Extraction of Lipid Soluble Antioxidants from Rosemary Leaves Using Vegetable Oils

Thesis

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Abstract

Antioxidants are bioactive chemical compounds shown to prevent and prolongate lipid oxidation. Both water – and lipid – soluble antioxidants are available synthetically and naturally. Because lipid – soluble antioxidants are needed to protect lipid – containing foods from deteriorating, a new technique for obtaining these compounds is necessary. Synthetic antioxidants are commonly used in food products; however, consumers do not want these lab - made chemicals in their foods. They want less processed, natural ingredients (Caleja et al. 2016). Therefore, the objective of this research is to extract the natural lipid - soluble antioxidants from rosemary leaves without the use of chemical solvents. Vegetable oils were used as the solvent for the extraction. Three different vegetable oils (high oleic soybean oil (HOSO), peanut oil (PO), and cottonseed oil (CO)) were selected based on researching different oils and concluding these were satisfactory. Both fresh and dried rosemary leaves were ground into fine particles, added to the three heated vegetable oils (in 20 – 40% w/w), mixed and heated for 1 - 3 hours. As a control, a solvent extraction using ethanol was performed as well. The solutions containing the rosemary antioxidant extraction (RA) were filtered from the rosemary particles and analyzed using high performance liquid chromatography (HPLC). HPLC analysis confirmed the extraction of carnosic acid and carnosol (the two major lipid-soluble antioxidants found in rosemary leaves) in all three vegetable oil solutions.

There was more carnosic acid found in the extraction from HOSO compared to the other two vegetable oils. However, all three extracts contained similar amounts of carnosol. Comparing the vegetable oil extractions to ethanol showed a higher level of carnosic acid in the vegetable oil solutions. To test the extracted rosemary antioxidants' efficacy, 2% (w/w) of the solutions were added to soybean oil (SO) and stored at 60°C for 22 days. After 22 days of storage the peroxide value (PV) and anisidine value (AV) were significantly lower in the soybean oil containing the obtained antioxidant solutions compared to the pure soybean oil. Therefore, vegetable oils are a suitable alternative to use as a solvent when extracting lipid – soluble antioxidants from rosemary leaves instead of using ethanol, a common chemical solvent used for extraction of antioxidants from rosemary leaves.

Dedication

This work is dedicated to my grandfather, Professor Gilbert Ginsburg, who always taught me to never give up on my dreams, work hard, and have a smile on my face. I love you and miss you Grandpa.

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Chapter 1. Introduction

Antioxidants are molecules that interfere with free radicals attacking more stable molecules. Antioxidants come in two forms, water - and lipid – soluble. Both are important in working in different parts of the body to protect healthy cells and organs. Antioxidants also help in delaying lipid oxidation from occurring in food systems. Lipid – soluble antioxidants are more effective in protecting fat - containing foods since they react with the fat better than water – soluble antioxidants. Lipid oxidation occurs when a hydrogen atom is abstracted from a fatty acid, forming a free radical. Once this free radical is formed, atmospheric oxygen reacts with the radical, producing a peroxy radical (Choe et al. 2005). This peroxy radical continues to react with other lipids and compounds found in the system, forming hydrogen peroxides, ketones, epoxides, aldehydes and other compounds. Lipid oxidation products, primarily secondary oxidation products, cause the food to go rancid, giving an off flavor and forming toxic compounds. Antioxidants react with the free radical before it can react with the fatty acid, quenching oxygen, and/or chelating metal ions (Riesche et al. 2008). Over 90% of food contain lipids, and it is essential that these molecules are preserved and protected (Yanishlieva-Maslarova et al 2001). Furthermore, the USDA recommends that less than 10% of a person's calories per day should come from solid, saturated fats (USDA 2015). Therefore, there is a need to replace saturated fats with liquid, unsaturated fats.

Unsaturated fats are more susceptible to lipid oxidation than saturated fats. They have double bonds with pi bonding that contain high electron density which allow for an easier abstraction of hydrogen atoms compared to hydrogens that are surrounded by all sigma bonds (Choe et al. 2005). Therefore, there is a need for more lipid – soluble antioxidants. Synthetic antioxidants such as butylated hydroxy anisole (BHA) and butyl hydroxytoluene (BHT), are lipid – soluble antioxidants that are commonly used in food products. However, many consumers do not want these lab - made chemicals in their foods. They want less processed, natural ingredients (Caleja et al. 2016). Consumers want to be able to pronounce, recognize, and not be worried about the additives in their food. Rosemary is an herb rich in lipid-soluble antioxidants, mainly carnosic acid. Carnosic acid has been shown to be an effective antioxidant, more effective than BHA and BHT (Birtic et al 2015). Furthermore, rosemary is cultivated and grown in the USA (USDA – NRCS 2019) and can be grown in many different areas, therefore there is not a limited abundance when needed. The common technique used to extract carnosic acid and the other antioxidants found in rosemary leaves involves the use of chemical solvents. Even though chemically extracted antioxidants undergo further treatment to remove the chemicals, often times there are residuals left in the antioxidant extraction (Tena et al. 1997). The cost of using chemicals to extract lipid – soluble antioxidants is high. The chemicals that are used need to be removed which is time consuming and costly. Once the solvent is removed it can no longer be used for another purpose and is lost since the common technique of removal is through evaporation of the solvent. The extra rosemary particles that were used for the extraction can no longer be used and must be discarded

since they are contaminated with chemical solvents. To avoid these concerns, a natural extraction process is preferable. Since many of the antioxidants found in rosemary leaves are lipophilic, a lipid source can be effective in extracting the antioxidants. Vegetable oils are therefore a good solution to extracting antioxidants from rosemary leaves.

Furthermore, consumers are familiar with vegetable oils and do not view them as a dangerous additive. Therefore, the objective of this research is to extract the lipid - soluble antioxidants from rosemary leaves with vegetable oils and without the use of chemical aids.

Chapter 2. Literature Review

1. Lipids and Lipid Oxidation

Fats and oils are made up of chemical compounds called lipids. Lipids are biological molecules soluble in nonpolar solvents (Vollhardt 2014). They are macromolecules composed of fatty acids and their derivatives. A subcategory of lipids are edible oils and fats which are mostly made up of triacylglycerols (Hu 2016). Triacylglycerols are compounds with structures of a glycerol backbone and three fatty acids connected to the backbone through esters. Fatty acids can be saturated, a carbon chain with all sigma bonds, or unsaturated, a carbon chain with at least one pi bond (Lawrence 2010). Fatty acids containing one pi bond are monounsaturated fatty acids (MUFAs), and those with two or more degrees of unsaturation are polyunsaturated fatty acids (PUFAs). Lipids are a source of energy, essential fatty acids, lipid-soluble vitamins and other lipid soluble compounds (Shahidi et 2006). Once fat is consumed, it is digested and metabolized into carbon dioxide and water. This metabolic process provides the body with energy, at a rate of 9 kcal/g (Lands 2005). Essential fatty acids are fatty acids that the body cannot synthesize from carbohydrates and proteins. Furthermore, essential fatty acids must be consumed from one's diet. Food benefits from lipids' flavor, texture, and mouthfeel (Shahidi et 2006). Therefore, lipids are essential to many food products such as pastries, confections, chocolates, and more.

A main concern when it comes to lipids is their stability against lipid oxidation. Lipids become undesirable and rancid through a process called lipid oxidation. Lipid oxidation causes the formation of hydroperoxides and its derivatives. Unsaturated, free fatty acids have a greater affinity to be oxidized than triacylglycerols and saturated fatty acids. Based on oxygen uptake, the relative autoxidation rate of oleic (C18:1), linoleic (C18:2) and linolenic acid (C18:3) is 1:40-50:100 (Choe et al 2005). As the level of unsaturation increases, the rate at which the fatty acid oxidizes increases due to the increase of allylic hydrogens available and electron density increase from the pi bonds. Overall, two types of lipid oxidation can occur: autoxidation and photosensitized oxidation (Choe and Min 2006).

1.1. Autoxidation

Autoxidation is a three-step process of initiation, propagation, and termination. Autoxidation requires a free radical to form from a hydrogen abstraction in the initiation step. Removing a hydrogen from a fatty acid is an unfavorable reaction; however, hydrogens located between two double bonds, allylic hydrogens, require less energy. Choe et al (2005) found that for linoleic acid (C18:2), the hydrogen located on C11 requires 50kcal/mol for removal, while the hydrogens at C8 and C14 require 75kcal/mol and the hydrogens bonded to C17 and 18 require 100kcal/mol for homolytic dissociation. Since the double bonds are located between C9 and C10, and C12 and C13, there is a high density of electron from the pi-bonds at C11, making the hydrogen abstraction a more favorable reaction than from other carbons. The free radical formed from the hydrogen abstraction reacts with an atmospheric triple oxygen ($^3\text{O}_2$) to form a peroxy radical. The peroxy radical reacts with a hydrogen from another lipid molecule, to form a

hydroperoxide and another radical lipid molecule. During this propagation step the oxidation continues, causing a chain reaction of more peroxides and other derivatives to form (Schaich 2013). Linoleic and linolenic acid form only conjugated products when undergoing autoxidation (Choe et al 2005). Finally, in the termination step, free radicals react with each other to leave no free radicals left in the oil or fat.

1.2. Photosensitized Oxidation

Unlike autoxidation which involves atmospheric oxygen ($^3\text{O}_2$), photosensitized oxidation is caused by a singlet oxygen ($^1\text{O}_2$) formation (Choe and Min 2006). The singlet oxygen reacts with unsaturated fatty acids directly and forms allylic hydroperoxides in the trans form (Frankel 1980). A free radical formation is not necessary for this peroxidation reaction to occur; however, singlet oxygen must be produced to start the reaction. Singlet oxygen can be produced by many mechanisms; however, the most prevalent is through exposure to light in the presence of a photosensitizer (Frankel 1980). Both conjugated and non-conjugated hydroperoxides are produced from this reaction because double bond migration and trans fatty acid occurs. In autoxidation, only conjugated hydroperoxides are formed.

1.3. Problems that Stem from Lipid Oxidation

Lipid oxidation causes unwanted reactions that result in disease and undesirable products. In the body, these reactions can lead to oxidative stress, causing the destruction of cells and DNA. This DNA destruction can cause cancer (Lawrence 2005). In food, the oxidation by-products cause off-flavors, nutritional deterioration, and toxicity. Secondary oxidation occurs when the hydroperoxides being formed from primary oxidation mainly deteriorate into aldehydes, ketones, alcohols, epoxides, and short chain

hydrocarbons. Aldehyde formation from lipid oxidation of unsaturated fatty acids in beer have been correlated with a stale flavor that is detected when beer oxidizes (Zhao et al 2006). Both hydroperoxides from autoxidation and photosensitized oxidation decompose and produce the same products with the same mechanisms (Choe and Min 2006). Consumption of secondary oxidation products such as ketones, aldehydes, and epoxides, has shown to be damaging to the liver (Kubow 1992). Lipid oxidation in fatty acids causes the undesirable odor and flavor in food commonly known as rancidity (Campo et al. 2006). Once food has gone rancid due to oxidation, the off-odor and flavor overpowers the food's natural odor and flavor, causing an aversion to the oxidized product. For these reasons, lipid-containing foods need help to prolong their shelf life by delaying the onset and hindering lipid oxidation.

