, R. J., Ausman, L. M. and Hegsted, D. M. 1991. Rice bran oil lowers serum total and low density lipoprotein cholesterol and Apo B levels in non-human primates. Atherosclerosis 88 133-142.

52. Nielsen, B. R.; Stapelfeldt, H. and Kibsted, L. H. 1997. Differentiation between 15 whole milk powders in relation to oxidative stability during accelerated Storage: Analysis of variance and canonical variance analysis. International Dairy Journal 7 (8/9) 589-599.

53. Norton, R. A. 1994. Isolation and identification of steryl cinnamic acid derivatives from corn bran. Cereal Chemistry 71 (2) 111-117.

54. Norton, R. A. 1995. Quantitation of steryl ferulate and p-coumarate esters from corn and rice. Lipids 30 (3) 269-274.

55. Nourooz-Zadeh, J. and Appelqvist, Lars-Ake 1988. Cholesterol oxides in Swedish foods and food ingredients: Milk powders. J. Food Sci. 53 (1) 74-79,87.

56. Ocke, M. C., Kromhout, D., Menotti, A., Aravanis, C., Blackburn, H., Buzina, R., Fidanza, F., Jansen, A., Nedeljkoviv, S. Nissinen, A., Pekkarinen, M. aand Toshima, H. 1995. Average intake of anti-oxidant (pro)vitamins and subsequent cancer mortality in the 16 cohorts of the seven countries study. International J. Cancer 61 480-484.

57. O'Connor, T. P. and O'Brien, N. M. 1995. Lipid oxidation. Page 309 *in* Fox, P. F. ed. Advanced dairy chemistry v.2: Lipids. Chapman & Hall London.

58. Patrick, P. S. and Swaisgood, H. E. 1976. Sulfhydryl and disulfide groups in Skim Milk as affected by direct ultra-high-temperature heating and subsequent storage. J. Dairy Sci. 59 (4) 594-600.

59. Peng, S. K., Taylor, C. B., Hill, J. C., and Morin, R. I. 1985. Cholesterol oxidation derivatives and arterial endothelial damage. Artherosclerosis 54 121-133.

60. Pryor, W. A., Stone, K., Zang, L. Y. and Bermudez, E. 1998. Fractionation of aqueous cigarette tar extracts: Fractions that contain the tar radical cause DNA damage. Chem. Res. Toxicol. 11 441-448.

61. Quinones, H. J.; Barbano, D. M. and Philips, L. G. 1998. Influence of protein standardization by UF on the viscosity, color and sensory properties of 2 and 3.3% fat milks. J. Dairy Science 81 (4) 884-894.

62. Qureshi, A. A., Bradlow, B. A., Salser, W. A. and Brace, L. D. 1997. Novel tocotrienols of rice bran modulate cardiovascular disease parameters of hypercholesterolemic humans. J. Nutritional Biochemistry 8 290-298.

63. Rogers, E. J., Rice, S. M., Nicolosi, R. J., Carpenter, D. R., McClelland, C. A. and Romanczyk, Jr. L. J. 1993. Identification and quantitation of γ-oryzanol components and simultaneous assessment of tocols in rice bran oil. J. American Oil Chemists Society 70 (3) 301-307.

64. Rong, N., Ausman, L. M. and Nicolosi, R. J. 1997. Oryzanol decreases cholesterol absorption and aortic fatty streaks in hamsters. Lipids 32 (3) 303-309.

65. Sander, B. D., Addis, P. B., Park, S. W. and Smith, D. E. 1989. Quantification of cholesterol oxidation products in a variety of foods. J. Food Protection 52 (2) 102-114.

66. Schaich, K. M. 1980. Free radical initiation in proteins and amino acids by ionizing and ultraviolet radiations and lipid oxidation. Part III. Free radical transfer from oxidizing lipids. CTR Crit. Rev. Food Sci. Nutrition 13 189-244.

67. Seetharamaiah, G. S. and Chandrasekhara, N. 1993. Comparative hypocholesterolemic activities of oryzanol, curcumin and ferulic acids in rats. J. Food Sci. and Tech. 30 (4) 249-252.

68. Shin, Tai-Sun, Godber, J. S., Martin, D. E. and Wells, J. H. 1997. Hydrolytic stability and changes in vitamin E vitamers and oryzanol of extruded rice bran during storage. J. Food Sci. 62 (4)704-708,728.

69. Silva, P.L., Robinson, E., Hearnsberger, J. O. and Silva, J. L. 1994. Effect of dietary vitamin E enrichment on frozen channel catfish (Ictalurus punctatus) fillets. J. Applied Aquaculture 4 (3) 45-55.

70. Stapelfeldt, H., Nielsen, B. R., and Skibsted, L. H. 1997a. Effect of heat treatment, water activity and storange temperature on the oxidative stability of whole milk powder. International Dairy J. 7 331-339.

71. Stapelfeldt, H., Nielsen, B. R., and Skibsted, L. H. 1997b. Towards use of electron spin resonance spectrometry in quality control of milk powder. Milchwissenschaft 52 (12) 682-685.

72. Steinberg, D. 1988. Metabolism of lipoproteins and their role in the pathogenesis of atherosclerosis. Atherosclerosis Rev. 18 1-22.

73. Sugano, M. and Tsuji, E. 1997. Rice bran oil and cholesterol metabolism. J. Nutritional Science 127 (3) 221S-224S.

74. Sugano, M. and Tsuji, E. 1996. Rice bran oil and human health. Biomedical and Environmental Sciences 9 242-246.

75. Swaisgood, H. E. 1996. Characteristics of milk. Page 841 *in* Fennema O. R., ed. Food chemistry 3rd Edition. Marcel Dekker Inc., New York.

76. Tamsma, A., Mucha, T. J.and Pallansch, M. J. 1963. Factors related to the flavor stability during storage of foam-dried whole milk. III Effects of antioxidants. J. Dairy Sci. 46 (2) 114-119.

77. Taylor, C. B., Peng, S. K., Werthessen, N. T., Tham, B. and Lee, K. T. 1979. Spontaneously occurring angiotoxic derivatives of cholesterol. The American J. Clinical Nutrition 32 (1) 40-57.

78. Tuohy, J. J., O'Leary, D. and Kelly, P. M. 1981. Packaging and storage of whole milk powder. Irish Journal of Food Science and Technology 5 82.

79. Urbach, G. 1990. Effect of feed on flavor in dairy foods. J. Dairy Sci. 73 3639-3650.

80. Van Lier, J. E. and Smith, L. L. 1968. Sterol metabolism: II Gas chromatographic recognition of cholesterol metabolites and artifacts. Analytical Biochemistry 24 419-430.

81. Verschuren, W. M. M., Jacobs, D. R., Bloemberg, B. P. M., Kromhout, D., Menotti, A., Aravanis, C., Blackburn, H., Buzina, R. Dontas, A. S., Fidanza, F., Karvonen, M. J., Nedeljkovic, S., Nissinen, A. and Toshima, H. 1995. Serum total cholesterol and long term coronary heart disease mortality in different cultures. J. Am. Medical Association 274 (2) 131-136.

82. Vuataz, G. 1988. Preservation of skim milk powders: Role of water activity and temperature in lactose crystallization and lysine loss. Page 201. *in* Seow, C. C. (ed.) Food preservation by water activity control. Elsevier Sci. Publ., Amsterdam, The Netherlads.

83. Walstra, P. and Jenness, R. 1984. Dairy Chemistry and Physics John Wiley and Sons, New York.

84. Weihrauch, J. L. 1988. Lipids of milk: Deterioration. Page 215 *in* Wong, N. P., Jenness, R., Keeny, M. and Marth, E. H., ed. Fundamentals of dairy chemistry, 3rd ed. Van Nostrand Reinhold Company, New York.

85. Weil, J. A., Boltz, J. R. and Wertz, J. E. Electron paramagnetic resonance: Elementary theory and practical applications. 1994. John Wiley &Sons New York.

86. Wewala, A. R. Manipulation of water activity: An important aspect of extending the shelf life of whole milk powder. Page 13. *in* New Zealand Dairy Research Institute Report 1990. Palmerston, New Zealand.

87. Won Park, S and Addis, P. B. HPLC determination of C-7 oxidized cholesterol derivatives in foods. J. Food Science 1985. 50 1437-1441, 1444.

88. Won Park, S and Addis, P. B. 1986. Identification and quantitative estimation of oxidized cholesterol derivatives in heated tallow. J. Agricultural and Food Chemistry 34 (4) 653-659.

