SEPARATION, IDENTIFICATION AND QUANTIFICATION OF THE UNSAPONIFIABLES OF COTTONSEED OIL AND ITS DEODORIZER DISTILLATE

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BY

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To the Dean of the Graduate School:

I am submitting herewith a thesis written by Priyanka Mathur entitled "Separation, identification and quantification of the unsaponifiables of cottonseed oil and its deodorizer distillate". I have examined this thesis for form and content and recommend that it be accepted in partial fulfilment of the requirements for the degree of Masters in Science with a major in Food Science.

Dr Clay King, Major Professor

We have read this thesis and recommend its acceptance:

Dr Dojin Ryu

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Dr Chandan Prasad, Department Chair

Accepted:

Dean of the Graduate School

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ABSTRACT

PRIYANKA MATHUR

SEPARATION, IDENTIFICATION AND QUANTIFICATION OF THE UNSAPONIFIABLES OF COTTONSEED OIL AND ITS DEODORIZER DISTILLATE

DECEMBER 2011

This study was conducted to separate, identify and quantify the unsaponifiables of cottonseed oil and its deodorizer distillate. The AOCS extraction method was used to separate the unsaponifiables from refined, bleached and deodorized (RBD) cottonseed oil and its deodorizer distillate. A silylation method was then used to identify and quantify the tocopherols and sterols which are the major components of the unsaponifiable materials of RBD cottonseed oil and its deodorizer distillate. Quantification of unsaponifiables was conducted using gas chromatography. The percentage of total unsaponifiables was found to be 2% in RBD cottonseed oil and 53.9% in the deodorizer distillate. The total tocopherols in RBD cottonseed oil were 3.6% of the total unsaponifiables (which included 2.3% α tocopherol and 1.37% β+γ tocopherol) and total sterols were 31.4% of total unsaponifiables (which included 1.8% stigmasterol, 2.3% campesterol and 27.2% β sitosterol). In the distillate, total tocopherols were 12.64% of the total unsaponifiables (which included 4.4% α tocopherol, 8.2 % β+γ tocopherols) and total sterols were 65.1% of total unsaponifiables (which included 1.2% stigmasterol, 6.6% campesterol and 57.3% β sitosterol).

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CHAPTER I

INTRODUCTION

Edible oils are primarily produced from the oilseeds of dicot plants. Crude oil extracted from oilseeds generally consists of free fatty acids, mono-, di-, and triglycerides, phosphatides, pigments, sterols, tocopherols, glycerol, hydrocarbons, vitamins, protein fragments, trace amounts of metals, glycolipids, pesticides and resinous materials (Cheryan, 1998).

Oilseed Processing

The first step in edible oil production is separation of the oil from other solid constituents of the seed by mechanical pressing or solvent extraction. The extracted oil is then refined to remove free fatty acids and the non-triacylglyceride components which contribute undesirable flavor, odor and appearance so that it becomes available for human consumption.

Refining of Crude Vegetable Oil

Refining of crude oil that is extracted from oilseeds is conducted in the following stages (Gunawan and Ju, 2009):

Degumming

This is the first step of refining where phospholipids are removed from crude oil. Phospholipids are present in two forms: hydratable and non-hydratable (phospholipid combined with calcium, magnesium or iron cations). The non hydratable forms are treated with acid to convert them to hydratable forms. These hydratable forms are then treated with water and converted to gums (which are removed by centrifugation).

Chemical Refining

Saponification: Firstly, phospholipids are precipitated out of the crude oil using phosphoric acid. Alkaline solution added next neutralizes the free fatty acids and excess phosphoric acid. Reaction of free fatty acids with the alkaline solution (NaOH) leads to formation of soap. This soap is removed from the crude oil by centrifugation (O'Brien et al., 1996). Chemical refining therefore helps in the removal of phospholipids as well as the free fatty acids present in the oil by converting them to soap. As a result, the refined oil so obtained is less prone to oxidation and deterioration. The biggest drawback of this process however is the disposal of the soapstock, or the chemical waste of soapstock acidulation which is a hazardous and expensive process. Another disadvantage of this process is that it leads to a loss of neutral oil, thereby reducing the overall yield of the refined product.

Physical Refining

This process involves dry degumming, dewaxing, separation, hydrogenation, steam distillation and deodorization. Physical refining is more environment-friendly as compared to chemical refining, since it eliminates pollution problems associated with acidulation of soapstock. Moreover, physical refining also leads to minimal refining losses. The free fatty acids are vaporized by steam during deodorization. The deodorizer distillate therefore contains mainly free fatty acids (>70%) with small amounts of unsaponifiables (5-10%) and is usually sold as a source of fatty acids.

In Chemical refining however, free fatty acids are neutralized by caustic solution and washed out of the oil before deodorization. Therefore, the deodorizer distillate obtained from chemical refining has lower free fatty acid contents (30-50%) and higher levels (25-33%) of unsaponifiables (De Greyt et al., 1999).

Bleaching

During refining of crude oil, neutralization is carried out by which all the free fatty acids are removed from the oil by neutralization with a base. This is followed by bleaching which helps in the removal of carotenoids, chlorophyll pigments, residual soap, phospholipids, trace metals and oxidized products by their adsorption onto the surface of bleaching agents or adsorbants. Types of adsorbants typically used include neutral earth, activated earth, activated carbon or heated clay which is mixed with the oil. An absorbant material such as bleaching earth or active carbon is used to reduce color pigments and contaminants to levels which are within acceptable limits.

Winterization

Winterization of the oil is conducted which refers to rapidly cooling of the oil to solidify traces of wax, which are then removed by filtration. These compounds lead to clouding of the oil. They have higher melting points and are therefore precipitated out when refined oil is chilled. This is followed by de-waxing of the oil which improves its palatability, transparency and brightness. Winterization is conducted to produce clear liquid oil which can then be used as salad oil.

Deodorization

Deodorization is a high-temperature, high-vacuum steam-distillation process to remove volatile, odoriferous materials present in edible fats and oils. Auto-oxidation of fat produces aldehydes, ketones, alcohols, other hydrocarbons and other volatile components that impart undesirable flavor and odor to the oil. These compounds along with free fatty acids are removed during deodorization. This process therefore produces bland flavored oil that contains 0.01-0.03% free fatty acids and a zero peroxide level (Gavin, 1978). The resulting oils possess improved flavor, odor, color, and stability, although the unsaponifiables and other volatile compounds are removed from the oil by this process.

Deodorization involves a steam distillation process in which the oil is heated to 230°C (446°F) under a vacuum of 2-10 mm of Hg. This is followed by purging of steam through the oil to bring about agitation in the oil and to carry away the volatile compounds. As a result, all the odor and flavor producing compounds are removed and the triglycerides are retained. The deodorized oils are then stored in an inert

environment of nitrogen to prevent oxidation (Jones and King, 1996). The samples for the present study were obtained from Pyco Industries, where deodorization of the oil is carried out at 480°F at 2-2.5 mm of Hg vacuum pressure. The amount of volatile compounds that evaporate depends on the temperature and pressure of deodorization.

The amount of free fatty acids depends on the contact time between the steam and oil during deodorization. This is due to the fact that steam is water vapor, which when comes in contact with the oil in the presence of heat leads to hydrolysis of the oil which in-turn leads to break down of the oil and production of free fatty acids.

When deodorization is carried out after chemical refining, most of the free fatty acids are removed from the oil as well as the distillate and therefore a higher percentage of unsaponifiables is obtained after chemical refining as compared to physical refining.

Many oil industries such as Pyco use corn derived citric acid during deodorization as a chelating agent for cottonseed oil. The citric acid acts as an emulsifying agent because it dissolves TBHQ. Citric acid contains an acid group which is a polar group; therefore it is able to dissolve TBHQ which is water soluble. Also, mono and di-glycerides are added along with the citric acid so that fatty acid groups are able to bind with the citric acid structure. Mono and di-glycerides are fat soluble compounds. Therefore, citric acid acts as an emulsifier because it helps in dissolving TBHQ in oil. TBHQ acts as an antioxidant in the oil and prevents oxidation of oil during processing.

tocopherols, fatty acids (30 to 60 wt%), sterols and sterol esters (10 to 30 wt%), hydrocarbons (10 to 30 wt%).

Composition of Vegetable Oil Deodorizer Distillate

The typical composition of vegetable oil deodorizer distillate consists of (Tables 1, 2 and 3):

Free Fatty Acids

The distillate obtained after chemical refining (saponification) of the oil contains a limited amount of free fatty acids, as most free fatty acids are eliminated during saponification. The free fatty acids in the vegetable oil deodorizer distillate are subjected to harsh conditions in the processing steps which result in reactions such as oxidation and cis-trans conversion (Mendes et al., 2002). In crude vegetable oils, double bonds are nearly always present in the cis configuration. Therefore, on exposure to elevated temperatures during deodorization and hydrogenation, trans fatty acids are produced as a result of geometric isomerization of the double bonds. Processing of the oil and the distillate at elevated temperatures during deodorization and hydrogenation leads to the formation of trans fatty acids. The rate of cis / trans isomerization of α-linolenic acid (C18:3) is about 10 and 100 times higher than that of linoleic (C18:2) and oleic acid (C18:1) respectively. This implies that the formation of trans fatty acids during refining would be higher in oils rich in α-linolenic acid such as soybean and rapeseed oil (De Greyt et al., 1999).

Table 1:

Typical Compositions (weight %) of Vegetable Oil Deodorizer Distillate

Deodorizer Distillate	**FFA**	**Mono-, Di-, and Triacyl-glycerols**	**Tocopherols**	**Sterols**	**Squalene**	**References**
1) Soybean Oil Deodorizer Distillate	32.0	11.63	18.01	18.81	2.09	Verleyen et al. (2001)
2) Rapeseed Oil Deodorizer distillate	39.2	13.52	4.19	13.36	0.40	Verleyen et al. (2001)
3) Sunflower Oil deodorizer distillate	39.2	5.31	5.06	13.9	0.73	Verleyen et al. (2001)
4) Olive Oil Deodorizer distillate	34.2	N.A	N.A	4.6	28.0	Bondioli et al. (1993)

Acylglycerols

Triacylglycerols, Diacylglycerols and Monoacylglycerols are also known as acylglycerols. Triacylglycerols are the major components in vegetable oil deodorizer distillate. Monoacylglycerols and diacylglycerols have detergent properties and hence they easily form miscelles in water solutions (Gunawan and Ju., 2009). Due to the high content of free fatty acids and triacylglycerols in vegetable oil deodorizer distillate, efficient separation of the other bioactive compounds such as tocopherols, sterols and squalene is a challenging problem.

