DEVELOPING AND USING A METHODOLOGY FOR RECOVERING AND SEPARATING UNSAPONIFABLES FROM COTTONSEED OIL DEODORIZER DISTILLATE USING A CENTRIFUGAL MOLECULAR DISTILLATION PROCESS

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To the Dean of the Graduate School:

I am submitting herewith a thesis written by Aniqua Jafri entitled "Developing and using a methodology for recovering and separating unsaponifiables from cottonseed oil deodorizer distillate using a centrifugal molecular distillation process". 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 Masters in Science with a major in Food Science.

Dr Cynthia Warren, Major Professor

We have read this thesis and recommend its acceptance:

Dr Kenneth Broughton

Department Chair, Dr Kenneth Broughton

Accepted:

Dean of the Graduate School

DEDICATION

For my loving parents, Tanveer and Durraiya Jafri,

and my brother, Wasim.

ACKNOWLEDGEMENT

First of all, I am grateful to the Almighty God for allowing me to complete this research. I would like to express my deepest appreciation to my committee for their continued support and encouragement: Dr. Cynthia Warren, my committee chair; and Dr. Shane Broughton. I am extremely grateful for the learning opportunities and guidance provided by my committee. I am also grateful to Dr Rene Scott, for her guidance in writing and editing my research.

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Finally, I would like to place on record, my sense of gratitude to one and all, who directly or indirectly, have lent a helping hand in completion of this research.

ABSTRACT

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DEVELOPING AND USING A METHODOLOGY FOR RECOVERING AND SEPARATING UNSAPONIFABLES FROM COTTONSEED OIL DEODORIZER DISTILLATE USING A CENTRIFUGAL MOLECULAR DISTILLATION PROCESS

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Cottonseed oil deodorizer distillate (CODD) is composed of highly volatile compounds such as tocopherols, phytosterols, and free fatty acids (FFA). Interest in extracting tocopherols and sterols from vegetable oils is increasing for use as antioxidants and additives by the food industry. In this study, recovery of tocopherols and sterols present in CODD was investigated using a centrifugal molecular distillation (CMD). Using a Lab-3 CMD, twenty-five runs were conducted using different evaporator temperatures (140°C-220°C) and feed flow rates (5-15 g/min). The unsaponifiable compounds were quantified using gas chromatography-flame ionization detector after derivatization and silylation to increase their volatility. The optimum condition to produce tocopherols and sterols with minimum FFA content was 165°C at 5 g/min flow rate. It was possible to obtain a material with 12.91% tocopherol and 29.48% sterols from CODD. This methodology resulted in a 85.26% of FFA elimination with an efficient recovery of sterols (95.40%) and tocopherols (81.79%).

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LIST OF ABBREVIATION

BHA	Butylated hydroxyanisole
BHT	Butylated hydroxytoluene
BSTFA	N,O-Bis(trimethylsilyl) trifluoroacetamide
CODD	Cottonseed oil deodorizer distillate
FAMEs	Fatty acid methyl esters
FFA	Free fatty acids
GRAS	Generally Recognized as Safe
GC	Gas chromatography
GC-FID	Gas chromatography-flame ionization detector
GC-MS	Gas chromatography-mass spectrometry
HPLC	High performance liquid chromatography
MSTFA	*N*-methyl-*N*-trimethyl-silyltrifluoroacetamide
SFC	Supercritical fluid chromatography
SODD	Soybean oil deodorizer distillate
TBHQ	tert-Butylhydroquinone
TLC	Thin-layer chromatography
TMCS	Trimethylchlorosilane
TMS	Trimethylsilyl

CHAPTER I

INTRODUCTION

Edible vegetable oils are widely consumed in the US and worldwide. Prior to being sold for human consumption, vegetable oils undergo processing to remove undesirable compounds. Processing includes neutralizing, refining, bleaching and deodorizing resulting in significant amounts of by-products such as soap stocks and deodorizer distillate. Deodorizer distillate is a complex mixture of volatile components, including free fatty acids (FFA), triglycerides, tocopherols, and phytosterols (Ramamurthi and McCurdy 1993). Cottonseed oil deodorizer distillate (CODD) may be of interest to the food, cosmetic and pharmaceutical industries due to a high portion of sterols (17-20 weight %), 11% tocopherols, and 40-50% free fatty acids.

Although various techniques have been developed for the recovery of tocopherols and sterols, few are published in the literature. These techniques are used alone or in combination with others, including solvent extraction, chemical treatment, crystallization, and supercritical fluid CO_2. In general, most processes are designed to remove or eliminate fatty acids or recover sterols in the initial step, followed by concentration of tocopherol by other methods. The differing techniques have various operating steps and are time consuming.

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To acquire a good yield of tocopherols and sterols of high purity, these techniques require constant monitoring. Currently, molecular distillation techniques and chromatography are used for enhanced recovery and product purity. Centrifugal molecular distillation is generally accepted as the most suitable technique for the purification, concentration and/or separation of thermally sensitive and high molecular weight compounds. It is also considered an efficient technique to separate FFA from tocopherols, and concentrate tocopherols with good recovery.

CHAPTER II

REVIEW OF LITERATURE

Cottonseed Oil

In the early 19th century, edible oil processing did not include refining. Food fat (e.g. Lard, milk fat, etc.) were primarily consumed unrefined (not processed) with their flavors a desirable characteristic. At the end of the 19th century, margarine development led to the edible oil deodorization process in europe (Lee and King 1937). Deodorization, the last processing step in refining edible oils results in improved oil quality by removing undesirable flavors and odors (Gavin 1978). In 1900, Wesson introduced the first vacuum deodorizing process in the US, becoming the world standard for edible oils (Nixon 1930).

In the US, cottonseed oil is primarily used as a salad or cooking oil. Due to its high level of saturated palmitic acid and very low unstable linolenic acid, cottonseed oil is valued for its shelf stability and flavor properties (Liu and others 2002). Production of high levels of cottonseed oil (880 million pounds worldwide) (NCPA 2015) result in large amounts of deodorizer distillate by-product. This by-product is produced during the purifying steps of cottonseed oil and is comprised of highly volatile compounds, such as sterols (β-sitosterol, stigmasterol, campesterol), tocopherols (vitamin E), and fatty acids (Erickson 1990).

Currently vitamins and sterols are produced synthetically for human consumption, though interest in extracting these natural compounds from seeds and vegetable oils is increasing (Mendes and others 2005). Tocopherols make up 6-11% of cottonseed oil deodorized distillate (CODD) and are used as antioxidants and additives in the food industry. Sterols make up 14-17% of the CODD and serve as raw material in the production of hormones and vitamins for the food industry (Mendes and others 2002). Sterol and vitamin content can increase the commercial value of vegetable oil deodorizer distillate (Gunawan and others 2009), while the fatty acids that make up 50% of the deodorizer distillate are considered low quality and are used primarily as biodiesel (Woerfel 1995).

Fats and Oils

Fats and oils are consumed by both humans and animals and are solid and liquid at room temperature, respectively (Damodaran and others 2007). Edible oils can be used in the production of soap and as other surface-active molecules. Currently, many edible oils are also used in the transportation industry as biodiesel after being used as a frying oil (Pinto and others 2005) to help reduce man's carbon footprint. This increased demand is also increasing the cost of certain edible oils, such as canola and soybean oil (Gunstone 2011).

Composition

Edible oils are primarily composed of triglycerides. A triglyceride consists of one molecule of glycerol attached to three fatty acids (Fig 2.1) (Paul and others 2014). Glycerol is referred to as the "backbone" of a triglyceride. Triglycerides are also called triacylglycerols. The prefix mono-, di- and tri- describe the number of fatty acids esterified to the glycerol. Monoacylglycerols and diacylglycerols also exist. Mono- and diacylglycerol have detergent properties, and form micelles in aqueous solutions (Gunawan and Ju 2009). An acylglycerol is formed via esterification of fatty acids to glycerol. The chemical and physical properties of the triacylglycerols depend on the position of the double bonds and the number of carbons contained in the specific fatty acid side chains (StAngelo 1996; Senanayake and Shahidi 2002; Don Banks 2015).

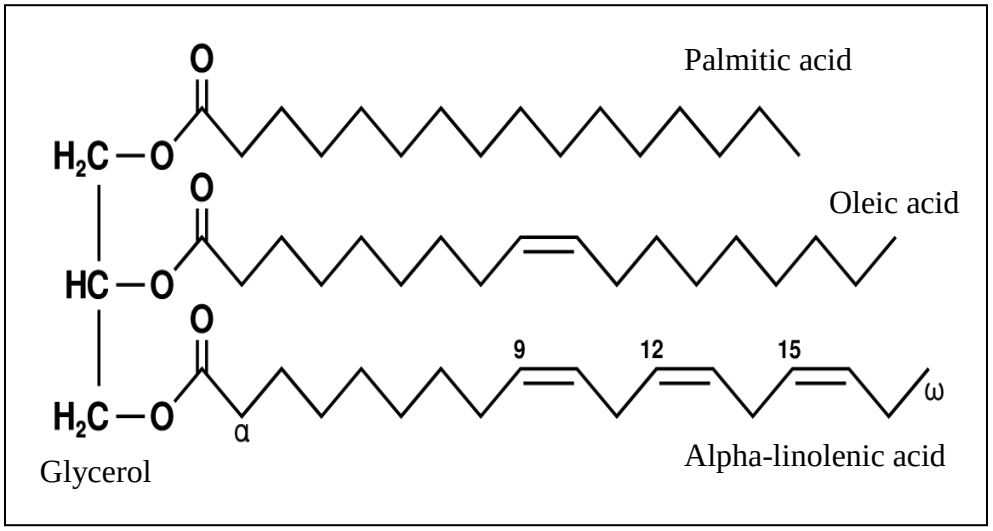

Figure 2.1-Triglyceride molecule (www.mybiofuels.org)

Classification of fatty acids is dependent upon the number of double bonds in the fatty acid. Common names are given to fatty acids based on the number of double bonds they posses and the number of carbons present (Belitz and others 2009). The most common fatty acids in edible oils are palmitic, stearic, oleic and α-linoleic acid (Gunstone 2013). There is some concern about linolenic acid because it is responsible for the rancidity of many edible oils. Cottonseed, sunflower, corn, and palm oil contain less than 1% linolenic acid.

Table 2.1-Fatty acid composition (%wt) of common oils.

	Soy	**Cottonseed**	Corn	Canola	Sunflower	Palm	High oleic sunflower
Palmitic, C16:0	11	**26**	13	4.3	6	46	4
Stearic, C18:0	4	**2**	3	1.7	5	4	5
Oleic C18:1, ω-9	22	**21**	31	59.1	20	40	81
Linoleic, C18:2, ω-6	53	**58**	52	26	60	10	8
α-Linolenic, C18:3, ω-3	8	**< 1%**	1	8.2	< 1%	1	< 1%

In general, the percent unsaturated fatty acids in an oil are directly proportional to its deterioration and instability (Bradley and Min 1992; St.Angelo 1996; Zambiazi and Zambiazi 2000). Table 2.1 shows the percent composition of specific fatty acids found in common oils (Gunstone 2013). Stable edible oils are the most marketable because of their increased shelf life. Cottonseed oil and corn oil contain saturated fatty acids which make them more shelf stable, adding to their value because hydrogenation of these oils is not necessary.

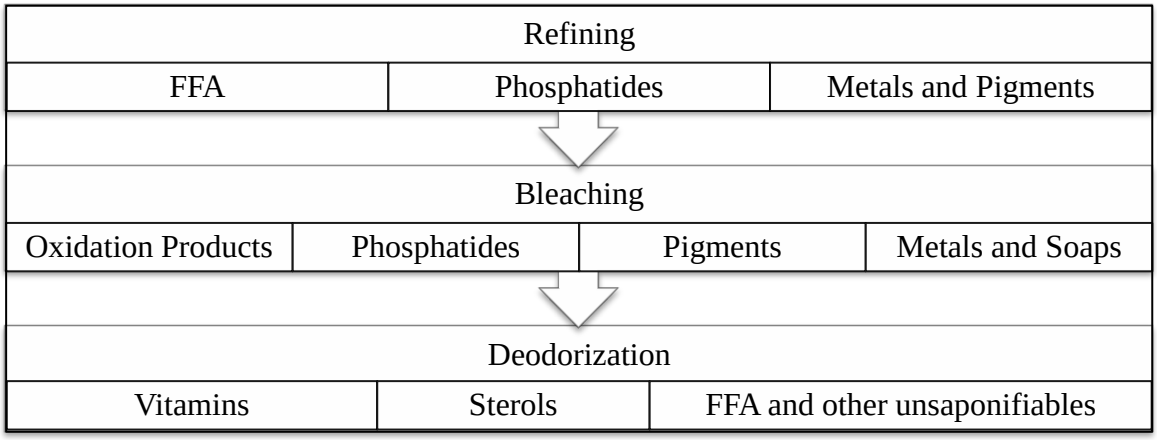

Figure 2.2-Removal of contaminants from edible oil through refining bleaching deodorization process.

Several costly processing steps are necessary to produce finished oils that meet consumer and market demands for taste and quality (Hodgson 1996). These include removal of phosphatides, sterols, fatty alcohols, and in some oils, modification of the triglyceride molecules by interesterification. Other processing includes refining, bleaching and deodorization (Berbesi 2006) (Fig. 2.2). The over-processing of oil can increase production costs, reduce product quality, and decrease tocopherol (Vitamin E)

content. Maximum tocopherol content is desirable because of its antioxidant and health benefits (Gunstone and Harwood 2007).

Edible oils typically consist of 96 to 98% triglycerides. The remaining 2-4% consists of non-triglyceride components (FFA, mono-and diglycerides, phospholipids, sterols, tocopherols, color, and trace metals) (Leung and Strezov 2014) that are responsible for the color and odor of unrefined oil. Cottonseed oil contains an additional pigment called gossypol that darkens when heated. These components are undesirable in good stable oil. Removal of the objectionable non-triglyceride components while minimizing loss of the desirable components is the objective of the refining process of these seed oils (O'Brien 2008).

A common first step in the refinement of oils is to use sodium hydroxide to neutralize the free fatty acids, forming soapstock. Soapstock and other impurities are then centrifuged and washed away from the oil (Daniel 1994). The beneficial components in oil, tocopherols and sterols, need to be considered to prevent their loss while refining (Norman 2015). Depending on the refining condition, the oil content of tocopherols and sterols may vary from 2-20% (Verleyen and others 2001; Buczenko and others 2003; Noqala-Kalucka and others 2004).

The next step in refinement is bleaching; a key process in oil refining, designed to remove not only pigments, but color bodies, trace metals, and residual soap (Hodgson 1996). The final step is deodorization. Deodorization rids an oil of volatile compounds such as FFA, glycerol, sterols, tocopherols, and pigments, producing bland flavored oil

that contains 0.01-0.03% FFA (Gavin 1978). Deodorization results in an oil with improved flavor, color, oxidative stability and deodorized distillate. The tocopherols and sterols, minor amount of mono-, di- and triglycerides collect in the deodorized distillate that remains after processing (Gavin 1978; Jones and King 1996).

All volatile compounds present in deodorized distillates and are insoluble in aqueous alkali after hydrolysis (saponification) are categorized as "unsaponifiable" (Chauhan BS 2008). Although fats and oils are a source of dietary lipids, they are also a source of other essential nutrients. These unsaponifiables include phospholipids, phytosterols (campesterol, stigmasterol and beta-sitosterol), tocols (tocopherols and tocotrienols, including vitamin E) and hydrocarbons (alkanes, squalene and carotenes), each essential to the human diet (Gunstone 2013).