2. Antioxidants

2.1. Preventing Lipid Oxidation

The main goal of antioxidants in food is to help in preserving the food and increase the foods' shelf life. Antioxidants are molecules that inhibit lipid oxidation from occurring. They come in many different forms and use different mechanisms to be effective. Antioxidants help stop the propagation of lipid oxidation (Lobo et al 2010). These compounds can be water or lipid soluble. Water-soluble antioxidants help chelate metal ions in aqueous food emulsions (Yanishlieva-Maslarova 2001). Most antioxidants are added to protect food from going rancid and changing colors due to lipid oxidation; therefore, the focus of this paper is lipid-soluble antioxidants.

2.2. Antioxidant Categories: Primary vs. Secondary

Antioxidant categories are based on the mechanism the antioxidant uses to prevent lipid oxidation. There are two antioxidant categories: primary and secondary. Primary antioxidants accept free radicals which delays the oxidation process by interrupting the propagation step of oxidation. Secondary antioxidants delay oxidation through many different mechanisms such as chelating metal ions, deactivating singlet oxygen, decomposing hydroperoxides to nonradical species, and replenishing and/or regenerating primary antioxidants and others (Riesche et al. 2008). Secondary antioxidants work synergistically with primary antioxidants to promote their antioxidant activity, but secondary antioxidants do not convert free radicals to stable compounds themselves (Riesche et al. 2008). Primary antioxidants will be the focus since they protect lipids against atmospheric oxygen and free radicals and are therefore the first defense mechanism in lipids. Primary antioxidants retard autoxidation by scavenging free radicals, breaking the oxidation chain reaction, and thus preventing lipid peroxides and their degradation compounds to form (Elias and Decker 2017). They are the most effective antioxidants since they interrupt the free radical chain reaction by donating a hydrogen atom to the free radical and accepting the free radical. While interrupting the chain oxidation reaction, the primary antioxidant becomes a radical and is stabilized through resonance delocalization of the electron. Antioxidants interrupt the radical from attacking a stable molecule because their reduction potential is lower than the free radical's reduction potential (Choe et al 2005). The primary antioxidant forms quinone structures after intercepting the free radical from attacking the lipid. This formed radical intermediate cannot be attacked by a molecular oxygen and therefore is less susceptible

to oxidation (Brewer et al. 2011). Furthermore, primary antioxidants are beneficial to add to lipid containing food products to protect the products, and per consumer demand, natural antioxidants are better to use in lipid containing food products.

2.3. Antioxidant Origins: Natural vs. Synthetic

There are two categorical sources of antioxidants: synthetic and natural. Natural antioxidants can be found in many different plant types. Synthetic antioxidants are man-made chemicals, synthesized in the lab. Examples of synthetic antioxidants commonly found in food, medical, and cosmetic products are tertiary-butyl-hydroquinone (TBHQ), butylated hydroxy anisole (BHA), and butylated hydroxytoluene (BHT) (Lobo et al. 2010). BHA and BHT are synthetically derived from petroleum and work very efficiently as antioxidants (Larson et al. 1997). In small amounts, these compounds are efficient and may even help to prevent cancer; however, in large doses BHA, BHT, and TBHQ have toxic effects (Papas 1999). Since high levels of synthetic antioxidants have been shown to be toxic, consumers are interested in finding antioxidants from natural sources on the label rather than synthetic antioxidants; even if the same dangers occur from high levels of antioxidants from natural sources, consumers are more accepting of naturally occurring compounds. Furthermore, many consumers who research and discover BHA and BHT are derived from petroleum do not like to imagine they are consuming gasoline. Consumers want their foods to have cleaner labels with natural ingredients and additives (Schevey et al. 2015). Furthermore, consumers do not like to see chemical sounding, unpronounceable names on labels; they like to see ingredients they can recognize and picture (Ashemann-Witzel et al 2019).

Phenolic compounds such as flavonoids, tocopherols, and phenolic acids are natural, effective chain-breaking antioxidants and have a better reputation than synthetic antioxidants (Riesche et al. 2008). Flavonoids are secondary metabolites from plant metabolism, and phenolic acids are precursors for flavonoid biosynthesis. Both can be extracted from plant material and serve as antioxidants in fats and oils. Some natural, plant-derived antioxidants, such as carnosic acid, has shown to protect lipid-containing foods, such as meat patties, and oil, more efficiently than synthetic antioxidants (Loussouarn et al. 2017). When tested in tara seed oil, the addition of carnosic acid decreased the hydroperoxide and malondialdehyde, primary and secondary oxidation product, formation over the course of 15 days significantly more than the addition of BHA and BHT (Li et al. 2018).

2.4. Natural Antioxidants

2.4.1. Benefits of Natural Antioxidant

Antioxidants are essential for plants' growth, reproduction, and defense system (Ghasemzadeh et al. 2011). Along with being crucial to the plants themselves, antioxidants are also beneficial in medicine, cosmetics, and food. Studies have shown that natural antioxidants can delay and even prevent the onset of neurodegenerative disease caused by oxidative stress such as Alzheimer's and Parkinson's disease (Zhao 2009). Phenolic compounds have a relatively low standard electron potential compared to free radicals; therefore, the polyphenols will donate an electron, scavenge the free radical, and stabilize itself with resonance (Fraga et al. 2010). This reaction helps to delay peroxide formation which helps to prevent disease, lipid oxidation, and rancidity.

2.4.2. Sources of Natural Antioxidants

Fruits, vegetables, legumes, nuts, oilseeds, cereals and herbs all contain natural antioxidant phenolic compounds such as phenolic acids and flavonoids that help them survive. Plant tissues have developed antioxidant systems to protect themselves against the oxidative stress they endure (Brewer 2011). When undergoing severe stress, plants' antioxidant enzymes are activated causing an increase in antioxidant biosynthesis (Agati, et al. 2012). Plants contain four major phenolic types: phenolic acids, phenolic diterpenes, flavonoids, and volatile oils. These compounds act as primary and secondary antioxidants by trapping free radicals, scavenging free radicals, and chelating metal ions. Herbs have been shown to produce antioxidant compounds as secondary metabolites. Among various herbs, such as thyme, sage, basil, rosemary, and dill, rosemary contains a greater antioxidant content in their leaves (Paur et al 2011).

2.4.2.1. Rosemary's Antioxidants

Among various herbs, rosemary contains many water- and lipid-soluble phenolic compounds. From the lipid-soluble antioxidants, the most abundant phenolic diterpene in rosemary leaves is carnosic acid (Wellwood 2004). Depending on the variety, environmental conditions, and stage of growth the rosemary leaves have gone through, they contain 3-50 mg/g dried weight of carnosic acid. Carnosic acid is found mostly in rosemary's leaves, particularly in the chloroplasts (Munne-Bosch et al. 2001). Carnosic acid is synthesized through a non-mevalonate isopentenyl diphosphate pathway. Pyruvate and glyceraldehyde 3-phosphate form the isopentenyl diphosphate providing 1-deoxy-D-xylose-5-phosphate. Phenyltransferases operate to produce geranylgeranyl pyrophosphate (C₂₀). C₂₀ goes through a series of internal addition to form copalyl pyrophosphate which

generates carnosic acid (Munné-Bosch et al. 2001). Carnosic acid, $C_{20}H_{27}O_4$, has an aldehyde group at C10 and two hydroxy groups in the ortho positions at C₁₁ and C₁₂, which has a significant impact on its ability to act as an antioxidant (Britic et al. 2015). Both hydroxy groups on C₁₁ and C₁₂ readily donate their hydrogen atom to interrupt the oxidation chain reaction. Furthermore, once carnosic acid is consumed and works to protect lipids from oxidation, carnosic acid degrades into carnosol (Zhang et al. 2012). Carnosol is similar in structure to carnosic acid, however the aldehyde group is replaced with an ester bonded to C7. Johnson (2011) found promising results showing carnosol targets tumorigenic cells, reduces them, and therefore have anti-cancer capabilities.

Other major, volatile components of rosemary leaves that contribute to rosemary's antioxidant capabilities are α -pinene, borneol, camphor, and 1,8-cineole (Singletary 2016). Borneol, a bicyclic organic compound, has been shown to prevent DNA damage from oxidative stress (Horvathova et al. 2012). Borneol contains a hydroxy group in the endo position of the cyclohexyl ring. α -pinene is an organic terpene that has been shown to have properties to be effective as an antiphotaging compound since it provides photoprotection against the free radicals induced by ultraviolet radiation from the sun (Karthikeyan et al 2019). α -pinene is an alkene containing a reactive four membered ring. 1,8-cineole, a monoterpene, has been shown to lower inflammation of umbilical vein endothelial cells through mediating NF-kB expression and maintains the balance of pro- and anti-inflammatory cytokines thus reducing the LPS-induced vascular endothelium injury (Linghu et al. 2019). The properties of carnosic acid mentioned above, along with the other phenolic and aromatic compounds found in rosemary work

synergistically to make it a good natural antioxidant source, and beneficial for human consumption.

3. *Extracting Natural Antioxidants from Herbs*

3.1. *Solvent and Distillation Extractions*

Many extraction methods have been developed to extract antioxidants from herbs. Organic solvents are commonly used to extract phenolic antioxidants from herbs such as rosemary leaves. Solvents have a range in polarity and therefore can be used to separate the lipophilic compounds biosynthesized from herbs. An accelerated solvent extractor (ASE), at a temperature of 129°C and 56% methanol, 44% water (v/v), was found to be optimal in extracting phenolic diterpenes from rosemary leaves, 10.986 and 5.647 mg/g carnosic acid and carnosol, respectively (Hossain et al. 2011). Using a Soxhlet extraction process with methanol as the solvent, Erkan et al (2008) extracted 6% carnosic acid from rosemary leaves. Delgado et al. (2014) performed two Soxhlet extractions with 200 ml of methanol or *n*-hexanes, and 10 g of rosemary leaves using a nine-cycle extraction. The mixture was filtered and evaporated under vacuum at 40°C. The *n*-hexane extracted 95.1 and 6.81 mg/100g extract carnosic acid and carnosol respectively, while the methanol extracted 59.3 and 7.05 mg/100g extract. Methanol was used in the three extractions mentioned, and while Hossain et al. (2011) and Erkan et al. (2008) found it to be successful, Delgado et al. (2014) found that using *n*-hexane is more effective in extracting carnosic acid. Along with the extraneous, nine-cycle extraction method, Delgado et al (2014) performed a direct extraction by combining 50:50 v/v methanol: water with 10 g of ground rosemary, leaving it to sit at room temperature for 8 hours, filtering and centrifuging the extract. This 50:50 mixture, direct extraction method