Tocopherols

Tocopherols are organic compounds that are present in plant materials. These compounds are important since they retard the oxidation and spoilage of plant matter. They are important components of Vitamin E. The general structure of Tocopherols consists of an aromatic head and a 16-carbon hydrocarbon tail (Figure 1). The number and position of the methyl groups in the aromatic ring gives rise to α, β, γ, and δ isomers (Gunawan and Ju., 2009). α -tocopherol is the most active form of Vitamin E in the body and is a powerful biological antioxidant. α-and γ- tocopherols account for most of the Vitamin E activity. The primary form of Vitamin E in dietary and animal feed supplements is α –tocopherols. However, γ- tocopherols are the major isomers of tocopherols that are present in soybean oil, canola oil and rapeseed oil deodorizer distillates (Gunawan and Ju, 2009).

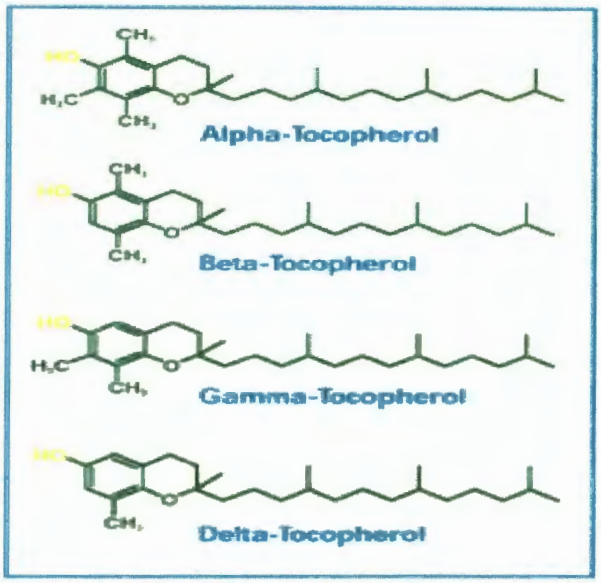


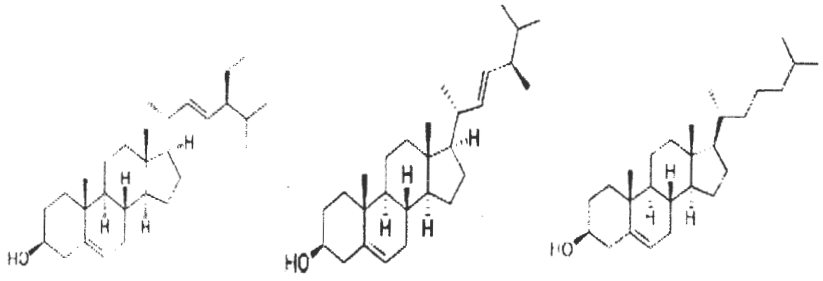
Figure 1: Structures of the isomers of Tocopherols (www.nutritiondynamics.com).

Phytosterols

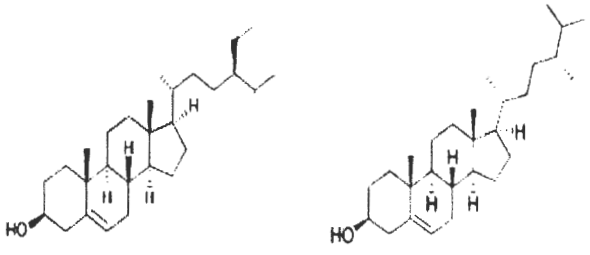
Phytosterols are members of the 'triterpene family' of natural products. The composition of free phytosterols in vegetable oil deodorizer distillate is frequently a mixture of sitosterol, campesterol, stigmasterol, and brassicasterol. Phytosterols, primarily beta-sitosterol, campesterol and stigmasterol are integral components of plant cell membranes that are abundant in vegetable oils, nuts and seeds (Figure 2).

Squalene

Squalene, a 30 carbon isoprenoid (Figure 3), is an intermediate product in cholesterol synthesis in animals and is present abundantly in shark liver oil and olive oil. Squalene has been shown to be an important dietary cancer chemopreventive agent. It can also serve as a powerful antioxidant (Ryan et al., 2007).



Stigmasterol Brassicasterol Cholesterol



β-sitosterol Campesterol

Figure 2: Structures of phytosterols found in vegetable oils (www.wikepedia.org)

Figure 3: Structure of Squalene (www.wikipedia.org)

Table 2: *Relative Compositions of Sterols in Some Vegetable Oils (Reina et al., 1999)*

Cottonseed oil	**Soybean oil**	**Corn Oil**
1. Campesterol: 8.8 %	1. Campesterol: 20.0 %	1. Campesterol: 18.6 %
2. Stigmasterol: 2.7 %	2. Stigmasterol: 17.4 %	2. Stigmasterol: 6.1 %
3. β-sitosterol: 82.8 %	3. β-sitosterol: 53.1 %	3. β-sitosterol: 67.5 %
4. Brassicasterol: ---	4. Brassicasterol: 0.1%	4. Brassicasterol: ----
Canola Oil	**Palm Oil**	**Virgin Olive Oil**
1. Campesterol: 35.3 %	1. Campesterol: 22.9 %	1. Campesterol: 3.2 %
2. Stigmasterol: 0.7 %	2. Stigmasterol: 12.2 %	2. Stigmasterol: 0.7 %
3. β-sitosterol: 51.0 %	3. β-sitosterol: 60.1 %	3. β-sitosterol: 86.8 %
4. Brassicasterol: 9.9%	4. Brassicasterol: ---	4. Brassicasterol: ----
Coconut Oil	**Peanut Oil**	**Safflower Oil**
1. Campesterol: 8.6 %	1. Campesterol: 16.8 %	1. Campesterol: 13.2 %
2. Stigmasterol: 12.0 %	2. Stigmasterol: 9.1 %	2. Stigmasterol: 6.9 %
3. β-sitosterol: 59.0 %	3. β-sitosterol: 65.0 %	3. β-sitosterol: 51.5 %
4. Brassicasterol: ----	4. Brassicasterol: ----	4. Brassicasterol: ----

Cottonseed Oil

Cotton crops are grown globally primarily for fiber production. As a result, cottonseed is available in large quantities around the world and about four million tons of cottonseed oil is produced annually by crushing these seeds, making it the sixth most important plant oil in commerce. Due to its high level of saturated palmitic acid

and extremely low levels of unstable linolenic acid, cottonseed oil is highly valued and is known for its stability and flavor properties (Liu et al., 2002).

Development of Cottonseed Oil Industry

The cotton crop is both a food (cottonseed oil) as well as fiber (cotton lint) crop. A cotton plant produces 150 kg of cottonseed for every 100 kg of cotton fiber produced. Cottonseed oil has dominated the American market for over 50 years. In the last 50 years, research scientists have developed through experimentation a colorless, odorless, clear and bland flavored cottonseed oil that has set the standards for edible fats and oils worldwide.

Table 3: *Unsaponifiables in Cottonseed Oil (Jones and King, 1996)*

Unsaponifiable compounds	Total content (mg/kg oil)	
TOCOPHEROLS	Crude oil	Refined oil
a) α-tocopherol	a) 402	a) 320
b) β-tocopherol	b) 1.5	b) ---
c) γ-tocopherol	c) 572	c) 313
d) δ-tocopherol	d) 75	d) ---
Total Tocopherols	1050.5	633
STEROLS		
a) Stigmasterol	a) 17.3	
b) β-sitosterol	b) 3348	
c) Campesterol	c) 276	
d) Brassicasterol	d) 0.5	

Early Hindu medical books have cited that oil was extracted by crushing of cottonseed which was used in medicine preparation. For many centuries the use of

cottonseed oil was confined to local areas of India, China and Egypt. However following the Industrial revolution in the nineteenth century, there was an increase in the demand for less expensive oils which led processing plants to extract oil from the seeds of the cotton plant. Cottonseed crushing and refining became a profitable venture in the United States around the year 1870. Most of the oil was usually exported for soap manufacturing. However crude cottonseed oil, due to its dark color, unpleasant odor and flavor and high free fatty acid content could not be used in food preparation. The development of oilseed milling led to a reduced triglyceride content in the oil as well as an improvement in its flavor and odor. As a result of technological advancements and progression of extraction technologies and hydraulic pressing over the years, the quality of the oil has improved considerably. The introduction of chemical refining methods, color removal, bleaching, deodorization, and catalytic hydrogenation of oil brought about a new era in oil refining methods.

Cottonseed oil was the edible oil of choice in the United States for many years as a salad oil, cooking oil as well as hydrogenated shortenings (crystallized cottonseed oil or CRISCO). However post World War 2, cottonseed oil lost its dominant position in the market and was taken over by soybean oil, since there was not enough quantity of cottonseed oil to supply to the market. Soon other vegetable oils started competing with soybean oil due to the consumer's appreciation for nutritional foods. The demand for cottonseed oil declined from 1950's till the 1980's, after which it started regaining its domestic market share due to an increase in consumer awareness about saturated fatty acids, cholesterols and trans fat. Cottonseed oil's functional properties such as pleasant and nutty flavor, good oxidative stability

due to low amounts of 18:3's and a beta prime crystal structure due to a high palmitic fatty acid content have helped to maintain it as a desirable vegetable oil (O'Brien et al., 1996; Jones and King, 1996).

Composition of Cottonseed Oil

Fatty Acid Composition

Cottonseed oil comprises 22% oleic acid and 52% linoleic acid and less than 1% linolenic acid (Table 4). Palmitic acid makes up 24% of the fatty acids. Trace amounts of other saturated fatty acids are also found in cottonseed oil (Spencer et al, 1976). It has a high content of 18:2's and 18:1's and a very low content of 18:3's, which make it the ideal frying oil. The fatty acid ranges in cottonseed oil, as given by the Food and Agricultural Organization of the World Health Organization (FAO/WHO) indicated in Table 4.

Non-Glyceride Composition of Cottonseed Oil

The non-glyceride constituents of crude cottonseed oil mainly consist of gossypols, phospholipids, sterols, resins, carbohydrates and pigments (Table 5). Most of these compounds are removed during the refining process (Sonntag, 1979).

Gossypols

Gossypol is a biologically active terpenoid compound present in the glands of the cotton plant and the cotton seed kernel. Processing of the seeds ruptures the glands and releases the gossypols which then mix with the proteins and oils of the kernel.

Gossypols provide a strong red-brown color which is characteristic of crude cottonseed oil. Caustic refining and bleaching of the oil almost completely eliminate the gossypol levels and therefore tend to reduce the color of the crude oil, thereby resulting in a light yellow or amber color of refined cottonseed oil. Gossypol levels in crude oil range from 0.05 to 0.42%, and in refined oil: around 0.01% (Jones and King, 1996).

Table 4: *Range of Fatty Acid Compositions for Cottonseed Oil adopted by FAO/WHO Codex Alimentarius Committee.*

S.NO	FATTY ACID	RANGE (%)
1.	C<14	<0.1
2.	C14:0 (Myristic)	0.5-2.5
3.	C14:1 (Myristoleic)	<0.2
4.	C15:0 (Pentadecanoic)	<0.1
5.	C16:0 (Palmitic)	17-29
6.	C16:1 (Palmitoleic)	0.5-1.5
7.	C18:0 (Stearic)	1.0-4.0
8.	C18:1 (Oleic)	13-44
9.	C18:2 (Linoleic)	33-58
10.	C18:3 (Linolenic)	0.1-2.1
11.	C20:0 (Arachidic)	<0.5
12.	C20:1 (Gadoleic)	<0.5
13.	C22:0 (Behenic)	<0.5
14.	C22:1 (Erucic)	<0.5

Phospholipids

Crude oil contains about 0.7 to 0.9% phosphatides. These compounds are partly soluble in both oil and water, and are hence used as emulsifying agents (Jones and King, 1996).