Deodorizer distillate is a composite mixture of free fatty acids, tocopherols and phytosterols, making it a good source of vitamin E and sterols (Ramamurthi and McCurdy 1993). Deodorizer distillate from cottonseed or any other unsaturated vegetable oil is considered to be a good source of tocopherols (0.8-10 wt%). It may contain 30 to 40 percent unsaponifiables which can consist of about 10 percent tocopherols, 14 to 20 percent sterols, and 3 to 4 percent stigmasterol (Winters 1990). Deodorizer distillate of most vegetable oils has a very high FFA content (>85%) as shown in Table 2.2 which is desirable for the production of soap and biodiesel (De Greyt 2013). Cottonseed oil deodorizer distillate (CODD) has a higher market value potential because of its lower

content of FFA (40-50%), and a higher content of tocopherols (10-11%) and sterols (17-20%).

Table 2.2-Typical compositions of deodorizer distillates from vegetable oil refining.

(%)	Soy[b]	Canola[a]	Sunflower[b]	Cottonseed[b]	Palm[a]
FFA	25-40			42	85-90
Tocopherols	11.1	8	9.3	11.4	0.15-0.30
Sterols	18	7	18	20	0.2-0.4
Stigmasterol	4.4	-	2.9	0.3	0.5-1.0

[a]Ref. (De Greyt and others 2013)
[b]Ref. (Winters RL 1989)

Fatty Acids

Refined cottonseed oil has 75% oleic (18:1) and α-linoleic acids (18:2), 24% palmitic acid (16:0), and less than 1% linolenic acid (18:3) (FAO/WHO). Because linolenic acid has 18 carbons with 3 double bonds, it is more susceptible to oxidation, which is undesirable in the food industry. Linolenic acid is also referred as 18:3 omega-3. This fatty acid creates problem with respect to storage and is very labile on oxidation (Halver 1978). Since cottonseed oil contains a low amount of linolenic acid, this composition makes cottonseed oil very stable without the need of hydrogenation (O'Brien and others 1996).

After distillation, CODD contains a high percentage of free fatty acids (40-55%), depending on the raw material and the refining process parameters. The FFA needs to be

separated from tocopherols for commercial use because they are considered a contaminant. These FFAs can be used for biodiesel production (Jiang and others 2006). Several extraction techniques exist for the separation of FFA from tocopherols.

Tocopherols

Natural tocopherols consist of a number of mixed isomers and serve as antioxidants in foods. The number and position of the methyl groups ($-CH_3$) in the aromatic ring give rise to α, β, γ, and δ isomers (Gunawan and Ju 2009). The γ- and δ-tocopherol present in a mixed tocopherols product are very useful because of their ability in preventing oxidation in fats and oils. Crude cottonseed oil typically contains about 1000 ppm tocopherols with a loss of up to 30% during processing. In cottonseed oil, γ-tocopherol accounts for 58% of the total tocopherols with α-tocopherol comprising 41% and the β and δ isomers collectively representing 1% of the total tocopherols (Muller-Mulot 1976). Natural tocopherols can be recovered from deodorizer distillate and marketed as Vitamin E to be used in foods and as supplements.

Tocopherol helps in improving the organoleptic characteristics of many foods by preventing oxidation and rancidity. They are preferred over artificial antioxidants, such as butylated hydroxytoluene (BHT), butylated hydroxyanisole (BHA) and tert-butylhydroquinone (TBHQ). Extracts of plant-derived antioxidants generally contain a mixture of natural compounds, which could have synergetic effects; therefore, they can have better effects and less toxicity (Chermahini and others 2011). Mixed tocopherols are a valuable ingredient in food processing due to high temperature resistance during food

processing step, and are lipid soluble. They are able to prevent off flavor formation attributable to oxidation and rancidity, and extend the shelf life of processed foods. Tocopherols are widely accepted as a safe food additive and have Generally Recognized as Safe (GRAS) status (Tomassi and Silano 1986; Haumann 1990).

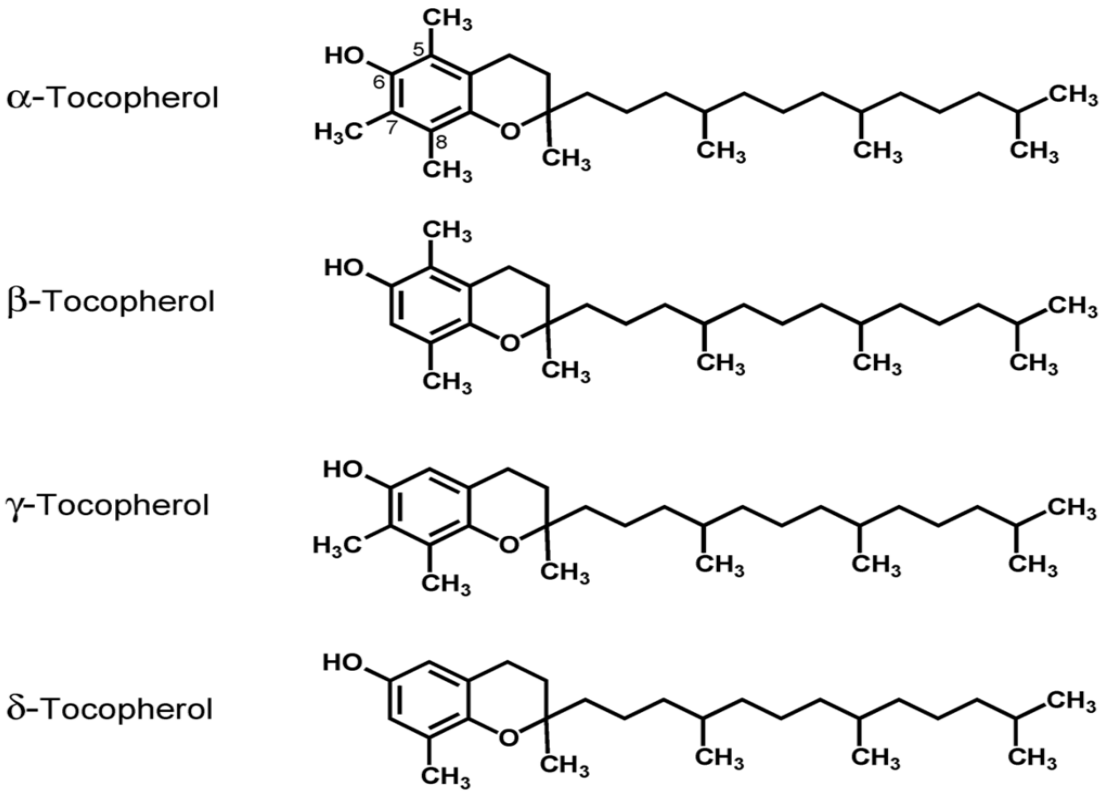

Figure 2.3-Structure of isomers of tocopherols (Smolarek and Nanjoo 2011)

Tocopherols are important nutritionally and physiologically due to their involvement in metabolic processes in the human body (Afonso and others 2013). Tocopherols provide the most concentrated source of γ-tocopherol homologue for use as a direct nutritional supplement (multivitamin capsules), single-dose nutrient capsules and in liquid dietary supplements as vitamin E (Ramamurthi and others 1991). Vitamin E

refers to a group of compounds that include both tocopherols and tocotrienlos. Among all four tocopherol isomers, alpha-tocopherol is the most biologically active form. Studies have demonstrated that tocopherols are helpful in reducing the risk of cancer (Kline and others 2007), cardiovascular disease, and cataracts (Rimm and others 1993; Block and others 1994).

Sterols

Phytosterols, also referred to as plant sterols, are a major component of unsaponifiable matter of cottonseed oil. In U.S. cottonseed cultivars, the average cottonseed phytosterol amount is typically 300 ppm (Reeves and Weirauch 1979). The primary sterol in any cottonseed oil is β-sitosterol (Van Niekerk and Burger 1985). The pharmaceutical industry uses sterols to produce steroids (DeMoraes and others 2006, Walsh and others 1998) and artificial vitamins (Bondioli and others 1993).

Phytosterol products have a high market potential. They serve as a substance for further modification or are used directly in the pharmaceutical industry (production of steroids), in nutrition (anti-cholesterol and anti-cancer properties), or as cosmetics (skin-conditioning agents, fragrances, lipstick) (Fernandes and Cabral 2007). As an additive to certain manufactured foods, phytosterols have been shown to have a anti-cholesterolemic property by reducing the intestinal absorption of dietary cholesterol (Richelle and others 2004). Phytosterols can be recovered from CODD and are marketed as provitamin A, oryzanol and natural tocopherols and sterols (Kusdiana and Osaka 2004).

Extraction Methods

Various combinations of physicochemical extraction methods have been published in the literature to eliminate FFA and recover tocopherols and sterols from different deodorizer distillates. These methods include: liquefied petroleum gas, lipase-catalyzed esterification, transesterification, solvent extraction and crystallization, and supercritical fluid CO_2 (Lee and others 1991; Ramamurthi and Mc Curdy 1993; Chang and others 2000; Buczenko and others 2003; Torres and others 2007; Carmona and others 2010; Lin and Koseoglu 2003; Moreira and Baltanas 2004). These techniques are either used alone or in combination with each other. Studies on deodorizer distillate from olive oil, rice bran oil, crude palm oil and soybean oil have evaluated sterol and tocopherol concentrations using supercritical fluid extraction (Snyder and others 1999; Ibanez and others 2002; Gast and others 2005; Sughira and others 2010). In general, most of the techniques are designed in order to separate out the FFA, followed by tocopherols and phytosterols fractions.

Buczenko and others (2003) studied the extraction of unsaponifiable matter from the vegetable oil deodorizer distillate. They used saponification as a pretreatment followed by tocopherol concentration using liquefied petroleum gas extraction. However, fatty acids are neutralized in saponification, resulting in a bad chromatogram. Chang and others (2000) performed a supercritical CO_2 extraction to recover the tocopherols and FFA from soybean oil deodorizer distillate (SODD). This employs high pressure for improved recovery. Crystallization followed by solvent extraction is another technique

for tocopherol and sterol recovery. The research using this technique is not environmental friendly and is limited to low recoveries of value-added compounds from different deodorizer distillates (Moreira and Baltanas 2004; Lin and Koseoglu 2003).

By esterification and/or transesterification, FFAs and glycerides are converted to fatty acid methyl esters (FAMEs) that are more easily removed by vacuum distillation due to their lower molecular weight. Therefore, the complete conversion of fatty acids to their methyl esters is critical to obtain a high concentrate of tocopherols using molecular distillation. However, this process needs special care to maintain high vacuum and is energy intensive. Complete separation is difficult as some of the other components (sterols, unesterified FFAs, and glycerides) are co-distilled along with the tocopherols. Usually, a combination of esterification and crystallization is used followed by molecular distillation to recover sterols, and thus enhance tocopherol purity of the final product (Jiang and others 2006).

Crystallization is also one of the frequently used methods to purify sterols from deodorizer distillate, either alone or proceeding other techniques. This technique involves phase separation between tocopherols and sterols. Methanol is the most common crystallization solvent used while ethanol, hexane, acetone, or petroleum ether may be used as well. Crystallization is usually carried out at a temperature between -20°C to 10°C for 20-24 hrs. Yan and others (2012) determined the optimum condition for phytosterol recovery from SODD with co-solvent petroleum ether/water (3.41:1 g/ml

feed solution to solvent ratio, 26.5 h ripening time, 4.5°C ripening temperature). The sterol yield and purity obtained with this condition was 6.64% and 94.7%, respectively.

There are several ways to breakdown the plant sterols from different plant matrices for extraction, including acid, alkaline and enzyme hydrolysis (Toivo and others 2000; Piironen and others 2002; 2003). Schwartz and others (2008) saponified different vegetable and industrial fats and oils extracting the tocopherols, tocotrienols, and plant sterols using heptane diethyl-ether followed by silylation using N,O-Bis(trimethylsilyl) trifluoroacetamide (BSTFA) as the silylating agent and trimethylchlorosilane (TMCS) as the silylation catalyst. The composition of fats and oils was analyzed using normal-phase HPLC (NP-HPLC) with fluorescence detection for tocopherols, and GC-FID (flame ionization detection) for plant sterols.

Several studies demonstrate that lipase-catalyzed hydrolysis and methyl esterification followed by vacuum distillation to separate the fatty acid methyl esters from tocopherols and sterols is an effective method for purifying useful components in vegetable oil deodorizer distillate (Ramamurthi and McCurdy 1993; Ghosh and Bhattacharyya 1996). Chu and others (2003) performed alkali neutralization following enzymatic hydrolysis using a commercial immobilized lipase, *Candida antartica* (Novozyme 435), to remove the FFA from the deodorized distillate. Large scale use of the lipase reaction is difficult commercially due to its high ratio of solvent to lipase, both of which are expensive. Moreover, the temperature profile suggested by this method is not appropriate and does not provide good resolution of peaks.

Tocopherols and sterols, present in CODD have a high molecular weight and are heat sensitive. Owing to similar volatility of these natural compounds, it is difficult to separate them even using fractional distillation. Exposure to high temperature leads to degradation of tocopherols. These behaviors hinder efficient separation of unsaponifiables through traditional and time consuming methods, as they decompose at high temperatures (De Moraes and others 2006). Although effective, these methods result in low recovery of the desirable end products.

Molecular Distillation

An alternative separation technique for tocopherols and sterols is molecular distillation, operating at low temperatures and low pressure (De Moraes and others 2006). In lipid chemistry, this technique has been used for the purification of monoacylglycerols (Szelag and Zwierzykowski 1983), the recovery of carotenoids from palm oil (Batistella and Maciel 1998), recovery of squalene (Sun and others 1997), and the recovery of tocopherols (Batistella and others 2002). This technique is used to process thermally sensitive compounds that do not tolerate long exposure to increased temperature and suffer thermal decomposition when heated to desired temperatures. This technique is efficient for the separation, purification and/or concentration of natural compounds.

The two types of molecular distillation available are: falling film and centrifugal disc (Batistella and others 1998). In the centrifugal disc scheme, liquid enters through the feed stream and flows uniformly around the evaporator by centrifugal force, forming a thin film. Film formation is induced through the use of a high vacuum and a small gap

between the condenser and the evaporator (Batistella and others 2000). Low molecular weight compounds are volatilized and condensed into the distillate stream while the heavier compounds are collected in the residue stream. FFA are volatilized and removed in the distillate stream attributable to their lighter molecular weight. Since they have a high molecular weight, tocopherols and sterols should concentrate in the residue stream (Martins and others 2006).

Centrifugal Distillation System

Separation of FFA and tocopherols can be performed using a Lab-3 Centrifugal Distillation System (Kittaning, PA). Figure 2.4 shows the scheme of a centrifugal molecular still. This system has a 3-inch diameter rotor with a total capability of about 0-2 lbs/h. The major part of the equipment is constructed with stainless steel allowing for easy disassembly/assembly and cleaning. It is equipped with a heated rotor, a water-cooled condensing surface, a chamber trap, a vacuum chamber with condenser, and a vacuum pump set.

