ABSTRACT

Title: CHARACTERIZATION OF OLIVE OILS COMMERCIALLY AVAILABLE IN THE UNITED STATES

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This research examined 30 olive oils commercially available in the United States for their fatty acid composition, oxidative stability, and antioxidant capacities. The olive oils in this study differed in their oxidative stability (23.64 – 212.1 h induction time) and concentrations of rancid byproducts (peroxide values: 40.46 – 65.07 μmol *t*-butyl peroxide equivalents/g oil; *p*-anisidine values: 2.70 – 8.79 OD_{350}/g oil; free fatty acid contents: 0.19 – 1.20 g oleic acid/100 g oil). These commercial olive oils also possessed varying concentrations of total phenolic contents (554 – 810 μg gallic acid equivalents/g oil) and oxygen radical absorbance capacities (1.4 – 11.7 μmol Trolox equivalents/g oil) ($P < 0.05$). These data suggest that olive oils commercially available in the United States differ in their quality, stability, and nutritional value. Furthermore, the retail price may not reflect the quality, stability, and nutritional value of the olive oil.

CHARACTERIZATION OF OLIVE OILS COMMERCIALLY AVAILABLE IN THE UNITED STATES

By

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Chapter 1: Literature Review

Significance, Rationale, and Hypotheses

According to the International Agreement on Olive Oil and Table Olives (IAOOTO), which is the current document used to govern the trade of olive oil internationally, olive oil is the oil obtained solely from the fruit of the olive tree (*Olea europaea* L.) to the exclusion of oils obtained using solvents, re-esterification processes, or mixtures of other oils (United Nations, 2003). In 2003, the production of olive oil worldwide was 2,766,773 metric tons (FAOSTAT data, 2004), which equates to ca. 4 % of the total worldwide vegetable oil production (Visioli & Galli, 2002a). As per the IAOOTO, virgin olive oil (VOO) is olive oil obtained from the olive fruit solely by mechanical and other physical means, under minimized thermal conditions that do not deteriorate the oil, and has not undergone any treatments other than crushing and malaxation of the olives (Angerosa et al., 2001) and washing, decanting, centrifugation, and filtration (United Nations, 2003). It is proposed that for this reason VOOs retain more health-beneficial phytochemicals as compared to other commercially available refined table oils which are generally further filtered and/or solvent extracted (Visioli & Galli, 1998a). The beneficial effects of VOO toward cardiovascular disease, when compared to more processed oil and oil consisting solely of fat in similar proportions to olive oil, are proposed to be linked to the phytochemical composition and antioxidant capacities of the oils (Scaccini et al., 1992, Visioli & Galli, 1998a). Additionally, oils obtained in a manner similar to that used to obtain virgin olive oil may exhibit acceptable oxidative stability and less safety concerns as compared to other conventional processing methods (Parry & Yu,

2004). The goal of this research is to assess the quality and stability of olive oils by measuring their fatty acid composition, concentrations of phytochemicals, oxidative stability, and antioxidant capacities

Only one study has been publish in a peer-reviewed journal that analyzed olive oil samples for their antioxidant capacities by the oxygen radical absorbance capacity assay using fluorescein ($ORAC_{FL}$) as the fluorescent probe, and the study only evaluated one olive oil sample (Ninfali et al., 2005). This assay is a widely accepted *in vitro* measure of antioxidant capacity towards the biologically-relevant peroxyl free radical (Cao & Prior, 1998; Ou et al., 2001; Ou et al., 2002; Huang et al., 2005).

Olive oils sold commercially in the United States differ in many individual characteristics. Those identified on some of the labels are the variety of olives used to make the oil, the country of origin of the olives, the amount of processing to which the oil has been subjected, and various other qualifiers (e.g. organic) (North American Olive Oil Association (NAOOA), 2002). With many combinations of these characteristics, the consumer may not know which brand is of the highest quality and is most health-beneficial, although there is one criterion that is internationally accepted by many nations. The IAOOTO establishes the classification of extra virgin olive oil (EVOO) as "a virgin olive oil which has a free acidity, expressed as oleic acid, of not more than 0.8 grams per 100 grams, and the other characteristics of which correspond to those fixed for this category in this standard" (United Nations, 2003). This low value upper limit results in just 10 % of the olive oils produced worldwide being of the extra virgin quality (Visioli et al., 2000a). Likewise, the IAOOTO states

that olive oil (formerly known as "pure" olive oil (POO)) is "the oil consisting of a blend of refined olive oil and virgin olive oils fit for consumption as they are and has a free acidity, expressed as oleic acid, of not more than 1 gram per 100 grams and its other characteristics correspond to those fixed for this category in this standard" (United Nations, 2003; The Olive Oil Source, 2005). POO can contain oil that has been subjected to further filtering with charcoal or other chemical or physical filters (The Olive Oil Source, 2005) and thus is less likely to retain as many phytochemicals as virgin quality olive oil, that by definition is not further filtered (Visioli & Galli, 1998a). As evidenced by these international trade standards, the general quality of olive oil samples, as measured by the lack of hydrolytic rancid byproducts such as free fatty acids (FFAs), is very important. Although virgin olive oils contain higher concentrations of phytochemical compounds that possess antioxidant activities (Boskou, 2000) and may have beneficial effects toward cardiovascular disease (Visioli & Galli, 1998a), they also tend to contain higher concentrations of FFAs as compared to more processed types of olive oils (United Nations, 2003). As FFAs have undesirable sensory properties (Meijboom, 1964), there is thus a paradox in the benefits of olive oil such that the "desirable" compounds are coupled with the "undesirable" compounds. The differences and trends in such identification need to be examined so that general comparisons among quality parameters can be evaluated and an overall statement of the quality of an oil sample can be determined.

Little research has been published in peer-reviewed journals addressing the concern with the quality and oxidative stability of olive oils commercially available in the United States. This is of utmost importance given that the most recent standards

for olive oils sold to consumers in the United States are from 1948 (United States Department of Agriculture (USDA), 1948) and that the terminology used to define olive oils commercially sold in the United States are rarely displayed on the label. The Food and Drug Administration (FDA) recently approved a new qualified health claim for olive oil which states that "limited and not conclusive scientific evidence suggests that eating about 2 tablespoons (23 grams) of olive oil daily may reduce the risk of coronary heart disease due to the monounsaturated fat in olive oil… to achieve this possible benefit, olive oil is to replace a similar amount of saturated fat and not increase the total number of calories you eat in a day" (FDA, 2004a). This was based on 12 intervention studies deemed useful in evaluating the claim, four of which were deemed most persuasive (FDA, 2004b). The results from these and several other intervention studies have indicated that diets high in monounsaturated fats from olive oil decrease the concentration of serum LDL cholesterol as compared to diets high in saturated fats (Denke & Grundy, 1991; Kris-Etherton et al., 1999). However, as some of the beneficial effects of olive oil toward coronary heart disease have been linked to its phytochemical composition (Visioli & Galli, 1998a; Hu, 2003), it is very important that the evaluation of the quality of olive oils sold in the United States is performed using up-to-date and appropriate methods and standards. Although there are methods for evaluation as addressed in the current United States standards (USDA, 1948), the official United States terminology for classification is not required on olive oil products and generally is omitted in favor of international standard naming conventions defined in the IAOOTO that are not officially recognized by the United States government. The use of terminology not governed by the United States

to classify the quality of olive oils commercially available in the United States may result in oils that vary greatly in overall quality.

Olive oil has been an intergovernmental regulated source of dietary international trade since the inception of the International Olive Oil Agreement in 1956 (United Nations, 2003). Shortly thereafter, the International Olive Oil Council (IOOC) was created to oversee the administration of this agreement and has governed its usage ever since. In 1986, the International Olive Oil Agreement was formally rewritten as the IAOOTO which is the current document used to govern trade by the IOOC (International Olive Oil Council, 2005a). The IOOC reviews this document maximally every two years to reflect the ever changing standards set forth by the Codex Alimentarius Commission (CAC) and the European Union (EU). The CAC was created in 1963 by the Food and Agriculture Organization (FAO) of the United Nations and the World Health Organization (WHO) to develop standards and guidelines intended to protect the health of consumers, ensure fair trade practices, and promote coordination of food standards (Codex Alimentarius, 2005). Unfortunately the IOOC guidelines do not pertain to the United States, since, as of May 2005, the United States was not a member of the IOOC and thus did not have to abide by the standards and naming conventions set forth in the IAOOTO (International Olive Oil Council, 2005b). The United States has its own set of standards enacted on March 22, 1948, that have neither been updated nor amended since their inception by the USDA over half a century ago (USDA, 1948). While the IOOC standardizes labeling vocabulary with a grading system that uses distinctions such as EVOO, VOO, and olive oil (United Nations, 2003), the United States has its own formal terminology

(USDA, 1948). Although not commonly used, this system labels oils as U.S. Grade A though D and with alternative titles of U.S. Fancy, U.S. Choice, U.S. Standard, and Substandard, respectively (USDA, 1948). Seemingly due to the increasing popularity of terms defined in the IAOOTO, most olive oils sold in the United States have decided to adopt the IOOC-accepted titles. However, since the United States is not required to recognize the definitions set forth in the IAOOTO, these definitions do not always ensure the assumed quality of the oils (California Olive Oil Council (COOC), 2004). By importing most of its olive oil from countries that are governed by the IAOOTO, the United States government does not have to be as strict in making and enforcing its own regulations for olive oils sold in the United States. The imported oils are guaranteed to be of a certain quality as defined by their classification at their time of importation. However there is no guarantee as to the quality of the oils by the time they are offered to consumers.

Less than 0.1 % of the worldwide production of olive oil was produced in the United States (900 metric tons), as compared to the top three producers Spain, Italy, and Greece with 1,329,500, 500,000, and 367,000 metric tons, respectively (FAOSTAT data, 2004). As can be expected, for the most recent data available – 2002, these three countries were also the top exporters worldwide (FAOSTAT data, 2004). Since the IOOC's enactment of the IAOOTO in 1986, the United States has increased its importation of olive oil by at least 9000 metric tons per year nine of the last 16 years where this trend only occurred once in the 25 years prior to 1986 (FAOSTAT data, 2004). This has equated to over 436 million dollars from this importation in 2002 alone (FAOSTAT data, 2004). Likewise, of the countries that

imported olive oil in 2002, the United States ranked second only to Italy in metric tons imported, 215,195 and 520,110, respectively (FAOSTAT data, 2004) and thus the United States gets over 99.5 % of its olive oil via importation. Ideal conditions for growing olive trees are between 35 and 45 degrees latitude (Visioli & Galli, 1998a) and so based on the temperature and climate found at these locations, growth is conceivable in the United States. Parts of California have been used to successfully grow olive trees in the United States (COOC, 2005; USDA, 2005) and approximately 99 % of the olive oil produced in the United States is from olives grown in California (USDA, 2004). By keeping growth and production of olive trees and olives in the United States to a minimum, the lack of enforcement of the United States standards by the United States government may go unnoticed and therefore bottlers and sellers of olive oils in the United States do not have to be overtly troubled with strict guidelines, evaluation procedures, and labeling concerns.

It should not be mistaken that olive oil sold in the United States is entirely free of governmental regulations as there are regulations as to what a commercial olive oil product can and must have on its label. Product descriptions on the principal display panel of food, including olive oil (NAOOA, 2002), are regulated by Section 403(a)(1) of the Federal Food, Drug and Cosmetic (FD&C) Act which was enacted to prevent food products from being misbranded (FDA, 1999). Thus an olive oil that was not obtained under the assumption that it was of an extra virgin quality could not be labeled as such (NAOOA, 2002). However, the importation of olive oil does not imply that (a) the correct naming terminology will be used for the product, (b) the oil that is bottled and sold still meets the standards by which it was imported, and (c) the

oil be sold and consumed in the lifetime of the olive oil, which is generally recognized by most producers as 12 – 18 months after bottling (Morelló et al., 2004a). Although an olive oil sample can be imported as an EVOO, by the time the oil is offered to consumers the oil could have degraded from storage or harsh conditions and thus no longer fall under the classification under which it was imported. This creates a problem in terms of assuring consumers that the product they bought is of the expected and implied quality.

As there is no United States body of government that oversees importation and labeling of olive oil, a private company, the NAOOA, regulates ca. 80 % of the olive oil imported into the United States (NAOOA, 2005). However, there are two problems with this. First, since the NAOOA is a private identity, consumers are not guaranteed the same level of strict regulatory enforcement as would be done by a United States government association. Second, the NAOOA guidelines solely regulate by qualitative aspects of olive oils, such as whether the oil is filtered or if it consists of a blend of different classifications of olive oils, and do not have definitive standards for classifying and labeling oils (NAOOA, 2002). This is not the case for many European bottlers and sellers that are regulated by the IAOOTO (United Nations, 2003). In the mid 1990s, the NAOOA petitioned the United States government to update its standards however the petition has been supposedly bogged down in the bureaucratic process since that time (NAOOA, 2005). Recently (November 4, 2004), the COOC submitted a petition to the USDA to update its current, 1948 standards (USDA, 2004). The petition is still under review as comments and supporting claims on the initial petition were not due until December

8, 2004 (USDA, 2004). Until guidelines change, it is important to analyze the FFA content of olive oils commercially available in the United States to verify that the oils abide by the most common international standards so that consumers are not misled as to the quality of the product they are purchasing.

Overall, olive oils commercially available in the United States need to be assayed for their fatty acid composition, concentrations of phytochemicals, oxidative stability, and antioxidant capacities. It is hypothesized that these olive oils will differ in their concentrations of rancid byproducts and total phenolic contents, oxidative stability, and antioxidant capacities, yet will possess similar fatty acid compositions. Obtaining the FFA value for each olive oil sample will allow the quality of olive oil products commercially available in the United States to be compared against their international counterparts as well as the internationally-accepted standards for olive oil labeling and trade. It is also hypothesized that some of these olive oils will exceed the international legal limit for their labeled classifications. In addition, further exploration of whether the price of an olive oil correlates well to its quality and stability will help determine if the price of an olive oil commercially available in the United States accurately portrays to the consumer the quality of the oil. In addition, it is hypothesized that the price of these olive oils will correlate to the above parameters where higher-priced oils will be associated with better quality parameters. This will aid consumers in their practical evaluation of the quality of olive oil products that are commercially available in the United States.

Chemical Composition

The chemical composition of olive oils varies greatly based on the many factors (di Giovacchino, 2000). Albeit there are many published concentration ranges for the compounds that comprise olive oil samples (Itoh et al., 1981; Montedoro et al., 1992; Jiménez de Blas & del Valle González, 1996; Manzi et al., 1998; Visioli & Galli, 1998a; Servili et al., 1999; Stefanoudaki et al., 1999; Boskou, 2000; Brenes et al., 2000; Owen et al., 2000a; Psomiadou et al., 2000; Giacometti, 2001; Boskou, 2002; Nenadis & Tsimidou, 2002; Psomiadou & Tsimidou, 2002a; Psomiadou & Tsimidou, 2002b; Beltrán et al., 2004), the findings tend to have some overall consistencies.

Fatty Acid Composition

Stefanoudaki and colleagues (1999) showed using canonical discriminant analysis that the fatty acid composition of olive oil samples depended on the altitude where the olives were grown as well as other environmental factors such as relative humidity and rainfall. Additionally, fatty acid composition of VOOs varies year-to-year and by the harvest date, which is probably due to differences in the amount of summer rainfall in the growing region and temperature during olive fruit ripening and oil biosynthesis (Beltrán et al., 2004). Regardless, the fatty acid composition of olive oil consists primarily of oleic acid (*cis* 18:1n-9) which ranges from 55.0 – 83.0 % of the fatty acid content in the sample (Codex Alimentarius, 2001) and where specific studies have found it to range from 74.66 to 81.02 % of the fatty acid content in the sample (Stefanoudaki et al., 1999; Beltrán et al., 2004). Morelló and colleagues

(2004a) noted that after 12 months of VOO storage in sealed bottles in the dark, the trend of percent of oil as oleic acid increased whereas the trend for polyunsaturated essential fatty acids (i.e. linoleic (18:2n-6) and linolenic (18:3n-3)) both decreased. This trend was also seen in oils that were made from olives harvested "first" (i.e. from November to January) versus "last" (i.e. January and beyond) (Morelló et al., 2004a). Regarding the remaining fatty acids found in olive oil, the next concentrated fatty acids are palmitic acid (16:0) and 18:2n-6 and they account for 7.5 – 20.0 % and 3.5 – 21.0 % of the fatty acid content in olive oils, respectively (Codex Alimentarius, 2001).

