2004

Quality evaluation of cheddar cheese containing Gamma-Oryzanol

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QUALITY EVALUATION OF CHEDDAR CHEESE CONTAINING GAMMA-ORYZANOL

A Thesis

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

in

The Department of Food Science

by
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B.S., Chemical Engineering, Universidad de San Carlos de Guatemala, 1999
August 2004

DEDICATION

To God for providing me everything I have, what I am and what I have achieved in my life, eternally grateful.

ACKNOWLEDGEMENTS

First of all, I want to express my everlasting gratitude to Dr. Witoon Prinyawiwatkul, my advisor and friend who has supported and guided me in every little matter throughout the entire time I have been studying at Louisiana State University; I would not think twice to keep studying under his supervision. I also want to thank all members of my committee, Dr. Marlene Janes, Dr. Charles Boeneke and Dr. Zhimin Xu for the assistance and suggestions they gave me during the time this project lasted.

I also would like to express thanks to Matthew Summers, Dr. Jack Losso, Miss Ting Zhang and Mr. Tac Kurihara for their time, friendly hands, help, ideas and materials supplied.

My studies and research at LSU would not have been the same, most importantly my life, without the charm and joy of my friends; from the bottom of my heart, thank you very much guys, Camile, Aisha, Maureen, Evi, Ligia, Ashley, Richelle, Shreya, Ray, Armen, Ivan, Rishipal, Sandeep, friends who are not around anymore and the ones that in spirit are beside me, you know who I am talking about.

Nothing would be completed without showing appreciation to the ones who brought me to this world, me beloved parents, to my sister, who emotionally and spiritually have always been in my thoughts and heart, to my girlfriend Florence for supporting me, encouraging me to do my best and for giving me what I had missing in my life, thanks baby, and to the rest of my relatives.

To all the people in the department of Food Science, thanks for everything, I had a great time in Baton Rouge, I will take you in my heart, and to the ones I did not mention, sorry about that, I do not have mind for everything now, I'm graduating soon.

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ABSTRACT

Health benefits from consuming rice bran oil (RBO) have been extensively studied in humans. RBO has been shown to decrease cholesterol absorption by inhibiting the oxidation of the LDL-cholesterol. γ-Oryzanol is one of the RBO components that possess antioxidative properties; it is a polyphenolic compound resembling tocopherol (vitamin E), which is another component of RBO. There are evidences that oryzanol has potential applications for enhancing shelf-life of foods containing fats processed at a high temperature, decreasing the total cholesterol, LDL-cholesterol and triglyceride levels in plasma lipids, and healing ulcers. In order to take advantage of the health benefits of oryzanol, food products fortified with oryzanol should be developed and characterized. Cheddar cheese containing γ-Oryzanol was developed, and the effects of oryzanol on cheese quality during ripening process were studied. Cheese was stored for 5 months and drawn every month for quality evaluation of color, texture, microbial growth, aroma development, bacterial identification by polymerase chain reaction, oryzanol retention, moisture content and free fatty acid profile. The consumer acceptance was evaluated after 5 months of maturation. The results indicated that by adding γ-Oryzanol to cheddar cheese changes occurred in the quality, but, they were not significant when stored for up to 4 months, and the γ-Oryzanol was well retained. Consumers were able to differentiate the cheddar cheese containing γ-Oryzanol from the control containing no γ-Oryzanol. Overall appearance, texture, taste and hardness were the most discriminatory sensory attributes. There was a significant positive increase 0.13 to 0.30 times higher in the purchase intent of cheddar cheese containing γ-Oryzanol after consumers had been informed about the health benefits of oryzanol. Overall liking was the attribute critical to purchase intent and the most important attribute that changed the acceptability of the cheese. Consumers preferred the cheese with γ-Oryzanol less than the

control but were willing to buy it and compromise the overall liking of cheese merely to get the health benefits of the product. Further studies need to be done to determine the direct effects of cheddar cheese containing γ-Oryzanol in human health, and to optimize the homogenization and fortification of γ-Oryzanol when incorporated in cheese products.

CHAPTER 1. INTRODUCTION

The functional foods industry is evolving at a rapid pace. Consumer interest in healthy eating and self-medication is not just a passing fad. The growing importance of health and wellness has significantly altered consumption and buying behaviors. In the United States and some other developed countries, functional and/or nutraceutical foods have already become part of consumers' regular diet as exemplified in the rush of energy bars, meal replacement beverages and fortified cereals, vitamin/mineral supplements, herbal extracts, etc. The global market for functional foods is estimated to be over $45 billion in 2002 (Institute of Food Technologists Functional Foods Newsletter, 2002). Nowadays consumers are more health-conscious and often search for health foods as part of their daily diets; this drastically alters their consumption and buying behaviors. Consumers also pay attention to the link between diet and health.

Health benefits from consuming rice bran oil (RBO) have been extensively studied in humans. RBO has been shown to decrease cholesterol absorption by inhibiting the oxidation of the LDL-cholesterol. γ-Oryzanol is one of the RBO components that possess antioxidative properties; it is a polyphenolic compound resembling tocopherol (vitamin E), which is another component of RBO (Godber *et al.*, 2002).

γ-Oryzanol has not been thoroughly investigated as a functional ingredient in food products, but its potential applications in human health such as antioxidant activity, reduction of serum cholesterol, cholesterol absorption as well as of early atherosclerosis risk, inhibition on platelet aggregation, inhibition of tumor promotion, menopausal syndrome treatment and antiulcerogenic activity have been thoroughly investigated.

In order to take advantages of the health benefits of γ-Oryzanol, food products fortified or enriched with γ-Oryzanol should be developed as functional foods that may interest consumers.

The natural cheese consumption per capita in the U.S. increased almost 2% from 2001 to 2002. More impressively, during the last quarter of the 20th century, when fluid milk consumption was rapidly declining, cheese consumption continued to grow. According to the 2003 HealthFocus® Trend Report, use of full-fat cheese is on the rise, but coming at the expense of low-fat, fat-free, lower-cholesterol and lower-calorie alternatives.

Usual value-added cheese have the addition of herbs, chopped nuts or seeds such as caraway to the curds of lightly flavored soft cheese. Hard and semi-hard cheeses are, in general, less suitable for development of value-added variants. Cheddar is the most popular cheese flavor in North America (Spanier *et al.*, 2001). It is by far the most important variety of hard cheese and is produced on an extremely large scale and on a world-wide basis (Varman and Sutherland, 2001). In addition, cheddar cheese should be a compatible food matrix with γ-Oryzanol because it contains the highest amounts of tocopherols (vitamin E) compared with other hard cheeses which scarcely contain vitamin E (Renner, 1989).

Knowing that cheddar cheese is a very acceptable and well-liked product on a world-wide basis and that it is compatible with the characteristics of γ-Oryzanol, a sharp cheddar cheese containing γ-Oryzanol was developed in the Dairy Store of the Department of Dairy Science at Louisiana State University, and the effects of γ-Oryzanol on its quality were studied during the 5-month ripening process which had not been done before.

The developed cheese was stored for up to 5 months and drawn for quality evaluation every month. The quality parameters evaluated in the cheese included color, texture, microbial growth, aroma development, bacterial identification by polymerase chain reaction, oryzanol retention, moisture content and free fatty acid profile together with consumer acceptance after 5 months of maturation.

In this thesis, first, a literature review (Chapter 2) is presented, including background information related to the topic, such as RBO, health benefits of γ-Oryzanol, cheddar cheese, its characteristics and manufacturing procedures, functional foods, cheese trends and a brief discussion of the research justifications. The materials and methods (Chapter 3) are extensively described with information related to each procedure, including the physicochemical and biological analyses, the consumer study design and the statistical analysis. The results and discussion (Chapter 4) of every quality parameter evaluated in this research are presented. The conclusions are presented in Chapter 5. The last section includes the appendices containing some figures, the consent form for the consumer study, the questionnaire used for the consumer study and SAS codes used for the statistical analysis of all experiments.

CHAPTER 2. LITERATURE REVIEW

2.1 Rice Bran Oil and γ-Oryzanol

Rice Bran Oil (RBO) is made from the pericarp and germ of the *Oryza sativa* seeds. Rice bran constitutes about 10% of rough rice grain and contains 18%-22% oil. RBO contains a small variable quantity of tocotrienols (72-612 ppm, especially β- and γ-tocotrienols) (Rukmini and Raghuram, 1991; Rogers *et al*., 1993). Moreover, RBO is naturally very rich in α-tocopherol (ca. 100 mg), similar to soybean oil, another vegetable oil with a well-known antihyperlipidaemic action (Changbumrung *et al*., 1980).

In contrast to most common refined vegetable oils, crude rice bran oil is richer in unsaponifiable matters such as steryl ferulates which have growth-promoting vitamin-like activity. Steryl ferulates consist of a mixture of esters of ferulic acid with plant sterol or triterpene alcohol, which are unique to rice oil, with important pharmacological actions (Jariwalla, 2001). The prototype member of the group is γ-Oryzanol, which has been extensively characterized and studied (Naruse and Takeshita, 1999) and was first isolated by Kaneko and Tsuchiya in the early 1950s (Kaneko and Tsuchiya, 1955).

The most accessible natural source of γ-Oryzanol is rice bran, but some components of γ-Oryzanol, mainly sitostanyl ferulate and campestanyl ferulate and lesser amounts of sitosteryl ferulate and campesteryl ferulate, can also be found mostly in the inner pericarp of corn, wheat, rye and triticale grains (Seitz, 1989).

2.1.1 Properties of γ-Oryzanol

γ-Oryzanol is a mixture of ferulic acid esters of triterpene alcohols such as cycloartenol and 24-methylene cycloartanyl (Figure 2.1), and about 1 to 3% is found in RBO. Its

fundamental molecular structure is the ferulic acid aromatic phenolic nucleus esterified to cyclopentanperihydrophenanthrene (Seetharamaiah and Prabhakar, 1986).

Gamma-oryzanol is a white or slightly yellowish, tasteless powder with little or no odor (O'Neil *et al*., 2001), and its industrial production is shown in the flowchart of Figure 2.2.

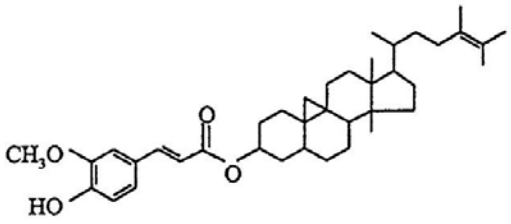

Figure 2.1: Oryzanol comprises any of several plant sterols esterified to ferulic acids. The example shown here is cycloartenyl ferulate.

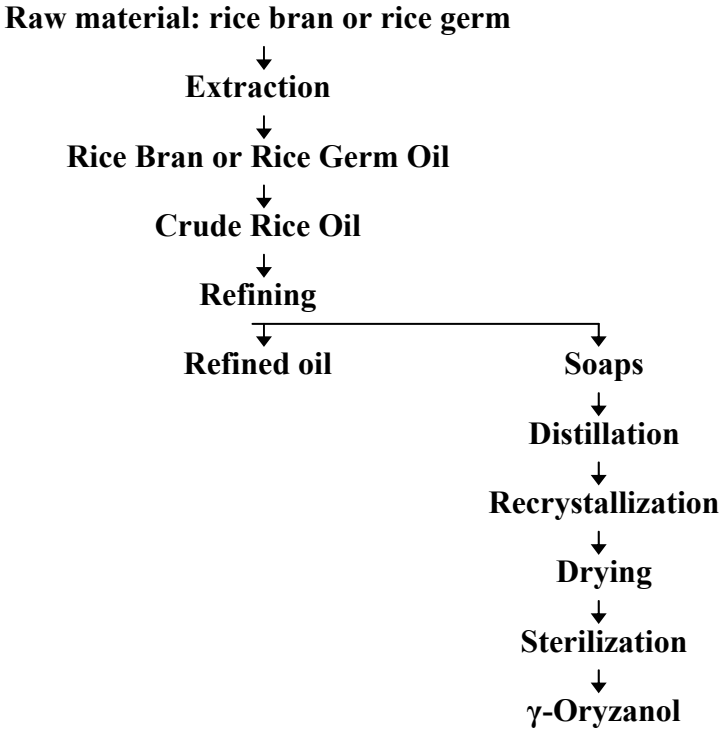

Figure 2.2: Production flowchart of gamma-oryzanol.

RBO and its main components are non-toxic and non carcinogenic (Deckere and Korver, 1996), and the γ-Oryzanol molecule may be poorly absorbed after oral administration (Scavariello and Arellano, 1998). Used commercially in Japan as a food, γ-Oryzanol protects

RBO from oxidation and inhibits peroxidation of lipids medicated by iron or UV irradiation. γ-Oryzanol is a polyphenolic compound with hydroxyl groups and resembles tocopherol (vitamin E) in its antioxidant properties. However, unlike tocopherol, it can increase the antioxidative ability of rice in a dose-dependent manner, at the same time manifesting resistance to high temperature (Jariwalla, 2001). Additionally, the antioxidant capacity of γ-Oryzanol is enhanced in a synergistic manner in the presence of α-tocopherol and other amino acids (Jariwalla, 2001).

2.1.2 Health Benefits of γ-Oryzanol

One of the most investigated properties of γ-Oryzanol is its antiulcerogenic property (Mizuta *et al*., 1978; Ichimaru *et al*., 1984). Different studies have been carried out on rat models to evaluate which pharmacological mechanism is mainly involved in γ-Oryzanols' antiulcerogenic property. One study investigated the antiulcerogenic effect of γ-Oryzanol in mice subjected to conditioned emotional stimuli (communication box method) and rapid eye movement (REM) sleep deprivations (flower pot method). The incidence of gastric lesions was reduced by a single administration of γ-Oryzanol at 100 and 200 mg/kg.

γ-Oryzanol has also found an application as a medical antioxidant when used in combination with α-tocopherol. Although γ-Oryzanol is widely employed in the cosmetic industry as an antioxidant, only one scientific study is available in the literature concerning its modulating effect on sebaceous gland secretion after the topical application (Ueda *et al*., 1976). Moreover, γ-Oryzanol is widely employed as an anabolic agent by bodybuilding athletes (Rosenbloom *et al*., 1992; Grunewald and Bailey, 1993).

When γ-Oryzanol (300mg/day) was administered for 3 months to hyperlipidaemic subjects, a significant decrease in plasma level of total cholesterol (TC) and low density lipoprotein cholesterol (LDL-C) was observed in both hypercholesterolaemic and

hypertriglyceridaemic patients, while a relevant increase in high density lipoprotein cholesterol (HDL-C) was observed only in the hypercholesterolaemic group, and no side effects were observed (Raghuram *et al*., 1989; Cicero and Gaddi, 2001; Yoshino *et al*., 1989a; 1989b). Similar results were obtained when chronic schizophrenic patients with dyslipidaemic who had been receiving neuroleptics for a mean of 10 years were treated with 100 mg of γ-Oryzanol three times daily for 16 weeks (Sasaki *et al*., 1990), and also when postmenopausal women having a diet enriched with test oils having γ-Oryzanol (Lichtenstein *et al*., 1994).

A decrease in early atherosclerosis and reduction of cholesterol absorption were observed by Rong *et al*. (1997). Research investigating the influence of oryzanol on platelet aggregation in rats concluded that oryzanol inhibited the aggregation (Seetharamaiah *et al.*, 1990).

Nair *et al*. (1984) concluded that RBO and γ-Oryzanol contribute to lowering the risk for colon cancer in humans. Yasukawa *et al*. (1998) reported that γ-Oryzanol and its four major components (cycloartenyl ferulate, 24-methylene cycloartanyl ferulate, campesteryl ferulate and sitosteryl ferulate) had an inhibitory effect on tumor in two-staged carcinogenesis in mouse skin. In similar studies, the modifying effects of phytic acid and γ-Oryzanol on the promotion stage of carcinogenesis were investigated using several two-stage carcinogenesis models in rats. The female rats were pretreated with a single intragastric dose of 7,12-dimethylbenz(a)anthracene (DMBA). There were no significant differences in the final incidences and multiplicities of mammary tumors; the average tumor diameter was significantly reduced, and the average survival time was increased with phytic acid and γ-Oryzanol but without significant difference (Hirose et al., 1999). These results indicate that phytic acid inhibits hepatic and mammary carcinogenesis.

A water-soluble oryzanol enzymatic extract (WSOEE) was developed recently and evaluated for its antioxidant potential. WSOEE has been shown to have the *in vitro* antioxidant capacity due to the extensive scavenging of peroxyl radicals. WSOEE is a new potential antioxidant agent from rice bran, being a new source of water-soluble oryzanol. This soluble product showed a high free-radical-scavenging capacity, preventing protein oxidation and lipid peroxidation when cells *ex vivo* were exposed to active-oxygen substances and/or free radicals. This property makes it potentially useful in the formulation of solid and liquid food for treatment and prevention of chronic pathological states associated with a high generation of active-oxygen substances and/or free radicals – such as atherosclerosis, neurodegeneration, and cancer – and for elderly persons and practitioners of sports (Parrado *et al*., 2003).

The effect of high oryzanol RBO on the oxidative stability of low-heat and high-heat whole milk powder (WMP) was investigated by Nanua *et al*., 2000. The results from this study indicated that RBO has potential for use as a natural antioxidant in WMP, due to the presence of tocopherols and tocotrienols as part of the unsaponifiable matter, which have well-studied antioxidative properties.

Antioxidant activities of vitamin E and γ-Oryzanol components purified from rice bran were studied in a cholesterol oxidation system accelerated by 2,2'-azobis(2-methylpropionamidine) dihydrochloride, and all components exhibited significant antioxidant activity in the inhibition of cholesterol oxidation (Xu *et al*., 2001).

2.1.3 Applications of γ-Oryzanol

γ-Oryzanol has suggested to have potential functionality such as antioxidant activity, reduction of serum cholesterol, reduction of cholesterol absorption and decrease of early

atherosclerosis, inhibition on platelet aggregation, inhibition of tumor promotion, menopausal syndrome treatment and antiulcerogenic activity.

Since the functionality of γ-Oryzanol is promising, rice bran or γ-Oryzanol may have great market potential and can be applied to a wide range of products and functional foods that may provide cholesterol-lowering and antioxidant effects.

2.2 Cheese

The word "cheese" is commonly used as a collective term for widely variable products such as matured and non-matured cheese made with rennet, acid curd cheese, fresh cheese, and even processed cheese. Most of these fit the definition established by the FAO/WHO, i.e., cheese is the fresh or matured solid or semi-solid product obtained by coagulating milk, skimmed milk, partly skimmed milk, cream, whey cream, or buttermilk, or any combination of these materials, through the action of rennet or other suitable coagulation agents, and by partially draining the whey resulting from such coagulation.

2.2.1 Cheese Principles

Cheese manufacturing is aimed at making an attractive and durable product in which important nutrients of the milk are concentrated. The cheese must be left to ripen to acquire desirable flavor and consistency. To achieve this, cheese is kept for a variable time under favorable conditions. Cheese making is a complicated process, involving many processing steps and several biochemical transformations. All of these variables affect yield, composition, and quality of the cheese and its byproducts (predominantly whey), and often in different directions (Walstra *et al*., 1999).

When milk is made into cheese, casein and fat are concentrated, whereas the other milk components, especially water, are mainly removed along with whey. None of the milk

components are fully retained, and other substances may be added, notably salt. This is illustrated in Figure 2.3. The yield and the composition of the cheese are determined by the properties of the milk, especially composition, and by the manufacturing practice.

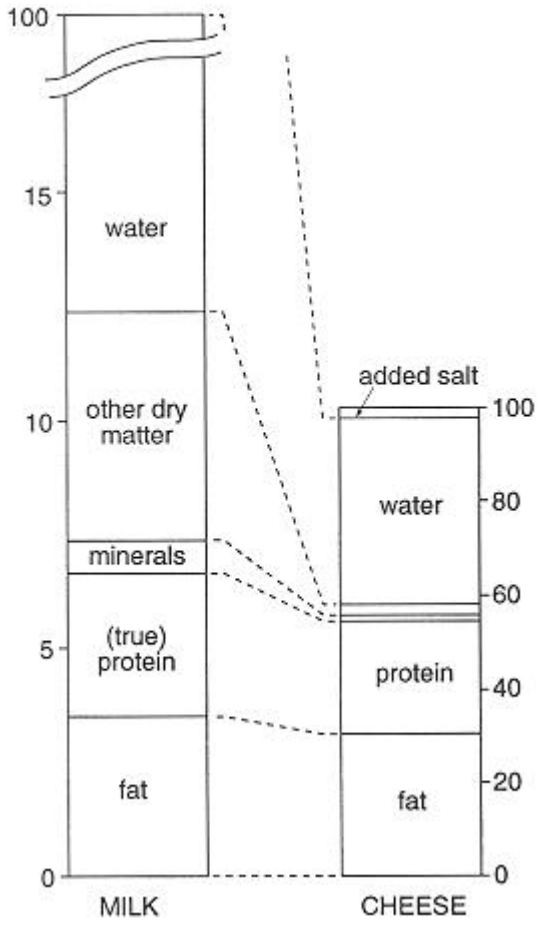

Figure 2.3: Example of the gross composition of milk and cheese and of the transfer of components from milk to cheese (Walstra *et al*., 1999).

The manufacture of cheese may involve many different processing steps, where some steps are essential for all cheese varieties: (1) clotting of the milk by means of enzymes or acid, or both (a gel is formed, due to the casein particles aggregating into a network, enclosing fat globules), (2) removal of whey (comparable to milk serum) by means of syneresis of the gel (the resulting curd makes up 10% to 30% of the original volume of milk; the drier the curd, the firmer

and the more durable the cheese will be), (3) acid production in the cheese during its manufacture due to the conversion of lactose into lactic acid by lactic acid bacteria (the resulting pH of curd and cheese affects such parameters as syneresis, consistency, and ripening of the cheese), (4) salting, (5) fusion of curd grains into a coherent loaf that is easy to handle, and (6) ripening (microbial, biochemical, chemical, and physical processes during ripening are responsible for changes in composition and structure of the cheese; hence flavor and texture).

Fusion of curd grains and ripening are typical processing steps of ripened cheese; when these are not carried out, the product is referred to as fresh cheese (Walstra *et al*., 1999).

With the objective of diminishing variation in the course of the manufacture of the cheese and in its properties, some additional process steps have been applied: pasteurization of the milk and addition of cultures of microorganisms to the milk.

By using different biochemical processes, the relatively tasteless dairy protein is converted into tasty and easily digestible cheese with different flavors. Cheese is rich in essential amino acids and it also binds large amounts of minerals and vitamins (Spreer and Mixa, 1998).

Worldwide, there are more than 2000 types of cheese, sometimes made by very different manufacturing processes. A classification can be based on several aspects, and is done in different countries according to different criteria. A general classification can be made for three major groups: rennet or natural cheese, fresh cheese or non ripened cheese and long-life cheese (processed cheese). For another classification into groups and types, different aspects and characteristics can be used, such as: type of process (rennet cheese, rennet acid cheese, acid curd cheese, processed cheese), type of consistency (hard, semi hard, semi soft, soft cheese), type of milk (cow, sheep, goat, buffalo), chemical composition (Calcium content in conjunction with

pH, dry matter, water, fat), ripening process (ripened cheese and non ripened/fresh cheese), variations in taste, type of hole formation (large, medium and small round holes, cracks, irregular holes, no holes) and surface characteristics (blue fungus or white fungus cheese, smear cheese, skinless cheese). A general and internationally recognized classification is based on the raw material, type of consistency, interior and exterior, interior hole formation and surface characteristics (Spreer and Mixa, 1998).

2.2.2 Cheddar Cheese Properties

Cheddar-type cheeses are characterized by the mixing of salt with the curd before pressing it into a coherent loaf. Salt considerably retards the growth of lactic acid bacteria. Because of this, most of the lactose in the curd should have been converted before the curd is salted. Moreover, salted curd tends to fuse poorly during pressing if its pH is still too high because the curd flows insufficiently (Walstra *et al*., 1999).

Cheddar cheese is classified as a hard cheese, with a long shelf life and without a surface flora. It is about 45% to 50% fat in the dry matter and minimum dry matter of 62% (Spreer and Mixa, 1998). It has a buttery but firm body with close texture and a clean nutty flavor (Varnam and Sutherland, 2001).

Cheddar cheese contains little active milk proteinase, active rennet, and a large pool of proteolytic enzymes from lactic acid bacteria; most of the fast acid-producing strains are also strongly proteolytic. At the low curing temperature (usually below 10°C) the proteolysis in the depth is relatively slow, whereas the degradation in the width is fast. It may be cured for varying lengths of time, from 3 to 15 months (Early, 1992). The curing room is around 85% percent relative humidity at 4°C (40°F) and the cheese is held for 60 days or longer. The peak flavor is usually attained in 9 to 12 months (Kosikowski and Mistry, 1997).

Defects that may occur in Cheddar cheese include: open texture which may lead to formation of cracks upon gas production during maturation, "seaminess" which refers to the appearance of whitish "veins" seen in a cross-section of the cheese, incomplete acid production that often is responsible for insufficient flavor and abnormal consistency, contaminating bacteria that may cause defects, especially at high pH, low salt content, and high ripening temperature, difficulties in cooling down the interior of the cheese when made in very large blocks, and bitter flavor development if the salt content is low and the curing temperature is high (Walstra *et al*., 1999).

2.2.3 Cheddar Cheese Manufacture

A big demand is put on the starter culture. The curd making should be as brief as possible for economical reasons, and this requires very fast acid production. Therefore, fast-growing, fast acid-producing and phage-resistant bacteria are needed. This combination is hard to fulfill; for that reason, mixtures of single-strain, fat starters are generally employed, and contamination of the starter by phages is rigorously prevented. Furthermore, the starter should be homofermentative (little gas production), and cause no bitterness in the cheese. Strains of *Lactococcus lactis* var. *cremoris* are usually used. Currently, a mixture of two strains (*Lactococcus lactis* subsp. *cremoris* and subsp. *lactis*) is often applied, e.g., in a ratio of 1:2 (Walstra *et al*., 1999). The former strain is fairly heat-tolerant (i.e., it keeps growing during scalding) and is responsible for a fast acid production; however, these bacteria form many bitter peptides which are decomposed poorly. The second strain is far less heat-tolerant (it does produce some acid during scalding, but it does not keep growing, and hence contributes little to the rate and extent of acid production), but has considerable "debittering" properties while not

producing many bitter peptides itself; hence, the proteolytic system of these bacteria is of great importance for a satisfactory maturation (Walstra *et al*., 1999).

Figure 2.4 outlines the manufacturing process of cheddar cheese. It represents a somewhat traditional way of manufacturing, although formerly the time from renneting to milling often was even much longer. Nowadays a fixed time schedule is usually maintained and the processing time is much shorter, e.g., 3 hours from renneting to milling.

After cutting, stirring and scalding, the curd settles and fuses into a rather compact mass. Then "cheddaring" starts, which is a process, characteristic of Cheddar and of most of its related types. The whey is drained off, and the curd mass cut into large strips that are piled up. The slabs fuse again and are allowed to spread slowly into thinner slabs that are turned, cut again into strips, piled up, etc.

The curd mass will only flow readily if its pH is lower than 5.8 and the temperature is not too low. The flow causes a "fibrous" curd structure. It has long been assumed to be essential for obtaining a characteristic Cheddar.

Paramount is the acid production during cheddaring. The water content and pH of the curd at that stage largely determine the composition of the cheese. The curd is pressed into a loaf when its acidity is low.

Prior to salting, the curd is milled, i.e., cut into strips about the size of a finger. Milling too finely leads to excessive loss of fat and curd fines in the press whey. Milling too coarsely causes a longer time for the diffusion of the salt into the strips to complete, resulting in a non homogeneous cheese texture. The salt is mixed with the curd and some time is allowed for salt absorption (mellowing); otherwise, excessive salt would be lost with the whey, which in normal cases already contains about 50% of the added salt (both milling and pressing cause a

considerable expulsion of whey). The salt should be evenly distributed, but this is hard to achieve. Acid production in the curd is insufficient if the cheese contains over 5% to 5.5% salt in the water, while at less than 4.5% salt the lactic acid bacteria ferment too fast. In either case the flavor development is unsatisfactory and contaminating organisms have a greater chance of growing out, which may cause strong off-flavors (Walstra *et al*., 1999).