89. Won Park, S and Addis, P. B. 1985. Capillary column gas-liquid chromatographic resolution of oxidized cholesterol derivatives. Analytical Biochemistry 275-283.

90. Wulf, D. M., Morgan, J. B., Saunders, S. K., Tatum, J. D., Smith, G. C. and Williams, S. N. 1995. Effect of dietary supplementation of vitamin E on storage and caselife properties of lamb retail cuts. J. Animal Science 73 399-405.

91. Yagi,K. and Ohishi, N. 1979. Action of ferulic acid and its derivatives as antioxidants. J. Nutritional Science and Vitaminology 25 127-130.

92. Yamaoka, M., Carrillo, M. J. H., Nakahara, T. and Komiyama K. 1991. Antioxidative activities of tocotrienols on phospholipid liposomes. J. the American Oil Chemists Society 68 (2) 114-118.

93. Yoon, S. H. and S. K. Kim. 1994. Oxidative stability of high-fatty acid rice bran oil at different stages of refining. J. American Oil Chemists Society 71 (2) 227-229.

94. Zangh, D., Yasuda, T., Yu, Y., Zheng, P., Kawabata, T., Ma, Y. and Okada, S. 1996. Ginseng extract scavanges hydroxyl radical and protects unsaturated fatty acids from decomposition caused by iron-mediated lipid peroxidation. Free Radical Biology and Medicine 20 (1) 145-150.

APPENDIX I: TASTE PANEL EXEMPTION FROM INSTITUTIONAL OVERSIGHT

Louisiana State University Agricultural Center

IRB accession #:_____ Proposal #:_____

LSU Ag Ctr: HUMAN RESEARCH SUBJECTS
APPLICATION FOR EXEMPTION FROM INSTITUTIONAL OVERSIGHT

ALL LSU Ag Ctr research/projects using living humans as subjects,
or samples or data, obtained from them, directly or indirectly,
with or without their consent, must be approved in advance by the
LSU Ag Ctr Institutional Review Board (IRB), unless they meet the
criteria for exemption from IRB oversight and are exempted.

This Form helps the PI determine if the project can be exempted,
and is used to request an exemption. NOTE: A determination of
Exempt status does not release the researcher from exercise of
prudent practice in protecting the interests of research
subjects, including obtaining informed consent. Exempt research
must be conducted in a manner consistent with the Ethical
Principles and Guidelines for the Protection of Human Subjects
(Belmont Report) and LSU Guide to Informed Consent; documents
available from Office of Sponsored Research or
http://www.osr.lsu.edu/osr/comply.html).

Instructions: Complete checklist, pp 2-4. If project appears to
qualify for exemption, send 2ccs of completed form and a brief
project protocol (adequate to evaluate risks to subjects and your
responses to Parts A & B) to Michael Keenan, School of Human
Ecology.

Principal Investigator _Jackin N.
Nanua_____ _____Student?__Yes

Department/Unit _dairy science dept._____Ph:84385_____

Project Title: Potential of High Oryzanol Rice Bran oil as an antioxidant in Milk Powder.

Agency expected to fund project:LSU_____

Subject pool (eg Psychology students)Food and dairy science departments (Students and Staff)._____

Are any of the following "vulnerable populations" to be used in the study: (children <18; the mentally impaired, pregnant women, prisoners, the aged, other)? (circle those applicable) Y/N_No_

I certify my responses are accurate and complete. If the project scope or design is later changed I will resubmit for review.

PI Signature _____ Date _____(no per signatures)

==========

Screening Action: Exempted ✓ Not Exempted

Recommended for: Full ___ IRB Review*
* PI: Obtain the IRB forms packet: send completed form to Bill Todd, Dept. of Veterinary Sciences plus 1 cc of any associated grant proposal.

Reviewer Michael Keeman Signature _____
Date 5-4-99

cc PI

Part A: DETERMINATION OF "RESEARCH" and POTENTIAL FOR RISK

This section determines whether the project meets the

Department of
Health and Human Services definition of "research" and if not,
whether it nevertheless presents more than "minimal risk" to humans
that makes IRB review prudent.

1. Is the project a systematic investigation designed to develop
or contribute to generalizable knowledge?

(Note "systematic investigation" includes "research development,
testing and evaluation"; therefore some instructional development
and service programs will include a "research" component).

YES_✓_ Go to Part B: Project constitutes research

NO_____ Go to 2

2. Does the project present physical, psychological, or legal
risks to the participants reasonably expected to exceed those risks
normally experienced in daily life or in routine physical or
psychological examination or testing?

YES____ Check C2 and stop here: IRB review required

NO_____ Check C1: Apply for exemption from IRB oversight

Part B: EXEMPTION CRITERIA FOR RESEARCH PROJECTS

This Part establishes whether the project is confined to categories
of research activity that may be exempted from IRB oversight.

Please answer each question 1-5; although a single exemption
criterion may be sufficient to exempt a project, some projects
contain several elements that may be met by different criteria.

1. Is this research conducted in established or commonly accepted educational settings, AND does the research involve normal educational practices (e.g. research on regular and special education strategies or research on the effectiveness of, or comparison among instructional techniques, curricula or classroom management methods)? (NOT "YES" merely because conducted at LSU).

YES____ Check C1 & go to 2: This exemption criterion is satisfied

NO✓____ Go to 2: This exemption criterion is not applicable

2. Will this research use educational tests (cognitive, diagnostic, aptitude, achievement), survey procedures, interview procedures or observation of public behavior?

YES ___ Go to 2.1

NO✓____ Skip to 3: (Criterion not applicable)

2.1 Will minors (<18y) be subjects AND does this research use survey procedures, interview procedures or observation of public behavior in which the observer participates?

YES____ Check C2, and skip to 3: IRB review probably required

NO✓____ Go to 2.2

2.2 Is the information recorded in such a manner that human subjects can be identified directly, or indirectly through identifiers (such as a code) linked to the subjects?

YES____ Go to 2.3

NO✓ Skip to 3: This exemption criterion is satisfied

2.3 Will any disclosure of the human subjects' responses have the potential to place the subjects at risk of criminal and civil liability, or be damaging to the subjects' financial standing, employability or reputation?

(The collection of sensitive data regarding the subjects' (or relatives' or associates') possible substance abuse, sexuality, criminal history or intent, medical or psychological condition, financial status, or similarly compromising information are examples of instances which will require an answer of YES):

YES____ Go to 2.4

NO_____ Skip to 3: This exemption criterion is satisfied

2.4 Are the human subjects elected or appointed public officials or candidates for public office?

YES____ Check C1 & go to 3: This exemption criterion is satisfied

NO_____ Check C2 and go to 3: IRB review probably required

3. Does this research involve the collection or study of existing* data, documents, records, pathological or diagnostic specimens?

YES____ Go to 3.1 (*"existing" implies a retrospective study)

NO✓ Skip to 4: (Criterion not applicable)

3.1 Is this material or information publicly available, or
will it be recorded in such a manner by the investigator that the
subjects cannot be identified directly, or indirectly through
identifiers linked to the subjects?

YES_____ Check C1 & go to 4: This exemption criterion is satisfied

NO____ Check C2 & go to 4: IRB review probably required.

4. Is this a taste or food evaluation or consumer taste or food acceptance study?

YES_✓_ Go to 4.1

NO_____ Skip to 5: (criterion not applicable)

4.1 Will only wholesome foods without additives be consumed?
OR any food ingredients (including additives) consumed will be demonstrably at or below the level, and for a use found to be safe;
are agricultural chemicals or environmental contaminants demonstrably at or below the level found to be safe by the Food and Drug Administration or approved by the Environmental Protection Agency or the USDA Food Safety and Inspection Service?

YES ✓ Check C1 & Go to 5: This exemption criterion is satisfied

NO, or unsure_____ Check C2 & go to 5: IRB review may be required

5. Does the project include ANY research activity with human subjects not exempted under one or more of the above criteria?

YES___ Check C2: IRB review required

NO_✓_ Check C1; Go to Part C and proceed accordingly

Part C: PRELIMINARY EVALUATION of EXEMPT STATUS by Investigator:

1. ✓ This project CAN be exempted from IRB Review unless C2 is also checked (you must have answered B1 thru B5). Forward 2 copies of this form and the protocol to Michael Keenan,

School of Human Ecology for a determination/grant of exemption.

2. ____ IRB review required (if C1 also checked, seek exemption)
* Send signed original IRB protocol forms plus one cc of any associated grant application to Bill Todd, IRB Chair, Dept. of Veterinary Sciences.

