THE IMPACT OF DEGREE OF MILLING ON THE CONTENTS OF RICE BRAN LIPIDS AND GAMMA-TOCOTRIENOL

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To the Dean of the Graduate School:

I am submitting herewith a thesis written by Kasturi S. Chitre entitled "The Impact of Degree of Milling on the Contents of Rice Bran Lipids and Gamma-Tocotrienol." I have examined this thesis for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Master of Science with a major in Food Science.

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We have read this thesis and recommend its acceptance:

Department Chair

Accepted:

Dean of the Graduate School

DEDICATION

To my parents, Dr. Kalpana and Sudhir Chitre, without you the entire journey of my life would not have been possible. You are my inspiration and encouragement. Thank you for the endless and selfless love, care and support at each and every stage of my life.

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ABSTRACT

KASTURI S. CHITRE

The IMPACT OF DEGREE OF MILLING ON THE OCNTENTS OF RICE BRAN LIPIDS AND GAMMA-TOCOTRIENOL

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The purpose of this research was to study the impact of milling on total lipids and γ-tocotrienol concentrations in rice brans of Cheniere and Francis varieties. The brown rice obtained after dehulling was milled to 4%, 6%, 8% and 9% milling degrees. Total lipids were analyzed using AACC method and γ- tocotrienol was quantified by reverse phase HPLC. The results indicated that Cheniere had significantly higher contents of lipids and γ- tocotrienol than Francis at all degrees of milling ($p \leq 0.05$). It was also found that lipids and γ- tocotrienol content decreased with increasing milling degree from 4 % to 9%. The lipid content of the bran at 4% milling was significantly higher than the bran at 9% milling. The lowest γ- tocotrienol content was found to be 344 μg/g bran in Cheniere and 331.1 μg/g bran in Francis. Thus, γ- tocotrienol rich bran could possibly be obtained by avoiding hard milling or 9% milling degree of brown rice.

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CHAPTER I

INTRODUCTION

Rice is a chief staple for almost half of the globe. Rice is believed to find its history back in India at around 3000BC. The native Indian population discovered it as a wild plant. Further research led to establishment of various cultivation and cooking methods of the grain. Soon, rice got the popularity and spread across the West. Rice is believed to make an entry in Europe in medieval period. The port of entry of rice to America was at Charleston, South Carolina. It was a gift to local farmer by captain of a ship which was destroyed by storm. In 1700, Charleston became a hub for export of rice from America (Essortment, 2002).

The clean white appeal of rice along with the bland taste and cooked soft texture made it popular all across the world. Rice can be called as one of the rare widely consumed food ingredients in the world which is completely allergy free, gluten free and easy to digest. In Today's date rice is grown in all the continents. The only exception is Antarctica where the environmental conditions are adverse for rice growth (Kahlon, 2009).

Asia is the chief rice producer, contributing to almost 60% to the total world rice production. The recent most official reports state that in the year 2008-2009, the world

rice production was approximately 662 million metric tons. Among all the Asian countries, China was the lead producer with 193 million metric tons production of rice and 130 million metric tons consumption, followed by India with 148 million metric tons production and 92 million metric tons consumption and then Indonesia with 57 million metric tons production and 37 million metric tons consumption. United States consumed only 4 million metric tons contributed to almost 1.4% of the total world rice production. In United States, Almost all the varieties are cultivated for the eating purpose (IRRI, 2009; USDA, 1977).

Rice bran is a chief byproduct of rice milling industry. Though designed as a byproduct, it is gaining a rising importance in variety of food applications. Rice bran is widely used in crackers, fiber rich snacks, pastas, bakery foods, beverages, breakfast cereals, juice enhancers, dough conditioner etc. It is equally in demand in pharmaceuticals, medical products and functional foods due to its high nutraceutical content. The recent trend is the nutritious meal replacement drinks which uses stabilized rice bran as one of the major constituents. Due to the high fiber content, it gives bulk to the drink and helps in achieving the satiety as well as its high nutritional profile including phytochemicals such as tocopherols, tocotrienols, γ-oryzanol (Kahlon, 2009).

Tocotrienols, members of the Vitamin E family, are gaining a rising attention for their wide spectrum health benefits. Among all the four isomers of tocotrienols, γ-tocotrienol is found to be the most stable isomeric form (Shin, Godber, Martin, & Wells,

1997). At the same time, rice bran is the second highest source of γ- tocotrienol followed by palm oil (Slover, 1971; Whittle, & Pennock, 1967).

Relevance of Research

Degree of milling is a critical factor affecting the nutritional value of the bran. Several researches have been conducted to study the impact of degree of milling on the protein, starch, mineral and thiamin content. However, the data on the impact on lipid soluble bioactive nutraceuticals such as γ- tocotrienol is still waiting to be explored completely.

Undermilling could result in incomplete extraction of the nutraceuticals from the brown rice into the bran due to partial removal of the bran layers. On the other hand, overmilling could result in the addition of starches and other substances to the bran which could decrease the relative concentration of γ- tocotrienol in the bran. Therefore, to optimize the contents of these valuable nutraceuticals in the bran, it is essential to understand how and to what extent, a degree of milling could affect the concentrations of total lipids as well as the lipid soluble phytonutrient, γ- tocotrienol.

Therefore, the purpose of this study is to determine the impact of different degrees of milling on the nutritional quality of the rice bran in terms of total lipid content and the γ- tocotrienol concentration.

The exact location of these lipid soluble phytonutrients is unknown. However, few researches have suggested that they might be present in the bran layers of a rice caryopsis. This information would be helpful to predict the location of these lipid bodies inside the rice kernel.

There is no single universally accepted method for the analysis of γ- tocotrienol from the rice bran sample. The analytical procedures for determination of γ-tocotrienol and γ-oryzanol are new and have not been fully established. Therefore, an additional purpose of this study is to determine the best suitable analytical method for quantification of γ-tocotrienol in the rice bran. Since, our research staffs including those in nutrition area have been conducting research in tocotrienols, their valuable knowledge and expertise combined with those of the outside laboratory staff will be employed to examine, verify and determine the best possible method for the analysis of these micronutrients in the rice bran.

Objectives

1. To select a methodology that can be adapted and implemented on the new High Performance Liquid Chromatography equipment in our facility for quantification of γ-tocotrienol content in rice bran.
2. To determine the impact of the four different degrees of milling on the total lipid content, using AACC approved method and concentrations of γ-tocotrienol using High Performance Liquid Chromatography (HPLC).

CHAPTER II

REVIEW OF LITERATURE

Rice Anatomy

Rice is a chief staple for more than half of the world's population and rice bran is a major by product of the rice processing industry. A rice kernel consists of various layers which are distinctively separated from each other. These layers include hull, pericarp, seed coat, nucellus, aleurone layer, embryo/germ, and endosperm. As shown in figure 1, hull is the outermost layer of the kernel which serves as a protective covering to the brown rice against insects and humid conditions. Hull contains high quantities of fiber and silica with trace amounts of some minerals. It contributes to 18-20% weight to a rough rice kernel. Beneath the hull lie 4 different layers of bran called as pericarp, seed coat also called as tegmen, nucellus and aleurone (Marshall, & Wadsworth, 1994). These four layers along with an embryo comprise the rice bran which contributes to 8-12 % of the brown rice weight (Cosmetic Ingredient Review, 2006). Aleurone is made up of one to five layers of parenchymal cells. The number of the cell layers in aleurone layer might vary for each variety. However, it is a constant finding that the dorsal side of the grain has thicker aleurone layer as compared to the ventral side (Del Rosario, Briones, Vidal, & Juliano, 1968). The soft germ or embryo comprises 2-4% of the brown rice caryopsis and contains high amounts of proteins and lipids.

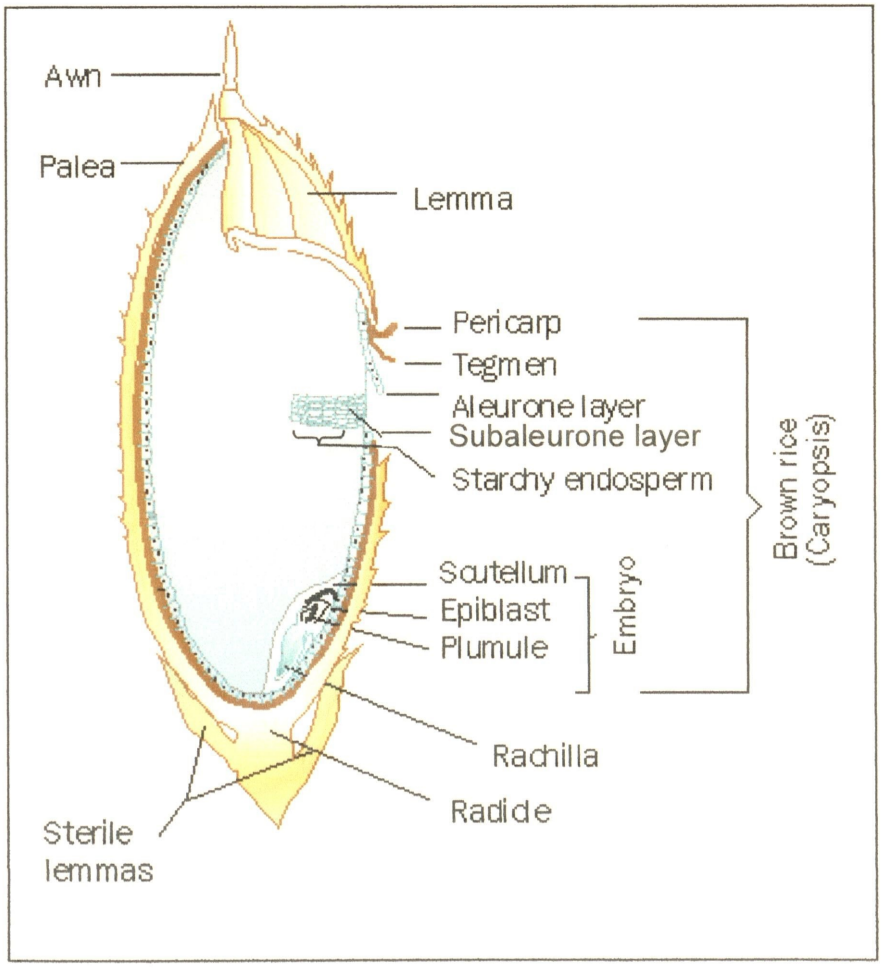


Figure 1. Rice kernel structure (Kennedy, Burlingame, & Nguyen, 2002)

During milling, germ is ground and mixed with other bran layers. Therefore, the lipid content of the rice bran is also contributed by embryo (Vasan, Venkatesan, Kousalya, Ganeshan, & Subramanyan, 1979). If milling is continued, it results in removal of the aleurone layer, leaving behind a subaleurone layer and a starchy endosperm which are collectively called as 'polish', comprising 3-4% of the brown rice on the weight basis. Subaleurone layer contributes to most of the protein content of the kernel with some lipid

bodies, whereas the starch content is attributed by the starchy endosperm. Endosperm has some amounts of proteins but not very significant amounts of lipids. The lipid found in endosperm is preliminary a structural part of the protein or starch molecule and it does not contain vital lipid soluble nutraceuticals such as tocotrienols, tocopherols, γ-oryzanol and other sterols which are found in appreciable amounts in bran layers. Rice bran thus contains the bran layers, germ and the polish (Marshall, & Wadsworth, 1994). The quantity of the bran layers in a rice kernel is dependent on a verity of factors such as environmental condition, agricultural practices, kernel maturity, kernel thickness etc. (Chen, & Bergman, 2005; Mohapatra, & Bal, 2007).

Rice Processing

When rice is harvested from the farm, it is called as a paddy or rough rice which has hull layer intact on the rice kernel. Typically, rice is harvested at moisture level of approximately 20%. It is then dried in farm scale column dryers to a desired moisture level which usually ranges from 12-14% (D.R. McCaskill, Personal Communication, January 14, 2010). Following the drying process, rough rice is dehulled to remove the inedible hull layer to give brown rice which still has the bran layers intact on it. Brown rice is then milled using commercial grain milling equipments to give white rice and bran as a byproduct. The chief purpose of milling is to free the starchy endosperm from all the surrounding layers and embryo (Wadsworth, 1994). Commercially, further processing such as polishing might be required to meet the customer demands and quality standards

(USA Rice Federation, 2010). The commercial processing of rice is illustrated in figure 2.