extracted 9.34 and 1.10 mg/100g extract of carnosic acid and carnosol respectively. The direct extraction method was much longer and extracted ten times less carnosic acid than the Soxhlet extraction method. Bicchi et al. (2000) used acetone as a solvent and sonicated 10 g of rosemary leaves in 100 ml acetone for 20 minutes in an ultrasonic bath to extract the antioxidants. This sonication method was successful in extracting $0.20 \pm 0.04\%$ carnosic acid and $0.015 \pm 0.004\%$ carnosol from dried rosemary leaves. Tena et al. (1997) used sonication with acetone, methanol, hexane and dichloromethane to extract antioxidants from rosemary leaves and found they extracted 26.2 ± 1.5 , 15.9 ± 1.3 , 1.9 ± 0.08 , and 7.9 ± 1.1 mg/g carnosic acid, respectively. Bicchi et al. (2000) and Tena et al. (1997) used acetone as the extraction solvent and found with assistance from ultrasonic and bath the solvent is successful in extracting the phenolic diterpenes from rosemary leaves. Rodríguez-Rojo et al. (2012) found that de-oiling, milling, and using a microwave-assisted extraction process with 96% ethanol, 4% water (v/v) maximized the amount of carnosic acid extracted, 2.46 ± 0.06 mg/mL, from rosemary leaves. All methods mentioned were successful in extracting carnosic acid from rosemary leaves. Overall, methanol is a common solvent to use for the extraction process, and including assistance from sonication, heat or microwaves improves the extraction efficacy. Solvents commonly used, such as methanol and acetone, can leave behind residues that are toxic and thus prohibited in food products (López-Sebastián et al. 1998). Therefore, including chemical solvents in the extraction process is undesirable for labeling purposes, contamination problems, and consumer perception.

3.2. Supercritical Fluid Extraction

A different approach to extract antioxidants from rosemary is to use supercritical carbon dioxide (SC-CO₂). SC-CO₂ uses pressure, temperature, and the flow rate of the CO₂ to extract the antioxidants from rosemary (Zermane et al. 2010). Using a supercritical fluid for the extraction (SFE) eliminates the need to remove a toxic and unwanted solvent. CO₂ is commonly used in its supercritical fluid form since it is nontoxic, nonflammable, and can easily be separated from the solutes extracted (Tena et al 1997). Supercritical fluid extraction gives high selectivity during the extraction process and therefore compounds of interest can be obtained with a reduced potential of undergoing oxidation (Baysal, T. et al 2000). When a solvent is used to extract antioxidants, oxidation occurs which degrades the antioxidants. When rosemary's antioxidants are extracted with solvents the carnosic acid degrades into carnosol. Performing an SFE does not cause oxidation to occur since it uses an inert gas (Bicchi et al. 2000). Tena et al (1997) found that using Supercritical CO₂ extracted 1.3 times the amount of carnosic acid, 35.7 mg/g, compared to performing an ultrasound assisted solvent extraction. However, the apparatus for a supercritical extraction method is large and expensive. Using SFE is a good alternative to performing solvent extractions, but it still has its drawbacks such as the price of the instrumentation. Therefore, an extraction of antioxidants without the use of harsh chemicals, and a more affordable option is needed. Furthermore, the extraction efficacy must be investigated to determine which method of extraction is best.

4. Methods to Identify Antioxidants' Chemical Composition

4.1. Gas Chromatography-Mass Spectroscopy

4.1.1. Different Parameters and What was Detected

Analyzing volatile antioxidants can be done using Gas Chromatography paired with a Mass Spectrometer (GC-MS). Moreover, the antioxidants extracted from herbs can be identified and quantified with GC-MS. Different heating, ramping, and timing parameters have been used for successful composition identification. Employing different oven parameters can affect how well the peaks are separated and therefore affect the ability to clearly identify and quantify the compounds found in the sample. Al-Owaisi (2014) identified tannins, glycosides, alkaloids, flavonoids, and steroids extracted from *Moringa peregrine* leaves by starting at an oven temperature of 60°C and ramping it to 280°C with a run time of 75 minutes. Using a run time of 73.3 minutes, Qadir (2014) identified smaller antioxidants such as, α -pinene, borneol, and 1,8-cineole, from *Artemisia amygdalina*'s essential oil using similar parameters to Al-Owaisi (2014) in rosemary extract. Kathirvel (2016) was able to detect vital phytochemicals essential to the antioxidant activity from *Dryopteris cochleata* leaves by starting at a temperature of 110°C and reaching 280°C for a total run time of 36 minutes. Daferera (2000) identified small antioxidant compounds such as α -pinene, borneol, and cineole from rosemary leaves, with the cineole being the most abundant by initially holding the oven temperature at 60°C and ramping up to 250°C for a total run time of 35.3 minutes. Tohidi (2017) used the GC-MS to identify the volatile compounds found in Thyme leaves' essential oil by setting the oven temperature at 60°C and ramping it to 260°C for a total run time of 54 minutes. These methods mentioned were used to identify smaller, volatile,

bioactive compounds. Most methods started at 60°C and ramped to over 280°C to ensure the volatile compounds all eluted and were well separated from each other.

4.1.2. Derivatization to Detect Less and Non-Volatile Antioxidants

GC is used to separate, identify, and quantify volatile molecules. However, rosemary contains both volatile and non-volatile antioxidants. Therefore, to completely analyze the antioxidants, volatile and non-volatile, found in rosemary extract, derivatization of the compounds can be performed. Derivatization chemically alters the compounds to improve their chromatography. Silylating the extract reacts an active hydrogen with a silyl group to make the non-polar compounds relatively more polar, and therefore be more volatile and be able to pass through the GC (Kyle 2017). Protic functional groups, such as -OH and -NH, bonded to the compound are converted to silyl ethers or esters (Segura et al. 1998). Common reagents used for silylation include Trimethylsilyl (TMS) derivatives, *N,O*-bis(trimethylsilyl) trifluoroacetamide (BSTFA) and *N*-methyl-*N*-trimethylsilyl-trifluoroacetamide (MSTFA). Zhang (2004) and Proestos (2013) added a mixture of trimethylchlorosilane (TMCS) and *N,O*-bis(trimethylsilyl) trifluoroacetamide (BSTFA) to the extracts, heated them, and then ran it through the GC-MS. This method allows for the detection of the larger, non-volatile phenolic compounds. However, the reagents used for derivatization can react with chemicals lining the column and thus inactivates the column (Kyle 2017). The beginning of the column near the inlet where the sample is being injected is affected first and therefore the column may require trimming more often. For this reason, derivatization should be used with caution and awareness of the inactivation of the column faster than non-derivatized samples.

4.1.3. Dynamic Headspace Method

When analyzing volatile compounds entrapped in a non-volatile medium such as oil, the volatiles should be desorbed out of the medium before injecting in the GC. Dynamic headspace (DHS) distinguishes between the non-volatile medium and volatile vapor phase by partitioning the vapor phase analytes above the medium it is in. The vapor phase is then transferred to the GC for analysis (Snow and Slack 2002). DHS uses an inert gas to extract the volatile compounds out of the medium (Kolb 1999). Incubating the samples with heat, usually 40-60°C, is used to disperse the volatile analytes equally between the medium and the gaseous headspace of the vial (Kyle 2017). Once the volatile compounds are trapped in the gaseous headspace, they get desorbed through thermal processing and get carried and transferred to the column by a carrier gas. DHS is a continuous technique that accounts for the medium's matrix and over time the volatile compounds will be captured and can be transferred to be analyzed (Wojnowski et al. 2017). DHS is a green way to analyze samples since there is no need for solvents to be used. Furthermore, DHS systems are made to be all-inclusive with the trapping and purging of the volatile compounds occurring automatically in the system.

4.2. Selected Ion Flow Tube -Mass Spectrometry

GC-MS has been the accepted, most often used technique to identify and quantify volatile compounds. However, sample preparation, column choices, and extraction techniques could add bias to the chromatographic and spectral results (Vendel et al. 2019). Different GC columns are made with chemicals varying in polarity. Therefore, compounds that are similar in polarity to the column's polarity will have more of an affinity to react and be displayed on the chromatogram. However, more recently, techniques have been developed that utilize soft chemical ionization with direct mass

spectrometry (Vendel et al 2019). One of these newer techniques is Selected Ion Flow Tube Mass Spectrometry (SIFT-MS). SIFT-MS uses positive ions, mainly NO^+ , H_3O^+ , and O_2^+ , that are created in an ion source to be injected into a fast-flowing inert carrier gas (N_2 or He) through a Venturi-type tube opening (Vendel et al 2019, Smith and Španel 2005). After the ions have flowed through the tube they flow through a differentially-pumped quadrupole mass spectrometer, where they are analyzed and detected by a channeltron multiplier/pulse counting system (Smith and Španel 2005). SIFT-MS analysis allows for a real-time quantification of volatiles injected since it monitors the precursor and product ion count rates as they move downstream towards the mass spectrometer detection system (Smith and Španel 2005). Furthermore, food products can be analyzed with SIFT-MS by placing the food directly at the entry port with no sample preparation necessary.

4.3. Liquid Chromatography

To detect larger, non-volatile phenolic compounds, such as carnosic acid, liquid chromatography (LC) may be used. Roby (2013) identified small and large terpenoid and flavonoid antioxidants found in Thyme, Sage, and Marjoram leaves that are also present in rosemary leaves by using high performance liquid chromatography (HPLC) paired with a UV detector at 280nm. Jayasinghe (2003) also used HPLC paired with a pulsed amperometric detector system (PAD) to identify and quantify small and large phenolic compounds found in his Basil leaves samples between 200-400nm. Rosmarinic acid was found to be the most abundant in Basil leaves, whereas little carnosic acid was detected, and no carnosol was detected. Tronsoso et al (2005) detected carnosic acid, carnosol, and rosmarinic acid from fresh rosemary leaves using HPLC and UV-vis spectrophotometer

at absorbances of 230, 270, 280 and 330nm; clear, well-separated chromatograms were seen at 230 and 330nm. Señoráns (2000) used LC-MS paired with diode array detection system (DAD) to identify and quantify the major antioxidants in Rosemary leaves such as carnosic acid and carnosol. Acetonitrile or methanol and acidified water were used as the solvents for the mobile phases and a reverse phase C18 column was used in all methods to detect and quantify the carnosic acid and carnosol in extracts. These methods can detect carnosic acid, but for HPLC to be used as an analysis method, the extract has to be in an aqueous, or organic solvent, not in lipids. Therefore, to analyze the carnosic acid and carnosol found in an oil-based Rosemary antioxidant extract, a solvent extraction technique must be employed before running the sample through the HPLC.