APPENDIX II: TASTE PANEL CONSENT FORM

TASTE PANEL CONSENT FORM

I, _____agree to participate in the Research entitled "The Potential of Rice Bran Oil as an Antioxidant in Milk Powder", which is being conducted by Jackin N. Nanua, of dairy science dept., LSU, telephone # (225) 388-4385.

I understand that the participation is voluntary and will not in any way affect how I am treated on my job. I reserve the right to withdraw my consent at any time and have the results of my participation returned to me, removed from the experimental records or destroyed.

The following has been explained to me:

1. The aim of the research is to carry out a sensory evaluation of milk powder fortified with rice bran oil. My participation will contribute to the understanding of consumer acceptance of rice bran oil fortified milk.

2. The procedure involves tasting and evaluating coded samples of milk by normal standard methods and recording on score sheets.

3. The only risk envisioned during my participation is that of an allergic reaction to the food being tasted. The food being tasted has been explained to me, hence the risk to allergic reaction is minimal.

4. It is my responsibility to make known to the investigators any allergies I may have towards food so as to be excluded from these panels.

5. The results of this participation are confidential and will not be released in any individually identifiable form without my prior consent unless required by Law.

6. The investigators will answer any other questions I may have about the research, now or during the course of the research project.

_____ _____

Signature of Investigator Signature of Participant

APPENDIX III: TRIANGLE TEST

Triangle Test

Reconstituted Milk

Your last four SSN # digits _____ *April 30, 1999*

Two of the three coded milk samples are the same and one is different. Please taste the samples in the order presented from left to right. Circle the code of the different sample. Rinse your mouth between samples with water.

_____ _____ _____

Give any comments.

_____

_____

_____

_____

Thank you for your participation.

APPENDIX IV: PAIRED PREFERENCE TEST

Paired Preference Test

Reconstituted Milk

Your last four SSN # digits _____ *April 30, 1999*

Please taste the two samples in the order presented, from left to right. Rinse your mouth with water between samples.

Circle the one you prefer

Give any comments.

_____

_____

_____

_____

Thank you for your participation.

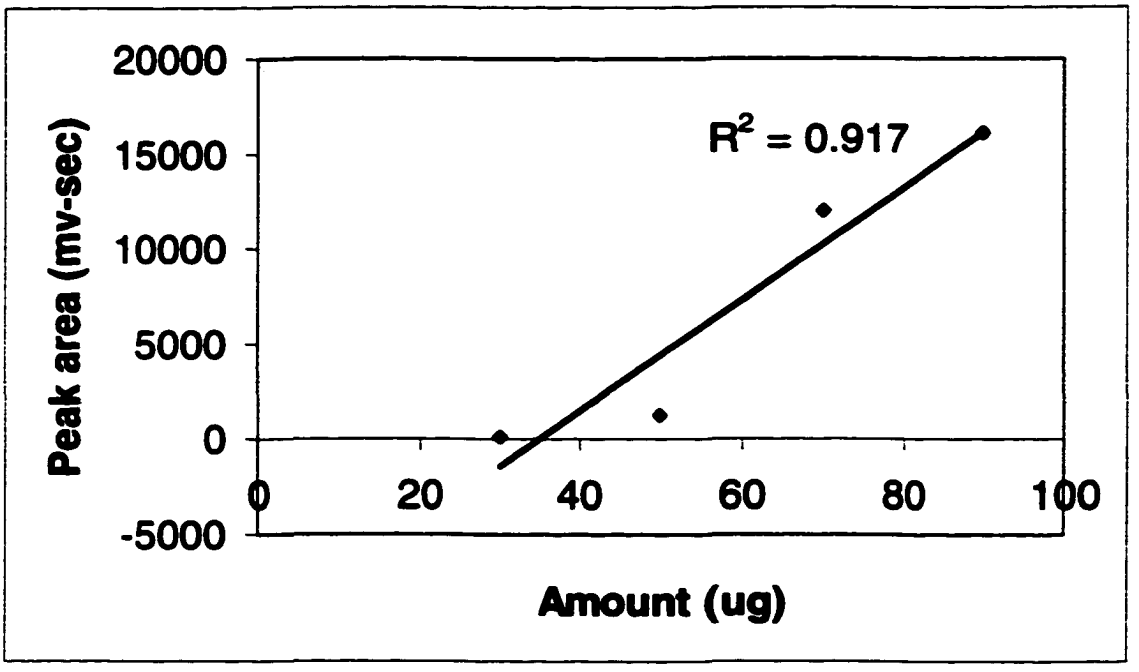
APPENDIX V: PAIRED PREFERENCE, DUO-TRIO AND TRIANGLE TESTS PROBABILITY TABLE

Minimum Numbers of Correct Judgments to Establish Significance at Probability Levels of 5% and 1% for Paired-Difference and Duo-Trio Tests (One-Tailed, $p=1/2$) and the Triangle Test (One-Tailed, $p=1/3$).

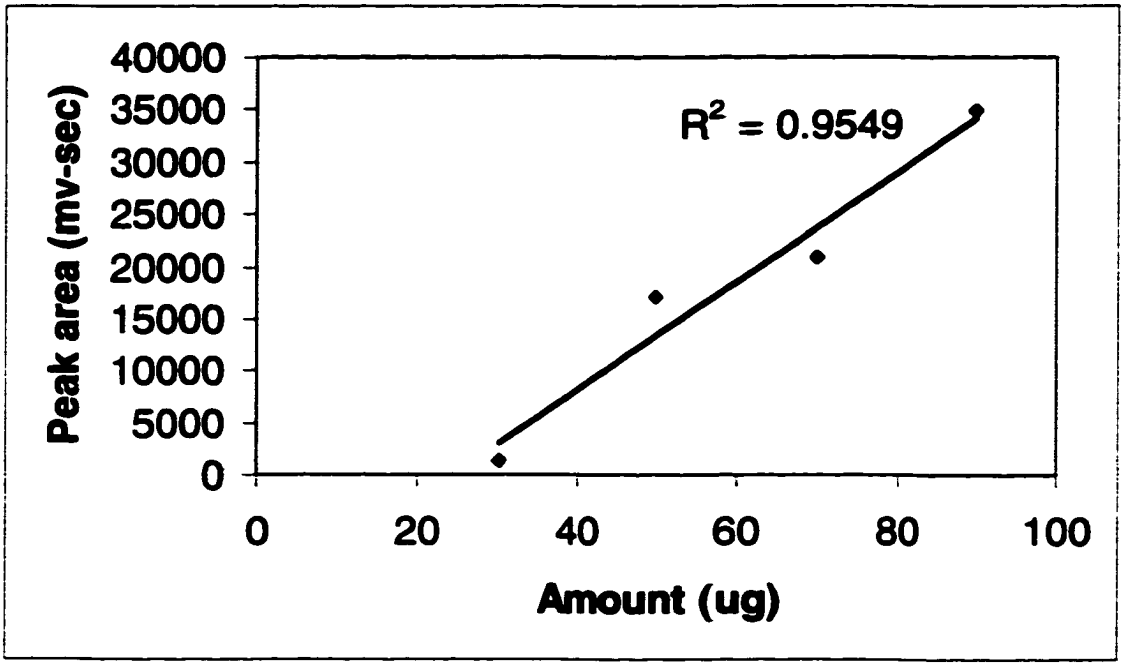
Paired difference and Duo-Trio Tests			Triangle Test		
Number of	Probability Levels		Number of	Probability Levels	
Trials (n)	0.05	0.01	Trials (n)	0.05	0.01
7	7	7	5	4	5
8	7	8	6	5	6
9	8	9	7	5	6
10	9	10	8	6	7
11	9	10	9	6	7
12	10	11	10	7	8
13	10	12	11	7	8
14	11	12	12	8	9
15	12	13	13	8	9
16	12	14	14	9	10
17	13	14	15	9	10
18	13	15	16	9	11
19	14	15	17	10	11
20	15	16	18	10	12
21	15	17	19	11	12
22	16	17	20	11	13
23	16	18	21	12	13
24	17	19	22	12	14
25	18	19	23	12	14
26	18	20	24	13	15
27	19	20	25	13	15
28	19	21	26	14	15
29	20	22	27	14	16
30	20	22	28	15	16
31	21	23	29	15	17
32	22	24	30	15	17
33	22	24	31	16	18
34	23	25	32	16	18
35	23	25	33	17	18
36	24	26	34	17	19
37	24	26	35	17	19
38	25	27	36	18	20