Simple Phenolics

Phenolic compounds are those with at least one hydroxyl group attached to an aromatic ring whose known benefits reside in their function as antioxidants. The polar fraction of VOO samples includes these phenolic compounds which have been reported to be 50 – 800 mg/kg olive oil (Visioli & Galli, 1998a). This has been supported by Montedoro and colleagues (1992) who found that the phenolic fraction of EVOO ranged from 50 – 1000 mg/kg. As already explored, this is generally due to many reasons, the most important of which are cultivar, soil, degree of olive fruit ripeness, means by which the oil is extracted, and storage conditions (Visioli & Galli, 1998a). Additionally, plants will increase their synthesis of phenolic compounds in response to pathogens and pollutants and thus the conditions of the environment where the crops were grown may result in varying concentrations of biosynthesized phenolic compounds (Zobel, 1996). The phenolic fraction of VOO consists of many

simple phenolics and secoiridoids. One of the most active as an antioxidant is hydroxytyrosol (HTyr), a compound that attenuates the formation of peroxides under accelerated oxidation in the presence of oxygen and heat (Papadopoulos & Boskou, 1991; Stupans et al., 2002). HTyr has a more complex form, oleosidic, found commonly in very high concentrations in the olive fruit, as well as oleuropein (Oleur) and some of its minor derivatives and aglyconic derivatives which are also very active antioxidants (Stupans et al., 2002). HTyr (3,4-(dihydroxyphenyl)ethanol) has one benzene ring with two hydroxyl groups attached *ortho* to each other (*o*-diphenolic) and is a metabolite of Oleur which is simply HTyr conjugated to elenolic acid by an ester bond. The increased antioxidant activity of these compounds may be due to the diphenolic structure of their aromatic rings (Rice-Evans et al., 1996; Visioli & Galli, 1998b). This structure allows them to become stable phenoxyl radicals via an intramolecular hydrogen bonding interaction between the hydroxyl group and the adjacent phenoxyl free radical (Visioli & Galli, 1998b) and may confer more delocalization of the free radical as is the case with many flavonoids and phenolic acids that possess *o*-diphenolic structures (Rice-Evans et al., 1996). Additionally, after two successive reductive hydrogen atom donations, *o*-diphenolic compounds form very stable diketones (Nawar, 1996; Visioli & Galli, 1998b). The ability of both of these compounds to be retained in olive oil even after washing with some water, as is often done in the extraction process, illustrates their amphipathic nature and possible antioxidant activities towards both lipophilic and hydrophilic substances. However in unilamellar liposomes made from soybean phospholipids, these main beneficial phenolics, HTyr and Oleur and its derivatives, did not penetrate into the

membrane and were instead associated with the outer surface of the phospholipid bilayer (Paiva-Martins et al., 2003). Yet, as these were synthetic liposomes, transport proteins may actually exist *in vivo* that can possibly transport phenolic compounds into the cell. Also, HTyr increases cytosolic Ca^{2+} in a dose-dependent manner in human lymphomonocytes suggesting its antioxidant role may involve intracellular release of calcium stores (Palmerini et al., 2005). Servili and colleagues (1999) identified HTyr and Oleur and its minor derivatives separated from VOO samples by high performance liquid chromatography (HPLC) and further supported their identification with 1D- and 2D-, ^{1}H and ^{13}C nuclear magnetic resonance (NMR). They were found in mean concentrations of 9.0 – 15.6 mg/kg and 365.6 – 752.3 mg/kg of olive oil, respectively, depending on the extraction method (Servili et al., 1999).

Lignans

Phytoestrogens are non-steroidal, diphenolic compounds that are biosynthesized in plants and are protective agents against cardiovascular disease and hormone-dependent cancers (Lephart et al., 2005). The phytoestrogen content of olive oil predominantly consists of the lignans (+)-pinoresinol and (+)-1-acetoxypinoresinol as evidenced by MS and ^{1}H and ^{13}C NMR (Brenes et al., 2000; Owen et al., 2000a) and are found at 2.7 – 66.9 mg/kg and 11.7 – 41.2 mg/kg of EVOO, respectively (Brenes et al., 2000). These compounds also contain a phenolic ring and thus may possess antioxidant activities (Dragsted, 2003). Identification of these compounds as major components of olive oil resulted in their ranging from 0.65

– 99.97 mg/kg of EVOO, yet they were virtually absent from more refined olive oils (Owen et al., 2000a).

Flavonoids

Flavonoids are phytoestrogenic compounds that are more complex than simple phenolics yet normally contain phenolic ring structures (Singleton et al., 1998). Bouaziz and colleagues (2004, 2005) found that the flavonoid content of olive fruit consists primarily of the flavones, including luteolin 7-*O*-glucoside, luteolin and apigenin, and the flavonols, including rutin, quercetin 3-arabino-glucoside and quercetin. All of these compounds contain phenolic ring structures and thus likely possess antioxidant activities (Dragsted, 2003). Of these, the predominant flavonoids were rutin, luteolin 7-*O*-glucoside, and quercetin 3-arabino-glucoside (Bouaziz et al., 2004). The first two compounds were more potent antioxidants in a liposome model based on percent inhibition of conjugated diene hydroperoxides (Morelló et al., 2005). Further, their IC_{50} values at 30 min reaction against the 2,2-diphenyl-1-picrylhydrazyl (DPPH) stable free radical were about 4 times that of butylated hydroxytoluene (BHT) (Bouaziz et al., 2005).

In terms of VOOs themselves, Mateos and colleagues (2001) identified flavones luteolin and apigenin. Also, Brenes and colleagues (1999) discovered that as the olives used to make olive oil ripened longer, luteolin concentrations increased whereas apigenin concentrations, which are very minor regardless, did not show a consistent trend.

Carotenoids and Tocopherols

Carotenoids and tocopherols are also found in olive oils in appreciable amounts. However tocopherol concentrations in olive oils are generally lower than in many other vegetable oils (Visioli et al., 2000a). Both carotenoids and tocopherols possess antioxidant capacities, however only the tocopherols contain a phenolic ring structure and may quench free radicals *in vivo* (Boskou, 2002). Carotenoids contain a large amount of conjugation that allows them to be excellent singlet oxygen quenchers and protect oil from photooxidation (Boskou, 2002). Su and colleagues (2002) found carotenoid content to be predominantly β-carotene and lutein and tocopherol content to be α- and γ-tocopherol. The concentration of α-tocopherol in EVOOs range from 98 – 370 mg/kg (Psomiadou et al., 2000; Psomiadou & Tsimidou, 2002a; Psomiadou & Tsimidou, 2002b) while β-carotene and lutein ranged from 1.0 – 2.7 and 0.9 – 2.3 mg/kg, respectively (Psomiadou & Tsimidou, 2002a; Psomiadou & Tsimidou, 2002b). Manzi and colleagues (1998) had similar results as measured by normal phase HPLC for α-tocopherol (160.95 – 206.76 mg/kg) and carotenes (1.25 – 4.39 mg/kg). Generally the concentrations of carotenoids and tocopherols in olive oils vary greatly as they tend to range from 1 – 20 mg/kg and 5 – 300 mg/kg oil, respectively (Boskou, 2000), with α-tocopherol representing about 95 % of the tocopherol fraction (Stark & Madar, 2002). Few olive oils have carotenoid concentrations above 10 mg/kg oil, while higher quality oils tend to have tocopherol values over 100 mg/kg oil (Boskou, 2000).

Terpenoids

The unsaponifiable fraction of olive oil is primarily squalene (~5451 mg/kg = ~86 %), followed by β-sitosterol (~718 mg/kg = ~11 %), and then relatively minor amounts (< 1 %) of other compounds including the phytosterols campesterol and stigmasterol (Giacometti, 2001). Nenadis and Tsimidou (2002) and Psomiadou and Tsimidou (2002a, 2002b) had similar results by reverse phase (RP)-HPLC for squalene, 2078 – 6520 mg/kg and 3882 – 5858 mg/kg, respectively. However, squalene only constitutes ~ 0.5 – 0.7 % of the olive oil (Newmark, 2000) and thus these unsaponifiables represent only a small fraction of the entire olive oil sample. Albeit, olive oil contains 7- to 300-fold more squalene than most other vegetable oils and up to 5000-fold more than some vegetable oils (Ostlund et al., 2002). Squalene is a precursor of phytosterols in plants and a precursor of cholesterol in humans (Ostlund et al., 2002). It significantly suppresses colonic azoxymethane (AOM)-induced colonic aberrant crypt foci (ACF) formation and crypt multiplicity in male F34 rats (Rao et al., 1998) and effectively inhibits 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK)-induced lung tumorigenesis in female A/J mice (Smith et al., 1998). However, it may also increase serum cholesterol in humans (Ostlund et al., 2002). More research needs to be conducted into squalene's disparate roles.

Steronoids

Phytosterols are compounds that can compete with cholesterol for absorption in human digestive systems, but may be 20 times more difficult to absorb than cholesterol itself (Boskou, 2000), thus possibly leading to decreased intake of

cholesterol and a possible decreased risk for atherosclerotic conditions. The phytosterol content of olive oil ranges from 1000 – 2000 mg/kg oil and is made up of four classes of sterols: 4-α-desmethylsterols, 4-α-methylsterols, 4,4-dimethylsterols, and triterpene dialcohols (Boskou, 2002). The 4-α-desmethylsterols are the predominant class found in olive oil (Boskou, 2002). Jiménez de Blas and del Valle González (1996) identified the trimethylsilyl derivatives of VOO sterols by gas chromatography (GC). They consist primarily of β-sitosterol (mean = 84.9 %), followed by Δ5-avenasterol (7.8 %) and campesterol (3.2 %), and with under one percent of many other sterols, including a minor amount of cholesterol (Jiménez de Blas & del Valle González, 1996). The general concentrations for these three most abundant 4-demethylsterols in olive oil samples range from 75 – 90 %, 5 – 36 %, and ~3 % of the total sterol fraction, respectively (Boskou, 2000). Itoh and colleagues (1981) showed that the sterol composition of VOOs by GLC consisted primarily of β-sitosterol (mean = 89.3 %), followed by 28-isofucosterol (5.6 %) and campesterol (3.0 %), and again with other minor sterols under one percent. Both of these studies showed similar values in refined olive oil as well and thus these phytochemical compounds seem more stable towards filtering than their phenolic counterparts.

Sensory Properties

Color

The color of olive oil is usually due to the lipophilic chlorophyll and pheophytin pigments present in the olive fruit, where pheophytin a is usually the predominant determining factor (Boskou, 2000). Transporting the olives quickly to

the olive mill for processing is important as well. Olives should be transported in plastic cases with special holes that allow for air circulation to help disperse the heat produced by the catalytic actions of the olives and should not be transported in jute sacks.

Flavor

The flavor of olive oil ranges from bitter/pungent to ripe/sweet as the ripeness of the olive fruit increases (di Giovacchino, 2000; Morelló et al., 2004b) and is primarily due to non-volatile compounds in the oil, particularly the bitter and pungent secoiridoid derivative of oleuropein (Visioli et al., 2002b; Mateos et al., 2004), the bitter and pungent phenolic constituents – specifically phenol and cinnamic acid (Visioli et al., 2002b), the pungent and burning secoiridoid derivative of ligstroside (Andrewes et al., 2003), and aldehydes. High concentrations of phenolic compounds in olive oils are very likely to result in oils having a high stability yet a strong, "fruity" flavor that may not be particularly preferred by consumers (Visioli et al., 2002b). On the other hand, "sweet" oils are almost devoid of phenolic compounds (Visioli et al., 2002b). Use of the centrifugation method for extraction of the olive oil from the olive fruit also results in less retention of volatile compounds, specifically those responsible for "fusty," "winey," and "vinegary" defects in the flavor of some olive oils due to the fermentation products isoamyl alcohol, acetic acid, ethyl acetate, respectively (di Giovacchino, 2000). Additionally, the removal of leafs and other foreign material by fans and washing is also essential to prevent the "green-leaf" flavor of certain olive oils (di Giovacchino, 2000).

Aroma

Unripe olive fruits also tend to produce an olive oil with an herbaceous aroma (di Giovacchino, 2000) as well as failure to remove most of the olive seeds included in the extraction process (Luaces et al., 2003). The aroma of an olive oil is mainly due to low molecular weight, relatively nonpolar compounds in the sample that are easily volatilized at room temperature. These are generally many short-chain compounds consisting solely of hydrocarbons or with a good amount of nonpolar character and a minimal number of polar functional groups (i.e. aldehydes, ketones, alcohols, carboxylic acids, and esters) (Sánchez & Salas, 2000). Variety of olive fruit is the most important factor in determining the volatile profile of olive oil while malaxation time and temperature are secondary (Tura et al., 2004). Although numerous (i.e. over 100) volatile hydrocarbon compounds have been identified in olive oil (Boskou, 2000), the main compound in virgin and EVOOs is usually trans-2-hexenal, an early lipoxygenase byproduct formed by the oxidation of α-linolenic acid (Angerosa et al., 1999; Tura et al., 2004; Zunin et al., 2004). Using ^{1}H-NMR, Sacchi (1996) showed that hexanal, also an early lipoxygenase byproduct formed by the oxidation of 18:2n-6, was consistently higher than hexenal.

Health Benefits

Keys and colleagues (1986) first hypothesized that the traditional Mediterranean diet has beneficial effects towards many diseases and provided

evidence based on cohort studies that the diet was associated with fewer deaths from coronary heart disease than subjects on non-Mediterranean European diets. Olive oil has long been the major fat source in the diet of people from most Mediterranean countries (Boskou, 2000) and thus was thought to play a role in the overall benefits of the diet. Recently its interest as a healthy fat source has been expanding to other countries, including the United States (Boskou, 2000), mainly due to the inherent health benefits associated with its high levels of monounsaturated fatty acids and phenolic compounds (Hu, 2003). Increased intake of olive oil lowers the incidence of cancers (Stark & Madar, 2002) and cardiovascular diseases through hypocholesterolemia, hypotension, and decreased lipid oxidation (Scaccini et al., 1992; Stark & Madar, 2002, Moreno & Mitjavila, 2003a), mediates the immune response (de la Lastra et al., 2001; Stark & Madar, 2002), and controls hypertriglyceridemia that accompanies diabetes (Giron et al., 1999) and other aging-associated health problems (Trichopoulou & Vasilopoulou, 2000a).

Cancer Risk Reduction

Boskou (2002) has reported an association between phenolic compounds and the beneficial roles of olive oil in human health. An inverse association between intake of monounsaturated fat and the risk of breast cancer (Trichopoulou et al., 2000b) supports the thought that the high level of monounsaturated fatty acids may play a role in the overall health benefits of olive oil consumption. This was further supported by a case-control study of Mediterranean populations where weak inverse associations between monounsaturated:saturated fatty acid ratio and large bowel,

breast, endometrium, and prostate cancer were observed (Trichopoulou et al., 2000b). The high concentrations of monounsaturated fat might account for these associations. Takeshita and colleagues (1997) found that in ICR mice fed various diets that either contained normal safflower oil, which is naturally low in oleic acid, or safflower oil from a mutant strain, resulting in high oleic acid content, there was no difference in 1,2-dimethylhydrazine-induced colon carcinogenesis as measured by tumor incidence and multiplicity. Thus, high intake of oleic acid may not be solely responsible for the chemopreventive affects of olive oil. As many phytochemicals are retained in olive oil due to its specific processing methods, these compounds may also play an important role in the overall health benefits of the oil in the context of a healthy diet.

Larger clinical and epidemiological studies have illustrated the inherent negative association between cancer risk and olive oil consumption. An ecological study using existing international databases and already published data was used to determine that 76 % of the intercountry variation in colorectal cancer incidence rates was explained by three of ten factors examined, where the only inversely associated factor was found to be intake of olive oil (Stoneham et al., 2000). A case-control study as part of the European Community Multicenter Study on Antioxidants, Myocardial Infarction, and Breast Cancer on risk for breast cancer in five centers across Europe (Germany, Netherlands, Northern Ireland, Spain, and Switzerland) showed that higher consumption of oleic acid, the predominant fatty acid in olive oil, was significantly related to a decreased risk of breast cancer only in the Spanish center, with an odds ratio of 0.40 (95 % confidence interval (CI) = 0.28 – 0.58 for the 75^{th} versus the 25^{th} quartile of intake) (Simonsen et al., 1998). Only in the

Netherlands was there another significant odds ratio for oleic acid intake at 2.36 (1.01, 5.50) where it was now directly correlated to breast cancer risk (Simonsen et al., 1998). In the other countries' centers pooled the odds ratio was 1.27 (0.88, 1.85). As oleic acid is the predominant fatty acid in olive oil and Southern Europeans tend to ingest most of their oleic acid from olive oil, it seems to beg the idea that additional compounds in olive oil may be responsible for its breast cancer-preventive functions (Simonsen et al., 1998). This idea was further supported in a case-control study of risk for breast cancer in Spain where higher consumption of olive oil, and neither total fat intake nor individual types of fat, was significantly related to a decreased risk of breast cancer with an odds ratio of 0.66 (95 % confidence interval (CI) = 0.46 – 0.97 for the highest versus the lowest quartile of intake) (Martin-Moreno et al., 1994). A similar trend was seen in a case-control study of risk for breast cancer in Greece where higher consumption of olive oil was significantly associated with a decreased risk of breast cancer, with an odds ratio of 0.75 (95 % confidence interval (CI) = 0.57 – 0.98 for eaten once a day versus more than once a day) (Trichopoulou et al., 1995). Willett (1997) claimed that from a review of international comparisons, case-control studies, and cohort studies there was not enough evidence to correlate increased dietary intake of monounsaturated fatty acids with increased risk of breast cancer. However, in at least three case-control studies conducted in Greece, Spain, and Italy, olive oil was associated with a decreased risk of breast cancer (Willett, 1997). Also, in a large pooled analysis of cohort studies there was no significant decrease in breast cancer risk from consuming monounsaturated fatty acids in the place of any other type of fat or carbohydrate (Smith-Warner et al., 2001) when the variable of location

was removed by pooling various studies. In a multi-center case-control study of risk for ovarian cancer in Italy higher consumption of olive oil was significantly related to reduced risk of ovarian cancer with an odds ratio of 0.68 (95 % confidence interval (CI) = 0.50 – 0.93 for the highest versus the lowest quintile of intake) (Bosetti et al., 2002). A case-control study of risk for squamous cell esophageal cancer in Northern Italy illustrated that olive oil intake showed a significant reduction of cancer risk with an odds ratio of 0.3 (95 % confidence interval (CI) = 0.1 – 0.5 for the highest versus the lowest quintile of consumption) (Bosetti et al., 2000). A similar trend was seen in a case-control study of risk for cancer of the oral cavity and pharynx in Italy where consumption of olive oil was significantly associated with a decreased risk of these cancers with an odds ratio of 0.4 (95 % confidence interval (CI) = 0.3 – 0.7 for the highest versus the lowest quintile of consumption) (Franceschi 1999).