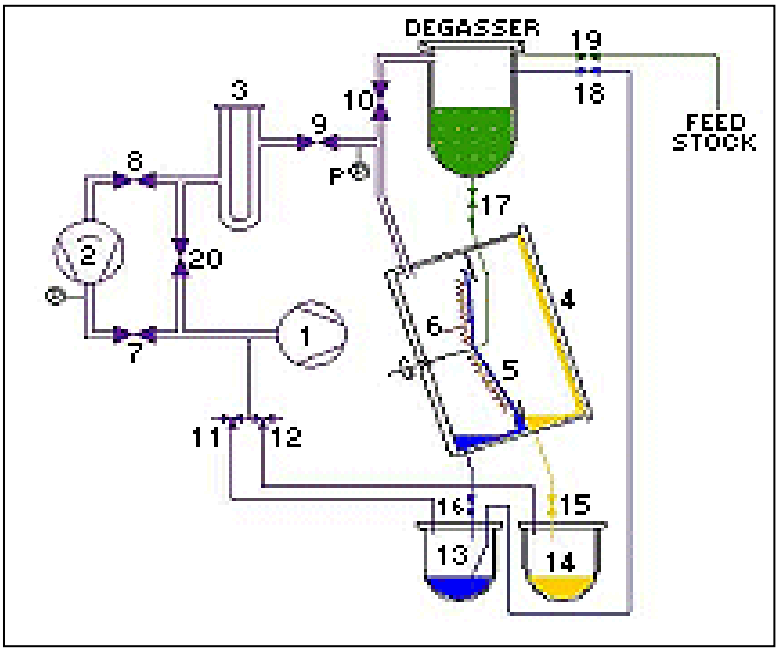

Figure 2.4-Scheme of centrifugal molecular still (www.myers-vacuum.com).

Operation

The vacuum still is designed as a batch/continuous distillation unit. Initially, the material flows through the degasser reservoir where it is degassed to remove the small amount of low boiling particles and gases which enables a more efficient process at the next stage. The material is then introduced into the feed vessel and flows via a rotor feed valve in the center of the heated, spinning rotor into the high distillation chamber. Figure 2.5 shows the feed stream inside the condenser.

The liquid film forms by centrifugal force and is then distributed uniformly around the periphery of the rotor. The vacuum still utilizes the heat of condensation as a source of radiant emission to heat the surface film on the evaporator. Thermal degradation to the organic material reduces to a greater extent due to a combination of

short residence time and lower distilling temperatures. As the materials travel the heated rotor surface, the material spreads out into a thin film (0.05 mm thick). The best way to create a uniform thin film is to feed material to the center of the heated disc.

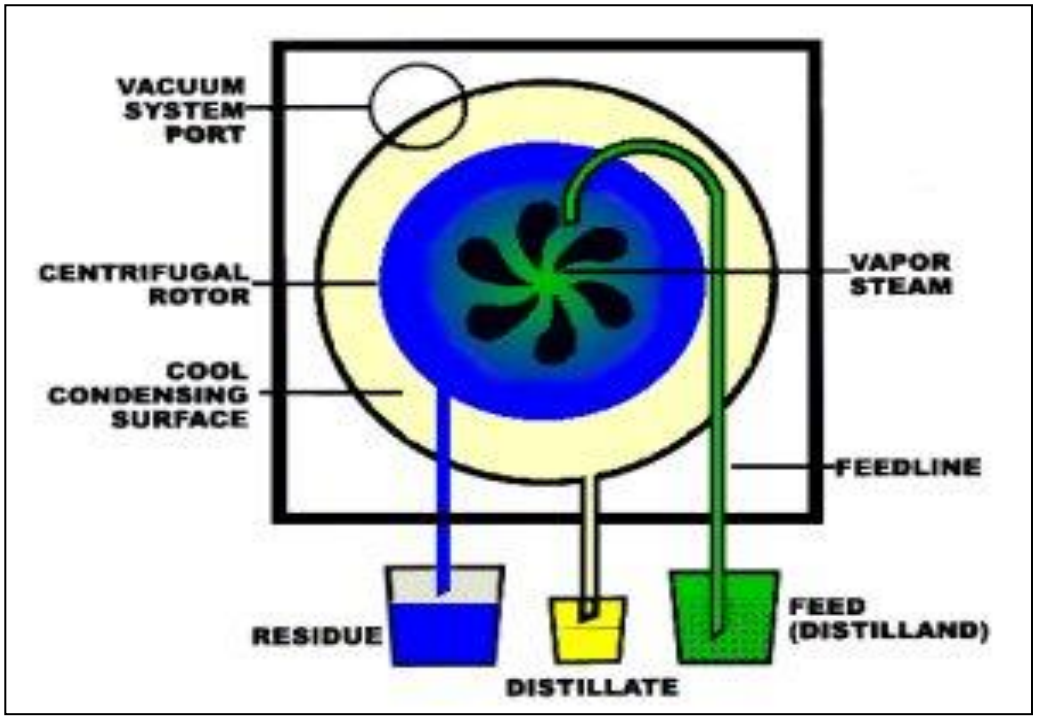

Figure 2.5-Schematic diagram of feed stream inside the condenser of centrifugal disc. (www.myers-vacuum.com)

Lightweight compounds evaporate and condense in a fraction of a second, on the condenser in the vacuum chamber and are collected in the distillate collector. The un-evaporated material, i.e. residue, moves off the edge of the rotor to be trapped, pumped into the residue chamber and discharged to the residue collector. The degree of separation is determined by the molecular weights of distilled mixture. The closer the molecular

weight of compounds, the less efficient the desired fractionation. This will lead to multiple runs of the distillate stream.

The purity of the distillate depends on the film thickness, which is governed by the feed flow rate. The film layer closest to the heaters will be subject to greater thermal hazard and vice-e-versa. Condensation begins whenever a sufficient temperature differential occurs between evaporator and condenser. Research needs to be done to find the suitable temperature and feed flow rate at which distillation will occur.

Analysis of Unsaponifiables

Following extraction of unsaponifiables, the resulting desirable products can be analyzed using various methods. Although tocopherols and sterols are two different components present in fats and oils, it is possible to analyze them simultaneously. A wide range of analytical techniques such as supercritical fluid chromatography (SFC), thin-layer chromatography (TLC), high-performance liquid chromatography (HPLC) and gas chromatography (GC) have been used to determine the tocopherol and sterol content in fats and oils (Kircher and Rosentein 1946; Slover and others 1983; Reina and others 1999; Snyder and others 1999; Lechner and others 1999; Carmona and others 2010).

Because of its complex composition the analysis of deodorizer distillates is a challenge (Verleyen and others 2001) and are commonly analyzed using HPLC or GC. The main advantage of GC over HPLC is that it is possible to separate tocopherols and phytosterols under the same conditions. It is difficult to process and analyze deodorizer distillates samples by HPLC due to high lipophilicity of sterols (Lagarda and others

2006), an artifact not recognized with GC. Therefore, GC is a more effective technique for the analysis of deodorizer distillates (Gunawan and Ju 2009). GC allows for better separation of tocopherols and sterols and detection of lower compound concentrations compared to HPLC (Schaeffler and Morel du Boil 1981). With HPLC, its difficult to identify individual peaks. GC-FID (flame ionization detection) or GC-MS (mass spectrometry) may be considered more convenient for determination of phytosterols when compared to HPLC methodology (Lagarda and others 2006). Capillary GCs columns allow for improved component resolution and high thermal stability due to shorter analysis times and less peak interference compared to packed columns (Abidi 2001).

To obtain improved peak shape, resolution and thermal stability, sterols are usually derivatized before GC analysis. Derivatization is done to increase the volatility, detectability, and stability of the compounds. Though it is possible to separate sterols without derivatization, resolution is optimized with trimethylsilyl derivatization (Mortan and others 1995). Trimethylsilyl (TMS) or acetate 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 (Lagarda and others 2006). The most commonly used derivatizing agents are *N*-methyl-*N*-trimethyl-silyltrifluoroacetamide (MSTFA) in anhydrous pyridine and BSTFA containing 1% TMCS (Lagarda and others 2006). Verleyen and others (2001) analyzed deodorizer distillates of different vegetable oils using vegetable oil deodorizer distillate samples for derivatization and silylation with

BSTFA + 1% TMCS solution. Derivatized sterols and tocopherols were then quantified using GC-FID.

These studies used a varying series of chemical and physical processing steps that are time-consuming and inefficient in the recovery of tocopherols and sterols. Ito and others (2006) evaluated soybean oil deodorizer distillate using only centrifugal molecular distillation to recover tocopherols. As the feed flow rate increases, the material also increases inside the evaporator, the efficiency of heat transfer between the material and the evaporator is reduced due to poor contact with the disc. This decreases the D/R (mass of distillate/mass of residue) split ratio resulting in a significant loss of tocopherols in the distillate due to high evaporator temperature and high feed flow rate. Martins and others (2006) eliminated this issue by lowering the evaporator temperature and feed flow rate to concentrate the tocopherols. Centrifugal molecular distillation is a simple and efficient process to remove and concentrate sterols and tocopherols from deodorizer distillate while preventing tocopherol oxidation when condensed at low temperature and feed flow rate with centrifugal molecular distillation.

Separation of FFA from tocopherols using molecular distillation is technologically viable due to their different molecular weights and different vapor pressures (Table 2.3). When the FFAs are separated from CODD, the remaining portion is a mixture of tocopherols and sterols. The FFAs are collected in the distillate and the tocopherols collected in the residue during molecular distillation.

Table 2.3-Molecular weight and vapor pressure of FFA and tocopherols. (Winters 1986)

Component	Molecular weight (g/mol)	Vapor pressure (Pa)
FFA	180	533.28
Tocopherols	415	20.00

In the proposed study, tocopherols and FFAs from CODD were recovered using a centrifugal molecular still. The CODD was obtained from Pyco Industries (Lubbock, TX), where deodorization of the oil was carried out at 480°F at 2-2.5 mm of Hg vacuum pressure. The unsaponifiables of CODD, including sterols and tocopherols were quantified using GC-FID after derivatization and silylation with BSTFA + 1% TMCS to increase their volatility.

Purpose of Study

The purpose of this study was to separate, identify and quantify total FFAs, tocopherols and sterols present in cottonseed oil deodorizer distillate (CODD) using centrifugal molecular distillation and gas chromatography.

Specific Aims:

- To separate and identify the valuable unsaponifiables from CODD using centrifugal molecular distillation.

- To achieve <1% FFA in residue and >90% mixed tocopherols/sterols mixture in the residue stream.
- To validate the technique of centrifugal molecular distillation to separate and recover the unsaponifiables from CODD.
- To determine the optimum condition for separating and recovering the unsaponifiables from CODD using five different temperatures and feed flow rates.

Null Hypotheses:

- There will be no effect of temperature on the % weight of tocopherols, sterols and FFAs identified.
- There will be no effect of feed flow rate on the % weight of tocopherols, sterols and FFAs identified.

CHAPTER III

MATERIALS AND METHODS

Cottonseed Oil Deodorizer Distillate

Cottonseed oil deodorizer distillate (CODD) was obtained from Pyco Industries (Lubbock, TX) and kept below 8°C during storage.

Chemicals

α-tocopherols, sterols (β-sitosterol and stigmasterol) and a cholesterol-internal standard were purchased from Fisher Scientific (Hanover Park, IL) for use as reference standards in qualitative and quantitative chromatographic analysis. All standards were stored in refrigerator at 4°C. Chromatographic grade silylating agent BSTFA (N, O-bis-(trimethylsilyl) trifluoroacetamide) containing 1% TMCS (Trimethyl chlorosilane) (Supelco, Bellefonte, PA), pyridine (Acros Organics, New Jersey, US) and chloroform (Fisher Scientific, Hampton, New Hampshire) was used for gas chromatography (GC) analysis. Ethyl alcohol, phenolphthalein and sodium hydroxide (Fisher Scientific) were used for free fatty acid derivatization.

Preparation of Samples

Each CODD sample was degassed using a vaccum degasser to remove small amounts of low boiling materials and trapped gases, enabling a more efficient vacuum distillation. When the liquid enters the tank it will flow and be distributed on a layer of internal baffle plates designed for the liquid to flow in thin laminar film and is exposed to a vacuum that forces the gas to escape and break out of the sample. The vacuum pump moves the escaping gas from the degasser discharging it to the feed line. Initial, feed vessel and rotor temperature was 60°C with the condenser temperature at 5°C. After degassing, condenser temperaure was raised to 60°C. The experiments were performed between 140°C and 220°C with varying feed flow rate from 5 to 15 g/min. The condenser temperature was maintained at 60°C to avoid solidification of distillate stream on the condenser (Figure 3.1).

100 g of CODD was melted to obtain a homogenous mixture before feeding the degasser, and flow rate adjusted to a predetermined value (5-15 g/min). After distillation, the distillate and residue fractions were weighed and transferred to amber bottles and kept below 4°C, for further analysis. Samples of both distillate and residue streams were collected and submitted for FFA, tocopherol and sterol analysis. Figure 3.1 shows the experimental design for analyzing the CODD samples using centrifugal molecular distillation for this study. The split ratio D/R (mass of distillate/mass of residue) was also used to evaluate the process of centrifugal distillation.

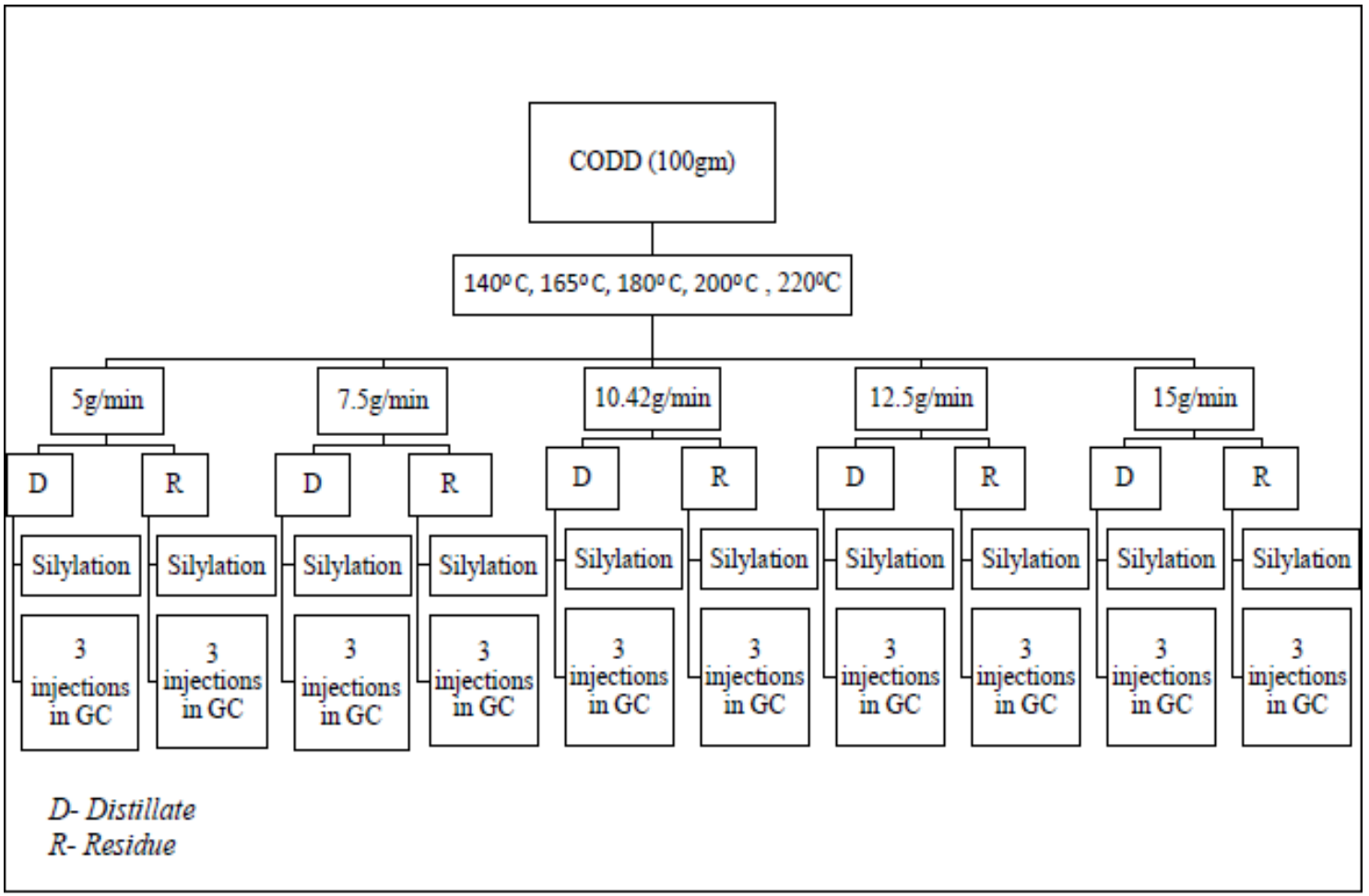

Figure 3.1 -Flow diagram showing the experimental design using centrifugal molecular distillation for CODD.