4.4. Nuclear Magnetic Resonance

Nuclear Magnetic Resonance (NMR) is a technique that can be utilized to identify phenolic compounds found in Rosemary leaf extract. NMR uses probes to irradiate atomic nuclei with magnetic fields and radio frequencies (Blumich 2003). Common nuclei that are used for NMR identification are Hydrogen, proton, and Carbon. Xiao et al. (2008) used proton NMR to identify the various secondary metabolites such as Carnosic Acid and Carnosol found in solvent extracted Rosemary antioxidants. Tawfeeq et al. (2016) examined Rosemary samples with proton NMR and identified smaller, volatile, aromatic antioxidants such as Camphor, 1,8-Cineole, and α -Pinene in Rosemary's composition. However, lipids have many hydrogens and carbons, therefore the proton and carbon NMR spectra of a combination of lipids and antioxidants is oversaturated with the lipids, and the phenolic compounds cannot be distinguished. Oversaturating the system with protons and carbons produces spectra with peaks from the lipids that overlay

the peaks from the antioxidants. Phosphorous-31 (^{31}P) NMR is another approach to consider when identifying phenolic compounds. ^{31}P is 100% abundant, and oxygen-phosphorous (O-P) bonds are formed from active hydrogens in a hydroxy (-OH) functional group (Verkade 1994). Therefore, reacting the antioxidants found in rosemary with phosphorous creates an O-P bond that can be detected by ^{31}P NMR, and can lead to the identification and quantifications of the phenolic compounds found in Rosemary leaf extract.

4.5. Comparison of Identification Methods

GC-MS requires the compounds being looked at to be volatile since it is going through the column as a gas and is separated based on volatility and interaction with the stationary phase. However, if the compounds being looked at are nonvolatile, derivatization can be done to modify the compounds making them suitable to pass through the GC (Grob 2004). Once the compounds pass through the GC, they go to the MS where they get ionized and fragmented. This fragmentation produces a spectrum of fragmentations and relative intensity peaks based on the mass to charge ratios (m/z) (Masucci et al. 2004). These fragmentation patterns and a well-established library is used to identify the compounds sent through the column. SIFT-MS uses chemical ionization to fragment and pass the volatile compounds to the mass spectrometer where they are analyzed (Vendel et al 2019). LC passes the compounds through a column and separates them based on the compounds' polarities (Anesi et al. 2015). This analytical technique is useful in getting relatively fast separation of compounds and then sending them to a detector to identify the compound. NMR applies a magnetic field to the sample being analyzed. This magnetic field causes NMR magnetic nuclei to spin with or against the

field. The spinning, along with nuclei coupling creates a spectrum that is used to identify the compound (Vollhardt 2014). NMR is a useful spectroscopy tool that can be used to identify antioxidants based on their nuclei's spin states.

5. Methods for Evaluating Antioxidants' Efficacy

Direct methods of measuring the antioxidant efficacy of extractions include adding the extract to food and conducting a storage study. Primary and secondary oxidation products formed over time can be measured using peroxide value (PV) and anisidine value (AV) respectively. PV quantifies the amount of hydroperoxide - the first compounds to form from oxidation degradation of lipids - formed in the sample. Peroxides are relatively stable at room temperature, however over time and under unfavorable conditions they degrade into aldehydes, ketones, epoxides and other volatile compounds (Barriuso and Astriasaràn. 2013). AV quantifies the amount of secondary oxidation degradation products such as toxic aldehydes and ketones, formed from the primary oxidation products (Gotoh et al. 2011). PV is measured with titration techniques, and AV is measured with spectrophotometric techniques. These analysis methods test the extract's efficacy in delaying lipid oxidation in foods.

6. Goals in Researching Rosemary

As discussed in Section 2.4.3, rosemary leaves are rich in water - and lipid – soluble antioxidants. Many of the antioxidants such as carnosol, α -pinene, 1,8-cineole, and more have been shown to have therapeutic and medicinal properties (Johnson 2011, Karthikeyan et al 2019, Linghu et al. 2019). Along with being important in sustaining a healthy body, the antioxidants found in rosemary leaves are effective in delaying lipid oxidation. Carnosic acid, the most abundant lipid – soluble antioxidant found in rosemary

leaves has been shown to be more effective in preventing lipid oxidation than synthetic antioxidants, BHA and BHT (Britic et al. 2015). Furthermore, rosemary is currently being cultivated in five states, California, Texas, North Carolina, South Carolina, and Oregon, in the United States of America (USDA-NRCS 2019). There is a great amount of rosemary being grown within the country that allows for fresh, home -grown materials to be used instead of importing them.

Consumer demands is leading for a push towards “clean label” (Aschemann-Witzel et al. 2019). When looking at labels, consumers want to read “all-natural ingredients,” “organic,” and “free from” ingredients that are known to be common allergens or additives that have negative associations such as BHA and BHT. Clean label foods are expected to be transparent; the consumer is able to read and picture every ingredient listed and know they will not be harmed when consuming the product.

For the extraction method developed here, vegetable oil will be used as the solvent. Carnosic acid, carnosol and other antioxidants found in rosemary are lipophilic (Britic et al 2015). This lipid-soluble characteristic of the antioxidants indicates that oil would be a good solvent to extract the antioxidants with. Consumers know what vegetable oil is when reading the ingredient list, and therefore are not concerned when consuming it. Therefore, choosing to use vegetable oil as a solvent is a good alternative to using organic solvents that can be expensive and toxic, and provides a clean label.

Chapter 3: Method Development for Rosemary Antioxidant Extractions and its Analysis

1. Selection of Vegetable Oils used for Extraction of Lipid-Soluble Antioxidants from Rosemary Leaves

Rosemary leaves are rich in both water - soluble and lipid - soluble antioxidants. Lipid - soluble antioxidants, such as carnosic acid, are more effective in protecting lipids from undergoing lipid oxidation compared to water – soluble antioxidants. Carnosic acid and carnosol have been shown to be the most potent and powerful antioxidants found in rosemary leaves. They are more effective than rosmarinic acid (Pérez-Fons et al 2010). Extraction of antioxidants from rosemary leaves is typically done with the use of chemical solvents or supercritical fluid extractions (Tena et al 1997). However, since carnosic acid and carnosol are lipophilic, oil could be a suitable alternative solvent to extract the antioxidants from rosemary leaves. Therefore, the aim of this work is to use oil to extract lipid - soluble antioxidants from rosemary leaves. In this study, three different vegetable oils were used to extract antioxidants from rosemary leaves, high oleic soybean oil (HOSO) donated by Malet (Carnegie, PA, USA), peanut oil (PO) (Kraft Heinz Company, Glenview, IL, USA) and cottonseed oil (CO) (Kedem Food Products, Bayonne, NJ, USA) both purchased from Amazon.com (Seattle, WA, USA).

The selection of the oils was made due to the expected fatty acid profiles of the vegetable oils; since different fatty acids may affect the extraction efficacy of lipid - soluble compounds. Lipids' fatty acid composition affects the efficiency of natural antioxidants (Pokorný et al 2005). Oils with a greater level of unsaturation have been

shown to be more polar than oils with higher saturated fatty acid content (Murali et al 1993). This increase in polarity could help to solubilize antioxidants that are somewhat polar, such as the antioxidants found in rosemary leaves (Pokorný et al 2005). Studies have shown oleic acid, (C18:1) enhances the activity of antioxidants (Hernandez 2016). Studies have shown that solubilizing antioxidants in oleic acid increases antioxidant activity compared to using the antioxidants alone (Hernandez 2016). Therefore, oils containing high amounts of oleic acid could be better at extracting antioxidants from rosemary leaves. Polyunsaturated fatty acids (PUFAs) such as linoleic and linolenic acid (C18:2 and C18:3 respectively) are relatively more susceptible to lipid oxidation compared to monounsaturated fatty acids such as oleic acid (Choe et al. 2005). Therefore, oils with a high percentage of linoleic and linolenic acid may oxidize to a greater extent in an antioxidant extraction process compared to the oils with a lower percentage of C18:2 and C18:3. This is particularly important because heat is involved in the extraction process developed in this study, and heat increases lipid oxidation by providing the energy of activation. Therefore, to better select the vegetable oils for extraction of antioxidants from rosemary leave the fatty acid profile of the cottonseed oil, peanut oil, and high oleic soybean oil has been studied. Oils' analysis was done through performing fatty acid methyl esterification (FAME) and profiling the derivatizations with gas chromatography paired with a flame ionization detector (GC-FID).

1.1. Analysis of the Vegetable Oils' Chemical Composition

1.1.1. Vegetable Oils' Fatty Acid Analysis Using Methyl Esterification Procedure

Derivatization of the oils to fatty acid esters was performed with a similar procedure to Carvalho and Malcata (2005). Briefly, the FAME procedure was as follows;

100 μ L of oil was aliquoted and mixed with 10 mL of hexane until it was completely dissolved. After adding 200 μ L of 2N potassium iodide dissolved in methanol, the mixture was vortexed for 20 seconds and left to sit for 1 minute. The top layer of the samples was collected, added to sodium thiosulfate anhydrous, centrifuged at a rate of 13.1 rotations per minute (rpm) for 15 minutes at 15°C to separate any residual water from the fatty acid methyl esters in hexane, and transferred to a 1.5 ml Thermo Scientific vial for further GC analysis.

1.1.2. Gas Chromatography Paired with Flame Ionization Detector Parameters for FAME Analysis

FAME samples were analyzed using an Agilent series 6890 CG (Agilent Technologies Inc., Santa Clara, CA) coupled with a split injection, a flame ionization detector (FID), an autosampler (Agilent Technologies Inc., model 7683), and an HP-88 capillary column (100 m x 0.25 mm internal diameter with 0.25 μ m film thickness (Agilent Technologies Inc., Santa Clara, CA)). The oven temperature was programmed to increase from 120 to 145°C at a rate of 8°C/min, maintained at 145°C for 26 min, increased to 220°C at a rate of 2°C/min, maintained at 220°C for 1 min, increased to 235°C at a rate of 30°C/min and maintained at 235°C for 1 min, for a total run time of 69.2 min. Nitrogen was used as the carrier gas with a constant flow of 2.0 mL/min. The inlet was set to 250°C with a split ratio of 25:1, and the detector was set to 260°C. Identification of the FAMEs were based on their retention times in comparison with fatty acid methyl ester standards purchased from Sigma Aldrich (St. Louis, MO, USA).

1.2. Evaluation of the different Vegetable Oils in Extraction of Lipid-Soluble Antioxidant

1.2.1. The Vegetable Oils' Fatty Acid Profile and Their Effects on Antioxidant Extraction

The GC-FID chromatograms for all three oils are shown in Figure 1. Since the chromatograms show a difference in peak areas and retention times, clearly the fatty acid profile of each vegetable oil was different. The results of the fatty acid profile are displayed in Table 1. As seen in Table 1 each oils' composition varies in fatty acids and their percentages. High oleic soybean oil is rich in oleic acid with its fatty acid composition being $80.78 \pm 0.05\%$ oleic acid, which is similar to what Napolitano et al (2018) found for high oleic soybean oil. It also has 1.4 times the amount of oleic acid, found in peanut oil and 4.5 times the amount found in cottonseed oil. Considering the low oxidation rate of oleic acid, a monounsaturated fatty acid (MUFA), compared to polyunsaturated fatty acids, it is more resistant to oxidation (Choe et al. 2006) and the high solubility in oleic acid, may confirm this vegetable oil as a good solvent. Furthermore, as mentioned in Section 1.1, antioxidants are more effective in preventing lipid oxidation when solubilized in oleic acid than when antioxidants are added alone to foods containing fat (Hernendaz 2016). In agreement with Hernandez (2005) findings, cottonseed oil is mainly made of linoleic acid (C18:2). As shown in Table 1, cottonseed oil contains twice the amount of PUFAs found in peanut oil, and 8 times the amount found in high oleic soybean oil. Moreover, cottonseed oil contains 1.9 and 2.2 times the amount of saturated fatty acids found in peanut oil and high oleic soybean oil, respectively. These variations of fatty acids found in cottonseed oil may make this vegetable oil a good option as a solvent for extracting antioxidants from rosemary leaves

as it may help to determine PUFAs and saturated fats role in extracting antioxidants.