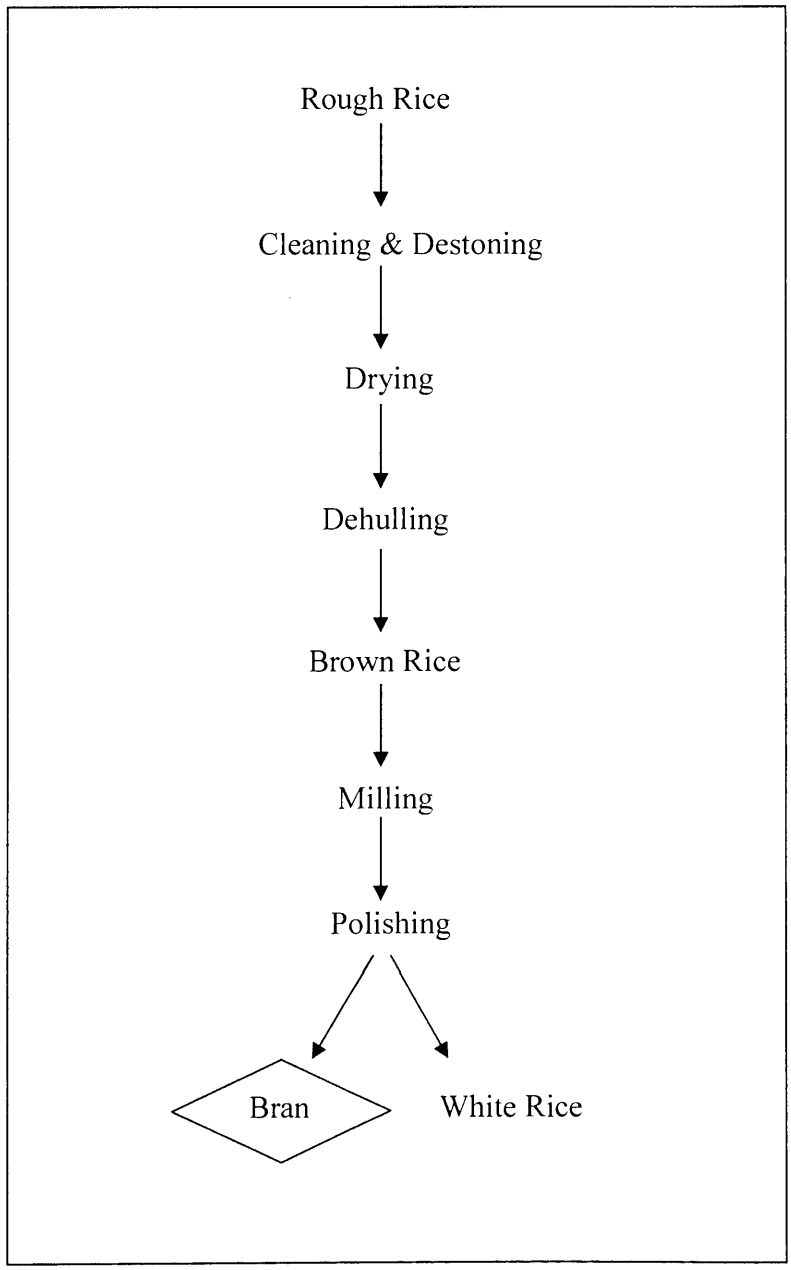


Figure 2. Significant steps involved in commercial rice processing (Orthoefer, 2005).

Degree of Milling

Definition

Degree of milling is defined as the extent to which the bran, germ and polish have been separated or removed from the brown rice kernels during the milling process (Chen, & Bergman, 2005).

Measurement of Degree of Milling

Various methods have been developed for determining the milling degree of the rice. The milling degree can be measured quantitatively such as percentage bran removal, surface lipid content or qualitatively such as visual judgment, staining methods etc. However, there is no single approved method for defining milling degree which is agreed by everybody (Pan, Amaratunga, & Thompson, 2007). Thus, selection of the method to judge the milling quality is subjected to individual discretion. Some of the commonly practiced methods for assessment of the degree of milling are as explained below.

Quantitative/ objective methods. The most widely practiced quantitative methods for estimation of milling degree are as follows.

1. *Percent bran removal:* Prediction of the degree of milling based on the percent weight loss of brown rice due to removal of bran is most popular technique practiced by research purposes. As the results are based on the changes in the weight of the samples before and after milling, it is one of the most

accurate, precise, reliable and reproducible method from scientific aspect. It is most commonly practiced where rice sample are milled in batches for research experiments (Wadsworth, 1994). Usually, 8-10% of the removal of kernel weight as bran is commercially acceptable (D.R. McCaskill, Personal Communication, January 14, 2010).

2. *Colorimetric methods:* Various colorimetric techniques are used to estimate the intensity of color of the milled rice. A well milled rice grain would be whiter and lighter and have higher *L* value (Lightness) as compared to an under milled rice.

3. *Surface lipid content:* Assay of the milled rice surface lipids is one of the commonly practiced techniques for the prediction of the milling degree. An organic solvent such as hexane is usually used to dissolve and extract lipids from the surface of the rice grain. Surface lipid content degreases with higher degree of milling. It is usually expressed as percent surface lipids of the milled rice.

4. *Satake milling meter:* This equipment measure the light reflected and absorbed by rice samples and gives the objective data for the prediction of milling degree, whiteness and transparency of the milled kernels (Satake, 2010; Wadsworth, 1994)

Qualitative/ subjective methods. The most widely practiced quantitative methods for estimation of milling degree are as follows.

1. *Direct visual estimation:* Estimation of the extent of milling by direct visual observation with naked eye or with the use of some magnification device is the most inexpensive methods. This is the official method adopted by Federal Grain Inspection Service (FGIS), USDA. Milled rice samples are observed carefully for their color and compared with the standards which are already set. FGIS has defines three standards for degree of milling; i.e. reasonably well milled, well milled and hard milled.

    a. Reasonably well milled rice would have germ and bran removed. However, some bran layers can still be visible as patches or streaks.

    b. Well milled has bran and germ completely removed along with some amount of endosperm.

    c. Hard milled rice can also be understood as the over milled rice in which all the bran layer and outer endosperm layer has been completely removed and thus, it is the whitest one among all the three variations (USA Rice Federation, 2010).

2. *Staining:* Various dyes can be used for staining such as methylene blue, congo red, Sudan III, alkaline alcohol, a mixture of eosine and methylene blue

etc. The principle behind this method is that if the milling is inadequate to remove all the bran from the kernel, the residual bran left behind on the surface of the grain would colored differently the endosperm.

3. *Iodine test:* In this test, rice kernel is immersed in iodine solution. This results in bran layer becoming yellow against the blue background which can be visually assessed to judge the milling degree.

4. *New May-Grunwalg (NMG) method:* It is a selective dye staining method in which each part of the rice kernel is shows different colors after staining. It uses a mixture of 2 dyes, eosin yellow and methylene blue dissolved in methanol. After the staining is complete, Outer bran layers are green, inner bran layer and endosperm are colored blue and endosperm is pink. Thus, the extent of milling can be assessed by the observation of the colors on the stained rice grain (Wadsworth, 1994).

Significance of Degree of milling

Impact on the functional and cooking properties. Rice is valued for its eating quality (USDA, 1977). Therefore, it becomes very essential to mill rice to an appropriate degree where it retains most of its functional and cooking properties. If the rice is under milled, it would have the bran layer covering the inner rice kernels. A bran layer is rich in fibers and contains less amount of starch. Thus, it reduces the water absorption during cooking. It has seen that, when bran layers are completely removed from the rice kernels,

it results in more water uptake during cooking increasing length and volume of a rice grain. On the other hand, over milled rice has higher starch content which affects the starch pasting properties of rice. Degree of milling is very critical in cooking properties of rice as it affects certain other parameters such as maintenance of the identity of the grain, interaction with other ingredients, cooking time etc. (Mohapatra, & Bal, 2007; USA Rice Federation, 2009).

Degree of milling also affects the shelf life and storage properties of rice. If rice is undermilled, the high lipid content coming from the bran layers on the surface of a kernel could contribute to the rancidity (Roberts, 1979).

Impact on the sensory properties. Milling degree plays an important role in determining the sensory properties of the rice. As the outer seed coat and the bran layers are tough and fibrous, they alter the texture, flavor, aroma and taste of rice which usually is not preferred by consumers. Therefore, commercially rice is well milled and then polished to remove the bran completely form the kernels (Roberts, 1979). Additionally the lipid content of the rice also affects the uptake of spices, seasoning and flavor components inside the kernel (USA Rice Federation, 2010).

Color is another important sensory attribute that affects the acceptability of the product. It has been observed that color pigments are accumulated more in bran layers as compared to an endosperm. As a result, higher the milling degree, whiter is the rice and whiter rice has more commercial value (Lamberts et al., 2007).

Impact on the nutritional properties. Milling degree is an important parameter determining the nutritional quality of white rice as well as its co-products, bran. Various researches are being conducted to evaluate the impact of degree of milling on specific nutrient content of the bran. Lamberts et al. (2007) found that when the bran was obtained from the brown rice milled at various degrees, the contents of protein and minerals was decreased whereas starch concentration was increased as the milling was increased. This indicates that proteins and minerals are found more towards the surface of the brown rice whereas starch is concentrated more in the endosperm. Similar results were reported by Roberts (1979) where the undermilled rice was found to contain more protein, fats, fibers, minerals and vitamins as compared to well milled white rice indicating that bran layers stores most of the nutrients of the rice kernel.

When rice is milled to a higher extent, it not only decreases the nutritional quality of the white rice reaching to the consumers but also reduces phytochemical contents of the bran by diluting it with more starch from the endosperm. Ha et al (2006) analyzed the changes in the lipid soluble nutraceuticals such as tocopherols, tocotrienols, γ-oryzanol, octasanol, squalene and other phytosterols at different degrees of milling. The results showed that the total lipids, tocopherol, tocotrienols, γ-oryzanol, squalene and octasanol concentrations in the rice bran decreased significantly with the increased milling. Similar findings were reported by few other researchers where the phytochemical concentrations especially tocopherols, tocotrienols and γ-oryzanol content was decreased with higher degree of milling. The extent of the impact of milling degree on the nutritional quality

might vary with the rice variety. (Chen & Bergman, 2005; Lloyd, Siebenmorgen, & Beers, 2000; Rohrer, & Siebenmorgen, 2004; Schramm, Abadie, Hua, Xu, & Lima, 2007). On the other hand, it is also seen that tocotrienols and γ-oryzanol exhibit higher concentration gradient than tocopherols throughout the various layers of bran (Chen, & Bergman, 2005). Therefore, to optimize the contents of these valuable nutraceuticals in the bran, it is essential to understand how and to what extent, a degree of milling could affect the concentration of total lipids as well as the lipid soluble phytonutrients such as tocotrienols, tocopherols and γ-oryzanol.

Nutritional Value of Rice Bran

Rice bran is perhaps nutritionally most essential part of a rice kernel. Rice bran stores almost 60% of the nutrients of a rice kernel. It is nature's way to store the nutrients in bran which could be used by the seed during germination and seed growth. Rice bran serves as a good source of proteins, fat, fibers, minerals and vitamins. The nutritional information of rice bran is given below in table 1 and 2.

Table 1

Proximate Composition of Rice Bran

Nutrients	Content
Calories	90 – 100 Kcal/oz bran
Moisture	8 – 12 %
Protein	12 – 16 %
Fat	17 – 22
Crude fiber	8 – 12 %
Ash	7 – 10 %

Source: Orthoefer, 2005

Rice Bran Lipids

The lipids present in a rice kernel can be identified as starch and nonstarch lipids. Starch lipids are located in starchy endosperm and mainly found as lipids attached to protein and starch molecule as a part of their structure. The most common starch lipids are lysophospholipids, glycolipids, along with some triacylglycerols and free fatty acids. (Choudhury, & Juliano, 1980; Orthoefer, 2005). However, the majority of the lipids of the brown rice kernels are nonstarch lipids which are located in the aleurone, subaleurone and germ layers. Lipids in the brown rice are found in the form of spherosomes or

droplets with varying sizes across the kernel. The lipids droplets in the aleurone layer are found to be less than 1.5 mm in diameter followed by less than 1.0 mm in subaleurone layer and less than 0.7 mm in a germ. Bran layers contain the highest concentration of non starch lipids which may vary from 37-41%. The major fatty acid in nonstarch lipids were 22–25% palmitic acid (16:0), 37–41% oleic acid (18:1), and 37–41% linoleic acid (18:2) (Shin, & Godber, 1996; Orthoefer, 2005).

Rice bran is also a rich source of minerals and vitamins. The minerals and vitamins profile of rice bran is given below in table 2.

Table 2.

Minerals, Vitamins and Unsaponifiable content of rice bran

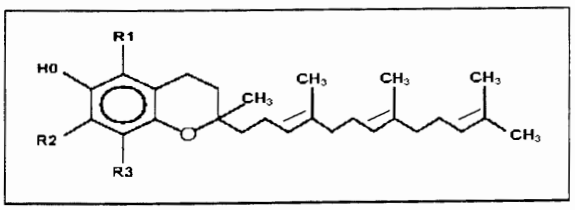
Category	Nutrients	Content
Minerals	Calcium	140 – 1310 µg/g
	Iron	190 – 530 µg/g
	Magnesium	8650 – 12300 µg/g
	Phosphorous	14800 – 28700 µg/g
	Potassium	13650 – 23900 µg/g
	Silicon	1700 – 16300 µg/g
	Sodium	0 – 290 µg/g
Vitamins	Vitamin A	4 µg/g
	Thiamine	10 – 28 µg/g
	Riboflavin	2 – 3 µg/g
	Niacin	236 – 590 µg/g
	Pyridoxine	10 – 32 µg/g
	Pantothenic acid	28 – 71 µg/g
	Folic acid	0.5 – 1.5 µg/g
	Vitamin B_{12}	0.005 µg/g
	Tocopherols	395 µg/g
	Tocotrienols	585 µg/g
Unsaponifiable Matter	Total unsaponifiables	6 %
	Gamma oryzanol	6.42 mg/g

Source: (Lloyd. Siebenmorgen, & Beers, 2000; Orthoefer, 2005; Saunders, 1990; Slover, 1971; Whittle, & Pennock, 1967)

As shown above, the rich nutritional profile of rice bran, particularly the presence of certain valuable phytochemicals such as tocopherols, tocotrienols and γ-oryzanol, makes this rice milling by product a specialty commodity. These unsaponifiable compounds have shown to impart a variety of health benefits. Therefore, efforts are being made for inclusion of rice bran in the diets through cereals, fiber drinks, crackers, pasta, bakery products etc. as a source of the nutraceuticals (Kahlon, 2009).