Parameters

- Vacuum- <10 mtorr
- Rotor temperature- 140°C, 165°C, 180°C, 200°C and 220°C
- Feed flow rates: 5 g/min, 7.5 g/min, 10.42 g/min, 12.5 g/min and 15 g/min.

Determination of free fatty acid (FFA)

The AOCS Official Method Ca 5a-40 (Free Fatty Acids) (AOCS 1990) was used to determined the % free fatty acid. Three gram samples were dissolved in 50 ml of hot neutralized alcohol (reagent ethyl alcohol and phenolphthalein indicator). To determine the unknown concentration of FFA, samples were titrated with a standard alkali, NaOH (0.1 N/1 N) to a faint permanent pink endpoint. The percentage of FFA in most fats and oils is expressed as oleic acid (C 18:1) using the formula:

$$\% \text{ FFA} = \frac{\text{ml of alkali} \times \text{alkali normality} \times 28.2}{\text{Weight of sample (g)}}$$

Simultaneous analysis of tocopherols and sterols in GC

The AOCS Official Method Ce7-87 (Total tocopherols in deodorizer distillate) was slightly modified to simultaneously quantify the amount of tocopherols and sterols in the CODD samples and the product streams obtained after centrifugal distillation, i.e. distillate and residue. Following distillation, 150 mg of homogeneous liquid sample was directly weighed in screw-capped test tubes. Samples were dissolved in 1 ml of chloroform followed by the addition of 0.5 ml of pyridine and uniformly mixed. One

ml BSTFA + 1% TMCS solution was then added to the samples as a silylating agent. Then each test tube was placed in an oven at 70 °C ± 1.0 for 20 min. This was followed by a 1 ml addition of silylated cholesterol solution (internal standard) to each test tubes. The samples were transferred to a GC vial and further diluted with 1.5 ml chloroform (Figure 3.2).

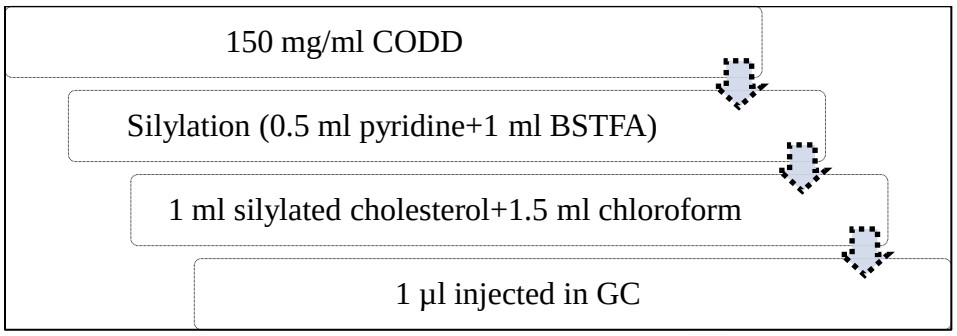

Figure 3.2-Methodology for silylation of CODD samples.

Determination of response factors

Response factor (FC) is defined as the ratio between the concentration of a compound being analysed and the response of the detector to that compound. They were determined by accurately weighing 10 mg of pure reference standards, followed by the derivatization procedure described in Figure 3.2. Different components present in cottonseed oil deodorizer distillate were quantified against an internal standard. Response factors for α-tocopherol, stigmasterol and β-sitosterol were identified with reference standards injected into the GC. Response factor for γ-tocopherols and campesterol were taken to be equivalent to that of α-tocopherol and β-sitosterol, respectively.

The response factor (FC) for reference standards was calculated via following equation;

$$FC = \frac{A_{IS} \times C_I}{A_I \times C_{IS}}$$

Where;

A_{IS}=area of internal standard
C_I= mg of standard compound
A_I= area of standard compound
C_{IS}= mg of internal standard

Gas Chromatographic Conditions

Quantification of unsaponifiable compounds was performed using an Agilent 6890 Series GC system equipped with a flame ionization detector (FID) and a CP-Sil 8 CB Low Bleed/MS column, 0.25 mm film thickness and 0.25 mm internal diameter capillary column. Nitrogen was used as the carrier gas with a split ratio of 10:1. Detector and injector temperatures were held at 360°C and 300°C, respectively. The GC temperature program was as follows: 240°C for 2 min, followed by heating to 275°C at 10°C/min, held for 5 min, then to 300°C at 20°C/min, held for 2.25 min, and then to 330°C at 15°C/min, held for 4 min giving a total run time of 20 min.

Raw sample of CODD and both residue and distillate samples from product streams were silylated and injected into the GC. Compounds detected in the chromatogram of CODD were compared and identified against the retention time of known amounts of reference standards. Each compound was quantified using a standard calibration curve.

Preparation of Reference Standard Solutions

All standards were dissolved in chloroform to a final concentration of 10 mg/ml and were silylated prior to injection in the GC. The internal standard-cholesterol was silylated, combined with the CODD samples and evaluated by GC (Figures 3.3).

Methodology for reference standards

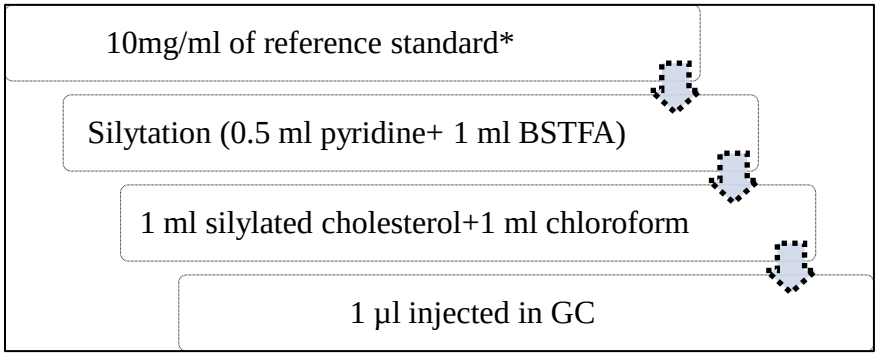

Figure 3.3-Methodology for reference standards.

*Reference standard: α-tocpherol, β-sitosterol, and stigmasterol.

Statistical Analysis

One-way MANCOVA was used to determine the effect of temperatures and feed flow rates on the unsaponifiable compounds present in the cottonseed oil deodorizer distillate using centrifugal molecular distillation. Feed flow rate in g/min was used as a covariant. The descriptive and post-hoc test were used to interpret the effect of temperatures on the compounds. Pillai's criterion was used to interpret the effect of temperatures for more than two groups.

CHAPTER IV

RESULTS AND DISCUSSION

Following centrifugal distillation technique, CODD was analyzed for FFA, tocopherol and sterol content in each sample. The CODD used for this study was brownish and semisolid at room temperature. The total weight percent of tocopherols and sterols in the raw CODD samples were found to be 9.16% and 17.92%, respectively. The analysis of raw cottonseed oil deodorizer distillate before distillation is shown in Table 4.1.

Table 4.1-Raw material characteristics of CODD.

Analysis	**Weight percent**
% FFA (as oleic acid)	34.60
Total tocopherols (%)	9.16
• α-tocopherol	2.72
• γ-tocopherol	6.44
Total sterols (%)	17.92
• β-sitosterol	16.58
• Stigmasterol	0.10
• Campesterol	1.24

Table 4.2-Peak areas and the retention times of reference standards.

Standards (Conc. 2.5 mg/ml)	**Peak area (average of 3 graphs)**	**Retention time (min) (average of 3 graphs)**
α-tocopherol	399.0743	10.043
β-sitosterol	456.309	12.445
Stigmasterol	643.963	11.873
Campesterol	254.864	11.545

Comparison of GC retention times with the standards were performed for peak identification. The peak areas and retention times of 2.5 mg/ml of reference standards used for this study are shown in Table 4.2. The chromatogram of raw CODD sample analyzed is shown in Appendix A. The evaporator temperature and feed flow rate are the main evaluated variables in the centrifugal molecular distillation process (Fregolente and others 2005, Batistella and others 2002).

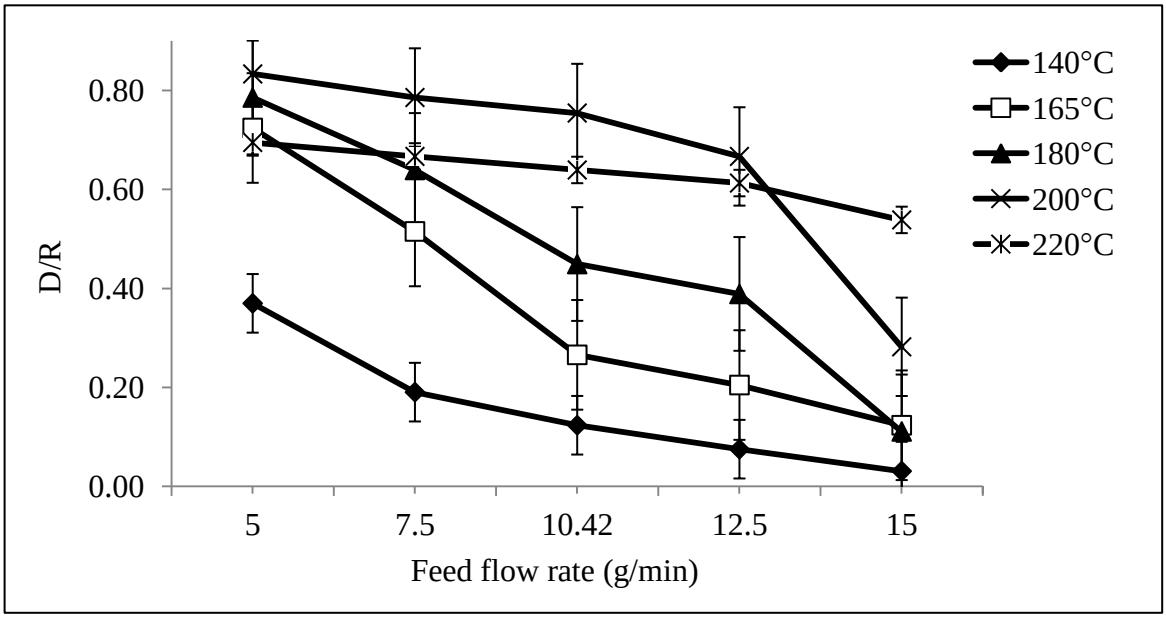

Figure 4.1-D/R versus the feed flow rate at different evaporator temperature.

The relation of D/R in functioning with different evaporator temperatures and feed flow rates used is shown in Figure 4.1. When a raw material flows through the molecular distillation process, it is fractionated into two streams, distillate (D) and residue stream (R). The split ratio D/R (mass of distillate/mass of residue) is considered an important parameter to evaluate the centrifugal distillation process (Martins and others 2006). These results show that as the split ratio decreased there was an increase in the feed flow rate across all temperatures evaluated (Figure 4.1). With an increase in the feed flow rate, the feed volume also increased inside the evaporator, leading to a reduction in efficiency of heat transfer between the material and the evaporator. This reduction in heat transfer resulted in a diminishment in the split ratio. At a constant feed flow rate, the split ratio increased with an increase in evaporator temperature. Contrarily, increasing the feed

flow rate while maintaining the same evaporator temperature resulted in a reduction in evaporation ratio D/R.

Results obtained at 165°C as a function of different feed flow rates are shown in Table 4.3. The maximum concentration (purified product) of tocopherols and sterols obtained in the residue stream was 12.91% and 29.48% at 5 g/min of feed flow rate. Good recovery (efficiency of purification) of tocopherols (81.79%) and sterols (95.40%) was obtained in the residue stream.

Table 4.3-Results obtained from 165^{o}C of evaporator temperature.

Feed flow rate (g/min)	**5**	**7.5**	**10.42**	**12.5**	**15**
Split ratio (D/R)	0.72	0.52	0.27	0.20	0.12
FFA content in distillate (%)	70.24	73.89	71.57	63.18	46.95
FFA content in residue (%)	9.7	11.56	25.4	30	33.18
FFA recovery in distillate (%)	**85.26**	**72.61**	**43.44**	**31.04**	**14.93**
Tocopherols content in distillate (%)	4.60	2.98	1.05	0.59	0.28
Tocopherols content in residue (%)	12.91	12.46	10.28	10.74	9.76
Tocopherols recovery in residue (%)	**81.79**	**89.82**	**88.73**	**97.38**	**94.84**
Sterols content in distillate (%)	5.86	3.58	1.19	0.76	0.67
Sterols content in residue (%)	29.48	25.06	21.99	21.45	20.07
Sterols recovery in residue (%)	**95.40**	**92.28**	**96.90**	**99.33**	**99.66**

Results obtained at 180°C in relation to different feed flow rates are shown in Table 4.4. The maximum output for tocopherol recovery in the residue stream was more than 100%. This might be due to human error while monitoring the distillation process or recording the mass of distillate and residue.

A preliminary study was conducted at ChemTech Services Inc, IL (2013) using a 2-stage wiped film molecular distillation process in order to evaluate the feasibility, efficiency, cost, and parameters effect on separation of unsaponifiables from CODD. Separation of FFA, tocopherols and sterols from CODD was investigated using evaporator temperature between 135^{o}C to 175^{o}C with a constant feed flow rate. The percentage FFA at Stage1Residue at 165^{o}C and 175^{o}C were 2.2% and 1.4%, respectively. Almost all of the tocopherols and sterols volatilized into residue, leaving minor amount in the distillate stream. Stage1 at 165^{o}C had a good separation of unsaponifiables consisting of 18.6% of tocopherols and 44.4% of sterols. Stage1Residue at 165°C was used as a feed for Stage2 with different temperature range between 195^{o}C to 235^{o}C. The final product was collected in a distillate stream. Stage2Residue at 205^{o}C had a good separation of unsaponifiables consisting of 24.80% tocopherols and 59.72% sterols, leaving 97.14% diglycerols with a negligible amount of tocopherols and 2% sterols in the residue.