39	26	28	37	18	20
40	26	28	38	19	21
41	27	29	39	19	21
42	27	29	40	19	21
43	28	30	41	20	22
44	28	31	42	20	22
45	29	31	43	20	23
46	30	32	44	21	23
47	30	32	45	21	24
48	31	33	46	22	24
49	31	34	47	22	24
50	32	34	48	22	25
60	37	40	49	23	25
70	43	46	50	23	26
80	48	51	60	27	30
90	54	57	70	31	34
100	59	63	80	35	38
			90	38	42
			100	42	45

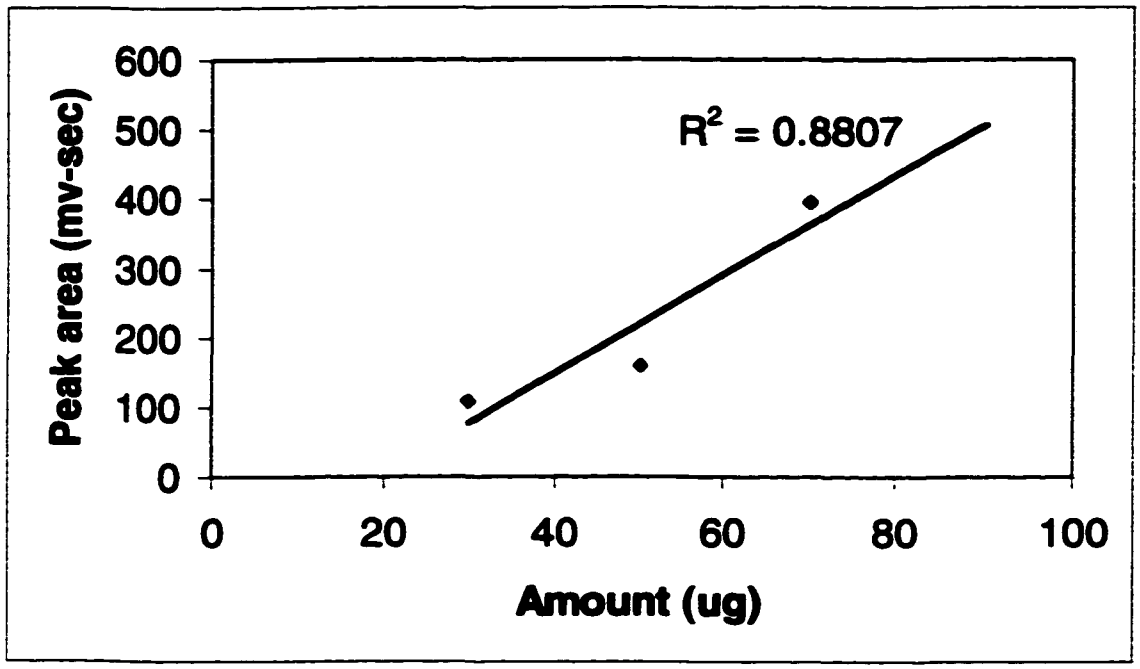
APPENDIX VI: STANDARD CURVES



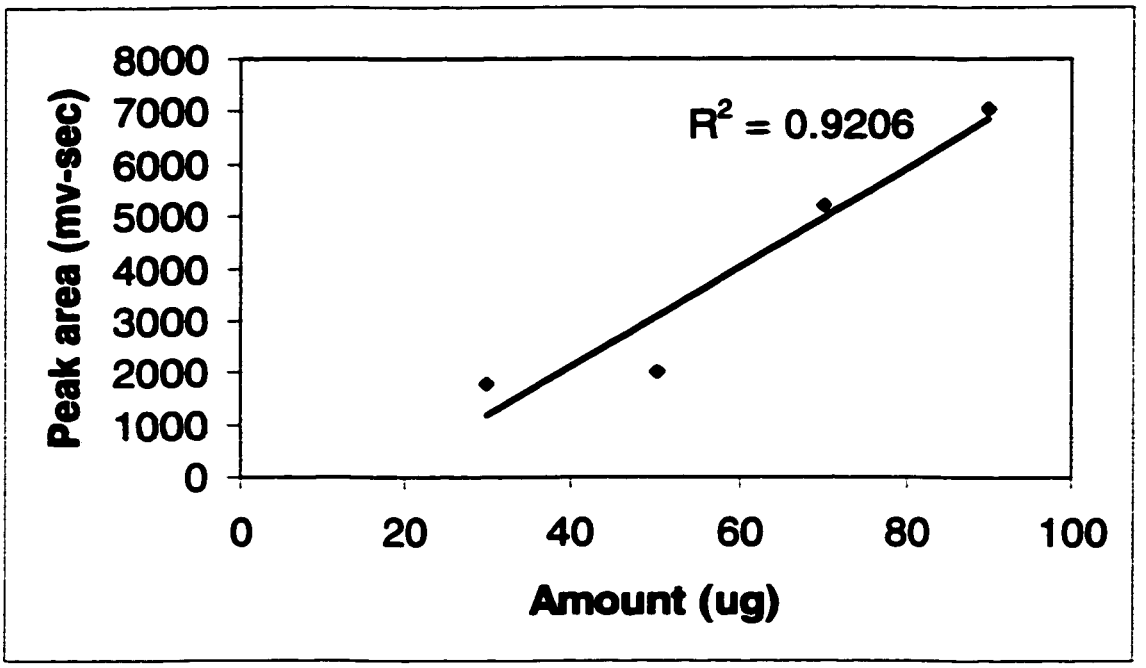
a) 25-Hdrroxycholesterol



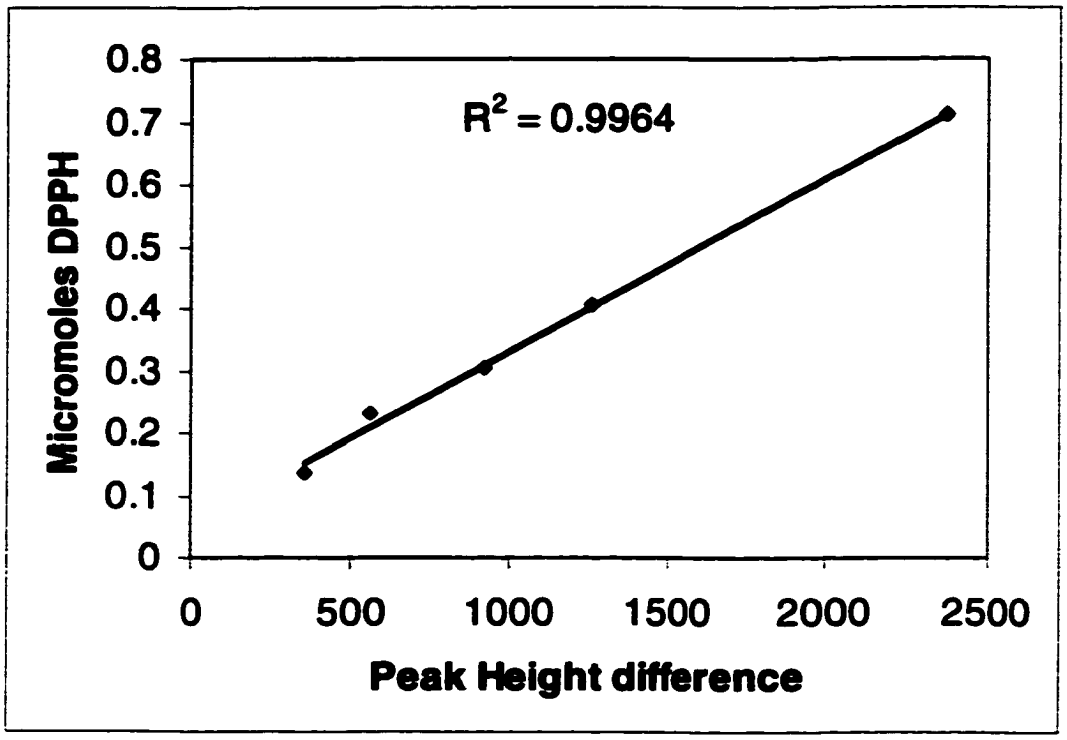
b) 5α,6α-Epoxycholesterol



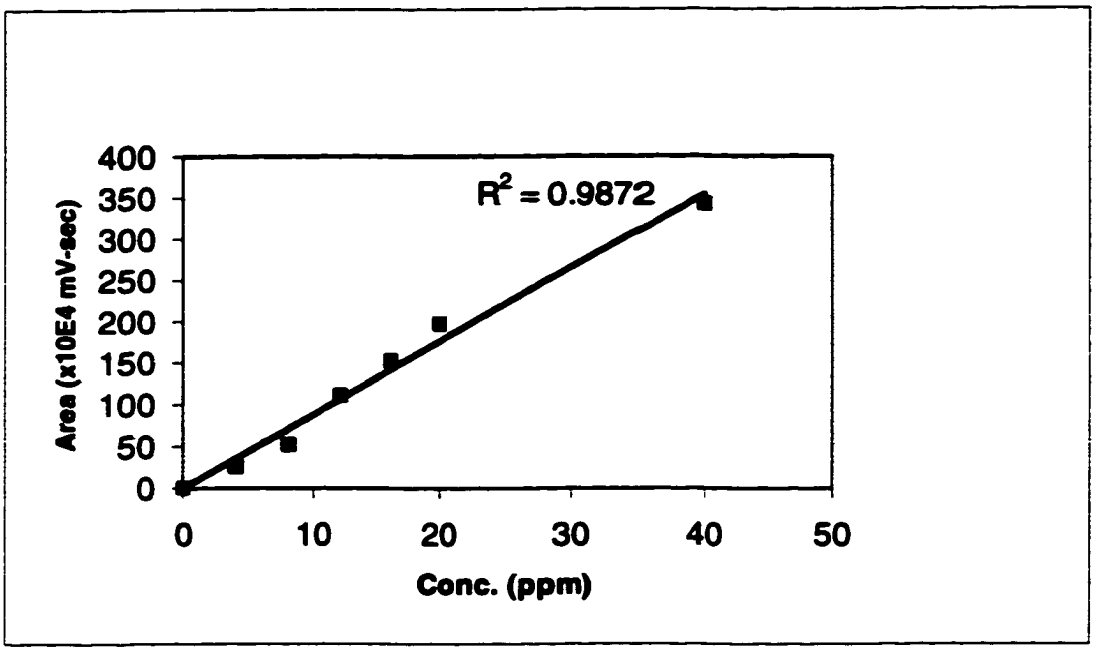
c) 7-Ketocholesterol



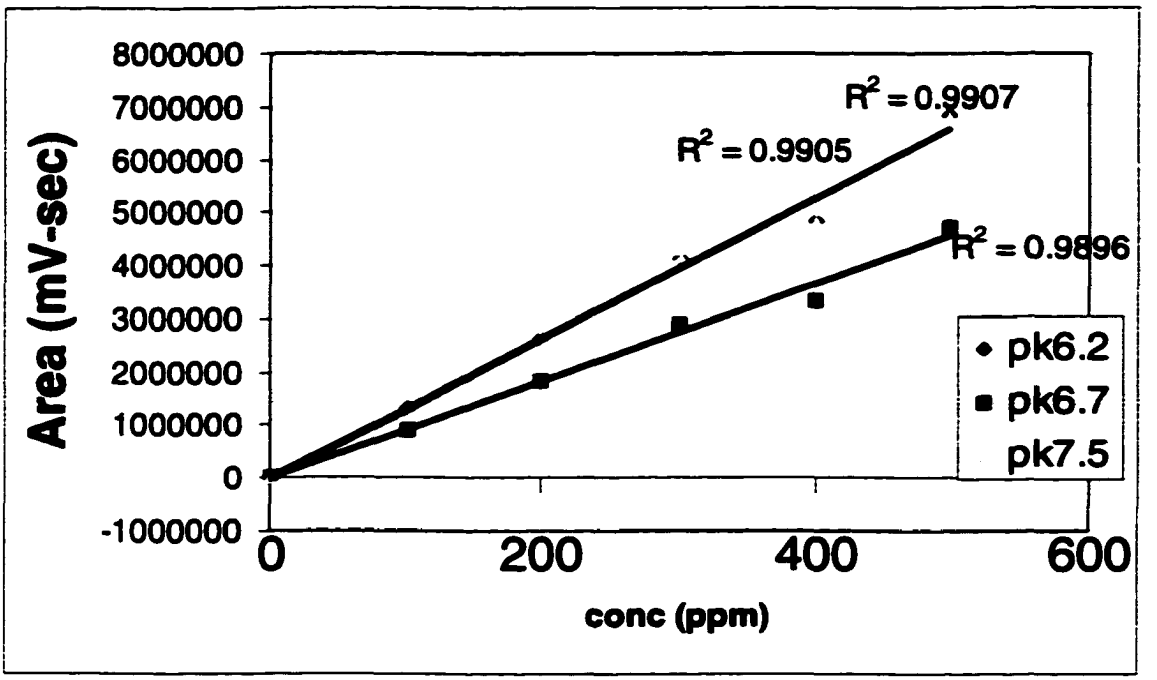
d) 5β,6βα-Epoxycholesterol



e) Free Radicals



f) Alpha Tocopherol



g) Gamma-oryzanol (Individual Peaks)

APPENDIX VII: GENERAL LINEAR MODELS PROCEDURES

a) Powder Color WHITE ANOVA

Dependent Variable: WHITE

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	11	21.94023056	1.99456641	8.05	0.0001
Error	24	5.94840000	0.24785000		
Corr. Total	35	27.88863056			

R-Square	C.V.	Root MSE	WHITE Mean
0.786709	0.521749	0.49784536	95.41861111

Source	DF	Type III SS	Mean Square	F Value	Pr > F
OIL	2	5.50493889	2.75246944	11.11	0.0004
BLOCK	1	10.22933611	10.22933611	41.27	0.0001
OIL*BLOCK	2	5.12757222	2.56378611	10.34	0.0006
REP	1	0.07562500	0.07562500	0.31	0.5858
OIL*REP	2	0.60171667	0.30085833	1.21	0.3146
REP*BLOCK	1	0.02946944	0.02946944	0.12	0.7332
oil*rep*block	2	0.37157222	0.18578611	0.75	0.4833

Tukey's Studentized Range (HSD) Test for variable: WHITE

NOTE: This test controls the type I experimentwise error rate, but generally has a higher type II error rate than REGWQ.

Alpha= 0.05 df= 24 MSE= 0.24785
Critical Value of Studentized Range= 3.532
Minimum Significant Difference= 0.5076

Means with the same letter are not significantly different.