Tocopherols

Tocopherols are naturally occurring isomers and antioxidants found in cottonseed. α- Tocopherol is also known as Vitamin E. γ-Tocopherol has greater effectiveness as an antioxidant but less vitamin activity. Crude cottonseed oil contains about 1000 ppm tocopherols but 50% of these can be lost during processing. The deodorization step removes tocopherols from the oils, but they can be recovered from the deodorizer distillate and can be marketed as a by-product. γ-Tocopherol accounts for 58 % of the total tocopherols and α- Tocopherol makes up 41%. The β and δ isomers collectively represent only 1% of the total tocopherols (Muller-Mulot, 1976).

Sterols

Phytosterols are a major component of the unsaponifiable matter of cottonseed oil. Refined cottonseed oil is known to contain an average of 300 ppm phytosterols. The primary sterol in cottonseed oil is β-sitosterol. Cholesterol is almost completely absent in cottonseed oil (Jones and King, 1996).

Unsaponifiable Components in Deodorizer Distillate

Unsaponifiables are compounds which are found dissolved in fats and oils and are soluble in fat and oil solvents but cannot form soap by caustic treatment. They are the minor components which accompany triacylglycerols and make up about 0.5-2.5%, exceptionally 5-6% of vegetable oils (Elmadfa, 1995). Unsaponifiable compounds include higher aliphatic alcohols, such as tocopherols and tocotrienols, sterols, carotenoids, squalene, pigments and other hydrocarbons.

Table 5: *Unsaponifiables in Cottonseed Oil (Jones and King, 1996)*

Unsaponifiable compounds	Total content (mg/kg oil)	
TOCOPHEROLS	Crude oil	Refined oil
e) α-tocopherol	a) 402	a) 320
f) β-tocopherol	b) 1.5	b) ---
g) γ-tocopherol	c) 572	c) 313
h) δ-tocopherol	d) 75	d) ---
Total Tocopherols	1050.5	633
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f) β-sitosterol	f) 3348	
g) Campesterol	g) 276	
h) Brassicasterol	h) 0.5	

Health Benefits of Unsaponifiables

Phytosterols, squalene and tocopherols are components present in the unsaponifiable lipid fraction of foods. Phytosterols have been known to have a wide spectrum of biological effects including anti-inflammatory, anti-oxidative and anti-carcinogenic activities (DeJong et al, 2004). Phytosterols have also shown to have anti-cholesterol activity. They inhibit the intestinal absorption of cholesterol, thereby lowering the total plasma cholesterol and low density lipoprotein levels.

Squalene is also considered to be a key intermediate in cholesterol biosynthesis. It is an essential dietary cancer chemopreventive agent. Squalene is also a powerful antioxidant. It is also capable of acting as an antidote to reduce accidental drug-induced toxicities (Senthilkumar et al, 2006).

Tocopherols are fat soluble antioxidants that function as scavengers of lipid peroxyl radicals. Tocopherol content in food is considered to be inversely proportional to the mortality from cardiovascular disease (Kushi et al. 1996). Also, due to their antioxidant effect and their ability to quench free radical damage, play a putative role in prevention of Alzheimer's disease and cancer.

Molecular / Short Path Distillation

Most of the substances present in oil have a high molecular weight and are thermally sensitive, due to which their separation and purification through traditional methods is hindered, since they decompose at higher temperatures. Molecular or Short Path Distillation is therefore used for separation and purification of these compounds,

since it operates under low pressure and relatively low temperatures. It minimizes the losses of thermally sensitive compounds and does not involve use of any toxic chemicals.

Molecular / Short Path Distillation is characterized by high vacuum in the distillation space, short exposure of the distilled liquid to elevated temperatures, and a small distance between the evaporator and the condenser, which is why it is referred to as 'Short path' distillation. This process is applied to lipid-containing products, including mono-glycerides production, recovery of carotenoids from palm oil, and purification of structured lipids. It can also be applied for production of biodiesel from castor oil, heavy petroleum characterization and recovery of tocopherols and free fatty acids from vegetable oil deodorizer distillate.

In a falling film distillatory apparatus, the distilled liquid continuously passes down the heated evaporating cylinder and evaporates partially and the vapors condense on the internally cooled condenser placed close to the evaporating cylinder. The liquid is made to pass through the evaporating cylinder in the form of a uniform thin film, which is responsible for the short residence time of the liquid in the evaporator.

CHAPTER II

REVIEW OF LITERATURE

Methodology for Seperation and Quantification of Unsaponifiables

Various studies have reported different methodologies for the quantification of the total unsaponifiables in different vegetable oils. Ryan et al., 2007 determined the unsaponifiable content of five types of seeds, grains and legumes by acid hydrolysis of the samples using ethanol and hydrochloric acid, followed by saponification of the extracts using potassium hydroxide. The phytosterols, tocopherols and squalene were then analysed using high-performance liquid chromatography (HPLC) system with a photodiode array detector. In another HPLC analysis, Park et al., 2004 examined tocopherols and tocotrienols from rice bran. They conducted saponification of the oils in a pilot scale extractor; and the unsaponifiable fractions were then separated out in hexane layers. The hexane layers were then removed by a vacuum evaporator and the unsaponifiables were collected and analysed in the HPLC. Further, Ito et al., 2007 used molecular/short path distillation to separate the tocopherols from soybean oil deodorizer distillate and used the HPLC with a fluorescence detector for the analysis of tocopherols.

Kalogeropoulos and Andrikopoulos, 2004 studied the squalene content in oils and fats from frying of potatoes. They prepared fatty acid methyl esters of the samples by cold alkaline methylation and analyzed them by capillary gas chromatography-mass spectrometry (GC-MS). Further Gast et al., 2005 purified tocochromanols from crude palm oil and soybean oil deodorizer distillate using gas chromatography. Moreover, Malecka, 2002 extracted the unsaponifiable matter from tomato seeds, oat grains and wheat germ oil by saponification followed by solvent extraction with diethyl ether. The compounds were then quantified using GC-MS. Also Kircher and Rosentein, 1946, performed analysis for campesterol, sitosterol and stigmasterol using Gas Liquid Chromatography (GLC) and Thin Layer Chromatography (TLC). Reina et al., 1999 reported an in-laboratory validated method for identification and quantification of sterols and triterpene diols by using GC-MS and TLC after saponification of the samples. Also, Schwartz et al., 2008 analyzed the tocopherol, tocotrienol and sterol contents of 14 vegetable oils and 9 industrial fats and oils using normal-phase HPLC (NP-HPLC) with fluorescence detection for tocopherols and GC-FID (flame ionization detection) for plant sterols, after saponification and extraction with heptanes – diethyl ether followed by silylation using N,O-Bis(trimethylsilyl)trifluoroacetamide (BSTFA) as the silylating agent and trimethylchlorosilane (TMCS) as the silylation catalyst. HPLC was also used by Anwar et al, 2008 for analysis of tocopherols and sterols in fruit seeds.

De Moraes et al., 2006, reported that molecular distillation is an alternative method for separation and purification of tocopherols since it operates under low temperature and pressure and does not require toxic or inflammable substances. Ito et

al., 2006 also used the centrifugal molecular distillation method to separate the unsaponifiables from soybean oil deodorizer distillate. Further, Snyder et al., 1999 used supercritical fluid chromatography to separate and determine the concentration of plant sterols from the extracts from samples of seed oils, margarine, corn germ oil and corn fiber oil. Moreover, Sugihara et al., 2010, also used Supercritical fluid extraction technology for fractionation and concentration of squalene and phytosterols in rice bran oil deodorizer distillate. In another study reported by Ghosh and Bhattacharyya, 1996, tocopherol and sterol concentrate was isolated from sunflower oil deodorizer distillate by biohydrolysis and bioesterification using Candida cylindracea and M. miehei lipase to hydrolyze the oil to remove the free fatty acids. This was followed by distillation of the ester fraction to separate the unsaponifiables. Carmona et al., 2010, isolated sterols from soybean oil deodorizer distillate by a method which first consisted of saponification of the samples, followed by separation of the unsaponifiable constituents by thin layer chromatography. This was followed by derivatization and silanization and then analysis in the GC.

In a study conducted by Torres et al, 2009 soybean oil deodorizer distillate was first subjected to a two step enzymatic reaction; the product obtained mainly comprised fatty acid ethyl esters, tocopherols, phytosterols, squalene, free fatty acids and triacylglycerols. The phytosterol esters were then purified from this mixture using supercritical carbon dioxide. In another study conducted by Wong et al., 2008, the phytosterol content was determined in grain processing residues. In their study, the samples were first saponified with potassium hydroxide and then derivatized using BSTFA as the silylating agent to make them more volatile. The analysis was then

conducted using Gas chromatography. Another study conducted by Bereau et al, 2003 used a similar methodology for derivatization of the unsaponifiable compounds. In this study, the sterols were first derivatized into silyl ethers with the addition of hexamethyldisilosane and trimethylchlorosilane as the silylating agents. This was followed by quantification using a GC-MS system. The tocopherols however were analyzed using an absorbance detector. Similarly, Ferrari et al., 1996 determined the sterol content in vegetable oils by preparing trimethylsilyl derivatives of the samples and then quantification using Gas Chromatography. The tocopherol content however was analyzed using HPLC. Moreover, Verleyen et al., 2001, performed analysis of the deodorizer distillates of different vegetable oils. They directly used their deodorizer distillate samples for derivatization and silylation using BSTFA with 1% TMCS solution. The samples were then analyzed using GC-FID.

The analysis of deodorizer distillates is a challenging problem. Tocopherols and phytosterols are two compounds in the deodorizer distillate which are commonly analyzed by GC or HPLC. The advantage of the HPLC method over the GC method is that derivatization and silylation to increase the volatility of the compounds is not necessary. Secondly, the HPLC can be operated under milder column temperatures and therefore is a suitable method for analysis of thermally unstable compounds like sterols. However the HPLC method has many limitations as compared to the GC method. Firstly, it is not possible to separate tocopherols and phytosterols under the same conditions. However this limitation can be overcome by using the GC since both phytosterols as well as tocopherols can be separated under the same conditions in the GC. Secondly, the high lipophilicity of sterols can make sample processing and

chromatography difficult (Lagarda et al., 2006). Therefore GC is the most appropriate technique for the analysis of deodorizer distillates (Gunawan and Ju, 2009). Lagarda et al., 2006 have also mentioned that GC is more sensitive than HPLC for analysis of sterols. They also reported that GC-FID or GC-MS may be considered for determination of phytosterols as compared to the HPLC method. This is also because capillary GC columns offer short analysis times and lesser peak interference, improvement in component resolution and high thermal stability compared with packed columns (Abidi, 2001).