From the preliminary study, it was concluded that 2-stage molecular distillation was an effective process in separation of unsaponifiables. Based on the results obtained, current study was proposed and conducted to see the effect of temperature and feed flow

rate on CODD samples using a centrifugal molecular distillation. It was found that centrifugal distillation is a cost effective process compared to wiped filmed distillation, with a higher recovery of tocopherols and sterols in single stage molecular distillation process.

Table 4.4-Results obtained from 180^{o}C of evaporator temperature.

Feed flow rate (g/min)	**5**	**7.5**	**10.42**	**12.5**	**15**
Split ratio (D/R)	0.79	0.64	0.45	0.39	0.11
FFA content in distillate (%)	62.1	73.17	74.87	56.41	72.38
FFA content in residue (%)	9.38	9.37	15.92	31.94	18.67
FFA recovery in distillate (%)	**78.97**	**82.47**	**67.08**	**45.65**	**20.92**
Tocopherols content in distillate (%)	3.46	4.45	0.67	0.29	0.81
Tocopherols content in residue (%)	8.78	10.44	11.28	10.78	11.62
Tocopherols recovery in residue (%)	**53.68**	**69.57**	**84.98**	**84.78**	**114.21**
Sterols content in the distillate (%)	4.39	8.64	1.55	0.84	1.54
Sterols content in residue (%)	21.67	24.07	23.85	22.83	19.89
Sterols recovery in residue (%)	**67.70**	**81.93**	**91.82**	**91.72**	**99.89**

Elimination of Free Fatty Acid (FFA)

During the distillation process, FFA concentrates into the distillate stream attributable to their lighter molecular weight when compared to tocopherols and sterols. The FFA content of raw CODD material was 34.60% (before centrifugal distillation). The FFA content in the distillate stream versus the split ratio D/R is shown in Figure 4.2 With a split ratio higher than 0.75 a more than 90% recovery can be obtained. Similar

results were found in a study done by Ito and others (2006) on soybean oil deodorizer distillate.

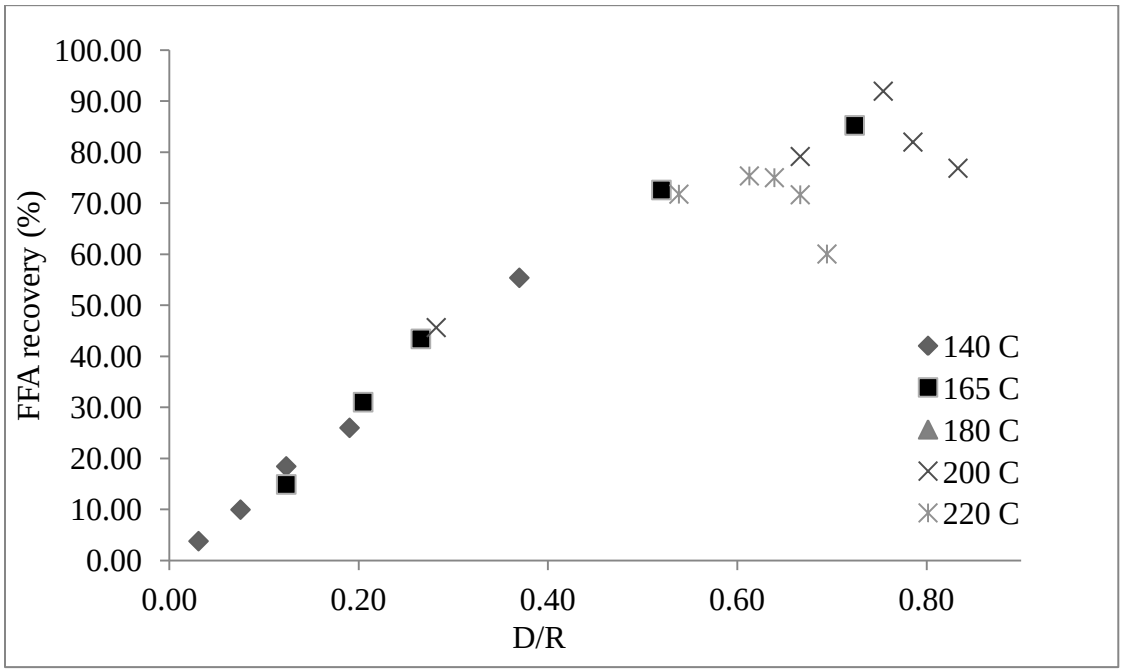

Figure 4.2-Percent FFA recovery in the distillate stream.

Concentration of FFA in the residue stream was directly proportional to the feed flow rate and evaporator temperature. The FFA content in the residue stream is shown in Figure 4.3, indicating a loss of FFA in the residue stream with an increase in the feed flow rate. Loss of FFA can occur due to an increase in the feed flow rate and decrease in evaporator temperature (Ito and others 2006, Martin and others 2006), indicating that a greater portion of FFA is eliminated in the residue stream. Increasing the temperature decreases the FFA content in the residue stream, leading to increases in the tocopherol content in the distillate stream. The highest FFA loss occurred in the residue stream at 140°C and 15 g/min was 35.3%. At 165°C, the loss of FFA increased from 9.7% to

33.18% with an increase in the feed flow rate at constant temperature. The loss of FFA is comparatively low at higher temperatures. Ito and others (2006) found high loss of FFA at lower temperature between 140°C and 160°C across all feed flow rates. To promote fractioning of FFA into the distillate stream, maximal efficiency occurred at 7.5 g/min with a temperature between 165°C and 200°C. D/R. Martins and others (2006) found similar results on SODD using wiped film molecular distillation, more than 90% recovery of FFA in distillate stream at 220°C with D/R higher than 2.

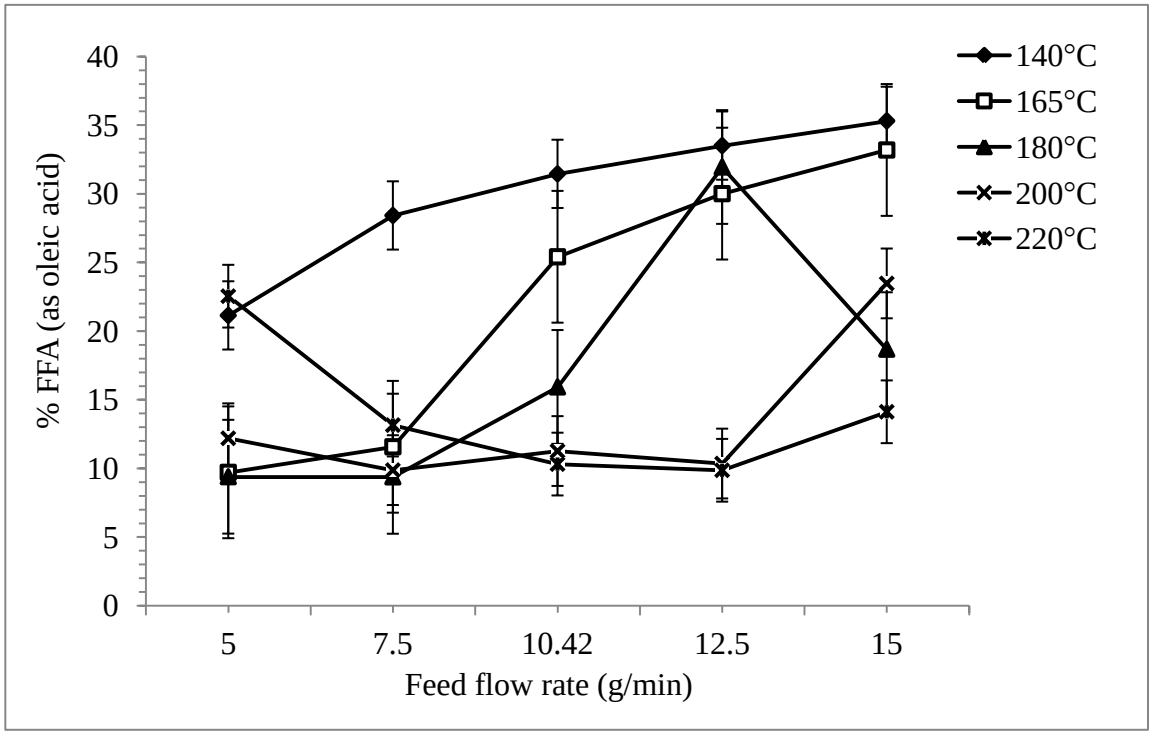

Figure 4.3-FFA content in the residue stream versus feed flow rate.

The best way to evaluate the FFA elimination in the distillate stream is to consider its recovery and the percentage of FFA in the raw material can be evaluated based on recovery in each stream (Martins and others 2006). A higher recovery of 91.27% was achieved at 200°C and 10.42 g/min in the distillate stream, as shown in Figure 4.4. Martins and others (2006) showed more than 90% recovery of FFA at low temperature (140°C-180°C) and feed flow rate varying between 5-23 g/min using a wiped film molecular distillation. To obtain a maximal efficiency of FFA in the distillate stream, a temperature higher than 165°C should be utilized in the current study. At a temperature of 165°C and a feed flow rate of 5 g/min, more than 85.26% of FFA was eliminated in the distillate stream. At a temperature of 180°C and 7.5 g/min, more than 82% of FFA was eliminated from CODD in the distillate stream. Overall, a good recovery of FFA was achieved in this study.

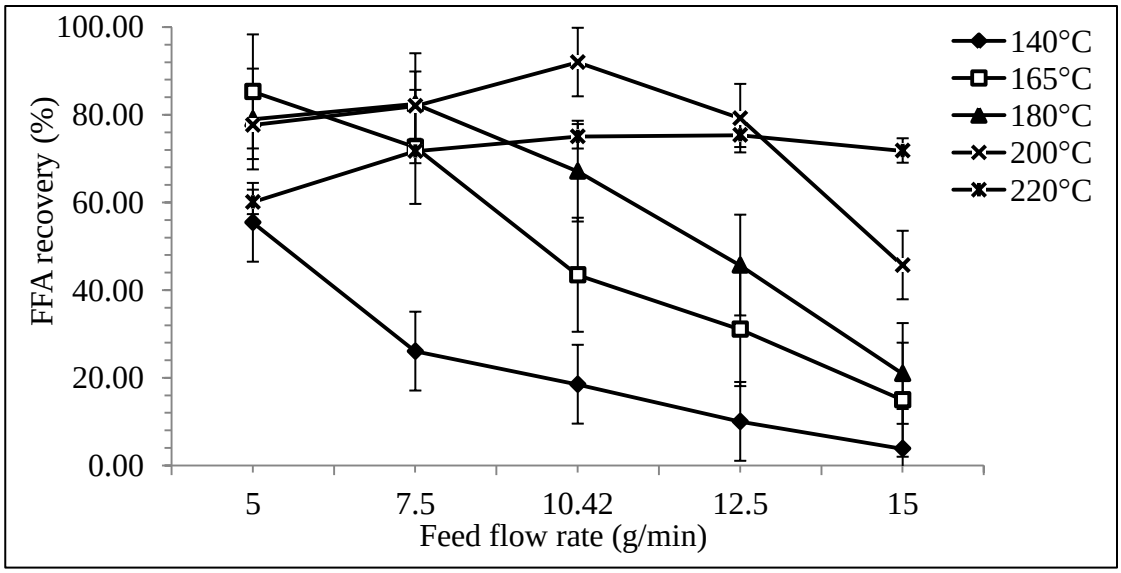

Figure 4.4-Recovery of FFA in the distillate stream.

Recovery of Tocopherols and Sterols

Total tocopherols and sterols content are to be recovered in the residue stream. In distillate stream, the concentration of tocopherols and sterols are inversely proportional to the feed flow rate. In this study, the tocopherol and sterol concentration changed widely with the temperature and feed flow rate in the residue stream (Figure 4.5).

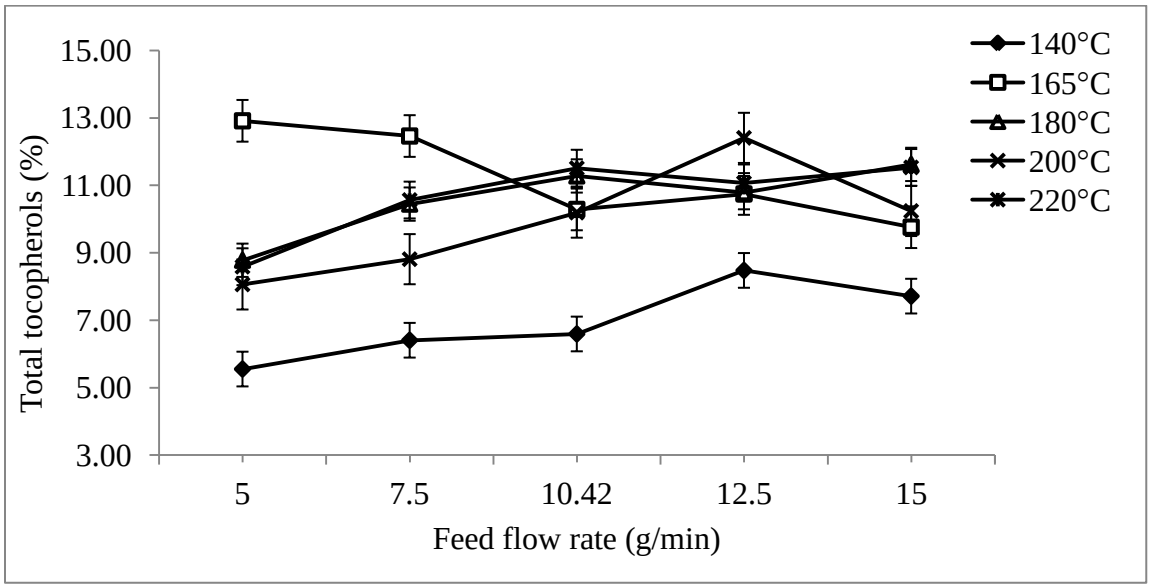

Figure 4.5-Total tocopherols in residue stream in function to temperature and feed flow rate.

The maximum output of tocopherols obtained in the residue stream was 12.91% using 165°C of evaporator temperature and at 5 g/min of feed flow rate (Figure 4.5). Martin and others (2006) were able to obtained 18.33% of tocopherols (twice the tocopherols concentration present in their raw material) at 160°C and 10.4 g/min of feed flow rate. Our study showed 9.7% FFA content at 165°C and 5g/min of feed flow rate, indicating that 85.26% of FFA eliminates into the distillate stream. There was wide

difference in tocopherol concentrations at 140°C and 165°C. The reason is unknown as to why there is large difference between these two temperatures (Ito and others 2006).