Tocotrienols

Tocotrienols and tocopherols together with their four isomers form the Vitamin E family. Tocopherols possess longer history in the scientific literature as compared to tocotrienols, which were discovered in 1964 by Pennock and coworkers. A tocotrienol moiety consists of a chromanol ring and an unsaturated farnesyl isoprenoid side chain which contains three double bonds. This unsaturated side chain is assumed to be responsible for various unique biological effects of tocotrienols (Ghosh, Hauer-Jensen, & Kumar, 2009). Tocotrienol exists in four different isomeric forms; alpha, beta, gamma and delta which differ in the number of methyl groups attached to the side chains illustrated in figure 3 (Schauss, 2009).



Isomers	R1	R2	R3
Alpha (α)	CH_3	CH_3	CH_3
Beta (β)	CH_3	H	CH_3
Gamma (γ)	H	CH_3	CH_3
Delta (δ)	H	H	CH_3

Figure 3. Tocotrienol molecules (Carotech. Inc, 2009)

Sources. Palm oil, barley, rice bran, oats are the rich sources of tocotrienols. Among all, palm oil and rice bran contain high amounts of γ-tocotrienol. 100 g of rice bran has 58.5 mg of total tocotrienols out of which 34.9 mg is the γ-tocotrienol.

Table 3.

Sources of Tocotrienols

Sources	**Tocotrienols (mg/1000g)**				**Total Tocotrienols (mg/1000g)**
	alpha	**beta**	**gamma**	**delta**	
Palm oil	205	-	439	94	738
Rice bran	236	-	349	-	585
Wheat germ	24	165	-	-	189
Barley	670	120	120	-	910
Oat	180	-	30	-	210

Source: Slover, 1971; Whittle, & Pennock, 1967

Tocotrienol content in rice bran is dependent on various factors such as environmental conditions, growing conditions, kernel maturity, kernel thickness, storage conditions, milling degree etc. (Chen, & Bergman, 2005). However, it has been observed that γ- tocotrienol is most stable among all the isomers and it can be preserved to a higher extent as compared to other tocols (Shin, Godber, Martin, & Wells, 1997).

Various spectrophotometric and chromatographic techniques have been designed to extract and quantify the concentrations of tocotrienols in rice bran as accurately as possible. The most recent technique is the use of high performance liquid chromatography (HPLC). It is one of the very sensitive and accurate techniques. For the quantification of tocotrienols both normal phase and reverse phase chromatography have been suggested by various researchers. However, reverse phase chromatography is most widely used for the analysis of the lipid soluble, non-polar components (Chen, & Bergman, 2005; Nielsen, & Hansen, 2008; Rogers et al., 1993; Sookwong, Nakagawa, Murata, Kojima, & Miyazawa, 2007).

Health benefits of Tocotrienols. Various researches have focused on the beneficial effects of tocotrienol on the health.

1. *Antioxidan.* Vitamin E isomers are well known for quenching free radicals and thus serving as potent antioxidants. However, all the isomers of tocopherols and tocotrienols differ in their activity. Though α-tocopherol is traditionally used as an antioxidant in a variety of food and cosmetic products, γ-tocotrienol was found to possess 27% higher antioxidant capacity than α-tocopherol (Qureshi, Packer, & Peterson, 2000). It is thought that due to the structural differences in the unsaturated side chain and the methyl groups, tocotrienols can penetrate better and be associated with the cell membrane more closely. It perhaps enhances their free radical quenching ability, serving as a first line of defense against any oxidative agent attacking the cell membrane (Qureshi, Packer,

& Peterson, 2000; Schauss 2009). γ-tocotrienol was also found to be effective than α-tocopherol in reducing triglycerides and fatty acid peroxidation and plasma cholesterol concentrations (Watkins et al., 1993).

2. *Anticancer.* The apoptotic and tumor suppressive properties of different isomers of tocotrienols are being extensively studied. Various researchers in 1990's suggested the anticarcinogenic effects of tocotrienols in human cells. Tocotrienols caused apoptosis in human breast cells and prevented the proliferation of cancerous cells (Yu, Menchaca, Gapor, Sanders, & Kline, 1999). Similar observations of tumor suppressive effects of γ-tocotrienol were reported at the same time. In 1999 an in vitro study in human and murine tumor cells suggested that apoptosis and cell cycle arrest could be initiated by isoprenoids, one of the possible mechanisms of tumor suppressive effects of tocotrienols (Mo, & Elson, 1999).

3. *Neuroprotective effect.* Oxidative damage to brain cells has been associated with a variety of abnormalities and diseases. Oral administration of tocotrienols has shown to inhibit the impact of toxins on the neurons and prevent strokes. This neuroprotective effect of the tocotrienols was found to be both, dependant as well as independent of the antioxidative mechanism (Khanna, Roy, Painandi, Maurer, & Sen, 2006; Sen, Khanna, & Roy, 2009).

4. *Reduction in cholesterol.* 3-hydroxy-3-methyl-glutaryl coenzyme A (HMG-CoA) reductase is a key enzyme in the biosynthesis of cholesterol in human body and inhibiting

the activity of this enzyme is the key step in regulating the cholesterol levels. γ-tocotrienol is found to have a strong inhibitory effect on HMG-CoA reductase which is 30 times greater than α-tocopherol (Pearce, Parker, Deason, Qureshi, & Wright, 1992). The antioxidative function of tocotrienols was also found to be responsible for reducing the harmful oxidative products of cholesterol (Xu, Hua, & Godber, 2001).

5. *Prevention of arterial blockage.* The blockage of the carotid artery is a serious step towards a risk of stroke. A tocotrienol complex derived from palm oil is one of the first few natural nutraceuticals which have shown a positive impact in reversing the atherosclerotic conditions in human. The consumption of 240 mg tocotrienol for 18-36 months have shown to decrease the plaque formation in carotid artery (Carotech Inc., 2009).

6. *Protective effect on gastric health.* Gastric mucosa can be damaged and injured by a wide variety of chemicals such as alcohol, acids, drugs etc. Aspirin is one of the common drugs causing gastric lesions mediated through lipid peroxidation. Due to the ability to inhibit lipid peroxidation, tocotrienol rich palm oil fractions have shown to protect the gastric mucosa from the aspirin induced gastric lesions (Nafeeza, Fauzee, Kamsiah, & Gapor, 2002).

7. *Anti-aging/ Cosmetic uses.* Tocotrienols are more effective antioxidants than tocopherols. They act as a first line of defense to neutralize the free radicals which are formed in our skin due to various factors such as exposure to UV rays, chemicals etc.

Thus, tocotrienols help to protect against the skim damage and aging signs. Tocotrienols enhances the effectiveness of sunscreens containing compounds that reduces the harmful effects of UV rays (Schaffer, Muller, & Eckert, 2005).

8. *Bone health.* γ-tocotrienol was found to exert a protective role on bone metabolism in rats. Vitamin D activation is an important step in bone metabolism homeostasis which is found to be inhibited in Vitamin E deficiency resulting in hypocalcaemia and bone loss. However, the supplements of γ-tocotrienol (60 mg/kg rat weight) and a mixture of α-tocopherol and the tocotrienol were found to normalize the vitamin D metabolism and calcium homeostasis (Norazlina, Ng, & Ima-Nirwana, 2005).

9. *Radiation countermeasure.* A study conducted at Armed Forces Radiology Research Institute suggested that γ-tocotrienol is more efficient in protecting against the radiation damages than α-tocopherol. In this study, a dose of (400 mg/kg body weight) of either α-tocopherol or γ-tocotrienol was given to the mice subcutaneously and after 24 hours of the does, they were exposed to irradiation with Cobalt-60. All the mice in α-tocopherol group died preliminary from gastrointestinal syndrome. However, 40% of the mice survived from γ-tocotrienol group. The protective effect of γ-tocotrienol against the radiation exposure could be attributed to more rapid intestinal epithelial cells absorption of γ-tocotrienol than α-tocopherol (Tsuzuki, Yunoki, & Yoshimura, 2007).

CHAPTER III

MATERIALS AND METHODS

Sample Selection

Two varieties of long grain rice namely, Cheniere and Francis selected for this research as both are high yielding and comparatively recent varieties. In order to have a high quality and reduce variability in the sample, seed rice for both the cultivars were obtained from Stratton Seed Co., Stuttgart, AR. The samples when acquired were packaged and sealed in 50 lb sacks in order to protect them from environmental conditions and thus preserve their quality. All the samples were obtained in the form of rough rice and were not exposed to any dehulling and/or milling and thus had the outer husk intact on the individual rice kernel.

Sample Preparation

250 g of rough rice was dehulled using a lab scale dehuller (Rimac MTH-35A.464-07) to remove the outer husk of the kernels, giving brown rice as an end product. However, to ensure the maximum husk removal from the rice kernels, the brown rice samples obtained after first dehulling were again passed through a second dehulling cycle. At the end of the two dehulling cycles, approximately 17-18% of the weight of rough rice was removed as husk. The immature and non-dehulled rice kernels from

samples were discarded. 200 g of the brown rice was weighed accurately and then milled using a lab scale Miller (Grainman 60-115-60-2AT) to four different degrees of milling i.e. 4%, 6%, 8% and 9% as listed below in the table 4. The degrees of milling were decided based on the bran yield during milling on weight basis which were also matched to Federal Grain Inspection Services, USDA standards for rice quality. Three milling standards were obtained from Federal Grain Inspection Services (FGIS), USDA; reasonably well milled, well milled and hard milled. The color values of these three standards were then recorded using Hunter Color LAB instrument, where *L* is a measure of brightness from black (0) to white (100), + *a* is red, - *a* is green and + *b* is yellow, - *b* is blue. The color values were L=64.76, a=3.16, b=17.16 for reasonably well milled rice, L=72.04, a=1.45, b=16.07 for well milled rice and L=72.95, a=0.97, b=15.63 for hard milled rice. Once the color values were established for three standards, samples of the brown rice were milled to the 3 different degrees that had color values corresponding to the USDA standards (±1 standard LAB reading). In addition, a fourth degree of milling which is 6% bran removal was also standardized as various researches emphasized on this milling degree to be a critical one. Table 4 explains the % bran removal, time of milling for all the four degrees of milling and their comparable USDA standard milling standards.

Table 4.

Degrees of Milling of Brown Rice

Degree of milling (%)	Milling time (seconds)	Approximate Bran yield (g) per 200g of brown rice	Comparable FGIS, USDA standard
4	20	8.0	Reasonably well milled
6	30	12.0	-
8	50	16.0	Well milled
9	60	18.0	Hard milled

To prevent the oxidation, the collected bran was immediately flushed with nitrogen, sealed and stored at -20°C until the analysis (refer figure 4).

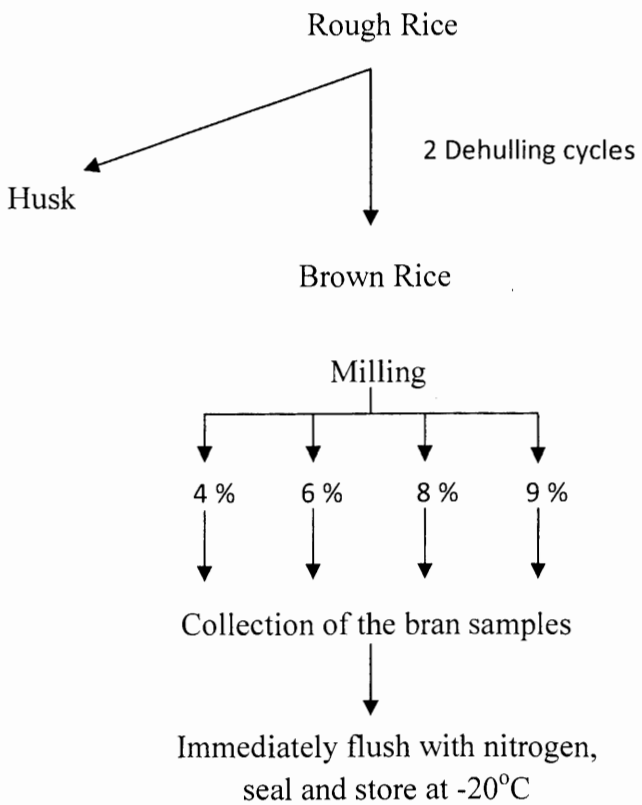


Figure 4. Schematic representation of the sample preparation procedure.