Peanut oil's fatty acid profile is consistent with Hammond's et al. (1997) findings. The only concern with the oils' fatty acid profile is their susceptibility to lipid oxidation during the extraction process. PUFAs contain allylic hydrogens which require less activation energy for free radicals to extract compared to non-allylic hydrogens.

Figure 1 Gas chromatography paired with a flame ionization detector (GC-FID) chromatograms of the different vegetable oils, a) high oleic soybean oil, b) peanut oil, and c) cottonseed oil used for extraction.

Therefore, PUFAs undergo more oxidation than MUFAs and saturated fatty acids (Choe et al. 2005). On the other hand, saturated fatty acids are more resistant to lipid oxidation (Choe et al. 2005) which may prevent the high level of degradation in cottonseed oil during the extraction process. Therefore, to better understand the effect of vegetable oils' fatty acid profile on the extraction efficacy of antioxidants from rosemary leaves, all these three oils (high oleic soybean oil, peanut oil, and cottonseed oil) were used after testing their oxidation stability.

Table 1 Fatty acid profile of vegetable oils (high oleic soybean oil, peanut oil, and cottonseed oil) used for extracting antioxidants from rosemary leaves.

Fatty Acid	Percentage		
	High Oleic Soybean Oil	Peanut Oil	Cottonseed Oil
Lauric acid	ND	ND	0.87±0.04
Palmitic acid	6.02±0.03	9.33±0.00	20.00±0.46
Heptadecanoic acid	0.75±0.00	ND	ND
Cis-10-heptadecanoic acid	1.30±0.01	ND	ND
Stearic acid	3.90±0.00	3.31±0.00	2.59±0.06
Oleic acid	80.78±0.05	57.67±0.01	17.88±0.31
Linoleic acid	5.46±0.03	22.16±0.14	58.66±0.33
Gamma-linolenic acid	ND	1.55±0.01	ND
Linolenic acid	ND	1.22±0.00	ND
cis-11-eicosanoic acid	1.80 ±0.00	ND	ND
cis-8,11,14-eicosatrienoic acid	ND	3.19±0.08	ND
cis-5,8,11,14,17-eicosatrienoic acid	ND	1.57±0.05	ND

*ND- Not detected; Detection is limited to above 0.50%

1.2.2. Evaluation of the Vegetable Oils' Oxidative Stability

The oxidation stability of the selected vegetable oils (high oleic soybean oil, peanut oil and cottonseed oil) used for the extractions were tested after exposing the oils to a heating process equivalent to what is used for the antioxidant extractions. The level of primary oxidation products, hydroperoxides, and secondary oxidation products,

ketones and aldehydes, were measured using peroxide value (PV) and *p*-Anisidine value (AV), respectively.

1.2.2.1. Peroxide Value Measurement

PV of the vegetable oils was measured using Association of Official Agricultural Chemists' (AOAC) official method 965.33. Fifteen (15) mL of 3:2 v/v acetic acid/chloroform was added to 2.50 ±0.05 g of vegetable oil and mixed until the oil dissolved. Two hundred and fifty (250) µL of saturated potassium iodine solution was added to the sample and mixed for one minute, at which time the sample changed from colorless to yellow. Fifteen (15) mL of distilled water was added to the sample and mixed for 30 seconds. Five hundred (500) µL of 1% starch indicator was added and mixed in, turning the mixture dark purple in color. The mixture was titrated with 0.01N sodium thiosulfate until the dark purple color disappeared. A blank titration was carried out using the same materials mentioned above, except without the oil sample. The PV, concentration of hydroperoxides in the sample, expressed in mEq/Kg, was calculated using the equation below:

$$PV = \frac{(S-B) \times N \times 1000}{M} \quad (1)$$

S- volume of titrant used for sample

B- volume of titrant used for blank

N- normality of titrant used

M- mass of sample

All solvents and chemicals were purchased from Fisher Scientific (Fair Lawn, NJ, USA).

1.2.2.2. p-Anisidine Value Measurement

AV was measured according to American Oil and Chemist Society's (AOCS) Cd 18-90 method. One (1.0) g of the vegetable oil sample was dissolved in 25 ml isooctane.

The UV absorbance of the vegetable oil dissolved in isooctane was measured against pure isooctane at 350 nm using a Spectronic GENESYS 20 Visible Spectrophotometer (Thermo Electron Corporation, Waltham, MA). Five (5) ml of the vegetable oil solution and 5 ml of the pure isooctane, as the blank, were transferred to test tubes, where 1 ml of 0.25g of *p*-Anisidine dissolved in 100 ml of glacial acetic acid was added to them. The test tubes were vortexed and allowed to sit in the dark for 10 minutes. After the 10-minute period, the UV absorbance of the vegetable oil-isooctane-*p*-Anisidine solution was measured against the UV absorbance of the isooctane-*p*-Anisidine solution at 350nm. The AV, concertation of aldehydes and ketones in the vegetable oil, expressed in AnV (an arbitrary unit given since AV is measured with absorbance, a unitless measurement) was calculated using the following equation:

$$p - A.V. = \frac{25 \times (1.2A_s - A_b)}{m} \quad (2)$$

As - absorbance of the vegetable oil solution after reaction with the *p*-Anisidine reagent

Ab - absorbance of the vegetable oil solution

m - mass of the vegetable oil in grams

All solvents and chemicals were purchased from Fisher Scientific (Fair Lawn, NJ, USA).

1.2.3. Vegetable Oil's Oxidative Stability Results

The results of the applied heating process on the selected vegetable oils' oxidative stability, primary and secondary lipid oxidation formation are shown in Figure 2. As shown in this figure all the samples show a minor level of primary and secondary oxidation products even before heating. The heating process changed the level of oxidation; however, its effects was not identical on the oxidation of the different vegetable oils. The high oleic soybean and peanut oils had significantly more

hydroperoxide formation after heating. The PV increased from 0.53 ± 0.12 to 2.26 ± 0.23 mEq/kg in the high oleic soybean oil after heating and from 2.01 ± 0.69 to 4.27 ± 0.67 mEq/kg after heating in the peanut oil. Among the three vegetable oils used, the cottonseed oil was the only vegetable oil that did not show a significant increase in primary oxidation after the heating process. Figure 2a also shows, no significant difference in PV between the peanut oil and cottonseed oil after heating, however they formed significantly more primary oxidation products than the high oleic soybean oil. This observation could be related to the presence of the higher content of PUFAs or short chain fatty acids in regular, non – high oleic vegetable oils. Similar results were found (Ali et al. 2013) when comparing the heat stability of high oleic sunflower oil to regular sunflower oil. The high oleic sunflower oil had significantly lower amounts of hydroperoxides, aldehydes, and ketones formed after being heated at 185°C for 4 hours compared to the regular sunflower oil (Ali et al. 2013).

The comparison of the oils' secondary oxidation products is shown in Figure 2b. As expected, high oleic soybean oil's AV remained constant, while AV increased in cottonseed oil and peanut oil. The increase in the secondary oxidation of the cottonseed oil was greater than in the peanut oil possibly due to its chemical composition. As shown in Table 1, cottonseed oil is relatively high in linoleic acid, $58.66 \pm 0.33\%$, compared to peanut oil and high oleic soybean oil, 22.16 ± 0.14 and 5.46 ± 0.03 respectively. Linoleic acid is known to be highly susceptible to lipid oxidation (Choe et al 2006); and when heated, secondary oxidation products form in cottonseed oil more rapidly than the other vegetable oils (Dowd et al. 2010). Although these observations confirm some level of oxidation in the vegetable oils, the amounts of PV and AV were still below 10 mEq/kg

and 10 AnV, respectively, the acceptable upper limit in vegetable oils (Yi et al. 2013 and Rossel 1994). Therefore, all three oils were deemed appropriate for further investigations of the best oil to use for the extraction of lipid-soluble antioxidants from rosemary leaves.

Figure 2 Peroxide (a) and Anisidine (b) values of the vegetable oils, High oleic soybean oil (HOSO), peanut oil (PO) and cottonseed oil (CO), used for extractions before and after the heating process. Superscript letters represent statistical differences between columns, $p < 0.05$.

2. Methods Developed for Extracting Lipid-Soluble Antioxidants from Rosemary Leaves

2.1. Antioxidant Extraction from Rosemary Leaves Using Vegetable Oils

Rosemary antioxidants can be extracted from fresh or dried leaves. In order to develop the best extraction method with the highest efficiency, both fresh and dried rosemary leaves were tested in this study. Fresh rosemary leaves were purchased from Marx Foods (USA), and used for the extraction of lipid-soluble antioxidants. In order to develop a drying process, the water level of the rosemary leaves was measured. The rosemary leaves were heated at 60°C for 12 hours and tested with a LabSwift-aw water activity meter (Novasina, Lachen, Switzerland). It was concluded that fresh rosemary leaves contain about 67% water and have a water activity of 0.975. This high level of water and water activity may apply more oxidation in the solvent vegetable oil during the

processing conditions. Damodaran (2017) showed water activity above 0.70 increases the rate of lipid oxidation and is sufficient for microorganisms to grow in that medium. Fresh leaves also start to deteriorate after 2 weeks (Lange et al. 1994) which limits time for extractions. The main advantage of using fresh leaves for extraction is that they require less processing time since the step of drying the leaves is not required. On the other hand, there are several benefits when using dried leaves. For instance, dried leaves can be kept longer than fresh leaves. This provides opportunities for a better selection of the rosemary origins, and types. The carnosic acid content in rosemary leaves varies depending on the environmental conditions which the rosemary grows in (Birtic et al. 2015). For example, there is a greater concentration of carnosic acid found in rosemary leaves grown in spring and summer conditions, compared to winter and fall conditions (Rodríguez-Rojo et al. 2012). Therefore, when the rosemary is purchased for extraction, instead of performing the extractions all at once, the leaves can be dried, stored, and used when needed while still containing the same concentration of carnosic acid in each extraction. Moreover, considering the objective of this research, it is important to keep the source of rosemary constant. There is a need to ensure that the starting material being used is the same every time an antioxidant extraction is performed. Drying rosemary leaves provided that opportunity. Therefore, for a complete evaluation of the developed extraction method, both fresh and dried leaves were used to determine which type of leaf is best for extraction. For the drying process, various heating conditions including heating time, temperature, and rate were tested. It was found that the best drying technique is using minimal heat, 20 - 40°C, for 40 - 48 hours. They were then ground in a Geno Grinder (Spex Sample Prep. Metuchen, NJ, USA) twice for 7 - 10 minutes each time. The fresh

leaves were ground with a handheld grinder, Microplane 48916 Herb Mill 2-in-1 (Russellville, AK, USA). The ground rosemary leaves (20 – 40% w/w) were fully immersed into the vegetable oil from 110 - 170°C to ensure the vegetable oils' lipid crystals were melted and remained at the same temperature during the extraction process. The oil and ground rosemary mixture was kept at that temperature and constantly stirred for 1 - 3 hours on a magnetic stirrer hot plate. After heating, the samples were run through a coffee filter to separate the solid residue from the solution. The oil - antioxidant solutions were stored at 4°C for further analysis of their antioxidant efficacy, identification, and quantification.