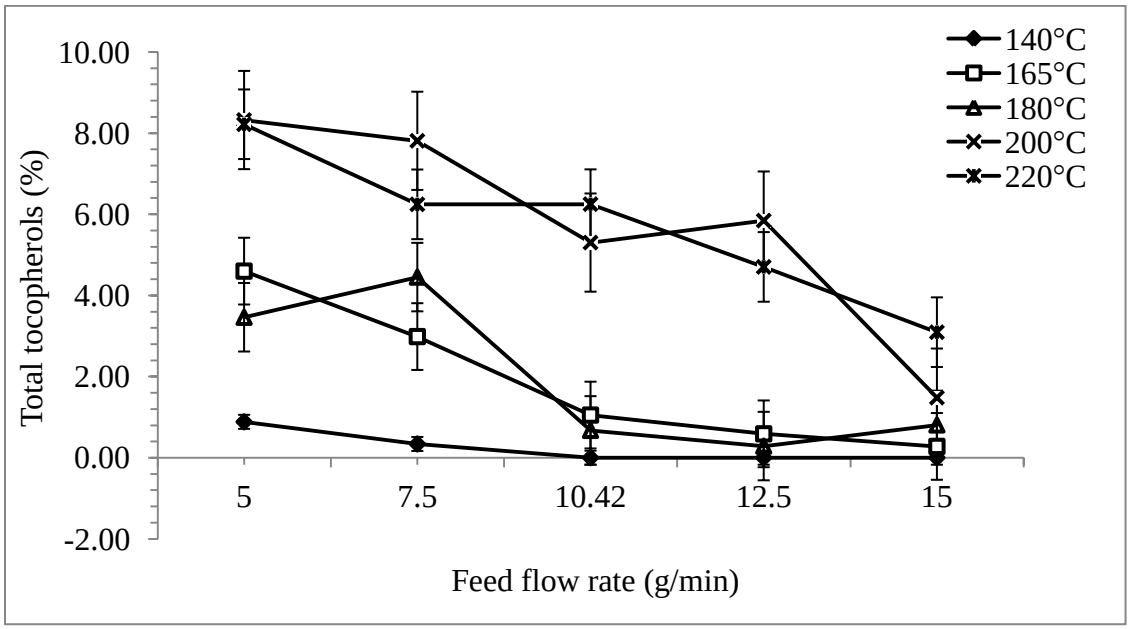

Figure 4.6-Total tocopherols in the distillate stream in function to temperature and feed flow rate.

At 140°C, the percentage of total tocopherols and total sterols were lower than 1% and 1.5%, for all the feed flow rates in the distillate stream (Figure 4.6 and 4.7). There was a considerable loss of tocopherols and sterols at a higher temperature (200°C and 220°C) in the distillate stream. Ito and others (2006) observed up to 2.5% loss of tocopherols at 200°C and 220°C, respectively. Increasing the feed flow rate effectively decrease the loss of both the compounds in the distillate stream. The optimum operating conditions for tocopherols and sterols were low evaporator temperature (140°C) and high feed flow rate (12.5g/min).

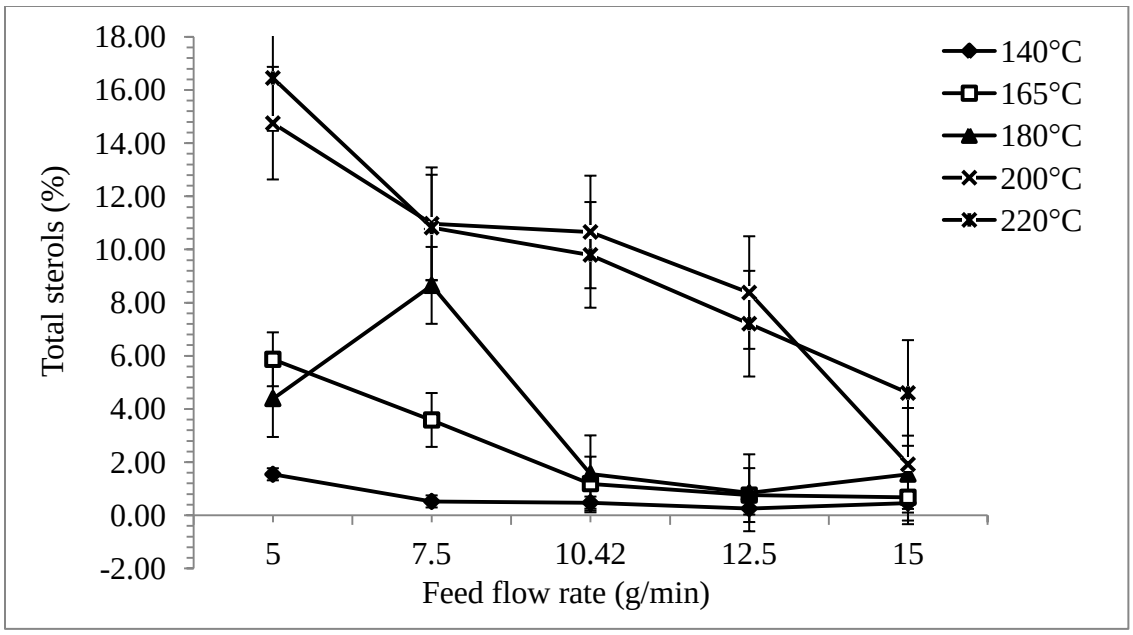

Figure 4.7-Total sterols in the distillate stream versus feed flow rate.

At 220°C, more than 16% of sterols were eliminated into the distillate at a low feed flow rate (5 g/min). The best way to evaluate the concentration of tocopherols and sterols is to consider its recovery (Figure 4.8-4.9). The FFA recovery can also be considered when evaluating the elimination of FFA in distillate (Figure 4.4). The recovery corresponds to the percentage of compounds present in the raw material which is present in their respective streams. Efficiency of recovery allows for the determination of optimum operating conditions for the recovery of tocopherols and sterols with high yield using centrifugal distillation. The recovery of a compound is calculated via the equation;

$$\text{Recovery} = \frac{\text{Sample mass (\%)} * \text{content of the substance in the sample (\%)} * 100}{\text{Raw material (\%)} * \text{content of the substance in the raw material (\%)}}$$

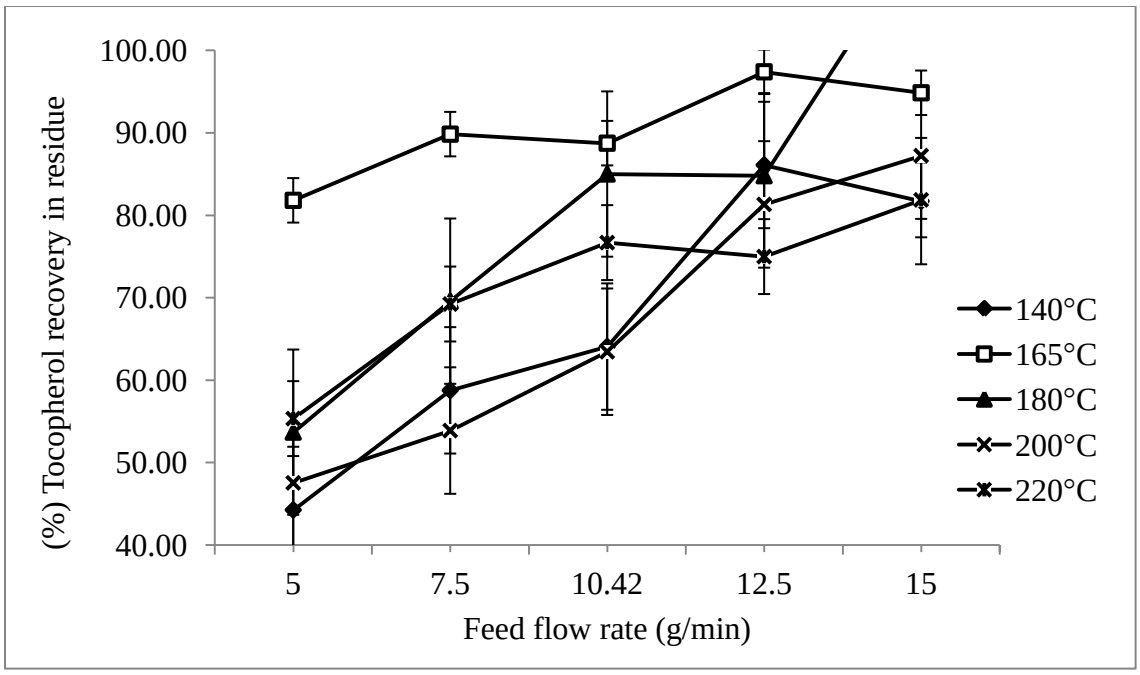

Figure 4.8-Recovery of tocopherols in the residue stream.

The percentage of total tocopherols in raw material can be evaluated based on recovery in each stream. Figure 4.8 shows the recovery of tocopherols in the residue stream. Higher tocopherol recoveries were found in the residue stream across all temperatures assessed. At 140°C and 5 g/min, almost 54% of the tocopherols were found in the distillate stream. At 165°C and a feed flow rate above 7.5 g/min, the total tocopherol content was recovered in the residue stream (97.38%). At 180°C and a feed flow rates above 12.5 g/min, 99.55 % of tocopherols were recovered.

Jiang and others (2006) were able to recover 50% tocopherol and 90% FAME, of the original content in raw material through acid-catalyzed esterification and crystallization of sterols followed by molecular distillation from rapeseed oil deodorizer distillate. They recovered more than 85% of tocopherol at 200°C and 230°C evaporator

temperature. However, part of mono- and diglycerides began to recover along with tocopherols. There is no information available in the literature regarding recovery of sterols and FAMEs in the residue stream.

Tocopherols are not expected to decompose or oxidize under this process due to short exposure time with heat. However, some of the tocopherols are inevitably lost during the centrifugal distillation process escaping with the uncondensed material in the cold traps or formed a thick film on the centrifugal disc due to low temperature and high feed flow rate. Due to a higher temperature of distillation, more tocopherol molecules could volatilize and the condenser would not be able to recover all molecules, allowing the molecules would escape and condense in the cold trap (Posada and others 2007).

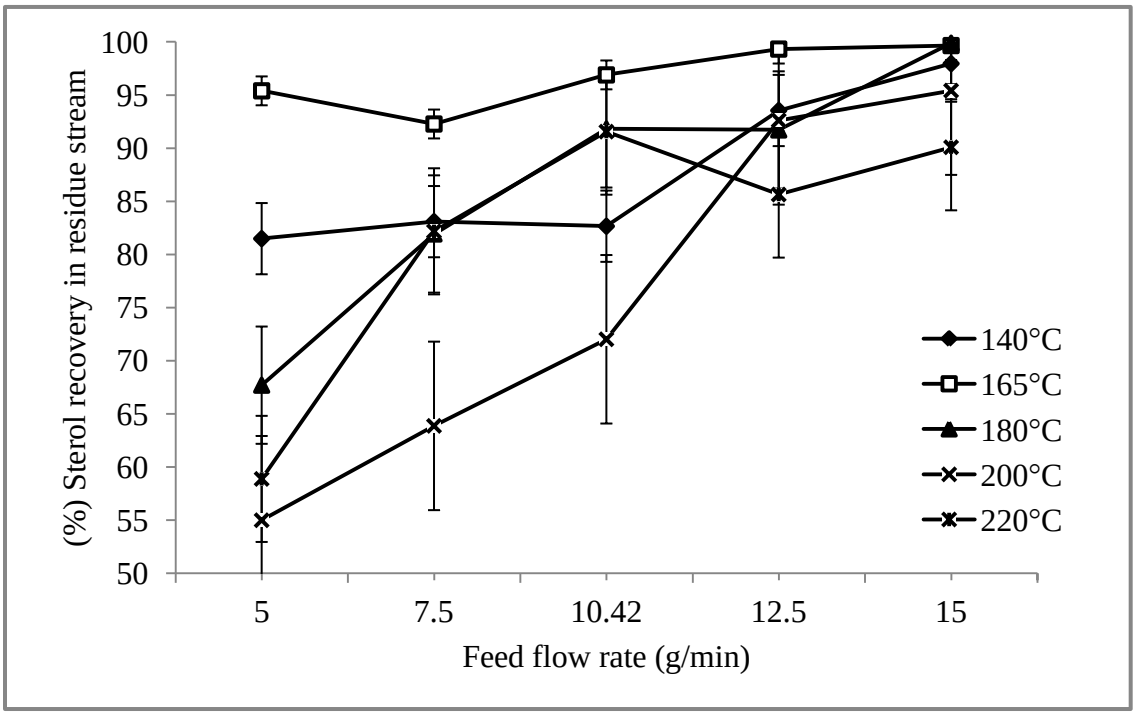

Figure 4.9-Recovery of sterols in the residue stream.

Figure 4.9 shows the recovery of sterols in the residue stream. The percentage of total sterols in the raw material can be evaluated based on their recovery in each stream. Sterol recovery exceeded 90% at 165°C for all feed flow rates in the residue stream. At 200°C and 5 g/min, there was a loss of 38% of the total sterols in the distillate stream. At 165°C and at a feed flow rate above 5 g/min, total sterols content were recovered in the residue stream (99.66%). A total of 99.89% sterol recovery was found at 180°C and a feed flow rate above 10.42 g/min.

No literature exists on the recovery of sterols using molecular distillation technique, but, solvent extraction technique had been proposed in the literature. Yan and others (2012) conducted a study on the recovery of phytosterols from SODD using methyl esterification followed by crystallization. Their study showed a 94.7% sterol recovery under optimized conditions (3.41:1 g/ml feed solution to solvent ratio, 26.5 hr ripening time, 4.5^{0}C ripening temperature). However, their method was more toxic and less efficient compared to the molecular distillation. In the current study, a 99.89% sterols recovery occurred at 180°C and 15 g/min using one-step molecular distillation process.

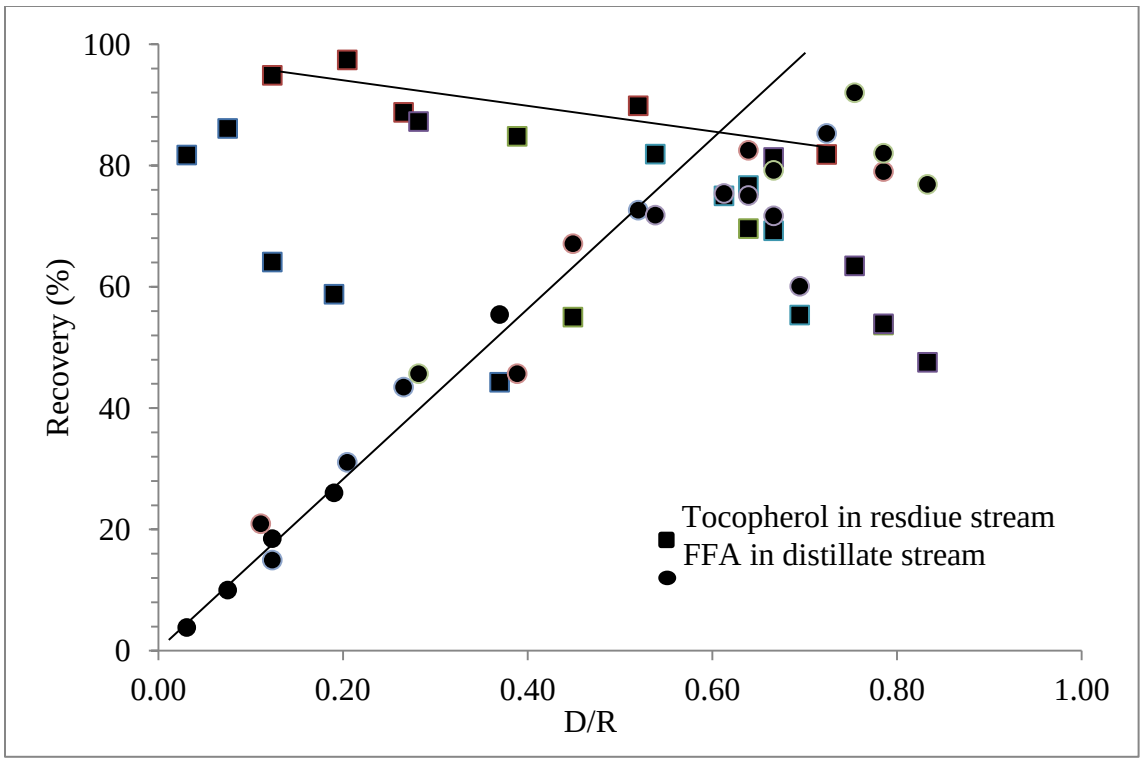

Figure 4.10-Optimized condition for Tocopherol and FFA recovery.