Tukey Grouping	Mean	N	OIL
A	95.9625	12	0
B	95.2333	12	0.1
B	95.0600	12	0.2

Tukey's Studentized Range (HSD) Test for variable: WHITE

NOTE: This test controls the type I experimentwise error rate, but generally has a higher type II error rate than REGWQ.

Alpha= 0.05 df= 24 MSE= 0.24785
Critical Value of Studentized Range= 2.919
Minimum Significant Difference= 0.3425

Means with the same letter are not significantly different.

Tukey Grouping	Mean	N	BLOCK
A	95.9517	18	L
B	94.8856	18	H

b) Powder Color-RED ANOVA

Dependent Variable: RED

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	11	747.72475556	67.97497778	0.97	0.4985
Error	24	1683.89133333	70.16213889		
Corr. Total	35	2431.61608889			

R-Square	C.V.	Root MSE	RED Mean
0.307501	2123.565	8.37628431	0.39444444

Source	DF	Type III SS	Mean Square	F Value	Pr > F
OIL	2	126.78433889	63.39216944	0.90	0.4185
BLOCK	1	73.10250000	73.10250000	1.04	0.3176
OIL*BLOCK	2	127.15551667	63.57775833	0.91	0.4175
REP	1	71.06490000	71.06490000	1.01	0.3243
OIL*REP	2	139.89401667	69.94700833	1.00	0.3838
REP*BLOCK	1	70.78417778	70.78417778	1.01	0.3252
oil*rep*block	2	138.93930556	69.46965278	0.99	0.3862

Tukey's Studentized Range (HSD) Test for variable: RED

NOTE: This test controls the type I experimentwise error rate, but generally has a higher type II error rate than REGWQ.

Alpha= 0.05 df= 24 MSE= 70.16214
Critical Value of Studentized Range= 3.532
Minimum Significant Difference= 8.5397

Means with the same letter are not significantly different.

Tukey Grouping	Mean	N	OIL
A	3.048	12	0
A	-0.914	12	0.2
A	-0.951	12	0.1

Tukey's Studentized Range (HSD) Test for variable: RED

NOTE: This test controls the type I experimentwise error rate, but generally has a higher type II error rate than REGWQ.