2.2. Antioxidant Extraction from Rosemary Leaves Using a Chemical Solvent

To test the efficiency of the developed extraction methods, an extraction using a solvent extraction technique was also designed. Dried, ground rosemary leaves were added to an ethanol - water mixture, 3:1 (v/v), in a 1:6 ratio (w/v). The mixture was heated at 40°C and shaken in a shaking water bath (Grant Instruments, Cambridge, UK) for 1.5 hours. After the shaking process was done, the liquid containing extracted antioxidants was filtered (pore size 20-25 µm) from the rosemary particles under vacuum. The filtrate was collected and stored at -20°C for further analysis such as identification and quantification of its antioxidants.

3. Methods Used for Analysis of the Extracted Rosemary Antioxidants

3.1 Analysis of Carnosic Acid and Carnosol Using High Performance Liquid Chromatography Paired with Photo Diode Array Detector

The identification and quantification of carnosic acid and carnosol in the extracted products were analyzed by High Performance Liquid Chromatography paired with a Photo Diode Array detector (HPLC-PDA). Prior to HPLC analysis, sample preparation

was done on both vegetable oil and chemical solvent extractions as explained in the following. In the vegetable oil solutions, the antioxidants were extracted using acetonitrile. Four (4) ml of acetonitrile, purchased from Fisher Scientific (Fair Lawn, NJ, USA), was added to 1 gram of the antioxidant – vegetable oil solution, vortexed and centrifuged. The top layer was collected for analysis. The addition of acetonitrile, vortexing and centrifuging was performed a total of three times to ensure all the antioxidants were being extracted from the antioxidant - vegetable oil solution. This extraction was repeated a total of three times since there was a faint yellow color in the third acetonitrile addition (from the carnosic acid), but fourth addition of acetonitrile was clear. Therefore, it was determined that extracting three times was sufficient to obtain all the carnosic acid. The resulting acetonitrile - antioxidant mixture was diluted with acetonitrile. One (1) ml of sample was added to 1 ml of acetonitrile. The diluted samples were then mixed with acidified water (pH 2.0) in a 1:1 (v/v) ratio and filtered through nylon, 0.22 μm filters, for analysis. The samples were stored at -20°C until the HPLC analysis was performed; each sample was run in triplicate.

For the extractions provided with the chemical solvent, 1 g of solvent extract was flushed with nitrogen to dryness. The powder was then reconstituted in 4 ml of acetonitrile. Five hundred (500) μL of the acetonitrile containing the extract was diluted with another 500 μL of acetonitrile, and that mixture was further diluted with acidified water (pH 2.0) in a 1:1 (v/v) ratio and filtered through nylon, 0.22 μm filters, for analysis. The samples were stored at -20°C until the HPLC analysis was performed; each sample was run in triplicate.

To identify and quantify carnosic acid and carnosol content of the prepared HPLC samples analysis was done by HPLC - PDA on a Shimadzu apparatus (Shimadzu Prominence series, Kyoto, Japan). The HPLC was equipped with a binary pump delivery system, OF-20AD for mobile phase A, CAN-20A for mobile phase B, and an autosampler, Sil-20AC. A reverse phase C₁₈ Kinetex column, 5µm particle, 4.6mm x 250mm (Phenomenex, Torrance, CA, USA) was used to separate the compounds. The mobile phase was a mixture of solvent A (10 mMol acetic acid) and solvent B (acetonitrile). The optimized chromatographic condition at 1 ml/min flow rate was: 0-1 min, 80-20% B; 1-11 min, 60-40% B; 11- 20 min, 40-60% B; 20- 45 min, 20-80% B; 45- 50 min, return to the initial conditions. The oven, CTO-20A (Shimadzu), temperature was held at 25°C. Detection was accomplished by using a Photo Diode Array (PDA) series SOD-M20A (Shimadzu) from 200 to 700 nm.

To examine the concentration of carnosic acid and carnosol found in each extraction, standard curves were built from carnosic acid and carnosol standards purchased from Fisher Scientific (Fair Lawn, NJ, USA) and Sigma Aldrich (Darmstadt, Germany), respectively. Standard curves were built with known amounts of the compounds of interest. The chromatograms obtained from those runs with the known amounts of sample show an area of the peak which can be used to determine the amount of the compounds in an unknown sample. External curves were built to quantify the amount of carnosic acid and carnosol found in the extracts. Using external curves to quantify the amount of carnosic acid found in extract is common practice (Bicchi et al 2000, Tena et al. 1997, Carvalho Jr. et al. 2005, etc.). Ten (10) mg of carnosic acid was dissolved in 10 ml of acetonitrile. Five hundred (500) µL of the

carnosic acid-acetonitrile mixture was diluted with 500 μL of acetonitrile and then mixed with 1mL of acidified water (pH 2.0). The carnosic acid-acetonitrile-acidified water mixture was filtered through a nylon, 0.22 μm filters, into 1.5mL vials and stored at -20°C until their analysis. A similar procedure was done for carnosol however, 5 mg of carnosol was dissolved in 8 mL of acetonitrile. Five-point curves were built with each compound by running 10, 25, 50, 100, and 200 μL aliquots of the carnosol solution and 10, 25, 50, 100 and 150 μL aliquots of the carnosic acid solution. Each dilution factor was run in triplicate.

3.2. Examination of the Extracted Antioxidants' Efficacy on Lipid Oxidation

The efficacy of the extracted antioxidant solution on vegetable oil oxidation stability was tested by adding them to a regular soybean oil (SO). Soybean oil, donated by Archer Daniels Midland Company (ADM) (Decatur, IL, USA) was chosen because it was minimally processed with no added antioxidants and is relatively susceptible to lipid oxidation. Moreover, soybean oil is a vegetable oil, but was not used as a solvent to extract rosemary's antioxidants. Lipid oxidation was examined on three different soybean oil samples prepared by adding 2% (w/w) rosemary antioxidant extract to them, which is equivalent to 0.02% (w/w) of the active antioxidants. These samples in addition to a control, soybean oil sample (without anything added) were incubated at 60°C for 22 days. Primary and secondary oxidation levels were monitored using peroxide value, *p*-Anisidine value and Iodine value (IV).

3.2.1. Primary and Secondary Oxidation Evaluation of the Soybean Oil Samples

At various timepoints the levels of primary oxidation and secondary oxidation formation in the soybean oil samples were measured using peroxide value and *p*-Anisidine,

respectively. These analyses were done based on the same technique described earlier in Sections 1.2.2.1 and 1.2.2.2.

To monitor the changes in the level of unsaturated fatty acids in the soybean oil samples during the oxidation, samples' double bond content was measured and calculated using the IV method according to AOCS Tg 14-64. The soybean oil sample was dissolved in 7.5 ml of a 1:1 (v/v) solution of acetic acid - cyclohexane. Twelve and a half (12.5) ml of Wijs solution was added to the soybean oil - solvent mixture, mixed and let to sit in the dark for 30 minutes. After being stored in the dark, 10 ml of an iodine solution was mixed in with the soybean oil – solvent - Wijs mixture, and then 50 ml of deionized water was mixed in with the solution. The mixture was titrated with 0.1M sodium thiosulfate solution. A blank mixture was prepared without the soybean oil, stored in the dark and titrated with 0.1M sodium thiosulfate solution as well. The Wijs solution, acetic acid, and cyclohexane were purchased from Fisher Scientific (Fair Lawn, NJ, USA) and 0.1M sodium thiosulfate solution was purchased from Sigma Aldrich (Darmstadt, Germany). The IV, measured in g/100g, was calculated using the following equation:

$$IV = \frac{(B-S) \times M \times 12.691}{m} \quad (3)$$

S- volume of titrant used for sample
 B- volume of titrant used for blank
 M- molarity of titrant used
 m- mass of sample

4. Statistical Analysis

All data are reported as mean values and standard deviations of replicated samples. Using GraphPad Prism 8 software, two-way ANOVA was used to determine significant differences between treatments based on a significance level of 0.05.

Chapter 4. Results and Discussion

1. Comparison of Antioxidants Extracted from Fresh vs. Dried Rosemary Leaves

As explained in Chapter 3, Section 2, the lipid-soluble antioxidants were extracted from fresh and dried rosemary leaves. To determine the best extraction process and to decide between using fresh and dried rosemary leaves, the extracts from both fresh and dried leaves were tested on corn oil's (CRO) oxidative stability. Two and a half (2.5) % (w/w) of each extract obtained in high oleic soybean oil was added to corn oil and heated at 65°C for 22 days. As the control, a corn oil sample without any antioxidant extraction added was kept at the same conditions. Corn oil was chosen to test the extracted antioxidant efficacy because it provides a new vegetable oil with a different chemical composition compared to the vegetable oils used for extractions. As shown in Figures 3a, on day zero the primary oxidation in all three corn oil samples were the same. However, after being heated for seven days the PV increased in the control oil but did not significantly increase in the corn oil with added antioxidants. After 18 days of storage, the same trend was seen, the PV in the control corn oil rose to 24.5 mEq/kg, whereas the corn oil with added antioxidants extracted from fresh and dried leaves rose to 5.2 and 4.5 mEq/kg, respectively. Finally, after 22 days of heating, the peroxide value was more than 3 times higher in the corn oil without any antioxidant extraction added to it, compared to the corn oil with added antioxidant extraction using both fresh and dried rosemary leaves. As shown in Figure 3b, a similar trend was seen with the *p*-Anisidine value. After 22 of

storage the AV was 1.8 times higher in the control corn oil compared to the corn oil with added antioxidants from both fresh and dried leaves. Furthermore, the results showed both extracts made from dried and fresh leaves had similar effects on their antioxidant efficacy. Both extracts from fresh and dried leaves significantly decreased the amount of hydroperoxide and secondary oxidation products formed from lipid peroxidation after

Figure 3 Peroxide values (a) and p-Anisidine values (b) of corn oil (CRO), corn oil with 2.5% w/w antioxidant extraction using fresh and dried leaves after being stored at 65°C for 22 days. Superscript letters represent statistical differences between columns, $p < 0.05$.

heating the corn oil for 22 days at 65°C.