Analyzing the profile of tocopherols and sterols in comparison to the split ratio, it was possible to obtain a recovery higher than 90% with a split ratio lower than 0.54 for tocopherols and sterols. The increase in tocopherol recovery implies the loss of FFA in the distillate stream. In Figure 4.10 the intersection of tocopherol and FFA recovery identifies a optimum ratio D/R when 86% of total tocopherol and FFA were recovered in a stream with split ratio higher than 0.6.

Comparison of the Operating Conditions

The best operating conditions for molecular distillation leading to the highest tocopherol concentration from CODD in the residue stream are reported in Table 4.5. The temperature and feed flow rate were found to be directly proportional to each other to reach approximately the same tocopherol and sterol concentration. Under these conditions, utilizing molecular distillation increases the loss of FFA in the residue stream. The FFA content was lower in the residue stream at 165^{o}C due to higher elimination of FFA in the distillate stream (85.26%). It can be seen that 81.79% and 81.30% of tocopherols were recovered from CODD in the residue stream at 165^{o}C and 200^{o}C, respectively.

Table 4.5-Best operating conditions for the recovery of tocopherols and sterols in residue stream.

Temperature °C	**140^{o}C**	**165^{o}C**	**180^{o}C**	**200^{o}C**	**220^{o}C**
Feed flow rate (g/min)	**12.5**	**5**	**10.42**	**12.5**	**12.5**
% Tocopherols	8.47	12.91	11.28	12.41	11.07
% Sterols	18.03	29.48	23.85	27.67	24.76
% FFA in residue	33.5	9.7	15.92	10.34	9.85
Recovery of FFA (%)	9.98	85.26	67.08	79.17	75.36
Recovery of tocopherols (%)	86.08	81.79	84.98	81.30	74.97
Recovery of sterols (%)	93.55	95.40	91.82	92.62	85.64

Conditions leading to a higher concentration of tocopherols resulted in a significant loss of tocopherols in the distillate stream. In the distillate stream, the tendency was that with an increase in the feed flow rate the loss of tocopherol decreases. In this study, other compounds such as mono-, di- and triglycerides, and unknown compounds, were also concentrated in the distillate stream at higher temperatures.

This fractionation was limited to a one-stage molecular distillation process. To achieve higher tocopherol and sterol concentrations, the use of multistep methods would be necessary to modify FFA into more volatile FAMEs before centrifugal distillation process. From a commercial point of view, the optimum condition for eliminating FFA in the distillate stream is at 200°C and a feed flow rate of 12.5 g/min. With a 2-stage distillation process, the FFA content could possibly be reduced from 10% to 5% or less, which is then acceptable.

Statistical Analysis

One way MANCOVA was used for analyzing the following variables:

- **Independent variable**: Temperature (140°C, 165°C, 180°C, 200°C and 220°C)
- **Covariate**: Flow rate in g/min
- **Dependent variable**: Tocopherol distillate and FFA distillate

The multivariate null hypothesis of the effect of temperature on the distillates was rejected using the Pillai's Trace criterion ($p<0.001$). The covariate (flow rate) also had an effect on the amount of the distillates, also using the Pillai's trace criterion ($p<0.001$).

Pillai's criterion and Wilks' Lambda are the two common tests of significance and is widely used in interpreting the results for more than two groups. For the current study, we have used Pillai's criterion, because it is more robust to violation of assumptions concerning homogeneity of the covariance matrix. They are not highly linked to assumptions about the normality of the distribution of the data.

Effect	Value	F	Hypothesis df	Error df	Significance
Temperature	1.030	5.047	8.000	38.000	0.000
Flow rate g/min	.725	23.677	2.000	18.000	0.000

The above results allow us to analyze the ANOVA's separately. Values <0.001 indicated variables were significant. For the tocopherol distillate, the null hypothesis of the effect of temperature was rejected ($p<0.001$) because a significant effect of temperature on tocopherol distillate content was found. The null hypothesis of the effect of temperature on the FFA distillate content were retained ($p>0.001$). There was also no effect of flow rate on tocopherol distillate content.

Effect	F	df	Significance	Remarks
Temperature Toc distillate	24.813	4, 19	0.000	Significant
Flow rate g/min Toc distillate	42.812	1,19	0.000	Significant

Sterol distillate was not analyzed because it was highly correlated with tocopherol distillate (***R*=.95, *p* < .001**) and using both as variables in the same analysis violates assumptions of MANOVA. All temperatures examined resulted different amounts of tocopherols distillate. Since the p-value was >0.01, the null hypothesis was retained. There was no difference in tocopherol distillate amount between 140°C and 165°C, 140°C and 180°C, 165°C and 180°C, and between 200°C and 220°C.

Since the p-value <0.01 among the temperatures, the null hypothesis was rejected. A significant effect on the difference in temperatures for recovery of tocopherol distillate from CODD using molecular distillation was found. As the temperature increased the amount of tocopherol distillate increased, 140°C=165°C=180°C<200°C<220°C. For FFA distillate, there was no significant difference between 140°C to 220°C.

Table 4.6-Descriptive statistics obtained for distillates using One-way MANCOVA.

Temperature_Category		Mean	Std. Deviation	N
Toc Distillate	140	.464	.3120	5
	165	1.736	1.4715	5
	180	1.598	1.3800	5
	200	4.320	1.9490	5
	220	4.344	1.2548	5
	Total	2.492	2.0355	25
FFA Distillate	140	55.74	10.205	5
	165	65.17	10.939	5
	180	67.79	8.094	5
	200	66.77	6.060	5
	220	63.77	8.021	5
	Total	63.85	9.172	25

One way MANCOVA was used for analyzing the effect of temperature on the residues, using the following variables:

- **Independent variables**: Temperature (140°C, 165°C, 180°C, 200°C and 220°C)
- **Covariate**: Flow rate in g/min
- **Dependent variable**: Tocopherol residue, sterol residue and FFA residue

Effect	Value	F	Hypothesis df	Error df	Significance
Temperature	1.206	3.191	12.000	57.000	0.002
Flow rate g/min	0.604	8.646	3.000	17.000	0.001

Using the Pillai's trace criterion, the multivariate null hypothesis that there is no effect of temperature on the amount of residues was retained ($p < .01$). There was also a significant effect of flow rate on the amount of the residues. These results allow interpretation of each dependent variable separately.

From the univariate test, it was shown that the 2 variables were subject to change due to temperature. The flow rate was only significant for FFA residue ($p<0.01$). Results as follows;

Effect	F	df	Significance	Remarks
Temperature				
Toc residue	8.826	4, 19	0.000	Significant
FFA residue	5.923	4, 19	0.003	Significant

Flow rate g/min FFA residue	10.189	1, 19	0.005	Significant

Table 4.7-Descriptive statistics obtained for residue using One-way MANCOVA.

Temperature_Category		Mean	Std. Deviation	N
FFA Residue	140	29.956	5.5590	5
	165	21.968	10.7335	5
	180	17.056	9.2659	5
	200	13.418	5.6835	5
	220	13.986	5.1094	5
	Total	19.277	9.3635	25
Tocopherol Residue	140	4.462	0.7453	5
	165	7.124	.8416	5
	180	6.398	.7416	5
	200	6.298	1.1832	5
	220	7.010	.7412	5
	Total	6.258	1.2560	25
Sterol Residue	140	18.10	1.214	5
	165	23.61	3.754	5
	180	22.46	1.724	5
	200	22.19	3.496	5
	220	23.79	3.432	5
	Total	22.03	3.393	25
FFA Residue	140	29.956	5.5590	5
	165	21.968	10.7335	5
	180	17.056	9.2659	5
	200	13.418	5.6835	5
	220	13.986	5.1094	5
	Total	19.277	9.3635	25

For tocopherol residue, the mean for 140°C was lower than 165°C and 220°C. Results showed a low concentration of tocopherol at 140°C compared to 165°C in the residue stream. For sterol residue, there was no significant effect of temperature between the groups. For FFA residue, the mean for 140°C was higher than 200°C and 220°C; no

other difference in temperatures was observed. Results showed a decrease in the amount of FFA with the increase in temperature in residue stream. Feed flow rate also had an effect on the product streams.

Future Work/Recommendations

The relationship between the operating conditions to obtain higher yield of tocopherols and sterols needs to be considered. Based on the preliminary study, it can be observed that a multistage process can lead to a high tocopherol and low FFA content in residue stream.

Table 4.8- Operating conditions at different temperatures in function with %FFA in residue stream.

Temperature °C	165	165	180	180	200	220
Feed flow rate g/min	5	7.5	5	7.5	12.5	15
%FFA in residue	**9.7**	**11.56**	**9.38**	**9.37**	10.37	**14.11**
%Tocopherol recovery	81.79	89.82	53.68	69.57	81.30	81.85
% Sterol recovery	95.40	92.28	67.70	81.93	92.62	90.09

For instance, if the purpose of the recovery was to obtain a product with the maximum recovery, the separation should be carried out at 165°C; while if a product with high recovery without sacrificing yield is the target, a better choice would be to distill the

residue obtained at optimum conditions, which is 200°C. There are few alternatives that can be use to achieve a higher concentration of tocopherols and sterols in the residue stream. The FFA content varies between 9% and 14%, is shown in Table 4.8.

Molecular distillation without pre-steps will probably require a second pass to meet the goals requested. The first stage residues will serve as feed/raw material for a 2nd stage. However, using these residues as a feed will reduce the FFA to between 5 - 1% with variations of losses. The pet food industry accepts up to 5% FFA in their products. This process is as a 2-stage molecular distillation process. Another alternative would be to involve an acid-catalyzed or base-catalyzed methyl esterification step followed by centrifugal molecular distillation. FFA can be distilled from deodorizer distillate or can be first converted into FAMEs for easy separation followed by 2-stage molecular distillation process. The advantage of this latter option is not only a decrease in the evaporator temperature by $\geq$20°C, but also the elimination of di-and triglycerides from the mixture. This procedure will give a clean product with a loss of 5% or less.

CHAPTER VI

CONCLUSION

A processing procedure was developed for the recovery and separation of tocopherols and sterols from cottonseed oil deodorizer distillate using centrifugal molecular distillation, investigating various processing parameters such as temperature and feed flow rate. The optimum operating conditions leading to higher free fatty acid elimination in the distillate stream was 165°C evaporator temperature at 5 g/min of feed flow rate which, consequently, lead to a higher concentration of tocopherol and sterol in the residue stream. There was a total tocopherol recovery of 81.79% and a sterol recovery of 95.40%, with an 85.26% of FFA elimination.

The optimal recovery of distillate and residue from CODD using centrifugal molecular distillation was dependent on the evaporator temperatures and feed flow rates used. More data is required to see the individual effect of flow rate on the distillate and residue stream. It is difficult to perform operation with a methylation of CODD in one step process because of the increased loss of tocopherols and sterols. The residue stream was a final product with FFA as a by-product for biodiesel or pet food purpose. The process requires at least two successive molecular distillation steps for residue at 165°C. These results might serve as a guideline for a scale-up of the molecular distillation

process, potentially to be used in a pilot plant or commercial plant for concentrating vitamin E and sterols from CODD.

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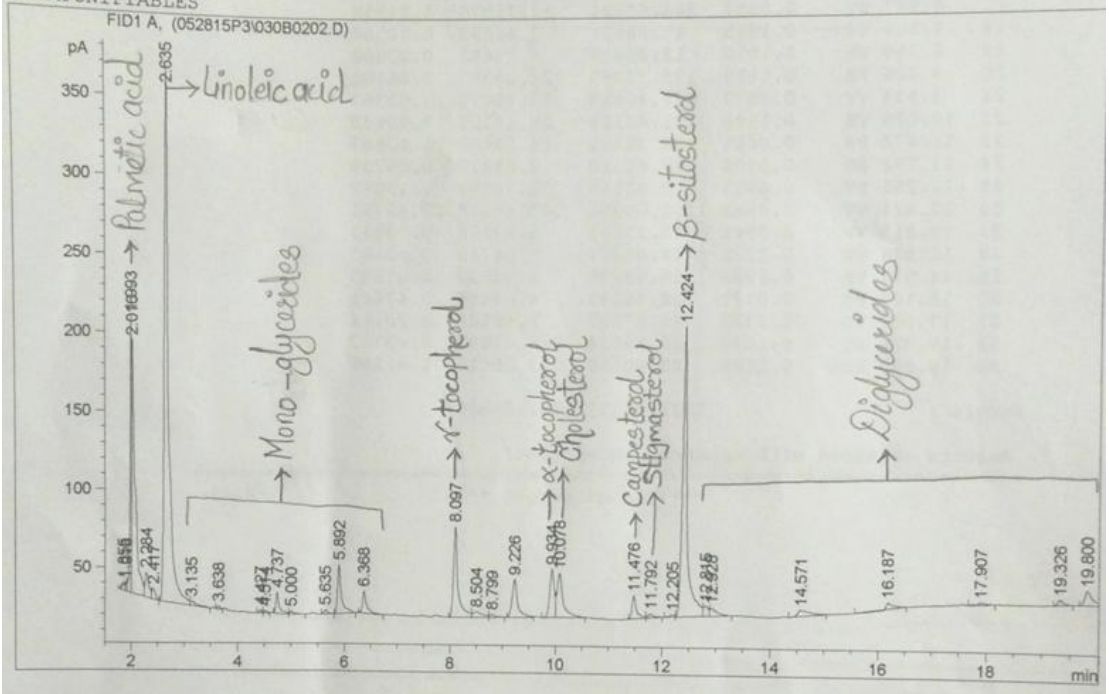
APPENDIX A

Gas Chromatogram of Cottonseed Oil Deodorizer Distillate

Injection Date : 5/29/2015 5:41:52 PM Seq. Line : 2
Sample Name : CODD Location : Vial 30
Acq. Operator : AJafri Inj : 2
Acq. Instrument : Instrument 1 Inj Volume : 1 µl
Sequence File : C:\HPCHEM\1\SEQUENCE\DEF_GC.S
Method : C:\HPCHEM\1\METHODS\PRIYANKA.M
Last changed : 5/28/2015 9:47:29 PM by AJafri
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	1.855	BV	0.0439	16.03532	4.94027	0.27078
2	1.916	VV	0.0260	9.91002	5.51639	0.16734
3	1.993	VV	0.0262	319.81409	176.38515	5.40048
4	2.016	VV	0.0555	669.74030	161.04915	11.30943
5	2.284	VV	0.0648	79.40645	15.76163	1.34088
6	2.417	VP	0.0698	29.95840	6.61923	0.50589
7	2.635	VV	0.0809	1879.31238	337.71158	31.73463
8	3.135	VP	0.1112	23.89938	2.75531	0.40357
9	3.638	VV	0.0690	8.40140	1.81431	0.14187

Data File C:\HPCHEM\1\DATA\052815P3\030B0202.D Sample Name: COD

Peak #	RetTime [min]	Type	Width [min]	Area [pA*s]	Height [pA]	Area %
10	4.427	PV	0.0554	7.33246	1.96969	0.12382
11	4.514	VV	0.0679	7.22088	1.50734	0.12193
12	4.737	VV	0.0621	59.07694	13.49313	0.99759
13	5.000	VB	0.0738	9.12687	1.69526	0.15412
14	5.635	PP	0.0735	13.35520	2.75879	0.22552
15	5.892	VB	0.0739	160.63457	31.29060	2.71252
16	6.368	BP	0.0740	74.74584	14.07142	1.26218
17	8.097	BV	0.0857	344.63324	57.71070	5.81958
18	8.504	VV	0.1635	41.68127	3.48593	0.70384
19	8.799	VB	0.1070	13.85639	1.77662	0.23398
20	9.226	PB	0.1025	175.34987	24.20957	2.96101
21	9.934	VV	0.0877	178.46823	30.75071	3.01367
22	10.078	VB	0.1180	231.45108	28.14127	3.90835
23	11.476	PB	0.0861	86.38191	14.59465	1.45867
24	11.792	BB	0.0934	17.61110	2.58827	0.29739
25	12.205	PV	0.0955	8.92545	1.34397	0.15072
26	12.424	VV	0.0862	1151.09961	183.48506	19.43781
27	12.815	VV	0.0941	43.25571	6.63078	0.73043
28	12.928	VB	0.1275	48.65311	5.04713	0.82157
29	14.571	VB	0.1995	59.93296	3.93830	1.01205
30	16.187	PB	0.0977	28.33099	4.04666	0.47841
31	17.907	VP	0.1333	14.87257	1.49184	0.25114
32	19.326	BB	0.1089	25.88024	3.47637	0.43702
33	19.800	BBA	0.1289	83.60688	9.29610	1.41181
Totals :				5921.96111	1161.35320	

Results obtained with enhanced integrator!