Alpha= 0.05 df= 24 MSE= 70.16214
Critical Value of Studentized Range= 2.919
Minimum Significant Difference= 5.7626

Means with the same letter are not significantly different.

Tukey Grouping	Mean	N	BLOCK
A	1.819	18	H
A	-1.031	18	L

c) Powder Color-YELLOW ANOVA

General Linear Models Procedure

Dependent Variable: YELLOW

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	11	298.41816389	27.12892399	4.96	0.0005
Error	24	131.22400000	5.46766667		
Corr. Total	35	429.64216389			

R-Square	C.V.	Root MSE	YELLOW Mean
0.694574	19.95660	2.33830423	11.71694444

Source	DF	Type III SS	Mean Square	F Value	Pr > F
OIL	2	90.50777222	45.25388611	8.28	0.0018
BLOCK	1	96.92402500	96.92402500	17.73	0.0003
OIL*BLOCK	2	65.33195000	32.66597500	5.97	0.0078
REP	1	16.28122500	16.28122500	2.98	0.0973
OIL*REP	2	7.32131667	3.66065833	0.67	0.5213
REP*BLOCK	1	14.12506944	14.12506944	2.58	0.1211
oil*rep*block	2	7.92680556	3.96340278	0.72	0.4947

Tukey's Studentized Range (HSD) Test for variable: YELLOW

NOTE: This test controls the type I experimentwise error rate, but generally has a higher type II error rate than REGWQ.

Alpha= 0.05 df= 24 MSE= 5.467667
Critical Value of Studentized Range= 3.532
Minimum Significant Difference= 2.3839

Means with the same letter are not significantly different.

Tukey Grouping	Mean	N	OIL
A	13.3133	12	0.2
A	12.2825	12	0.1
B	9.5550	12	0

Tukey's Studentized Range (HSD) Test for variable: YELLOW

NOTE: This test controls the type I experimentwise error rate, but generally has a higher type II error rate than REGWQ.

Alpha= 0.05 df= 24 MSE= 5.467667
Critical Value of Studentized Range= 2.919
Minimum Significant Difference= 1.6087

Means with the same letter are not significantly different.

Tukey Grouping	Mean	N	BLOCK
A	13.3578	18	H
B	10.0761	18	L

d) Fat Content ANOVA

Dependent Variable: FAT

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	11	38.51452333	3.50132030	2.86	0.0162
Error	23	28.18071667	1.22524855		
Corr. Total	34	66.69524000			

R-Square	C.V.	Root MSE	FAT Mean
0.577470	3.248355	1.10690946	34.07600000

Source	DF	Type III SS	Mean Square	F Value	Pr > F
OIL	2	18.08150513	9.04075256	7.38	0.0033
BLOCK	1	10.33331267	10.33331267	8.43	0.0080
OIL*BLOCK	2	3.78343333	1.89171667	1.54	0.2349
REP	1	1.16336067	1.16336067	0.95	0.3400
OIL*REP	2	3.20838718	1.60419359	1.31	0.2894
REP*BLOCK	1	0.37600067	0.37600067	0.31	0.5849
oil*rep*block	2	0.82396667	0.41198333	0.34	0.7179

General Linear Models Procedure
Least Squares Means

Means with the same letter are not significantly different.

	OIL	FAT LSMEAN
A	0	33.1766667
AB	0.1	34.1850000
B	0.2	34.9545833

	BLOCK	FAT LSMEAN
A	H	33.5586111
B	L	34.6522222

e) Cholesterol oxidation ANOVA

General Linear Models Procedure

Dependent Variable: COP

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	11	27.86019048	2.53274459	0.27	0.9865
Error	23	218.00666667	9.47855072		
Corr. Total	34	245.86685714			

R-Square	C.V.	Root MSE	PPM Mean
0.113314	52.58926	3.07872550	5.85428571

Source	DF	Type III SS	Mean Square	F Value	Pr > F
OIL	2	0.88166667	0.44083333	0.05	0.9546
BLOCK	1	3.22666667	3.22666667	0.34	0.5653
OIL*BLOCK	2	4.07448718	2.03724359	0.21	0.8082
REP	1	1.38240000	1.38240000	0.15	0.7060
OIL*REP	2	9.74205128	4.87102564	0.51	0.6049
BLOCK*REP	1	0.18026667	0.18026667	0.02	0.8915
oil*block*rep	2	10.30051282	5.15025641	0.54	0.5881

Tukey's Studentized Range (HSD) Test for variable: PPM

NOTE: This test controls the type I experimentwise error rate, but generally has a higher type II error rate than REGWQ.

Alpha= 0.05 df= 23 MSE= 9.478551
Critical Value of Studentized Range= 2.926
Minimum Significant Difference= 2.1539
WARNING: Cell sizes are not equal.
Harmonic Mean of cell sizes= 17.48571

Means with the same letter are not significantly different.

Tukey Grouping	Mean	N	BLOCK
A	6.129	17	L
A	5.594	18	H

Tukey Grouping	lsmeans	N	Oil
A	5.90	11	0
A	6.09	12	0.1
A	5.71	12	0.2

f) Thiobarbituric acid reactive substances MIXED PROCEDURE (All days)

Tests of Fixed Effects

Source	NDF	DDF	Type III F	Pr > F
OILTRT	2	2	2.98	0.2514
HEATTRT	1	1	42.81	0.0966
oiltrt*heattrt	2	2	2.07	0.3255
AGEDAYS	4	4	35.74	0.0022
oiltrt*agedays	8	8	1.31	0.3540
heattrt*agedays	4	4	3.80	0.1121
oiltrt*heattr*ageday	8	8	1.76	0.2210

Least Squares Means

Effect	AGEDAYS	LSMEAN	Std Error	DF	t	Pr > |t|	Alpha
AGEDAYS	1	0.10944444	0.11032928	4	0.99	0.3774	0.05
AGEDAYS	10	0.25555556	0.11032928	4	2.32	0.0815	0.05
AGEDAYS	20	1.49000000	0.11032928	4	13.51	0.0002	0.05
AGEDAYS	30	1.34777778	0.11032928	4	12.22	0.0003	0.05
AGEDAYS	40	0.45777778	0.11032928	4	4.15	0.0143	0.05

Least Squares Means

Lower	Upper
-0.1969	0.4158
-0.0508	0.5619
1.1837	1.7963
1.0415	1.6541
0.1515	0.7641

Differences of Least Squares Means

Effect	AGEDAYS	_AGEDAYS	Difference	Std Error	DF	t	Pr > |t|
AGEDAYS	1	10	-0.14611111	0.15162637	4	-0.96	0.3898
AGEDAYS	1	20	-1.38055556	0.15162637	4	-9.10	0.0008
AGEDAYS	1	30	-1.23833333	0.15162637	4	-8.17	0.0012
AGEDAYS	1	40	-0.34833333	0.15162637	4	-2.30	0.0832
AGEDAYS	10	20	-1.23444444	0.15162637	4	-8.14	0.0012
AGEDAYS	10	30	-1.09222222	0.15162637	4	-7.20	0.0020
AGEDAYS	10	40	-0.20222222	0.15162637	4	-1.33	0.2532
AGEDAYS	20	30	0.14222222	0.15162637	4	0.94	0.4014
AGEDAYS	20	40	1.03222222	0.15162637	4	6.81	0.0024
AGEDAYS	30	40	0.89000000	0.15162637	4	5.87	0.0042

General Linear procedure

Tukey's Studentized Range (HSD) Test for variable: TBA (Day one)
Alpha= 0.05 df= 30 MSE= 0.003408
Critical Value of Studentized Range= 3.487
Minimum Significant Difference= 0.0588

Means with the same letter are not significantly different.

Tukey Grouping	Mean	N	OIL
A	0.10083	12	0
A	0.09917	12	0.2
A	0.08583	12	0.1

Tukey's Studentized Range (HSD) Test for variable: TBA
Alpha= 0.05 df= 30 MSE= 0.003408
Critical Value of Studentized Range= 2.888
Minimum Significant Difference= 0.0397

Means with the same letter are not significantly different.

Tukey Grouping	Mean	N	BLOCK
A	0.11611	18	H
B	0.07444	18	L

Tukey's Studentized Range (HSD) Test for variable: TBA (day Ten)
Alpha= 0.05 df= 30 MSE= 0.011336
Critical Value of Studentized Range= 3.487
Minimum Significant Difference= 0.1072

Means with the same letter are not significantly different.