Cardiovascular Disease Risk Reduction

Changes in lipid composition and an overall decreased risk for coronary and ischemic heart disease are correlated with intake of olive oil. Olive oil intake is inversely correlated with plasma low density lipoprotein (LDL) concentrations and directly correlated with plasma high density lipoprotein (HDL) concentrations (Moreno & Mitjavila, 2003a). Yet olive oil's role goes beyond the reshuffling of the lipoprotein fractions as olive oil possesses antioxidant activities and specifically can decrease LDL oxidation and LDL peroxidation in cardiovascular tissues (Boskou, 2000; Kris-Etherton et al., 2002; Moreno & Mitjavila, 2003a; Vissers et al., 2004). Krzeminski and colleagues (2003) showed that when rats ate a high olive oil and high

cholesterol diet, as compared to rats on simply the high cholesterol diet, they had significantly lower plasma total cholesterol, LDL cholesterol, triacylglycerols, total phospholipids, and hepatic total cholesterol and significantly higher plasma antioxidant level, pancreatic bile flow, bile cholesterol, bile acids, and fecal dry output. In all cases, the VOO diet group showed superior results over a more processed olive oil diet group as VOO is believed to retain more beneficial phytochemicals since it is not filtered. Simply the intake of high monounsaturated fatty acid oils is beneficial in terms of serum triacylglycerol and lipoprotein concentrations and thus cardiovascular health. Kris-Etherton and colleagues (1999) showed in a randomized, double-blind, crossover study design that subjects who ate high monounsaturated fatty acid oil as olive oil and peanut oil showed a significant decrease in serum total cholesterol, LDL cholesterol, triacylglycerols, and apolipoprotein B as compared to an Average American diet. Larsen and colleagues (1999) showed in a randomized, double-blind, crossover study design that subjects who ate diets enriched with VOO, versus refined sunflower or rapeseed oil, had a slightly significant decrease ($P < 0.10$) in non-fasting peak concentrations of activated blood coagulation factor VII, which is known to be a key protein in thrombogenesis and ischemic heart disease. The fact that the olive oil was not refined may support the finding that additional phytochemicals maintained in olive oil from its lack of refinement may have health-beneficial effects.

Additionally, the role of the inflammatory response in disease progression has lead researchers to explore olive oil's role in inhibiting it. In macrophage cells some of the major compounds found in olive oil (i.e. squalene, β-sitosterol, and tyrosol

(Tyr)) were all capable, to some extent, of decreasing the expression of cyclooxygenase-2, prostaglandin E_2/leukotriene B_4 synthesis, and nuclear factor kappa B expression (Moreno, 2003b) which are all involved in the inflammatory response. Also, in these inflammatory cells (i.e. macrophages) damaging reactive free radicals can be generated *in vivo* and olive oil compounds could, to some extent, decrease the undesirable anionic superoxide free radical concentration, hydrogen peroxide production, arachidonate release, and nitric oxide free radical release – as measured by nitrite accumulation (Moreno & Mitjavila, 2003a). Miles and colleagues (2005) showed that in human blood cultures containing mononuclear cells various phenolic compounds derived from olive oil could decrease the concentrations of the inflammatory cytokine interleukin-1β as well as the inflammatory eicosanoid prostaglandin E_2. Thus, olive oil phytochemicals may play a role in decreasing the inflammatory response that accompanies the progression of various diseases. Olive oil phenolic compounds inhibit platelet aggregation, reduce pro-inflammatory thromboxane B_2, and inhibit respiratory burst of neutrophils (Visioli et al., 2000a). Thus, olive oil has the potential to reduce atherosclerotic plaque formation by decreasing the inflammatory response and reducing platelet aggregation to sites of oxidation in smooth muscle cells.

Antioxidant Activities

Because reactive oxygen species (ROS) and the arachidonate lipoxygenase pathway have been linked to the pathogenesis of cancer (Floyd, 1990), cardiovascular disease (Ross, 1993; Moreno & Mitjavila, 2003a), and brain ischemia (Floyd, 1990),

olive oil's involvement in their inhibition has also been studied. *In vivo* studies with rats have illustrated the proposed beneficial health effects of olive oil phytochemicals. Rats have a greater resistance to LDL oxidation when they were fed EVOO versus 75 % oleic acid in the form of triolein (Scaccini et al., 1992). Hydroxytyrosol (HTyr) is one of the strongest antioxidants in olive oil on a per molar basis and found to be more potent than the typical synthetic antioxidant used as a preservative, BHT (Wiseman et al., 1996). When rats were administered HTyr it resulted in an increase in their plasma antioxidant capacity (Visioli et al., 2000b). Additionally, a low dose of HTyr was able to decrease urinary excretion of the F_2-isoprostane 8-*iso*-prostaglandin (PG) $F_{2\alpha}$, a marker of arachidonic acid free radical-induced peroxidation in phospholipids seen in passive smoking-induced oxidative stress in rats (Visioli et al., 2000c). In human volunteers, urinary excretion of 8-*iso*-$PGF_{2\alpha}$ was inversely correlated to the amount of phenolics ingested with olive oil in a dose-dependent manner (Visioli et al., 2000d). Also, Owen and colleagues (2000b) showed that the fecal matrix of adenoma patients produced various ROS and that the phenolic compounds from olive oil were able to scavenge them as evidenced by the formation of dihydroxybenzoic acids from salicylic acid that were identified by HPLC. β-sitosterol, the predominant phytosterol found in olive oil, was able to attenuate growth of LNCaP cells, a human prostate cancer cell line (Jones, 2002). Also, in the treatment of males with benign prostatic hyperplasia, β-sitosterol improved flow measures and urological symptoms, both favorable alterations (Jones, 2002). Additionally, the hypoxanthine/xanthine oxidase (XOD) assay was utilized in Caco-2 cells, a human colorectal adenocarcinoma cell line, where preincubation with

as little as 100 μmol/L of HTyr, or olive oil high in *o*-diphenolic compounds, completely protected the cells from oxidative stress induced by xanthine oxidase (i.e. superoxide free radicals and hydrogen peroxide as measured by malondialdehyde content (Manna et al., 1997)) and greatly increased cell viability (Manna et al., 2002). A similar trend was seen with the application of olive oil samples containing high concentrations of *o*-diphenolics like HTyr where they decreased the percent hemolysis of human erythrocytes (Manna et al., 2002).

Bioavailability

There has also been much research and analysis on urinary excretion of phenolic metabolites to support the hypothesis that the most abundant and most potent olive oil phenolics, Tyr and HTyr, respectively, are bioavailable in both rats (Tuck et al., 2001; Tuck et al., 2002; Visioli et al., 2003) and humans (Visioli et al., 2000e; Miró-Casas et al., 2001a; Miró-Casas et al., 2001b; Covas et al., 2003; Miró-Casas et al., 2003; Visioli et al., 2003; Vissers et al., 2004). Vissers and colleagues (2002) confirmed that HTyr, and its oleosidic from Oleur, and Tyr, and its oleosidic from ligstroside (Ligstro), were absorbed and processed in the intestines of humans and were not metabolized by colonic bacteria. Also, Manna and colleagues (2000) showed that in Caco-2 human intestinal epithelium cancer cells HTyr is absorbed by passive diffusion and that this passage is bidirectional.

Although this information points to olive oil's phenolic antioxidant fraction as a major reason for the above health benefits, Vissers and colleagues (2004) reviewed

that the concentration of phenolic compounds needed to exhibit antioxidant activity *in vitro* was at best about 1000-fold higher than could be achieved by eating 50 g of olive oil per day. Thus, they claim that the consumption of olive oil phenolics alone cannot account for the decreases in LDL oxidation and oxidation of other compounds as evidenced by biomarkers that are seen with increased olive oil consumption (Vissers et al., 2004). Thus another unexplored mechanism may be responsible.

Analytical Methods

Various analyses have been conducted on olive oil samples. These can be categorized as follows: characterization tests, antioxidant assays, and stability tests. More details are described below.

Fat Characterization Tests

The predominant components of olive oil samples are fats. The fatty acid composition of olive oil samples has been tested numerous times in past studies but generally by the same procedure. Briefly, the method begins with saponification of the bound fatty acyl sidegroups to free them from their ester bonds. Then, each free fatty acid is then methylated to a fatty acid methyl ester (FAME). FAMEs are created as they are much more easily volatilized by GC so they can be separated and then accurately quantified by a detector. As certain fatty acids are associated with changes in health status, it is vital to the research goals of this proposal to obtain this information.

The iodine value (IV) is a measure of the number of carbon-carbon double bonds in a sample, the saponification value is a measure of the concentration of saponifiable material in a sample, and the phosphorus content is a good predictor of the total concentration of phospholipids in a sample. However, all three of these tests are unspecific for particular fatty acids and not as descriptive as the fatty acid composition.

Phytochemical Characterization Tests

The polar fraction of olive oil samples and olive oil waste water from the pressing process contain primarily non-lipid compounds that are generally more hydrophilic or amphipathic in nature such as phenolics and flavonoids. Since the polarity of such compounds results in enough variation in their partition coefficients (K_p), many of these compounds are easily separated via RP-HPLC using a C-18 column. Additionally, the small size of some of these compounds allows them to more easily be volatilized and thus analyzed by high resolution GC (Del Carlo et al., 2004) or to be separated by electrophoresis and thus analyzed by capillary zone gel electrophoresis (Pancorbo et al., 2004). Verification of the compounds has been performed by numerous methods including NMR – which isotopically identifies the location of specific isotopic atoms within molecules due to their electron shielding from neighboring atoms, mass spectroscopy (MS) – which very accurately measures the mass over charge ratios of the unknown compounds, and ultraviolet spectroscopy (UV-Spec) – which simply identifies the UV absorption spectrum of unknown compounds which vary based on the overall electronic structure of the compounds.

More general spectrophotometric tests for the determination of the total phenolic contents (TPC) and total *ortho*-diphenolic contents (ToDPC) are performed almost exclusively by the Folin-Ciocalteu (FC) method. Compounds containing phenolic structures are thought to react with the FC reagent by transferring an electron onto a molybdenum atom contained within the FC reagent (Huang et al., 2005). This reduction converts the rather yellow FC reagent to a blue species (Huang et al., 2005) whose absorbance can then be measured spectroscopically at a wavelength of 765 nm. The same reaction takes place when measuring ToDPC, however when a diphenolic compound reduces two molecules of FC reagent, it itself becomes a stable quinone (Singleton et al., 1998). To measure ToDPC the absorbance is measured spectroscopically at a wavelength of 370 nm, as *ortho*-quinones absorb light at around this wavelength (Land et al., 2003). In particular, the TPC procedure is a commonly accepted assay that is routinely performed in dietary antioxidant research laboratories throughout the world (Huang et al., 2005). Chemically, TPC's measurement of the absorption at a long wavelength is a favorable feature as it minimizes interference from the visibly-colored compounds in the sample matrix (Huang et al., 2005). Although the TPC procedure usually correlates well with many free radical scavenging capacity assays, this is not always the case as the FC reagent does react with tyrosine side-chains even though these groups do not possess free radical scavenging capacities (Huang et al., 2005). Specifically, TPC measures the total concentration of compounds that contain aromatic rings that can dissociate a proton at pH ~10 and thus appropriately approximates the phenolic fraction of sample extracts (Huang et al., 2005). However, free radical scavenging

assays like $ORAC_{FL}$ and DPPH free radical scavenging ability do not necessarily correlate with TPC values as every antioxidant/phenolic compound differs in their free radical scavenging ability. The effectiveness of an antioxidant is related to many factors including its kinetic and thermodynamic willingness to reduce a free radical based on its bond strength and reduction potential (Buettner, 1993), its stability as a free radical itself, and its solubility in the desired medium (Nawar, 1996). Thus, although TPC is probably a valid estimation of the concentration of phenolic compounds in sample extracts, some of the detected compounds actually have insignificant free radical scavenging abilities while others may have large amounts of free radical scavenging activity.

Characterization Tests for Unsaponifiables

The unsaponifiable fraction of olive oil samples contains nonpolar compounds which are generally more lipophilic in nature or have large domains of lipophilicity, such as carotenoids, phytoestrogens/lignans, phytols, phytosterols, stigmastadienes, squalene, secoiridoids, tocopherols, and other hydrocarbons. Since most of these compounds are not solely hydrocarbon in nature, there is enough variation in the K_ps of the compounds so that most of them can also be separated via HPLC. Additionally, after silylation of the compounds they can all successfully be separated by GC and identified regardless of their lipophilicity (Jiménez de Blas & del Valle González, 1996; Giacometti, 2001). Verification of the compounds has been performed by numerous methods including NMR, MS, and tandem MS-MS – which separates the compounds in the first MS chamber and then bombards a single

compound so it fractures into ionized pieces that can then be measured in the second MS. Measurement of the concentration of these compounds focuses narrowly on individual components that are proposed to contain antioxidant capacities and does not paint a general overall depiction of the antioxidant capacity of the olive oil samples.

Organoleptic Characterization Tests

Certain compounds present in olive oil samples add to the organoleptic properties of the oils. These include color, flavor, and aroma. The color of olive oil samples has been measured by numerous methods each with their own scales for color (e.g. brightness, bromothymol blue color, chroma, DIN99d Color System, hue, integral color index, L*a*b*, lightness, Naudet's color index, and tristimulus colorimetry). Other tests simply measure the concentration of certain highly conjugated colored compounds that are known to absorb light at known wavelengths in the visible spectrum (i.e. carotenoids, chlorophylls, and pheophytins). In general, a worldwide, industrially-accepted method is to determine the L*a*b* color indices using a colorimeter (Mínguez-Mosquera et al., 1991; Ceballos et al., 2003; Melgosa et al., 2004). The ripeness index of olive fruits can also be determined as a measurement of their organoleptic properties.

The aroma and flavor of olive oil samples has also been determined. The aroma of a sample is due to low molecular weight, relatively nonpolar compounds in the sample that are easily volatilized at ambient temperature. These are obtained by

solid phase microextraction (SPME) (Luaces et al., 2003) or by headspace extraction and are most easily separated by GC and classified by MS. Some compounds in olive oil tend to leave a bitter flavor in the olive oil samples that has been measured by a trained panel as well as by a bitterness index which is simply the absorbance of the oil at 225 nm (Tovar et al., 2001; Romero et al., 2003; Morelló et al., 2004a; Morelló et al., 2004b).

These tests are generally performed to measure the organoleptic quality of olive oil samples. However, they are rarely used to quantitatively characterize the quality or stability of olive oil samples.

Other Characterization Tests

There are many other more general tests to characterize olive oil samples. Some determine various compounds in the sample (i.e. polypeptides, saturated alcohols, and waxes) while others are general physicochemical measurements that characterize the oil (i.e. carbonyl index, density, dissolved oxygen, moisture, K_P by HPLC, refractive index (RI), turbidity, and viscosity). Others simply identify carcinogens present in the oil and look for unhealthy levels of these compounds (i.e. specifically benzo(a)pyrene and also other polycyclic aromatic hydrocarbons). Benzo(a)pyrene was found in retail virgin and refined olive oil samples at 0.54 – 1.23 and 0.58 – 1.63 μg/kg, respectively, using GC-MS-MS (Abdulkadar et al., 2003) and the accuracy of detecting benzo(a)pyrene in olive oil samples was confirmed with both GC-MS and HPLC (Bogusz et al., 2004).

Characterization of the olive oil samples should be rather general. In this, the general identification of the overall concentrations/percentages of fat and phenolic content of the samples, and not specific compounds, is important. Additionally, a few parameters that characterize oils overall should be included so as to verify that the oils are indeed generally the same. Thus, of the methods presented above, the ones that are deemed important to this study are fatty acid composition, TPC-FC, and density and refractive index of the olive oil samples. This should represent an overall identification of the samples and not focus on individual aspects of the samples.

Antioxidant Property Assays

A goal of this proposed research is to identify the antioxidant capacity of olive oil samples and relate this capacity to the ability of the olive oil samples to quench free radicals *in vivo*. It is important to identify the procedures that have been conducted to date, as well as those that have not, and identify those that are measuring the quenching of biologically-relevant free radicals. First, the definition of an antioxidant as it pertains to health-related biological research must be clarified. Halliwell and Gutteridge (1999) defined an antioxidant as "any substance that, when present at low concentrations compared with those of an oxidizable substrate, significantly delays or prevents oxidation of that substrate" where an oxidizable substrate includes every type of molecule that can be found *in vivo*. Typically the most damaging of these oxidizing compounds are free radicals, which are compounds that contain one or more unpaired electrons and exist independently (Halliwell et al., 1995) (i.e. they exist for a length of time greater than a molecular vibration, ca. 10^{-13}

sec, and are thus intermediates and not simply transition states in reaction mechanisms). Antioxidants help stabilize these free radical electrons by resonating these free radical electrons through their extensive conjugation thus decreasing the reducing potential of these scavenged electrons and their abilities to induce harm. The reductive stabilization of free radicals in exchange for oxidation and concurrent creation of a free radical on the reducing compound is more energetically favorable when the reducing compound is an antioxidant versus an unsaturated fatty acid (Buettner, 1993). Thus antioxidants attenuate the damage inflicted by free radicals on other compounds by preferentially donating at least an electron to the compounds containing the free radical.