Analyses

γ-Tocotrienol

Determination of γ- tocotrienol was performed using methods suggested by Rogers et al (1993) and Chen and Bergman (2005) for extraction of tocotrienols from the bran and analysis using reverse phase high performance liquid chromatography (RP-HPLC) with necessary modifications in the sample size and additional centrifugation during extraction procedure.

Preparation of calibration curve. A standard of γ- tocotrienol was purchased from ChromaDex.inc, California. A stock solution was prepared by dissolving γ-tocotrienol standard in HPLC grade methanol. This stock solution was then carefully diluted to make different standard concentrations. Three concentration points, 0.02 μg/ml, 0.1 μg/ml and 0.5 μg/ml were used to plot a calibration curve were (figure 7).

Extraction. A Direct Solvent Extraction Procedure described by Chen and Bergman (2005) was used to extract tocotrienols from the rice bran samples with necessary modifications. In the original procedure 50 mg of rice bran was used for extraction procedure. However, in this study 50 mg of rice bran was found to produce more concentrated and cloudy extract. Therefore, sample size was decreased to 25 mg of raw rice bran for extraction. This resulted in an extract that was clearer and had more appropriate concentration of γ- tocotrienol for RP-HPLC analysis.

25 mg of rice bran was mixed thoroughly with 1 ml methanol by vortex mixing at room temperature for 1 minute. It was then centrifuged at 900 x g for 10 minutes. The supernatant was carefully transferred to another vial leaving the rice bran residue at the bottom of the vial. This procedure of methanol addition, centrifugation and supernatant collection was repeated twice to get a total of approximately 3 ml of extract. However, the final extract obtained at the end of the extraction procedure was found to be turbid and had suspending rice bran residues. Therefore, an additional centrifugation was necessary to settle down all the remaining impurities at the bottom of the tube to obtain a clearer extract. This was achieved by centrifugation of the final extract at 14,000 rpm for 10 minutes. The supernatant was filtered through 0.45 μm nylon membrane syringe filter. It was then followed by dilution of the 0.5 ml of the clear extract obtained after the filtration with 4.5ml methanol to give total 5ml of the diluted extract (1:10 dilution). This extract was then flushed with nitrogen, sealed, covered with aluminum foil and stored at -20°C until RP-HPLC analysis. The extraction procedure was performed in triplicates for each sample.

Reverse Phase High Performance Liquid Chromatography (RP-HPLC) analysis. The High Performance Liquid Chromatography system was purchased from Shimadzu Corporation, United States.

1. *HPLC Equipment components.* It included A degassing unit (DGU 20A5, Shimadzu Corporation) and LC 20AD pump (Shimadzu Corporation). A 20 μl of sample

was injected through a manual injector (Shimadzu Corporation) attached to 20 µl injection loop. Separation was achieved on Hypersil ODS column (4.6 mm x 250 mm, 5 µm particle size, Thermo Fisher Scientific Inc.). Hypersil ODS C18 (10 mm length x 4 mm ID, 5 µm particle size, Thermo Fisher Scientific Inc.) was used as a guard column to filter out unwanted components and other impurities that might interfere with the analysis. The output was detected with Fluorescence Detector (RF 10AXL, Shimadzu Corporation). The wavelengths for the detector were set at 298 nm for excitation and 328 nm for emission. Each sample was analyzed a minimum of three times and the best three chromatograms were selected for further calculations.

2. *Mobile Phase.* All the solvents used for analysis were HPLC grade. Mobile phase consisted of a volumetric mixture of acetonitrile: methanol: isopropanol: water (45:45:5:5, v/v) for first 6 minutes. A gradient was achieved by changing the composition of mobile phase linearly over 4 minutes to acetonitrile: methanol: isopropanol (25:70:5, v/v). This condition was maintained for 5 minutes before returning to the initial concentrations. The flow rate was adjusted to 0.8 ml/minute and the total duration of the run was 20 minutes.

3. *Software.* The data was collected and analyzed using LC solution software (Shimadzu Corporation). The HPLC system was controlled by CBM 20 Alite system controller. The results obtained were expressed as µg of tocotrienol/100g of rice bran

Total Lipid Content

Lipid content of the rice bran was determined by solvent extraction procedure using a Soxhlet apparatus as approved by American Association of Cereal Chemists (AACC) method no. 30-20 for estimation of crude fat in grains and stock feeds with required modifications (refer Appendix B). Petroleum ether was used as a solvent for the analysis. As per the requirement of the procedure, moisture content was removed from the rice bran samples by dehydrating them in a convection oven at 105°C for 8 hours. The samples were then removed from the oven and cooled in a desiccator before recording the weight. The procedure was repeated until 3 consecutive constant readings were obtained for the weight of the dehydrated samples. 5 g of the dehydrated bran was weighed carefully and used for further analysis as described by AACC method 30-20. The results obtained were expressed as % lipid content of the rice bran.

Statistical Analysis

Statistical analyses were conducted using SPSS 15.0 software. Two Way Analysis of Variance (ANOVA) was performed on 2x4 factorial design of the study where the independent variable cultivar had 2 levels and degree of milling had 4 levels. Post hoc tests were performed for further analysis of data. The level of significance was set as $P<0.05$.

CHAPTER IV

RESULTS AND DISCUSSION

Total Lipid Content

Total lipids from the rice bran milled at various degrees of milling were measured as % total lipids. As shown in Table 5, the results showed that the % total lipids in bran milled to various degrees ranged from 20.5 to 27.0%. These values agree with the literature where the lipid content of the rice bran has been reported to vary from 16.0 to 28.3 % (Abdul-Hamid, Raja Sulaiman, Osman, & Saari, 2007; Goffman, Pinson, & Bergman, 2010; Saunders, 1990). Overall, Cheniere had significantly higher lipid content than Francis at all degrees of milling.

A Two Way ANOVA with a 2x4 factorial design was performed to analyze the effect of two independent variables; Degree of milling and cultivar/ rice type on the lipid content. The results suggested that the main effect of the variable, rice type or cultivar was significant at the p value of 0.05. However, the effect of cultivar was almost similar for each level of degree of milling. Therefore, there was no significant interaction (p=0.062) between cultivars and degrees of milling (figure 5). The results also suggested that the main effect of the variable, degree of milling was significant at the p value of 0.05 (refer Appendix A).

Table 5.

Total Lipid Content (%) of Raw Rice Bran for Two Cultivars at 4 Degrees of Milling.

Degree of milling (%)	**Mean‡ ± SD**		
	Cheniere	**Francis**	**Average**
4	$27.0 \pm 0.4^{a,x}$	$25.3 \pm 0.6^{a,y}$	$26.2^{a} \pm 1.0$
6	$26.5 \pm 0.5^{a,x}$	$23.6 \pm 0.5^{b,y}$	$25.1^{b} \pm 1.6$
8	$25.7 \pm 0.5^{a,x}$	$22.4 \pm 0.4^{b,y}$	$24.1^{c} \pm 1.8$
9	$23.5 \pm 0.5^{b,x}$	$20.5 \pm 0.7^{c,y}$	$22.1^{d} \pm 1.7$
Average	25.7 ± 1.4^{x}	23.0 ± 1.9^{y}	24.3 ± 2.2

Note:

‡ mean values expressed as % lipids in the raw rice bran

^{a,b,c,d} means values with different superscripts in the same column differ significantly ($p \leq 0.05$)

^{x,y} means values with different superscripts in the same row differ significantly ($p \leq 0.05$)

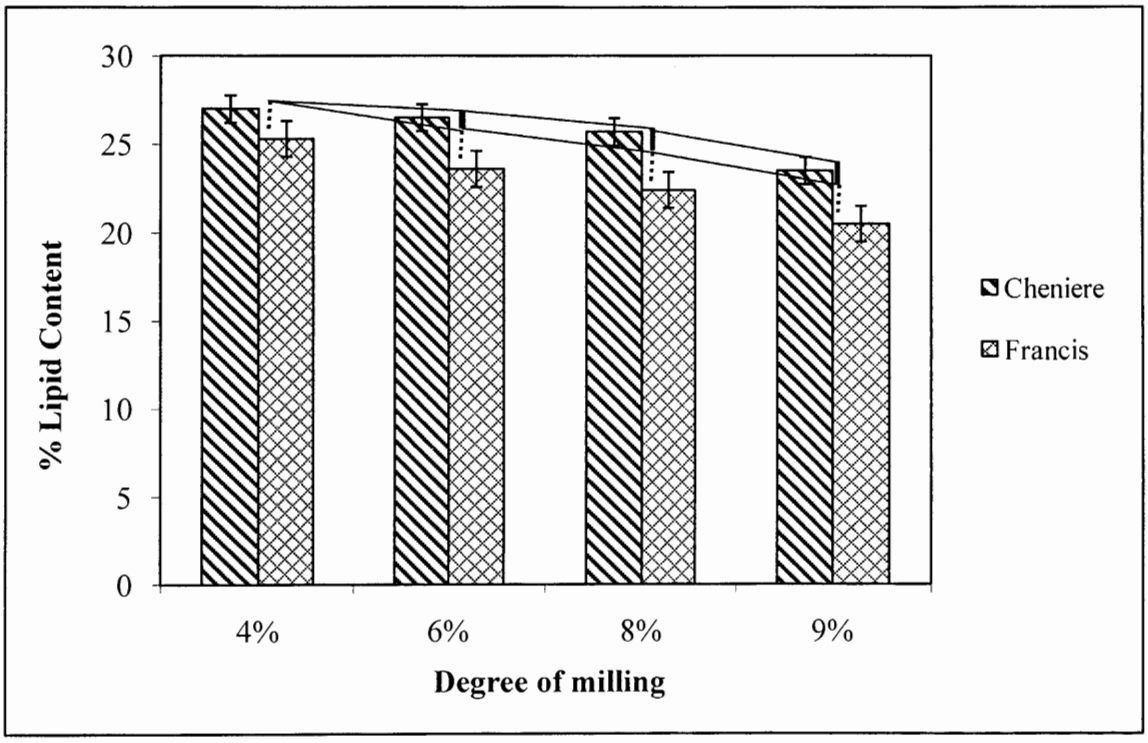


Figure 5. A graphical representation of the % lipid content at all degrees of milling and rice types along with the interaction between the degrees of milling and the cultivars.

The results indicated that the highest % lipids were obtained at 4% degree of milling for both the cultivars which were 27.0% for Cheniere and 25.3 % for Francis (refer table 5). The reason for highest lipid content at 4% milling could be that the bran produced from 4% milling (reasonably well-milled rice) of the brown rice contains a large part of the bran layers only, without any endosperm material (USA Rice Federation, 2010).This makes the rice bran more concentrated in lipids, as the aleurone layer, the innermost of the bran layers is believed to contain a large quantity of lipid bodies (Bechtel, & Pomeranz, 1977).

The overall trend indicated that the % lipid content decreased with the increasing milling degree. Comparing the changes in lipid content from 4 % to 9% milling degrees, Cheniere had 27.0 % lipids at 4% milling degree which decreased to 23.5 % at 9% milling. Similarly, for Franics, the lipid content decreased from 25.3 to 20.5% as the milling degree was increased from 4% to 9%. This reduction in the lipid content from 4% to 9% milling was found to be statistically significant ($p \leq 0.05$). Similar trends were reported by Abdul-Hamid, Raja Sulaiman, Osman, & Saari (2007) where the bran lipid content decreased as the milling was increased.

The lowest lipid contents for both the cultivars were obtained at 9% degree of milling. This milling degree was found be comparable to the 'hard milled' standard defined by Federal Grain Inspection Services, USDA. This milling degree also includes the outer layers of the starchy endosperm (polish), along with the bran layers, making the lipid bodies in the rice bran more diluted. In other words, the bran obtained from the brown rice milled at 9% contains more amyloplasts from the starchy endosperm containing more starch granules which could have resulted in lower % lipid content (Rohrer, & Siebenmorgen, 2004; USA Rice Federation, 2010).

Overall, it could be said that the rice lipids are primarily located in the bran layers and the germ. Germ layer mainly contains 19-24% lipids. Rice bran includes both germ and aleurone layer, that are concentrated in lipids. This makes the total lipid content in bran about 16-28% (Abdul-Hamid, Raja Sulaiman, Osman, & Saari, 2007; Goffman,

Pinson, & Bergman, 2010; Marshall, & Wadsworth, 1994; Saunders, 1990). Aleurone layer, which is the innermost of the bran layers, is believed to store the highest amount of lipid bodies (Bechtel, & Pomeranz, 1977). At 4% and 6% degrees of milling, almost all the bran layers and the germ is believed to be removed from the rice surface. Therefore, rice bran that is produced from 4% and 6% degrees of milling includes bran layers, germ and barely any endosperm material. With further milling, a part of the starchy endosperm is incorporated into the bran which predominantly contains starch granules with very few lipid bodies. Therefore, the % lipid content was found to be high for 4 and 6% milling levels, compared to 8% and 9%. Well-milled rice (8-10% bran removal) has endosperm, regarded as polish, as a part of bran. This dilutes the lipid percentage of the rice bran produced from well milled rice. This could be the reason for the lower % lipid content in the bran obtained at 9% milling degree due to inclusion of non lipid substances at higher milling (USA Rice Federation, 2010).