The derivatizing agents that are usually used *N*-methyl-*N*-trimethyl-silyltrifluoroacetamide (MSTFA) in anhydrous pyridine and bis(trimethylsilyl)-trifluoroacetamide (BSTFA) containing 1% TMCS (Lagarda et al., 2006). Prior to capillary GC, sterols and stanols are usually transformed into derivatives that yield improved peak shape, resolution and sensitivity and a higher thermal stability. Although it is possible to separate sterols and stanols without derivatization, (Mortan et al., 1995) their resolution is not as good as that of their trimethylsilyl derivatized counterparts. Therefore sterols are mostly analyzed as their trimethylsilyl (TMS) or acetate derivatives, (Lagarda et al., 2006) since silylation and derivatization improves the volatility of these compounds by making them more thermally stable and suitable for characterization by the GC-MS or GC-FID system. The analysis of sterols is mostly conducted by a GC-FID system; however the GC-MS system is used to confirm the peak identities (Lagarda et al., 2006).

In the present study, the silylation method that was used first was using BSTFA and TMCS with HDS (Heptadecyl stearate) solution. However the graphs obtained from gas chromatography after silylation with these chemicals introduced a lot of unwanted peaks in the chromatogram. This could have been due to the fact that HDS solution interfered with the silylation of the unsaponifiables and as a result there were too many peaks obtained which were difficult to remove. As a result the silylation method was modified and applied in this study. Since previous research showed that the use of BSTFA and TMCS without HDS solution yielded better silylation of unsaponifiable compounds in soybean oil, the same method was applied to cottonseed oil in the present study. Therefore in this study, the unsaponifiables of cottonseed oil including sterols, tocopherols and squalene were analyzed using the GC after derivatization and silylation with BSTFA and TMCS to increase their volatility.

CHAPTER III

PURPOSE AND OBJECTIVES

Separation and Quantification of the Unsaponifiables of Cottonseed Oil and its Deodorizer Distillate

Purpose

The purpose of this research is to develop a method to separate and quantify the unsaponifiable constituents present in RBD (refined, bleached and deodorized) cottonseed oil and its deodorizer distillate. Tocopherols (α, β, γ, δ), sterols (stigmasterol, sitosterol, brassicasterol, campesterol), and squalene are classified as 'Unsaponifiables'. These are lipid-like compounds which are soluble in organic solvents. However these compounds cannot be saponified, which is why they are called unsaponifiables. These compounds are known to have many diverse health benefits. These individual components will be extracted using solvent extraction procedures, separated and quantified using Gas Chromatography (GC), after increasing their volatility by silylation. The standards for all these unsaponifiable compounds will also be silylated and quantified using the GC.

Objectives

- To examine in detail the methodology for silylation and chromatography for separation, identification and quantification of the unsaponifiables of cottonseed oil and cottonseed oil deodorizer distillate.

- To use seven unsaponifiable standards (α, β, γ, δ tocopherols and sterols-sitosterol, stigmasterol and campesterol) to identify and quantify the unsaponifiable components in cottonseed oil and cottonseed oil deodorizer distillate.
- To use the methodology that had been developed in the Food Science laboratory of Texas Woman's University in the past one year, for separation of total unsaponifiables from cottonseed oil and the deodorizer distillate.

CHAPTER IV

MATERIALS AND METHODS

Study Design

Three different samples of cottonseed oil were used for this study. These three samples were obtained from Pyco Industries from 3 different storage tanks containing RBD (refined, bleached, deodorized) cottonseed oil. The three tanks (numbered as 811, 815 and 816) were used as storage tanks for storing the RBD cottonseed oil. Three samples were collected from the three RBD cottonseed oil containing storage tanks and were numbered as 811, 815 and 816 respectively.

A common deodorizer distillate sample (common for all the three storage tanks) was also collected from the deodorization of cottonseed oil (Figure 4). This deodorizer distillate sample was obtained from the oil that was used to fill all the three storage tanks. The processing parameters were as follows: Temperature of deodorization: 480°F, Vacuum pressure: 2-2.5 mm of Hg).

The three oil samples and the common deodorizer distillate sample (total: 4 samples) were collected in amber bottles and were stored in the refrigerator. Before extraction, each of the four samples was mixed well to dissolve any saturated fatty acids that would have settled at the bottom of the bottle during storage. Three extractions were carried out for all the four samples. The three extracted residues from

each of the four samples were silylated. Each of the silylated residues were then analyzed using the GC three times (Figure 5 and 6).

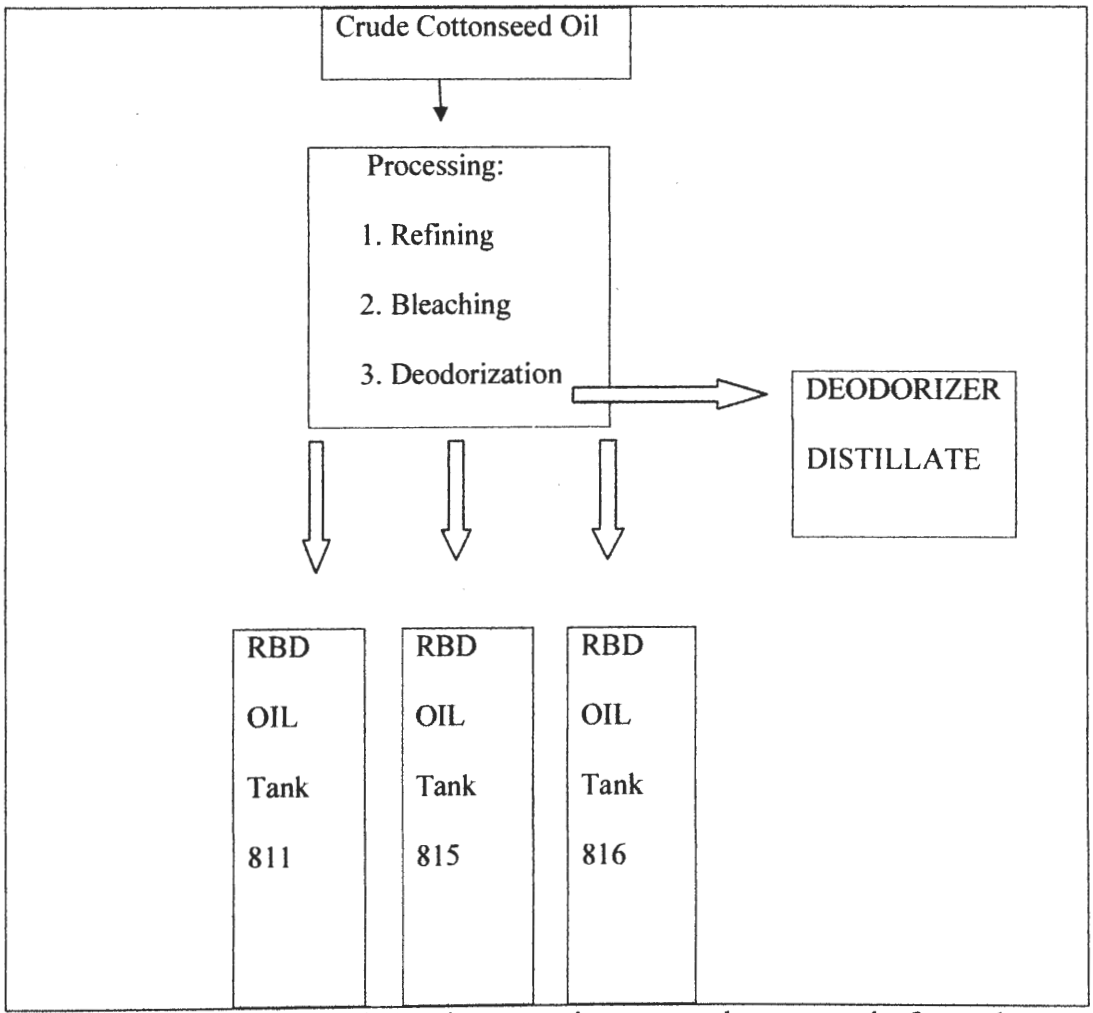

Figure 4: Flow diagram depicting the processing steps and storage tanks from where the oil and distillate samples were collected.