Free radicals that are present in humans are typically those generated by ROS which include the $O_2^{\bullet-}$ (anionic superoxide free radical), $ROO^{\bullet}$ (neutral peroxyl free radical), $HO^{\bullet}$ (neutral hydroxyl free radical), $RO^{\bullet}$ (neutral alkoxyl free radical), $CO_3^{\bullet-}$ (anionic carbonate free radical), and $CO_2^{\bullet-}$ (anionic carbon dioxide free radical) (Halliwell & Whiteman, 2004). High concentrations of some of these reactive species are often formed *in vivo* typically as byproducts of either electron transport or lipid peroxidation, as intermediates in biochemical reactions, or as signaling molecules (Nawar, 1996; Acworth & Bailey, 1996; Leray, 2005). The major biologically-relevant free radicals that are highly capable of redox reactions are the anionic superoxide radical, neutral peroxyl radical, and neutral hydroxyl radical (Ou et al., 2002; Roginsky & Lissi, 2005). Roginsky and Lissi (2005) also added the neutral nitric oxide radical to this list. Thus, specifically only tests against the three

agreed upon free radicals mentioned above or generally only tests against either negatively or neutrally-charged free radicals may have the most biological relevance.

Many antioxidant tests have been performed on olive oil samples in the past. Some are spectroscopic assays that measure free radical-induced damage, such as an antioxidant assay that measures the bleaching of β-carotene using a β-carotene-linoleate model system (AA-β-carotene) (Gorinstein et al., 2003) and the ferric thiocyanate (FTC)-linoleate assay (Larrosa et al., 2003) that measures linoleate oxidation, where in both cases changes in absorbance are measured over time. Some are spectroscopic assays using metal reduction, such as the cupric copper (Cu(II)) reduction and chelation assay against phenolics present in the Mediterranean diet (Briante et al., 2003) and the ferric reducing-antioxidant power (FRAP) assay which measures the reduction of the ferric iron-2,4,6-tripyridyl-1,3,5-triazine (Fe(III)-$TPTZ_2$) complex (Manna et al., 2002; Pellegrini et al., 2003). Others are spectroscopic assays against cationic free radicals such as the Trolox equivalent antioxidant capacity (TEAC) assay against the long-lived cationic 2,2'-azinobis-(3-ethylbenzothiazoline-6-sulfonate) (ABTS) free radical (Gorinstein et al., 2003; Pellegrini et al., 2003) and the N,N-dimethyl-*p*-phenylenediamine dihydrochloride (DMPD) free radical scavenging assay using the cationic DMPD free radical (Briante et al., 2003). However, none of these assays are against the aforementioned biologically-relevant free radicals or even free radicals with the same charge state. Thus, these assays are most likely not as informative when evaluating the *in vivo* antioxidant potentials of olive oil samples.

In terms of biologically-relevant free radical assays, the following have been conducted: a RNS assay that measures changes in nitric oxide radical formation in macrophage cells measured by nitrite accumulation as an indicator of antioxidant concentrations (Moreno, 2003b); ROS assays that measure (a) the free radical scavenging of superoxide radicals produced by the chelation of the potassium cation by 18-crown-6 from potassium superoxide (KO_2) and the free radical scavenging of hydroxyl radicals produced by the Fenton system at a physiological pH both where subsequent formation and measurement of the 5, 5-dimethyl-1-pyrroline-N-oxide (DMPO)-radical adduct was performed by electron paramagnetic (spin) resonance (EPR/ESR) (Valavanidis et al., 2004), (b) the antioxidant capacity of EVOO phenolics against the superoxide radical and the semiquinonic radical by the diaphorase (DIA)/nicotinamide adenine dinucleotide (NADH)/juglone assay, which is usually used in phytotherapy (Lavelli, 2002), (c) the antioxidant capacity of olive oil phenolics against the superoxide radical and hydrogen peroxide by the XOD assay (Lavelli, 2002; Moreno, 2003b), which can then also be coupled to the addition of ferric iron and ethylenediaminetetraacetic acid (EDTA) and allows additional testing of the antioxidant activity against the reaction of the hydroxyl radical with salicylic acid (Owen et al., 2000b), and (d) the total radical-trapping antioxidant potential/parameter (TRAP) of EVOO against the peroxyl radical initiated by either 2,2'-azobis(2-amidinopropane)dihydrochloride (AAPH) (Pellegrini et al., 2003) or 2,2'-azobis(2,4-dimethylvaleronitrile) (AMVN) (Cabrini et al., 2001). Although these assays measure antioxidant activity against biologically-relevant free radicals, the fluorometric ORAC assay measures free radical absorbance capacity against the

peroxyl radical which is very relevant in lipid peroxidation reactions and is a very critically evaluated and analyzed method. As olive oils consist mainly of lipids, this assay is ideal for use with olive oil samples. Additionally, the DPPH free radical scavenging assay can be measured by spectroscopy or EPR/ESR (Gorinstein et al., 2003; Valavanidis et al., 2004). Although it is not a physiologically-relevant free radical, it is a stable, neutral free radical and could be important to study.

The ORAC assay was developed by Cao and colleagues (1993) and measures antioxidant scavenging activity against peroxyl radicals induced by AAPH at a physiological pH using B-phycoerythrin (B-PE) as the fluorescent probe, where the loss of fluorescence of the probe is due to its oxidation by the peroxyl radical. The antioxidant activity is determined by calculating the difference of the area under the fluorescence decay curve for a given sample and the area for a blank negative control at an excitation wavelength of 490 nm and an emission wavelength of greater than 515 nm. This allows the antioxidant activity to be assessed as the reaction goes to completion. Although Ninfali, Bacchiocca, and colleagues (Bacchiocca et al., 2001; Ninfali et al., 2001; Ninfali et al., 2002a; Ninfali et al., 2002b; Bacchiocca et al., 2003; Ninfali et al., 2005) have measured the ORAC values for olive oil samples, they had only done so using B-phycoerythrin (B-PE) as the fluorescent probe until recently when for one EVOO sample they used fluorescein (Ninfali et al., 2005). Ou and colleagues (2001) have since shown that B-PE is not an ideal probe to obtain results as it is not photostable and after sufficient exposure to excitation light it can be photobleached (Ou et al., 2001). Additionally, B-PE interacts with polyphenols due to non-specific protein binding (Ou et al., 2001). Even the originator of the method,

Cao and Prior (1999), have shown that the use of B-PE as the fluorescent probe results in inconsistent data. Thus, the use of fluorescein as the fluorescent probe is superior and removes the aforementioned problems with B-PE and thus $ORAC_{FL}$ is superior to $ORAC_{B\text{-}PE}$ (Ou et al., 2001).

In regards to other free radical scavenging assays, it has been argued that hydrogen atom transfer reactions are vital to assessing the possible *in vivo* free radical scavenging, and thus antioxidant, power of compounds as they measure free radical chain-breaking activity (Huang et al., 2005) which is typically the case when phenolic compounds react with peroxyl radicals (Foti et al., 2004). $ORAC_{FL}$ is such an *in vitro* assessment of a rather hydrophilic sample's free radical scavenging ability. It possesses interlaboratory (three laboratories) validation and industrial recognition (Huang et al., 2005) as it is intended to mimic phenolic free radical scavenging ability of peroxyl radicals *in vivo*. ORAC values for individual phenolic compounds are in reasonable agreement with their structures (Roginsky & Lissi, 2005). FRAP has drawbacks of interference due to reduction by non-physiological antioxidants, slow reaction kinetics, and non-physiological pH requirements that significantly inhibit the one electron reduction transfer (Ou et al., 2002). Additionally, TEAC and FRAP spectrophotometrically only measure partial reducing capabilities of antioxidants against the stable cationic ABTS radical and the Fe(III)-$TPTZ_2$ complex, respectively (Aruoma, 2003), and thus do not maximize the potential assessment of hydrogen atom transfer antioxidant reaction mechanisms.

Since the development of $ORAC_{FL}$, Huang and colleagues (2002) and Wu and colleagues (2004) have further improved the method to include a lipophilic solubility

enhancer. This randomly methylated β-cyclodextrin (RMCD), which is also utilized in TPC measurements, helps to dissolve the lipophilic antioxidants in the aqueous reaction solution (Huang et al., 2002). Wu and colleagues (2004) analyzed many samples for their $ORAC_{FL}$ and $ORAC_{FL}$ with RMCD to assess hydrophilic and lipophilic radical scavenging capacity, respectively. Although their results identified a significant free radical scavenging capacity using the lipophilic $ORAC_{FL}$ procedure (up to 28.6 % of the combined scavenging ability from the two tests for avocados), the use of both tests, $ORAC_{FL}$ for hydrophilic antioxidants and $ORAC_{FL}$ for lipophilic antioxidants, and subsequent summation of the results to derive the total antioxidant capacity may not have been appropriate. The extraction procedures were not always performed in a stepwise fashion (i.e. a stepwise fashion is where extraction number one is used for hydrophilic $ORAC_{FL}$ and then a subsequent extraction of the remaining unanalyzed sample from the first extract is used for the lipophilic $ORAC_{FL}$). The assays may have measured some of the same compounds and thus overlapped in their readings. By using different extraction methods and solvents it is likely that certain compounds were extracted by both extraction methods and then were analyzed by both $ORAC_{FL}$ procedures. Thus, the reported total antioxidant capacity actually represented an over-estimation of the antioxidant capacity and actual results were probably less than those obtained. An independent laboratory has reported that the addition of RMCD in the $ORAC_{FL}$ procedure increased the correlation of $ORAC_{FL}$ values to the concentration of the entirely hydrocarbon compound lycopene, in a dose-dependent manner (Bangalore et al., 2005). However, this increase was only seen up to a certain concentration at which time the further

addition of RMCD resulted in a statistically significant decrease in correlation (Bangalore et al., 2005). Thus, although this $ORAC_{FL}$ method with RMCD has promise, it has yet to be accepted as an enhancement to the $ORAC_{FL}$ procedure sans RMCD and has neither been widely used nor successfully and thoroughly validated. Thus, it seems as though $ORAC_{FL}$ sans RMCD is a reliable and tested method for measuring antioxidant capacity against a biologically-relevant free radical and helps characterize the capacity of a sample to act as an antioxidant source *in vivo*.

Extracts have free radical scavenging abilities in both a time- and dose-dependent manner. Although ORAC could be used for these analyses as well, it is a common practice to obtain data from more than one free radical scavenging assay. Plus the ORAC assay takes ca. 70 minutes per sample which is a lot longer than many other antioxidant assays (Cao & Prior, 1998) and would take a considerable amount of time to show the desired trends. Thus, as the DPPH free radical scavenging assay is commonly used to measure time- and concentration-dependent reaction kinetics of antioxidants, it will be employed to measure the time- and dose-dependent action of the olive oil antioxidants against stable free radicals in this research. The DPPH free radical scavenging assay measures the loss of absorbance at 517 nm due to the quenching of the DPPH stable free radical over time. The DPPH free radical scavenging assay was recently suggested to not measure radical chain-breaking activity via a physiologically-relevant hydrogen atom transfer mechanism in alcoholic solvents (Foti et al., 2004). Additionally, it correlates to the number of hydroxyl groups in a sample and has advantages over the similarly simple method against the cationic ABTS radical, as it will react with many phenolics that the ABTS free radical

will not (Roginsky & Lissi, 2005). This is another reason why this method was chosen to accompany the ORAC assay to measure the antioxidant capacity of the olive oil samples.

The capacity of some compounds, especially *o*-diphenolic compounds, to chelate free divalent transition metal cations (e.g. cobalt, copper, iron, manganese, and nickel (Nawar, 1996)) has been used as a method for the study of the antioxidant activity of compounds by another mechanism of action. Metal ions may induce oxidative reactions by catalyzing hydroperoxide decomposition, directly oxidizing other compounds, or activating molecular oxygen to form the superoxide free radical which then can give rise to singlet oxygen or the peroxyl free radical (Nawar, 1996). Specifically ferric iron and cupric copper exhibit pro-oxidant effects when reduced under certain conditions (Floyd, 1990), cupric copper more so than ferric iron, however ferric iron exists in physiologically higher concentrations than cupric copper (Paiva-Martins & Gordon, 2002). Paiva-Martins and Gordon (2002) have shown that phenolic components of olive oil have high antioxidant capacities in the pH range 3.5 – 7.4. However they also mention that their activity is reduced or may even be pro-oxidant in the presence of ferric iron. Of the metal chelation assays available, ones against iron (Paiva-Martins & Gordon, 2005) and copper (Visioli et al., 2000b; Visioli & Galli, 2002a; Visioli et al., 2002b; Briante et al., 2003) have been studied directly with olive oil's phenolic and secoiridoid compounds and the results have indicated that these compounds can indeed chelate metal cations by various mechanisms dependant on the pH of the solution (Briante et al., 2003; Paiva-Martins & Gordon, 2005). Indirectly, the deoxyribose assay has been used to measure

chelating activity against ferric iron. Specifically it measures the ability of antioxidants to chelate ferric iron and reduce it to its ferrous state (Fe(II)). This procedure measures the antioxidant's pro-oxidative capacity as iron in the ferrous state more readily reacts with hydrogen peroxide which creates damaging hydroxyl radicals by a Fenton-like reaction (Aruoma, 2003). However, it then measures the subsequent antioxidant activity against physiologically-relevant free radicals. Yet these measurements cannot be separated. Although this assay measures various physiologically important occurrences (iron chelation, pro-oxidant capacities, antioxidant hydroxyl radical quenching), its lack of specificity to antioxidant or pro-oxidant measurements makes it less of a useful test. This test underscores the variability of compounds to act as pro-oxidants and antioxidants. When the chelation of a specific metal in certain conditions results in a change in the metal's reducing potential and subsequent change in the lability of an electron from the metal, the chelator has now either acted as a pro-oxidant or an antioxidant. If the chelator increases the reductive electrical potential of an electron in the metal cation, it acted as a pro-oxidant (Buettner, 1993). If it decreased this electrical potential, its role was that of an antioxidant (Buettner, 1993). Thus, although certain compounds can chelate highly reactive free metals in an antioxidative sense, this chelation can lead to pro-oxidative effects if this chelated product is more reactive than when it was free. Overall, most metal chelation assays do not distinguish between pro-oxidant and antioxidant activities of the compounds being studied.

It should be noted that no tests on olive oil samples have been performed to measure or assay oxidative DNA damage. Although this has important applications

toward cancer risk reduction, the fact that olive oil contains mostly fat and rather lipophilic compounds which are not fully soluble in the nucleus of cells where DNA is compartmentalized is probably a reason for absence of such studies.

Quality and Stability Tests

As this research intends to identify the antioxidant capacity of olive oil samples so as to determine their possible *in vivo* health benefits, it is also a goal of this proposed research to identify whether increases in antioxidant compounds result in increased stability of the olive oil samples. The overall quality and oxidative stability of an oil sample depends not only on its fatty acid composition but also on the content of other compounds present in the oil such as antioxidants, pro-oxidants, and byproducts of prior hydrolytic and oxidative reactions. These can be measured by several tests of rancidity whose results indicate the prior oxidation history, the current oxidation state, or the expected shelf-life of the oils. Collectively these define the quality, stability, and overall value of the oils. The quality of olive oil decreases due to the formation of rancid byproducts that alter sensory and nutritional characteristics of the oil (Meijboom, 1964). Such rancidity can be seen by increased FFA content, the production of highly reactive carbonyl compounds, a decrease in the α-tocopherol concentration, and the generation of off-flavor compounds (Meijboom, 1964). Compositional stability is of vital importance to olive oil producers as oil with a greater oxidative stability usually results in superior organoleptic properties, a higher concentration of antioxidant compounds, and a longer storage time for the consumer (NP Analytical Laboratories, 2005).

Lipid rancidity in olive oils is a common and undesired change that begins while the olive fruit is still growing on the tree and continues to occur during olive fruit harvesting, olive fruit pressing and/or processing, and oil bottling and storage. It also occurs during pressing, as lipolytic enzymes (i.e. lipases) are released from within the food matrix (Hamilton, 1994) and they catalyze lipid hydrolysis reactions. In addition, the lipoxygenases, which activate during crushing of olives, catalyze the creation of nascent C5 and C6 saturated and unsaturated aldehydes, alcohols, and esters, all of which are oxidation byproducts that quickly incorporate into the oily phase (Angerosa et al., 2001). In order to minimize such byproducts, Servili and colleagues (2003) determined the optimal time of exposure of olive pastes to air contact and the optimal temperature during malaxation by responsive surface modeling. For two Italian cultivars the results varied, however, it was found that to minimize certain volatiles and maximize desirability, either the temperature or the time should be at a minimum (i.e. 22 °C or zero minutes) (Servili et al., 2003). The chemical compounds produced from such reactions are responsible for the rancid flavors and aromas of lesser quality oils while at the same time usually indicating that the concentrations of antioxidants usually found in the oil are depleted by highly reactive intermediates from the lipid oxidation process. The mechanisms for such rancidity resulting from lipid degradation occurs by various paths, the two most recognized being hydrolytic rancidity and oxidative rancidity (Mauron, 1977).

Hydrolytic rancidity results in the formation of FFAs due to the hydrolysis of tri-, di-, and mono-acylglycerols and their derivatives containing at least one fatty acyl group (NP Analytical Laboratories, 2005). This can generally be controlled with

good transportation and careful packaging of the oil samples (Hamilton, 1994). However, the kinetics of this hydrolysis reaction is increased by either chemical or enzymatic activity, resulting in a more rapid accumulation of FFAs (Mauron, 1977). *Candida Rugosa*, a nonsporogenic yeast fungus that secretes an extracellular lipase enzyme, increases its lipase activity when supplied solely with olive oil as a nutrient source, but only after metabolizing most of the glycerol present in the oil (Del Río et al., 1990). This shows that olive oil, with certain bacteria present, may possess increased lipolytic activity after exhausting all other carbon sources thus resulting in more lipase-driven hydrolytic rancidity of the oil sample.