RP-HPLC Analysis of γ- Tocotrienol

A calibration curve (figure 6) was plotted successfully with 3 concentration points 0.02 μg/ml, 0.1 μg/ml and 0.5 μg/ml using LC solution software.

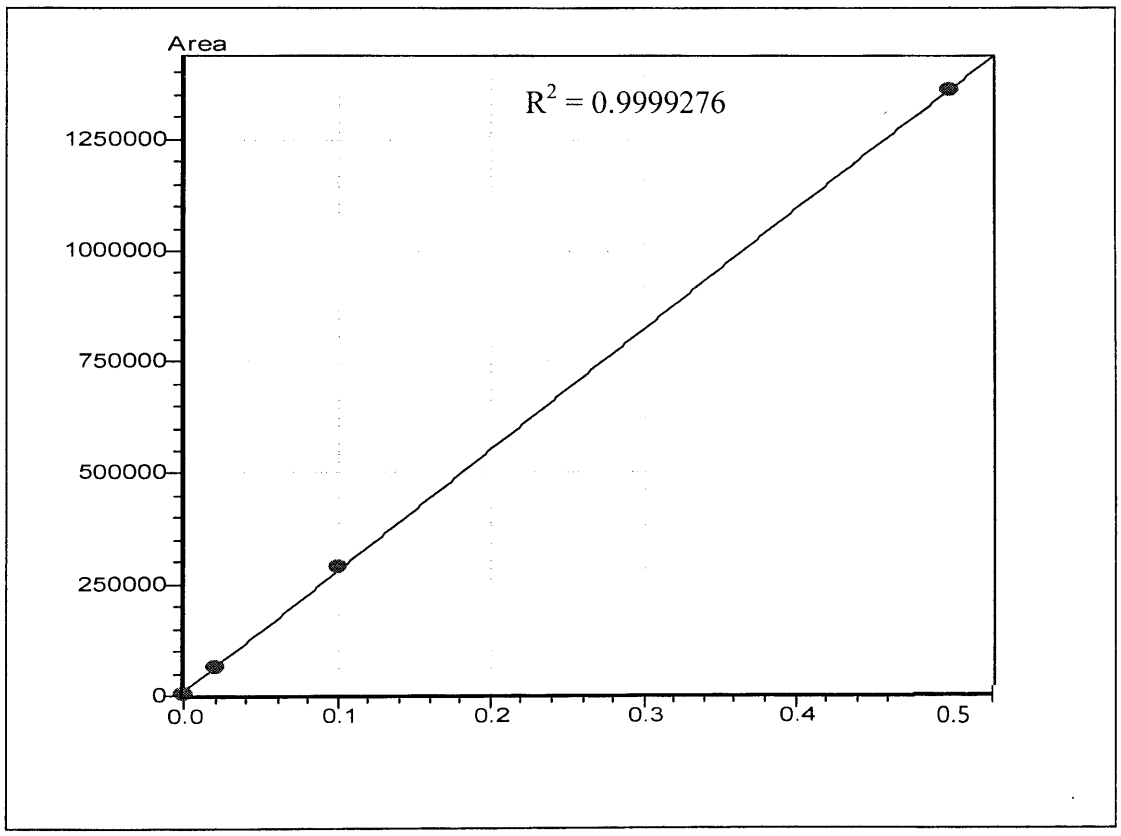


Figure 6. A calibration curve for γ- tocotrienol

Equation for the calibration curve was y = ax+ b, where a = 2705078, b = 10721.68.

R square (R^2) is a popular measure in statistics which is used to define "how closely a regression line approximates the real data point" (Kolobe, 2006). It takes a value between 0 and 1 where 1.0 indicates the best value of a regression analysis. Thus, the R^2 value of 0.9999 for the above Calibration curve was considered to be a very high value for a regression line.

The chromatograms for the three concentrations are illustrated below in figure 7.

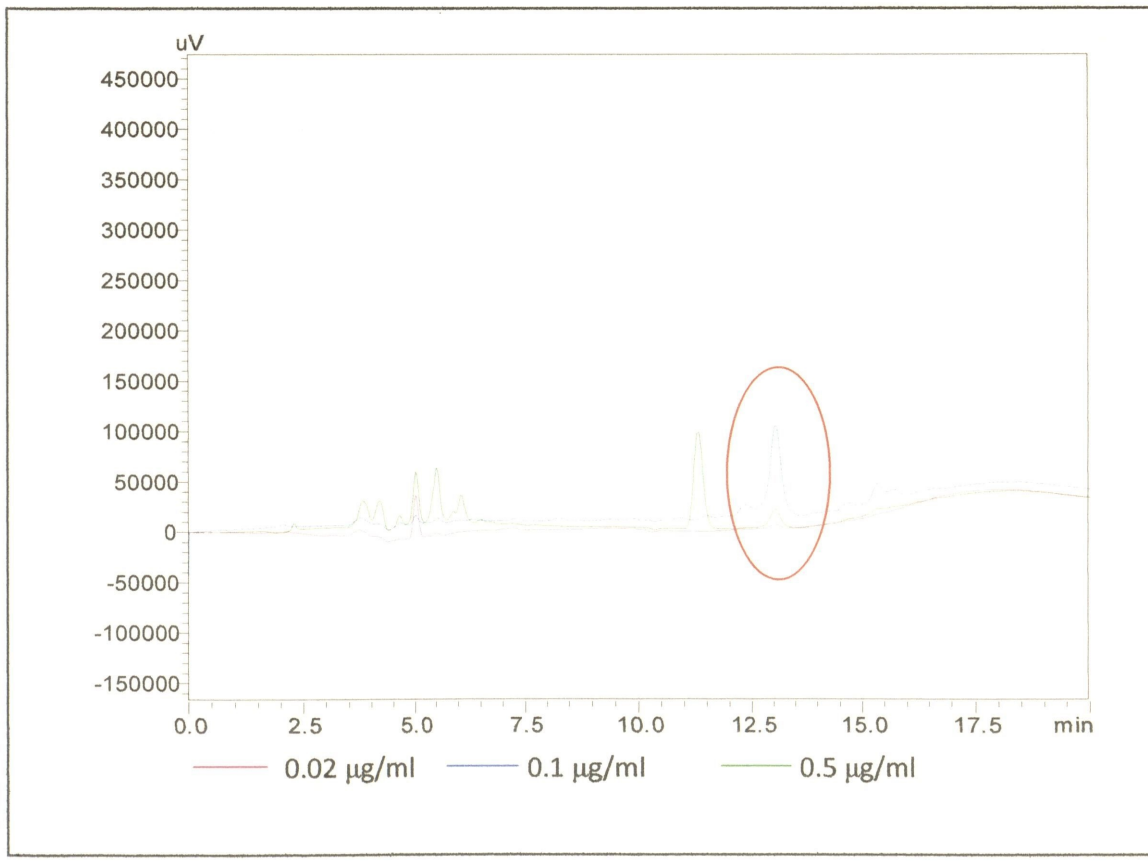


Figure 7. Chromatograms of the three concentrations of the calibration curve

γ-Tocotrienol Levels

The concentration of γ- tocotrienol was measured from the rice bran milled at four different degrees of milling i.e. 4%, 6%, 8% and 9%. The quantification was achieved using reverse phase HPLC. As shown in Table 7, the γ- tocotrienol concentration ranged from 331.1 to 349.7 µg/g of rice bran. According to the literature, γ- tocotrienol in the rice bran ranges from 123.9 to 349.0 ppm or µg/g of bran weight (Chen & Bergman,

2005a; Chen & Bergman, 2005b; Slover, 1971; Whittle, & Pennock, 1967). Sookwong, Nakagawa, Murata, Kojima & Miyazawa (2007) reported 567.0 μg of γ- tocotrienol/g dry weight of rice bran. The wide variation in the concentration of tocotrienols could be attributed to different rice varieties, growing conditions, kernel thickness, kernel maturity, extent of bran removed from the kernel etc (Chen, & Bergman, 2005). The chromatograms of all the four degrees of milling for both the cultivars are illustrated below in figures 8 to 15.

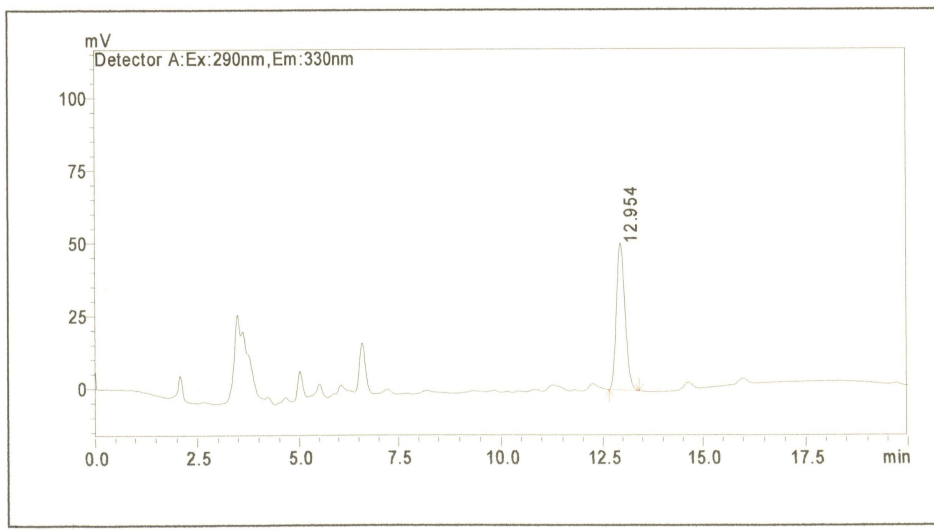


Figure 8. Chromatogram of γ- tocotrienol in Francis at 4% milling degree.

2005a; Chen & Bergman, 2005b; Slover, 1971; Whittle, & Pennock, 1967). Sookwong, Nakagawa, Murata, Kojima & Miyazawa (2007) reported 567.0 μg of γ- tocotrienol/g dry weight of rice bran. The wide variation in the concentration of tocotrienols could be attributed to different rice varieties, growing conditions, kernel thickness, kernel maturity, extent of bran removed from the kernel etc (Chen, & Bergman, 2005). The chromatograms of all the four degrees of milling for both the cultivars are illustrated below in figures 8 to 15.

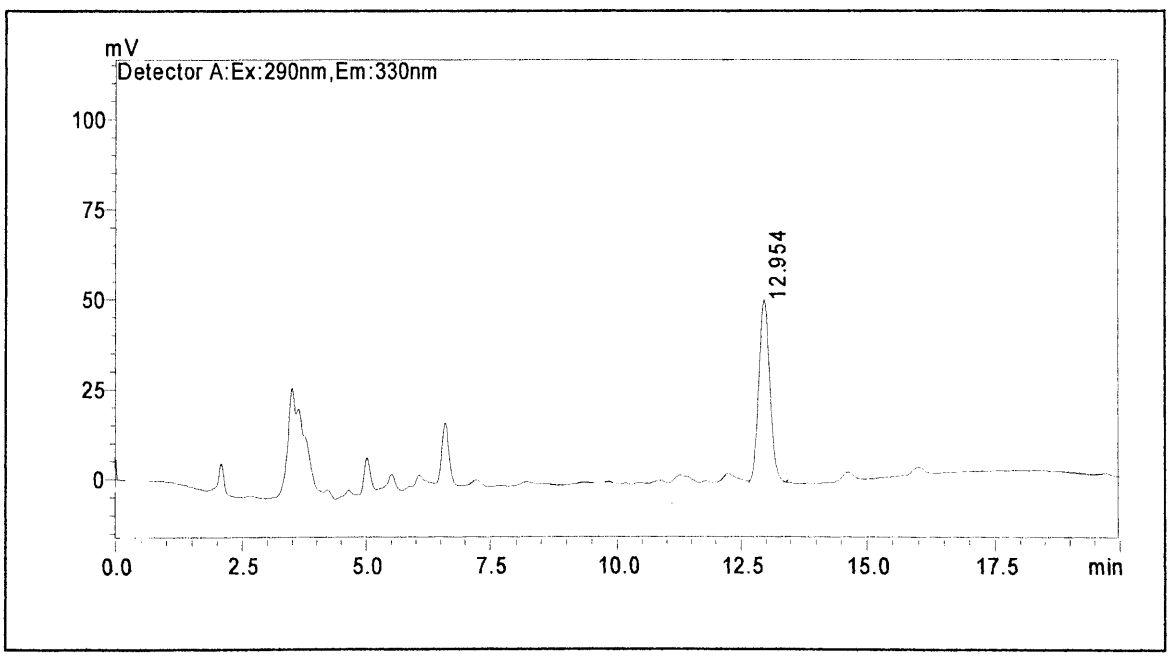


Figure 8. Chromatogram of γ- tocotrienol in Francis at 4% milling degree.