Tukey Grouping	Mean	N	OIL
A	0.27583	12	0
A	0.24583	12	0.2
A	0.24500	12	0.1

General Linear Models Procedure

Tukey's Studentized Range (HSD) Test for variable: TBA

Alpha= 0.05 df= 30 MSE= 0.011336
Critical Value of Studentized Range= 2.888
Minimum Significant Difference= 0.0725

Means with the same letter are not significantly different.

Tukey Grouping	Mean	N	BLOCK
A	0.38389	18	H
B	0.12722	18	L

Tukey's Studentized Range (HSD) Test for variable: TBA (Day 20)

Alpha= 0.05 df= 30 MSE= 0.205062
Critical Value of Studentized Range= 3.487
Minimum Significant Difference= 0.4558

Means with the same letter are not significantly different.

Tukey Grouping	Mean	N	OIL
A	1.7133	12	0
A	1.3842	12	0.2
A	1.3725	12	0.1

Tukey's Studentized Range (HSD) Test for variable: TBA

Alpha= 0.05 df= 30 MSE= 0.205062
Critical Value of Studentized Range= 2.888
Minimum Significant Difference= 0.3083

Means with the same letter are not significantly different.

Tukey Grouping	Mean	N	BLOCK
A	1.9339	18	H
B	1.0461	18	L

Tukey's Studentized Range (HSD) Test for variable: TBA (Day 30)

Alpha= 0.05 df= 30 MSE= 0.052138
Critical Value of Studentized Range= 3.487
Minimum Significant Difference= 0.2298

Means with the same letter are not significantly different.

Tukey Grouping	Mean	N	OIL
A	1.51583	12	0
B	1.27250	12	0.2
B	1.25500	12	0.1

Tukey's Studentized Range (HSD) Test for variable: TBA

Alpha= 0.05 df= 30 MSE= 0.052138
Critical Value of Studentized Range= 2.888
Minimum Significant Difference= 0.1554

Means with the same letter are not significantly different.

Tukey Grouping	Mean	N	BLOCK
A	1.62889	18	H
B	1.06667	18	L

Tukey's Studentized Range (HSD) Test for variable: TBA (Day 40)

Alpha= 0.05 df= 30 MSE= 0.059691
Critical Value of Studentized Range= 2.888
Minimum Significant Difference= 0.1663

Means with the same letter are not significantly different.

Tukey Grouping	Mean	N	BLOCK
A	0.72111	18	H
B	0.19444	18	L

g) ESRS ANOVA Day One

General Linear Models Procedure

Dependent Variable: RADICALS

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	11	0.71554167	0.06504924	1.73	0.1257
Error	24	0.90053333	0.03752222		
Corr. Total	35	1.61607500			

R-Square	C.V.	Root MSE	RADICALS Mean
0.442765	8.181902	0.19370654	2.36750000

Source	DF	Type III SS	Mean Square	F Value	Pr > F
OIL	2	0.11281667	0.05640833	1.50	0.2426
BLOCK	1	0.00902500	0.00902500	0.24	0.6283
OIL*BLOCK	2	0.48011667	0.24005833	6.40	0.0059
REP	1	0.01480278	0.01480278	0.39	0.5359
OIL*REP	2	0.05907222	0.02953611	0.79	0.4665
BLOCK*REP	1	0.00046944	0.00046944	0.01	0.9119
oil*block*rep	2	0.03923889	0.01961944	0.52	0.5994

Tukey's Studentized Range (HSD) Test for variable: RADICALS

NOTE: This test controls the type I experimentwise error rate, but generally has a higher type II error rate than REGWQ.

Alpha= 0.05 df= 24 MSE= 0.037522
Critical Value of Studentized Range= 3.532
Minimum Significant Difference= 0.1975
Means with the same letter are not significantly different.

Tukey Grouping	Mean	N	OIL
A	2.40750	12	0.2
A	2.40667	12	0
A	2.28833	12	0.1

Tukey's Studentized Range (HSD) Test for variable: RADICALS

NOTE: This test controls the type I experimentwise error rate, but generally has a higher type II error rate than REGWQ.

Alpha= 0.05 df= 24 MSE= 0.037522
Critical Value of Studentized Range= 2.919
Minimum Significant Difference= 0.1333
Means with the same letter are not significantly different.

Tukey Grouping	Mean	N	BLOCK
A	2.38333	18	H
A	2.35167	18	L

h) ESR Spectroscopy (day ten) ANOVA
General Linear Models Procedure

Dependent Variable: RADICALS

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	11	1.17012222	0.10637475	1.98	0.0779
Error	24	1.28706667	0.05362778		
Correc. Total	35	2.45718889			

R-Square	C.V.	Root MSE	Radical Mean
0.476204	7.544581	0.23157672	3.06944444

Source	DF	Type III SS	Mean Square	F Value	Pr > F
OIL	2	0.16937222	0.08468611	1.58	0.2268
BLOCK	1	0.21160000	0.21160000	3.95	0.0585
OIL*BLOCK	2	0.60305000	0.30152500	5.62	0.0099
REP	1	0.00004444	0.00004444	0.00	0.9773
OIL*REP	2	0.05767222	0.02883611	0.54	0.5910
BLOCK*REP	1	0.00401111	0.00401111	0.07	0.7868
OIL*BLOCK*REP	2	0.12437222	0.06218611	1.16	0.3306

Tukey's Studentized Range (HSD) Test for variable: RADICALS

NOTE: This test controls the type I experimentwise error rate, but generally has a higher type II error rate than REGWQ.

Alpha= 0.05 df= 24 MSE= 0.053628
Critical Value of Studentized Range= 3.532
Minimum Significant Difference= 0.2361
Means with the same letter are not significantly different.

Tukey Grouping	Mean	N	OIL
A	3.12083	12	0
A	3.11500	12	0.1
A	2.97250	12	0.2

Tukey's Studentized Range (HSD) Test for variable: RADICALS

Alpha= 0.05 df= 30 MSE= 0.049106
Critical Value of Studentized Range= 2.888
Minimum Significant Difference= 0.1509

Means with the same letter are not significantly different.

Tukey Grouping	Mean	N	BLOCK
A	3.14611	18	H
B	2.99278	18	L

i) Gamma-oryzanol ANOVA

General Linear Models Procedure

Dependent Variable: ORYZANO

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	11	224138.88500833	20376.26227	4.86	0.0006
Error	24	100595.62446667	4191.484353		
Corr. Total	35	324734.50947500			

R-Square	C.V.	Root MSE	ORYZANO Mean
0.690222	84.71177	64.74167400	76.42583333

Source	DF	Type III SS	Mean Square	F Value	Pr > F
OIL	2	208022.44951667	104011.22475833	24.81	0.0001
BLOCK	1	6619.72080278	6619.72080278	1.58	0.2210
OIL*BLOCK	2	3801.90910556	1900.95455278	0.45	0.6407
REP	1	51.81600278	51.81600278	0.01	0.9124
OIL*REP	2	126.64503889	63.32251944	0.02	0.9850
REP*BLOCK	1	1769.18380278	1769.18380278	0.42	0.5221
oil*rep*block	2	3747.16073889	1873.58036944	0.45	0.6448

Tukey's Studentized Range (HSD) Test for variable: ORYZANO

NOTE: This test controls the type I experimentwise error rate, but generally has a higher type II error rate than REGWQ.

Alpha= 0.05 df= 24 MSE= 4191.484
Critical Value of Studentized Range= 3.532
Minimum Significant Difference= 66.005

Means with the same letter are not significantly different.

Tukey Grouping	Mean	N	OIL
A	180.11	12	0.2
B	49.16	12	0.1
B	0.00	12	0

Tukey's Studentized Range (HSD) Test for variable: ORYZANO

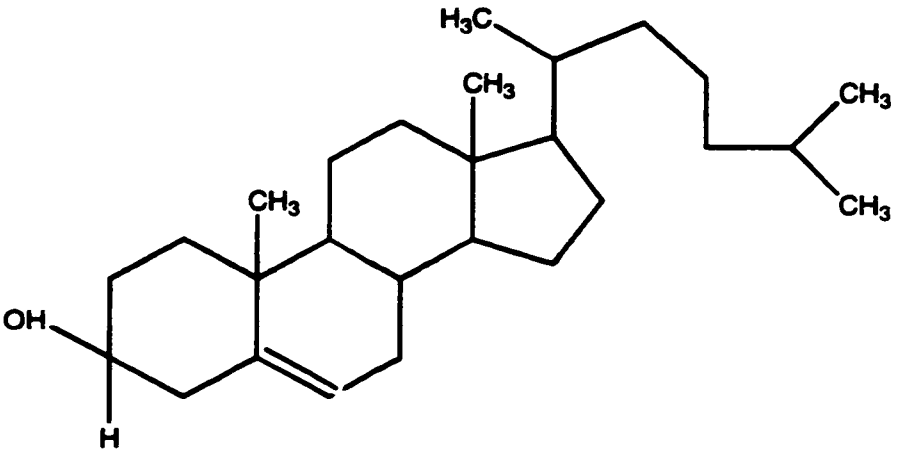
NOTE: This test controls the type I experimentwise error rate, but generally has a higher type II error rate than REGWQ.