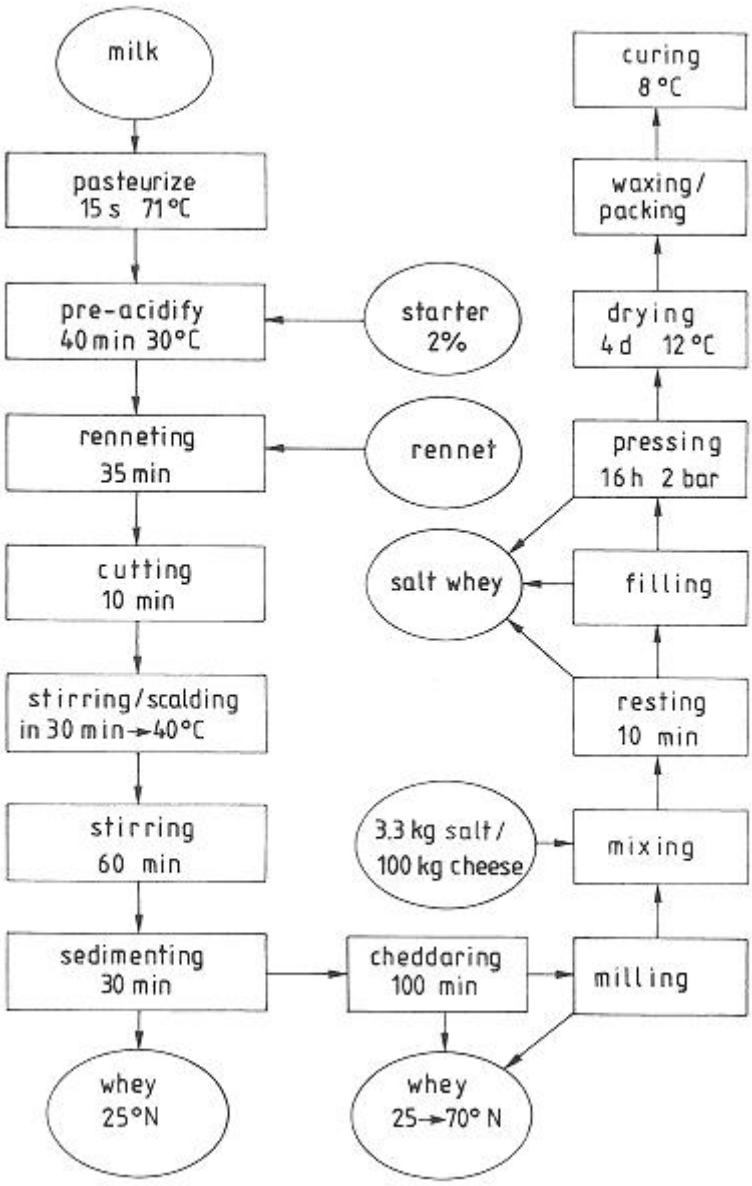

Figure 2.4: An example of a traditional method for manufacture of Cheddar cheese. Simplified from Walstra *et al*. (1999).

The curd, after salting, is usually pressed under vacuum and lower pressure. The lower the temperature and the pH during pressing, the more difficult it is to transform the salted curd into a coherent loaf. Soon after pressing, the cheese (often in the shape of large blocks) is supplied with a surface coating (e.g., paraffin), after which it needs little further care; but it is also common to find processors making rindless cheddar cheese (Kosikowski and Mistry, 1997).

2.3 Functional Foods

2.3.1 Definitions

Japan is the birthplace of the term "functional foods". The term "Foods for Specific Health Use (FOSHU)" was established in 1991 after large-scale research programs were launched and funded by the Japanese government in the early 1980s on systematic analysis and development of food functions, analysis of physiological regulation of the function of food and analysis of functional foods and molecular design (Ashwell, 2002). Afterwards, a variety of terms, more or less related to FOSHU have appeared world-wide. These include more exotic terms, such as nutraceuticals, designer foods, pharmafoods, medifoods, vitafoods and the more traditional dietary supplements and fortified foods. All are foods or food products marketed with the message of a benefit to health.

The term "nutraceutical" was coined from "nutrition" and "pharmaceutical" by Stephen DeFelice, founder of Foundation for Innovation in Medicine, in 1989. He defined a nutraceutical as a food, dietary supplement, or medical food that has a medical or health benefit, including the prevention and treatment of disease (DeFelice, 1995).

For FDA, functional foods can fall into a number of existing categories of the Federal Food, Drug and Cosmetic Act of 1938 (FDCA), as amended. If the product is determined to be a

food and not a drug, it can be regulated as conventional food (including food for special dietary use), dietary supplements, medical food, or as infant formulas.

In Canada, two definitions emerged. A nutraceutical is a product isolated or purified from foods that is generally sold in medicinal forms not usually associated with food. A nutraceutical is demonstrated to have a physiological benefit or provide protection against chronic diseases. A functional food is similar in appearance to, or may be, a conventional food, is consumed as part of a usual diet, and is demonstrated to have physiological benefits and/or reduce the risk of chronic disease beyond basic nutritional functions (Dentali, 2002).

The European Commission (EC) Concerted Action known as FUFOSE (Functional Food Science in Europe) states that a food can be regarded as functional if it is satisfactorily demonstrated to affect beneficially one or more target functions in the body, beyond adequate nutritional effects, in a way that is relevant to either an improved state of health and well-being and/or reduction of risk of disease. Functional foods must remain foods, and they must demonstrate their effects in amounts that can normally be expected to be consumed in the diet. They are not pills or capsules, but part of a normal food pattern (Ashwell, 2002).

Since 1989, the nutraceutical or functional food industry has evolved into a market worth $20.2 billion in the U.S. and a global market estimated to be $47.6 billion in 2002 (Institute of Food Technologists Functional Foods Newsletter, 2002).

According to a Business Communications Co., Inc. report, the nutraceuticals market's growth is attributed to different factors: consumers have an increasing interest in maintaining better health, there has been a rise in available information regarding the link between diet and health, many consumers wish to avoid spending money on health care and prescription medications, there have been several changes in food laws that have affected label and product

claims and an increasing sector of the public is quickly aging and purchasing functional food products (Business Communications Co., Inc., 2003). This report clearly shows that there is a real gap to introduce more functional foods in the U.S. market, and consumers will be interested in them.

2.3.2 Cheese Trends

The growing importance of health and wellness has significantly altered consumption and buying behaviors. Low-carbs diets, concerns about trans-fat and obesity, and greater demand for organic and natural products are requiring a shift in what it is marketed and how it is marketed. The consumer demand for convenience has always been a driving factor in the food industry. But as the pace of life quickens, consumer expectations continue to rise, whether it's for ease of preparation, greater portability or the convenience of single-serve packages.

Consumers learned during the 1990s that, for the most part, to enjoy cheese they could not cut back on fat grams. In fact, when most consumers choose cheese, they are not too worried about the Nutrition Facts label, the emphasis is on flavor (Berry, 2004).

The natural cheese consumption per capita in the U.S. increased almost 2% from 2001 to 2002. More impressively, during the last quarter of the 20th century, when fluid milk consumption was rapidly declining, cheese consumption continued to grow. In fact, since 1980, consumption of natural cheese per capita has increased roughly 75% (USDA/ERS, 2003) and about 60% for cheddar cheese (USDA/ERS, 2004).

According to the 2003 HealthFocus® Trend Report, use of full-fat cheese is on the rise, but coming at the expense of low-fat, fat-free, lower-cholesterol and lower-calorie alternatives. In 2002, only 26% of shoppers used low-fat, low-cholesterol or low-calorie cheese weekly, down

19 points from 45% in 1994. Use of regular or full-fat cheese was 61%, up 8 points from 53% in 1994. Use of fat-free cheese, which has only been tracked since 2000, was down 2 points.

Cheesemakers continue to be innovative in the range of flavors added to cheese. They are taking natural cheese favorites and giving them exciting new flavor profiles, Havarti, for instance, is now available with horseradish and chives, Cheddar is spiked with Pinot Grigio or Shiraz, and Smoked Gouda is enhanced with garlic (Berry, 2004). Convenience is another trend that continues on an upward trajectory.

Cheddar and Hispanic-style cheeses are the most preferred cheeses on burritos and quesadillas, with almost three-fourths of all consumers preferring multiple cheeses on these Mexican foods. Indeed, the popularity of Hispanic foods, along with Hispanic cheeses, is at an all-time high. Manchego, asadero, queso blanco, all Hispanic cheeses, promise to become as familiar to the U.S. consumer as Cheddar and Mozzarella.

Though still a relatively small niche, organic dairy products, including cheeses, are among the fastest growing food categories. Consumer demand for products perceived to be healthful and natural is fueling the trend, as is cheesemaker and dairy producer interest in reaping the often significantly higher margins that organic products command.

Vitamin D is important in human bone health. Leading public health experts are getting concerned that the general population is not getting enough vitamin D as a result of decreased consumption of milk and increased used of sunblock (the body makes vitamin D when unprotected skin is exposed to sunlight). Researchers at the Minnesota-South Dakota Dairy Foods Research Center, St. Paul, Minn., have successfully fortified pasteurized processed cheese with vitamin D3 (100 IU per serving). There was no loss of vitamin D3 during manufacturing,

and the vitamin was uniformly distributed. No loss of the vitamin occurred during storage over a nine-month period (Lovette, 2004).

Recent media attention surrounding cholesterol created a new market for dairy products that work to reduce the level of low-density lipoproteins (LDL) levels found in the body. Glanbia Dairies (Dublin, Ireland) introduced an innovative product in the United Kingdom called Heartily Healthy cholesterol-reducing cheese alternative. Since cheese products typically contain high levels of cholesterol, the company states that the Cheddar-style product is made using milk that has had the dairy fat replaced with wheat germ oil-–an ingredient known to combat cholesterol (Kuhn, 2004).

2.4 Research Justifications

γ-Oryzanol utilization, as a functional ingredient, has not been thoroughly investigated. Its potential applications in human health such as antioxidant activity, reduction of serum cholesterol, cholesterol absorption and risk of early atherosclerosis, inhibition on platelet aggregation, inhibition of tumor promotion, menopausal syndrome treatment and antiulcerogenic activity, are excellent reasons to take advantage of the health benefits of γ-Oryzanol consumption. In order to take advantage of these benefits, food products fortified or enriched with γ-Oryzanol should be developed as functional foods that may interest consumers.

In order to find the best food product fitting the potential functionalities of γ-Oryzanol, its properties must be taken into account. γ-Oryzanol is liposoluble, and its antioxidant activity is enhanced synergistically in the presence of α-tocopherol as described in section 2.1.1.

A product which delights the taste of a big percentage of the population and is healthy by itself is a good potential target, regardless of race, religion, background or social stratus. Hard cheeses contain high amounts of fat, are an excellent source of Calcium for lactose intolerant

people, and also contain proteins with essential aminoacids necessary for a normal body metabolism. Hard cheeses also contain microorganisms which benefit human intestinal flora. Adding a nutritive value to a hard cheese with health benefits creates consumer awareness of the functional cheese products.

Several studies suggest that dairy foods such as cheese and milk prevent demineralization of enamel and favor remineralization related with dental caries. Silva *et al.* (1986) using intraoral caries models (i.e., models that use sections of human or bovine enamel placed at interproximal sites in fixed appliances), found that consumption of 5 g of aged Cheddar cheese immediately following sucrose intake (i.e., a 10 percent sucrose challenge) reduced, by an average of 71%, sucrose-induced demineralization of experimental enamel slabs. Likewise, using a new *in situ* caries model developed by Featherstone and Zero (1992), enamel demineralization occurred in the absence of Cheddar cheese. In contrast, in the presence of cheese, a significant trend toward remineralization of enamel was evident. In addition, components in cheese or milk such as protein (casein and whey), lipids, calcium, and phosphorus may be partly responsible for the beneficial effects of these foods on oral health (Miller *et al*., 1995).

Cheddar is the most popular cheese flavor in North America (Spanier *et al*., 2001); it is by far the most important variety of hard cheese and is produced on an extremely large-scale and on a world-wide basis (Varnam and Sutherland, 2001). In addition, cheddar cheese shows a compatible food matrix with γ-Oryzanol because it contains the highest amount of tocopherols (vitamin E) compared to other hard cheeses which scarcely contain vitamin E (Renner, 1989), and thus its antioxidant effects can be enhanced.

In a search performed in Medline (PubMed), in Agricola and in Ingenta with key words combining “rice bran oil”, “RBO”, “oryzanol”, “gamma-oryzanol”, “γ-oryzanol”, “nutraceutical” or “functional” with “cheese”, it could be noticed that there has not been any research on cheese containing RBO components or γ-Oryzanol, nor or the possible effects of these compounds in the quality of the cheese.

Usually value-added cheeses have had the addition of herbs, chopped nuts or seeds such as caraway to the curds of lightly flavored soft cheese. Hard and semi-hard cheeses are, in general, less suitable for development of value-added variants. Cheddar cheese and related cheeses, may, however, be supplemented with various materials including chopped walnuts and pickles. Both hard and soft types are involved and may be blended with a range of beverages including beer, whisky, port wine and liqueurs (Varnam and Sutherland, 2001). Nutritionally modified cheeses are mostly related with low-fat soft cheese varieties or hard cheeses using unmodified technology which lacks of the characteristic ripened flavor of the full fat equivalent and has bitter taste characteristics.

With one in four Americans suffering from some form of coronary heart disease, 108 million with high cholesterol levels, it is not surprising that almost every consumer behavior related to improving heart health has escalated (AHA, 2004). Four out of ten of households (43%) are treating a member for high cholesterol (Sloan, 2004) and with 44% of shoppers saying that reducing the risk of heart disease or helping to maintain healthy cholesterol levels is an extremely/very important food claim. Marketers have begun differentiating heart-healthy products based on their ingredients (HealthFocus®, 2003).

For all the reasons mentioned above, cheddar cheese containing γ-Oryzanol was developed, which could potentially have better positive effects in humans than other products with a different nutritional profile.

2.5 Objectives

2.5.1 General

The main goal of this study was to develop a sharp cheddar cheese containing γ-Oryzanol and study its effects on cheese quality during and after the ripening process.

2.5.2 Specific

Determine quality differences of sharp cheddar cheese containing γ-Oryzanol by evaluating color, texture, microbial growth, aroma development, moisture content, free fatty acid profile, starter culture performance and the oryzanol retention together with a consumer study after 5 months of maturation.

CHAPTER 3. MATERIALS AND METHODS

3.1 Cheddar Cheese Manufacturing

The process used to produce the cheddar cheese containing γ-Oryzanol was similar to the method shown in Figure 2.4 (section 2.2.3).

The facilities of the creamery in the Dairy Science department in the LSU campus were used to produce the cheddar cheese containing γ-Oryzanol.

Milk obtained from the LSU farm (6201 Lb) was pasteurized on October 28th, 2003 at 177°F (77°C) for 20 seconds, then kept overnight below 40°F (4.5°C) until the next day for the cheese making. The milk contained 3.46% of fat and 3.06 % of protein.

On October 29th, 2003, early in the morning, the pasteurized milk was poured in a vat and heated slowly with constant stirring. The heating process was continued by increasing the temperature at a rate of 1°F every 2 minutes for approximately 1.5 hours until the temperature reached 88°F (Figures 3.1 and 3.2).

At this point, the starter culture, mesophilic direct vat set (DVS) from CHR Hansen (Milwaukee, WI) was added at 2.1% by weight (Figures 3.3 and 3.4), which contains selected strains of *Lactococcus lactis* subsp. *cremoris* and subsp. *lactis*. Together with the starter culture, the color (annatto food color) was added, at 0.03% by weight (Figures 3.5 and 3.6).

The milk with the starter culture and the color was stirred at 88°F for 45 minutes. Immediately afterward, calcium chloride food-grade ($CaCl_2$ aqueous solution 32%) from DSM (DSM Food Specialties, Menomonee Falls, WI) was added at 0.02% w/v (Figure 3.7).

Figure 3.1: Stirring milk in the vat while being heated up.

Figure 3.2: Stirring milk in the vat while being heated up before adding culture.

Figure 3.3: Adding starter culture to milk when temperature reached 88°F.

Figure 3.4: Different view of the addition of the starter culture to the heated milk.

Figure 3.5: Adding color to heated milk after starter culture was added.

Figure 3.6: Color added to heated milk while stirring.

Figure 3.7: Addition of $CaCl_2$ to the milk after the culture and color were added.

Figure 3.8: Adding the rennet into the milk after the culture, color and $CaCl_2$ were added.

The process continued with agitation at 88°F for 15 minutes then the rennet from CHR Hansen (Milwaukee, WI) was added at 0.01% with a dilution 1/50 in water. The rennet consisted of a 100% fermentation produced chymosin, Sodium Chloride (NaCl), Sodium Benzoate and caramel color. The addition of $CaCl_2$ and rennet is shown in the Figures 3.7 and

3.8, respectively. The stirring was continued for a few seconds just to mix the rennet, then the agitation was stopped, and the milk was allowed to settle down for 35 minutes as the renneting took place. Figure 3.9 shows that the vat was covered while the renneting process took place.

After 35 minutes, the hardness of the curd formed from the renneting process was checked to see if it was hard enough to start cutting (Figure 3.10). As soon as the curds' hardness was desirable, all the curd was cut as shown in Figures 3.11 and 3.12 and then was allowed to heal for 5 minutes. The cutting process was done with extreme carefulness to avoid destroying the curd cubes since they are still fragile and soft.

Figure 3.9: Renneting taking place in the vat while the temperature was still 88°F.

Healing the curd can be observed in the Figure 3.13 where the curd was left undisturbed without agitation, and the cooking of the curd is shown in the Figure 3.14 when agitation was started again.

The cooking process is also referred to as scalding which involves an increase of temperature from 88°F to 102°F at a rate of 3°F every five minutes with constant agitation. The temperature was held at 102°F for about one hour.

Figure 3.10: Cutting the curd as a pre-test to check if it was hard enough to continue the process.

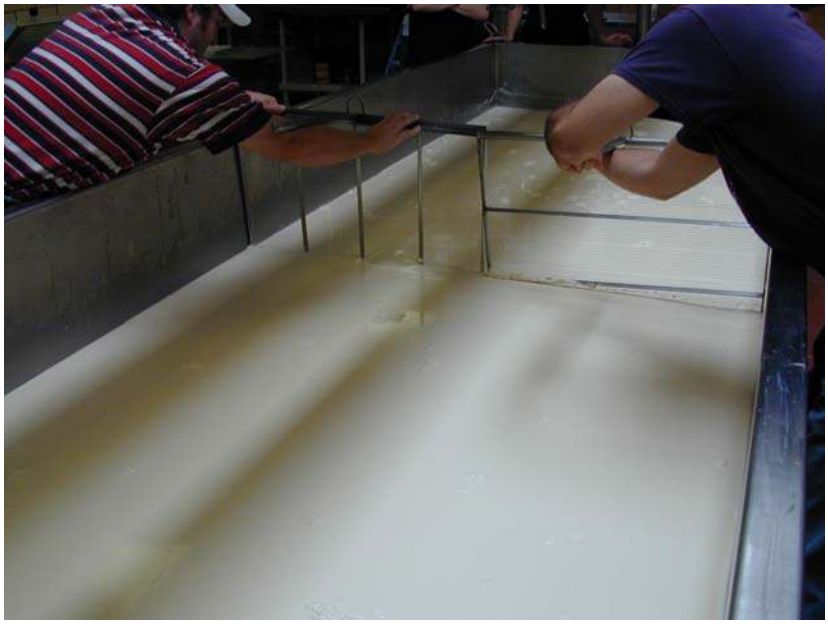

Figure 3.11: Cutting the curd after renneting.

Figure 3.12: Another view of the process of cutting the curd after renneting.

Figure 3.13: The curd is healed by letting it sit without stirring.

After letting the curd sediment to complete, the whey protein was drained out of the vat (Figures 3.15 and 3.16) while keeping the curd in the vat as shown in Figure 3.17.

Figure 3.14: Cooking the curd with agitation and increasing the temperature.

To allow the whey protein to come out of the curd, the curd was cut in loaves (Figure 3.18). Then the loaves were separated as shown in Figure 3.19 and flipped over every 15 minutes to facilitate the separation of the whey protein from the curd.

Figure 3.15: Draining of whey proteins out of the vat.

Figure 3.16: Draining of whey protein, while keeping the curd inside vat.

Figure 3.17: Draining of whey protein, while keeping the curd inside vat (continued).

Figure 3.18: Cutting curd into loaves of 20 inch long and 10 inch wide.

Figure 3.19: Flipping loaves over to help drain whey protein out of the curd.

Figure 3.20: Two loaves of curd stacked to help release of whey protein in vat.

Stacking loaves over loaves increases pressure to release the whey protein still trapped in the curd, and flipping over also helps to drain whey protein. Turning the loaves up side down took about 1.5 hours.

The temperature of the vat was still kept at 88°F. Once the titrable acidity reached 0.45%, the loaves were no longer flipped over and subsequently were milled in pieces of approximately one inch by one inch by two to three inches (Figure 3.22).

Once all the loaves were milled and spread over the surface of the vat, the curd was allowed to rest for 15 minutes, then salt was added at 2.8% w/w in 3 additions every 10 minutes and mixed (Figure 3.23).

Figure 3.21: Four loaves of curd stacked to help release of whey protein in vat.

Figure 3.22: Milling the curd loaves into smaller pieces (1x1x2-3 inches).

Figure 3.23: Mixing salt with the milled curd which lasted approximately 30 minutes.

At this point, a part of the salted curd was separated from the rest and the γ-Oryzanol (Maypro Industries, Inc., Purchase, NY) of 98.8% purity was added in amount to attain a concentration of 100mg per 28 grams of curd (see Appendix E for certificate of analysis of γ-Oryzanol). The mixing of γ-Oryzanol was done manually. The salted curd without γ-Oryzanol was used as the control.

The curd was allowed to rest for about 10 minutes and then filled into a hoop of 40 pounds having cheesecloth wrapped to avoid loss of curd during subsequent pressing (Figure 3.24).

In the Figure 3.25, the pressing process using a pneumatic method can be seen where the curd was pressed and left overnight under a pressure of 50 psi.

Following the overnight pressing step, the pressed curd was taken out of the hoops, separated from the cheesecloth and vacuum packed in plastic bags (Figure 3.26). The plain curd (the control sample) and the curd containing γ-Oryzanol were packed separately. After the curd

was packed in vacuum in plastic bags, the cheese is ready to be maturated in cold humid rooms for up to 5 months.

Figure 3.24: 40 pound hoops with cheesecloth wrapping the salted curd.

Figure 3.25: Hoops filled with the curd pressed under 50 psi pressure overnight.

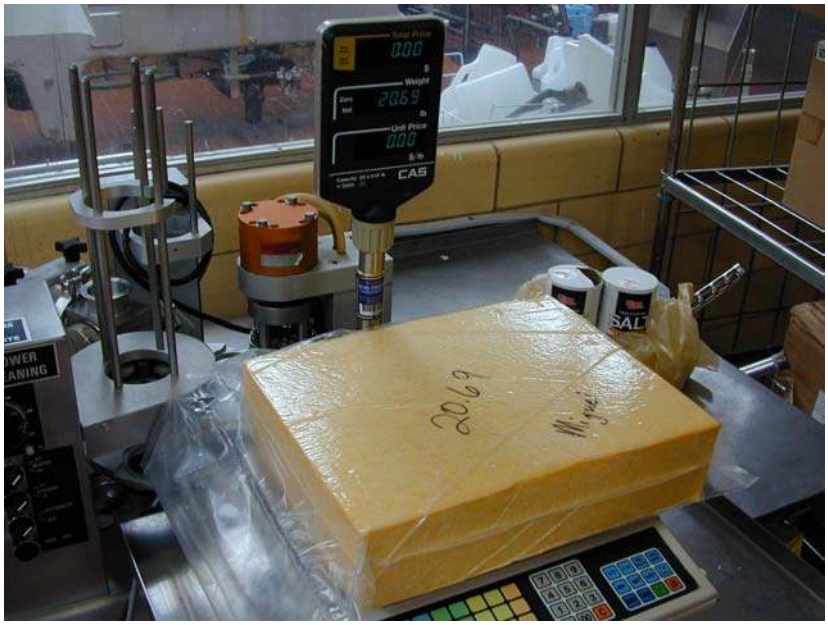

Figure 3.26: Vacuum packed curd ready to be maturated.

3.2 Cheese Ripening

The ripening process allows flavor, texture and aroma to develop. For the cheddar cheese, an appropriate storage condition would be relative air humidity (RH) between 75% and 80%, temperatures between 5°C and 16°C, and ripening time for 150 days (Walstra *et al.*, 1999).

Figure 3.27: Storage of the cheddar cheese in the Dairy Store at LSU.

Our cheese products were stored in the cooler at 40°F (4.4°C) and RH of 85% for up to 5 months (Figure 3.27). Prior to storage, the cheese was packed in labeled small plastic bags after being cut to small pieces to facilitate the sampling for subsequent experiments and to avoid confusion.

3.3 Samples and Sampling

The cheddar cheese samples consisted of one control which was the normal sharp cheddar cheese and one treatment of cheddar cheese with γ-Oryzanol targeting 100mg per serving size (28g). Two batches of cheeses were collected for this experiments.

Monthly sampling up to three months was performed during ripening in order to measure different aspects of the quality of the cheddar cheese. The cheese was separated in different bags for different analyses (Figures 3.28 and 3.29).

Figure 3.28: Cheese separated in small bags for different analyses.

Figure 3.29: Cheese packed in vacuum in small bags for different analyses.

3.4 Analysis

Different analyses were performed for both treatment and control to determine differences in the quality of cheddar cheese containing γ-Oryzanol such as: color, texture, microbial growth, aroma development, bacterial identification by polymerase chain reaction (PCR), oryzanol retention, moisture content, free fatty acid profile and at the end of maturation, a consumer study.

3.4.1 Color

The color spectrum is a combination of different parameters which are visualized in Figure 3.30, where L* is lightness, a* is redness and b* is yellowness.

In a 2 dimension form, the color spectrum can be seen in Figure 3.31, where a* and b* were defined above and the hue angle (H°) equals $\tan^{-1}$ (b*/a*).

The L*, a* and b* parameters express the color on the basis of luminance, which is the descriptor of color not visible to human eyes. a* and b* are chroma coordinates, c* is the derived quantities saturation (chroma) defined as a right triangle $(a^{*2}+b^{*2})^{1/2}$. L* may have

values between 0 and 100, a* and b* may have values between around -80 and +80 (Berger-Schunn, 1994) but usually are between -60 and +60. The negative values of a* and b* show the greenness and blueness of the sample, respectively.

The vocabulary used for color analysis is the internationally accepted nomenclature of the CIE (Commission Internacionale de l'Eclairage; International Commission on Illumination).