The FFA value of an oil sample quantifies the extent of hydrolytic rancidity by directly measuring the products of hydrolysis of tri-, di-, and mono-acylglycerols and their derivatives containing at least one fatty acyl group. This is accomplished by titrating the oil sample with a strong base until the carboxylic acid groups of the free fatty acids become fully deprotonated. At that time the phenolphthalein indicator in the mixture will begin to become deprotonated, as its phenolic buffering groups have higher pK_a values than the carboxylic acid groups. As the conjugate base of the fully protonated indicator is a pink compound, the amount of base used in the titration should be recorded and used to calculate the FFA content of the oil sample. The method used to determine the FFA values will be according to the American Oil Chemists' Society (AOCS) method Cd 3d-63 (1999) with minor modifications in the solvent system. This method is one of two accepted as official procedures for determination of the FFAs in olive oil samples per the IAOOTO; the other is ISO 660, "Determination of acid value and acidity" (International Olive Oil Council,

2003). Thus the results from either can be directly compared to the limits set forth in the IAOOTO that determine the classification of olive oils internationally as EVOO, VOO, POO, etc.

The smoke point also measures the hydrolytic rancidity of an oil sample. It is the temperature (°C) that each oil sample begins to continuously smoke (AOCS, 1997a). As the smoke point of oil samples should relate to their FFA content (AOCS, 1999), its use as an evaluation tool could also be viable in determining the extent of hydrolytic rancidity.

The other most recognized path of lipid degradation is by oxidative rancidity that results in not only unpalatable compounds but is nutritionally unhealthy due to the possible formation of toxic compounds (Sanders, 1994; Morales & Przybylski, 2000). This path proceeds by either autooxidation, due to a reaction with molecular oxygen, or photooxidation, due to the influence of light (Morales & Przybylski, 2000). Autooxidation is termed as such because even with proper care, this type of oxidation continues to occur as the first two steps in this chemical reaction have very low activation energies, 4-5 kcal mol^{-1} and 6-14 kcal mol^{-1}, respectively (Hamilton, 1994). Since it cannot be completely suppressed, attenuation of these oxidative reactions is desired. Oxidative rancidity results from complex free-radical mediated lipid oxidation mechanisms that occur in three phases: initiation, propagation, and termination. The initiation phase is the generation of the first few free radicals. The subsequent auto-oxidative propagation phase occurs whereby these reactive products then react with additional lipid molecules to form other reactive chemical species or decompose to smaller less reactive hydroperoxides. In the final termination phase of

lipid oxidation, rancid compounds are formed that include hydrocarbons, alcohols, aldehydes, ketones, and carboxylic acids. External sources of energy increase the rate of lipid oxidation during all of the aforementioned phases (NP Analytical Laboratories, 2005).

Unfortunately, it is hard to quantify the amount of oxidation based on the concentration of substances at each of these stages individually as the concentrations of each vary over time. The levels of primary oxidation products (peroxides, hydroperoxides, and peroxyl free radicals) are simply transitory intermediates as their concentrations decrease as these products undergo further oxidation. This further oxidation leads to secondary oxidation products that have a tendency to escape the liquid oil sample, as they may be extremely volatile. Unfortunately, the products of each of these phases will both increase over time, as more substrates, initiators, and catalysts are present, and decrease over time, as those primary products undergo further oxidation and possibly become volatile and escape the liquid oil sample. As there is not one particular test for all products of this type of lipid oxidation (both primary and secondary), the sufficient quantification of oxidative rancidity involves multiple, interrelated tests. These include, but are not limited to, the *p*-anisidine value (pAV), thiobarbituric acid reactive substances (TBARS), the peroxide value (PV), headspace volatiles, fatty acid composition, IV, and the oxidative stability index (OSI) (Pike, 1998).

The pAV and TBARS both determine the prior oxidation that has occurred in the oil samples by directly measuring the secondary products of lipid peroxidation produced during the termination phase. The pAV represents the amount of α- and β-

unsaturated aldehydes (AOCS, 1997b) whereas the TBARS quantifies mainly the malondialdehydes, as well as some saturated aldehydes, 2-enals, and 2,4-dienals present in the oil samples resulting from past oxidation of the oil samples (NP Analytical Laboratories, 2005). Although both tests are valid determinants of secondary oxidation products, the pAV is seen as a better predictor of oil quality as it measures a more extensive assortment of non-volatile compounds and compounds from more advanced secondary hydroperoxide oxidation (NP Analytical Laboratories, 2005). Additionally, Kanavouras and colleagues (2004) determined that hexanal, nonanal, (*E*)-2-decenal, (*E*)-2-heptenal, and 2-pentyl furan best describe the oxidation of VOOs. Of these compounds, pAV should specifically measure the presence of the two unsaturated aldehydes while TBARS may respond to all four aldehydes but with little specificity as it is intended to primarily detect malondialdehyde (Janero, 1990). On the other hand, since TBARS does include various non-specific reactions, it frequently will react with compounds that are not secondary lipid oxidation byproducts thus resulting in false positives (Janero, 1990). Therefore, lesser TBARS values may be the result of primary oxidation products that have not yet been converted to secondary aldehydes, loss of the rather volatile aldehydes during the processing or usage of the oils, or the fact that TBARS does not react specifically with many secondary lipid oxidation byproducts. Higher TBARS values may be the result of reactions with nonlipid-derived compounds. Regardless, TBARS has many drawbacks. As a consequence, pAV will be used in its place. This method relies on the reaction between *p*-anisidine and unsaturated aldehydes under acidic conditions which forms a yellow product. The absorbance of the resulting

compound can then measured spectroscopically at a wavelength of 350 nm to determine the relative amounts of specific unsaturated aldehydes in the oil sample.

As the pAV and TBARS assay measure the secondary products of lipid oxidation which describe the past oxidation of the oil samples, the PV measures the current state of the oil by directly measuring primary oxidation products (i.e. peroxides) that are formed during both the initiation and propagation phases of lipid oxidation. A trend of increased rancidity from storage and autooxidation was shown by Psomiadou and Tsimidou (2002a) for EVOO samples. There was a trend for a marked increase in PV over a 24 month storage period in the dark and even more so when the bottles were periodically opened. This was not surprisingly accompanied by a decreased trend in TPC and with smaller decreases in lutein, α-tocopherol, squalene, chlorophyll, and pheophytin contents with little change in β-carotene content (Psomiadou & Tsimidou, 2002a). For these same EVOO samples, Psomiadou and Tsimidou (2002b) also tried to identify the effects of rancidity from photooxidation however the trend for an increase in PV over 10 to 22.5 hours of photooxidation was not as apparent. The trend for TPC was also not as apparent, as it was only slightly decreasing (Psomiadou & Tsimidou, 2002b). However there was an apparent decreased trend in α-tocopherol content, albeit there was no change in lutein and β-carotene content (Psomiadou & Tsimidou, 2002b). The variability in the PV and its indication of the current status of oil samples underlie its importance towards the research goals of this proposal and thus will be measured by the PV-FOX method, version II which is prominently used. This method relies on iron in the reduced ferrous state that becomes oxidized by peroxides present in the oil sample via a

Fenton-like reaction. This oxidized iron that is now in the ferric state forms a complex with xylenol orange that is also in the solution to form a purple complex whose absorbance can then be measured spectroscopically at a wavelength of 560 nm (Parry & Yu, 2004).

Most olive oil researchers use the European Official Methods of Analysis described in the regulations of the Commission of the European Union (1991) and not the FOX assay, version II, while the official procedure for determination of the FFAs in olive oil samples per the IAOOTO is ISO 3960, "Determination of the peroxide value" or AOCS method Cd 8b-90 (International Olive Oil Council, 2003). To make any true comparisons, at least to previously reported ranges of typical EVOO sample values, the same method must be utilized. This is not a major concern in this research as the FFA value is the most important value that must be measured by IAOOTO standards so as the classification of the olive oil samples can be compared to international standards. As the PV is not used as the primary indicator of classification of olive oil samples by the IAOOTO standards, use of any relevant method should suffice and thus the PV-FOX method, version II will be employed.

Similarly to the pAVs and TBARS results, high peroxide values are a definite indication of oxidized oils that may lead to further rancidity, whereas moderate to low values may be the result of decomposition of peroxides to secondary oxidation products after reaching high concentrations (NP Analytical Laboratories, 2005). The importance of measuring the PV in addition to the pAV was seen by Gutiérrez and Fernández (2002a) who found that over 180 days of mild oxidation the PV, as milliequivalents of active oxygen per kg of oil, initially increased (ca. 10 days) but

then decreased by about 3.5 – 4.0 by the end of the experiment (although these changes were much smaller in the samples stored at 2 °C in the dark where only an increase of about 1.0 was seen). Thus, the pAV and PV results are often combined to indicate a more overall current state of an oil sample when the consumer purchases the product, all the while reflecting the history of the oil. The combined values are referred to as the totox number (TN) as per the following equation (AOCS, 1997b): $TN = pAV + (2 \times PV)$. The TN is often a better indicator of current overall value of the oil as it takes into consideration the recent oxidation products from the initiation and propagation phases as well as the fully oxidized secondary products from the termination phase due to further oxidation of the oil. The formula is as such due to Holm and Ekbom's discoveries (1972) that when an oil was heated at 200 °C under a vacuum, 1 PV unit decomposed to form 2 pAV units. Although uncommonly used in the United States, the pAV and TN are frequently determined in Europe (Pike, 1998). Presentation of simply the pAV or PV does not fairly classify the degree of instability in the oil samples. Thus, TN values should be considered.

As the pAV, TBARS, PV-FOX, and TN illustrate the past and present oxidative status of the oil, headspace volatiles, IV, fatty acid composition, and OSI relate to the future lifetime of the oil and predicted stability of the oils over time. Several factors including fatty acid composition, presence of other chemicals, and storage conditions may slow or increase the rate of lipid oxidation in oils (Yu et al., 2002) whose affects will be measured by the following procedures. Headspace analysis characterizes and quantifies the amounts of volatile secondary oxidation products in a sample by GC. Although headspace analysis is recognized as a valuable

method for the determination of the initial concentration of secondary lipid oxidation products in a sample, its common use of heat may generate more volatile compounds than were initially present and thus actually measures some of the predicted stability. Likewise, its brief and discontinuous use of heat does not measure the full oxidation potential of the oil samples as well as other tests.

As explained above, the IV measures the number of double bonds present in the unsaturated fatty acids of an oil sample which are known to undergo oxidation more readily than single bonds. However, the IV can also be obtained from the fatty acid composition.

The procedure for obtaining the fatty acid composition was also explained above and was explained to be an important factor in determining the health benefits of the oils. However, as the first step in lipid oxidation involves a reaction between molecular oxygen and an unsaturated fatty acid, the fatty acid composition of oil samples is also a possible indicator of the oxidative stability and quality of oils. The kinetics of this lipid peroxidation is affected by the number of double bonds present in the fatty acid as 18:2n-6 is oxidized 64 times faster than oleic while 18:3n-3 is oxidized 100 times faster than oleic acid (Hamilton, 1994). Thus, simply in terms of the fatty acid fraction, the more unsaturation present in this fraction of an oil sample, the more susceptible it is to being oxidized. Its determination is vital to the research goals of this proposal.

The OSI more accurately correlates to the predicted longevity of an oil sample in general. It is defined by the induction period, which is the length of time before the rate of lipid oxidation of an oil sample rapidly accelerates (Pike, 1998). This is

indicated by an extremely rapid increase in the concentration of low molecular weight rancid byproducts (i.e. aldehydes, ketones, alcohols, carboxylic acids, and esters) (Sánchez & Salas, 2000). The degree of rancidity is determined by continuously measuring an increase in conductivity due primarily to the ionizable short-chain fatty acids, formic acid (Pike, 1998) and acetic acid (Morales & Przybylski, 2000) resulting from the induced oxidation of the oil sample. The measurement is vital for olive oil samples as olive oil tends to contain lipoxygenase enzymes that catalyze the peroxidation of polyunsaturated fatty acids and carotenes (Sánchez & Salas, 2000). These enzymes catalyze oxidative reactions and may result in a decreased shelf-life of the oil.

Morelló and colleagues (2004a; 2004b) noted that there was trend for a decrease in OSI after 12 months of VOO storage in sealed bottles in the dark and when oils from a "first" (i.e. from November to January) and "last" (i.e. January and beyond) harvest were compared as well as compared every two weeks from mid-September to mid-November. This was not surprisingly accompanied by a similar decreased trend in HTyr, TPC, ToDPC, carotenoid content, and α-tocopherol content with over-ripening of the olive fruit and increases in storage time (Roca & Mínguez-Mosquera, 2001; Gallardo-Guerrero et al., 2002; Morelló et al., 2004a; Morelló et al., 2004b; Rotondi et al., 2004). Also, tocopherols have been identified as antioxidants, especially in keeping unsaturated fatty acids from oxidizing in the presence of peroxides, as they are fat-soluble compounds. However, the oxidative stability of olive oil samples, as measured by the acceptable OSI with the Rancimat instrument, showed no significant correlation to the concentration of total tocopherols in the

sample (r = 0.05), whereas OSI correlated well to the concentration of both individual and total phenolic compounds (r = 0.97) (Baldioli et al., 1996). Consequently, the TPC and OSI of the olive oil samples, and not concentration of tocopherols, will be determined in this research. Also, Hasenhuettl and Wan (1992) showed that the temperature at which the Rancimat method for obtaining the OSI is run is highly correlated to the log of the OSI value ($R^2 > 0.99$) for olive oil samples when run at temperatures between 100 and 140 °C. This allows comparison of OSI values obtained when run at different final temperatures if so desired.

Lastly, the physicochemical properties of oils can provide useful data to compare the samples and can aid in assessing their overall quality. Both the density and refractive index can be affected by past oxidation of the oil samples. However the differences may be less pronounced than for the more specific procedures explained above.

There are a myriad of other tests that can be performed to evaluate and acquire data for similar characteristics obtained by the above methods such as measuring conjugated dienes (K_{232}) which is simply the absorbance of the oil at 232 nm, conjugated trienes (K_{270}) which is simply the absorbance of the oil at 270 nm, spectroscopic index (ΔK) which is simply the maximum variation in the absorbance of the oil at near 270 nm (Codex Alimentarius, 2001), hexanal content by headspace analysis, the concentration of oxidized triacylglycerols, triacylglycerol dimers and oligopolymers, and OSI by the Activated Oxygen Method (AOM), Oven Test with PV measure, or Swift's Test. Another test not included above that is very useful quantifies the activity of enzymes present in the oils themselves. One such test is for

lipoxygenase activity and results in a measure of the rate of fatty acid peroxidation and volatile compound formation (Salas et al., 1999). Although valuable, this test, and the others mentioned directly before it, would not add greatly to the characterization of the stability of the olive oil samples as many other procedures assessing various properties are already planning to be employed in this research.

Objectives

In summary, much information and many methods on analyzing olive oil have been presented. As it would be an insurmountable task to analyze these oils by most of the tests listed above, a select few have been purposefully chosen to help evaluate the aforementioned hypotheses. The objectives for this preliminary research are to (a) examine and compare properties of commercial olive oils in the United States based on their fatty acid composition, physicochemical properties, concentrations of phenolic compounds, oxidative stability, and antioxidant capacities and (b) elucidate whether the price of an olive oil sample is associated with certain variables that are related to any of its beneficial effects. This research is important as many olive oils are sold in the United States under assumptions that the consumer can be assured of the advertised quality based on label descriptions; however, this may not always be the case. Quantification of parameters that should correlate to the perceived quality of a product will be assessed and compared to help better elucidate any misconceptions in the quality of olive oils commercially available in the United States.

Chapter 2: Quality and Stability of Olive Oils

Introduction

The classification system for olive oils in the United States and in most olive oil-producing countries (e.g. Greece, Italy, and Spain) is based primarily on quality parameters as determined by measures of rancidity in the oils (United States Department of Agriculture (USDA), 1948; United Nations, 2003). Although there are methods for evaluation of olive oils commercially available in the United States (USDA, 1948), the United States standard graded naming convention is not required on olive oil products. It is generally omitted in favor of international standard naming conventions that are officially neither recognized nor regulated by the United States government. This paradox illustrates that the United States standards are being overlook and thus that olive oils bought commercially in the United States may exhibit a wide range of varying qualities. The Food and Drug Administration (FDA) recently approved a new qualified health claim for olive oil which states that "limited and not conclusive scientific evidence suggests that eating about 2 tablespoons (23 grams) of olive oil daily may reduce the risk of coronary heart disease due to the monounsaturated fat in olive oil… to achieve this possible benefit, olive oil is to replace a similar amount of saturated fat and not increase the total number of calories you eat in a day" (FDA, 2004a). As some of the beneficial effects of olive oil toward coronary heart disease have been linked to its phytochemical composition (Visioli & Galli, 1998a; Hu, 2003), it is imperative that olive oils sold commercially in the United States are assessed for their fatty acid composition, physicochemical properties, oxidative stability, and overall quality.

The quality and oxidative stability of oil samples can be assessed by several tests of food rancidity that measure the prior oxidation history, the current state of rancidity, or the expected shelf-life of the oils. Collectively these define the quality, stability, and overall value of the oils. These include, but are not limited to, free fatty acid (FFA) content, smoke point, *p*-anisidine value (pAV), thiobarbituric acid reactive substances (TBARS), peroxide value (PV), headspace volatiles, fatty acid composition, iodine value (IV), and oxidative stability index (OSI).