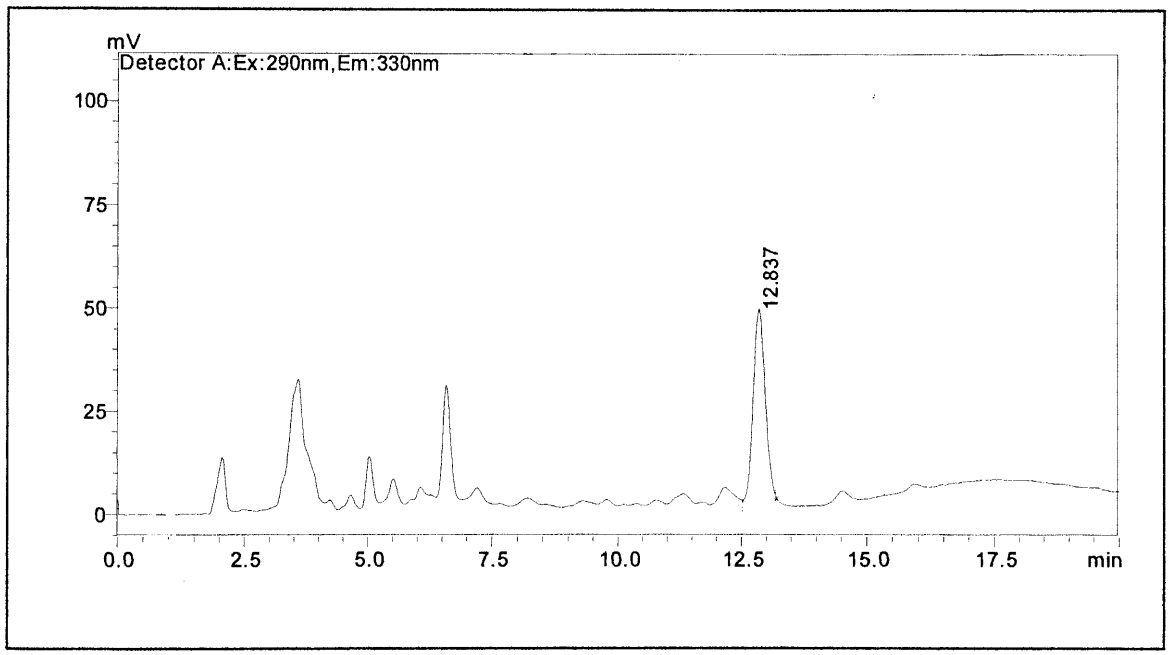


Figure 9. Chromatogram of γ- tocotrienol in Francis at 6% milling degree.

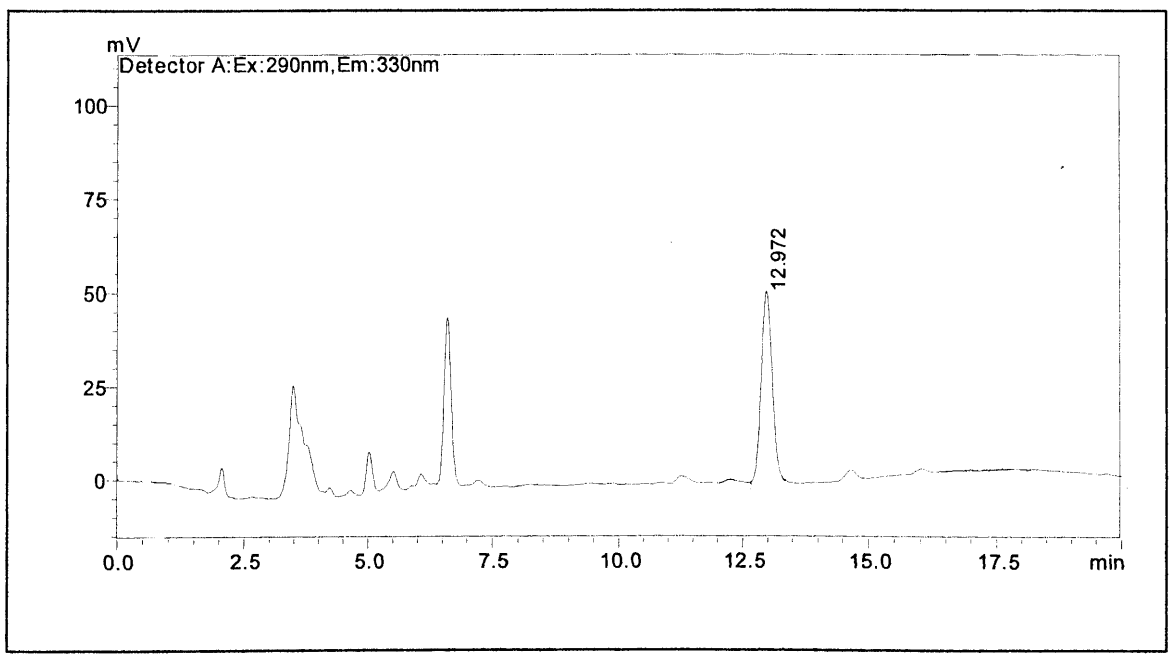


Figure 10. Chromatogram of γ- tocotrienol in Francis at 8% milling degree.

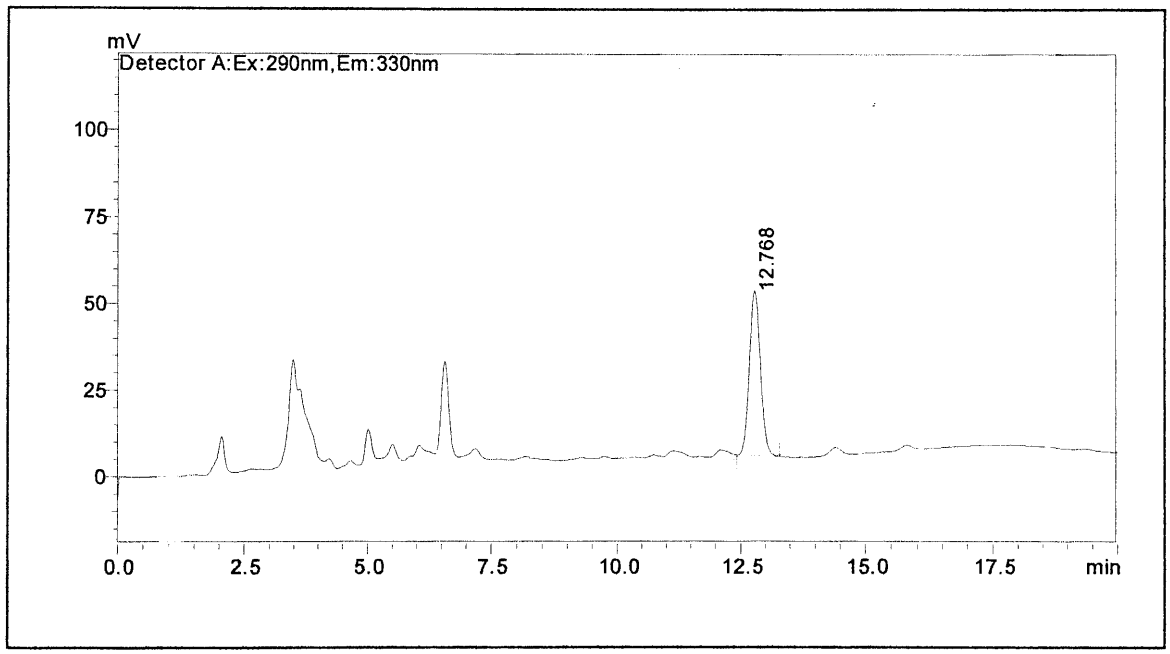


Figure 11. Chromatogram of γ- tocotrienol in Francis at 9% milling degree.

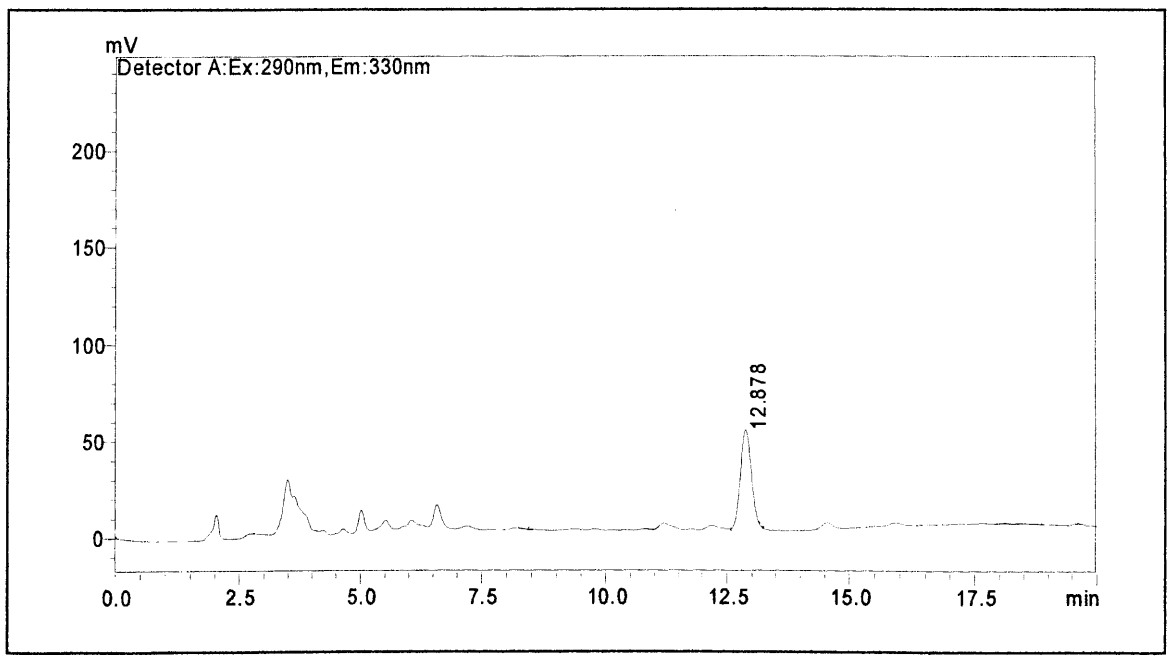


Figure 12. Chromatogram of γ- tocotrienol in Cheniere at 4% milling degree.

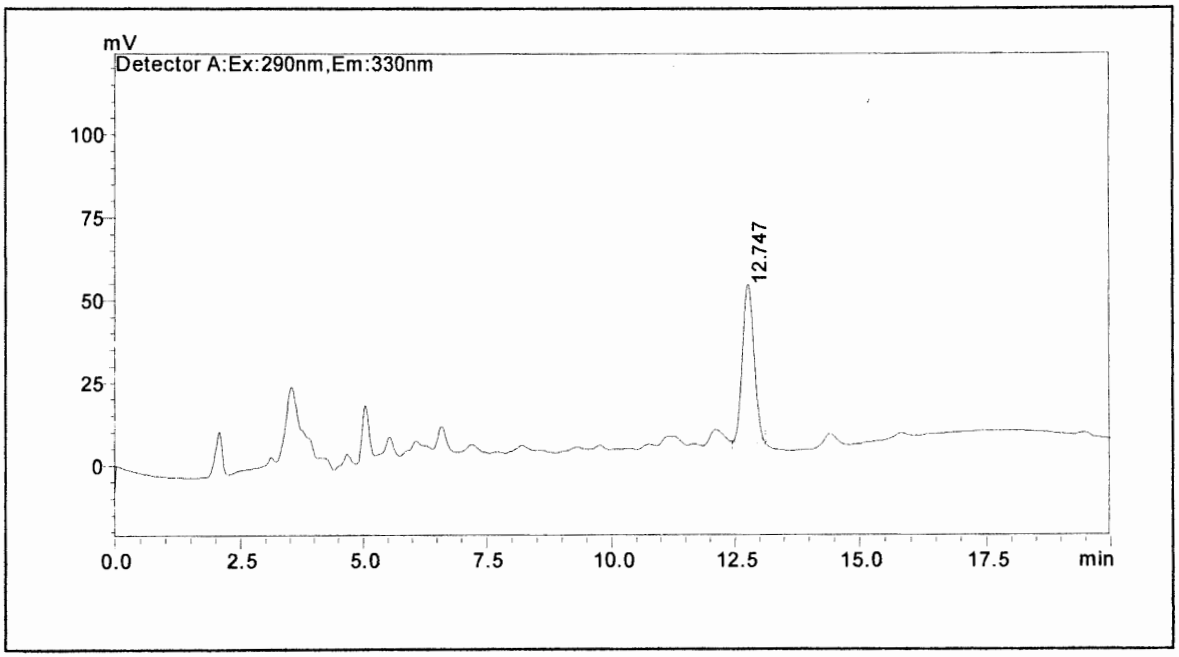


Figure 13. Chromatogram of γ- tocotrienol in Cheniere at 6% milling degree.