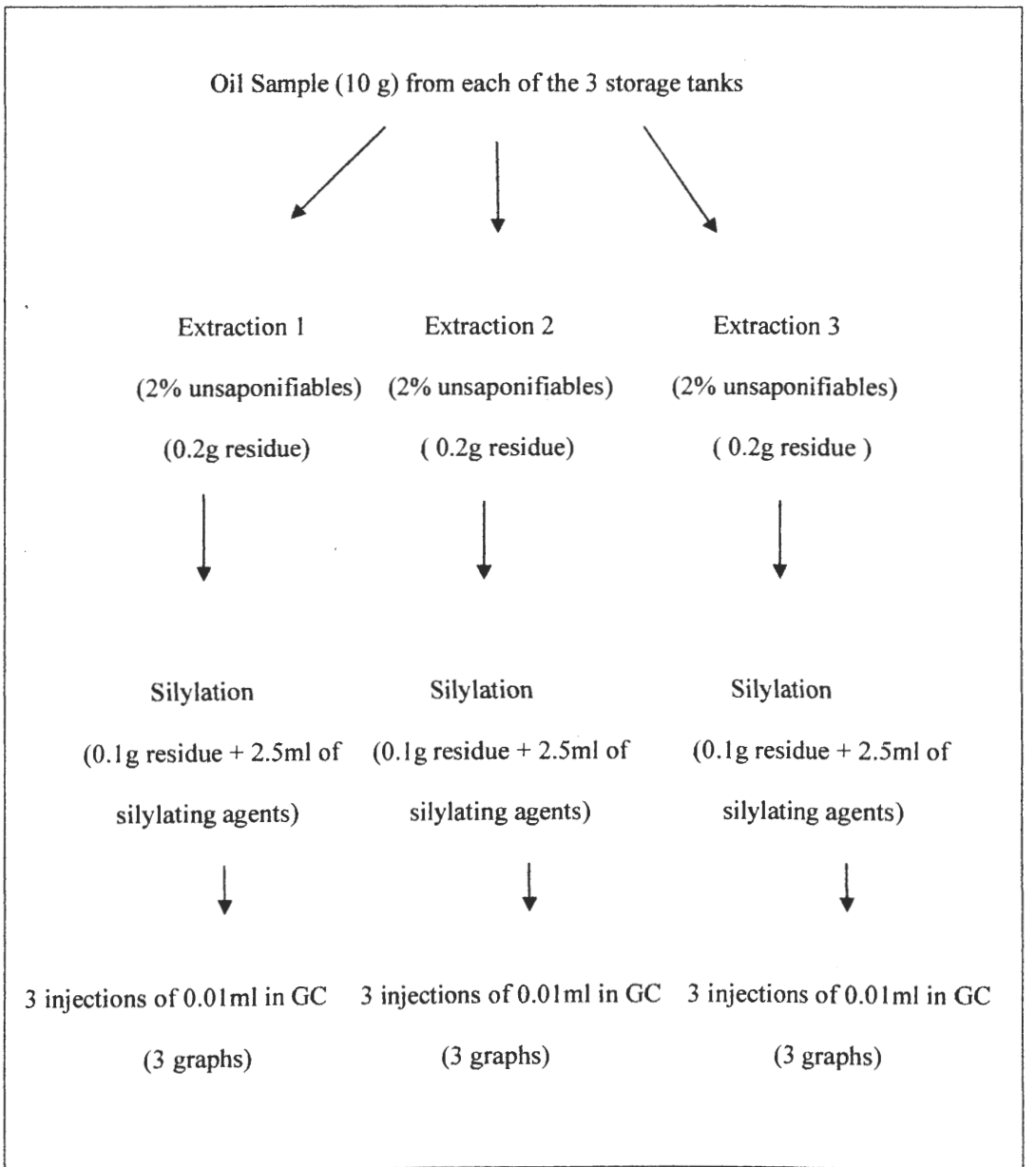

Figure 5: Flow diagram depicting the experimental design for cottonseed oil

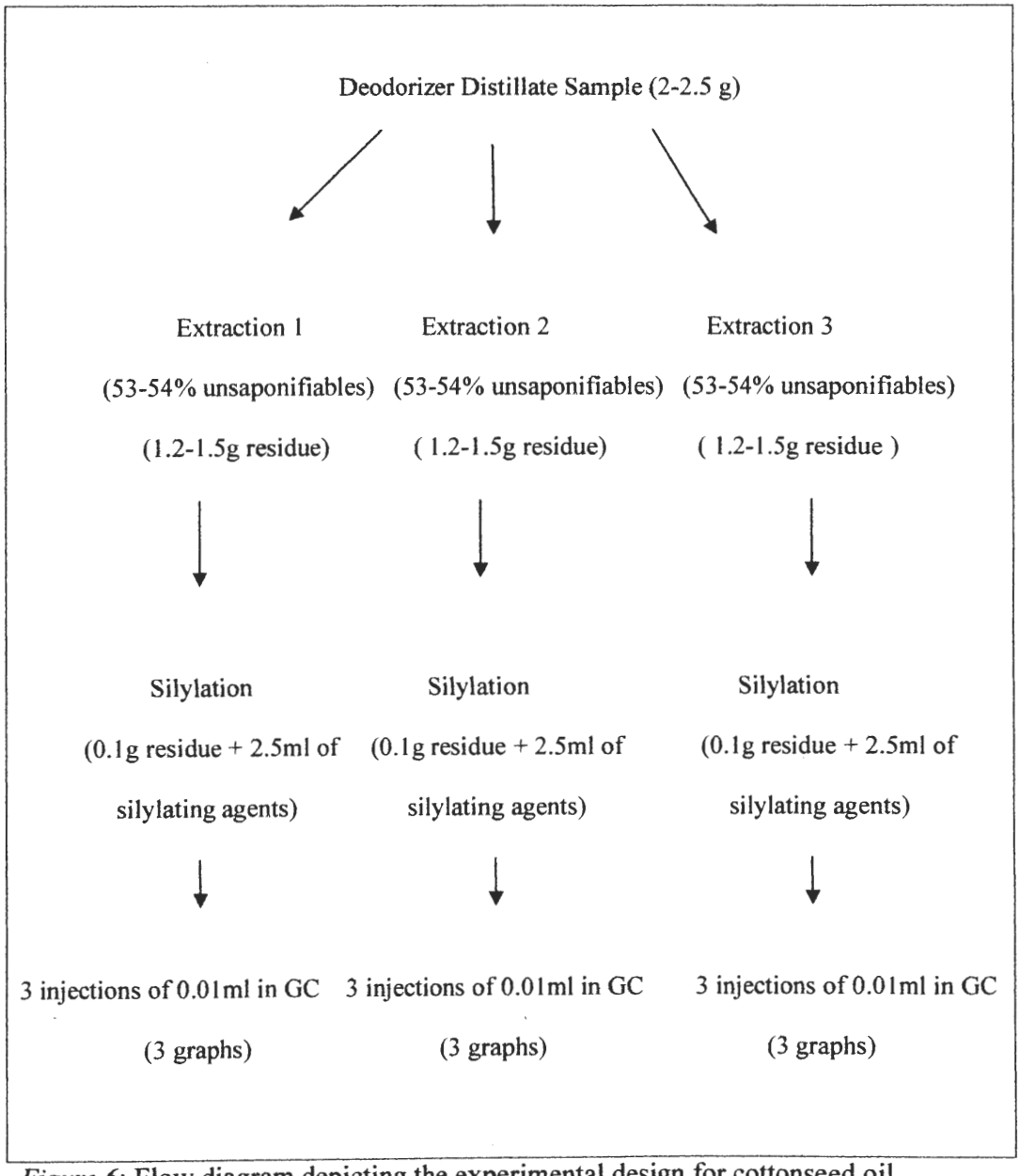

Figure 6: Flow diagram depicting the experimental design for cottonseed oil deodorizer distillate.

Materials

The samples of cottonseed oil and the deodorizer distillate of cottonseed oil were obtained from Pyco Industries, located in Lubbock, Texas. Pyco Industries is the largest cottonseed cooperative serving southern United States, with over 60 member gins. It has been in the cottonseed processing business for over 60 years. Pyco was formed by the merger of two cooperatives, 'Plainsman' and 'Yahoo', which is where 'PYCO' gets its name from. This cooperative majorly produces cottonseed oil for cooking and a salad oil, whole cottonseed and the by-products of cottonseed processing which include cottonseed meal, hulls and linters. Whole cottonseed is the raw material used for production of all their products. It is sold to Pyco by their gin members. The local gins process the cotton grown in local Texas farms surrounding the Lubbock area. The production of cotton in the surrounding area may provide up to 20 – 25% of the total cotton grown in the United States. The oil and distillate samples that were used in this study were obtained locally from the Lubbock area from Pyco Industries. The oil that was used in this study was refined, bleached and deodorized (RBD) oil.

All the analytical grade solvents were purchased from Fisher Scientific. The analytical grade reference sterol standards (β-sitosterol, stigmasterol, campesterol) and tocopheol kit (α, β, γ, δ tocopherols) were purchased from Sigma Aldrich. Squalene standard solution was purchased from Fisher Scientific. The internal standard - cholesterol was purchased from Sigma Chemicals.

Methodology

Saponification of Cottonseed oil and its Deodorizer Distillate Samples (Procedure given by the American Oil Chemists' Society (AOCS)).

The samples were weighed (2 to 2.5 g) and saponified with 25mL ethyl alcohol and 1.5 mL of 50% KOH solution under reflux. The potassium group from the potassium hydroxide attaches to the carboxylic group of the fatty acid molecule and this leads to the formation of a potassium salt of the fatty acid. The saponified sample was then transferred to a separatory funnel using 50 mL of water and 50mL of diethyl ether. The funnel was then shaken vigorously after which the lower aqueous layer was removed and the upper ether layer was retained. The soap formed after saponification came out in the water layer, while the unsaponifiables were retained in the ether layer, since they are fat-like compounds.

This extraction was repeated two more times using 50mL portions of diethyl ether every time. After the extraction, the ether layers were combined together and washed with 20mL of water three times. Washing repeatedly with water removes any traces of soap left behind after saponification with potassium hydroxide. This was followed by washing the sample with 20mL of 0.5N KOH solution followed by washing with 20mL of water. These washings were repeated alternately three times. Consequently, the sample was washed three to four times with 20mL of water until the washings are no longer alkaline to phenolphthalein.

Potassium hydroxide gives a pink color on reaction with water. If there is any unreacted potassium hydroxide left behind in the extraction mixture, it would be

removed after consecutive washings with water. The complete removal of potassium hydroxide is indicated by no pink color formation with phenolphthalein.

The extract so obtained was transferred to a tared beaker and was evaporated to dryness in a water bath. The drying was then completed in a vacuum oven at 75 - 80°C and an internal pressure of 200 mm of Mercury. After weighing, the residue was dissolved in 2mL of ether and 10mL of 95% alcohol will be added to it. It was then analyzed for its free fatty acid content by titrating it with sodium hydroxide solution. The same extraction procedure was conducted for the blank, without any fat or oil present.

Calculations were done as follows:

Unsaponifiable matter % = [A – (B + C) / (mass of sample)] * 100

Where A = mass of residue (g)

B = mass of fatty acid (g)

C = mass of blank (g)

Derivatization of the Sample for GC Analysis (Verleyen et al., 2001)

Derivatization of the samples was done to increase the volatility of the components. 0.1- 0.15g of the sample extracted by the above procedure was dissolved in 0.5mL of pyridine and 1mL of BSTFA (N, O-bis-(trimethylsilyl) trifluoroacetamide) containing 1% TMCS (Trimethyl chlorosilane) solution was added to it. This would act as the derivatizing and silylating agent. The silylating agent adds a silyl group (R_3Si) to the long hydrocarbon chains of the tocopherol, sterol and

squalene molecules. The silyl group makes these unsaponifiable compounds more volatile and thermally stable, therefore making their detection and quantification by the GC much more efficient. Unsaponifiable compounds consist of long hydrocarbon chains with hydroxyl groups. The trimethylsilyl group (R_3Si) has three methyl groups attached to a Si group. This trimethylsilyl group replaces the acidic hydrogen (H^+) from the dissociated hydroxyl group in the hydrocarbon chain and attaches to the oxygen group. Since the acidic hydrogen is removed, the compound becomes less polar. The introduction of three more methyl groups and one Si group in the hydrocarbon chain makes the molecule much heavier, non polar and volatile. Methylation of free fatty acids on the other hand, introduces only one methyl group by replacing the hydrogen atom in the fatty acid group. Therefore, methylated fatty acid is a lighter molecule than a silylated unsaponifiable molecule and is therefore comparatively less heat stable as compared to silylated unsaponifiable molecules.

The test tube was then placed in an oven at 70°C for 20 min for completion of the silylation. This was followed by addition of 1mL of silylated cholesterol solution (internal standard). The sample was then transferred to a GC vial and diluted with chloroform. 1µL of the derivatized sample was then injected in the GC for analysis (Figure 7).

Standard Solutions

All the standards were dissolved in chloroform and had a concentration of 10 mg/ mL (Dumont and Narine, 2007). They were silylated and injected in the GC along

with the internal standard (cholesterol). Standard calibration curves were then generated using the GC (Figures 8, 9 and 10).

GC Conditions

Separation of the unsaponifiable fractions was performed on a Hewlett-Packard gas chromatograph.