To further study extractions from fresh and dried leaves, the samples' carnosic acid and carnosol content was also measured. As shown in Table 2, both extractions from fresh and dried leaves contained similar carnosol content. However, the antioxidants extracted from fresh and dried rosemary leaves contained 3.91 ± 0.15 , and 10.66 ± 0.28 mg carnosic acid/g extract, respectively. The concentration of carnosic acid, the most abundant lipid-soluble antioxidant found in rosemary leaves, was significantly greater in the extraction using dried leaves than using fresh leaves. These results are in agreement

with Rodríguez-Rojo et al. (2012) who reported a higher content of carnosic acid in extract from dried rosemary leaves compared to extracts from fresh rosemary leaves. These observations about the difference in presence of antioxidants from fresh and dried rosemary leaves provides additional benefits when developing the extraction method. Dried rosemary leaves can be stored for a longer period of time compared to fresh rosemary leaves. Fresh herbs have a shelf life of around two weeks when kept at room temperature, and only around three days when kept at refrigeration temperature due to chilling injury (Lange et al. 1994). Furthermore, as discussed in Chapter 3 Section 2.1, the ability to store the rosemary leaves for an extended period of time is important for consistency reasons. Carnosic acid content varies in rosemary leaves depending on the environmental conditions the leaves are grown in (Britic et al 2015). Using the same source of leaves for each extraction is important in ensuring the same concentration of carnosic acid is present in each extraction. Therefore, in continuation of this study, dried leaves were used.

Table 2 Concentration of lipid-soluble antioxidants, carnosic acid and carnosol, extracted from dried and fresh rosemary leaves.

	Carnosic acid (mg/g extract)	Carnosol (mg/g extract)
Extract from Dried Rosemary Leaves	10.66±0.28 ^a	0.68±0.12 ^a
Extract from Fresh Rosemary Leaves	3.91±0.15 ^b	0.53±0.06 ^b

Superscript letters represent statistical differences between columns, $p < 0.05$.

2. Comparison of the Different Vegetable Oils on Extracting Antioxidants from Dried Rosemary Leaves

2.1. Effects of Different Extracted Antioxidants on Soybean Oil's Oxidative Stability

Considering the presence of higher carnosic acid content in the extract obtained from dried rosemary leaves for the rest of the research performed in this study was with

the use of dried rosemary leaves. As explained in Chapter 3 Section 3.3, antioxidant extracts from high oleic soybean oil (HOSO), peanut oil (PO), and cottonseed oil (CO) were added to soybean oil, 2% w/w, and stored in an incubator at an elevated temperature to test the extractions' antioxidant efficacy. As a control, soybean oil without any added antioxidants was stored in the incubator as well. The iodine value (IV), peroxide value (PV) and *p*-Anisidine value (AV) were measured at various time points to quantify any changes in the oil's unsaturation fatty acid content, primary, and secondary oxidation products, respectively.

As shown in Figure 4a, during the oxidation process, the level of unsaturation of the fatty acids in soybean oil decreased in all samples. In the control, the soybean oil without any additives had a significantly greater reduction of the unsaturated fatty acids; the IV dropped from 132 to 105 g/100g in the control after 22 days of storage compared to the others that dropped from 134 to 122, 135 to 126 and 141 to 131 g/100g in the soybean oils samples with antioxidants extracted from high oleic soybean oil, peanut oil, and cottonseed oil, respectively. These changes in the oils' IVs were plotted against the storage time and the curves' slopes were measured to compare the oxidation rate differences. The slope of the control is much greater compared to the slopes from the soybean oils with added antioxidants. IV in the control decreased at a rate of 1.25 g/100g per day, whereas the soybean oil with antioxidants from high oleic soybean oil, cottonseed oil and peanut oil decreased by 0.58, 0.48, and 0.46 g/100g per day, respectively. These values of the iodine value indicate a much higher rate of oxidation in the control samples compared to others. Among the three different antioxidant vegetable oil solutions, interestingly, the oxidation rate was higher in the soybean oil sample

containing antioxidants extracted with high oleic soybean had the greatest decrease in slope. This could be explained based on cottonseed oil and peanut oil's fatty acid profile and their high amount of linoleic acid content. Linoleic oil is comparatively more susceptible to oxidation than MUFAs and saturated fatty acids, and when it oxidizes it forms conjugated dienes (Choe et al 2005). Therefore, the formed conjugated dienes, can be detected in the IV measurements and are accounted for, whereas high oleic soybean oils' oxidation products were not. To better analyze the soybean oils' oxidation and effect of antioxidant addition along with IV, peroxide and *p*-Anisidine values were measured as well and shown in Figure 4.

After seven days of storage, shown in Figure 4b, the PV increased slightly in all the samples. After 11 days of storage, the control sample showed a significantly higher PV increase (4.3mEq/kg per day) compared to the soybean oil with extracted antioxidants (0.80mEq/kg increase per day). This significant increase starts a trend of the control increasing significantly compared to the soybean oils with extracted antioxidants added to them. Sadeghi et al. (2016) reported similar findings. In that study, after storing soybean oil with and without added antioxidants for 6 day in the dark at 65°C, the PV did not significantly change in any samples compared to day one. However, after 12 days of storage, Sadeghi et al. (2016) reported a significant increase in PV in the soybean oil without antioxidants compared to the soybean oil with added antioxidants. Figure 4b also shows after 14 days of storage, while the hydroperoxide formation remained constant in the soybean oils with antioxidants (0.81 mEq/kg per day increase) there was a 7.20 mEq/kg per day increase in hydroperoxide formation in the control. Furthermore, after 22 days of storage, the PV was much greater in the soybean oil without any additives

(96.1 ± 13.1 mEq/kg). The level of PV is much lower in the soybean oil with antioxidant extracted using high oleic soybean oil, peanut oil and cottonseed oil, 16.0 ± 4.3 , 15.0 ± 5.5 , and 21.2 ± 4.7 mEq/kg, respectively. To compare the vegetable oils on antioxidant extractions the PV figures' slopes were also measured and analyzed. All these curves show the slopes to be around 1.21 mEq/kg per day increase indicating there was no increasing PV based on the vegetable oils used for extraction of antioxidants from rosemary leaves. The increase in PV in the control (6.60mEq/kg per day) was much steeper than the 1.21mEq/kg per day increase in the soybean oil with extracted antioxidant added to them. There was a significant increase in the amount of hydroperoxides produced in the control compared to soybean oil with added antioxidant extracts. When comparing the soybean oils with extracted antioxidants added to them, there was no difference in PV seen based on the three different vegetable oils used for the extractions.

The same trend that occurred with PV was seen with AV. As shown in Figure 4c, after 14 days of storage, the AV increased by 0.15 AnV per day in the control, whereas it decreased by 0.06 AnV per day in the soybean oil samples with extracted antioxidants. A decrease in AV indicates that the vegetable oil did not undergo secondary oxidation at the time. Moreover, the aldehydes and ketones that had been produced earlier interacted with other molecules in the system and therefore are not accounted for in the AV measurement (McClements and Decker et al. 2008). Furthermore, after 22 days at 60°C, the AV increased in the control - the soybean oil without any added antioxidants - from 4.10 ± 0.44 AnV (after 14 days of storage) to 33.20 ± 0.70 AnV; while the soybean oils containing antioxidants using high oleic soybean oil, peanut oil and cottonseed oil

increased from 0.93 ± 0.03 , 1.22 ± 0.16 and 1.32 ± 0.04 to 8.19 ± 0.01 , 8.20 ± 0.16 and 9.62 ± 0.38 AnV, respectively. This lower level of AV shows that the control had significantly higher secondary oxidation products formed compared to the other samples. This is in agreement with Sadeghi et al (2016) findings who reported a higher level of secondary oxidation (AV) in soybean oil stored at 65°C after 12 days of storage without added antioxidants compared to soybean oil with added antioxidants.

Opposite to the PV results, there was a difference in the amount of secondary oxidation products formed in the soybean oil based on the different vegetable oils used in the antioxidant extractions. The soybean oil with antioxidants extracted from cottonseed oil had significantly more ketones and aldehydes formed than the soybean oils with antioxidant extract using high oleic soybean oil and peanut oil. These findings are consistent with findings described in Chapter 3 Section 1.2.3 that discussed the vegetable oils' oxidative stability. The cottonseed oil had a significantly greater increase in secondary oxidation products formed after the heating process used for extracting the antioxidants than the peanut oil and high oleic soybean oil. This could be due to cottonseed oil's oxidative instability, or it could be due to a difference in the antioxidant content of the antioxidant extraction from cottonseed oil compared to the antioxidant extraction from peanut oil and high oleic soybean oil. Therefore, the identity and quantity of the extracted antioxidants were investigated.

Figure 4 Iodine value (a), peroxide value (b) and anisidine value (c) of Soybean oil (SO) and soybean oil with 2% w/w of extracted antioxidants (RA) using high oleic soybean oil (HOSO), peanut oil (PO), or cottonseed oil (CO) added and kept at 60°C for 22 days.

2.2. Identification and Quantification of the Antioxidants Content in Different Extractions Extracts Using the Different Vegetable Oils

Before identifying and quantifying the antioxidant compounds found in the extractions using oils, the oils by themselves were tested for their antioxidant

composition. These oils were analyzed to determine that the antioxidants detected in the extracts are coming from the rosemary leaves and not the oils themselves. Studies have shown vegetable oils contain natural antioxidants, such as tocopherols, but to the best of our knowledge vegetable oils do not contain any carnosic acid or carnosol (the main lipid-soluble antioxidants in rosemary leaves) (Fan and Eskin et al. 2015). Figure 5 is representative of the HPLC analysis of the vegetable oils and confirms that neither of the non-volatile, carnosic acid nor carnosol, antioxidants were present in the vegetable oils used for antioxidant extractions. Therefore, if there were any peaks shown in the

Figure 5 Chromatograms from high performance liquid chromatography of the oils used for extraction a) high oleic soybean oil, b) peanut oil and c) cottonseed oil.

chromatograms from rosemary antioxidant extractions, it can be concluded that the peaks are displaying compounds extracted from the rosemary and not peaks that correspond to compounds found in the vegetable oils.

Figure 6 shows similar analysis (HPLC chromatograms) of the vegetable oils after extracting rosemary's antioxidant compounds and their comparison to the solution obtained from using the chemical solvent for extraction. As shown in this figure, samples' HPLC chromatograms show a number of different peaks at different retention

Figure 6 Chromatograms from high performance liquid chromatography (HPLC) of the extractions using a) high oleic soybean oil, b) peanut oil, c) cottonseed oil and d) solvent with carnosic acid (1), carnosol (2) and carnosic acid quinone (3) identified.

times which have not been shown in Figure 5. This observation confirms the presence of other compounds mainly extracted from rosemary leaves. Based on the literature (Bentayeb et al 2007, Masuda et al 2002) and quantification of the standard samples (eluted using the mobile phase parameters discussed in Chapter 3 Section 3.2.4), peaks 1, 2 and 3 represent the carnosic acid, carnosol, and carnosic acid quinone, respectively. A comparison of Figure 6a-c shows that the difference in fatty acid profiles among the different vegetable oils used did not affect the oils' ability to extract the lipid - soluble antioxidants. However, to have a better detailed analysis of the peaks' area and intensity is required. To do so, after clearly separating and identifying the lipid - soluble antioxidant compounds using retention times and UV spectra based on the compounds' standards, standard curves of carnosic acid and carnosol were constructed. Quantification of compounds by building calibration curves is common practice and has been used for carnosic acid (Bicchi et al 2000, Tena et al. 1997, Carvalho Jr. et al. 2005, etc.).