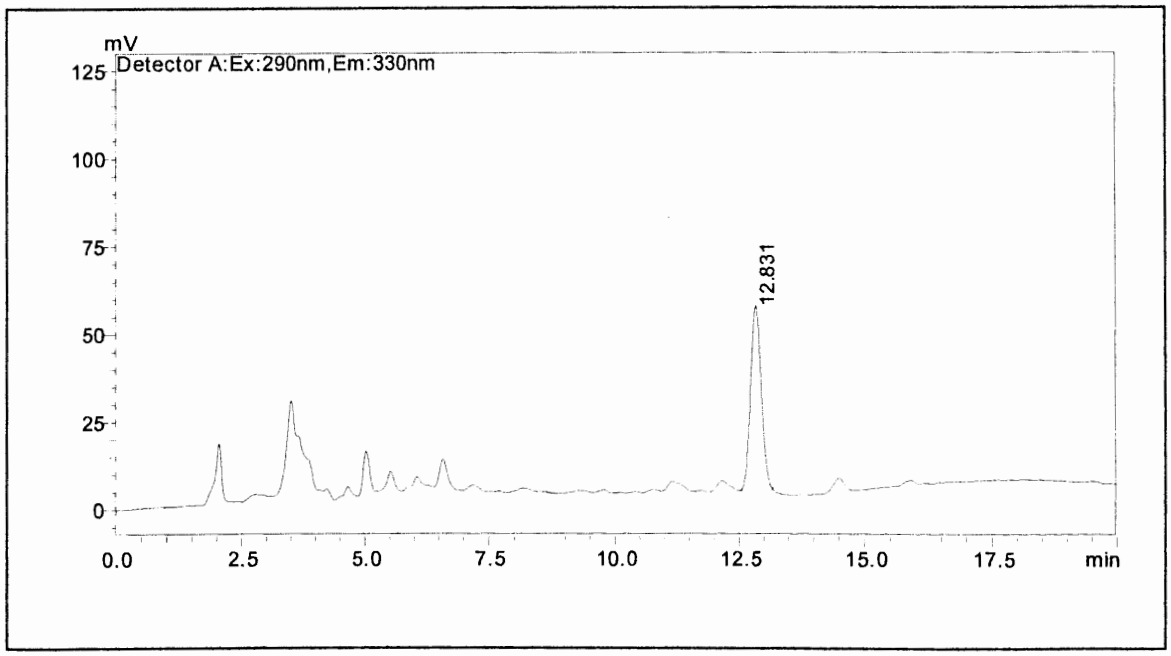


Figure 14. Chromatogram of γ- tocotrienol in Cheniere at 8% milling degree.

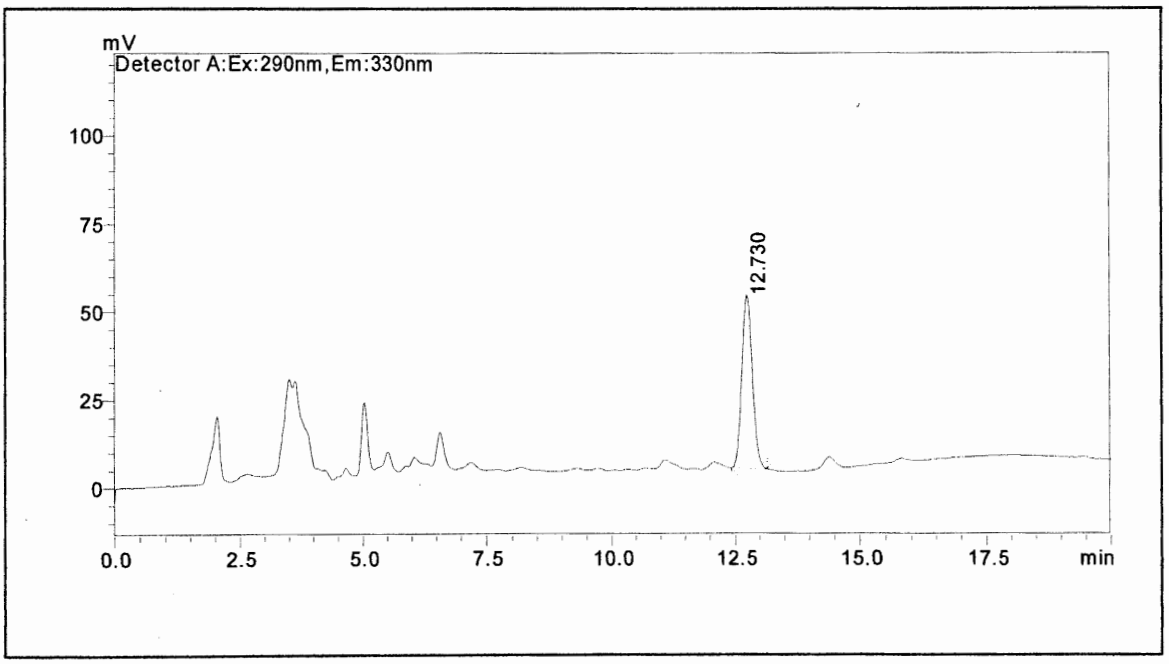


Figure 15. Chromatogram of γ- tocotrienol in Cheniere at 9% milling degree.

A Two Way ANOVA with a 2x4 factorial design was performed to analyze the effect of two independent variables; Degree of milling and cultivar/ rice type on tocotrienol content. Table 6 summarizes the statistical details of the data.

Table 6.

Total Statistical Model for γ- Tocotrienol Concentration

Source	Type III Sum of Squares	Degree of freedom	Mean Square	F value	signifi cance
Corrected Model	2809.542(a)	7	401.363	20.103	.000
Intercept	8441255.681	1	8441255.681	422796.806	.000
RiceType	2167.014	1	2167.014	108.539	.000
Millperc	608.486	3	202.829	10.159	.000
RiceType * Millperc	34.042	3	11.347	.568	.638
Error	1277.778	64	19.965		
Total	8445343.000	72			
Corrected Total	4087.319	71			

a R Squared = 0.687 (Adjusted R Squared = 0.653)

The 'sum of the squares' is shown as Type III, along with the associated degrees of freedom (df). The total evaluations (N) are 72 which represent 8 separate cells having 9 replications. The corrected model degree of freedom is 7, which is calculated by adding all the statistical degrees of freedom, including the two main and one 2-way interaction effects, along with the intercept. This total (8) is then subtracted by 1 to give 7 degree of freedom for this corrected model. The degree of freedom, error term is given by subtracting 8 from 72 (N), which equals 64. The statistical 'significance' values have

been calculated, using the degrees of freedom, for the main and interaction effects of the 2 variables on tocotrienol content of raw rice bran. For example, the 2-way interaction of cultivar x degree of milling has an F-value of 11.347 and the level of significance is above 0.05, indicating that there is no interaction between the two variables. On the contrary, the main effects of cultivar and degree of milling have the level of significance below 0.000, indicating that there are significant differences at p value of 0.05.

Degree of milling had 4 levels, 4%, 6%, 8%, and 9% and cultivar had 2 levels, Cheniere and Francis (2x4=8 cells). As evident from Table 6, the main effect of the variable, rice type or cultivar was found to be significant at p value of 0.05. However, this effect of cultivar was almost similar at each level of degree of milling. Therefore, it can be said that there was no significant interaction effect between the cultivars and degrees of milling (figure 16). The results also indicated that the main effect of the variable, degree of milling was significant at p value of 0.05. Further, the post-hoc tests were performed to understand specifically that which groups were significantly different from each other (Refer Appendix A).

The analysis was conducted comparing all the degrees of milling for each cultivar separately. This was performed as indicated in Appendix A by a pairwise comparison of all the combinations of degrees of milling. As Shown in Table 7, the results indicated that for Francis, γ- tocotrienol concentration at only 9% was significantly different as compared to other three degrees of milling i.e. 4, 6 and 8%. Cheniere had no significant differences across all the degrees of milling.

Table 7.

Mean γ- tocotrienol Content (μg/g of raw rice bran) for 2 Cultivars at 4 Milling Degrees.

Degree of milling (%)	**Mean‡ ± SD**		
	Cheniere	**Francis**	**Average**
4	349.7 ± 5.8 ^{a,x}	340.7 ± 4.6 ^{a,y}	345.2 ± 6.9 ^{a}
6	349.4 ± 3.6 ^{a,x}	338.4 ± 3.1 ^{a,y}	343.9 ± 6.5 ^{a}
8	348.4 ± 4.9 ^{a,x}	337.4 ± 4.5 ^{a,y}	342.9 ± 7.3 ^{a}
9	344.0 ± 4.1 ^{a,x}	331.1 ± 4.5 ^{b,y}	337.6 ± 7.8 ^{b}
Average	347.9 ± 5.0 ^{x}	336.9 ± 5.4 ^{y}	342.4 ± 7.6

Note:

‡ mean values are expressed as μg/g of raw rice bran

^{a,b} means values with different superscripts in the same column differ significantly ($p \leq 0.05$)

^{x,y} means values with different superscripts in the same row differ significantly ($p \leq 0.05$)

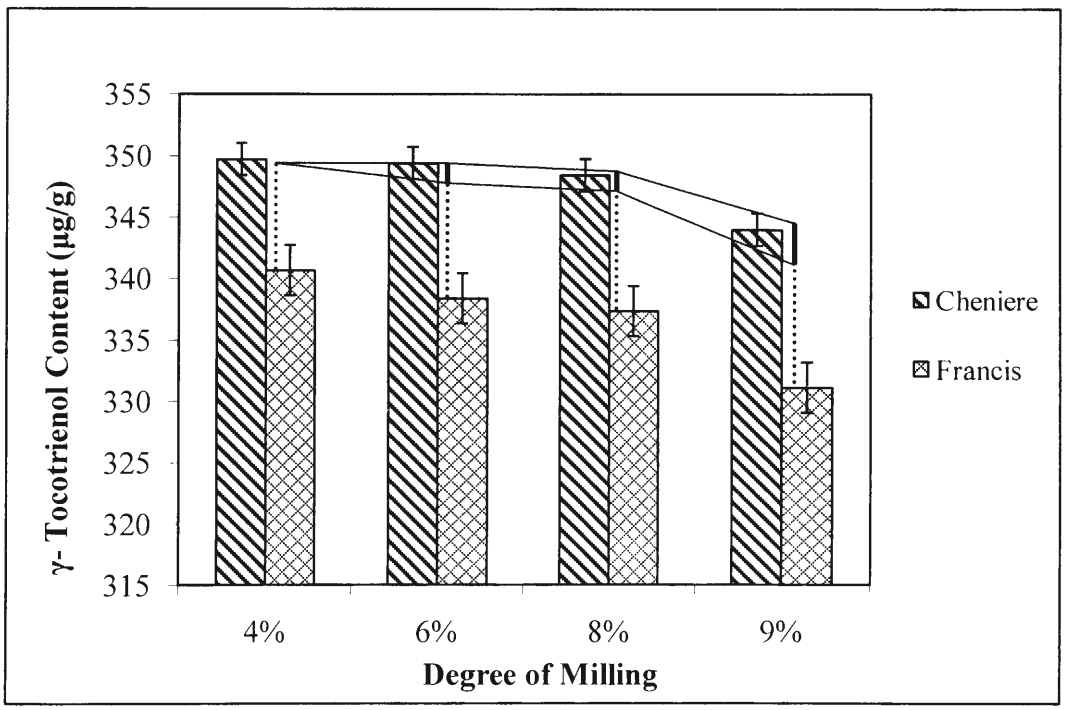


Figure 16. A graphical representation of the γ- tocotrienol content (µg/g of raw rice bran) at all degrees of milling and rice types along with the interaction between the degrees of milling and the cultivars.

In addition, considering the degree of milling changing from 4% to 9%, it was found that for both the cultivars, γ- tocotrienol concentrations decreased with the increasing milling degree and the lowest concentration was obtained at 9% milling degree. However, for Cheniere, this reduction in the γ- tocotrienol content from 4% to 9% degree of milling was not statistically significant ($p \leq 0.05$). The results obtained in this study were consistent with the findings reported by Schramm, Abadie, Hua, Xu, & Lima (2007) where it was found that as the milling duration was increased, the mean

Vitamin E content (α-tocopherol + γ-tocopherol + α-tocotrienol + γ-tocotrienol) did not decrease significantly for Cheniere variety.

The γ- tocotrienol concentrations followed the similar trend as the % lipid content resulting in the highest concentrations at 4% degree of milling (Refer table 7). As discussed earlier, rice bran produced from 4% milling is more concentrated in lipids from the aleurone layer and the germ (Bechtel, & Pomeranz, 1977). These lipid bodies, especially those from the aleurone layer, are assumed to be a storehouse of the natural antioxidants such as tocopherols, tocotrienols, γ-oryzanol etc. These phytochemicals are considered to perform their role of natural antioxidants and protect the lipids in a maturing kernel against the oxidation (Suarna, Dean, & Stocker, 1992). Thus, 4% or reasonably well milled stage, being rich in lipid bodies, is also a milling level that probably contains the highest γ- tocotrienol levels, compared to other milling degrees that include starch (endosperm) also, along with the lipid bodies.

It is also assumed that tocotrienols might be distributed more homogeneously throughout the rice bran layers than tocopherols and γ-oryzanol (Lloyd, Siebenmorgen, & Beers, 2000). This could be the reason for nonsignificant reduction in the γ- tocotrienol concentration in the bran obtained from 4%, 6% and 8% degree of milling showing that γ- tocotrienol concentration changed less across these milling degrees. At the same time, considering the milling degree changing form 4% to 8%, the total lipid content decreased significantly even though the tocotrienol content was not reduced significantly. Thus, it

could be said that though γ- tocotrienol might be distributed evenly throughout the rice bran layers but might not be distributed evenly throughout the lipid fractions of the bran layers.