The mechanisms for rancidity development in oils from lipid degradation occur by various paths, the two most recognized being hydrolytic rancidity and oxidative rancidity (Mauron, 1977). Hydrolytic rancidity results in the formation of FFAs due to the hydrolysis of tri-, di-, and mono-acylglycerols and their derivatives containing at least one fatty acyl group (NP Analytical Laboratories, 2005). This can generally be controlled with good transportation and careful packaging of the oil samples (Hamilton, 1994). This measure is of utmost importance when classifying and evaluating olive oils as the standards for labeling and classifying olive oils into different categories of quality in both the United States and internationally are primarily based on their FFA content (USDA, 1948; United Nations, 2003). As the smoke point of oil samples should relate to their FFA content (American Oil Chemists' Society (AOCS), 1999), its use as an evaluation tool could also be viable in determining the extent of hydrolytic rancidity.

The other most recognized path of lipid degradation is by oxidative rancidity that results in not only unpalatable compounds but is nutritionally unhealthy due to the possible formation of toxic compounds (Sanders, 1994; Morales & Przybylski,

2000). Oxidation of lipids in olive oils is a common and undesirable change that begins while the olive fruit is still growing on the tree and continues to be formed during olive fruit harvesting, olive fruit pressing and/or processing, and oil storage (Hamilton, 1994). Oxidative rancidity results from more complex free-radical mediated lipid oxidation mechanisms that occur in three phases: initiation, propagation, and termination. The initiation phase is the generation of the first few free radicals. The subsequent auto-oxidative propagation phase occurs whereby these reactive products then react with additional lipid molecules to form other reactive chemical species or decompose to smaller less reactive hydroperoxides. During lipid autooxidation, many rancid compounds including hydrocarbons, alcohols, aldehydes, ketones, and carboxylic acids are formed. Unfortunately, it is hard to quantify the amount of oxidation based on the concentration of substances at each of these stages individually as the concentrations of each vary over time. As there is not one particular test for all products of this type of lipid oxidation (both primary and secondary), the sufficient quantification of oxidative rancidity involves multiple, interrelated tests such as pAV and PV, and OSI. By using these three analyses, one can evaluate the lipid peroxidation status of the oil samples and current primary products, and future oxidative stability of the oil samples, respectively.

It is widely accepted that the kinetics of lipid peroxidation is increased by the number of double bonds present in the fatty acid (Hamilton, 1994) and thus the more unsaturation present in the fatty acid fraction of an oil sample, the more susceptible it is to being oxidized. Thus, the fatty acid composition of an olive oil sample is also a possible indicator of the oxidative stability and quality of the olive oil. Additionally,

the physicochemical properties of oils can provide useful data to compare the samples and are important for their food applications. Both the density and refractive index can be affected by past oxidation of the oil samples and are useful means of generally characterizing oil samples.

The objectives of this research are to examine and compare properties of commercial olive oils in the United States for their oxidative stability and physicochemical properties.

Materials and Methods

Materials

Extra virgin olive oils (EVOOs) and "pure" olive oils (POOs) were obtained from local food stores (supermarkets and health food stores) in the United States or donated by the olive oil suppliers. The olive oil samples were stored under nitrogen, in the dark, and at ambient temperature between experiments, and were handled during experimental analysis so as to minimize their contact with oxygen, light, and heat. Each sample's growing conditions, extraction history, prior storage conditions, and overall age were not known. Potassium hydroxide and sodium hydroxide were purchased from Fisher Scientific Chemicals USA, Inc. (Fairlawn, NJ, USA). Fatty acid methyl ester standards for fatty acid composition determination were purchased from Nu-Chek Prep, Inc. (Elysian, MN, USA). All other chemicals and reagents were purchased from Sigma-Aldrich, Inc. (St. Louis, MO, USA). All chemicals and solvents were of highest commercial grade and used without further purification.

Free Fatty Acid (FFA) Value

The FFA value of each olive oil sample was determined according to the AOCS method Cd 3d-63 (1999) with minor modifications in the solvent system. The results were expressed as percent FFA content in each oil sample by weight as oleic acid equivalents (g oleic acid/100 g oil). This method is one of two accepted as official procedures for determination of the FFAs in olive oil samples per the International Agreement on Olive Oil and Table Olives (IAOOTO) (International Olive Oil Council, 2003). Fifteen grams of each olive oil sample was placed into a 250 mL Erlenmeyer flask and dissolved in 70 mL reagent grade alcohol containing phenolphthalein indicator and then each oil solution was subsequently titrated with the potassium hydroxide solution. Measurements were conducted in triplicate for each oil sample. The neutralization and subsequent standardization of the potassium hydroxide solution was performed using potassium acid phthalate monobasic. Measurements were conducted in triplicate for this neutralization of the potassium hydroxide solution and the neutralization of the reagent grade alcohol solvent. All measurements were conducted in the same day and with the same reagent preparations.

Smoke Point

The smoke point of each olive oil sample, which is the temperature (°C) that each oil sample begins to continuously smoke, was determined according to the

AOCS method Cc 9a-48 (1997a). Eight mL of each olive oil sample was placed into a 10 mL beaker and the metallic tip of a thermometer was fully submerged in the oil so as not to touch the beaker bottom or sides. Measurements were conducted in duplicate for each oil sample.

p-Anisidine Value (pAV)

The pAV of each olive oil sample estimates the amount of secondary aldehydes - predominantly 2-alkenals and 2,4-dienals (Pike, 1998) - from lipid oxidation present in the olive oil sample. In the present study pAV was determined using a spectrophotometer (Genesys 20 Model 4001/4; ThermoSpectronic, Rochester, NY, USA) according to the AOCS method Cd 18-90 (1997b). The results were expressed as OD_{350}/g oil. About 500 mg of each olive oil sample was placed into a 15 mL glass test tube with a screw cap and dissolved in 5 mL isooctane. Absorbance was determined at 350 nm at ambient temperature against a blank of isooctane. These measurements were conducted only once for each oil sample solution as a pre-reaction reading. The assay reaction was initiated by mixing 200 µL of 0.25 % (w/v) *p*-anisidine in glacial acetic acid with 1.0 mL of the isooctane blank or each oil sample solution. Absorbance was again determined at 350 nm following 10 min of reaction time at ambient temperature against *p*-anisidine solution in isooctane. These measurements were conducted in triplicate for each oil sample solution as the post-reaction readings. All measurements were conducted in the same day and with the same reagent preparations. The pAV was calculated for each oil sample by the

following equation: pAV (OD_{350}/g oil) = $\frac{(1.2 \cdot abs_2 - 1.0 \cdot abs_1) \times df}{dens}$ (AOCS, 1997b), where *1.2* is the dilution factor of the oil solution used in the post-reaction (mL total solution/mL oil sample solution), abs_2 is the absorbance of the oil solution at 350 nm after the reaction with *p*-anisidine, *1.0* is the dilution factor of the oil solution used in the pre-reaction (mL total solution/mL oil sample solution), abs_1 is the absorbance of the oil solution at 350 nm before the reaction with *p*-anisidine, *df* is the dilution factor of the oil sample used in the procedure (mL solution/mL oil sample), and *dens* is the density of the oil sample obtained by the procedure described below (g/mL).

Ferrous-Oxidation in Xylenol Orange (FOX) Peroxide Value (PV)

The concentration of lipid peroxides present in each olive oil sample was determined using a spectrophotometer (Genesys 20 Model 4001/4; ThermoSpectronic, Rochester, NY, USA) according to a laboratory procedure previously described by Parry and Yu (2004). *tert*-Butyl peroxide was used as the standard at concentrations of 25, 50, 100, 200, 400, 600, and 800 μM in pure water and oil samples were diluted 1:100 with reagent grade alcohol. The reaction mixture contained 950 μL FOX reagent and 50 μL of either *t*-butyl peroxide solution or oil sample solution. Absorbance was measured at 560 nm following 10 min of reaction time at ambient temperature against a mixture of 950 μL FOX reagent and 50 μL methanol. Measurements were conducted in duplicate for each *t*-butyl peroxide solution and in triplicate for each oil sample solution. The PV-FOX of each oil sample was calculated from the standard curve and was expressed as concentration of

tert-butyl peroxide equivalents (tBPE) (μmol/g oil). All measurements were conducted in the same day and with the same reagent preparations.

Preparation of Fatty Acid Methyl Esters (FAMEs)

The preparation of FAMEs for analysis of fatty acid composition was performed according to a laboratory procedure previously described by Yu and colleagues (2003). FAME preparations were performed in triplicate for each oil sample.

Gas Chromatographic (GC) Analysis of FAMEs

The fatty acid composition of each olive oil sample was determined from the FAMEs using a GC with a flame ionization detector (GC-FID) (Model GC-2010; Shimadzu Scientific Instruments Inc., Columbia, MD, USA). The results were expressed as g individual fatty acid/100 g of total fatty acids. Analysis was carried out by GC-FID with a fused silica capillary column SP^{TM}-2380 (30 m × 0.25 mm with a 0.25 μm film thickness) (Model SP^{TM}-2380; Supelco Park, Belletonte, PA, USA) using an auto sampler (Model AOC-20s; Shimadzu Scientific Instruments Inc., Columbia, MD, USA). Helium was used as the carrier gas at a flow rate of 0.84 mL/min. Injection volume was 1 μL at a split ratio of 10:1. Initial oven temperature was 140 ºC and increased 6 ºC/min to 184 ºC which was then held for 3 min followed by an increase of 6 ºC/min to a final temperature of 244 ºC. Fatty acids were identified by comparing the retention time of peaks in the sample spectra with those

in the standards. The compounds were further verified by GC-mass spectroscopy (GC-MS). A representative GC-FID chromatogram and GC-MS mass spectrum can be found in the Appendix. Measurements were conducted once per FAME solution; the FAME procedure was conducted in triplicate for each oil sample.

Oxidative Stability Index (OSI)

The oxidative stability of each olive oil sample was determined using a Rancimat instrument (Model 734; Metrohm Ltd., Herisau, Switzerland) according to a procedure previously described by Parker and colleagues (2003). The results were expressed as the induction time (h) of each sample, which is the length of time that passes before an oil sample obtains a significant measurable rancidity defined as the time point where an oil sample's oxidation becomes rapidly accelerated (Pike, 1998). Four mL of each olive oil sample was placed into a glass reaction tube and oxidation was carried out at a temperature of 80 °C with an airflow rate of 7 L/h (Chen & Ho, 1997; Parker et al., 2003). The OSI for each oil sample was determined by the 743 Rancimat Control (version 1.00, Build 79; Metrohm Ltd., Herisau, Switzerland) software application for Windows which extrapolated the two linear parts of the conductivity plot and identified their point of intersection (Laubli & Bruttel, 1986). Measurements were conducted in triplicate for each oil sample except for a few samples where they were only conducted in duplicate due to very similar results.

Relative Density

The relative density of each olive oil sample, which is the ratio of the density of the oil sample to the density of deionized water, was determined gravimetrically at the observed ambient temperature of 25 °C (Model AB104-S; Mettler Toledo International Inc., Greifensee, Switzerland).

Refractive Index (η^{25}_{D})

The refractive index at 25 °C of each olive oil sample was determined using an ABBE Refractometer (Model 10450; American Optical Corporation, Southbridge, MA, USA) according to the AOCS method Cc 7-25 (1997c). Two drops of either deionized water or each olive oil sample was placed on the prism and the refractive index (η^{T}_{D}), based on light of a wavelength of 589 nm (sodium D-line), was recorded concomitantly with the temperature (°C) at ambient temperature. Measurements were conducted in triplicate for the deionized water and each oil sample. The deionized water was used to standardize the refractometer as its refractive index at 20 °C (η^{20}_{D}) is known to be 1.3330. Calculation of η^{20}_{D} for the deionized water and η^{25}_{D} for each oil sample took into account the varying ambient temperatures.

Statistical Analyses

All analyses were conducted using SPSS (version 10.0.1; SPSS Inc., Chicago, IL, USA) software application for Windows. Data were reported as means ± standard

deviations (SDs) unless noted otherwise, in which case data were reported as means ± standard errors of the means (SEMs) due to unequal sample sizes in the data being compared.

Assumptions for some of the statistical analyses were homoscedasticity and normality of the data. Homoscedasticity was determined by the Levene's test. Determination of whether the data followed a Gaussian distribution was performed using the Kolmogorov-Smirnov test.

Individual olive oil sample data sets were analyzed for significant differences among the means for various response variables. For homoscedastic data that followed a Gaussian distribution, the one-way analysis of variance (ANOVA) was used; for heteroscedastic data or if the data were not normally distributed, the non-parametric Kruskal-Wallis H test was used and the Games-Howell test was used to analyze pair-wise comparisons as it neither requires homoscedasticity nor normality of the data.

Simple linear regression analysis and correlation analysis were used to analyze relationships between two variables for individual olive oil samples. For homoscedastic data that followed a Gaussian distribution, simple linear regression analysis was used where each individual olive oil sample's mean explanatory variable value was paired with all of the response variable values and the Pearson's correlation coefficient (r) and the coefficient of determination (R^2) were calculated; for heteroscedastic data, weighted least squares linear regression was used where the weights were based on the reciprocals of the variance in the response variable; if the data were not normally distributed, they were not analyzed by linear regression,

however the non-parametric Kendall's correlation coefficient (τ_b) was calculated as it does not require normality of the data.

Extreme outliers, defined as any data points greater than 2.5 standard deviations away from the upper or lower quartile values, were excluded from all data analyses, however in no tests did this affect the significance of the results. Statistical significance was generally declared at $P < 0.05$ unless noted otherwise.

Results and Discussion

The FFA value for an oil sample quantifies the extent of hydrolytic rancidity via a titration, by directly measuring the concentration of products of triacyglycerol and phospholipid hydrolysis. The mean and median for FFA content were 0.55 and 0.56 g oleic acid/100 g oil, respectively, and ranged from 0.19 to 1.20 g oleic acid/100 g oil (Table 1). Additionally, the FFA values for each of the EVOO samples significantly differed from one another (Figure 1; Table 1; statistical analysis not shown for clarity) and thus the consumer may never be fully guaranteed that one type or brand of olive oil has the absolute best quality in terms of decreased levels of the byproducts of hydrolytic rancidity.

The smoke point also measures the hydrolytic rancidity of the oil samples as it tends to correlate inversely to the FFA content (AOCS, 1999). For the data values in this research, the FFA content was rather highly correlated with the smoke point with Kendall's correlation coefficient of $\tau_b = -0.65$ and a coefficient of determination of $R^2 = 0.42$ ($P < 0.01$), due to lack of normality of the data; the mean and median smoke

points were 207.7 and 204.0 ºC, respectively, and ranged from 185.5 to 245.5 ºC (Table 1). In comparing the smoke point and FFA value, the smoke point showed less variation between individual samples and thus may not exhibit differences in olive oil sample quality as sensitively as the FFA value.

The pAV signifies the prior oxidation that has occurred in an oil sample by directly measuring the secondary products of lipid peroxidation produced during the termination phase of lipid oxidation. Morales and Przybylski (2000) claim that a pAV less than ten is expected for freshly refined oil, and thus it is implied that oils that are not processed may contain even higher values. Under the experimental conditions, the pAVs ranged from 2.70 to 8.79 OD_{350}/g oil with mean and median of 5.25 and 4.53 OD_{350}/g oil, respectively (Table 1). This variability can most likely be explained by the fact that the compounds that affect the pAV are secondary, but not terminal, products of lipid peroxidation and that they themselves can be broken down to lower molecular weight compounds that are more volatile and leave the sample. Moreover, the pAVs from this research are generally within the range found for olive oil samples (Labrinea et al., 2001; Pereira et al., 2002; Gomes et al., 2003; Del Carlo et al., 2004).

As the pAV measures the secondary products of lipid oxidation which presents the past oxidation of the oil samples, the PV measures the current state of the oil by directly measuring primary oxidation products (i.e. peroxides) that are formed during both the initiation and propagation phases of lipid oxidation. Experimentally, the PV was determined using the FOX method, version II (Parry & Yu, 2004). The PVs-FOX found in this research ranged from 40.34 to 64.87 μmol tBPE/g oil with

mean and median of 50.53 and 49.66 μmol tBPE/g oil, respectively (Table 1). The PVs obtained in the current study are rather high when compared to those obtained by other olive oil researchers whose values ranged from 1.8 to 24 milliequivalents of active oxygen per kg of oil and where most values were at or below about 10 (Salvador et al., 1998; Gutiérrez et al., 1999; Gutiérrez & Fernández, 2002a; Gutiérrez et al., 2002b; Lavelli, 2002; Okogeri & Tasioula-Margari, 2002; Pereira et al., 2002; Gomes et al., 2003; Del Carlo et al., 2004; Rotondi et al., 2004). However, the method and standard compounds differed from those used in this research. Erroneous PVs will exist in the presence of ascorbic acid (Wolff, 1994), however considerable amounts of ascorbic acid should not have been present in this experiment. The possible interference from ascorbic acid may be due to its metal chelating ability and/or reducing potential as it contains two neighboring hydroxyl groups that can both be oxidized to form a resulting α-diketone. Hydroxytyrosol, the most potent simple phenolic antioxidant found in olive oil (Papadopoulos & Boskou, 1991; Stupans et al., 2002), also contains two neighboring hydroxyl groups that can become oxidized by a mechanism identical to that of ascorbic acid (Espín et al., 2001) and thus possibly it is to blame for the interference and inflated values seen in this experiment. Similarly to the precautions in drawing overt conclusions from the pAV results, high peroxide values are a definite indication of rancid oils whereas moderate to low values may be the result of depletion of peroxides to secondary oxidation products after previously having been at higher concentrations (NP Analytical Laboratories, 2005).