*** End of Report ***

APPENDIX B

Figure Showing %Sterols in Residue Stream

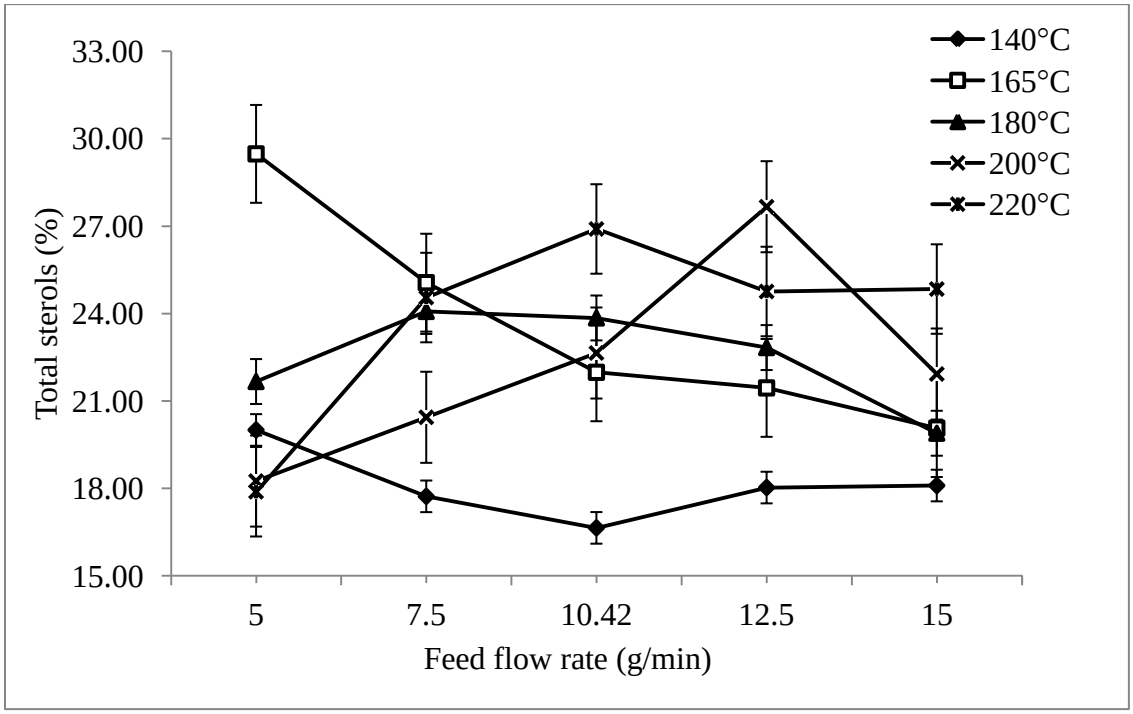


Figure-Percent sterols in residue stream in function of operating conditions.

APPENDIX C

Figure Showing % FFA Recovery in Distillate Stream

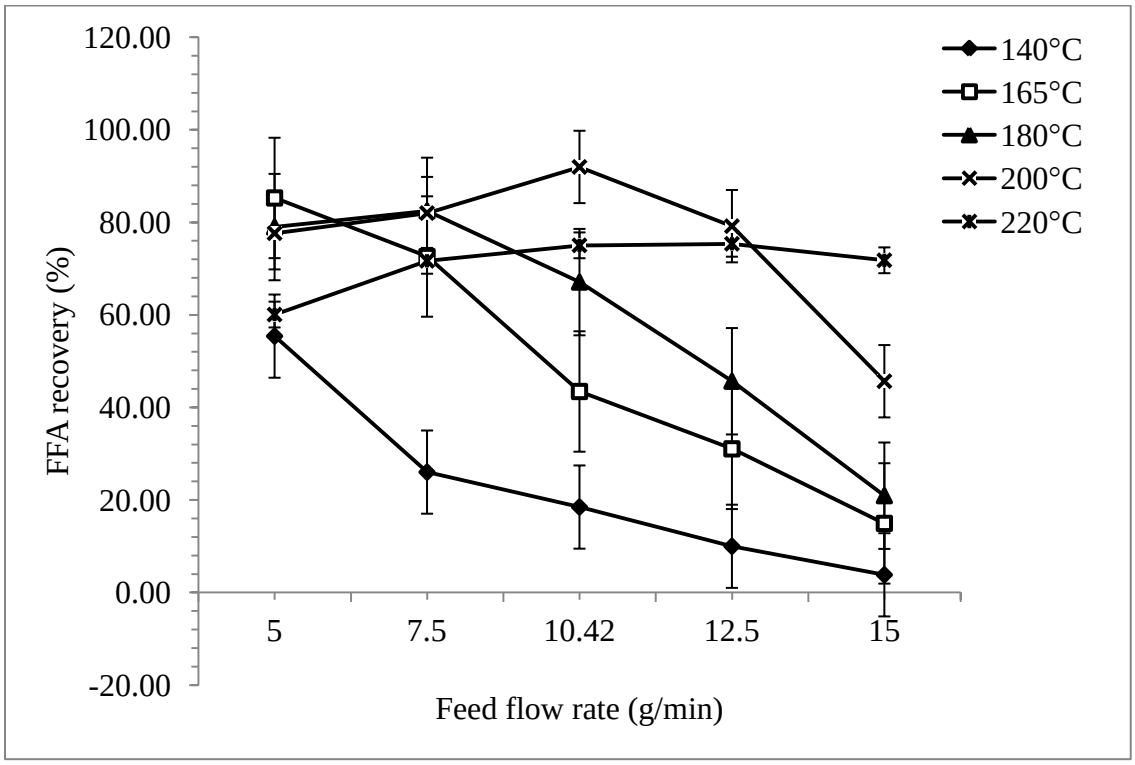


Figure-Percent recovery of FFA in distillate stream in function of operating conditions.

APPENDIX D

AOCS Official Methods Ca 5a-40 and Ce 7-87

AOCS Official Method Ca 5a-40

Free Fatty Acids

Reagents and apparatus:

(a) ethyl alcohol,

(b) phenolphthalein (1% soln, in alcohol),

(c) sodium hydroxide (NaOH) (0.1 N and 1 N),

(d) Erlenmeyer flasks, 250 ml, and Hot plate.

Method:

(a) Melt the CODD samples to obtain a homogenous mixture.

(b) Weigh 3 gm of sample into a 250 ml Erlenmeyer flask. Add 50 ml of neutralized alcohol (reagent alcohol and phenopthalein indicator-2 ml) into the flask with a volumetric dispenser.

(c) Titrate slowly with NaOH, shaking, until a pale pink color appears for 30 seconds with shaking.

(d) The percentage of free fatty acids (FFA) in most fats and oils is expressed as oleic acid using the formula:

$$\% \text{ FFA} = \frac{\text{ml of alkali} * \text{alkali normality} * 28.2}{\text{Weight of sample (gm)}}$$

Where;

ml of alkali=ml of NaOH

alkali normality= 0.1N/1 N

molecular weight of oleic acid= 28.2 g/mol

AOCS Recommended Practice Ce 7-87

Total Tocopherols in Deodorizer Distillate (with slight modifications)

Apparatus:

(a) Capillary gas chromatography- Agilent 6890 Series equipped with flame ionization detector and a 0.25 mm CP-Sil 8 CB low bleed capillary column,

(b) Electronic balance,

(c) Regular laboratory glassware- consisting of transfer pipets and Erlenmeyer flasks with stoppers.

Reagents:

(a) Pyridine- available from Acros Organics

(b) Silylating kit (BSTFA + 1% TMCS)- available from supelco

(c) Silylated Cholesterol, internal standard solution- final concentration of approximately 10 mg/ml.

(d) α-tocopherol reference standard- final concentration of approximately 10 mg/ml.

(e) Sterol reference standard kit (campesterol, stigmasterol and β-sitosterol)*- final concentration of approximately 10 mg/ml.

(f) Chloroform-reagent grade.

Procedure:

1. Capillary gas chromatographic conditions-

   (a) Capillary column, 50 m Cp-Sil 8 CB, 0.25-mm film thickness, 0.25 mm internal diameter.

(b) Oven temperature program-240^{0}C for 2 min, followed by heating to 275^{0}C at 10^{0}C/min, held for 5 min, then to 300^{0}C at 20^{0}C/min, held for 2.25 min, and then to 330^{0}C at 15^{0}C/min, held for 4 min giving a total run time of 20 min

(c) Carrier gas- Nitrogen.

(d) Split ratio, 10:1

(e) Injector temperature, 360 C.

(f) Detector temperature, 300 C.

2. Detemination of response factor for α-tocopherol-

(a) Accurately weigh 10 mg of α-tocopherol into a 50-ml Erlenmeyer flask.

(b) Add 0.5 ml of pyridine and 1 ml of BSTFA +1 % TMCS vial. Heat contents to 70 C for approximately 20 min in an oven.

(c) Allow flask to cool slightly. Add 1 ml of internal standard (Reagent, 3) and 1.5 ml of chloroform. Mix well

(d) Inject 1µl aliquot in gas chromatography set at previously described conditions (Procedure, 1).

(e) Calculate the response factor (FC):

$$FC = \frac{A_{IS} * C_I}{A_I * C_{IS}}$$

Where-

A_{IS}=area of internal standard
C_I= mg of standard compound
A_I= area of standard compound
C_{IS}= mg of internal standard

(f) Response factor of γ-tocopherol is taken to be equivalent to that of α-tocopherol.

3. Preparation of sample-

(a) Warm the sample and shake well. Accurately weigh 150 mg of sample into a 50-ml Erlenmeyer flask.

(b) Add 0.5 ml of pyridine and 1 ml of BSTFA +1 % TMCS vial. Heat contents to 70 C for approximately 20 min in an oven.

(c) Allow flask to cool slightly. Add 1 ml of internal standard (Reagent, 3) and 1.5 ml of chloroform. Mix well

(d) Inject 1µl aliquot in gas chromatography set at previously described conditions (Procedure, 1).

Calculations:

1. Total tocopherol content of a sample is expressed as the total weigh percent of α-tocopherol:

$$\% X = \frac{A_x \times FC \times C_{IS}}{A_{IS} \times S} \times 100$$

Where-

% X= weight percent of α-tocopherol/γ-tocopherol
A_x = area of α-tocopherol/ γ-tocopherol
FC = response factor from procedure, 2, (e)
C_{IS} = mg of cholesterol
S = sample weight (mg)

2. Total tocopherols % = % α + % γ

Notes: Response factor for sterol reference standards were conducted with same procedure.

APPENDIX E

Technical Procedure for GC-FID

Technical Procedure for Gas Chromatography (GC-FID)

Silylation:

1. Preheat the oven to 70°C.
2. Take (10-200) mg of liquid sample and place in a glass container with lid.
3. Add 0.5 ml of pyridine to the sample.
4. Mix the sample and pyridine well.
5. Add 1 vial of 1 ml BSTFA +1% TMCS (silylating agent) to the sample.
6. Immediately put the sample in the over for 20 mintues at 70°C±2.
7. Add 1.5 ml of chloroform to the sample.
8. Mix the mixture uniformly.
9. Transfer 1.5 ml of sample in GC vial and seal.
10. Label the vial with date, sample name and your name.
11. Sample is ready to inject in GC.

Program parameters:

1. Injection-Using the autosampler with a 10 μL syringe injection, perform eight solvent washes from each wash bottle. Inject a 1.0 μL sample with a fast plunger speed, three sample washes, and three sample pumps.
2. Inlet –Run in split mode with a 10:1 split ratio.
3. Carrier gas-Nitrogen
4. Oven- Run a temperature program starting at 240°C for 2 min. Then ramp at 10°C/min until 275°C is obtained. Hold for 5 min. Then ramp to 20°C/min until

300°C is obtained. Hold for 2.25 min and then to 330°C at 15°C/min, hold for 4 min, giving a total run time of 20 min.

5. Coulmn- CP-Sil 8 CB Low Bleed/MS column, 0.25 mm film thickness and 0.25 mm internal diameter capillary column.
6. Flame ionization detector-The detector and injector temperatures shall be set at 360°C and 300°C, respectively. The flow rate for hydrogen is 40 ml/min, for air is 450 ml/min and the makeup flow of Nitrogen is 45 ml/min.

Procedure:

1. Before starting up the instrument, open the main valve on the gas cylinders Hydrogen, Nitrogen and Oxygen) to check that carrier gas (Nitrgen) is being supplied to the gas chromatograph.
2. Turn on the GC and then the computer.
3. Make sure the GC has the correct column, and the injector is clean.
4. If necessary clean the column and injector with hexane and run a cooldown method using steps 11-15.
5. Place the GC vial (Ssilyation step 9)in the auto-sampler.
6. Run the GC software (Instrument 1) from the desktop.
7. Go to the *Sequence* and select the *Sequence parameter*. A new window will appear.
8. Locate the *Operator* and type in (your) username and date.

9. Locate the sequence details and type in all information about the sample (type of sample, method used to prepare sample, date prepared, date sample in run in GC, if there were any errors when preparing the sample). Be sure to be as specific as possible. Select *ok.*
10. Go to *Sequence* and select the *Squence Table*. A new window will appear.
11. Locate the position in which your sample is located in the auto-sampler and insert vial location in append line.
12. Type in the name of the sample and related information in the comment box.
13. Select a mwthod to use. Enter the number of injections of your sample. This represent the number of times the sample will run for each sample.
14. Next select Append. A new row will appear. Repeat steps 10-13 as needed.
15. Wait until the instrument shows green signal (Ready). Press start button in the upper left corner of the screen.
16. Perform analysis.
17. When shutting down the instrument, perform Shutdown by selecting cooldown method from *Load method.*
18. Once the machine is cooldown, turn off the GC software and GC. Close the main valve on the gas cylinders.