The L*, a*, b* (CIELAB) color space and the color differences (ΔE) that result from this color space are described with the following equation (coordinates from standardized color spaces and the resulting color differences are marked with an asterisk):

$$\Delta E^* = [(\Delta L^*)^2 + (\Delta a^*)^2 + (\Delta b^*)^2]^{1/2}$$

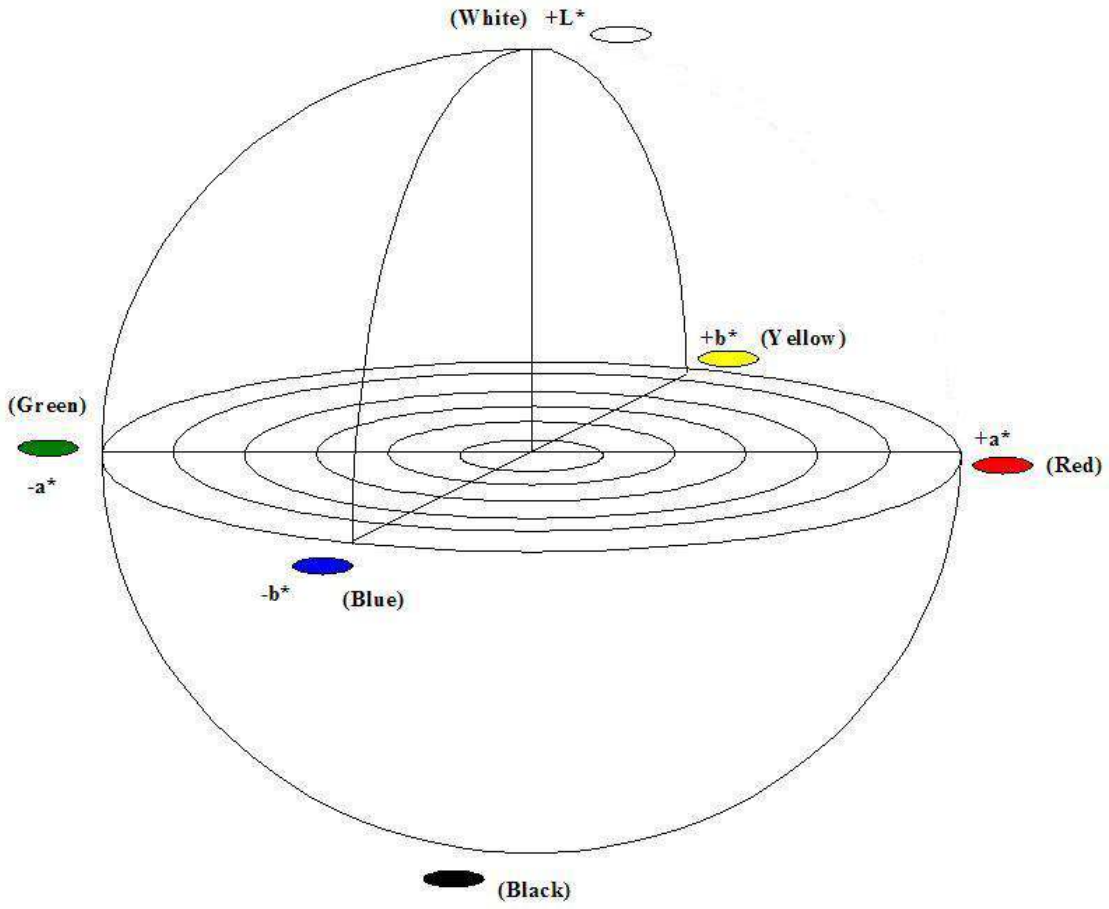

Figure 3.30: 3-D color spectrum.

The ΔE* is the distance between two samples in a visual uniform color space corresponding to the color difference between the two samples. (Δ is the mathematical symbol for difference). In the calculation of color difference, ΔE, with the help of all color difference formulas, the values of the standard always are subtracted from the values of the sample.

The color of the cheese was measured with a spectrophotometer Minolta model CM-508d Series (Osaka, Japan) with a 10° standard observer and D_{65} illuminant. The following parameters were recorded from the machine: L*, a*, b*, c* and H°. The spectrophotometer was calibrated to white with a standard supplied by the company each time a different replication was analyzed.

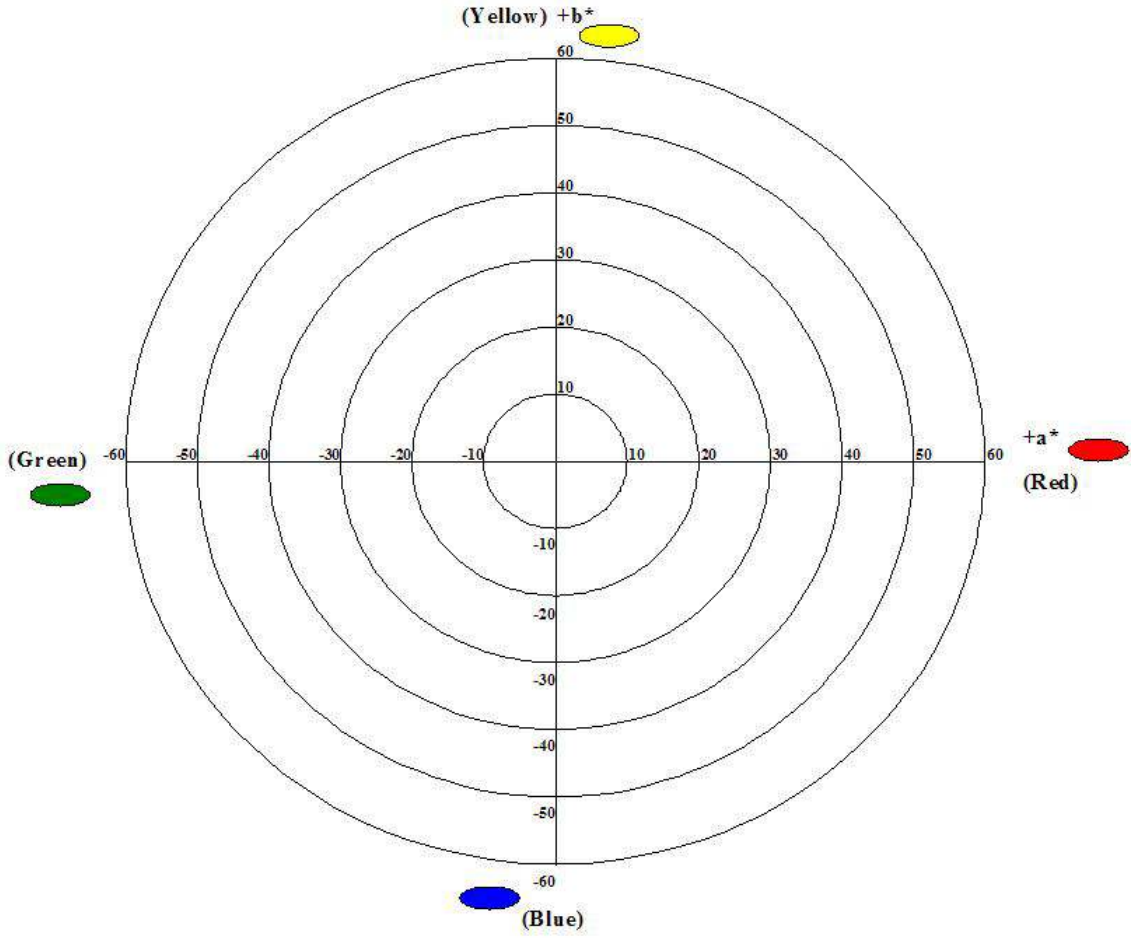

Figure 3.31: 2-D color spectrum.

Cheese was cut in cubes of 2cm by 2cm, allowed to cool down to room temperature and then color-measured by the spectrophotometer. Twelve different cubes were analyzed for each replication of each sample.

3.4.2 Texture

Texture profile analysis (TPA) imitates the grinding action of the jaw; it is performed by subjecting a specimen to a two-step compression. The first compression step, known as the "first bite", is followed by a second compression, the "second bite" (Figure 3.32). This is to simulate the first two bites taken during chewing of the food (Gunasekaran and Mehmet, 2002). The two compression steps may be separated by an optional wait time.

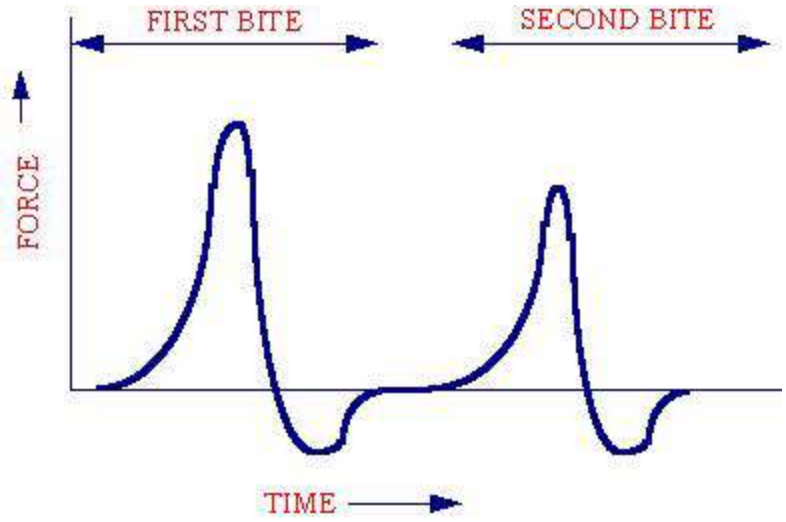

Figure 3.32: Two bite compression test as performed in a typical texture profile analysis (www.texturetechnologies.com/texture_profile_analysis.html).

A typical TPA test performed using a UTM (Universal Testing Machines) would generate a force-time profile as shown in Figure 3.33.

The many textural parameters determined from the TPA force-time (or deformation) curve are: hardness, cohesiveness, adhesiveness, chewiness, gumminess, springiness, and fracturability.

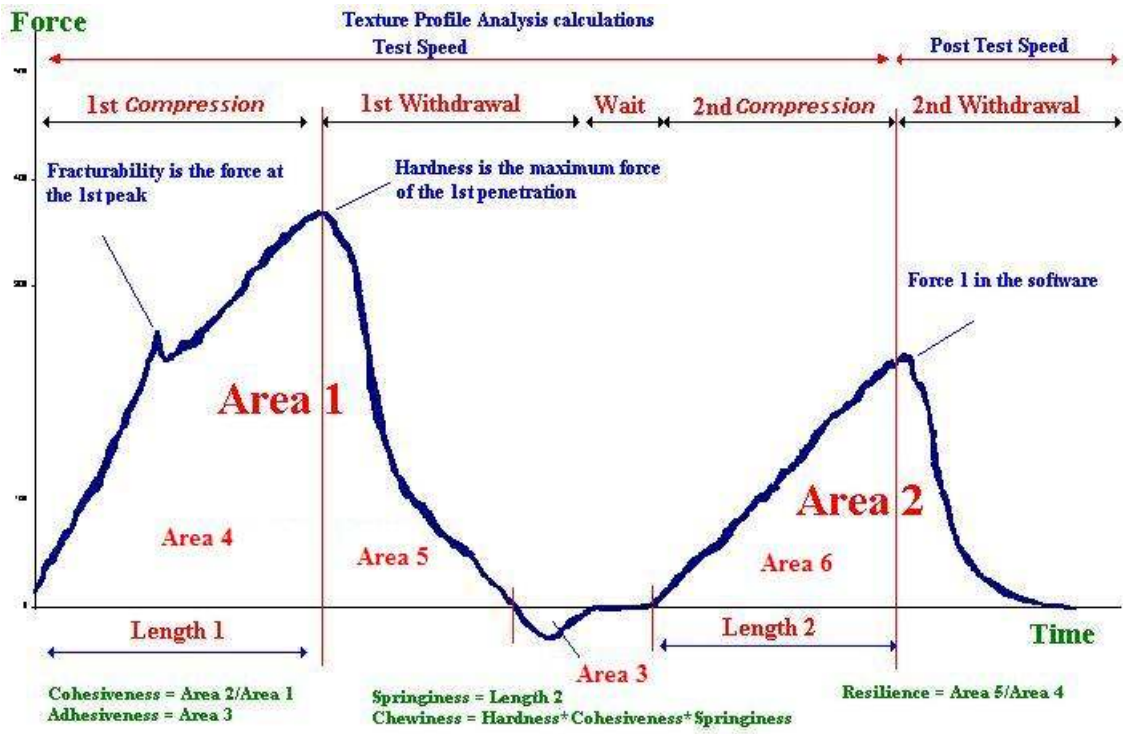

Figure 3.33: Schematic of a typical two-bite texture profile analysis force-time curve (www.texturetechnologies.com/texture_profile_analysis.html).

Hardness (N) is the force necessary to attain a given deformation. Fracturability (N) is the force at significant break in the curve on the first bite (originally known as "brittleness"). Cohesiveness (no units) is the strength of the internal bonds making up the body of the product (it can be Area 2/Area 1 or also Area 6/Area 4 from Figure 3.33). Adhesiveness (J) is the work necessary to overcome the attractive forces between the surface of the food and the surface of other materials with which the food comes in to contact. Gumminess (N) is the energy needed to disintegrate a semisolid food until it is ready for swallowing. Chewiness (J) is the energy needed to chew a solid food until it is ready for swallowing. Springiness (m) is the distance recovered by the sample during the time between the end of first bite and the start of second bite (originally known as "elasticity" – rate at which a deformed material goes back to its undeformed condition after the deforming force is removed). Resilience (no units) is the measure of how well a product "fights to regain its original position" (Gunasekaran and Mehmet, 2002).

For the TPA of the cheddar cheese, gumminess is not used since cheese is a solid and not a semisolid even that gumminess may be a better term than chewiness for cheese and other semisolid foods (Lee *et al.*, 1978). Also, for most cheeses and other soft foods, fracturability is either unidentifiable or not meaningful (Gunasekaran and Mehmet, 2002).

The TPA of the cheese was done with a texture analyzer Stable Micro Systems model TA.XT.plus (Texture Technologies Corp., New York) using a cylindrical probe TA-30 compression platen having a diameter of 3 inches and a TA-90A flat plate with a TA-90 heavy duty platform. A calibration of height and weight was performed every day the machine was used with the following values: height of 25mm, speed of 2 mm/sec and weight of 5 g for the height calibration, and a standard of 2Kg for the weight calibration.

The TPA configuration parameters were as follows: pre-test, test and post test speed of 2 mm/sec, compression by distance of 16mm (80% compression), a trigger system using force of 5g, and a waiting time between cycles of 5 seconds.

Cheese cubes of uniform 2cm by 2cm size and shape were analyzed by the texture analyzer to obtain the TPA. All cubes were in the same position and direction on the platform. Twelve different cubes were analyzed for each replication of each sample. After each cheese cube was analyzed, the probe and the base of the machine were cleaned to remove residues.

The TA.XT.plus was equipped with the texture exponent software which provides several textural parameters such as: hardness, fracturability, cohesiveness, adhesiveness, gumminess, chewiness, springiness and resilience; all of these parameters were determined from generated curves, similar to the one presented in the Figure 3.33, for each of the twelvecheese cube analyzed.

3.4.3 Microbial Growth

The basis for the microbial plate count is to dilute the bacteria to a certain level and then trap them in or on a solid medium where the individual cells will divide and produce macroscopic colonies, which can be counted through the transparent plate and medium (Goff *et al.*, 2003). A very well known nomenclature for bacteria count is a Colony Forming Unit (CFU).

Decimal dilutions (10^{-1} to 10^{-8}) were made from an original solution of 11g of cheese in 99ml of PBS (phosphate-buffered saline) solution which was then put in a stomacher (Seward model stomacher 80, Seward, England) at high speed for 120 seconds. The samples were decimally diluted and plated on to 3M™ Petrifilm™ (3M Microbiology Products, St. Paul, MN). The following analyses were performed: total aerobic count, *E. coli* and coliforms, and yeasts and molds. The 3M™ Petrifilm™ for total aerobic plate count, and *E. coli* and coliform plates were incubated for 48 hours at 37°C, and yeasts and molds plates were incubated for 72 hours at 30°C.

By convention it is determined that a plate containing 25 to 250 colonies is ideal for counting. The cheese must be sufficiently diluted to allow one plate in the dilution sequence to contain 25 to 250 colonies. The bacteria were counted in a Quebec Darkfield colony counter (Reichert-Jung, Buffalo, NY). And the results of these counts were presented in logarithmic numbers of CFU per gram that were calculated from the following equation:

$$\log \text{CFU/g} = \log (\text{Initial dilution} \times \text{Subsequent dilution} \times \text{Volume plated})^{-1}$$

3.4.4 Polymerase Chain Reaction

Polymerase chain reaction (PCR) is a common method of replicating specific fragments of DNA which rapidly amplifies a single DNA molecule into many billions of molecules. The PCR can be visualized in Figure 3.34.

PCR is a useful tool to identify the presence of specific microorganisms due to its specificity to certain DNA sequences. In this study, PCR was performed in order to determine the effects of γ-Oryzanol in the starter culture of cheddar cheese which was a mixture of *Lactococcus lactis* subsp. *cremoris* and subsp. *lactis*.

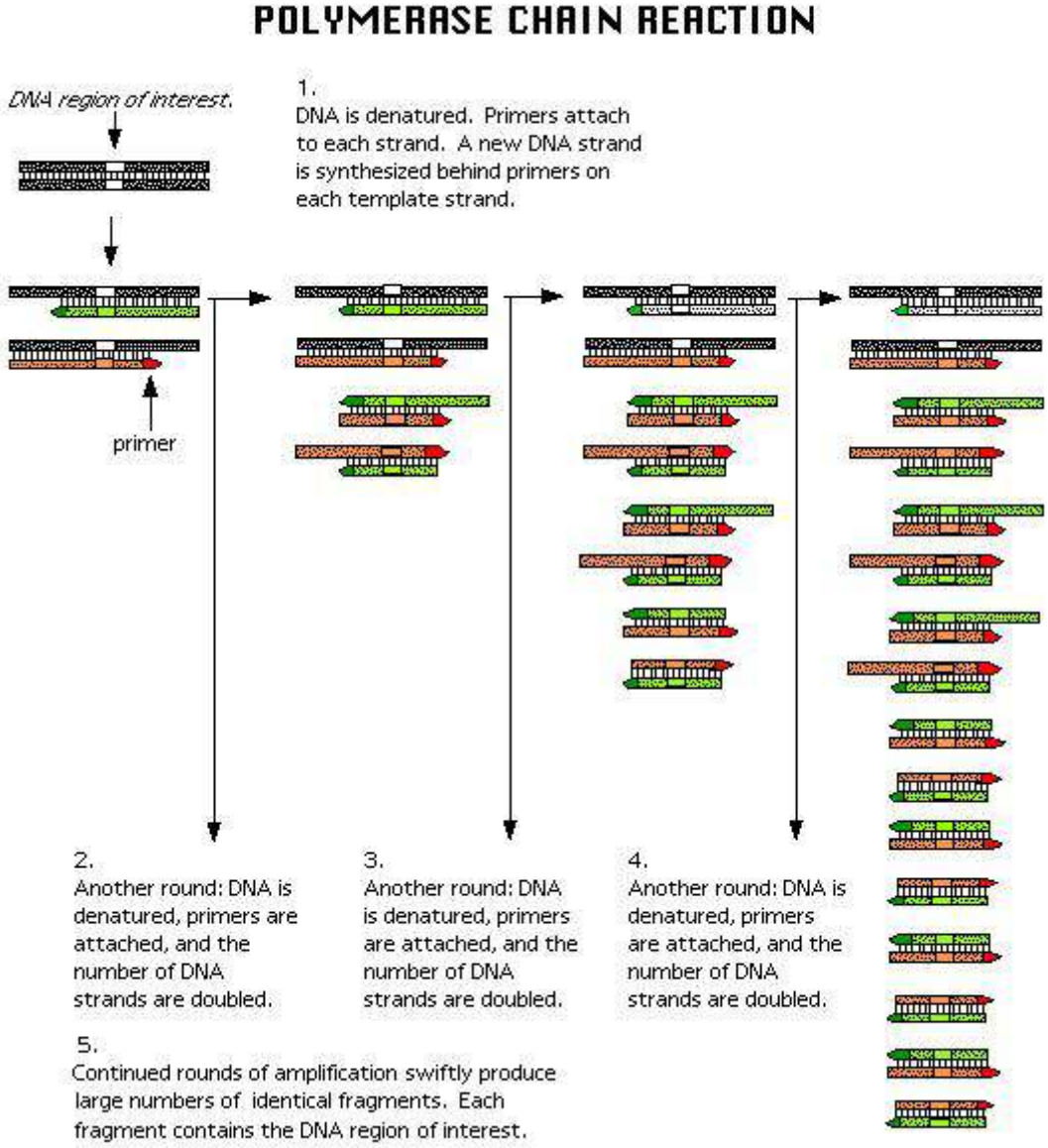

Figure 3.34: PCR (www.accessexcellence.org/AB/GG/polymerase.html)

Bacteria of the genus *Lactococcus* are used world-wide for the manufacture of fermented dairy products. Of particular importance are the two *Lactococcus* subspecies *lactis* and

cremoris. It is necessary to differentiate *L. lactis* strains as either *lactis* or *cremoris* because of their different characteristics in cheese manufacturing. Recently, comparison of DNA sequence data has shown genetic differences between the two subspecies (Ward *et al.*, 1998).

The method used in the cheddar cheese containing γ-Oryzanol to identify and differentiate both strains relied on the PCR amplification of a 340-bp region from within the 16S rRNA sequence using primers Y1 and Y2 (Young *et al.*, 1991) which anneal to highly conserved regions of the 16S rRNA sequence. This amplified region contains the sequence differences reported between *lactis* and *cremoris* (Salama *et al.*, 1991).

First, using the PCR amplification product, the ligase chain reaction (LCR) was used to differentiate a single base pair difference between the *lactis* and *cremoris* sequences. Secondly, differences in restriction endonuclease digest patterns of the PCR amplification product were used to differentiate between the two subspecies.

The procedure to extract the bacteria from the cheese was as follows: 11g of shredded cheddar cheese (after 4 month-storage) in 99ml PBS (phosphate-buffered saline) in dilution 1/10 was put in a stomacher Seward model stomacher 80 (Seward, England) at a high speed for 120 seconds. A part of the resulting mixture was passed through cheesecloth to filter cheese particles. One ml of the solution was passed through cheesecloth then centrifuged in eppendorf tubes in a Eppendorf centrifuge model 5415C (Eppendorf-Netheler-Hinz GmbH, Hamburg, Germany) at 3000 RPM for 3 minutes. The liquid was kept, the solid was discarded and the liquid part was centrifuged again at 3000 RPM for another 3 minutes. The liquid part was kept and centrifuged again at 10000 RPM for 3 minutes. The liquid part was discarded and the solid part was resuspended by vortexing in 0.5ml of PBS until total dissolution; this part was repeated

two more times. The final pellet was resuspended in 500µl of deionized water. Then the sample was heated for 5 minutes at 95°C and put in ice or frozen until performing the amplification.

The GeneLab PCR primers Y1 and Y2 (BioMMED, Baton Rouge, LA) used to amplify the 348-bp fragment from the 16S rRNA gene had the following DNA sequence: 5'-TGG CTC AGG ACG AAC GCT GGC GGC-3' and 5'-CCT ACT GCT GCC TCC CGT AGG AGT-3' respectively (Ward *et al.*, 1998). Each symbol in the sequence stands for a primary α-amino acid, A is alanine, C is cysteine, G is glycine and T is threonine (Fennema, 1996).

Before the amplification started, the primers Y1 (546µM) and Y2 (623µM) diluted at a ratio of 1:20 with deionized water. Next the amplification of the samples is described. In PCR tubes, 25µl of Taq PCR master mix kit (Qiagen GmbH, Hilden, Germany), 5µl of sample, 3µl of each primer and 14µl of water were mixed by vortexing to make a solution of 50µl.

This solution was subjected to an amplification in a Perkin Elmer gene amplifier model PCR system 2400 (Perkin-Elmer, Wellesley, MA) to one cycle of 94°C for 5 minutes and 60°C for 4 minutes followed by 24 cycles of 94°C for 1 minute and 60°C for 2 minutes plus 72°C for 2 minutes and kept at 4°C after the amplification was over.

The amplified Y1-Y2 product was digested with restriction endonucleases *MboII* as recommended by the manufacturers (New England BioLabs, Beverly, MA). For this digestion, 25µl of the amplified product was mixed with 1µl of *MboII* (5000units/ml) and 24µl of NE buffer 2 (10X concentrate) from New England BioLabs and incubated for 1 hour at 37°C.

The resulting digestion products were separated in a 1% agarose gel (by electrophoresis) for 2.5 hours and run together with non-digested products in order to determine the presence of both *lactis* subspecies. A 5X nucleic acid sample loading buffer (Bio-Rad Laboratories, Richmond, CA) was mixed in amount of 2µl with 8µl of the amplified product to be separated to

get a total of 10µl; each lane of the gel was loaded with 5µl of this mixture from different samples. In one of the lanes, 5µl of a EZ load™ 100-bp molecular ruler of 500µg/ml (Bio-Rad Laboratories, Richmond, CA) was loaded.

The 1% agarose gel was prepared by dissolving 0.24g of agarose I (Amresco, Solon, OH) into 30ml of 1X TBE buffer, the mixture was brought to boil while stirring, allowed to cool for about 5 minutes and then poured into a tray for electrophoresis. The 1X TBE buffer is a 1:10 dilution of a 10X TBE buffer solution prepared with 218g of Tris base, 110g of Boric acid and 9.3g EDTA in 2L of deionized water at pH 8.3 using NaOH. The 1X TBE buffer had to be kept in refrigeration, and the 10X TBE buffer was kept at room temperature. The gel was run in a Pharmacia Biotech Electrophoresis Power Supply model EPS 3500 (Pharmacia Biotech, Sweden).

Immediately after the electrophoresis was completed, the DNA in the gel was stained with a diluted SYBR® green I nucleic acid gel stain, 10,000X concentrate in DMSO (Molecular probes, Eugene, OR). This diluted stain was prepared by dissolving 30µl of the concentrated stain in a volume of 300ml. The gel was immersed in the diluted stain and agitated for 15 minutes at 40 RPM and 25°C in a Forma Scientific Orbital Shaker.

The resulting stained gel was observed under the UV light with a Hoefer machine model MacroVue UVis-20 (Pharmacia Biotech Inc., San Francisco, CA) to identify bands which represent the presence of the *lactis* subspecies. Since the *cremoris* PCR product is only digested with *MBoII* and the Y1-Y2 amplified fragment from *lactis* is not digested with *MboII* (Ward *et al.*, 1998), it is expected to see in the gel two bands for the digested products, only one band for the non-digested ones; these bands in the gel will acquaint with the presence of each subspecies of *Lactococcus lactis*.

3.4.5 Aroma Development

A common challenge when analyzing flavors and aromas from complex mixtures involves finding the best method. Fortunately, most aroma chemicals are volatile, and procedures for their isolation from foods and flavors have been established. An ideal approach to flavor isolation and analysis would provide an analytical sample whose composition is identical to the chemical mixture within the matrix, which is free of solvents and other impurities and could be completed within a few minutes with no intermediate processing of the samples. Solid-phase microextraction (SPME) approaches this ideal.

Figure 3.35 describes the apparatus used to extract the volatiles from cheese. The bottom centimeter of the fused silica fiber is coated with a relatively thin film of any of several stationary phases. This film serves as the organic "solvent" during the absorption of the volatile compounds from the analytical matrix. The needle functions to puncture the septa sealing of both the sample container and the GC injection port and to protect the fragile fused silica fiber during storage and use. A Supelco fiber of 100μm PDMS (polydimethylsiloxane) for Merlin Microseal™ (Supelco, Bellefonte, PA) was used for the study of aroma development in cheese.

In a 50ml volumetric flask, one gram of shredded cheese was placed; then 1ml of distilled water was added, afterwards, 0.5ml of internal standard (4-methy-2-pentanone solution of 0.005μl/ml of hexane) was added. The mixture was thoroughly mixed for a few seconds (equilibration rate increases), sealed with the septum type cap and the fiber inserted through the septa. Immediately afterward, the flask was put in the water bath at 65°C for 20 minutes.

The SPME process is illustrated in Figure 3.36 which is similar to the one we used in the analysis of cheese. During headspace sampling the PDMS fiber is extended into the vapor phase above the sample. After 20 minutes, the fiber was taken out of the flask and injected into the

GC-MS Varian model Saturn 2200 with a gas chromatograph Varian model CP-3800 (Varian Chromatography Systems, Walnut Creek, CA) with FID (flame ionization detector) for separation and analysis of the volatiles.