- Column type: CP-Sil 8 CB Low Bleed/MS column. A low bleed column was used since it does not give elevated baselines at higher temperatures. The film thickness of the column is 0.25 μm which is used for effective retention of compounds. A CP-Sil 8 column has a very high temperature range which can go up to 350°C. It is a very inert and non polar column and has a higher affinity for non polar compounds. Therefore it was used for the analysis and quantification of unsaponifiables which are lipids in nature and are non polar compounds.
- Column dimensions: 15m * 0.25 mm and 0.25mm film thickness.
- Detector: Flame Ionization Detector.
- Temperature program: injection temperature: 60°C, detector temperature: 360°C. Initial oven temperature: held at 80°C for 3 min, rising to 150°C at 10°C/ min, to 250°C at 5°C / min, and then to 340°C at 10°C / min, where it is held for 20 min.
- Carrier gas: nitrogen.
- For ignition of flame: hydrogen and oxygen

- Split injection: 1:50
- Injection volume: 1 µL
- Flow rate: 1mL per minute

Methodology for Silylation of Unsaponifiables

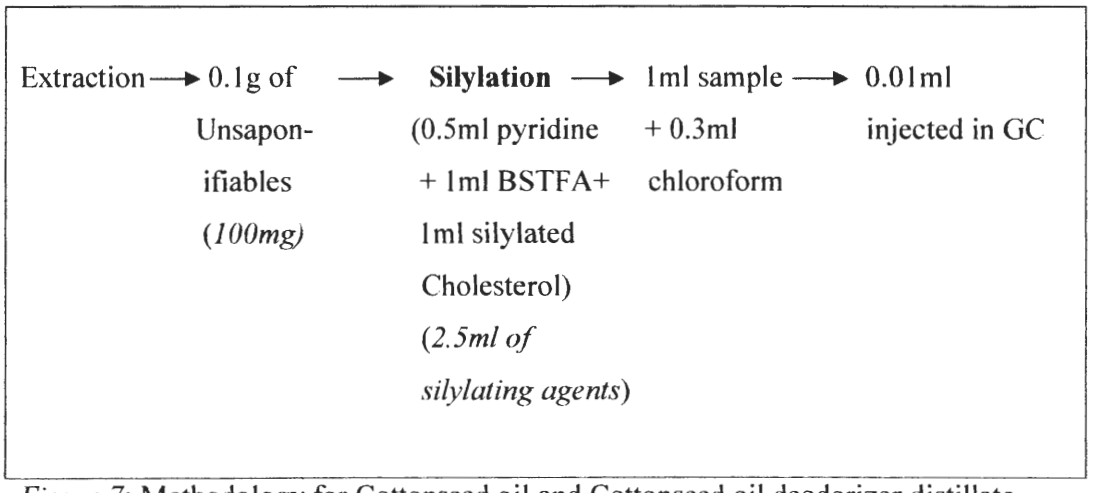

Figure 7: Methodology for Cottonseed oil and Cottonseed oil deodorizer distillate

Methodology for Standards

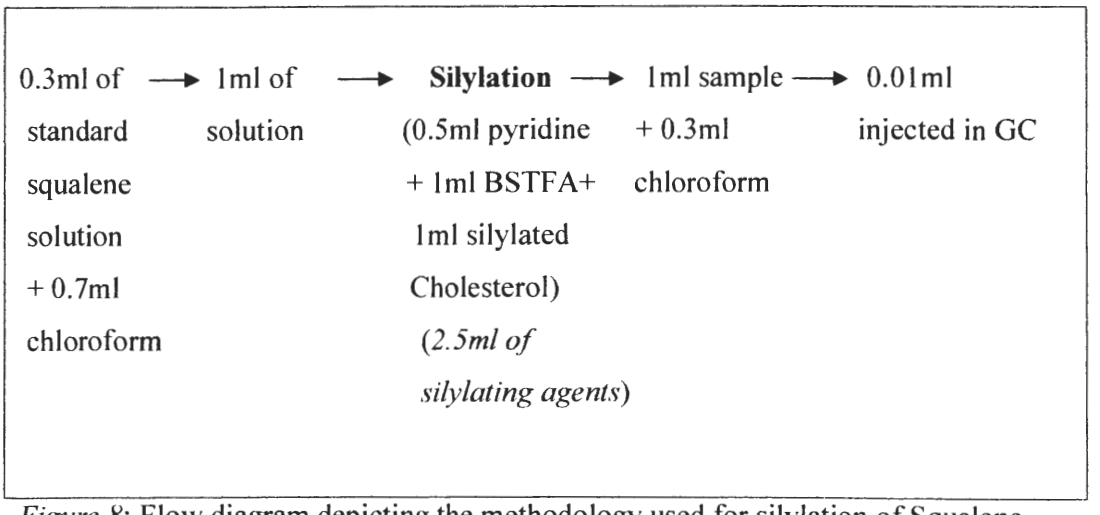

Figure 8: Flow diagram depicting the methodology used for silylation of Squalene standard.

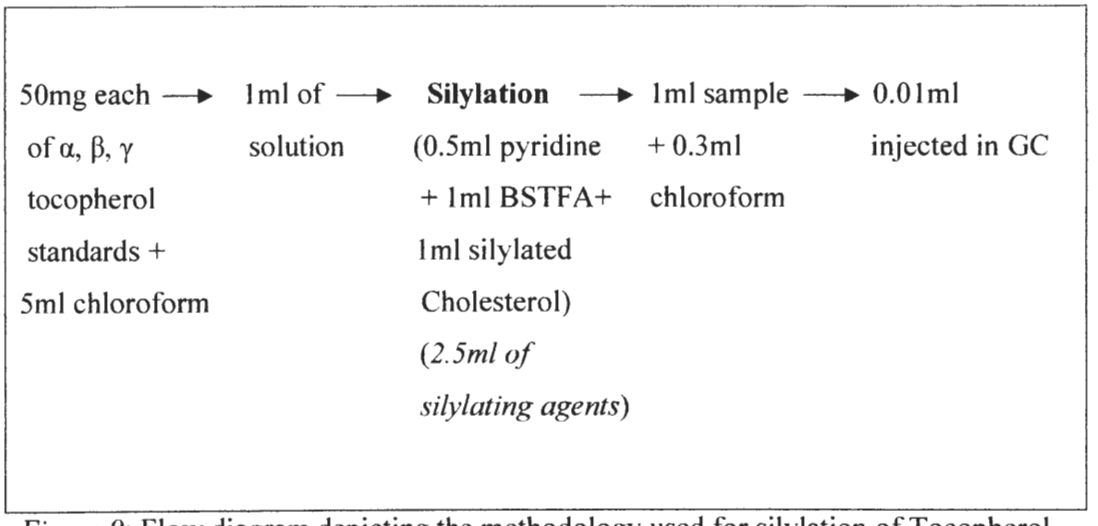

Figure 9: Flow diagram depicting the methodology used for silylation of Tocopherol standards

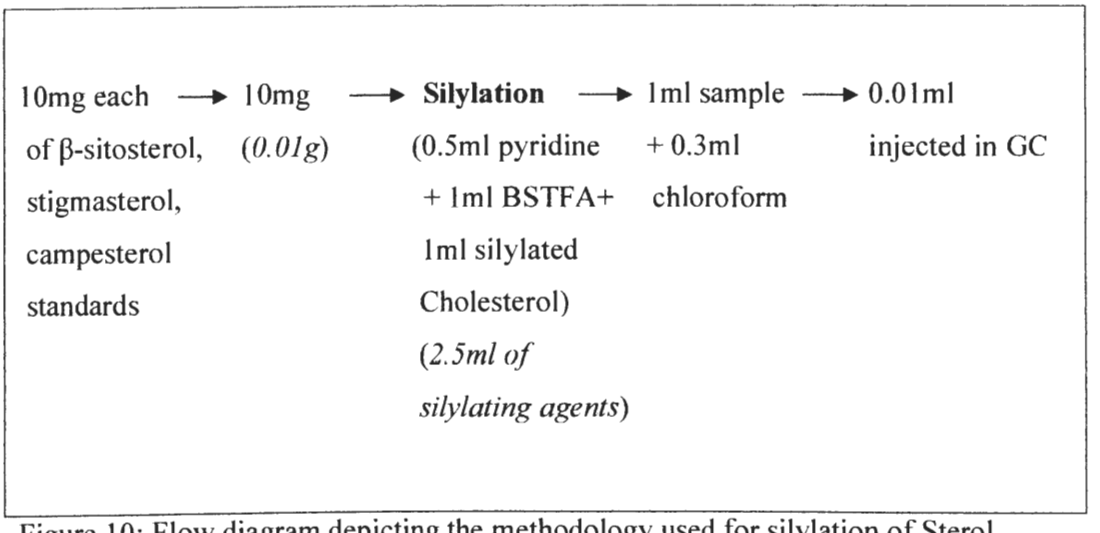

Figure 10: Flow diagram depicting the methodology used for silylation of Sterol standards

CHAPTER V

RESULTS

The percentage of the total unsaponifiable matter of Cottonseed oil from the three storage tanks numbered as 816, 815 and 811, was found to be 1.8%, 2.1% and 2.3% respectively (Tables 6,7 and 8). The percentage of the total unsaponifiable matter in Cottonseed oil deodorizer distillate was found to be 53.9% (Table 9). Table 10 indicates the peak area and the retention times of all the unsaponifiable standards. The total percentages of tocopherols and sterols in the unsaponifiable matter of the deodorizer distillate were found to be 12.64% and 65.07% respectively. The total tocopherol and sterol percentages in cottonseed oil sample number 815 were 4.41% and 31.3% respectively of the total unsaponifiable matter. Similarly, for cottonseed oil sample number 816 they were 2.94% and 26.79% respectively, and for cottonseed oil sample number 811 they were found to be 3.5% and 36.07% respectively (Table 11).

β sitosterol was found to be present in the highest concentration in the total unsaponifiable matter of both the distillate (57.3%) as well as the three oil samples 816, 815 and 811 (23%, 27.7% and 31.0% respectively). This was followed by α tocopherol; 4.4% in the distillate and 1.7%, 3.3% and 1.8% in the three oil samples respectively. γ tocopherol was found to be 4.4% in the distillate and 0.9%, 0.8%

and1.3% in the three oil samples (816, 815 and 811) respectively (Table 12). No peak for δ tocopherol was observed in all the four samples indicating that δ tocopherol was absent in both cottonseed oil as well as cottonseed oil deodorizer distillate. This was followed by Campesterol which was found to be 6.57% in the distillate and 2.6%, 2.6% and 1.8% in the three oils respectively. Stigmasterol and β tocopherol were present in small amounts in both the distillate and the oils (Table 12). The ratios of the different sterols and the different isomers of tocopherols are indicated in Table 13.