The fatty acid composition of each of the individual olive oil samples are shown in the Appendix. The predominant fatty acid was monounsaturated 18:1n-9 which ranged from 64.06 to 80.12 g/100 g total fatty acids with mean and median of 75.38 and 77.01 g/100 g total fatty acids, respectively (Figure 2; Appendix – Table 1). The lower, but still considerable, concentrations of 18:2n-6 found in the olive oil samples (Appendix – Table 1) help to perturb the oxidation from the lipoxygenase enzyme found commonly among plants and animals as the enzyme prefers 18:2n-6 as a substrate and 18:2n-6 is not as readily oxidized as polyunsaturated fatty acids containing a greater number of unsaturated carbon-carbon bonds (Hamilton, 1994). Since the stability of oils to lipid oxidation correlates directly with the number of double bonds in the fatty acids (Hamilton, 1994), the average percent of fatty acid molecules containing a hydrocarbon double bond was calculated according to the following equation:

$$\text{“\%C=C bonds”} = (\%16{:}1n\text{-}7 * 1) + (\%18{:}1n\text{-}9 * 1) + (\%18{:}2n\text{-}6 * 2) + (\%18{:}3n\text{-}3 * 3) + (\%20{:}1n\text{-}9 * 1)$$

The average number of fatty acid molecules containing a hydrocarbon double bond ranged from 90.6 to 99.3 % with mean and median of 94.6 and 94.2 %, respectively (data not shown). Therefore, although hydrocarbon pi bonds within olefins do undergo oxidation more readily than hydrocarbon sp^3 hybridized single bonds, the degree of unsaturation must not be the only factor that influences the stability of different processing types of olive oils.

The OSI, which indirectly quantifies the time remaining until an oil sample becomes rancid under accelerated oxidation conditions, more accurately correlates to

the predicted longevity and shelf-life of an oil sample. In this research, the OSIs ranged from as low as ca. one day (i.e. 23.64 h) to almost nine days (i.e. 212.1 h) under the experimental conditions (Figure 3). The mean and median for OSI were 110.5 and 105.8 h, respectively, and were supported by previous research findings for olive oil samples (Salvador et al., 1998; Brenes et al., 2002; Pereira et al., 2002; Beltrán et al., 2004; Cañizares-Macías et al., 2004). Most of the individual OSIs for each of the olive oil samples were significantly different from one another although there were too many interrelationships to illustrate them on the figure itself (Figure 3; statistical analysis not shown for clarity).

Lastly, the relative density and refractive index of oil samples can be useful means of comparing the samples and can aid in assessing their overall quality. Both of these values can be affected by past oxidation of the oil samples. The differences may be less pronounced than for the more specific procedures and research conclusions shown above. The mean and median for relative density, at 25 °C, were both 0.924 and ranged from 0.917 to 0.932 and did not significantly differ among the oil samples ($P > 0.05$) (Table 1). The mean and median for refractive index, measured as η^{25}_{D}, were both 1.4677 and ranged from 1.4672 to 1.4682 (Table 1).

The International Agreement on Olive Oil and Table Olives (IAOOTO) states that EVOO is "a virgin olive oil which has a free acidity, expressed as oleic acid, of not more than 0.8 grams per 100 grams, and the other characteristics of which correspond to those fixed for this category in this standard" (United Nations, 2003). The EVOO samples used in the above experiments were compared to this value as the method used to analyze FFA content of the samples in this experiment was one of the

official methods accepted by the IAOOTO (International Olive Oil Council, 2003), with minor modifications in the solvent system. Of the 25 EVOO samples, four had FFA values significantly above the limit set forth in the IAOOTO ($P > 0.05$) (Figure 1; Table 1). The mean ± standard deviation FFA values (n = 3) for these samples, measured as g oleic acid/100 g oil, were 0.85 ± 0.01, 0.99 ± 0.01, 0.83 ± 0.01, and 1.20 ± 0.01 (samples #9, 15, 16, 24) (Table 1; Figure 1). Although each of these values was above the maximum standards set forth in the IAOOTO for EVOO samples, each did fall well below the upper limit of 1.4 g oleic acid/100 g oil that equates to the highest grade of olive oil (U.S. Grade A or U.S. Fancy) according to the outdated United States standards for FFA content (USDA, 1948). Thus, these four olive oil samples could have been acceptably labeled as the highest available grade according to United States standards (U.S. Grade A or U.S. Fancy), however labeling using European standard terminology, which was the case with all four samples, was not appropriate and highly misleading based on the FFA values obtained by this research procedure. Such extreme values for FFA content expressed as percent oleic acid were not found for various commercially available EVOO samples analyzed in other laboratories (Gutiérrez & Fernández, 2002a; Gutiérrez et al., 2002b; Rotondi et al., 2004; Lavelli, 2002). The olive oils used in this study were not measured for their FFA content immediately upon purchase, however they were stored under acceptable conditions until they were analyzed. Future studies should address this issue by analyzing the olive oils immediately upon purchase.

Additionally, Gutiérrez and colleagues (1999, 2000) have shown that FFA values are negatively correlated to the ripeness and storage time and temperature of

conventionally grown Spanish olives used to make the olive oil. Also, Spyros and colleagues (2004) showed that heat and light can affect FFA content. When olive oil samples were exposed directly to light or higher temperatures the concentration of FFAs in the oil samples increased at a greater rate than those hidden from light and heat (Spyros et al., 2004). These results illustrate that many factors can influence the FFA content of an olive oil sample.

Some of the properties of olive oils studied in this research were significantly correlated to the OSIs. Due to heteroscedasticity of the OSI data, all correlation and regression analyses were conducted using weighted least squares regression. The most significant correlation that was appropriate was between the monounsaturated:polyunsaturated fatty acid ratio and OSI. These parameters were directly correlated with weighted Pearson's correlation coefficient of $r = 0.40$ and a coefficient of determination of $R^2 = 0.16$ ($P < 0.01$). This was followed by a direct correlation between the saturated:polyunsaturated fatty acid ratio and OSI ($r = 0.35$, $R^2 = 0.12$, $P < 0.01$) and inverse correlations between 18:2n-6 fatty acid composition and OSI ($r = -0.34$, $R^2 = 0.11$, $P < 0.01$), polyunsaturated fatty acid (PUFA) content and OSI ($r = -0.33$, $R^2 = 0.11$, $P < 0.01$), and FFA content and OSI ($r = -0.27$, $R^2 = 0.07$, $P < 0.05$). These indicate that the PUFA content in the oil, and most specifically 18:2n-6 which is a major PUFA found in the oil, is inversely associated with the oxidative stability of the oil, as would be expected (Hamilton, 1994). Interestingly, the FFA content is also associated with the OSI, although rather weakly, and as such the FFA content may be a good predictor of the stability and shelf-life of the oils. However, there were also significant associations that were

related in the unexpected fashion: 20:0 fatty acid composition inversely with OSI, pAV directly with OSI, monounsaturated fatty acid content directly with OSI, 18:1n-9 fatty acid composition directly with OSI, the monounsaturated:saturated fatty acid ratio directly with OSI, saturated fatty acid content inversely with OSI, and 16:0 fatty acid composition inversely with OSI ($P < 0.05$). As the relationship between the 20:0 fatty acid composition and OSI was most significant overall, it suggests that some of these associations may simply be the result of mere chance coincidence. Also, as all significant associations had $R^2 < 0.20$ and thus only less than 20 % of the variation in the OSIs was explained by any one of the above parameters, the strength and relevance of these associations is in question.

Conclusions

This research shows that olive oils commercially available in the United States differ in their quality and oxidative stability. Variability in the indicators of hydrolytic rancidity and lipid oxidation was also found.

Human health and quality of life may be greatly influenced by diet (Dreher, 1997). The desire for better health and less occurrence of disease in consumers lead them to make more educated decisions when purchasing commercially available food products. People who consume olive oils sold in the United States should be informed of the variation in the quality and shelf life of the product they are purchasing.

Figures and Tables

Table 1: Physicochemical Properties – free fatty acid (FFA) content, smoke point(pt), *p*-anisidine value (pAV), peroxide value(PV)-FOX, relative density, and refractive index (RI) of olive oil samples[*]

	FFA (g/100 g)	Smoke Pt (°C)	pAV (OD_{350}/g)	PV tBPE (μmol/g)	Density (25 °C/water)	RI (η^{25}_{D})
1	0.20 ± 0.02	239.5 ± 6.4	3.38 ± 0.17	50.42 ± 1.39	0.925 ± 0.005	1.4672 ± 0.0002
2	0.19 ± 0.01	245.5 ± 6.4	4.06 ± 0.05	50.19 ± 6.12	0.922 ± 0.004	1.4676 ± 0.0001
3	0.39 ± 0.01	230.0 ± 2.8	3.70 ± 0.04	40.34 ± 4.84	0.917 ± 0.005	1.4675 ± 0.0001
4	0.50 ± 0.01	226.0 ± 4.2	3.69 ± 0.02	58.90 ± 3.55	0.923 ± 0.007	1.4677 ± 0.0001
5	0.58 ± 0.01	215.0 ± 2.8	4.43 ± 0.06	53.42 ± 1.49	0.923 ± 0.001	1.4675 ± 0.0001
6	0.32 ± 0.01	225.5 ± 2.1	8.79 ± 0.06	48.91 ± 11.99	0.919 ± 0.007	1.4678 ± 0.0001
7	0.65 ± 0.01	212.5 ± 10.6	4.37 ± 0.08	51.52 ± 1.91	0.922 ± 0.003	1.4679 ± 0.0002
8	0.32 ± 0.01	225.0 ± 2.8	3.70 ± 0.09	45.74 ± 3.65	0.924 ± 0.008	1.4675 ± 0.0001
9	0.85 ± 0.01	200.0 ± 2.8	4.22 ± 0.14	64.87 ± 2.58	0.927 ± 0.002	1.4680 ± 0.0000
10	0.35 ± 0.00	215.0 ± 1.4	3.20 ± 0.02	47.29 ± 2.82	0.917 ± 0.006	1.4678 ± 0.0002
11	0.31 ± 0.02	221.0 ± 2.8	7.76 ± 0.12	48.88 ± 1.49	0.922 ± 0.005	1.4677 ± 0.0001
12	0.70 ± 0.01	196.0 ± 7.1	5.05 ± 0.07	55.10 ± 1.68	0.924 ± 0.002	1.4682 ± 0.0002
13	0.55 ± 0.01	202.0 ± 4.2	8.04 ± 0.05	52.40 ± 2.64	0.925 ± 0.009	1.4678 ± 0.0001
14	0.34 ± 0.01	205.0 ± 1.4	5.88 ± 0.06	54.02 ± 2.22	0.920 ± 0.002	1.4677 ± 0.0001
15	0.99 ± 0.01	191.5 ± 0.7	3.92 ± 0.03	52.38 ± 1.63	0.923 ± 0.005	1.4676 ± 0.0001
16	0.83 ± 0.01	190.0 ± 1.4	8.59 ± 0.28	46.62 ± 3.78	0.917 ± 0.008	1.4675 ± 0.0001
17	0.67 ± 0.01	195.5 ± 3.5	3.99 ± 0.07	55.85 ± 0.44	0.926 ± 0.007	1.4676 ± 0.0000
18	0.54 ± 0.00	204.0 ± 1.4	7.63 ± 0.23	47.85 ± 4.28	0.926 ± 0.008	1.4680 ± 0.0001
19	0.74 ± 0.01	194.0 ± 4.2	2.70 ± 0.11	45.73 ± 1.51	0.931 ± 0.003	1.4675 ± 0.0001
20	0.48 ± 0.00	207.0 ± 2.8	4.81 ± 0.02	42.77 ± 3.24	0.928 ± 0.000	1.4678 ± 0.0001
21	0.34 ± 0.00	204.0 ± 1.4	8.50 ± 0.10	40.73 ± 10.40	0.932 ± 0.001	1.4680 ± 0.0001
22	0.71 ± 0.01	200.0 ± 1.4	5.51 ± 0.01	47.73 ± 3.41	0.924 ± 0.005	1.4676 ± 0.0001
23	0.65 ± 0.02	195.5 ± 3.5	3.83 ± 0.08	49.13 ± 1.17	0.926 ± 0.005	1.4677 ± 0.0001
24	1.20 ± 0.01	185.5 ± 4.9	7.02 ± 0.05	43.51 ± 1.58	0.926 ± 0.008	1.4676 ± 0.0001
25	0.69 ± 0.02	192.5 ± 4.9	4.84 ± 0.11	58.65 ± 4.42	0.926 ± 0.003	1.4676 ± 0.0001
26	0.73 ± 0.02	189.0 ± 0.0	5.95 ± 0.08	59.92 ± 0.52	0.930 ± 0.007	1.4676 ± 0.0001
27	0.20 ± 0.00	223.0 ± 1.4	4.21 ± 0.02	46.36 ± 2.72	0.930 ± 0.001	1.4677 ± 0.0000
28	0.65 ± 0.00	196.0 ± 5.7	7.08 ± 0.19	44.21 ± 2.07	0.924 ± 0.002	1.4677 ± 0.0001
29	0.57 ± 0.00	197.0 ± 4.2	4.10 ± 0.08	59.43 ± 3.11	0.921 ± 0.003	1.4677 ± 0.0001
30	0.30 ± 0.00	208.0 ± 2.8	4.63 ± 0.15	53.01 ± 1.17	0.923 ± 0.002	1.4677 ± 0.0001

*: The data for individual samples are expressed as means ± standard deviations (n = 3), except for smoke point (n = 2). Free fatty acid (FFA) content is a representation of the FFA concentration in an oil sample and was expressed as g oleic acid/100 g oil. Smoke point is the temperature that an oil sample begins to continuously smoke. *p*-anisidine value (pAV) is a measure of the aldehyde content in an oil sample using *para*-anisidine. The pAV was expressed as 100 times the optical density at 350 nm of 1 g of oil in 100 mL of solvent and reagent. PV was determined by the FOX-II method and is a measure of the peroxide concentration in an oil sample. The PV-FOX value was expressed as μmoles of *t*-butyl peroxide equivalents (tBPE) per g of oil. The relative density was determined at 25 °C. Refractive index (RI, η) is a measure of the ratio of the speed of light in air to the speed of light in an oil sample. The RI was expressed as this ratio of light at 589 nm (the sodium D-line) at 25 °C. All pair-wise comparisons were not shown to retain legibility of the figure; relative density was the only property that did not significantly differ among the oil samples ($P > 0.05$) by the nonparametric Kruskal-Wallis H test due to heteroscedasticity.

Figure 1 – Free fatty acid (FFA) content of extra virgin olive oil samples. Data are expressed as means ± standard deviations (n = 3). Bars are labeled with a minus sign, a plus sign, or an asterisk indicates if they are significantly less than, significantly greater than, or not significantly different from the IAOOTO standard of 0.8 g oleic acid per 100 g oil ($P < 0.05$; one-tailed) for extra virgin olive oil samples based on the ANOVA Games-Howell pair-wise comparisons test due to heteroscedasticity, as this test does not require homoscedasticity of the data. All pair-wise comparisons were not shown to retain legibility of the figure as even at a $P < 0.01$ significance level there were too many unique differences between the means to report.

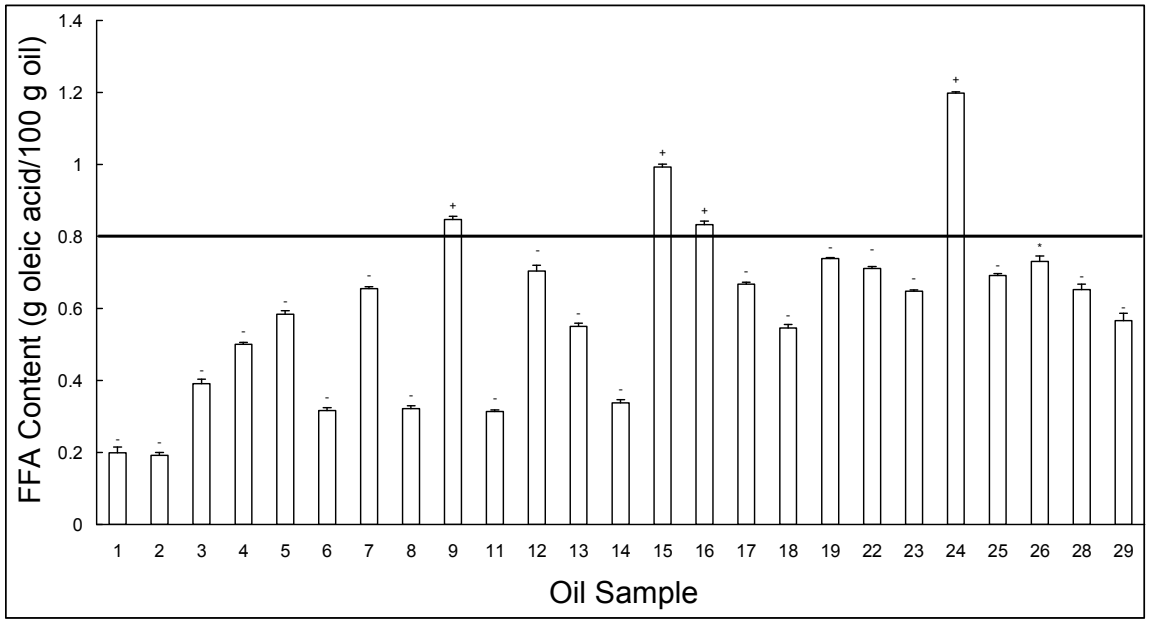


Figure 1

Figure 2 – 18:1n-9 fatty acid composition of olive oil samples. The clear and hashed bars represent the extra virgin olive oil samples (n = 25) and pure olive oil samples (n = 5), respectively. Data are expressed as means ± standard deviations (n = 3). All pair-wise comparisons were not shown to retain legibility of the figure as even at a $P < 0.01$ significance level there were too many unique differences between the means to report.