As shown in Figure 7, standard curves were built with a coefficient of determination (R^2) values greater than 0.99. The R^2 value is a measurement of variance that determines the significance of validation based on a regression line (Zhang et al 2018). For the standard curves built for carnosic acid and carnosol, R^2 is the proportion of variance in the dependent variable - the area under the peak - that is predictable from the independent variable - the mass of sample injected into the HPLC. Therefore, the closer the R^2 value is to 1, the more linear the graph is, the less variance there is among the peak area corresponding to the mass of sample injected, and the more accurate the curves. The accuracy of the curves better describes the relationship between the mass of carnosic acid

and carnosol based on the area under the peaks. Moreover, since the curves had R^2 values greater than 0.99, they are deemed successful and ready to be used.

Figure 7 Standard curves built, using High Performance Liquid Chromatography (HPLC), of antioxidant compounds of interest, Carnosic acid (a) and Carnosol (b), used to quantify the amount extracted in the Rosemary Antioxidant Extractions.

Using the standard curves, the concentration of carnosic acid and carnosol extracted from the rosemary leaves was calculated. These calculations were performed by fitting the area under the carnosic acid and carnosol peaks of the vegetable oils and solvent extractions' chromatogram along the calibration curve to obtain the mass of each compound per the amount of extract injected into the HPLC (300 μ L). Once the amount of carnosic acid and carnosol in the injection volume was obtained, it was multiplied by

the dilution factors to have the mass of carnosic acid and carnosol per gram extract and per gram rosemary leaves.

As shown in Table 3, high oleic soybean oil extracted significantly more carnosic acid than peanut oil and cottonseed oil. High oleic soybean oil extracted 10.66 ± 0.28 mg/g extract of carnosic acid, and there was no significant difference among the concentrations of carnosic acid in peanut oil and cottonseed oil (9.51 ± 0.78 and 8.94 ± 0.86 mg/g extract, respectively). The observed lowered concentration of carnosic acid in peanut oil and cottonseed oil compared to the high oleic soybean oil could be related to a possible oxidation process. Both cottonseed oil and peanut oil showed a higher oxidation rate during the extraction processing compared the high oleic soybean oil. As discussed earlier in Chapter 3 Section 1.2.3, and shown in Figure 2, peanut oil and cottonseed oil had significantly higher PV values than high oleic soybean oil during the applied heat processing. When these oils were used for antioxidants extraction a portion of the carnosic acid was perhaps used to protect the peanut oil and cottonseed oil from further oxidation and degradation. Moreover, one may connect the higher concentration of carnosic acid in high oleic soybean oil to the oil's high oleic acid concentration. As discussed in Chapter 3, Sections 1.1 and 1.1.4, the high oleic soybean oil contained 1.4 and 4.5 time the amount of oleic acid than peanut oil and cottonseed oil, respectively. Studies have shown oleic acid to enhance antioxidant efficacy (Hernandez 2016).

As opposed to the oils' carnosic acid content, Table 3 shows a comparable amount of carnosol in the extractions from all three vegetable oils. Carnosol is a degradation product of carnosic acid and therefore is found in small amounts in rosemary leaves (Loussouarn et al. 2017). Because carnosic acid degrades into carnosol, there was

an expectation that lower levels of carnosic acid would lead to higher levels of carnosol. This was not found to be true. Possibly, because carnosic acid degrades into many derivatives, not only carnosol. The extractions from peanut oil and cottonseed oil have a lower concentration of carnosic acid compared to the extraction from high oleic soybean oil. This lower level of carnosic acid did not lead to a greater production of carnosol in these vegetable oils since carnosic acid degrades into many derivatives, not only carnosol. Another predominant carnosic acid derivative is carnosic acid quinone (Zhang et al 2012). Although carnosic acid quinone peak (peak 3) has a relatively high intensity, it was not further analyzed to quantify the amount in each extract due to their being a lack of resources. Carnosic acid quinone was not available commercially as a standard to perform HPLC testing and building standard curves from. Furthermore, carnosic acid quinone lacks antioxidant activity. Carnosic acid quinone has been shown to be an intermediate product of the degradation process of carnosic acid to carnosol and has no antioxidant activity (Masuda et al. 2002).

Table 3 Concentration (mg/ g extract) of non-volatile antioxidants, carnosic acid and carnosol, in the different mediums used for extracting- high oleic soybean oil (HOSO), peanut oil (PO), cottonseed oil (CO) and solvent.

Medium for Extraction	Non-Volatile Antioxidants			
	Carnosic acid		Carnosol	
	mg/g extract	mg/g Rosemary Leaves	mg/g extract	mg/g Rosemary Leaves
HOSO	10.66±0.28 ^a	53.28±1.41 ^a	0.68±0.12 ^a	3.40±0.59 ^a
PO	9.51±0.78 ^b	47.56±3.88 ^b	0.80±0.18 ^a	4.00±0.90 ^a
CO	8.94±0.86 ^b	44.72±4.32 ^b	0.70±0.01 ^a	3.49±0.06 ^a
Solvent	6.75±0.30 ^c	40.52±1.79 ^c	2.59±0.06 ^b	15.55±0.38 ^b

Superscript letters represent statistical differences within each column, p < 0.05

2.3. Comparison of the Antioxidant Content in Extractions Made with the Vegetable Oils and Chemical Solvents

Since the main objective of this study was to develop an extraction method of antioxidants from rosemary leaves, it is vital to examine the method's efficacy compared to a solvent extraction technique. Currently, various solvents including methanol, ethanol, acetone, hexane and more are being used for extractions. As a control, a solvent extraction using ethanol was performed to compare the extraction efficacy of using vegetable oils as the solvent compared to using a common solvent extraction method. The results of this comparison of these methods are shown in Figure 6 and Table 3. As shown in Table 3, all the vegetable oil extracted between 44.72-53.28 mg carnosic acid/g rosemary leaves, and ethanol could extract only 40.52 mg carnosic acid/g rosemary leaves. This level of carnosic acid extraction by vegetable oil also shows the advantages of using the developed vegetable oil extraction method compared to the other techniques. For example, supercritical fluid extractions and methanol extractions provided 35.7, 12.6 and 14.76 mg carnosic acid/g rosemary, which is 28-67% lower than the amount of carnosic acid obtained by vegetable oils in this study (Tena et al. 1997, Bicchi et al. 2000, and Rodríguez-Rojo et al. 2012, respectively). Table 3 also shows a significantly higher carnosol content in the solvent extracted solution compared to the vegetable oil extracts. Vegetable oil extracts contained between 3.40-4.00 mg carnosol/g rosemary leaves, while the solvent extract contained 15.55 mg carnosol/g rosemary leaves. Although the discrepancy of carnosol content could be related to the carnosic acid degradation during the chemical solvent extraction, further investigation is needed to explain the drastic increase of carnosol content in this method. The carnosic acid quinone peak as shown in Figure 6 is smaller in the chemical solvent extraction compared to the vegetable oil

extractions. However as discussed earlier, carnosic acid quinone is an intermediate product between carnosic acid and carnosol. Therefore, the carnosic acid quinone peak in Figure 6 is smaller in the solvent's HPLC chromatogram compared to the vegetable oils' HPLC chromatograms because the extraction process using ethanol caused more oxidation to occur. Therefore, the carnosic acid quinone had further degraded into carnosol in the ethanol solution.

Chapter 5. Conclusion and Future Studies

Vegetables oils are a good substitute for chemical solvents to extract lipid – soluble antioxidants from rosemary leaves. Vegetable oils are mostly fatty acids that entrap and solubilize the carnosic acid and other lipid-soluble antioxidants found in rosemary leaves. Both fresh and dried rosemary leaves were used as a starting material. After performing high performance liquid chromatography (HPLC) analysis, the data showed extractions using dried leaves contained 63% more carnosic acid than extractions using fresh leaves as the starting material. All of the three different vegetable oils used for the extraction of antioxidants (high oleic soybean oil (HOSO), cottonseed oil (CO), and peanut oil (PO)) can be used as a solvent for extracting antioxidants. Even though the HOSO extracted more carnosic acid than the other two vegetable oils, all three of the antioxidant vegetable oil solutions had comparable antioxidant efficacy. The vegetable oils extracted 10-24% more carnosic acid than the chemical solvent (ethanol) extracted. However, the ethanol extract contained 75-78% more carnosol than the vegetable oil extracts. The proposed method of extraction does not require the antioxidants extracted to be reconstituted into oil since the antioxidants are in the vegetable oil at the end of the extraction process. The proposed developed method has been shown to be successful in extracting antioxidants, however further research is needed to improve the antioxidant - vegetable oil solutions' method development.

In the HPLC chromatograms there were many peaks, not just the carnosic acid, carnosol and carnosic acid quinone peaks. Those peaks need to be further analyzed to determine if the peaks are coming from the antioxidant - vegetable oil solutions or from the sample preparation necessary for HPLC analysis. Furthermore, the peaks have to be analyzed to determine that the compounds they are representative of are not toxic or dangerous for human consumption. The volatile antioxidants present in the solutions have yet to be fully analyzed. Based on the literature and preliminary experimentation, some of the volatiles have been identified. However, these volatile antioxidants have not been quantified in the antioxidant - vegetable oil solutions. To identify and quantify the volatiles found in the solutions, the volatiles have to be extracted from the oil since oil cannot be injected into a Gas Chromatographer for analysis. Therefore, a more complete evaluation of the antioxidant – vegetable oil solutions obtained must be performed before claiming its antioxidant potency. Furthermore, the vegetable oil - antioxidant solutions currently have a green color due to chlorophyll and other non - antioxidant compounds found in the solutions. Therefore, the extractions need to go through further purification before they can be used. Consumers and product developers do not want to incorporate a green mixture into their products that would cause the final product to be discolored. The removal process cannot use chemicals since including the use of chemicals would take away the claim that the final product falls under the “clean label” category. Therefore, a non-chemically based method for removing the residuals and color from the antioxidant vegetable oil solutions must be developed. Furthermore, the vegetable oil - antioxidant solutions have a strong odor from the rosemary leaves’ extracted compounds and need to be deodorized before being added to food products. These odorizing compounds from

the rosemary leaves are mostly volatile antioxidants included in the extractions.

Therefore, if these compounds are removed, some of the solutions' antioxidant efficacy would decrease. Therefore, a method must be developed to deodorize the solutions without interrupting their antioxidant activity.

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