Similar to % lipid content, the lowest γ- tocotrienol concentrations were obtained at 9% degree of milling. As discussed earlier, 9 % milling degree or the 'hard milled' stage as defined by Federal Grain Inspection Services, USDA results in production of a bran diluted to a greater extent with starch granules and other non lipid components which are low in γ- tocotrienol content This could have resulted in the lower γ- tocotrienol concentrations at 9% milling. (Rohrer, & Siebenmorgen, 2004; USA Rice Federation, 2010).

CHAPTER V

CONCLUSIONS

The concentrations of γ- tocotrienol were found to be in the range of 331.1 to 349.7 μg/g of raw rice bran and lipids were in the range of 20.5 to 27.0% which were in agreement with the values in literature (Abdul-Hamid, Raja Sulaiman, Osman, & Saari, 2007; Chen & Bergman, 2005a; Chen & Bergman, 2005b; Goffman, Pinson, & Bergman, 2010; Saunders, 1990; Slover, 1971; Whittle, & Pennock, 1967). The results showed significant differences among both the rice varieties, i.e. Cheniere and Francis. It was also found that Cheniere had higher contents of total lipids as well as γ- tocotrienol than Francis at all degrees of milling. Therefore, it could be concluded that concentrations of lipids and γ- tocotrienol of the bran could differ depending on the rice variety. These findings were consistent with the literature, where the nutraceutical content of the bran is reported to differ depending on the rice varieties and certain other factors such as growing conditions, thickness of the kernel, maturity of the kernel etc. (Chen, & Bergman, 2005).

The general trend showed that the total lipids and γ- tocotrienol content decreased as the degree of milling was increased. For Francis, % lipids decreased from 25.3 % to 20.5% as the degree of milling was increased from 4% to 9% respectively. Similarly, at

4% milling, γ- tocotrienol concentration was found to be 340.7 μg/g of raw rice bran which was decreased to 331.1 μg/g of raw bran at 9% milling in Francis. The highest concentrations of lipids and γ- tocotrienol were obtained at 4% degree of milling which could be a result of inclusion of lipid concentrated aleurone layer and the germ (Rohrer, & Siebenmorgen, 2004). The lipid bodies in the aleurone layer are assumed to be a source of antioxidants such as tocopherols, γ- tocotrienol, γ-oryzanol which is a natural defense mechanism to protect the lipids against the oxidation in a growing kernel (Suarna, Dean, & Stocker, 1992). Further milling to 9% resulted in the lowest lipids and γ- tocotrienol levels. It could be related to the fact that longer milling results in inclusion of starch granules in the bran and thus bran gets more diluted with other non-lipid components (Rohrer, & Siebenmorgen, 2004; USA Rice Federation, 2010).

From the above findings it could be concluded that to obtain bran which has the maximum concentration of lipids and γ- tocotrienol, 9% milling degree or hard milled stage of milling should be avoided. Isolation of the bran obtained after initial milling could result in the γ- tocotrienol rich bran which could further be used for the extraction and nutraceutical purposes.

The present study was conducted with the objective of selecting a most suitable method for the quantification of γ- tocotrienol using new HPLC equipment at our facility. This objective was achieved as the literature on the HPLC analytical procedures was extensively studied and the procedure described by Rogers et al (1993) and Chen and

Bergman (2005) was assumed to be the most suitable for the present laboratory and HPLC set up. An additional objective was to study the impact of the four different degrees of milling on the total lipid and γ- tocotrienol content in rice bran. This objective was accomplished by studying the impact of 4%, 6%, 8% and 9% milling degree on the lipid and γ- tocotrienol content of bran of two varieties; Cheniere and Francis.

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APPENDIX A

Statistical Output Sheets

STATISTICAL OUTPUT FOR % TOTAL LIPIDS

Table 1. Total statistical output/ Type III sum of squares table

Tests of Between-Subjects Effects

Dependent Variable: PercLipid

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	102.465(a)	7	14.638	56.846	.000
Intercept	14200.935	1	14200.935	55149.262	.000
RiceType	44.282	1	44.282	171.968	.000
MillPercent	55.875	3	18.625	72.330	.000
RiceType * MillPercent	2.308	3	.769	2.988	.062
Error	4.120	16	.258		
Total	14307.520	24			
Corrected Total	106.585	23			

a R Squared = .961 (Adjusted R Squared = .944)

Table 2. Tukey HSD

	N	Subset			
MillPercent	1	2	3	4	1
9.00	6	22.0167			
8.00	6		24.0667		
6.00	6			25.0500	
4.00	6				26.1667
Sig.		1.000	1.000	1.000	1.000

Means for groups in homogeneous subsets are displayed.
Based on Type III Sum of Squares
The error term is Mean Square(Error) = .258.
a Uses Harmonic Mean Sample Size = 6.000.
b Alpha = .05.

Table 3. Tukey HSD Multiple Comparisons

Dependent Variable: PercLipid

(I) MillPercent	(J) MillPercent	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Upper Bound	Lower Bound
4.00	6.00	1.1167(*)	.29297	.008	.2785	1.9549
	8.00	2.1000(*)	.29297	.000	1.2618	2.9382
	9.00	4.1500(*)	.29297	.000	3.3118	4.9882
6.00	4.00	-1.1167(*)	.29297	.008	-1.9549	-.2785
	8.00	.9833(*)	.29297	.019	.1451	1.8215
	9.00	3.0333(*)	.29297	.000	2.1951	3.8715
8.00	4.00	-2.1000(*)	.29297	.000	-2.9382	-1.2618
	6.00	-.9833(*)	.29297	.019	-1.8215	-.1451
	9.00	2.0500(*)	.29297	.000	1.2118	2.8882
9.00	4.00	-4.1500(*)	.29297	.000	-4.9882	-3.3118
	6.00	-3.0333(*)	.29297	.000	-3.8715	-2.1951
	8.00	-2.0500(*)	.29297	.000	-2.8882	-1.2118

Based on observed means.
* The mean difference is significant at the .05 level.

Table 4. Multiple Pairwise Comparisons of 2 cultivars at 4 degrees of Milling

MillPercent	(I) RiceType	(J) RiceType	Mean Difference (I-J)	Std. Error	Sig.(a)	95% Confidence Interval for Difference(a)	
						Upper Bound	Lower Bound
4.00	Cheniere	Francis	1.667(*)	.410	.015	.529	2.804
	Francis	Cheniere	-1.667(*)	.410	.015	-2.804	-.529
6.00	Cheniere	Francis	2.900(*)	.404	.002	1.778	4.022
	Francis	Cheniere	-2.900(*)	.404	.002	-4.022	-1.778
8.00	Cheniere	Francis	3.267(*)	.371	.001	2.236	4.297
	Francis	Cheniere	-3.267(*)	.371	.001	-4.297	-2.236
9.00	Cheniere	Francis	3.033(*)	.467	.003	1.738	4.329
	Francis	Cheniere	-3.033(*)	.467	.003	-4.329	-1.738

Dependent Variable: PercLipid
Based on estimated marginal means
* The mean difference is significant at the .05 level.
a Adjustment for multiple comparisons: Bonferroni.

STATISTICAL OUT FOR γ-TOCOTRIENOL CONTENT

Table 1. Total statistical out/ Type III sum of square table

Tests of Between-Subjects Effects

Dependent Variable: Tocotrienol

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	2809.542(a)	7	401.363	20.103	.000
Intercept	8441255.681	1	8441255.681	422796.806	.000
RiceType	2167.014	1	2167.014	108.539	.000
Millperc	608.486	3	202.829	10.159	.000
RiceType * Millperc	34.042	3	11.347	.568	.638
Error	1277.778	64	19.965		
Total	8445343.000	72			
Corrected Total	4087.319	71			

a R Squared = .687 (Adjusted R Squared = .653)

Post Hoc tests

Table 2. Tukey HSD

	N	Subset	
Millperc	1	2	1
9.00	18	337.5556	
8.00	18		342.9444
6.00	18		343.9444
4.00	18		345.1667
Sig.		1.000	.448

Means for groups in homogeneous subsets are displayed.
Based on Type III Sum of Squares
The error term is Mean Square(Error) = 19.965.
a Uses Harmonic Mean Sample Size = 18.000.
b Alpha = .05.

Table 3. Tukey HSD Multiple Comparisons

Dependent Variable: Tocotrienol

(I) Millperc	(J) Millperc	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Upper Bound	Lower Bound
4.00	6.00	1.2222	1.48942	.845	-2.7066	5.1511
	8.00	2.2222	1.48942	.448	-1.7066	6.1511
	9.00	7.6111(*)	1.48942	.000	3.6823	11.5400
6.00	4.00	-1.2222	1.48942	.845	-5.1511	2.7066
	8.00	1.0000	1.48942	.908	-2.9288	4.9288
	9.00	6.3889(*)	1.48942	.000	2.4600	10.3177
8.00	4.00	-2.2222	1.48942	.448	-6.1511	1.7066
	6.00	-1.0000	1.48942	.908	-4.9288	2.9288
	9.00	5.3889(*)	1.48942	.003	1.4600	9.3177
9.00	4.00	-7.6111(*)	1.48942	.000	-11.5400	-3.6823
	6.00	-6.3889(*)	1.48942	.000	-10.3177	-2.4600
	8.00	-5.3889(*)	1.48942	.003	-9.3177	-1.4600

Based on observed means.

* The mean difference is significant at the .05 level.

Table 4. Multiple Pairwise Comparisons of 2 cultivars at 4 degrees of milling

Dependent Variable: Tocotrienol

Millperc	(I) RiceType	(J) RiceType	Mean Difference (I-J)	Std. Error	Sig.(a)	95% Confidence Interval for Difference(a)	
						Upper Bound	Lower Bound
4.00	Cheniere	Francis	9.000(*)	2.483	.002	3.736	14.264
	Francis	Cheniere	-9.000(*)	2.483	.002	-14.264	-3.736
6.00	Cheniere	Francis	11.000(*)	1.601	.000	7.607	14.393
	Francis	Cheniere	-11.000(*)	1.601	.000	-14.393	-7.607
8.00	Cheniere	Francis	11.000(*)	2.206	.000	6.323	15.677
	Francis	Cheniere	-11.000(*)	2.206	.000	-15.677	-6.323
9.00	Cheniere	Francis	12.889(*)	2.037	.000	8.570	17.208
	Francis	Cheniere	-12.889(*)	2.037	.000	-17.208	-8.570

Based on estimated marginal means

* The mean difference is significant at the .05 level.

a Adjustment for multiple comparisons: Bonferroni.

APPENDIX B

AACC Method for Total Lipids

CRUDE FAT IN GRAIN AND STOCK FEEDS

Final approval 4-13-61; revised 10-30-75; reviewed 10-27-82 and 10-26-94

Apparatus

1. Soxhlet, Butt-type, Goldfisch, or similar extractor.
2. Fat flasks.
3. Filter paper, S&S No. 597, Reeve Angel No. 211, Whatman No. 2.
4. Whatman extraction thimbles 22 × 80 mm, Norton RA 98 alundum thimbles 22 × 80 mm, or equivalent.
5. Absorbent cotton, free from ether extract, or Pyrex glass wool.
6. Vacuum oven or vacuum desiccator for nonoxidative drying of sample.

Reagent

Anhydrous ethyl ether. *Caution.* See Note. Wash ether with two or three successive portions water to remove alcohol. Add solid NaOH or KOH and let stand until most of water is removed from ether. Decant into dry bottle, add shavings of metallic sodium (or preferably sodium wire), and let stand until there is no further evolution of hydrogen. Store in loosely stoppered bottle in cool place until used.

Procedure

(Large amounts of water-soluble components such as carbohydrates, urea, lactic acid, and glycerol may interfere with extraction of fat; if present, extract 2-g sample on small paper in funnel with five 20-ml portions water prior to drying for ether extraction.)

1. Extract with dry ethyl ether from 2 to 5 g (depending on fat content) sample that has been dried in vacuum oven at 95–100° under pressure not to exceed 100 mm Hg (about 5 hours [hr]) or in vacuum desiccator over prepared H_2SO_4 for about 24 hr under pressure not over 10 mm Hg. Extraction period may vary from 4 hr at condensation rate of 5–6 drops/second (sec) to 16 hr at 2–3 drops/sec.
2. Remove excess ether (may be done by placing glass thimble or test tube in place of sample) and dry extract and previously dried and tared beaker in oven at 100° for 30 minutes, desiccate, cool, and weigh.
3. Repeat heating, cooling, and weighing to constant weight.
4. Correct this weight by blank determination on reagents used.
5. Report as percent crude fat or ether extract.

Calculation

$$\text{Crude fat or ether extract, } \% = \frac{\text{weight of fat}}{\text{weight of sample}} \times 100$$

Crude Fat in Grain and Stock Feeds (continued)

Note

Ethyl ether is an extremely flammable solvent. Do not let vapors concentrate to a flammable level in the work area, since it is nearly impossible to eliminate all chance of sparks from static electricity even though the electrical equipment is grounded. Use an effective fume removal device to remove these vapors when released.

Store ethyl ether protected from light. Unstable peroxides can form upon long standing or exposure to sunlight in bottles. These can react explosively with Cl, O_3, $LiAlH_4$, or strong oxidizing agents.

Reference

Association of Official Analytical Chemists. 1990. Official Methods of Analysis, 15th ed. Sec. 920.39, p. 79.