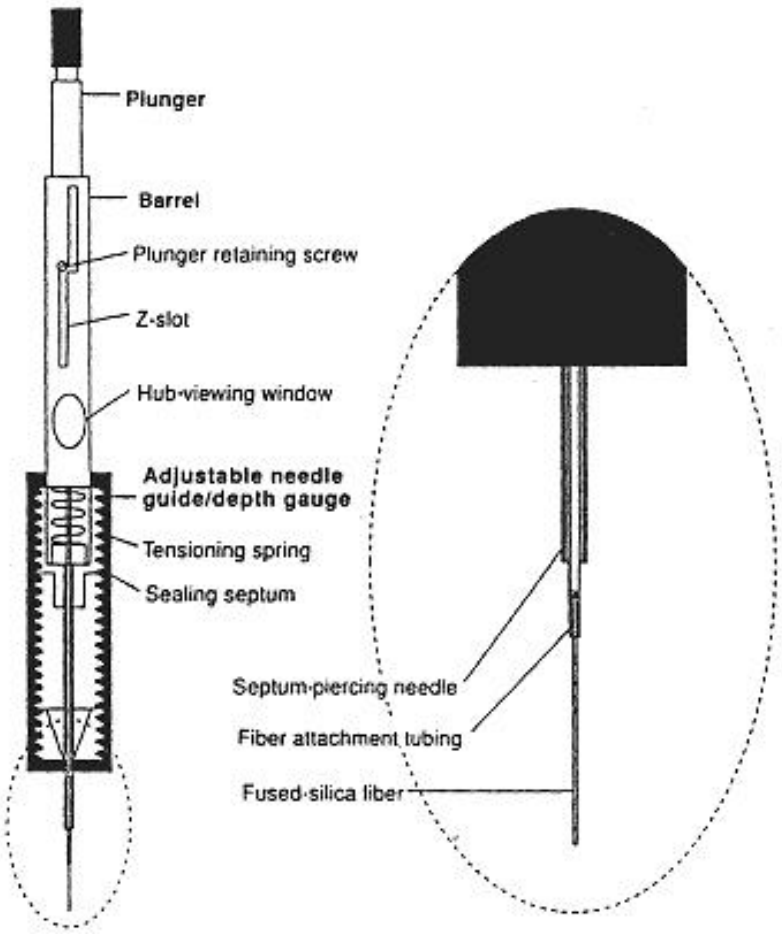

Figure 3.35: Graphic representation of a solid-phase microextraction (SPME) device (Zhang *et al.*, 1994).

The mobile phase of the system was helium at a flow rate of 0.8ml/min. The oven temperature started at 30°C and held for 2.5 minutes, then it was increased to 45°C at a rate of 2°C/min; as soon as the temperature reached 45°C, the increase rate was of 5°C per minute until the oven temperature reached 180°C. Then the temperature was increased to 200°C at a rate of

20°C per minute, and this temperature was held for 2 minutes to make a total analysis time of 40 minutes.

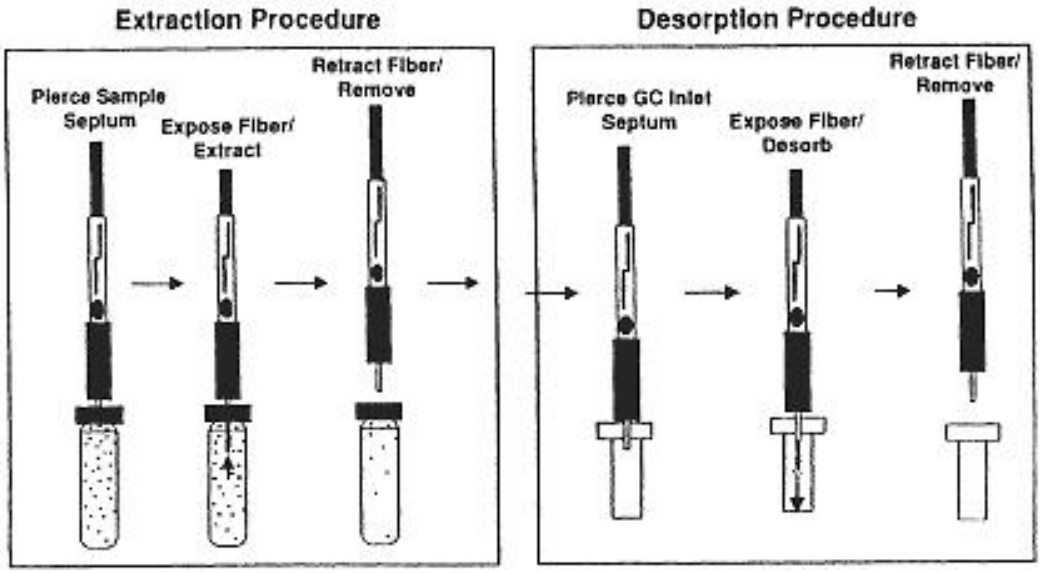

Figure 3.36: Sequence of events showing extraction steps and desorption (injection) steps followed to perform an analysis using SPME. The fiber is inserted directly into a liquid sample with the subsequent absorption of most of the analyte molecules (small circles) from the solution (Marsili, 1997).

The chromatograms obtained from the GC-MS helped to determine the most important aroma and flavor peaks in the samples and their changes during ripening, which were measured by their height (kilocounts) since the software available could not quantify the peaks.

3.4.6 Oryzanol Retention

Since γ-Oryzanol is fat soluble, the first part of the analysis was to extract the lipid fraction of the cheese. 5ml of a Chloroform-Methanol 2:1 solution (solvent) was added to a flask containing 1g of shredded cheese and then mixed in a vortex for several seconds (until total destruction of cheese texture). This mixture was heated and agitated constantly in a water bath at 40°C for 10 minutes, then mixed in a vortex again for a few seconds. The blend was then centrifuged at 2500 RPM and 20°C for 5 minutes.

The liquid part was put in another flask in a rotary evaporator under vacuum at 60°C to obtain crude oil (Xu *et al.*, 1999). The solid part was treated as follows: 5ml of hexane (solvent) was added and then mixed in a vortex for several seconds the new blend was centrifuged again at 2500 RPM and 20°C for 5 minutes, and the liquid part was separated from the solid. This whole process was repeated two more times.

Once the three extractions with hexane were done, the solid part was discarded, and after all the solvent was evaporated, the samples were prepared to be analyzed by HPLC. The extracted liquid which contained all the lipids (crude oil) from the cheese was dissolved in 5ml of hexane and put in vials for analysis.

The dissolved samples were injected into the HPLC system consisting of a Waters™ 486 tunable absorbance detector, a Waters™ 717plus autosampler and a Waters™ 470 scanning fluorescence detector for separation and analysis of γ-Oryzanol in the lipid extraction. The chromatograms obtained from the HPLC were used to determine the concentration of γ-Oryzanol retained in the cheese. The software calculated the area under the oryzanol peaks and its concentration was calculated using the following parameters:

The calibration curve between area under the peak and oryzanol content is:

$$\text{Peak area} = 123368 \times \text{oryzanol content } (\mu g)$$

Injection volume: 25µl of extracted lipid sample

Dilution factor: 5ml of hexane

Serving size of cheddar cheese: 28g

With these parameters mentioned above and making conversions between µg and mg, and µl and ml, the following equations were used to determine the concentration of oryzanol in a serving size of cheddar cheese (mg/serving size):

$$\text{Oryzanol content}(\mu g) = \frac{\text{Peak area}}{123368}$$

$$\text{Oryzanol} = \text{Oryzanol content}(\mu g) \times \frac{5ml \times 1000\mu l / ml}{25\mu l \times 1000\mu g / mg} \times \frac{28g}{\text{Sample weight}(g)}$$

3.4.7 Moisture Content

The moisture content was determined by means of a microwave oven which uses microwave energy to heat samples to remove moisture. Factors affecting the drying are time, sample size, placement of sample in oven, and energy of microwaves (Richardson, 1985).

The microwave oven used for this analysis was a CEM model Smart System 5 (CEM Corp., Matthews, NC) with the following parameters set: weight range of 2-4 grams, a power of 100%, delta weight of 0.5mg, delta time of 10 seconds, maximum time of 4 minutes, maximum temperature of 130°C and no weight compensation.

Shredded cheese (2 to 3 grams) was put between CEM square sample pads specific for this machine (part # 200150, CEM Corp., Matthews, NC) and then analyzed.

3.4.8 Free Fatty Acid Profile

The method used to analyze the free fatty acid profile of the cheese was similar to the one used for the determination of the oryzanol retention (section 3.4.6), however, with slight changes.

The first part of the analysis was to extract the lipid fraction of the cheese. 5ml of a Chloroform-Methanol 2:1 solution (solvent) was added to a flask containing 1g of shredded cheese and 1ml of a solution of internal standard consisting of 0.4652g of heptadecanoic acid (C17:0) in 50ml of hexane (C_6H_{14}). The mixture was mixed in a vortex for several seconds until total destruction of cheese texture. This mixture was heated and agitated constantly in a water bath at 40°C for 10 minutes, and then mixed in a vortex again for a few seconds. The blend was

then centrifuged at 2500 RPM and 20°C for 5 minutes in a Hermle Labnet centrifuge model Z383K (Hermle Labortechnik, Wehingen, Germany).

The liquid part was poured in another flask in a Labconco rotary evaporator model CentriVap Console under vacuum at 60°C to obtain crude oil (Xu *et al.*, 1999). The solid part was treated as follows: 5ml of hexane (solvent) was added and then mixed in a vortex for several seconds; the new blend was centrifuged again at 2500 RPM and 20°C for 5 minutes, and the liquid phase was separated from the solid. The evaporation process was performed two more times.

Once the three extractions with hexane were done, the solid part was discarded, and after all the solvent was evaporated, the samples were prepared to be analyzed by GC. The extracted liquid which contained all the lipids (crude oil) from the cheese was dissolved in 6ml of hexane together with 2ml of a solution of Boron trichloride (BCl_3) and methanol (Supelco, Bellefonte, PA) and 1ml of 2,2-dimethoxypropane (Sigma-Aldrich Co., St. Louis, MO). After agitating in vortex for a few seconds, the upper layer (hexane) was transferred to a test tube where a few crystals of sodium sulfate anhydrous ($NaSO_4$) were added to absorb moisture, and then the hexane extract was transferred to GC sample vials ready for analysis.

The hexane extract samples were injected into the gas chromatograph Hewlett Packard model 5890A (HP, San Fernando, CA) for separation and analysis of free fatty acids between C4 and C18 in the lipid extraction. The mobile phase of the system was Helium. The chromatograms obtained from the GC were used to help determining the concentration of the different fatty acids present in the cheese. The software calculated the areas under the peaks and their concentrations were calculated using the following parameters:

The relationship between peak areas and concentrations (conc.) of a free fatty acid (FFA) and internal standard (I.S.) is:

$$\frac{\text{Area FFA}}{\text{Area I.S.}} = \frac{\text{FFA conc.}}{\text{I.S. conc.}}$$

Injection volume: 3µl of extracted lipid sample

Dilution factor: 6ml of hexane

I.S. concentration: 0.4652g/50ml hexane

I.S. solution volume: 1ml

With these parameters mentioned above and making conversions between µg and mg, grams and Kg, and µl and ml, the following equations were used to determine the concentration of each free fatty acid in the cheese (mg/Kg of cheese):

$$\text{I.S. conc.}(\text{mg}/\text{Kg}) = \frac{0.4652\text{g} \times 1\text{ml} \times 3\mu\text{l} \times 1000\text{mg}/\text{g} \times 1000\text{g}/\text{Kg}}{50\text{ml} \times 6\text{ml} \times 1000\mu\text{l}/\text{ml} \times \text{Sample weight(g)}}$$

$$\text{FFA conc.}(\text{mg}/\text{Kg}) = \text{I.S. conc.}(\text{mg}/\text{Kg}) \times \frac{\text{Area FFA}}{\text{Area I.S.}}$$

The results for the free fatty acid profile were expressed in percentage based on the total amount of each FFA from C4 to C18, and the total amount of all FFA together.

3.5 Consumer Study

With the aim of understanding prospective buyers of a specific product and how this product can be introduced into the market, the use of sensory affective tests can assist food scientists and developers to understand the behavior of different consumers groups (Piggot, 1988). This type of affective test is a very accurate tool in recognizing consumer preferences.

Quantitative affective tests determine responses of a large group of consumers to a set of questions regarding preference, liking, sensory attributes, etc. (Meilgaard *et al.*, 1999). They obtain more data and information in less time than do qualitative tests.

The hedonic scale is a common tool to quantify consumer acceptance. The 9-point hedonic scale is a rating scale that has been used for many years in sensory evaluation in the food industry to determine the acceptance of a food and to provide a bench mark for comparison. Its use has been validated in the scientific literature (Stone *et al.*, 1993). The number of scale categories that have been used include the 5-, 7- and 9-point scale. The 3-point scale is not recommended for use with adult consumers, because adults tend to avoid using the extreme points of the scale in rating food product samples (Resurreccion, 1998).

Overall appearance includes all visible sensory attributes such as color, size, shape and surface texture. Appearance is commonly used by consumers to infer food product quality; frequently this is the only cue available, especially at the moment of purchase (Schröder, 2003). Flavor involves attributes like taste, specific flavors, aroma, etc. Aroma is the odor of a food resulting from the process that involves the passing of volatiles through the nasal passages located in the nose when a person inhales them (Meilgaard *et al.*, 1999). Overall liking can be defined as a complex expression of liking of the product as a whole (Pavon, 2003).

In order to find if consumers can detect overall difference and/or difference in a specific attribute(s) between two or more samples, discriminative sensory testing can be utilized. The applications of these tests are to determine if products differ due to changes in ingredients, processing, storage, etc., if an overall difference exists where no specific attribute(s) can be identified as having been affected, if a specific attribute(s) of the products differ or if the consumer is able to discriminate between test samples.

The traditional R-Index method and the bipolar and/or weighted R-Index method are discriminatory sensory tests from which the researcher can determine if panelists can detect differences in specific attributes between samples. Both methods present a signal (S) sample that is the new, reformulated, or improved product and the noise (N) sample which is the current, existing, or control product. The panelists' task is to indicate if he/she is sure or not sure that samples are different or the same. They can also indicate if the samples are more or less intense in specific attributes. The R-Index is a probability value of a given judge distinguishing correctly between 2 samples.

Research was conducted including four parts, a consent form (see Appendix B), a demographic and socioeconomic survey, an affective test, and the R-index with the traditional method and the bipolar method (see Appendix C). The consent form included some general explanation of the questionnaire to be filled by the panelist, stating the presence of potential allergens, contact information in case of interest in further knowledge about the topic and the purpose of the study; the demographic and socioeconomic survey asking age, gender, race, level of education and household income; the affective test included the rating of the overall appearance, smoothness of color (yellowness), odor/aroma, taste, overall texture/mouthfeel and overall liking with a 9-point hedonic scale (1=dislike extremely, 5=neither like nor dislike, and 9=like extremely) (Peryam *et al.*, 1957). The affective test asked consumers to evaluate the detection of a bitter aftertaste, the acceptance and the interest in buying a cheddar cheese containing γ-Oryzanol, an antioxidant and potential cholesterol reducing compound from rice bran using a yes/no scale (Moskowitz, 1994). The R-Index included the overall appearance, overall color and yellowness for the visual evaluation, odor for the aroma evaluation, taste, overall texture, hardness and chewiness for the gustatory evaluation. The yellowness, hardness

and chewiness were evaluated as a bipolar R-index, the rest were treated as the traditional R-Index.

Untrained consumers (n=100) were randomly recruited from Louisiana State University (LSU) campus and vicinities in Baton Rouge. The recruitment consisted of flyers, phone calls, emails and posting advertisements on LSU campus. Requirements to be a panelist were: (1) to be at least 18 years old, (2) not having allergic reactions to cheese and rice products and (3) positive attitude with disposition of time to complete a questionnaire. The consumer session was carried out on May 29th, 2004, between 10 a.m. and 4 p.m. in the dairy store of the Dairy Science department in LSU.

Consumers were instructed on the procedure to be followed. They were informed that each sample was randomly coded with a 3-digit number, 705 for control and 485 for treatment. They were familiarized with the (S) signal and (N) noise samples, and asked whether they were sure or not sure that samples were different or the same. They were also asked whether they were sure or not sure that samples were more or less intense in specific attributes.

The sample preparation consisted of trimming the outer portion of the block cheese (Figure 3.37). Samples were not allowed to dry out by wrapping them with plastic to prevent loss of moisture, and they were kept refrigerated. Samples were cut to 2cm cubes with a cheese wire (Figure 3.38). Although cheese should be stored in the refrigerator, cheese was removed to allow enough time to equilibrate twenty to thirty minutes before being served to the consumers at ambient temperature (Resurreccion, 1998).

The samples were served to the seated panelists on a tray with a cup of drinking water at room temperature, unsalted crackers, toothstick and a napkin as shown in Figure 3.39, in a room with controlled light, positive airflow and free of distracting odors, together with an explanation

of the procedure to follow and the questionnaire including the four parts mentioned above in this section.

Figure 3.37: Trimming outer part of block cheese.

Figure 3.38: Cutting cheese samples to 2cm-cubes with a cheese wire.

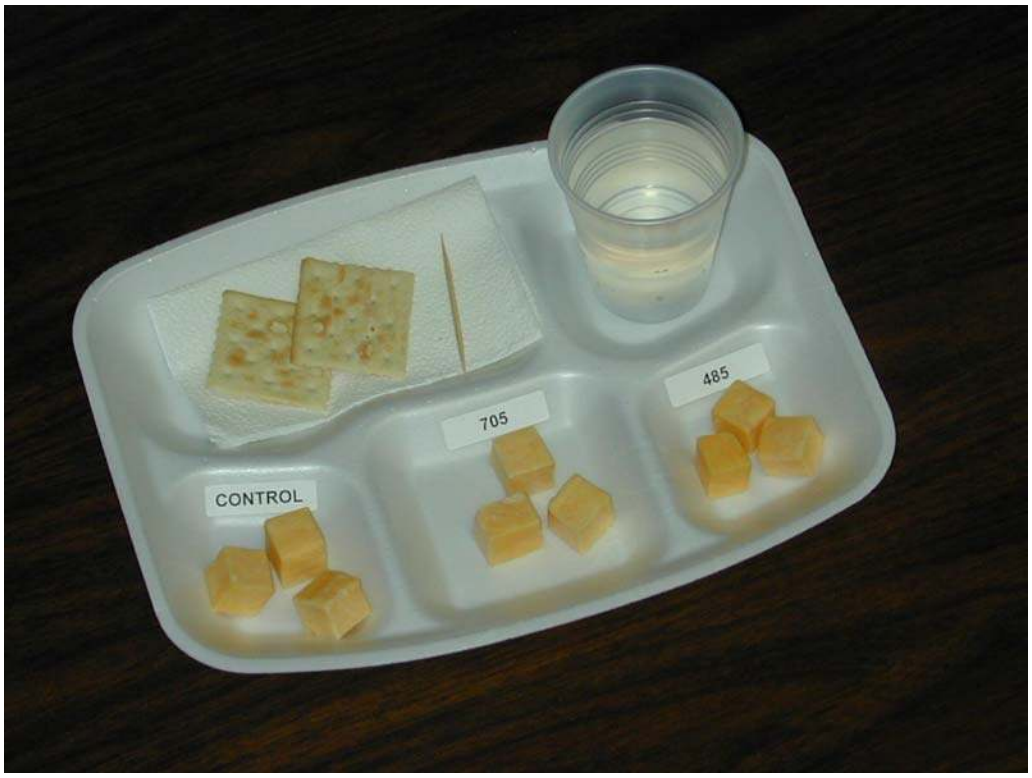

Figure 3.39: Sample presentation for consumer study.

3.6 Statistical Analysis

3.6.1 Physicochemical and Biological Analysis

The statistical analysis of color, texture, microbial growth, aroma development, oryzanol retention, moisture content and free fatty acid profile was performed using the software SAS version 9.00, 2002 (SAS Institute Inc., Cary, NC) (SAS codes are in Appendix D).

The GLM or ANOVA test was used to test the null hypothesis H_0: $\mu_{control} = \mu_{treatment}$ with an alternative hypothesis H_1: $\mu_{control} \neq \mu_{treatment}$, and Tukey's honestly significant difference (HSD) test with the studentized range was applied to determine significant differences in each sample (control and treatment analyzed separately) within different ripening times. The significance level (α) for this test was set at 0.05.

A two-sample t-test was used for the null hypothesis H_0: $\mu_{control} - \mu_{treatment} = 0$ and an alternative hypothesis H_1: $\mu_{control} - \mu_{treatment} \neq 0$ to determine if there were significant differences

between samples (control and treatment). This test, by the F test of variances with a significance level (α) of 0.2, judged if two estimates of variance from different populations could be combined; pooling variances together turns into a single more powerful variance and the following assumptions are taken: the differences are normally distributed, the observations and samples are independent and the variance is homogenous (Freund *et al.*, 1997). Whether the variances can be pooled together or not, SAS gives probabilities for equal and unequal variances. The second part of the two-sample t-test uses a significance level (α) of 0.05 for the t-test which determine significant differences found between samples using the hypotheses mentioned at the beginning of this paragraph.

3.6.2 Sensory Evaluation

3.6.2.1 R-Index

To determine if an overall difference exists in the cheese containing γ-Oryzanol and whether the cheese differs due to the presence of γ-Oryzanol, the discriminative sensory test, i.e., R-Index was used (traditional and bipolar tests). The traditional R-Index indicates the degree of difference between the S (signal) and N (noise) samples; a higher R-Index value indicates better discrimination.

For the traditional R-Index, the data obtained from the questionnaire were: consumer was certain that the sample was the treatment (S), that perhaps the sample was the treatment but not sure (S?), that perhaps the sample was the control but not sure (N?) or that definitely the sample was the control (N). With this data tabulated, the Table 3.1 was built.

From data tabulated as shown in Table 3.1, the traditional R-Index in percentage could be calculated with the following equation:

$$\text{R} - \text{Index} = \frac{\left[\text{a} \times (\text{f} + \text{g} + \text{h}) + \text{b} \times (\text{g} + \text{h}) + \text{ch}\right] + \left[0.5 \times \left(\text{ae} + \text{bf} + \text{cg} + \text{dh}\right)\right]}{\left(\text{a} + \text{b} + \text{c} + \text{d}\right) \times \left(\text{e} + \text{f} + \text{g} + \text{h}\right)} \times 100$$

Table 3.1: Tabulated information needed to calculate the traditional R-Index.

Sample (code)	Consumer's responses				Total
	S	S?	N?	N	
S (485)	a	b	c	d	n_s=a+b+c+d
N (705)	e	f	g	h	n_N=e+f+g+h

S (485) = cheese containing γ-Oryzanol; N (705) = cheese without γ-Oryzanol; S= different sure; S?= different unsure; N?= same unsure; N= same sure.

The Table with critical values of R-Index expressed in % of R-Index-50% (Bi *et al.*, 1995) was used to determine the critical R-Index value by using the number of signal or noise samples (N) to obtain the R-Index in the equation above and the significance level (α) for a 1-tailed or 2-tailed test. If the researcher knows that the signal can only be stronger or weaker than the noise then the test is 1-tailed, otherwise it is a 2-tailed test. For the traditional R-Index, the calculations were made based on a 2-tailed test since assumptions were not made about differences between samples. The null hypothesis (H_0: the %R-Index is equal to a chance (50%)) tells whether the consumer detected a difference in the samples by guessing or by chance.

If the R-Index value obtained from the equation above was equal to or greater than the critical value, then the consumer noticed a difference between samples not by chance. If this value was smaller than the critical value, then the consumer either noticed a difference between samples most probably by chance or could not differentiate the samples.

For the bipolar R-Index, the data obtained from the questionnaire was: in one part, that the consumer was certain the sample had more intensity of the attribute than the control (S+), that perhaps the sample had more intensity of the attribute than the control (S+?), that perhaps the sample had the same intensity of the attribute than the control but not sure (N?) or that definitely the sample had the same intensity than the control (N); in the other part, that the

consumer was certain the sample had less intensity of the attribute than the control (S-), that perhaps the sample had less intensity of the attribute than the control (S-?), that perhaps the sample had the same intensity of the attribute than the control but not sure (N?) or that definitely the sample had the same intensity than the control (N). With this data tabulated, tables like Table 3.2 and 3.3 could be built.

From data tabulated as shown in the Tables 3.2 and 3.3, the bipolar R-Indices (R_{more} and R_{less}) could be calculated with the same equation used for the traditional R-Index. The critical R-Index value for the bipolar method was calculated the same way as the traditional R-Index was done with the only change that it was a 1-tailed test.

Table 3.2: Tabulated information needed to calculate the bipolar R-Index (R_{more}).

Sample (code)	**Consumer's responses**				**Total**
	S+	**S+?**	**N?**	**N**	
S (485)	a	b	c	d	n_s=a+b+c+d
N (705)	e	f	g	h	n_N=e+f+g+h

S (485) = cheese containing γ-Oryzanol; N (705) = cheese without γ-Oryzanol; S+= more sure; S+?= more unsure; N?= same unsure; N= same sure.

Table 3.3: Tabulated information needed to calculate the bipolar R-Index (R_{less}).

Sample (code)	**Consumer's responses**				**Total**
	S-	**S-?**	**N?**	**N**	
S (485)	a	b	c	d	n_s=a+b+c+d
N (705)	e	f	g	h	n_N=e+f+g+h

S (485) = cheese containing γ-Oryzanol; N (705) = cheese without γ-Oryzanol; S-= less sure; S-?= less unsure; N?= same unsure; N= same sure.

The decision of taking R_{more} or R_{less} as the real R-Index value was based on the frequency of repetition of S+ and S+?, and S- and S-?. The ones with the highest proportion would be the R-Index value taken. If there was not a big difference in the frequency of repetition of S+ and S+?, and S- and S-?, either R-Indices values could be taken, or the calculations could be done as a traditional R-Index method, like mentioned above.

3.6.2.2 The McNemar Test

With the intention of analyzing the change in probability of purchase intent before and after consumers had been informed about the potential health benefits of the cheese, the McNemar test was performed using proc freq/agree of software SAS version 9.00, 2002 (SAS Institute Inc., Cary, NC) (code is in Appendix D).

The McNemar test represents a comparison of dependent proportions for binary response variables, and it is a two-related sample difference test that follows a Chi-square (χ^2) distribution with a degree of freedom (df) of one (Agresti, 1996). Consumers are categorized in two categories: before they have been informed about health benefits of cheese containing γ-Oryzanol and after they have been informed about the health benefits (O'Mahony, 1986).

The null hypothesis (H_0: $\pi_{+1} = \pi_{1+}$) states that there is no significant difference in the probability of buying the cheese before and after consumers had been informed about its health benefits ($\pi_{+}1$ is the probability of those who answered yes after, and π_{1+} is the probability of those who answered yes before). The aim of this test is to know if participants were influenced or not by the health benefits of this product and, therefore, to determine if their opinions changed from a "before" status to an "after" status.

To complement McNemar test and obtain more detailed understanding about changes in consumer purchase decision, a 95% confidence interval for the difference of proportions was

calculated. The difference of two sample marginal proportions (p_{+1} - p_{1+}) estimates the true difference (π_{+1} - π_{1+} or $\pi_{21} - \pi_{12}$). The sample proportions were calculated using the next equation:

$$p_{ij} = n_{ij}/N$$

where n_{ij} is the number of subjects making response i at the first question (before), and response j at the second question (after knowing that the product contained health promoting ingredients), and N is the total number of responses. The confidence interval of difference of proportions is calculated as follows:

$$(p_{+1} - p_{1+}) \pm z_{\alpha/2}(\text{ASE})$$

where (p_{+1} - p_{1+}) indicates the difference between the proportion of participants that answered "yes" after knowing that the cheese contained health promoting compounds p_{+1}, and the proportion of participants that answered "yes" before knowing that the product contained health promoting ingredients (p_{1+}); the $z_{\alpha/2}$ denotes the standard normal percentile having a right-tail probability equal to α/2; ASE is the estimated standard error for the proportion difference.