Table 6: *Average Percentages of Total Unsaponifiables in the Three Oil Samples and the Deodorizer Distillate Samples.*

% total unsaponifiables from oil sample number 816 (average of 3 values)	% total unsaponifiables from oil sample number 815 (average of 3 values)	% total unsaponifiables from oil sample number 811 (average of 3 values)	% total unsaponifiables from deodorizer distillate (average of 3 values)
1.8	2.1	2.3	53.9

Table 7: *Peak Areas and Retention Times of Tocopherol and Sterol Standards*

Standard	Peak area (average of 3 graphs)	Retention time (minutes)
1. α Tocopherol	2126.18	34.4
2. β Tocopherol	1586.26	33.2
3. γ Tocopherol	1369.71	33.3
4. δ Tocopherol	1781.39	32.4
5. β Sitosterol	1143.06	35.8
6. Stigmasterol	1046.68	35.46
7. Campesterol	1091.74	35.2

Table 8: *Peak Areas and Percentages of Sterols and Tocopherols in Cottonseed Oil and the Deodorizer Distillate Samples*

Sample	Unsaponifiable matter	Peak area (average of 9 graphs)	Relative Percentage	Total Tocopherols and Total Sterols
Cottonseed Oil Deodorizer Distillate	1) α Tocopherol	1286.23	4.40 %	Total Tocopherols: 12.64% Total Sterols: 65.07%
	2) β Tocopherol	838.73	3.82 %	
	3) γ Tocopherol	840.74	4.42 %	
	4) β Sitosterol	6546.95	57.30 %	
	5) Stigmasterol	123.21	1.20 %	
	6) Campesterol	717.77	6.57 %	
Cottonseed Oil (Sample: 815)	1) α Tocopherol	974.20	3.3 %	Total Tocopherols: 4.41% Total Sterols: 31.3%
	2) β Tocopherol	70.49	0.32 %	
	3) γ Tocopherol	149.86	0.79 %	
	4) β Sitosterol	3168.30	27.7 %	
	5) Stigmasterol	104.87	1.0 %	
	6) Campesterol	281.53	2.6 %	
Cottonseed Oil (Sample: 816)	1) α Tocopherol	500.55	1.7 %	Total Tocopherols: 2.94% Total Sterols: 26.79%
	2) β Tocopherol	83.74	0.38 %	
	3) γ Tocopherol	160.53	0.86 %	
	4) β Sitosterol	2629.96	23 %	
	5) Stigmasterol	128.64	1.23 %	
	6) Campesterol	280.01	2.56 %	
Cottonseed Oil (Sample: 811)	1) α Tocopherol	531.61	1.8 %	Total Tocopherols: 3.5% Total Sterols: 36.07%
	2) β Tocopherol	95.81	0.44 %	
	3) γ Tocopherol	239.60	1.26 %	
	4) β Sitosterol	3544.95	31.01 %	
	5) Stigmasterol	341.96	3.27%	
	6) Campesterol	195.46	1.79 %	

Table 9: *Tocopherol and Sterol Percentages of the Unsaponifiables in the Oil and Distillate Samples*

Unsaponifiable	Deodorizer Distillate	Oil Sample 816	Oil Sample 815	Oil Sample 811
α Tocopherol	4.4	1.7	3.3	1.8
β Tocopherol	3.8	0.4	0.3	0.4
γ Tocopherol	4.4	0.9	0.8	1.3
β Sitosterol	57.3	23.0	27.7	31.0
Stigmasterol	1.2	1.2	1.0	3.3
Campesterol	6.6	2.6	2.6	1.8
Others	22.3	70.2	64.3	60.4

Table 10: *Ratios of the Different Isomers of Tocopherols and the Different Sterols in Cottonseed Oil and Cottonseed Oil Deodorizer Distillate*

Sample	Ratios of percentages of Sterols	Ratios of percentages of Tocopherols
Cottonseed Oil Deodorizer Distillate	Sitosterol/Stigmasterol= 47.75 Sitosterol/Campesterol= 8.72 Stigmasterol/Campesterol= 0.183	α tocopherol:β tocopherol = 1.15 α tocopherol:γ tocopherol = 1.0 β tocopherol:γ tocopherol = 0.86
Cottonseed Oil (Sample 815)	Sitosterol/Stigmasterol= 27.7 Sitosterol/Campesterol= 10.65 Stigmasterol/Campesterol= 0.39	α tocopherol:β tocopherol = 10.31 α tocopherol:γ tocopherol = 4.2 β tocopherol:γ tocopherol = 0.41
Cottonseed Oil (Sample 816)	Sitosterol/Stigmasterol= 18.7 Sitosterol/Campesterol= 9.0 Stigmasterol/Campesterol= 0.48	α tocopherol:β tocopherol = 4.5 α tocopherol:γ tocopherol = 1.98 β tocopherol:γ tocopherol = 0.44
Cottonseed Oil (Sample 811)	Sitosterol/Stigmasterol= 9.48 Sitosterol/Campesterol= 17.32 Stigmasterol/Campesterol= 1.83	α tocopherol:β tocopherol = 4.1 α tocopherol:γ tocopherol = 1.43 β tocopherol:γ tocopherol = 0.35

Table 11: *Percent Unsaponifiable Compounds in 43,200 Pounds of Cottonseed Oil Deodorizer Distillate as Reported by Pyco Industries*

Unsaponifiables	**Percentage**
Total Sterols	14.1
Campesterol	1.3
Stigmasterol	0.4
Sitosterol	12.2
Total Tocopherols	7.95
Alpha tocopherol	2.8
Beta + Gamma Tocopherol	4.9
Delta Tocopherol	0.2
Water	1.6

Statistical Analysis

The total unsaponifiable content from the three oil samples was statistically analyzed using one way ANOVA. The results indicate that there was no significant difference between the values of the total unsaponifiables ($p>0.05$) of the three oil samples.

The nine GC graphs of the individual unsaponifiable compounds from the extraction from each of the three oil samples were also statistically analyzed using one way ANOVA. There was no significant difference ($p>0.05$) between the nine graphs for beta sitosterol, campesterol, and beta tocopherol from the three oil samples.

However a significant difference was observed in the values for alpha tocopherol, gamma tocopherol and stigmasterol. This could be due to the deodorization selectivity for various unsaponifiables which could result in the removal of different unequal amounts from the oil during deodorization, thereby resulting in some variations in the unsaponifiables remaining in the three oil samples. Also the procedure used for the extraction of the unsaponifiables might also have led to variations in the amount of unsaponifiables extracted from the different samples. Some variations could also have resulted from the methodology for silylation of the unsaponifiables, since it was a method that was adapted for cottonseed oil and its deodorizer distillate in our laboratory. Some amounts of analytical errors could have also contributed to the variations.

CHAPTER VI

DISCUSSION

The deodorizer distillate was found to contain a higher percentage of total unsaponifiables (53.9%) as compared to the oil samples (1.8-2.3%). This is because most of the unsaponifiable compounds and other volatile compounds are lost from the oil during deodorization, due to exposure to very high temperature and low pressure/vacuum. RBD Cottonseed oil contains 1.5-2.5% unsaponifiables. Deodorization of oil extracts these unsaponifiables and concentrates them in the distillate. This is because the molecular weight of unsaponifiables is much less than triglyceride molecules and they are also more volatile. Hence they are more likely to be extracted out with steam and vacuum during deodorization. The deodorizer distillate therefore contains a higher percentage (53%) of unsaponifiables as compared to RBD oil.

The ratios of the different individual compounds of the unsaponifiables of the deodorizer distillate were found to be very close to the ratios of the individual unsaponifiables that were determined from the values reported by the analysis of the Pyco oil samples (Table 14) of RBD cottonseed oil and deodorizer distillate (Table 15). The ratio of sitosterol: stigmasterol was found to be 8.72 in this study and was very close to the ratio from Pyco (9.38). Similarly, the ratio of Stigmasterol:

Campesterol was 0.2 in this study and 0.3 from Pyco's analysis. Further, the ratio of α tocopherol: β+γ tocopherol was also close-0.54 in this study and 0.57 in Pyco's analysis. The ratio of sitosterol: stigmasterol (47.75) was found to be higher as compared to the ratio from Pyco Industries (30.50). This difference could be attributed to the fact that this analysis was conducted by Pyco a year before this study on a different sample of deodorizer distillate. The method of extraction and derivatization used by Pyco for the analysis of unsaponifiables is also different than what was used for this study. Therefore the variability of the results from this study can be attributed to different samples of deodorizer distillates, processed at different times and under different conditions.

According to the quantification conducted by Pyco Industries, the total unsaponifiable content present in RBD cottonseed oil was found to be 1.2 %. In this study, the total unsaponifiable content of cottonseed oil was found to range from 1.8 % - 2.3%. This difference in the values is a result of many factors. Firstly, the different growing conditions, different geographical locations and different cultivars of cotton have an impact on the composition of the oil. Also, the processing conditions also affect the composition of the oil and the distillate. The processing of cottonseed oil is known to reduce unsaponifiables up to 50 %. The samples that were used in the present study were samples of refined, bleached and deodorized (RBD) oil. The processing conditions of the samples used by Pyco were probably different compared to the samples used in this study. Further, the different methodology of extraction of the unsaponifiables can also attribute to the different compositions of different samples. The method that was followed in the current study was the official AOCS

wet chemistry method of extraction. However, the procedure that was followed by the laboratories of Pyco was the TLC (thin layer chromatography) method of separation of unsaponifiables from the oil and distillate.

Table 12: *Ratios of the Different Unsaponifiable Compounds of Cottonseed Oil Deodorizer Distillate from this Study as Compared to the Laboratory Results from Previous Analysis of the Ratios of Unsaponifiables from Pyco Industries*

Ratios of unsaponifiables from this study	Ratios of unsaponifiables from Pyco Industries
1. Sitosterol/Stigmasterol= 47.75 2. Sitosterol/Campesterol= 8.72 3. Stigmasterol/Campesterol= 0.20 1. α tocopherol:β+ γ tocopherol = 0.54	1. Sitosterol/Stigmasterol= 30.50 2. Sitosterol/Campesterol= 9.38 3. Stigmasterol/Campesterol= 0.31 4. α tocopherol:β+ γ tocopherol = 0.57

The unsaponifiable content of cottonseed oil in this study was also found to be similar to the sterol content of cottonseed oil from the literature. Reina et al., 1999 indicated that the relative percentages of different sterols in cottonseed oil are as follows: Campesterol, 8.8% of total sterols; Stigmasterol, 2.7% of total sterols; and β sitosterol, 82.8% of total sterols (Table 2). In this study, the average percentages of the different sterols in the three RBD oil samples were as follows: Campesterol, 7.4% of total sterols; stigmasterol, 5.8% of total sterols; and β sitosterol, 86.7% of total sterols.