Alpha= 0.05 df= 24 MSE= 4191.484
Critical Value of Studentized Range= 2.919
Minimum Significant Difference= 44.54

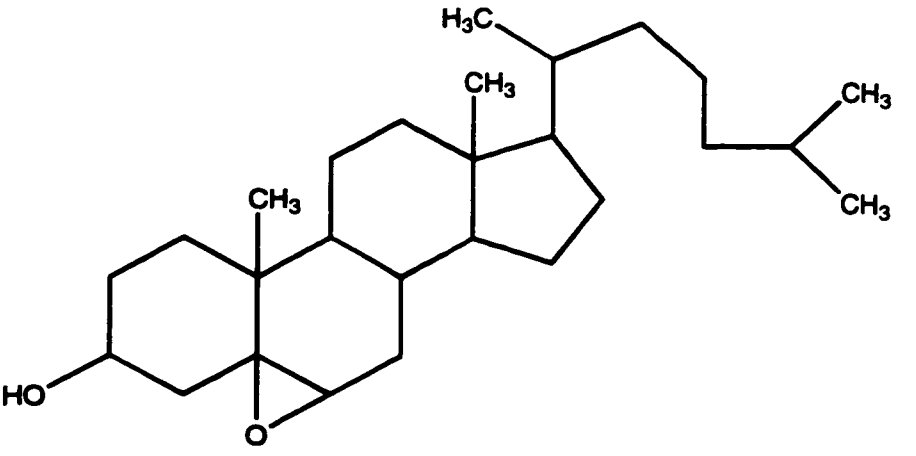
Means with the same letter are not significantly different.

Tukey Grouping	Mean	N	BLOCK
A	89.99	18	L
A	62.87	18	H

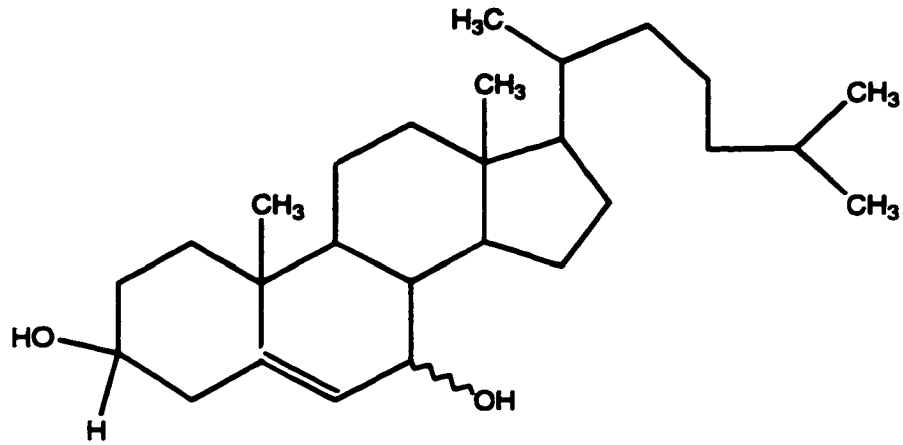
APPENDIX VIII: CHEMICAL STRUCTURES OF CHOLESTEROL AND SOME CHOLESTEROL OXIDATION PRODUCTS



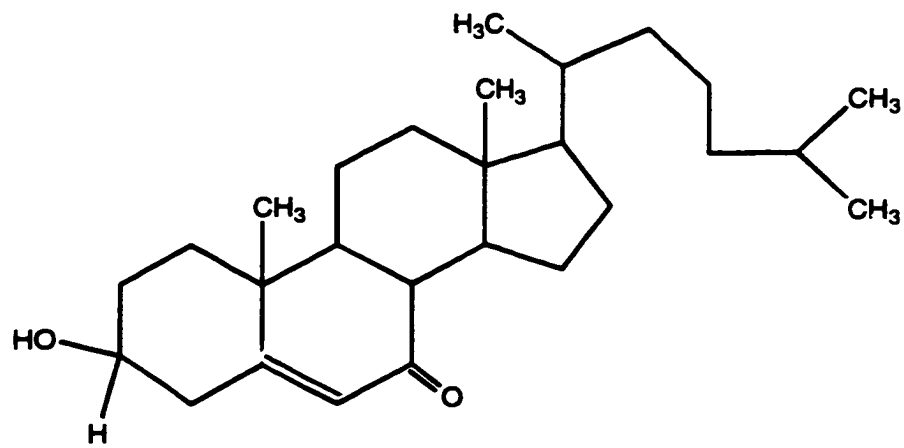
Cholesterol



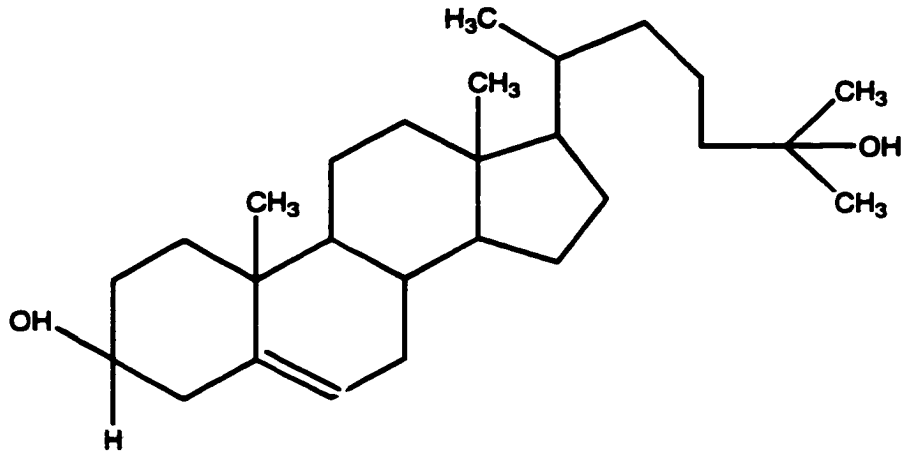
5,6-Epoxide



7-Hydroxycholesterol



7-Ketocholesterol



25-hydroxycholesterol

VITA

The author, Jackin N. Nanua, was born in Kenya on 20th April, 1953, to Evangeline and Fredrick M'Nanua. He attended Mutembe and Rubate Primary schools (Kenya) from 1961 to 1969, after which he proceeded to Chuka High School where he graduated in 1973. He then joined the Alliance High School for his advanced level studies majoring in physics, chemistry and biology from 1974 to 1975. In 1976, he joined the University of Nairobi in Kenya, where he was awarded a bachelor of science degree in Food Science and Technology in December 1979. He attended Reading University in the United Kingdom from 1988 to 1989, where he graduated with a Master of Science in Food Science degree (Dairy option) in December 1989. In August 1996, Nanua joined the Louisiana State University and Agricultural and Mechanical College to study for his doctorate degree, specializing in dairy foods technology.

The author briefly worked as a high school teacher in 1976. He also worked for the Republic of Kenya, Ministry of Agriculture, from 1979 to 1981, as lecturer at the Embu Institute of Agriculture. He joined Egerton University from 1981 where he has been a lecturer up to 1996. He is a committed believer in the Lord Jesus Christ as written in the Holy Bible, a fact that has greatly influenced his life. He has also been involved in many community development projects in Kenya and USA, especially Church related activities. He expects to receive the degree of Doctor of Philosophy in December of 1999. After his graduation, he intends to go back to Kenya and resume his teaching and research duties at Egerton University.

DOCTORAL EXAMINATION AND DISSERTATION REPORT

Candidate: Jackin Njagi Nanua

Major Field: Dairy Science

Title of Dissertation: The Potential of High Oryzanol Rice Bran Oil as an Antioxidant in Whole Milk Powder

Approved:

Major Professor and Chairman

Dean of the Graduate School

EXAMINING COMMITTEE:

Date of Examination:

30 August 1999