ASE is calculated with the following equation:

$$\text{ASE} = \sqrt{\frac{p_{1+} \times (1 - p_{1+}) + p_{+1} \times (1 - p_{+1}) - 2 \times (p_{11} \times p_{22} - p_{12} \times p_{21})}{N}}$$

where p_{11} is the proportion of subjects that answered "yes" before knowing and "yes" after knowing, p_{22} is the proportion of subjects that answered "no" before knowing and "no" after knowing, p_{12} is the proportion of subjects that answered "yes" before knowing and "no" after knowing, and p_{21} is the proportion of subjects that answered "no" before knowing and "yes" after knowing. When 0 is included in the confidence interval, then there is no significant difference.

3.6.2.3 Consumer Acceptance Test

Univariate and multivariate statistical analyses were performed to determine consumers' perceptions and preferences in the acceptability of each different sensory attribute as well as in overall liking of the cheese samples by using the software SAS version 9.00, 2002 (SAS Institute Inc., Cary, NC) (codes are in Appendix D).

An analysis of variance (ANOVA) was used to determine differences in acceptability for each of the sensory attributes among the samples, and Tukey's honestly significant difference (HSD) test with studentized range, which can be applied regardless of whether the overall test for differences is significant among the samples (Meilgaard et al., 1999), was applied to determine if the samples were significantly different. The significance level (α) for this test was set at 0.05.

A multivariate analysis of variance (MANOVA) was performed as an extension of the ANOVA test. However, more than one variable is tested to detect differences in groups across multiple dependent variables at the same time (Pavon, 2003). MANOVA can determine overall differences in the acceptability among the samples, including all sensory attributes simultaneously.

Descriptive discriminant analysis (DDA) was done to identify any discriminating sensory acceptability attributes that may have contributed to differences among samples and to the cheese in general.

Logistic regression models are useful to describe the effect of predictors (independent variables) on a binary dichotomous response variable (dependent variable), which follows an S-shaped curve (Agresti, 1996). Logistic regression was used to predict acceptance by using what is known as the odds ratio estimate.

When an estimated odds ratio equals 1.0, it means that there is no significant association between the two variables (Agresti, 1996). To be precise, the probability of success remains constant through the change of the independent variables. The higher the odds ratio estimate is, the more contribution from that specific attribute exists to the acceptability of the sample.

For the detection of a bitterness aftertaste, the acceptability of the cheese, and the interest in buying the cheddar cheese containing γ-Oryzanol before and after knowing the potential health benefits of it, a simple frequency analysis was performed.

CHAPTER 4. RESULTS AND DISCUSSION

4.1 Color

The analysis of variance and a post-hoc test indicated an existence of significant differences ($p<0.0001$) in all color parameters in cheese during ripening process. Pairwise tests between some months based on whiteness (L*), redness (a*), yellowness (b*) and huge angle (H°) measurements showed significant differences ($p<0.05$) for both control and treatment samples (Tables 4.1 and 4.2). L* and a* decreased for both control and treatment samples during time, and b* and H° increased in both samples, which means that there was less red and white and more yellow in the sample during ripening. The significant differences were noticed after one and two months of maturation for both samples but the treatment also showed differences in the month 4.

Table 4.1: Means and standard deviations of L* (lightness), a* (redness), b* (yellowness) and H° (hue angle) of control samples without γ-Oryzanol at different stages of maturation.

Month	Parameter			
	L*	a*	b*	H°
0	78.11 (1.37)[a]	10.46 (1.27)[a]	34.56 (1.64)[c]	73.22 (1.26)[b]
1	75.74 (1.27)[b]	11.22 (1.02)[a]	37.15 (2.21)[b]	73.22 (0.92)[b]
2	75.65 (1.79)[b]	7.79 (0.98)[b]	39.45 (2.30)[a]	78.80 (1.12)[a]
3	75.78 (1.63)[b]	7.81 (1.16)[b]	39.29 (2.07)[a]	78.79 (1.28)[a]
4	74.83 (1.38)[b]	8.09 (1.05)[b]	40.93 (3.16)[a]	78.83 (1.06)[a]

[a,b,c] Means within same column followed by different superscripts are significantly different ($P<0.05$).

When comparing color parameters between samples (control and treatment) at different stages of maturation, the significant differences were observed (Table 4.3). L* and b* showed

less significant differences between samples than a* and H°, and the former two parameters had significant differences up to the third month of maturation, L* had them between the first and third month and b* just in the first and third month.

Table 4.2: Means and standard deviations of L* (lightness), a* (redness), b* (yellowness) and H° (hue angle) of treatment samples with γ-Oryzanol at different stages of maturation.

Month	Parameter			
	L*	a*	b*	H°
0	77.46 (1.54)^{ab}	9.60 (1.06)^{b}	34.30 (1.82)^{c}	74.40 (1.21)^{c}
1	76.96 (0.80)^{ab}	10.35 (0.61)^{a}	35.65 (1.61)^{bc}	73.81 (0.60)^{c}
2	76.63 (0.89)^{b}	6.93 (0.72)^{d}	39.23 (2.75)^{a}	79.99 (0.51)^{a}
3	77.85 (0.59)^{a}	6.53 (0.50)^{d}	36.93 (2.02)^{b}	79.97 (0.64)^{a}
4	75.58 (2.07)^{c}	7.65 (1.15)^{c}	39.46 (3.29)^{a}	79.04 (1.32)^{b}

^{a,b,c,d} Means within same column followed by different superscripts are significantly different ($P<0.05$).

Table 4.3: Probabilities of significant differences (Pr>|t|) in L* (lightness), a* (redness), b* (yellowness) and H° (hue angle) between control (without γ-Oryzanol) and treatment (with γ-Oryzanol) samples during ripening.

Month	Parameter			
	L*	a*	b*	H°
0	0.1279	0.0142*	0.6153	0.0019*
1	0.0003*	0.001*	0.0103*	0.0112*
2	0.0235*	0.0014*	0.7608	<0.0001*
3	<0.0001*	<0.0001*	0.0002*	0.0003*
4	0.1456	0.1719	0.1210	0.5444

*Probabilities smaller than 0.05 (α) indicate significant differences between samples.

Total color difference was calculated in two different ways: comparing control and treatment samples at different stages of ripening (Figure 4.1), and comparing each sample at different months with the control sample at month 0 (Figure 4.2). From these figures, it can be seen that there is a slight increase in color difference (ΔE*) during the ripening of the cheese.

It cannot be said that there is a clear tendency of the color difference to increase during maturation of cheese when comparing the ΔE* in Figure 4.1, just that there is a difference in color between control and treatment samples at different stages of maturation.

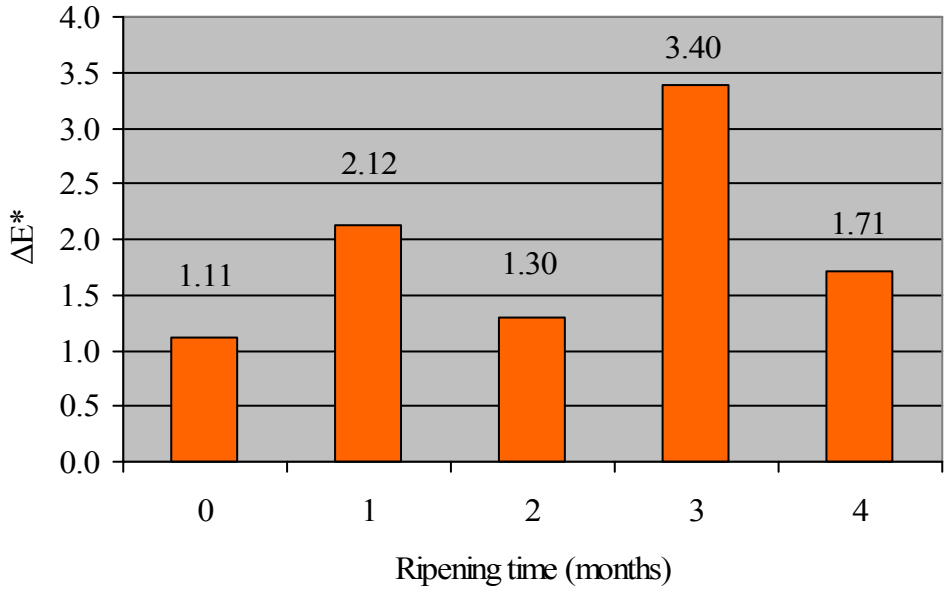

Figure 4.1: Total color difference (ΔE*) between control (without γ-Oryzanol) and treatment (with γ-Oryzanol) samples at different stages of cheese ripening.

It can be concluded that the difference in color for months 1, 2, 3 and 4 is higher than month 0. This means that the difference between control and treatment is larger.

In Figure 4.2, it can be noted that during the ripening process, the color difference of each sample at each stage of ripening compared with the control sample at month 0 tends to be smaller for the treatment samples than for the control.

From the obtained results of color parameters (L*, a*, b* and H°) and total color difference (ΔE*), and the subsequent statistical analysis, it can be concluded that γ-Oryzanol was not an important factor affecting the color difference of cheddar cheese. Also time was a significant factor affecting the color changes in cheese since both comparisons (Figure 4.1 and 4.2) showed an increase of color differences during time.

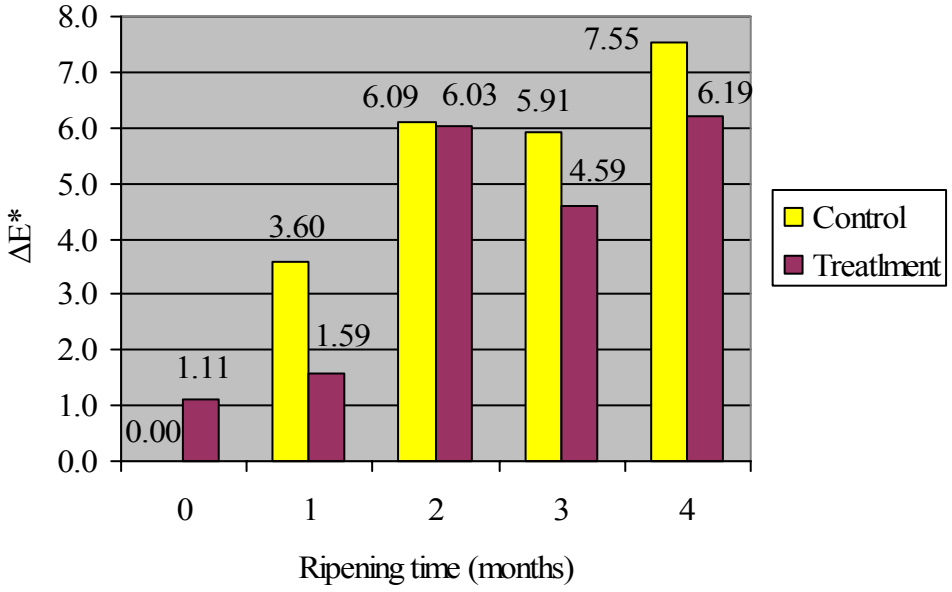

Figure 4.2: Total color difference (ΔE*) comparing samples with γ-Oryzanol and without γ-Oryzanol at all times with the control sample (without γ-Oryzanol) at month 0.

4.2 Texture

The results of textural parameters obtained from the texture exponent software for both control and treatment samples are presented in Tables 4.4 and 4.5, respectively. These tables also show the significant differences when comparing each sample within different months of ripening. The analysis of variance and a post-hoc test indicated an existence of significant differences ($p<0.0001$) in all textural parameters evaluated in cheese during the ripening process. Pairwise tests between some months within the same sample based on hardness, cohesiveness, adhesiveness, gumminess, chewiness, springiness and resilience results were significantly

different ($p<0.05$) for both control and treatment samples. The most important changes were in the hardness and gumminess which decreased during time over the four months of the study for both control and treatment samples. The other parameters also decreased during time but in a smaller ratio.

Springiness and resilience showed to have more differences between months for both control and treatment than the other textural parameters evaluated. Cohesiveness showed more significant differences in treatment samples; hardness had less significant differences than the other parameters in question for both samples, and chewiness and gumminess showed the same degree of difference between months for both samples, being between springiness and resilience, and hardness.

Table 4.4: Means and standard deviations of textural parameters of control samples without γ-Oryzanol at different stages of maturation.

Month	Parameter					
	Hardness (N)	**Cohesiveness (-)**	**Gumminess (N)**	**Chewiness (J)**	**Springiness (m)**	**Resilience (-)**
0	215.33 (34.68)^{a}	0.27 (0.03)^{a}	58.25 (12.31)^{a}	0.78 (0.18)^{a}	0.01 (0.00)^{a}	0.13 (0.01)^{a}
1	157.18 (72.42)^{b}	0.22 (0.03)^{b}	36.52 (23.02)^{b}	0.44 (0.26)^{b}	0.01 (0.00)^{b}	0.11 (0.02)^{b}
2	165.73 (57.13)^{b}	0.20 (0.02)^{bc}	34.23 (13.72)^{bc}	0.32 (0.12)^{bc}	0.01 (0.00)^{c}	0.09 (0.01)^{c}
3	129.72 (32.25)^{b}	0.19 (0.03)^{c}	24.97 (7.23)^{c}	0.22 (0.05)^{c}	0.01 (0.00)^{c}	0.08 (0.01)^{d}
4	145.94 (30.49)^{b}	0.21 (0.02)^{bc}	30.63 (8.42)^{bc}	0.25 (0.06)^{c}	0.01 (0.00)^{d}	0.08 (0.01)^{cd}

^{a,b,c,d} Means within same column followed by different superscripts are significantly different ($P<0.05$).

The adhesiveness is not included in the Tables 4.4 and 4.5 because both samples, control and treatment, gave an adhesiveness of 0 Joules at all times. The fracturability is also not

included in the results because it was present in just few samples and replications, and it was not representative of the behavior of textural changes.

Table 4.5: Means and standard deviations of textural parameters of treatment samples with γ-Oryzanol at different stages of maturation.

Month	Parameter					
	Hardness (N)	Cohesiveness (-)	Gumminess (N)	Chewiness (J)	Springiness (m)	Resilience (-)
0	170.73 (53.37)^{a}	0.26 (0.04)^{a}	45.39 (21.01)^{a}	0.58 (0.26)^{a}	0.01 (0.00)^{a}	0.13 (0.02)^{a}
1	146.87 (43.66)^{ab}	0.22 (0.03)^{bc}	32.28 (13.48)^{bc}	0.37 (0.16)^{b}	0.01 (0.00)^{b}	0.11 (0.01)^{b}
2	179.85 (50.32)^{a}	0.20 (0.03)^{c}	36.52 (14.83)^{ab}	0.33 (0.14)^{b}	0.01 (0.00)^{c}	0.10 (0.02)^{c}
3	121.93 (27.20)^{b}	0.17 (0.02)^{d}	21.30 (6.22)^{c}	0.19 (0.06)^{c}	0.01 (0.00)^{cd}	0.08 (0.01)^{d}
4	161.44 (46.23)^{a}	0.22 (0.03)^{b}	36.36 (11.94)^{ab}	0.28 (0.07)^{bc}	0.01 (0.00)^{d}	0.09 (0.01)^{c}

^{a,b,c,d} Means within same column followed by different superscripts are significantly different ($P<0.05$).

When comparing the results of hardness, cohesiveness, gumminess, chewiness, springiness and resilience between samples (control and treatment) at different stages of maturation, the differences seemed to be not very significant, as shown in Table 4.6. Just few pair-wise comparisons between control and treatment at a specific stage of the maturation give the impression to be significantly different, and the rest does not show any significant difference at all. Cohesiveness showed more differences at the third and fourth month, hardness, gumminess and chewiness at month 0, and resilience at the last month of analysis; springiness showed significant changes in the first month, and chewiness also showed changes in month 3.

Hardness, cohesiveness, gumminess, chewiness, springiness and resilience showed significant differences when compared between months within the same sample; they barely showed significant differences when compared between samples of the same maturation time.

Adhesiveness showed to be the only textural parameter which did not show a significant difference at all, 0 Joules for all times, samples and replications.

Table 4.6: Probabilities of significant differences (Pr>|t|) in texture parameters between control (without γ-Oryzanol) and treatment (with γ-Oryzanol) samples during ripening.

Month	Parameter					
	Hardness	Cohesiveness	Gumminess	Chewiness	Springiness	Resilience
0	0.0014*	0.1953	0.0137*	0.0043*	0.2044	0.4699
1	0.5540	0.5180	0.4418	0.2397	0.0123*	0.2700
2	0.3726	0.3945	0.5866	0.8895	0.3572	0.2465
3	0.3714	0.0078*	0.0685	0.0272*	0.4994	0.1628
4	0.1779	0.0216*	0.0614	0.1020	0.2979	0.0125*

*Probabilities smaller than 0.05 (α) indicate significant differences between samples.

Texture profile analysis showed some differences during ripening within the same samples but it did not show any major differences between control and treatment cheese samples during this time; all of which leads to the conclusion that γ-Oryzanol did not change textural conformation of cheddar cheese significantly.

All textural parameters evaluated, with exception of springiness, tended to decrease during time until the third month, and the fourth month showed a slight increase. For springiness there was not any increase in the fourth month, including both control and treatment samples (see Figures 4.5 to 4.10 in Appendix A for graphical trend of data in Tables 4.4 and 4.5).

4.3 Microbial Growth

The pattern of aerobic bacteria and coliform growth in both control and treatment samples during maturation is presented in Table 4.7. In this table, it can be seen the significant differences existing when comparing each sample within different months of ripening.

The analysis of variance and a post-hoc test indicated an existence of significant differences ($p<0.0208$ for aerobic bacteria and $p<0.0001$ for coliforms) in microbial growth in cheese during the ripening process. Pairwise comparisons between some months based on

aerobic plate count and coliform count were significantly different ($p<0.05$) for both control and treatment samples.

Table 4.7: Means and standard deviations of microbial growth of control (without γ-Oryzanol) and treatment (with γ-Oryzanol) samples at different stages of maturation.

Month	Microbial count			
	Aerobic Plate (log CFU/g)		Coliform Plate (log CFU/g)	
	Control	Treatment	Control	Treatment
0	6.74 (0.36)^{a}	6.71 (0.23)^{a}	4.36 (0.31)^{a}	4.53 (0.07)^{a}
1	6.44 (0.26)^{ab}	6.68 (0.06)^{a}	2.83 (0.18)^{b}	1.48 (2.09))^{ab}
2	5.29 (0.12)^{ab}	5.66 (0.08)^{b}	2.50 (0.28)^{b}	2.39 (0.55)^{ab}
3	5.32 (0.72)^{ab}	5.34 (0.37)^{b}	ND	ND
4	5.02 (0.04)^{b}	5.19 (0.07)^{b}	ND	ND

^{a,b,c} Means within same column followed by different superscripts are significantly different ($P<0.05$).
ND = not detectable; detection limit: 10 CFU/g.

The decline in both coliforms and aerobic bacteria for both samples over time could have been due to the acid production of the lactic acid bacteria used in the starter culture which slowly decreased the aerobic bacteria and coliform counts over time.

Yeasts and molds were not present in any of the samples at different stages of maturation with the method and conditions used in the experiment. *E. coli* was not present in any of the samples either; Reddy *et al.*, (1995) did not detect *E. coli* either, but Reitsma et al., (1996) found that *E. coli* O157:H7 survived during manufacture and for more than 60 days of curing of cheddar cheese.

When comparing the results of aerobic bacteria and coliforms between samples (control and treatment) at different stages of maturation, these did not show any significant differences, as shown in Table 4.8, which means that treated samples did not diverge on its quality from control samples during maturation.

The aerobic plate count in both samples, control without γ-Oryzanol and treatment with γ-Oryzanol, was significantly lower than the counts presented by Reddy *et al.*, (1995), at least 3 orders of magnitude at all stages of maturation.

Table 4.8: Probabilities of significant differences (Pr>|t|) in aerobic bacteria and coliform population between control (without γ-Oryzanol) and treatment (with γ-Oryzanol) samples during ripening.

Month	Microbial count	
	Aerobic Plate	Coliform Plate
0	0.9303	0.5298
1	0.3248	0.5275
2	0.0674	0.8253
3	0.9753	-
4	0.1003	-

Probabilities smaller than 0.05 (α) indicate significant differences between samples.

Microbial growth patterns for aerobic bacteria, coliforms and yeasts and molds showed some significant differences when compared at different ripening times within the same sample. When compared between samples at different ripening times, there were no significant differences at all, which established that γ-Oryzanol did not affect the microbial growth of aerobic bacteria, coliforms, yeast and molds in cheddar cheese during its ripening time. All microbial growth parameters reported tended to decrease during time for the whole experiment

(see Figures 4.11 and 4.12 in Appendix A for graphical trend of data in Table 4.7); coliforms did not show any growth after the second month.

4.4 Polymerase Chain Reaction

The purpose of performing PCR in the cheese was to determine if the starter culture of cheddar cheese was affected by γ-Oryzanol. The starter culture used in the manufacture of the cheddar cheese was a mixture of *Lactococcus lactis* subsp. *cremoris* and subsp. *lactis*. The method used for the PCR was expected to identify and separate both subspecies which was achieved as shown in Figure 4.3 (Ward *et al.*, 1998).

In Figure 4.3, lanes 1 to 4 are the control samples, lanes 6 to 9 are the treatment samples, lanes 5 and 10 are the 100-bp molecular ruler; lanes 1, 3, 6 and 8 are the digested samples with the restriction endonuclease enzyme *MboII* and lanes 2, 4, 7 and 9 are the non-digested samples. As indicated in Figure 4.3 the non-digested samples (lanes 2, 4, 7 and 9) show just one bright band and that the digested samples (lanes 1, 3, 6 and 8) have two bands which represent both subspecies of *lactis*.

The blurry bands in the digested samples represent the subsp. *cremoris* (348-bp) and the bright bands represent the subsp. *lactis* (257-bp) which means that subsp. *lactis* was in higher amount than *cremoris* in the cheese manufactured for this experiment; these results are similar to those of Reddy et al., (1995) who reported that using *Lactococcus lactis* subsp. *lactis* and *Lactococcus lactis* subsp. *cremoris* as starter cultures, the *Lactococcus lactis* subsp. *lactis* together with *Lactobacillus casei* were the predominant bacteria.

Based on the obtained gel from the PCR, it can be concluded that *Lactococcus lactis* subsp. *cremoris* (348-bp) and subsp. *lactis* (257-bp) are both present in the cheddar cheese containing γ-Oryzanol after 4 months of maturation.

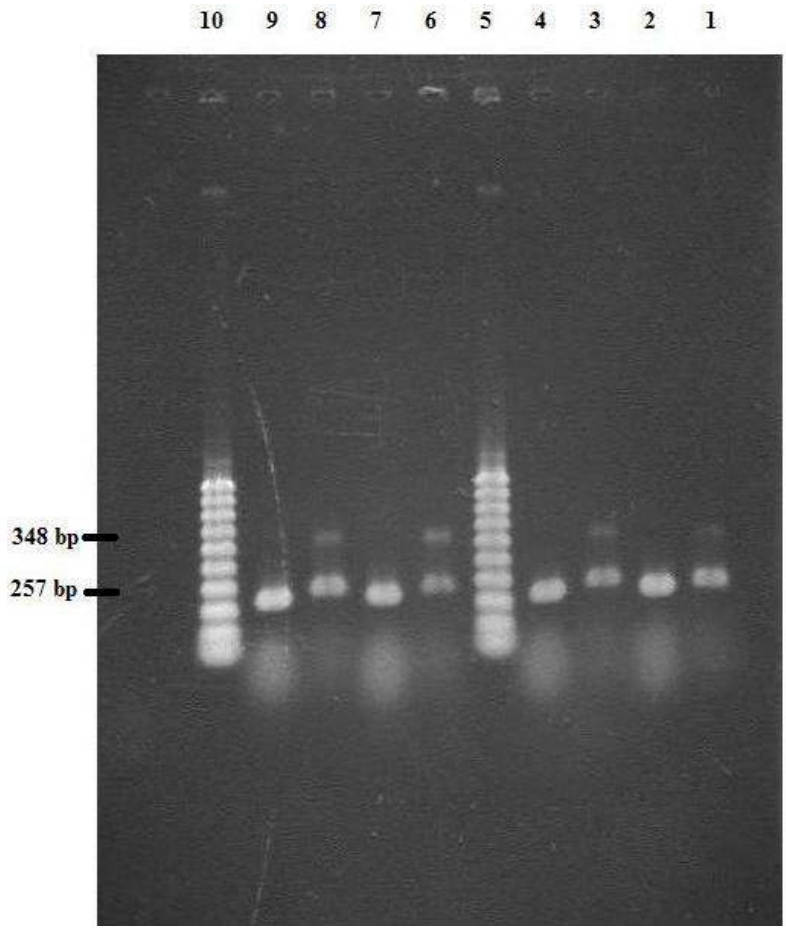

Figure 4.3: Agarose gel (4%) of digested and non-digested products of the Y1-Y2 amplified fragment from cheese extraction after 4 months of maturation.

4.5 Aroma Development

The pattern of aroma development during maturation in both control and treatment samples yielded the observation of six peaks in the chromatograms obtained (Tables 4.9 and 4.10). The software used to identify the peaks could not accurately identify them but based on data reported by Christensen and Reineccius (1995), the six peaks obtained in the experiments mostly represent butyric acid, ethyl butyrate, ethyl caproate, 2- or 3- methyl butanal, hexanoic acid and propionic acid, respectively, or ethyl esters of these compounds.

These tables show that there are not significant differences existing when comparing different months of ripening in each sample within the same sample with just one exception in

peak 2 of the control sample. The analysis of variance and a post-hoc test pointed out a lack of significant differences in aroma development in cheese between months during the ripening process within each peak formation. These values show that changes in the aroma development of cheese between months based on the peaks found in the chromatograms were not significantly different ($p<0.05$) for both control and treatment samples.

Table 4.9: Means and standard deviations of heights of aroma peaks of control samples without γ-Oryzanol at different stages of maturation.

Month	Peak					
	1	2	3	4	5	6
	(Kilocounts)					
0	9.43 (3.97)[a]	24.63 (6.18)[ab]	15.50 (3.91)[a]	15.10 (5.94)[a]	16.15 (10.39)[a]	31.00 (26.67)[a]
1	8.87 (2.80)[a]	8.60 (2.44)[b]	13.40 (4.91)[a]	9.00 (2.55)[a]	10.17 (2.75)[a]	16.60 (4.81)[a]
2	15.23 (7.68)[a]	42.17 (19.42)[a]	20.20 (8.92)[a]	15.70 (7.25)[a]	11.90 (4.85)[a]	10.10 (1.27)[a]

[a,b] Means within same column followed by different superscripts are significantly different ($P<0.05$).
Peaks 1, 2, 3, 4, 5 and 6 most probably represent butyric acid, ethyl butyrate, ethyl caproate, 2- or 3- methyl butanal, hexanoic acid and propionic acid, respectively.

Table 4.10: Means and standard deviations of heights of aroma peaks of treated samples with γ-Oryzanol at different stages of maturation.

Month	Peak					
	1	2	3	4	5	6
	(Kilocounts)					
0	6.87 (1.60)[a]	6.47 (0.50)[a]	9.27 (1.27)[a]	8.93 (1.85)[a]	8.70 (1.45)[a]	8.47 (0.92)[a]
1	5.00 (0.00)[a]	10.25 (0.0)[a]	8.70 (0.00)[a]	5.80 (0.00)[a]	7.00 (0.00)[a]	6.20 (0.00)[a]
2	5.80 (0.00)[a]	31.17 (23.41)[a]	11.87 (2.76)[a]	9.10 (2.10)[a]	6.77 (1.70)[a]	6.80 (1.44)[a]

[a] Means within same column followed by different superscripts are significantly different ($P<0.05$).
Peaks 1, 2, 3, 4, 5 and 6 most probably represent butyric acid, ethyl butyrate, ethyl caproate, 2- or 3- methyl butanal, hexanoic acid and propionic acid, respectively.