The values of the unsaponifiables of refined cottonseed oil (but not bleached and deodorized) in parts per million (or mg unsaponifiables per kg oil) were reported by

Jones and King, 1996. According to the calculations of this study, there are 2% unsaponifiables in cottonseed oil. Therefore the tocopherol content in cottonseed oil from the values reported by Jones and King, 1996 (Table 5) would be as follows: α tocopherol, 1.6% of total unsaponifiables; β tocopherol, 0 % of total unsaponifiables; γ tocopherol, 1.6% of total unsaponifiables; δ tocopherol, 0% of total unsaponifiables. In this study, the average tocopherol content (from the three oil samples) was as follows: α tocopherol, 2.2% of total unsaponifiables; β tocopherol, 0.36% of total unsaponifiables; γ tocopherol, 1% of total unsaponifiables; δ tocopherol, 0% of total unsaponifiables. Similarly, the sterol content of cottonseed oil, as recorded by Jones and King, 1996 was as follows: Stigmasterol, 0.1% of total unsaponifiables; β sitosterol, 16.7% of total unsaponifiables; Campesterol, 1.4% total unsaponifiables; Brassicasterol, 0.003% of total unsaponifiables. From this study, the average sterol content (average of the three samples of cottonseed oil) is as follows: Stigmasterol, 1.8% of total unsaponifiables; β sitosterol, 27.2% of total unsaponifiables; Campesterol, 2.3% of total unsaponifiables; Brassicasterol, 0% of total unsaponifiables.

The variability in the results could be due to the fact that there could have been some losses of unsaponifiables during extraction. There could also have been some impurities in the extracted sample which could have influenced the results. These impurities could be triglycerides that could have been present in the extracted residues. Further research can be conducted to quantify the amount of triglycerides and other compounds that could be present in the extracted unsaponifiables.

CHAPTER VII

CONCLUSION

In conclusion, the method developed in this study is an efficient method for the separation, identification and quantification of the unsaponifiables of cottonseed oil and the deodorizer distillate of cottonseed oil. The unsaponifiable content of the oil and the distillate from this method was similar to the content of unsaponifiables obtained from Pyco Industries and from previously conducted research. The concentration of the standard solutions was accurate and the variations were minimum for the deodorizer distillate. There was some amount of variability in the ratios for RBD cottonseed oil. Therefore it can be concluded that the procedure developed in this study was successful in the identification and quantification of the unsaponifiables from cottonseed oil and its deodorizer distillate.

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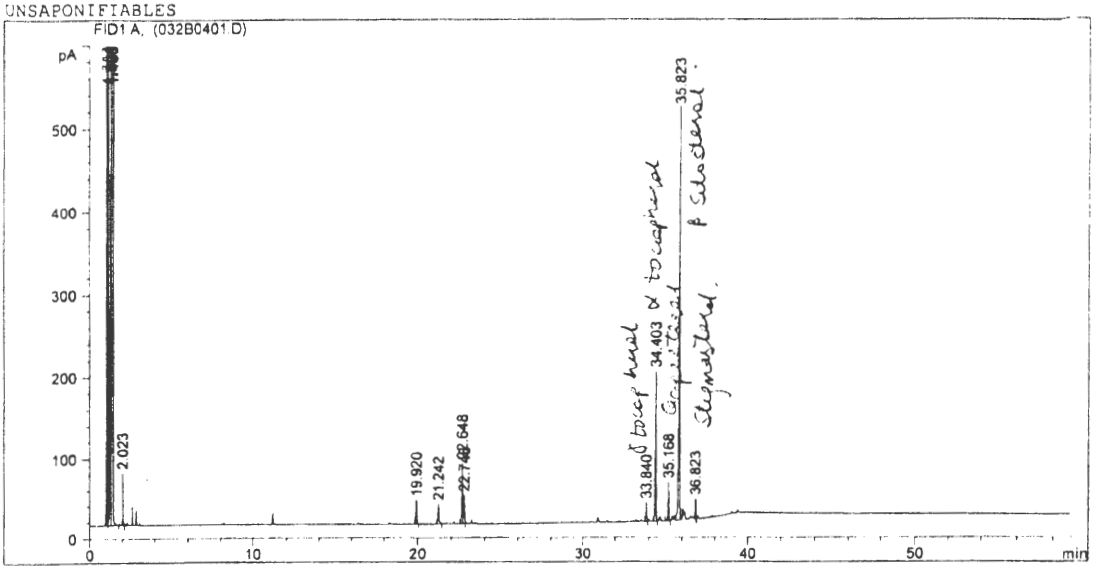
APPENDIX A

GC graph for cottonseed oil

Data File C:\HPCHEM\1\DATA\032B0401.D 815 (1) (4) Sample Name: 815

Injection Date : 8/13/2011 10:36:45 PM Seq. Line : 4
Sample Name : 815 Location : Vial 32
Acq. Operator : PK Inj : 1
Acq. Instrument : Instrument 1 Inj Volume : 1 µl
Method : C:\HPCHEM\1\METHODS\PRIYANKA.M
Last changed : 8/13/2011 6:06:34 PM by PK
UNSAPONIFIABLES



Area Percent Report

Sorted By : Signal
Multiplier : 1.0000
Dilution : 1.0000
Use Multiplier & Dilution Factor with ISTDs

Signal 1: FID1 A,

Peak #	RetTime [min]	Type	Width [min]	Area [pA*s]	Height [pA]	Area %
1	1.053	VV S	0.0217	2.01203e4	1.54309e4	13.26767
2	1.161	VV S	0.0127	3.74111e4	4.47975e4	24.66953
3	1.315	BV T	0.0328	1631.72729	661.32635	1.07599
4	1.400	VB S	0.0407	8.88993e4	2.74879e4	58.62176
5	2.023	BB	0.0241	98.17834	65.17146	0.06474
6	19.920	BB	0.0594	115.83513	29.73781	0.07638
7	21.242	BB	0.0753	121.14050	24.21947	0.07988
8	22.648	PV	0.0637	317.65924	76.06509	0.20947
9	22.748	VV	0.0619	143.17288	35.58111	0.09441
10	33.840	VV	0.0558	84.93996	23.13589	0.05601
11	34.403	VV	0.0502	622.55377	185.33331	0.41052
12	35.168	VV	0.0527	163.97934	48.24104	0.10813
13	35.823	VV	0.0518	1842.65698	502.07217	1.21508
14	36.823	VV	0.0491	76.45791	24.70869	0.05042
Totals :				1.51649e5	8.93918e4	

Results obtained with enhanced integrator!

*** End of Report ***

APPENDIX B

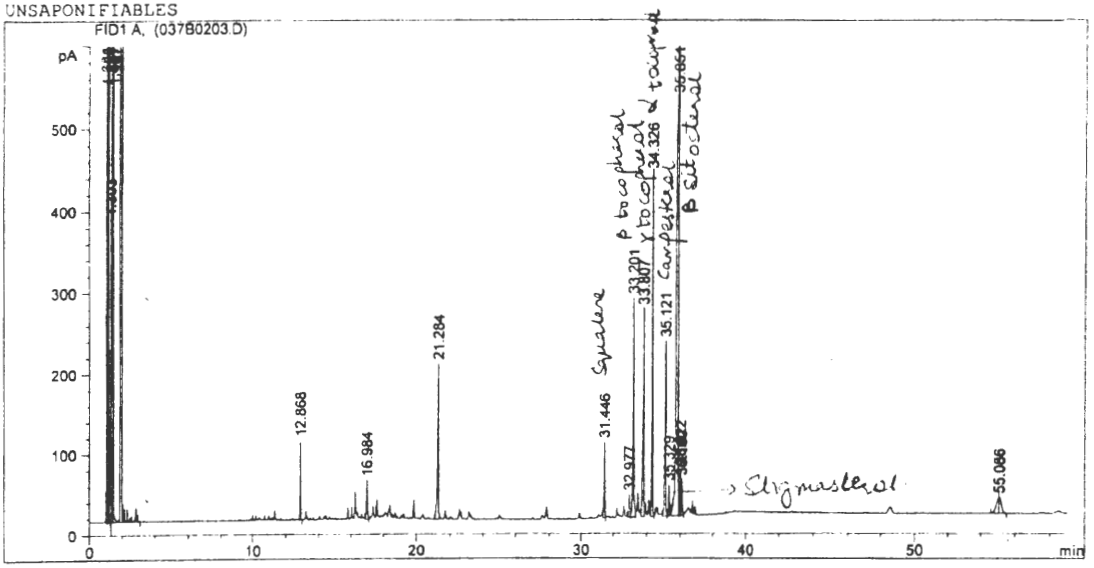
GC Graph Cottonseed Oil Deodorizer Distillate

Data File C:\HPCHEM\1\DATA\037B0203.D Sample Name: Distillate 1

(3)

Injection Date : 9/20/2011 4:03:15 PM Seq. Line : 2
Sample Name : Distillate 1 Location : Vial 37
Acq. Operator : PK Inj : 3
Acq. Instrument : Instrument 1 Inj Volume : 1 µl
Acq. Method : C:\HPCHEM\1\METHODS\PRIYANKA.M
Last changed : 8/13/2011 6:06:34 PM by PK
Analysis Method : C:\HPCHEM\1\METHODS\PRIYANKA.M
Last changed : 9/22/2011 3:39:08 PM by PK
(modified after loading)

UNSAPONIFIABLES



Area Percent Report

Sorted By : Signal
Multiplier : 1.0000
Dilution : 1.0000
Use Multiplier & Dilution Factor with ISTDs

Signal 1: FID1 A,

Peak #	RetTime [min]	Type	Width [min]	Area [pA*s]	Height [pA]	Area %
1	1.001	VV S	8.16e-3	1282.06421	2644.16772	0.79219
2	1.048	VV S	0.0222	2.22559e4	1.66991e4	13.75198
3	1.150	VV S	0.0113	1.72953e4	2.42216e4	10.68681
4	1.303	BV T	0.0336	984.47272	388.12253	0.60831
5	1.381	VV S	0.0432	9.12100e4	3.51785e4	56.35888
6	1.427	VB S	0.0250	1.31802e4	8786.95020	8.14407
7	12.868	VV	0.0460	298.41553	99.47222	0.18439
8	16.984	VV	0.0761	239.69038	48.98608	0.14811
9	21.284	PV	0.0649	880.96088	194.42761	0.54435
10	31.446	VV	0.0550	349.68185	94.96062	0.21607
11	32.977	VV	0.0596	132.72714	28.34596	0.08201
12	33.201	VV	0.0555	1066.13696	273.15799	0.65877
13	33.807	VV	0.0610	1043.49573	259.11734	0.64478

Data File C:\HPCHEM\1\DATA\037B0203.D Sample Name: Distillate 1

Peak #	RetTime [min]	Type	Width [min]	Area [pA*s]	Height [pA]	Area %
14	34.326	VV	0.0588	1600.63416	426.09955	0.98904
15	35.121	VV	0.0566	890.64307	218.25659	0.55033
16	35.329	VV	0.0579	149.67714	38.93332	0.09249
17	35.851	VV	0.0776	8196.53027	1414.35779	5.06465
18	36.022	VV	0.0520	200.74023	58.50398	0.12404
19	36.100	VV	0.0574	182.79474	45.00818	0.11295
20	55.056	BV	0.1317	212.65099	19.57750	0.13140
21	55.086	VB	0.1152	185.16031	19.59600	0.11441
Totals :				1.61838e5	9.11572e4	

Results obtained with enhanced integrator!

*** End of Report ***