The comparison between control and treatment samples at different stages of maturation did not show any significant differences either, with one exception, as shown in Table 4.11, which was at month 0 from peak 2, but the control samples showed peaks greater than the treatment samples. This means that γ-Oryzanol may have interfered in the flavor and aroma development in cheese. According to Parrado *et al.*, (2003), oryzanol derived from RBO showed capacity to inhibit lipid peroxidation and protect protein from oxidation; and according to Nanua *et al.*, (2000), a high-oryzanol RBO at 0.1% significantly reduced the oxidation of low-heat whole milk powder.

Table 4.11: Probabilities of significant differences (Pr>|t|) in heights of aroma peaks between control (without γ-Oryzanol) and treatment (with γ-Oryzanol) samples during ripening.

Month	Peak					
	1	2	3	4	5	6
0	0.3578	0.0357*	0.0982	0.2073	0.4943	0.2677
1	0.3578	0.6179	0.4945	0.3909	0.4242	0.3280
2	0.3988	0.5650	0.2425	0.2517	0.1588	0.0801

*Probabilities smaller than 0.05 (α) indicate significant differences between samples.
Peaks 1, 2, 3, 4, 5 and 6 most probably represent butyric acid, ethyl butyrate, ethyl caproate, 2- or 3- methyl butanal, hexanoic acid and propionic acid, respectively.

Aroma development pattern for cheddar cheese containing γ-Oryzanol did not show significant differences when compared at different ripening times within the same sample, and the same results when compared between samples at different ripening times.

When comparing the means of the different peaks from the chromatograms between control and treatment samples, it can be noticed that with just one exception, all the peak heights of aroma coming from treatment samples were lower than the control samples at the same ripening time. This may suggest that γ-Oryzanol may have inhibited the growth of bacteria that contribute to the odor development or that the antioxidant activity of γ-Oryzanol may have inhibited the lipolysis or proteolysis needed in the aroma development in cheddar cheese until

certain point even that statistically speaking there were not significant differences. There is no congruent tendency of peak heights for the main six compounds found to produce aroma in cheddar cheese, some increased during time and some others decreased (see Figures 4.13 to 4.18 in Appendix A for graphical trend of data in Tables 4.9 and 4.10).

4.6 Oryzanol Retention

In order to compare control and treatment samples, both were run in HPLC even though the control did not have γ-Oryzanol. The control samples did not show any peak in the chromatograms, and the treatment samples showed two joined peaks (see Figure 4.19 in Appendix A for an example of chromatograms obtained). The results of γ-Oryzanol retained in the cheese are shown in the Table 4.12; this table also shows the significant differences within treatments at different maturation times. ANOVA and a post-hoc test indicated that γ-Oryzanol retention analysis had significant differences ($p<0.0001$).

Table 4.12: Means and standard deviations of γ-Oryzanol retained in treated cheese at different stages of maturation (per serving size).

Month	γ-Oryzanol retained (mg/28g)
0	181.41 (5.94)[a]
1	99.30 (0.00)[b]*
2	172.36 (10.94)[a]
3	129.85 (21.67)[b]

[a,b] Means followed by different superscripts are significantly different ($P<0.05$).
* Only one sample was obtained.

The γ-Oryzanol retained showed significant differences between following months. The results seem to show a tendency to decrease in time during maturation but without any clear pattern (see Figure 4.20 in Appendix A for graphical tendency of data in Table 4.12 compared to

the target value) which could be due to a poor homogenization of γ-Oryzanol at the time of manufacture of the cheese or that the analytical method was not accurate nor precise. It was visually evident that the cheddar cheese containing γ-Oryzanol was not well mixed since white spots could be seen spread all over the cheese, some were bigger than others.

Even if the results of γ-Oryzanol retention in cheese do not show any expected values, it is very important to keep in mind that the target concentration of γ-Oryzanol added in cheese was 100mg per serving size of cheddar cheese (28g). The results presented in Table 4.12 show that the γ-Oryzanol was very well retained in the cheese and that there was some loss of it during ripening but still above 100mg/28g of cheese as expected.

4.7 Moisture Content

Table 4.13 presents the results of moisture content of both control and treatment samples together with significant differences between ripening times within the same sample. The analysis of variance and a post-hoc test indicated an existence of significant differences ($p<0.0378$ for control samples and $p<0.0026$ for treatment samples) in moisture contents in cheese during the ripening process. These values show that changes in moisture content of cheese between some months were without exception significantly different ($p<0.05$) for both control and treatment samples; between some other months, there were no significant differences at all.

The statistical analysis performed to compare the means between samples (control and treatment) produced the data presented in Table 4.14. The comparison of means in moisture content between control and treatment samples look very similar and with similar behaviors, with a slight decrease during ripening process, as it can been seen in Table 4.13, and the

probabilities seen in Table 4.14 show that there are not significantly differences between control and treatment samples at different ripening times.

Table 4.13: Means and standard deviations of moisture content in control (without γ-Oryzanol) and treatment (with γ-Oryzanol) samples at different stages of maturation.

Month	Moisture content (%)	
	Control	Treatment
0	44.55 (0.89)[a]	43.86 (0.49)[ab]
1	43.83 (0.09)[ab]	44.58 (0.42)[a]
2	41.65 (0.96)[b]	40.74 (0.40)[c]
3	42.45 (0.18)[ab]	42.45 (0.32)[b]

[a,b,c] Means within same column followed by different superscripts are significantly different ($P<0.05$).

Table 4.14: Probabilities of significant differences (Pr>|t|) in moisture content between control (without γ-Oryzanol) and treatment (with γ-Oryzanol) samples during ripening.

Month	Probability (Pr>|t|)
0	0.4353
1	0.1311
2	0.3415
3	1.0000

Probabilities smaller than 0.05 (α) indicate significant differences between samples.

Albeit there were some significant differences in moisture content between months within each sample and there were no significant differences between control and treatment samples, it can be concluded that γ-Oryzanol did not affect the moisture content of cheddar cheese in a significant way (see Figure 4.21 in Appendix A for graphical trend of Table 4.13).

The maximum moisture content in cheddar cheese is 39 percent by weight (USFDA, 2002), both samples have a moisture content of 39% which means that something was wrong with the process used or that the pressing step should have been done longer or with higher pressure.

4.8 Free Fatty Acid Profile

The concentration of each free fatty acid was calculated based on the chromatogram peaks and the software the GC was equipped with, the results of the free fatty acid profile for both control and treatment samples are presented in Tables 4.29 and 4.30 in Appendix A.

ANOVA and a post-hoc test indicated that there were significant differences ($p<0.05$) in the analysis of some free fatty acids, such as C4:0, C8:0, C14:0, C16:0, C18:0 and C18:2, for both samples; C6:0, C12:0, C18:1 and C18:3 gave significant differences just in control samples, C16:0 just in treatment samples, and C10:0 did not show any differences at all.

Tables 4.29 and 4.30 also show the significant differences between months within the same sample, and it can be seen that there are significant differences between some contiguous months and in some other contiguous months this differences do not exist.

The free fatty acid profile did not show any pattern during ripening when comparing the free fatty acid content between ripening times, but it did show that the long chain free fatty acids are more concentrated than the middle chain and short chain fatty acids.

When comparing the free fatty acid profile between samples (control and treatment) at different stages of the maturation, the significant differences are tabulated as probabilities in Table 4.15. It can be seen from this table that there were no significant differences in any pair-wise comparison between control samples and treatment samples at any time.

Table 4.15: Probabilities of significant differences (Pr>|t|) in texture parameters between control (without γ-Oryzanol) and treatment (with γ-Oryzanol) samples during ripening.

Month	Free fatty acid												
	C4:0	C6:0	C8:0	C10:0	C12:0	C14:0	C14:1	C16:0	C16:1	C18:0	C18:1	C18:2	C18:3
0	-	0.5239	0.4073	0.3482	0.4507	0.2824	0.5956	0.5785	0.9613	0.3936	0.6372	0.2154	0.5477
1	-	0.6726	0.3283	0.5725	0.1388	0.0539	0.4665	0.1139	0.6105	0.2348	0.3159	0.3508	0.5444
2	-	-	0.4395	0.3833	0.2756	0.2165	0.9440	0.6235	0.4975	0.6491	0.5094	0.6152	0.4961
3	0.9017	0.7462	0.7955	0.7576	0.1225	0.6861	0.7460	0.5916	0.2692	0.6383	0.6398	0.9670	0.4828

Probabilities smaller than 0.05 (α) indicate significant differences between samples.

Results of the free fatty acid profile show too much variability, maybe due to the changes in cheese quality during the ripening process due to γ-Oryzanols' presence. γ-Oryzanol can be interfering with the GC analysis in some way which does not allow the quantification of the free fatty acids efficiently or changing the interactions between the solvent and the lipid extract.

In this experiment, the total FFA content increased during the first month of maturation but then decreased in the following two months (Figure 4.4). It is known from another study that the total FFA content of cheese made from raw milk and pasteurized milk increases from day 1 to day 60 (Buffa *et al.*, 2001) which puts in contradiction both studies.

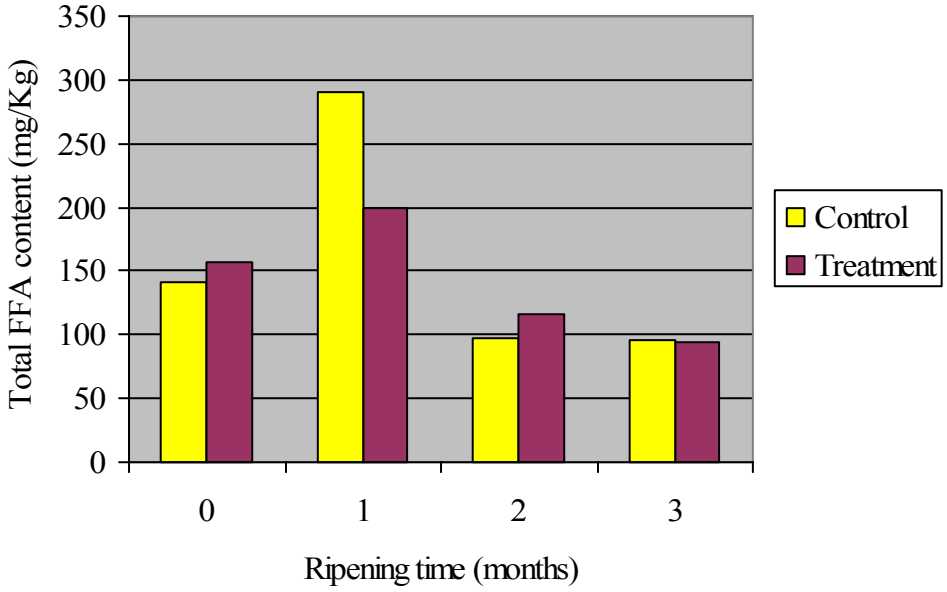

Figure 4.4: Total free fatty acid content in cheese samples during ripening per Kg of cheese.

The main FFA observed in the cheese during its maturation were myristic (C14:0), palmitic (C16:0), stearic (C18:0) and oleic (C18:1) acids (Table 4.16), representing together approximately 80% of total FFA content, coinciding with Buffa *et al.* in 2001, and confirming that the percentages of the FFA profile were similar in cheddar cheese containing γ-Oryzanol to normal cheddar cheese evaluated in other studies.

Another explanation for the decrease in FFA during time could be due to the fact that γ-Oryzanol had to do with this free fatty acid behavior during ripening affecting the tendency of FFA to increase during time. This seems to be the most logical explanation since γ-Oryzanol is known to have a good antioxidant activity (Xu *et al.*, 2001) which could have diminished the lipolysis in the cheese and the subsequent increase in free fatty acids. However, the control samples also showed this behavior which does not support the explanation above.

Table 4.16: Percentages (%) of free fatty acids (FFA) in cheese samples during ripening.

Month	**0**		**1**		**2**		**3**	
FFA	**C**	**T**	**C**	**T**	**C**	**T**	**C**	**T**
c4:0	0.00	0.00	0.00	0.00	0.00	0.00	6.14	6.08
c6:0	5.95	6.50	16.49	17.70	0.00	0.00	1.20	1.19
c8:0	2.39	2.33	3.25	2.83	2.95	2.30	0.76	0.75
c10:0	1.46	1.46	1.79	0.91	1.69	1.33	1.80	1.77
c12:0	3.32	3.22	2.75	2.76	3.83	3.01	1.70	3.79
c14:0	6.92	8.16	3.46	3.37	4.57	3.56	9.46	9.25
c14:1	1.44	1.11	1.26	0.14	1.72	1.44	1.47	1.45
c16:0	16.90	16.11	12.92	12.71	13.38	14.10	30.76	29.78
c16:1	0.03	0.02	0.52	0.62	5.79	0.71	1.10	1.94
C18:0	54.87	54.11	40.51	41.42	44.92	46.03	13.98	13.61
c18:1	2.41	1.94	1.90	2.08	2.64	12.84	26.99	26.29
c18:2	1.69	1.90	0.95	1.05	13.17	14.27	3.59	3.50
c18:3	2.63	3.13	14.21	14.40	5.35	0.41	1.05	0.60

C: control samples, T: treatment samples and c: carbon atoms in FFA.

From Table 4.16, it can also be seen the differences existing between saturated free fatty acids and unsaturated free fatty acids in the cheese throughout the whole ripening process. The main FFA observed are mostly saturated (no double bonds between carbon atoms) with exception of oleic acid which is a monosaturated fatty acid.

The only conclusion that can be reached from all these analysis and results is that both control and treatment samples of cheddar cheese in this experiment presented a similar FFA profile in percentages basis compared to data found in other experiments (Buffa *et al.*, 2001; Lindsay, 1982), and that the effects of γ-Oryzanol did not have any significant effects in the quality of cheddar cheese during maturation.

Fat plays an essential part in the flavor of cheese, albeit an indirect part, and the most important flavor compounds originating from the fat are the free fatty acids formed by lipolysis (Walstra *et al.*, 1999) which can explain part of the aroma development in cheese. The free fatty acids at the first month of maturation may have influenced more the aroma development of cheese than at the other times since at this stage was where the highest amount of free fatty acids was present.

The free fatty acids cause a somewhat pungent flavor. Probably, the distribution of aroma compounds over fat and aqueous phases enhances a balanced flavor (Walstra et al., 1999). This distribution was mostly influenced by the long chain fatty acids rather than small chain fatty acids at all times of maturation.

4.9 Consumer Study

4.9.1 R-Index

For the traditional R-Index, the data obtained from the questionnaire was: consumer was certain that the sample was the treatment (S), that perhaps the sample was the treatment but not

sure (S?), that perhaps the sample was the control but not sure (N?) or that definitely the sample was the control (N).

The calculated R-Indices and the critical R-Indices for the traditional method, including the frequencies of S, S?, N? and N for all the attributes evaluated by the traditional R-Index which were the overall appearance, overall color, odor, taste and overall texture are condensed in the Table 4.17.

Table 4.17: R-Index values of traditional method for the consumer study in cheese containing γ-Oryzanol with the attributes evaluated.

Attribute	Sample	Different		Same		R-Index (%)	Critical R-Index (%)
		S	S?	N?	N		
Overall Appearance	485 (S)	69	10	10	10	82.1	59.7
	705 (N)	16	4	23	56		
Overall Color	485 (S)	34	14	17	34	69.4	59.7
	705 (N)	10	5	18	66		
Odor	485 (S)	37	16	20	24	68.1	59.7
	705 (N)	13	9	28	47		
Taste	485 (S)	68	13	8	9	73.7	59.7
	705 (N)	28	14	21	36		
Overall Texture	485 (S)	59	12	13	13	75.3	59.7
	705 (N)	19	8	28	44		

485 (S) = cheese containing γ-Oryzanol; 705 (N) = cheese without γ-Oryzanol; S= different sure; S?= different unsure; N?= same unsure; N= same sure.

As seen in the Table 4.17, without any exception, the calculated R-Indices for all the attributes evaluated with the traditional method (overall appearance, overall color, odor, taste and overall texture) were greater than the critical R-Index value of 59.7 which means that the consumer significantly noticed a difference between control and treatment samples not by chance. The most discriminatory attributes were overall appearance (82.1%) and overall texture (75.3%) which tells that consumer noticed better differences in these attributes than in the others, especially for the overall appearance.

The data obtained for calculation of the R-Index by the bipolar method are tabulated in the Table 4.18. From the Table 4.18, the consumers noticed that the treatment samples (S) were more yellow than the control samples (N), they also were able to indicate that the treatment sample was harder than the control sample.

Table 4.18: Frequencies of attributes evaluated with R-Index by the bipolar method and directions of the differences.

Attribute	Sample	More		Same		Less		Total		R-Index to be used
		S+	S+?	N?	N	S-	S-?	More	Less	
Yellowness	485 (S)	27	13	12	21	19	5	40	24	More
	705 (N)	15	9	14	48	9	3	24	12	
Hardness	485 (S)	40	21	12	13	9	5	61	14	More
	705 (N)	14	9	22	41	9	5	23	14	
Chewiness	485 (S)	22	8	14	17	24	13	30	37	More/Less
	705 (N)	13	5	23	43	9	5	18	14	

485 (S) = cheese containing γ-Oryzanol; 705 (N) = cheese without γ-Oryzanol; S+= more sure; S+?= more unsure; N?= same unsure; N= same sure; S-= less sure; S-?= less unsure.

The calculated R-Indices and the critical R-Indices for the bipolar method with positive direction, including the frequencies of S+, S+?, N? and N for the yellowness and hardness are condensed in the Table 4.19.

For the chewiness, which did not show any direction for the R-Index, the calculated R-Index and critical R-Index combining S+ with S- and S+? with S-? are in the Table 4.20.

The calculated R-Indices observed in Tables 4.19 and 4.20 for all the attributes evaluated with the bipolar method, (yellowness, hardness and chewiness) were greater than the critical R-Indices values which means that the consumer, with a significant difference, noticed a difference between control and treatment samples not by chance (probability).

Table 4:19: R-Index values of bipolar method for the consumer study in cheese containing γ-Oryzanol with the attributes evaluated with positive direction.

Attribute	Sample	More		Same		R-Index (%) more	Critical R-Index (%)
		S+	S+?	N	N?		
Yellowness	485 (S)	27	13	12	21	66.1	59.5
	705 (N)	15	9	14	48		
Hardness	485 (S)	40	21	12	13	73.9	58.8
	705 (N)	14	9	22	41		

485 (S) = cheese containing γ-Oryzanol; 705 (N) = cheese without γ-Oryzanol; S+= more sure; S+?= more unsure; N?= same unsure; N= same sure.

It can also be said that consumers noticed more presence of the attribute in the treatment sample than in the control sample for the yellowness and hardness, and even if consumers could notice differences in the chewiness between the control and treatment samples, they were not able to notice whether these differences were more or less of the attribute in question.

The most discriminatory attribute by the bipolar method was the hardness of the samples which tells that consumer noticed better differences in this attribute than in the others.

Table 4:20: R-Index values of bipolar method for the consumer study in cheese containing γ-Oryzanol with the attributes evaluated with no direction (more/less).

Attribute	Sample	Combined		Same		R-Index (%)	Critical R-Index (%)
		S+, S-	S+?, S?	N	N?		
Chewiness	485 (S)	22+24	8+13	14	17	69.0	58.2
	705 (N)	13+9	5+5	23	43		

485 (S) = cheese containing γ-Oryzanol; 705 (N) = cheese without γ-Oryzanol; S+= more sure; S+?= more unsure; N?= same unsure; N= same sure; S-= less sure; S-?= less unsure.

As a conclusion of the R-Index study in the cheddar cheese containing γ-Oryzanol (treatment), consumers were able to differentiate between the treatment and the control samples (normal cheddar cheese) based on overall appearance, overall color, odor, taste, overall texture, yellowness, hardness and chewiness. The most discriminating attribute of the cheese by consumers was the overall appearance, and the least discriminating was the yellowness.

4.9.2 The McNemar Test

Table 4.21 describes counts, frequencies and percentages of purchase intent responses by consumers before and after knowing the potential health benefits they could get from the γ-Oryzanol in cheddar cheese. These data were used to calculate the difference in proportions, the probability in purchase intent, the statistic χ^2, the estimated standard error and the confidence interval by the McNemar test (see Appendix D for the codes in SAS).

Table 4.22 shows data obtained from the McNemar test. It can be seen from this table that a significant difference existed between the two responses, with a χ^2 value of 19.2 which is

greater than the critical $\chi^2_{df\ 1}$ of 3.84. The decision of buying the cheddar cheese containing γ-Oryzanol was influenced by the fact that consumers had been informed about the potential health benefits of the product.

Table 4.21: Purchase intent responses for cheddar cheese containing γ-Oryzanol before and after consumers were informed that the product contained a potentially health promoter compound.

Purchase intent (before)	**Purchase intent after knowing of health benefits**		
	Yes	No	Total
Yes	18 (18.18%)	1 (1.01%)	19 (19.19%)
No	22 (22.22%)	58 (58.59%)	80 (80.81%)
Total	40 (40.40%)	59 (59.60%)	99 (100.00%)

Table 4.22: Variables obtained in the McNemar test.

Difference of proportions	**95% confidence interval for the difference**	**Statistic χ^2**	**Pr> χ^2**
0.21	(0.13,0.30)	19.2	<0.0001

The 95% confidence interval for the difference in proportions was calculated in order to obtain a better understanding of the association between the two questions' responses (Pavon, 2003). This confidence interval for the cheese explains that the probability that consumers would buy the product after they had been informed about the potential health benefits was 0.13 to 0.30 times higher than the probability of consumers buying it before they had been informed about the health benefits. In conclusion, it can be said that there was a significant positive

increase in the purchase intent of cheddar cheese containing γ-Oryzanol after consumers had been informed about the health benefits of this compound.

4.9.3 Consumer Acceptance Test

Table 4.23 reports the mean scores and ANOVA results for the acceptability of overall appearance, smoothness of color, odor/aroma, taste, overall texture/mouthfeel and overall liking of the control sample and the treatment sample containing γ-Oryzanol (see Appendix D for the codes in SAS). The analysis of variance and a post-hoc test indicated an existence of differences in acceptability of odor/aroma, taste, overall texture/mouthfeel and overall liking of both samples.

Table 4.23: Means, standard deviations and analysis of variance for acceptability attributes of the control (without γ-Oryzanol) and treatment (with γ-Oryzanol) samples.

Attribute	Cheese sample		Pr>F
	Control	Treatment	
Overall Appearance	6.04 (1.73)[a]	5.62 (2.00)[a]	0.1146
Smoothness of color	6.22 (1.65)[a]	5.82 (1.75)[a]	0.0988
Odor/Aroma	6.21 (1.63)[a]	5.24 (1.50)[b]	<0.0001
Taste	5.52 (2.16)[a]	4.04 (1.94)[b]	<0.0001
Mouthfeel	5.84 (1.97)[a]	4.68 (1.72)[b]	<0.0001
Overall Liking	5.60 (1.79)[a]	4.07 (1.82)[b]	<0.0001

[a,b] Means within same row followed by different superscripts are significantly different (P<0.05).

The scores in Table 4.23 show, without any exception, that for all the attributes, the consumers gave lower scores to the treatment samples than to the control samples. This means that they liked the cheddar cheese containing γ-Oryzanol less than the normal cheddar cheese. These differences in the scores between both samples are significant for the aroma, taste,

mouthfeel and overall liking but not for the overall appearance and color. Therefore, consumers liked in the same scale the overall appearance and color, but they differentiate between samples when evaluating the aroma, taste, mouthfeel and overall liking.

Multivariate analysis of variance (MANOVA) indicated that both the control and the treatment samples were significantly different when the effects sensory acceptability scores were considered simultaneously with probabilities of less than 0.0001 for all, Wilks' Lambda, Pillai's Trace, Hotelling-Lawley Trace and Roy's Greatest Root statistic. These probabilities prove the results obtained from ANOVA.

To get a better understanding of such a difference, Descriptive Discriminant Analysis was performed. The first dimension of pooled within canonical structure (CAN 1) were used to identify discriminating attributes; these values are shown in Table 4.24.

Table 4.24: Canonical structure of attributes describing the cheese samples[a].

Attribute	CAN 1
Overall Appearance	0.21
Smoothness of Color (yellowness)	0.24
Odor/Aroma	0.64*
Taste	0.76*
Overall Texture/Mouthfeel	0.66*
Overall Liking	0.88*

[a] CAN1 is based on pool within group variances.
* Discriminating sensory attributes.

The canonical coefficients seen in Table 4.24 indicate that overall appearance and smoothness of color were not critical discriminant attributes in the sensory evaluation of cheese, whereas, aroma, taste, mouthfeel and overall liking were discriminant attributes with overall

liking being the most discriminant attribute with the highest canonical coefficient of 0.88 and overall appearance the least discriminant with a canonical coefficient of 0.21. This means that overall liking is the attribute giving more contribution to the sensory evaluation of cheese, followed by taste, then mouthfeel and aroma.

For the attributes evaluated with a yes/no scale in the questionnaire, the data is tabulated in the Table 4.25, from which, part was used for McNemar test and other for a logistic analysis.

Table 4.25: Frequencies and percentages of attributes evaluated in control (without γ-Oryzanol) and treatment (with γ-Oryzanol) cheese samples with a yes/no scale.

Question	Sample			
	Control		Treatment	
	Yes	No	Yes	No
Did you detect undesirable bitterness aftertaste?	45 (50.00%)	45 (50.00%)	74 (81.32%)	17 (18.68%)
Is this product acceptable?	74 (74.75%)	25 (25.25%)	36 (36.73%)	62 (63.27%)
Would you buy this product if it were commercially available?	56 (56.00%)	44 (44.00%)	19 (19.00%)	81 (81.00%)
Would you buy this product if it contained oryzanol, an antioxidant and potential cholesterol reducing compound from rice bran?	66 (66.67%)	33 (33.33%)	40 (40.40%)	59 (59.60%)

The logistic analysis performed using the acceptability of the product as a response variable produced the point estimates and probabilities presented in Table 4.26 when comparing with all sensory attributes.

The highest point estimate from the logistic analysis in Table 4.26 is the overall liking and with significant difference; the odds ratio estimate of overall liking has a value of 2.85 which means that this attribute is the most important for changing the acceptability of the product, and if the overall liking score increased 1.0 unit from the 9-point hedonic scale, the probability of the product to be acceptable would increase 2.85 times.

Table 4.26: Odds ratio estimates and probabilities ($Pr>\chi^2$) for logistic regression models using acceptability as a response variable.

Attribute	$Pr>\chi^2$	Odds ratio estimate
Overall Appearance	0.1020	1.38
Smoothness of color	0.0893	0.70
Odor/Aroma	0.6598	0.92
Taste	0.7981	1.06
Overall Texture/Mouthfeel	0.5187	1.13
Overall Liking	<0.0001*	2.85

* Probabilities smaller than 0.05 (α) indicate significant differences.

When using the responses in the questionnaire of buying the product if it were commercially available (before consumer are informed of the potential health benefits of the product) as a response variable for the logistic analysis of the product, the data is presented in the Table 4.27 with point estimates and probabilities when all sensory attributes are evaluated.

From the Table 4.27, it can be seen that overall liking and overall texture/mouthfeel have the highest point estimates with significant differences. This means that these attributes are the most important for changing the purchase intent of the product; if the overall liking score increased 1.0 unit from the 9-point hedonic scale, the probability of the product to be purchased would increase 3.39 times, and if the overall texture/mouthfeel score augmented 1.0 unit, the probability of the product to be purchased would increase 2.24 times.

Table 4.27: Odds ratio estimates and probabilities ($Pr>\chi^2$) for logistic regression models using purchase intent before knowing about health benefits as a response variable.

Attribute	$Pr>\chi^2$	Odds ratio estimate
Overall Appearance	0.1365	1.43
Smoothness of color	0.0673	0.59
Odor/Aroma	0.1345	0.71
Taste	0.6421	1.12
Overall Texture/Mouthfeel	0.0036*	2.24
Overall Liking	0.0005*	3.39

* Probabilities smaller than 0.05 (α) indicate significant differences.

After knowing the prediction in purchase intent of potential consumers before they have been informed of the health benefits of the product, knowing which attributes significantly differentiate and predict the purchase intent and at what ratio these changes after consumers have been informed of the health benefits of the product completes the logistic analysis.

When using the responses in the questionnaire of buying the product if it were commercially available (after consumer have been informed of the potential health benefits of the product) as a response variable for the logistic analysis of the product, the data is presented in the Table 4.28 with point estimates and probabilities when all sensory attributes are evaluated.

Table 4.28: Odds ratio estimates and probabilities ($Pr>\chi^2$) for logistic regression models using purchase intent after knowing about health benefits as a response variable.

Attribute	$Pr>\chi^2$	Odds ratio estimate
Overall Appearance	0.1657	1.24
Smoothness of color	0.9793	1.00
Odor/Aroma	0.5992	1.08
Taste	0.2920	1.21
Overall Texture/Mouthfeel	0.7400	0.95
Overall Liking	0.0316*	1.58

* Probabilities smaller than 0.05 (α) indicate significant differences.

The highest point estimate from the logistic analysis in Table 4.28 is the overall liking followed by the overall appearance, but just the former shows significant differences, thus overall appearance is not useful; the odds ratio estimate of overall liking has a value of 1.58 which means that this attribute is the most important for changing the purchase intent of the product after consumers have been informed of the potential health benefits of it; if the overall liking score increased 1.0 unit from the 9-point hedonic scale, the probability of the product to be acceptable would increase 1.58 times after knowing the health benefits of it.

If the estimated odd ratios used to predict consumer purchase intent that were identified for buying the product if it were commercially available for both before and after consumer have been informed of the potential health benefits of the product are compared to each other, it can be seen that just the attribute overall liking is common for both purchase intents (before and after), and that the mouthfeel is not an important predictor for purchase intent after knowing the health benefits. This leaves the overall liking attribute as the only predictor that is critical in the purchase intent.

The odds point estimate for predicting the purchase intent before knowing of the health benefits is 3.39 and after consumers are informed of the potential health benefit of the product, it decreases to 1.58, which shows that consumers are willing to compromise the overall liking of the cheese merely to get the health benefits of the product.

The overall liking score for the cheese containing γ-Oryzanol was 5.6 (between like slightly and neither like nor dislike), and the prediction for purchase intent would be that consumers are willing to go to an overall liking score of 3.5 (between dislike moderately and dislike slightly) and still purchase the cheese in order to get the health benefits of it.

The attributes were analyzed individually to predict changes in acceptability and purchase intent in case all attributes together did not show any significant responses. All of them showed significant differences when compared with the acceptability and with buying intent before and after consumers know about the health benefits of the product.

CHAPTER 5. CONCLUSIONS

Color analysis showed changes in the quality of the cheese during ripening. Some were significant some others were not significant, but there is not a clear tendency or pattern.

Texture profile analysis showed that there were changes in the quality of the cheese during maturation for control and treatment samples, but that there were no significant changes when γ-Oryzanol was added to cheese and compared to a control.

Microbial growth based on aerobic bacteria presented non significant changes for all the period of evaluation of the cheese; however, coliform counts showed differences because there was no growth of coliforms after the second month of evaluation.

Aroma development in cheese did not present any significant differences during maturation nor between control and treatment samples at different stages of ripening, but the peaks for the control samples were slightly larger than the treatment samples. The aroma development may have been inhibited by the antioxidant activity of γ-Oryzanol decreasing the lypolisis, proteolysis precursors of part of the flavor and aroma in cheese.

The polymerase chain reaction confirmed that *Lactococcus lactis* subsp. *cremoris* and subsp. *lactis* are both present in the cheddar cheese containing γ-Oryzanol.

Even if the results obtained of γ-Oryzanol in cheese do not show any clear pattern, γ-Oryzanol was very well retained in the cheese and there were some losses of it during ripening but the target of 100mg per serving size of cheddar cheese (28g) was reached.

γ-Oryzanol did not affect the moisture content of cheddar cheese in a significant way. The manufacture procedure used to make the cheddar cheese yielded a product that will not comply the FDA regulations because it must have a maximum moisture content in cheddar cheese is 39 percent by weight which is not the case for the cheese made for this experiment.

This situation may limit the potential use of γ-Oryzanol in cheese as a functional ingredient or the manufacturing procedure used.

The free fatty acid profile of both control and treatment samples of cheddar cheese in this experiment did not show any pattern or behavior that can give some idea of the effects of γ-Oryzanol in the cheese. The only thing that can be said is that the FFA profile obtained in this experiment presented a similar FFA profile in percentages basis when compared to data found in other experiments. Therefore, the effects of γ-Oryzanol did not make any significant differentiation in the quality of cheddar cheese during maturation.

ANOVA and post-hoc test determined that all quality parameters evaluated in control and treatment cheese samples had significant differences during maturation. However, the two-sample t-test indicated that in most cases, γ-Oryzanol did not produce significant differences in the quality of the cheddar cheese.

Both R-Index methods (traditional and bipolar) produced some important facts about the sensory evaluation of cheddar cheese containing γ-Oryzanol. First, the consumer, with a significant difference noticed a difference between control and treatment samples not by any chance (probability); the most discriminatory attributes were overall appearance and overall texture which tells that consumer noticed better differences in these attributes than in the others, especially for the overall appearance. The consumers noticed that the treated samples were more yellow than the control; they also were able to differentiate that the treatment sample was harder than the control sample; and last, but not less important, that for the chewiness of the samples, the consumers were not able to differentiate between control and treatment samples.

It can also be said that consumers noticed more presence of the attribute in the treatment sample than in the control sample for the yellowness and hardness. Also, even if consumers

could notice differences in the chewiness between the control and treatment samples, they were not able to notice whether these differences were more or less of the attribute in question.

The most discriminatory directional attribute was the hardness of the samples which tells that consumer noticed better positive or negative differences in this attribute than in the others. The most discriminating attribute of all was the overall appearance, and the least discriminating was the yellowness.

In other words, consumers were able to differentiate between the treatment containing γ-Oryzanol and the control (normal cheddar cheese), based on overall appearance, overall color, odor, taste, overall texture, yellowness, hardness and chewiness, but mainly with overall appearance, texture, taste and hardness.

By means of the McNemar test, it was found out that the decision of buying the cheddar cheese containing γ-Oryzanol was influenced with the fact that consumers had been informed about the potential health benefits of the product. This test also found out that the probability consumers would buy the product after they had been informed about the health benefits of it was 0.13 to 0.30 times higher than the probability of consumers buying it before they had been informed about the health benefits. So, there was a significant positive increase in the purchase intent of cheddar cheese containing γ-Oryzanol after consumers had been informed about the health benefits of this compound.

The consumer acceptance test of cheese containing γ-Oryzanol indicated an existence of significant differences in the attributes of odor/aroma, taste, overall texture/mouthfeel and overall liking of both samples but not for overall appearance and smoothness of color.

Consumers gave lower scores to the cheese containing γ-Oryzanol than to the control samples which means that they liked less the cheddar cheese with extra health benefits with γ-

Oryzanol than the normal cheese. Consumers liked at the same level the overall appearance and color of the cheeses, but they differentiated between sample when evaluating the aroma, taste, mouthfeel and overall liking.

From the DDA, it was found out that the overall liking attribute was the most discriminant attribute for cheese which suggests that this attribute is contributing more to the sensory evaluation of cheese, followed by taste, then mouthfeel and aroma. The least discriminant attribute for cheese was the overall appearance meaning that this attribute does not contribute in anything for the sensory evaluation of it.

The logistic regression analysis has shown overall liking is the most important attribute for changing the acceptability of the product, and if the overall liking scores increased 1.0 unit from the 9-point hedonic scale, the probability of the product to be acceptable would increase 2.85 times.

Evaluating the purchase intent before consumers are informed of the health benefits of the product determined that overall liking is still the most important attribute for changing the purchase intent, followed by overall texture/mouthfeel; however, if the overall liking score increased 1.0 unit from the 9-point hedonic scale, the probability of the product to be purchased would increase 3.39 times which is higher than 2.85 for acceptability. Also, if the overall texture/mouthfeel score augmented 1.0 unit, the probability of the product to be purchased would increase 2.24 times.

When evaluating the purchase intent after consumers are informed of the health benefits of the product, the logistic regression determined that overall liking was still the most important attribute for changing the purchase intent, followed by the overall appearance. However, only overall liking shows significant differences so overall appearance is not useful; and if the overall

liking score increased 1.0 unit from the 9-point hedonic scale, the probability of the product to be acceptable would increase 1.58 times after knowing the health benefits of it.

This value of 1.58 is smaller than the values when purchase intent before knowing about the health benefits was evaluated (3.39), which shows that consumers are willing to compromise the overall liking of the cheese merely to get the health benefits of the product.

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APPENDIX A. FIGURES AND TABLES OF ANALYSES RESULTS

a. TEXTURE

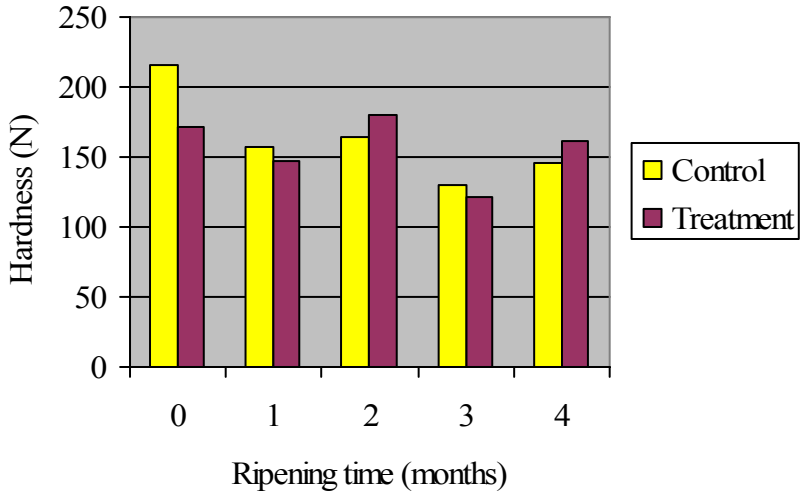


Figure 4.5: Hardness of control and treatment cheese samples during maturation.

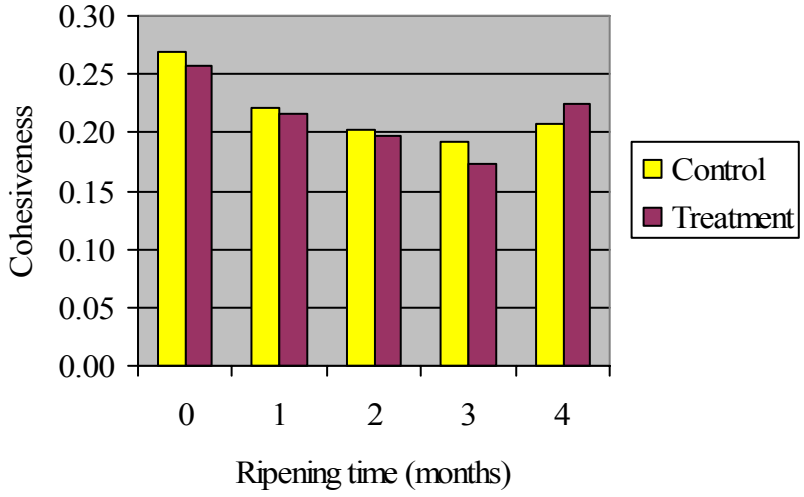


Figure 4.6: Cohesiveness of control and treatment cheese samples during maturation.

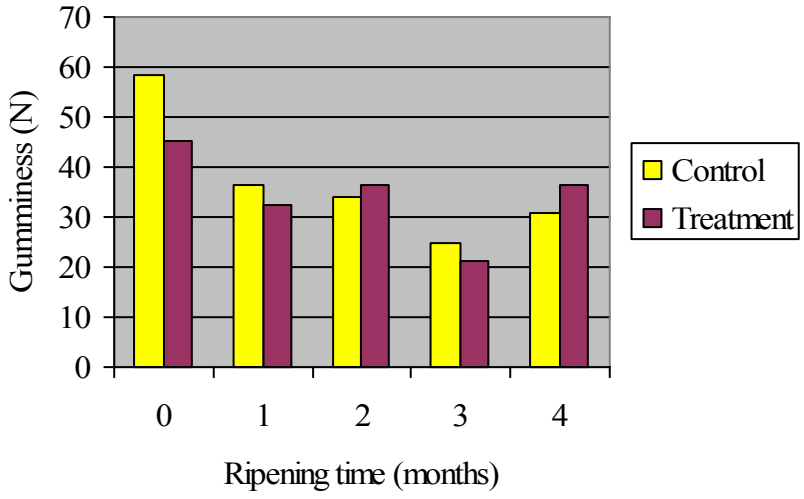


Figure 4.7: Gumminess of control and treatment cheese samples during maturation.

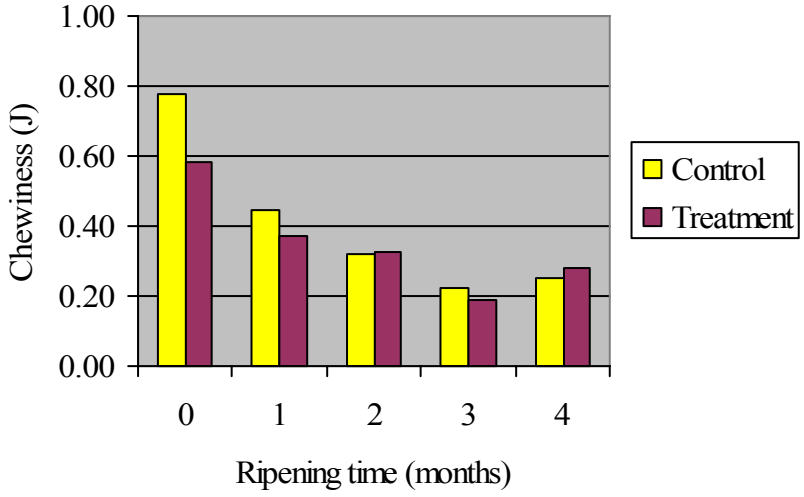


Figure 4.8: Chewiness of control and treatment cheese samples during maturation.

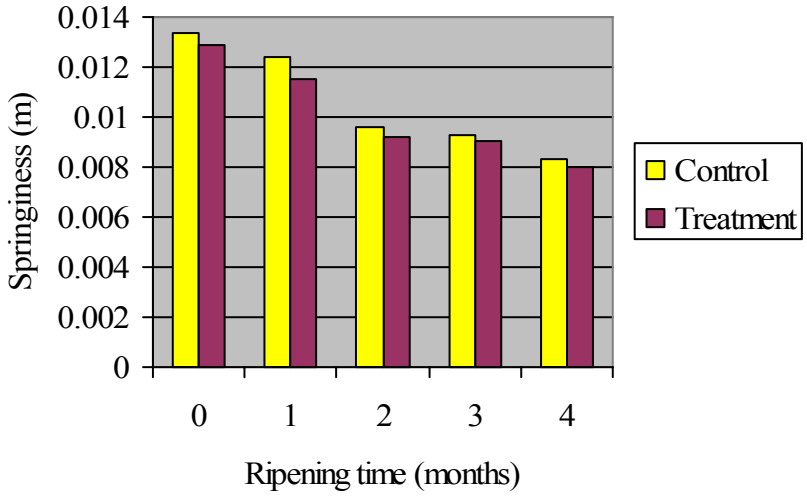


Figure 4.9: Springiness of control and treatment cheese samples during maturation.

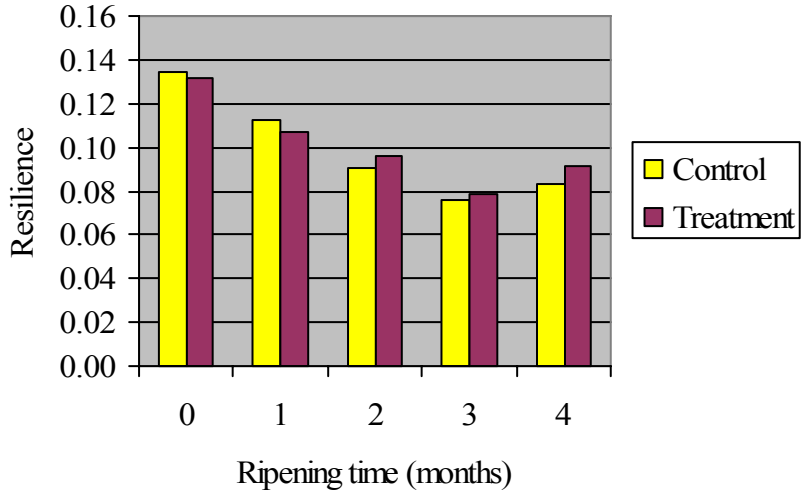


Figure 4.10: Resilience of control and treatment cheese samples during maturation.

b. MICROBIAL GROWTH

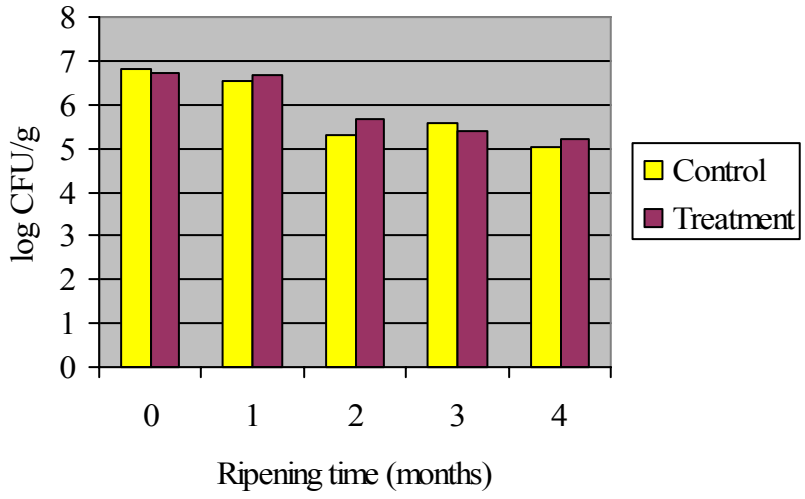


Figure 4.11: Total aerobic bacteria growth in control and treatment cheese samples during ripening.

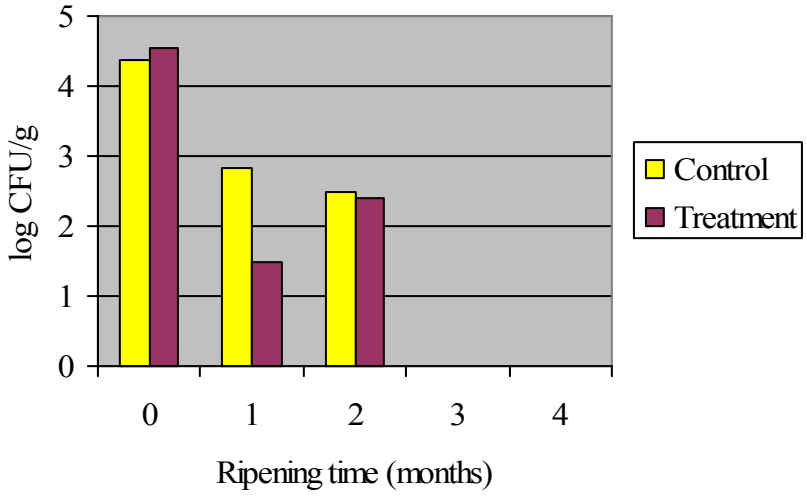


Figure 4.12: Total coliform growth in control and treatment cheese samples during maturation.

c. AROMA DEVELOPMENT

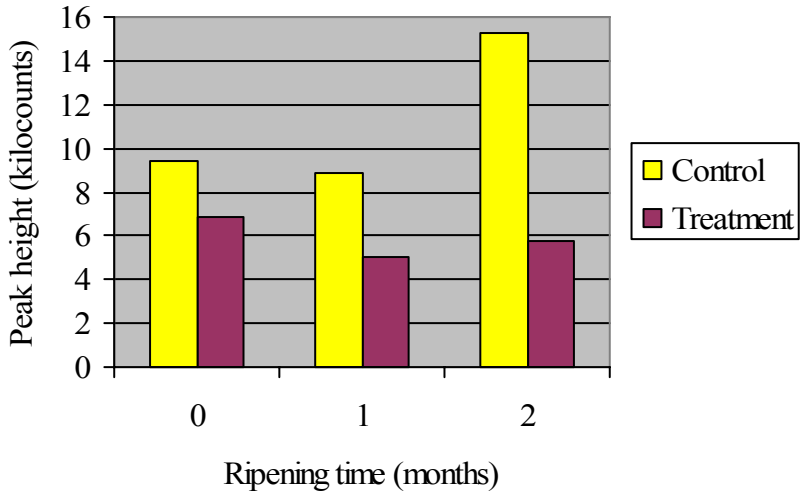


Figure 4.13: Aroma development of peak 1 in control and treatment samples during ripening.

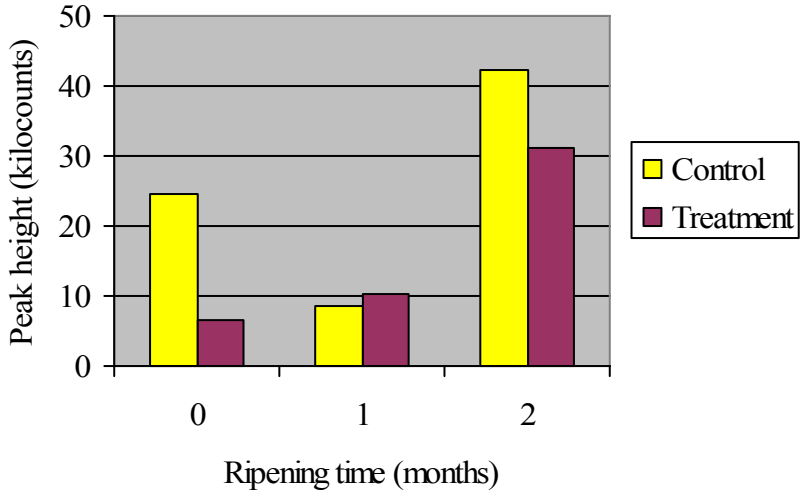


Figure 4.14: Aroma development of peak 2 in control and treatment samples during ripening.

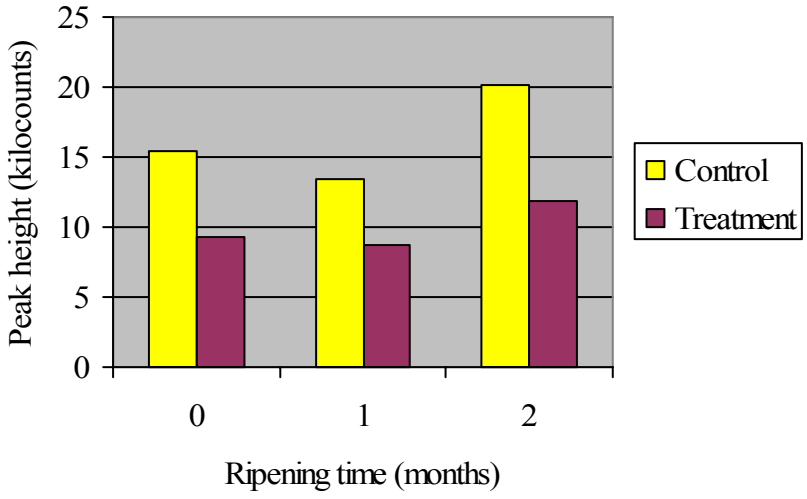


Figure 4.15: Aroma development of peak 3 in control and treatment samples during ripening.

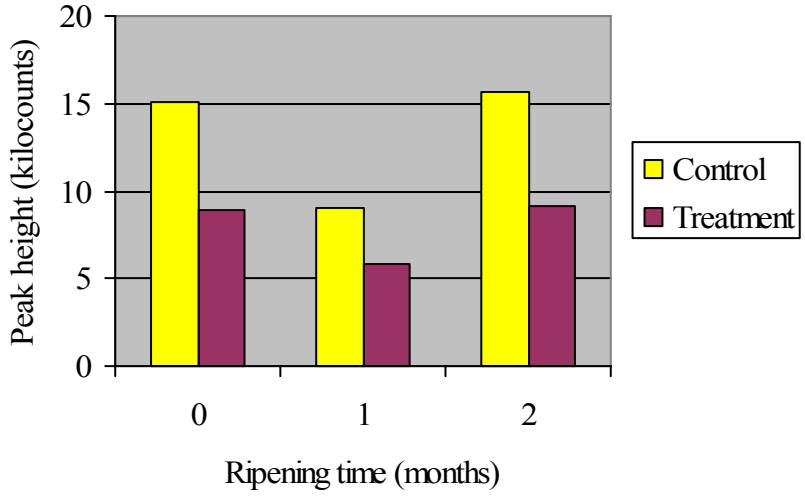


Figure 4.16: Aroma development of peak 4 in control and treatment samples during ripening.

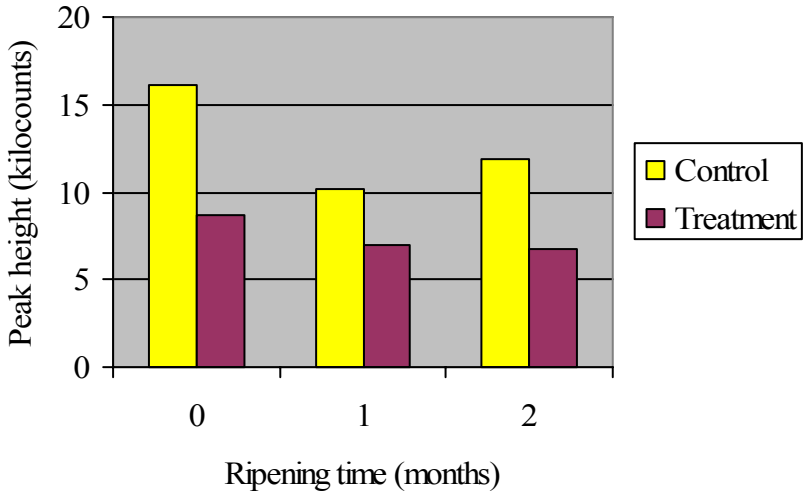


Figure 4.17: Aroma development of peak 5 in control and treatment samples during ripening.

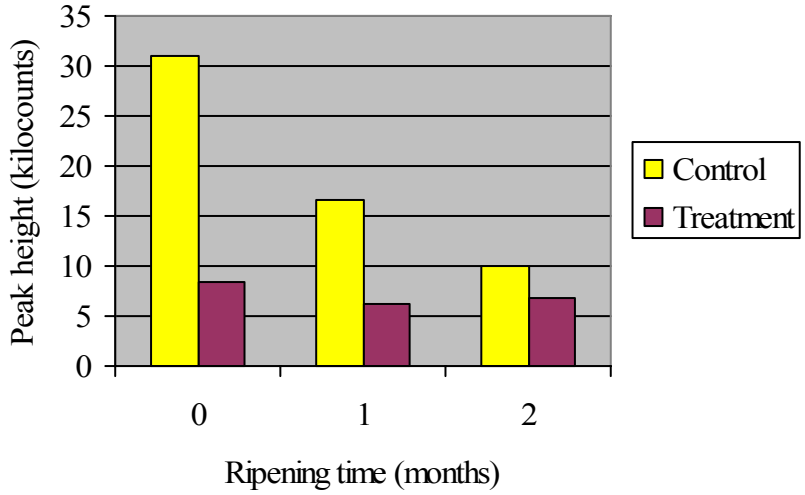
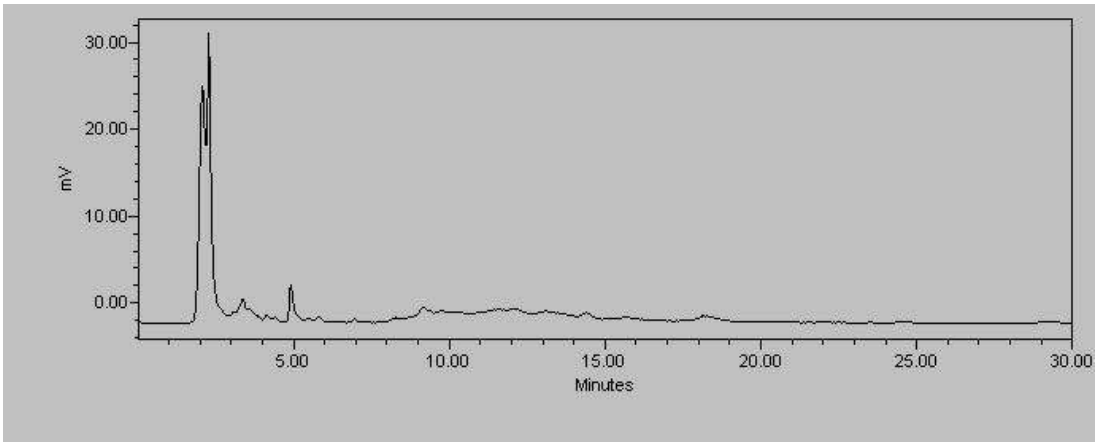
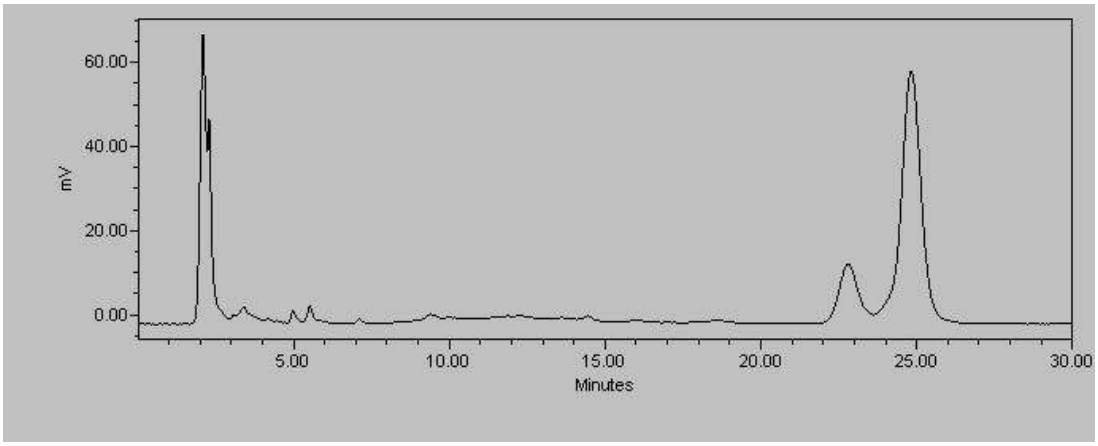


Figure 4.18: Aroma development of peak 6 in control and treatment samples during ripening.

d. ORYZANOL RETENTION



Control



Treatment

Figure 4.19: Chromatography of oryzanol in cheese.

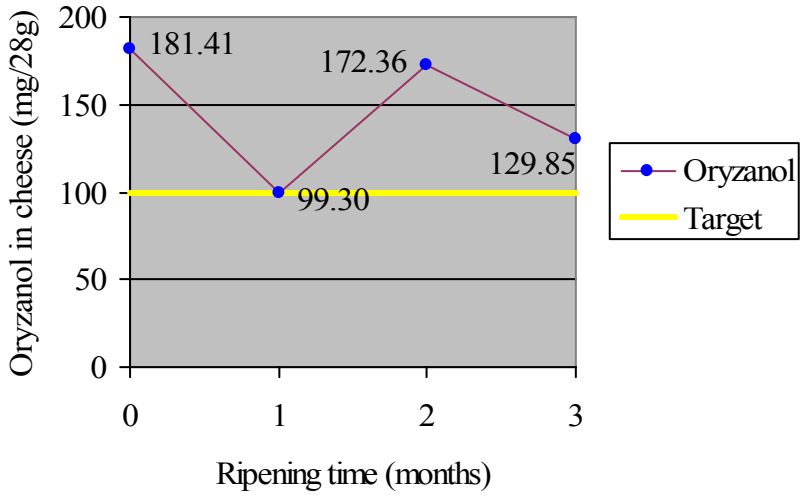


Figure 4.20: Oryzanol retention in treatment samples during maturation (Target=100mg).

e. MOISTURE CONTENT

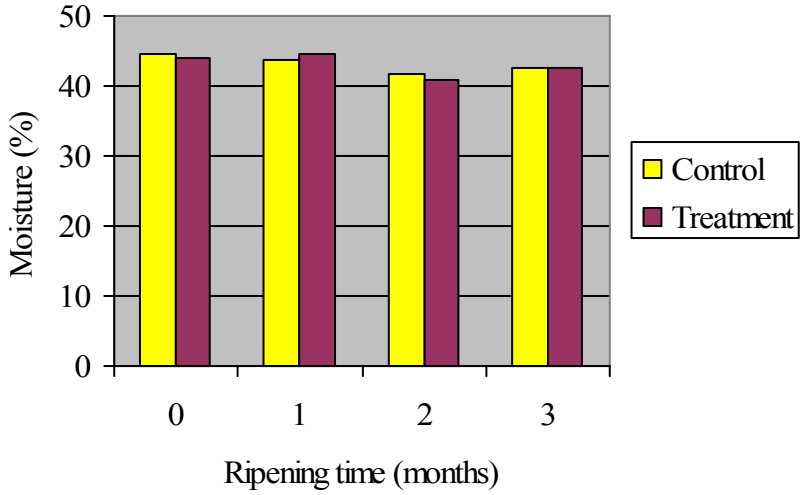


Figure 4.21: Moisture content in control and treatment cheese samples during ripening.

f. FREE FATTY ACID PROFILE

Table 4.29: Means and standard deviations of main free fatty acids in control samples of cheese without γ-Oryzanol at different stages of maturation.

Month	Free fatty acid (mg/Kg)*												
	C4:0	C6:0	C8:0	C10:0	C12:0	C14:0	C14:1	C16:0	C16:1	C18:0	C18:1	C18:2	C18:3
0	0.00 (0.00)^{b}	8.53 (2.77)^{b}	3.39 (0.38)^{ab}	2.06 (0.22)^{a}	4.71 (0.56)^{b}	9.82 (1.44)^{a}	2.00 (0.46)^{a}	23.94 (2.64)^{ab}	0.04 (0.01)^{a}	77.76 (9.44)^{ab}	3.42 (0.38)^{b}	2.38 (0.01)^{a}	3.66 (0.68)^{b}
1	0.00 (0.00)^{b}	0.00 (0.00)^{b}	2.84 (0.11)^{ab}	1.63 (0.05)^{a}	3.70 (0.11)^{bc}	4.41 (0.16)^{b}	1.66 (0.02)^{a}	37.25 (4.90)^{a}	1.53 (0.49)^{a}	117.79 (24.24)^{a}	5.48 (0.78)^{b}	2.78 (0.67)^{a}	42.08 (15.90)^{a}
2	0.00 (0.00)^{b}	0.00 (0.00)^{b}	2.84 (0.11)^{ab}	1.63 (0.05)^{a}	3.70 (0.11)^{bc}	4.41 (0.16)^{b}	1.66 (0.02)^{a}	13.35 (5.08)^{b}	5.11 (6.05)^{a}	44.89 (17.58)^{bc}	2.54 (0.08)^{b}	13.22 (5.83)^{a}	4.69 (5.91)^{b}
3	5.88 (1.58)^{a}	1.14 (0.07)^{b}	0.72 (0.06)^{b}	1.71 (0.14)^{a}	1.65 (0.86)^{c}	8.99 (0.76)^{a}	1.40 (0.12)^{a}	29.23 (2.43)^{ab}	1.07 (0.49)^{a}	13.29 (1.17)^{c}	25.66 (2.20)^{a}	3.37 (0.80)^{a}	0.97 (0.54)^{b}

^{a,b,c} Means within same column followed by different superscripts are significantly different ($P<0.05$).

* Also parts per million (PPM) in cheese wet basis.

Table 4.30: Means and standard deviations of main free fatty acids in treated samples of cheese with γ-Oryzanol at different stages of maturation.

Month	Free fatty acid (mg/Kg)*												
	C4:0	C6:0	C8:0	C10:0	C12:0	C14:0	C14:1	C16:0	C16:1	C18:0	C18:1	C18:2	C18:3
0	0.00 (0.00)^{b}	10.28 (1.65)^{a}	3.67 (0.09)^{b}	2.30 (0.17)^{a}	5.08 (0.11)^{a}	12.92 (2.64)^{a}	1.76 (0.30)^{a}	25.39 (0.26)^{a}	0.04 (0.01)^{c}	85.24 (2.67)^{a}	3.08 (0.78)^{a}	2.98 (0.30)^{b}	4.99 (2.53)^{a}
1	0.00 (0.00)^{b}	37.82 (26.40)^{a}	5.24 (0.44)^{a}	1.39 (1.88)^{a}	5.37 (1.06)^{a}	6.49 (0.97)^{b}	0.25 (0.06)^{b}	24.64 (4.42)^{ab}	1.24 (0.46)^{ab}	81.11 (19.11)^{a}	4.12 (1.22)^{a}	2.06 (0.51)^{b}	30.04 (17.34)^{a}
2	0.00 (0.00)^{b}	0.00 (0.00)^{a}	2.60 (0.34)^{b}	1.50 (0.15)^{a}	3.39 (0.27)^{a}	4.00 (0.28)^{b}	1.64 (0.29)^{a}	15.76 (0.65)^{b}	0.80 (0.09)^{bc}	51.57 (2.88)^{ab}	17.28 (21.46)^{a}	16.69 (5.94)^{a}	0.46 (0.00)^{a}
3	5.71 (0.09)^{a}	1.12 (0.07)^{a}	0.71 (0.04)^{c}	1.67 (0.10)^{a}	3.57 (0.61)^{a}	8.70 (0.45)^{ab}	1.36 (0.07)^{a}	27.99 (1.33)^{a}	1.82 (0.08)^{a}	12.79 (0.57)^{b}	24.71 (1.09)^{a}	3.32 (1.20)^{b}	0.56 (0.02)^{a}

^{a,b,c} Means within same column followed by different superscripts are significantly different ($P<0.05$).

* Also parts per million (PPM) in cheese wet basis.

APPENDIX B. CONSENT FORM FOR CONSUMER STUDY

RESEARCH CONSENT FORM

I, _____, agree to participate in the research entitled "Sensory Difference and Consumer Acceptance of Cheddar Cheese," which is being conducted by Witoon Prinyawiwatkul of the Department of Food Science at Louisiana State University, phone number (225)578-5188.

I understand that participation is entirely voluntary and whether or not I participate will not affect how I am treated on my job. I can withdraw my consent at any time without penalty or loss of benefits to which I am otherwise entitled and have the results of the participation returned to me, removed from the experimental records, or destroyed. One hundred consumers will participate in this research. For this particular research, about 30-35 min participation will be required for each consumer.

The following points have been explained to me:

1. In any case, it is my responsibility to report prior participation to the investigators any allergies I may have.

2. The reason for the research is to gather information on consumer sensory acceptability of cheddar cheese containing oryzanol (rice bran component). The benefit that I may expect from it is a satisfaction that I have contributed to solution and evaluation of problems relating to such examinations.

3. The procedures are as follows: One control and two coded samples will be placed in front of me, and I will evaluate them by normal standard methods and indicate my evaluation on score sheets. All procedures are standard methods as published by the American Society for Testing and Materials and the Sensory Evaluation Division of the Institute of Food Technologists.

4. Participation entails minimal risk: The only risk which can be envisioned is that of an allergic reaction to cheese and rice products. However, because it is known to me beforehand that the foods to be tested contain common food ingredients, the situation can normally be avoided.

5. The results of this study will not be released in any individual identifiable form without my prior consent unless required by law.

6. The investigator will answer any further questions about the research, either now or during the course of the project.

The study has been discussed with me, and all of my questions have been answered. I understand that additional questions regarding the study should be directed to the investigators listed above. In addition, I understand the research at Louisiana State University AgCenter that involves human participation is carried out under the oversight of the Institutional Review Board. Questions or problems regarding these activities should be addressed to Dr. David Morrison, Assistant Vice Chancellor of LSU AgCenter at 578-8236. I agree with the terms above.

_____	_____
Signature of Investigator	Signature of Participant
Date: _____	Witness: _____

APPENDIX C. QUESTIONNAIRE FOR CONSUMER STUDY

DEMOGRAPHIC SURVEY: All information collected will not be identified with your name.

1. What is your age group? (Please check one)

18-24 years____ 25-34 years____ 35-44 years_____
45-54 years____ 55-64 years____ Over 64 years_____

2. What is your gender? Male_____ Female_____

3. Which do you consider yourself to be? (Please check one)

African-American_____ Hispanic/Spanish_____ Other (Please specify)
Asian-American_____ White (Caucasian)_____ _____
Asian_____

4. Level of education? (Please check one)

Less than high school_____ Some college_____ Graduate (M.S., M.A., Ph.D., Ed.)_____
High school_____ Completed college_____

5. Which of these categories best describes your gross 2003 household income? (Please check one)

Under $9,999_____ $10,000 - 19,999____ $20,000 – 29,999____ $30,000 – 39,999____
$40,000 – 49,999_____ $50,000 – 59,999____ $60,000 – 69,999____ $70,000 – 79,999____
$80,000 – 89,999_____ $90,000 – 99,999____ Over $100,000_____

SAMPLE No.__705____

Please evaluate the following attributes of this cheddar cheese.

1. How would you rate the **OVERALL APPEARANCE** of this product?

Dislike Extremely	Dislike Very much	Dislike Moderately	Dislike Slightly	Neither Like nor Dislike	Like Slightly	Like Moderately	Like Very much	Like Extremely
[ ]	[ ]	[ ]	[ ]	[ ]	[ ]	[ ]	[ ]	[ ]
1	2	3	4	5	6	7	8	9

2. How would you rate the **SMOOTHNESS of COLOR (yellowness)** of this product?

Dislike Extremely	Dislike Very much	Dislike Moderately	Dislike Slightly	Neither Like nor Dislike	Like Slightly	Like Moderately	Like Very much	Like Extremely
[ ]	[ ]	[ ]	[ ]	[ ]	[ ]	[ ]	[ ]	[ ]
1	2	3	4	5	6	7	8	9

3. How would you rate the **ODOR/AROMA** of this product?

Dislike Extremely	Dislike Very much	Dislike Moderately	Dislike Slightly	Neither Like nor Dislike	Like Slightly	Like Moderately	Like Very much	Like Extremely
[ ]	[ ]	[ ]	[ ]	[ ]	[ ]	[ ]	[ ]	[ ]
1	2	3	4	5	6	7	8	9

4. How would you rate the **TASTE** of this product?

Dislike Extremely	Dislike Very much	Dislike Moderately	Dislike Slightly	Neither Like nor Dislike	Like Slightly	Like Moderately	Like Very much	Like Extremely
[ ]	[ ]	[ ]	[ ]	[ ]	[ ]	[ ]	[ ]	[ ]
1	2	3	4	5	6	7	8	9

5. How would you rate the **OVERALL TEXTURE/MOUTHFEEL** of this product?

Dislike Extremely	Dislike Very much	Dislike Moderately	Dislike Slightly	Neither Like nor Dislike	Like Slightly	Like Moderately	Like Very much	Like Extremely
[ ]	[ ]	[ ]	[ ]	[ ]	[ ]	[ ]	[ ]	[ ]
1	2	3	4	5	6	7	8	9

6. Did you detect undesirable bitterness aftertaste? YES [ ] NO [ ]

7. Please rate your **OVERALL LIKING** of this product?

Dislike Extremely	Dislike Very much	Dislike Moderately	Dislike Slightly	Neither Like nor Dislike	Like Slightly	Like Moderately	Like Very much	Like Extremely
[ ]	[ ]	[ ]	[ ]	[ ]	[ ]	[ ]	[ ]	[ ]
1	2	3	4	5	6	7	8	9

8. Is this product **ACCEPTABLE**? YES [ ] NO [ ]

9. Would you **BUY** this product if it were commercially available? YES [ ] NO [ ]

10. Would you **BUY** this product if it contained oryzanol, an antioxidant and potential cholesterol reducing compound from rice bran?

YES [ ] NO [ ]

SAMPLE No.__485____

Please evaluate the following attributes of this cheddar cheese.

1. How would you rate the **OVERALL APPEARANCE** of this product?

Dislike Extremely	Dislike Very much	Dislike Moderately	Dislike Slightly	Neither Like nor Dislike	Like Slightly	Like Moderately	Like Very much	Like Extremely
[ ]	[ ]	[ ]	[ ]	[ ]	[ ]	[ ]	[ ]	[ ]
1	2	3	4	5	6	7	8	9

2. How would you rate the **SMOOTHNESS of COLOR (yellowness)** of this product?

Dislike Extremely	Dislike Very much	Dislike Moderately	Dislike Slightly	Neither Like nor Dislike	Like Slightly	Like Moderately	Like Very much	Like Extremely
[ ]	[ ]	[ ]	[ ]	[ ]	[ ]	[ ]	[ ]	[ ]
1	2	3	4	5	6	7	8	9

3. How would you rate the **ODOR/AROMA** of this product?

Dislike Extremely	Dislike Very much	Dislike Moderately	Dislike Slightly	Neither Like nor Dislike	Like Slightly	Like Moderately	Like Very much	Like Extremely
[ ]	[ ]	[ ]	[ ]	[ ]	[ ]	[ ]	[ ]	[ ]
1	2	3	4	5	6	7	8	9

4. How would you rate the **TASTE** of this product?

Dislike Extremely	Dislike Very much	Dislike Moderately	Dislike Slightly	Neither Like nor Dislike	Like Slightly	Like Moderately	Like Very much	Like Extremely
[ ]	[ ]	[ ]	[ ]	[ ]	[ ]	[ ]	[ ]	[ ]
1	2	3	4	5	6	7	8	9

5. How would you rate the **OVERALL TEXTURE/MOUTHFEEL** of this product?

Dislike Extremely	Dislike Very much	Dislike Moderately	Dislike Slightly	Neither Like nor Dislike	Like Slightly	Like Moderately	Like Very much	Like Extremely
[ ]	[ ]	[ ]	[ ]	[ ]	[ ]	[ ]	[ ]	[ ]
1	2	3	4	5	6	7	8	9

6. Did you detect undesirable bitterness aftertaste? YES [ ] NO [ ]

7. Please rate your **OVERALL LIKING** of this product?

Dislike Extremely	Dislike Very much	Dislike Moderately	Dislike Slightly	Neither Like nor Dislike	Like Slightly	Like Moderately	Like Very much	Like Extremely
[ ]	[ ]	[ ]	[ ]	[ ]	[ ]	[ ]	[ ]	[ ]
1	2	3	4	5	6	7	8	9

8. Is this product **ACCEPTABLE**? YES [ ] NO [ ]

9. Would you **BUY** this product if it were commercially available? YES [ ] NO [ ]

10. Would you **BUY** this product if it contained oryzanol, an antioxidant and potential cholesterol reducing compound from rice bran?

YES [ ] NO [ ]

Part I: VISUAL. Please evaluate each cheddar cheese sample by **LOOKING** and comparing it with the labeled **CONTROL** sample.

OVERALL APPEARANCE				
Sample ID	Same I am sure	Same I am not sure	Different I am not sure	Different I am sure
705				
485				

OVERALL COLOR				
Sample ID	Same I am sure	Same I am not sure	Different I am not sure	Different I am sure
705				
485				

YELLOWNESS						
Sample ID	More I am sure	More I am not sure	Same I am not sure	Same I am sure	Less I am sure	Less I am not sure
705						
485						

Part II: ODOR. Please evaluate each cheddar cheese sample by **SMELLING** and comparing it with the labeled **CONTROL** sample.

ODOR				
Sample ID	Same I am sure	Same I am not sure	Different I am not sure	Different I am sure
705				
485				

Part III: TASTE AND TEXTURE. Please evaluate each cheddar cheese sample by **TASTING** and **CHEWING** and comparing it with the labeled **CONTROL** sample.

TASTE				
Sample ID	Same I am sure	Same I am not sure	Different I am not sure	Different I am sure
705				
485				

OVERALL TEXTURE				
Sample ID	Same I am sure	Same I am not sure	Different I am not sure	Different I am sure
705				
485				

HARDNESS						
Sample ID	More I am sure	More I am not sure	Same I am not sure	Same I am sure	Less I am sure	Less I am not sure
705						
485						

CHEWINESS						
Sample ID	More I am sure	More I am not sure	Same I am not sure	Same I am sure	Less I am sure	Less I am not sure
705						
485						

APPENDIX D. SAS CODES

a. COLOR AND TEXTURE

```
dm 'log;clear;output;clear';
data one;
input sample $ rep month hard cohes adhes gummi chewi spring resil
        LL      aa      bb      cc      hh;
datalines;
inserted data;
proc sort; by sample month;
proc means mean std n maxdec=2;by sample month;
var hard--hh;
proc sort; by sample;
proc glm;by sample;
class month;
model hard--hh = month;
means month/tukey lines;
proc sort; by month;
proc ttest; by month;
**//independent ttest;
class sample;
var hard--hh;
run;
```

b. MICROBIAL GROWTH

```
dm 'log;clear;output;clear';
data one;
input sample $ rep month logAPC logCOL;
datalines;
inserted data;
proc sort; by sample month;
proc means mean std n maxdec=2;by sample month;
var logAPC--logCOL;
proc sort; by sample;
proc glm; by sample;
class month;
model logAPC--logCOL = month;
means month/tukey lines;
proc sort; by month;
proc ttest; by month;
**//independent ttest;
class sample;
var logAPC--logCOL;
run;
```

c. AROMA

```
dm 'log;clear;output;clear';
data one;
input sample $ month peak1 peak2 peak3 peak4 peak5 peak6;
datalines;
inserted data;
proc sort; by sample month;
proc means mean std n maxdec=2;by sample month;
var peak1--peak6;
proc sort; by sample;
proc glm; by sample;
class month;
model peak1--peak6 = month;
means month/tukey lines;
proc sort; by month;
proc ttest; by month;
**//independent ttest;
class sample;
var peak1--peak6;
run;
```

d. ORYZANOL RETENTION

```
dm 'log;clear;output;clear';
data one;
input sample $ rep month oryzanol;
datalines;
inserted data;
proc sort; by month;
proc means mean std n maxdec=2;by month;
var oryzanol;
proc glm;
class month;
model oryzanol = month;
means month/tukey lines;
run;
```

e. MOISTURE CONTENT

```
dm 'log;clear;output;clear';
data one;
input sample $ rep month moisture;
datalines;
inserted data;
proc sort; by sample month;
```

```
proc means mean std n maxdec=2;by sample month;
var moisture;
proc sort; by sample;
proc glm; by sample;
class month;
model moisture = month;
means month/tukey lines;
proc sort; by month;
proc ttest; by month;
**//independent ttest;
class sample;
var moisture;
run;
```

f. FREE FATTY ACID PROFILE

```
dm 'log;clear;output;clear';
data one;
input sample $ rep month C4 C6 C8 C10 C12 C14 C141 C16
          C161 C18 C181 C182 C183;
datalines;
inserted data;
proc sort; by sample month;
proc means mean std n maxdec=2;by sample month;
var C4--C183;
proc sort; by sample;
proc glm; by sample;
class month;
model C4--C183 = month;
means month/tukey lines;
proc sort; by month;
proc ttest; by month;
**//independent ttest;
class sample;
var C4--C183;
run;
```

g. THE MCNEMAR TEST

```
dm 'log;clear;output;clear';
data one;
Input sample buy buyif count;
datalines;
inserted data;
run;
proc freq; weight count;
```

```
tables buy*buyif/agree; by sample;
run;
```

h. CONSUMER ACCEPTANCE (MANOVA, DDA AND LOGISTIC REGRESSION)

```
dm 'log;clear;output;clear';
data one;
input consumer sample gender appear color  aroma
        taste mouthf bitter olike accept buy buyif;
datalines;
inserted data;
proc sort; by sample;
proc means mean std n maxdec=2;by sample;
var appear color aroma taste mouthf olike;
proc freq; by sample;
tables bitter accept buy buyif;
proc anova;
class sample;
model appear color aroma taste mouthf olike = sample;
means sample/tukey lines;
Proc candisc out=outcan mah;
class sample;
var appear color aroma taste mouthf olike;
Proc logistic data = one;
model accept = appear color aroma taste mouthf olike;
Proc logistic data = one;
model accept = appear;
Proc logistic data = one;
model accept = color;
Proc logistic data = one;
model accept = aroma;
Proc logistic data = one;
model accept = taste;
Proc logistic data = one;
model accept = mouthf;
Proc logistic data = one;
model accept = olike;
Proc logistic data = one;
model buy = appear color aroma taste mouthf olike;
Proc logistic data = one;
model buy = appear;
Proc logistic data = one;
model buy = color;
Proc logistic data = one;
model buy = aroma;
Proc logistic data = one;
```

```
model buy = taste;
Proc logistic data = one;
model buy = mouthf;
Proc logistic data = one;
model buy = olike;
Proc logistic data = one;
model buyif = appear color aroma taste mouthf olike;
Proc logistic data = one;
model buyif = appear;
Proc logistic data = one;
model buyif = color;
Proc logistic data = one;
model buyif = aroma;
Proc logistic data = one;
model buyif = taste;
Proc logistic data = one;
model buyif = mouthf;
Proc logistic data = one;
model buyif = olike;
run;
```

APPENDIX E. CERTIFICATE OF ANALYSIS OF GAMMA-ORYZANOL

MAYPRO

Certificate of Analysis

REF# 12312

GAMMA ORYZANOL
LOT # B0309

Product Date: May 14, 2003

CHARACTERISTICS	RESULTS
Appearance	To Pass Test
Identification	To Pass Test
Content of Oryzanol	98.8%
Arsenic	Not more than 1 ppm
Heavy Metals	Not more than 10 ppm
Loss on Drying	0.07%
Ignition Residue	0.01%
Standard Plate Count (Max. 3000 cells/g)	To Pass Test
Molds & Yeast (Max. 1000 cells/g)	To Pass Test
Coliforms	Negative

Maypro Industries, Inc.
August 14, 2003

*Above data is based on the certificate of analysis as provided by the supplier The information contained herein is offered as a service to our customers and is not intended to relieve a customer from its responsibility to determine the suitability of this information or of the materials described herein for purchaser's purposes to investigate other sources of information, to comply with all laws and procedures regarding the safe use of these materials and to use these materials in a safe manner. No warranty, expressed or implied, is made of the merchantability or fitness of any product; and nothing herein waives any of the Seller's conditions of sale.

Maypro Industries, Inc. 2700 Westchester Avenue, Purchase NY 10577 Tel: 914. 251.0701 Fax: 914.251.0746

VITA

Miguel Ángel Gutiérrez Barberena was born on December 7th, 1975, in Guatemala City, Guatemala. He graduated from high school at Liceo Guatemala, in 1993 and attended Universidad de San Carlos de Guatemala in Guatemala City, where he graduated as a chemical engineer in June 1999, with a thesis in an evaluation of a plant purifier of water for pharmaceutical purposes. Between 2001 and 2002, he completed part of the studies for a Master of Science in food science and technology at Universiteit Ghent in Belgium. In 2002 he was awarded with a Fulbright scholarship by the U.S. government to pursue a Master of Science degree in the U.S. and in the fall of the same year with that scholarship he joined the graduate school in the department of Food Science at Louisiana State University and Agricultural and Mechanical College in U.S. He is a candidate for the degree of Master of Science in food science in August 2004. He has worked for Bayer AG in Guatemala and also holds experience in food and pharmaceuticals quality assurance, unit operations laboratory, and in cattle and coffee farm management. Miguel enjoys traveling, hiking, camping, biking, swimming, playing basketball and socializing.