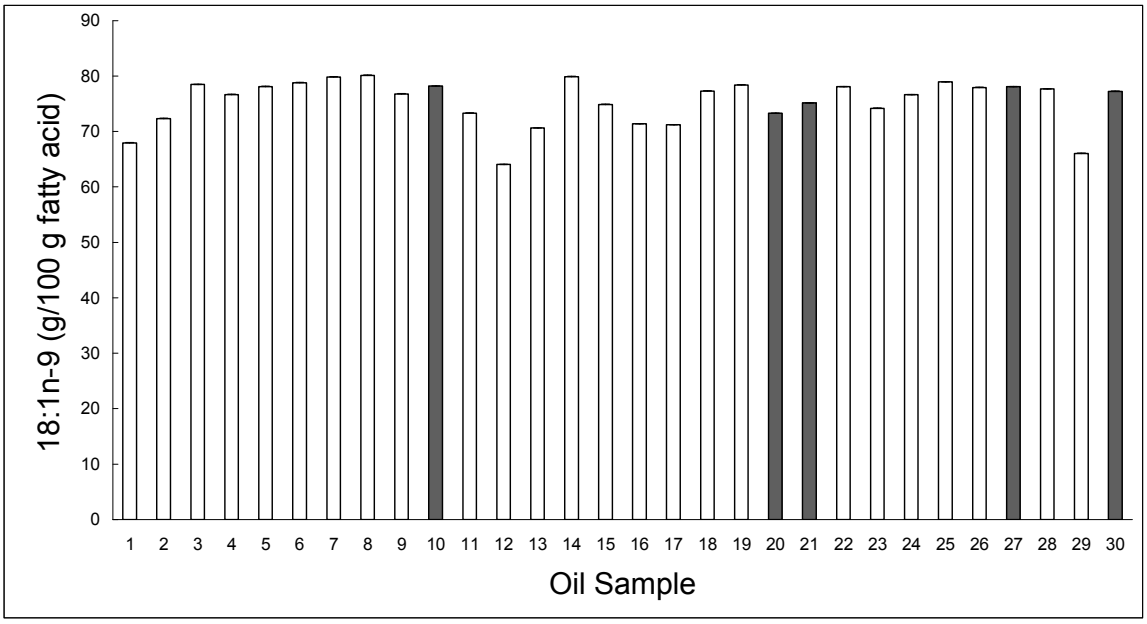


Figure 2

Figure 3 – Oxidative stability index (OSI) of olive oil samples. The clear and hashed bars represent the extra virgin olive oil samples (n = 25) and pure olive oil samples (n = 5), respectively. OSI was measured at 80 °C with an airflow rate of 7 L/h. The OSI procedure was conducted in triplicate for each sample except in a few cases where it was only conducted in duplicate due to very similar results. Data are expressed as means ± standard errors of the means (n = 3 or n = 2). All pair-wise comparisons were not shown to retain legibility of the figure as even at a $P < 0.01$ significance level there were too many unique differences between the means to report.

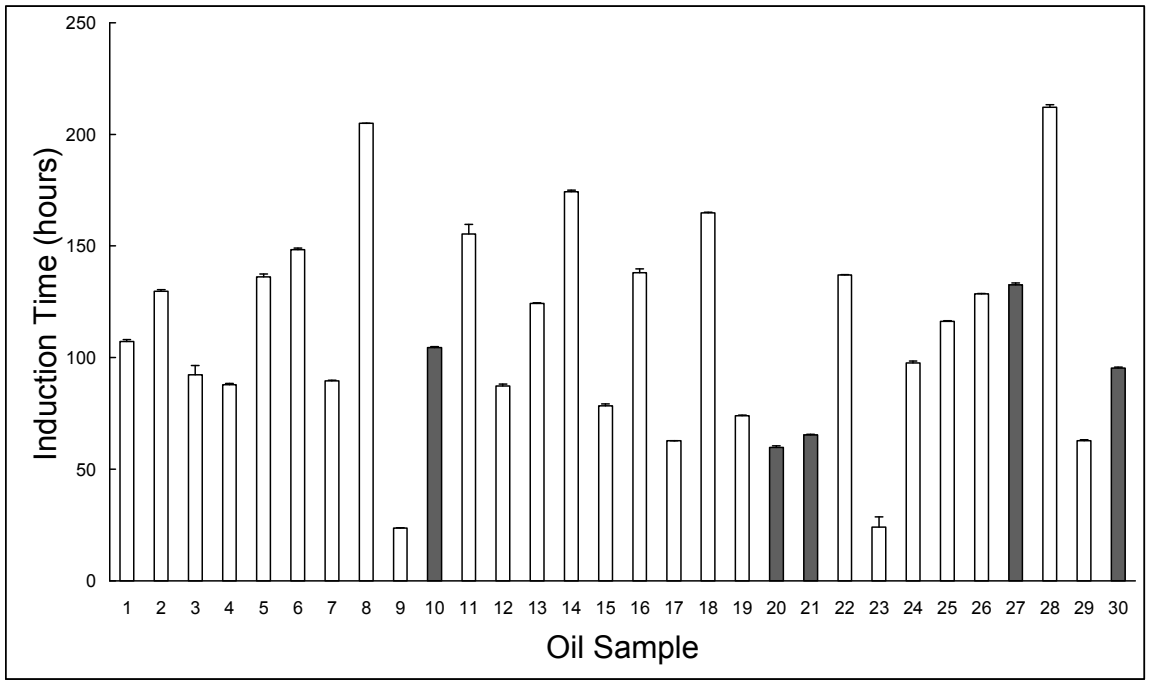


Figure 3

Chapter 3: Antioxidant Properties of Olive Oils

Introduction

Keys and colleagues (1986) first hypothesized that the traditional Mediterranean diet has beneficial effects towards a myriad of diseases. They provided evidence based on cohort studies that the diet was associated with fewer deaths from coronary heart disease than subjects on non-Mediterranean European diets. Olive oil has long been the major fat source in the diet of people from most Mediterranean countries (Boskou, 2000), thus it was thought to play a role in the overall benefits of the diet. Recently olive oil's interest as a healthy fat source has been expanding to other countries, including the United States (Boskou, 2000). This has been mainly due to the inherent health benefits associated with its high levels of monounsaturated fatty acids and phenolic compounds (Hu, 2003).

Intake of olive oil lowers the incidences of cancers (Stark & Madar, 2002) and cardiovascular diseases through hypocholesterolemia, hypotension, and decreased lipid oxidation (Scaccini et al., 1992; Stark & Madar, 2002, Moreno & Mitjavila, 2003a), mediates the immune response (de la Lastra et al., 2001; Stark & Madar, 2002), and controls hypertriglyceridemia that accompanies diabetes (Giron et al., 1999) and other aging-associated health problems (Trichopoulou & Vasilopoulou, 2000a). Specifically, extra virgin olive oils (EVOOs) are thought to be superior to most other vegetable oils, which are generally further filtered and/or solvent extracted. EVOOs may retain more health-beneficial phytochemicals in the oils due to their method of extraction from the olives and purification process (Visioli & Galli, 1998a). An association between phenolic compounds found in olive oil and the

beneficial roles of olive oil in human health exists (Boskou, 2002). Several studies have evaluated the antioxidant capacities of olive oils commercially available or produced in various countries; mainly in Spain (Ninfali et al., 2002a; Gorinstein et al., 2003), Italy (Owen et al., 2000b; Bacchiocca et al., 2001; Cabrini et al., 2001; Lavelli, 2002; Ninfali et al., 2002a; Bacchiocca et al., 2003; Pellegrini et al., 2003; Ninfali et al., 2005), or Greece (Valavanidis et al., 2004). These olive oil extracts and/or phytochemicals possess antioxidant capacities against the long-lived cationic 2,2'-azinobis(3-ethylbenzothiazoline-6-sulfonate) (ABTS) free radical (Gorinstein et al., 2003; Pellegrini et al., 2003), the cationic N,N-dimethyl-*p*-phenylenediamine dihydrochloride (DMPD) free radical (Briante et al., 2003), and the 2,2-diphenyl-1-picrylhydrazyl (DPPH) stable free radical (Gorinstein et al., 2003; Valavanidis et al., 2004) as well as the physiological free radicals of $O_2^{\bullet -}$ (anionic superoxide free radical) (Owen et al., 2000b; Lavelli, 2002; Moreno, 2003b; Valavanidis et al., 2004), $ROO^{\bullet}$ (neutral peroxyl free radical) (Cabrini et al., 2001; Pellegrini et al., 2003), $HO^{\bullet}$ (neutral hydroxyl free radical) (Owen et al., 2000b; Valavanidis et al., 2004), and $QH^{\bullet}$ (neutral semiquinonic free radical) (Lavelli, 2002).

No research has been published in peer-reviewed journals addressing total phenolic contents and antioxidant capacities of olive oils commercially available in the United States. This is of utmost importance given that the most recent standards for olive oils sold to consumers in the United States are from 1948 (United States Department of Agriculture (USDA), 1948) and that the terminology used to define olive oils commercially sold in the United States are rarely displayed on the label. The Food and Drug Administration (FDA) recently approved a new qualified health

claim for olive oil which states that "limited and not conclusive scientific evidence suggests that eating about 2 tablespoons (23 grams) of olive oil daily may reduce the risk of coronary heart disease due to the monounsaturated fat in olive oil… to achieve this possible benefit, olive oil is to replace a similar amount of saturated fat and not increase the total number of calories you eat in a day" (FDA, 2004a). However, as some of the beneficial effects of olive oil toward coronary heart disease have been linked to its phytochemical composition (Visioli & Galli, 1998a; Hu, 2003), it is very important that the evaluation of the total phenolic contents and antioxidant capacities of olive oils sold commercially in the United States are performed.

It remains unknown whether the total phenolic contents and antioxidant capacities of each olive oil are related to the cost of the olive oil to the consumer. Although a higher priced product may be of a greater quality, this may not always be the case, especially when the standards used to assess the product are neither up-to-date nor closely regulated. Further exploration of whether the price of an olive oil correlates well to its quality and stability will help determine if the price of an olive oil commercially available in the United States accurately portrays to the consumer the quality of the oil. This will aid consumers in their practical evaluation of the quality of olive oil products that are commercially available in the United States.

The objectives for this research are to examine and compare commercial olive oils in the United States for their total phenolic contents and antioxidant capacities and to assess whether the cost to the consumer is related to these parameters.

Materials and Methods

Materials

EVOOs and "pure" olive oils (POOs) were obtained from local food stores (supermarkets and health food stores) in the United States or donated by the olive oil suppliers. The olive oil samples were stored under nitrogen, in the dark, and at ambient temperature between experiments, and were handled during experimental analysis so as to minimize their contact with oxygen, light, and heat. Each sample's growing conditions, extraction history, prior storage conditions, and overall age were not known. 2,2'-azobis(2-aminopropane)dihydrochloride was purchased from Wako Chemicals USA, Inc. (Richmond, VA, USA). Randomly methylated β-cyclodextrin was purchased from CycloLab Cyclodextrin Research & Development Laboratory, Ltd. (Budapest, Hungary). Sodium carbonate was purchased from Fisher Scientific Chemicals USA, Inc. (Fairlawn, NJ, USA). All other chemicals and reagents were purchased from Sigma-Aldrich, Inc. (St. Louis, MO, USA). All chemicals and solvents were of highest commercial grade and used without further purification.

Preparation of Antioxidant Extracts

One thousand five hundred mg of each olive oil sample was placed into a 15 mL glass test tube with a screw top and mixed with 4.5 mL of methanol. After centrifugation of each oil sample solution at 2000 g for 3 min, the supernatant was collected in a clean 15 mL glass test tube with a screw top while 3 mL of methanol was added to the residue oil and mixed. Again, after centrifugation of each oil sample solution at 2000 g for 3 min, the supernatant was collected in the same 15 mL

glass test tube with a screw top as before while 3 mL of methanol was again added to the residue oil and mixed. Finally, after centrifugation of each oil sample solution at 2000 g for 3 min once again, the supernatant was collected in the same 15 mL glass test tube with a screw top as before and the test tube was filled to a final volume of 15 mL with methanol and stored under nitrogen and in the dark until analysis.

Total Phenolic Contents (TPC)

The TPC of each olive oil sample methanolic extract, which measures the total concentration of phenolic compounds in an extract (Singleton et al., 1998), was determined using a spectrophotometer (Genesys 20 Model 4001/4; ThermoSpectronic, Rochester, NY, USA) according to a procedure previously described by Parry and colleagues (2005). Gallic acid was used as the standard at concentrations of 5, 10, 20, 40, 80, and 100 μg/mL in methanol. The reaction mixture contained 800 μL of 7 % randomly methylated β-cyclodextrin (RMCD), 50 μL of gallic acid solution or oil extract, 2200 μL doubly-distilled water, 250 μL of the Folin-Ciocalteu reagent, and 750 μL of 20 % (w/v) disodium carbonate. Absorbance was measured at 765 nm following 2 hr of reaction time at ambient temperature and in the dark against a mixture of 800 μL of 7 % RMCD, 50 μL of methanol, 2200 μL of pure water, and 1000 μL of 20 % (w/v) disodium carbonate. Measurements were conducted in duplicate for each gallic acid solution and in triplicate for each oil extract. The TPC of each oil extract was calculated from the standard curve and was expressed as concentration of gallic acid equivalents (GAE) (μg/g oil). All

measurements were conducted in the same day and with the same reagent preparations.

2,2-diphenyl-1-picrylhydrazyl (DPPH) Free Radical Scavenging Ability

The free radical scavenging ability of each olive oil sample methanolic extract against the stable DPPH free radical ($DPPH^{\bullet}$) was determined using a spectrophotometric method according to a procedure previously described by Parry and colleagues (2005). The assay reaction was initiated by mixing 1 mL of methanol or oil extract (50 mg olive oil equivalents (OOE) per mL of the radical-antioxidant reaction mixture) with 1 mL of 200 mM $DPPH^{\bullet}$. The % $DPPH^{\bullet}$ remaining was calculated as the fraction of the absorbance at 517 nm obtained at 10 min antioxidant-radical reaction in the dark for the control and each oil extract divided by the absorbance at 517 nm obtained at 45 sec for the control reaction. No adjustment of the % $DPPH^{\bullet}$ remaining was needed for differences in the mass of each oil sample used in the extraction procedure and the dilution factor of each oil sample from the extraction procedure as the masses were within 0.004 g of each other (< 0.3 % differences; mean = 1.50 g with SD < 0.01 g) and each dilution volume was identical. Measurements were conducted in triplicate for the control and each oil extract. All measurements for each experiment were conducted in the same day and with the same reagent preparations.

Additionally, the $DPPH^{\bullet}$ scavenging assay was used to assess time- and dose-dependence of a single, randomly selected oil extract on the amount of $DPPH^{\bullet}$

scavenging. The selected oil extract was kept at its original 100 % concentration (50 mg OOE/mL reaction mixture) and also diluted to concentrations of 45, 40, 33, 25, 18, and 10 mg OOE/mL reaction mixture. Absorbance was measured at 517 nm following 0.75, 4, 10, 20, 40, and 60 min of reaction time at ambient temperature against a blank of methanol for the control and each dilution of the single oil extract. Measurements were conducted in triplicate for each dilution of the selected oil sample extract solution.

Oxygen Radical Absorbance Capacity ($ORAC_{FL}$)

The ORAC of each olive oil sample methanolic extract, which measures radical scavenging capacity against the peroxyl free radical (Cao & Prior, 1999), was determined using a fluorometer (Model FM109526; Barnstead International, Dubuque, IA, USA) according to a procedure previously described by Ou and colleagues (2001). Trolox was used as the standard at concentrations of 10, 40, 80, and 120 μM in methanol and oil extracts were diluted as needed with methanol. The reaction mixture contained 2380 μL phosphate buffer (10 mM, pH 7.4), 20 μL of 10 μM fluorescein (FL), 300 μL of methanol, Trolox solution, or oil extract, and 300 μL of known free radical initiator 600 mM 2,2'-azobis(2-amidinopropane)dihydrochloride (AAPH). Fluorescence was measured at an excitation wavelength of 490 nm and an emission wavelength of greater than 515 nm every minute until there were less than 100 fluorescence units being emitted at ambient temperature. The area under each sample's curve (AUCs) was then calculated by the trapezoidal method and was then used to calculate the relative AUC

by subtracting the control AUC from each oil extract's AUC. Measurements were conducted in duplicate for each Trolox solution and in triplicate for each oil extract except for a few samples where they were only conducted in duplicate due to very similar results. The $ORAC_{FL}$ of each oil extract was calculated from the standard curve and was expressed as concentration of Trolox equivalents (TE) (μmol/g oil). All measurements were conducted with the same reagent preparations.

Statistical Analyses

All analyses were conducted using SPSS (version 10.0.1; SPSS Inc., Chicago, IL, USA) software application for Windows. Data were reported as means ± standard deviations (SDs) unless noted otherwise, in which case data were reported as means ± standard errors of the means (SEMs) due to unequal sample sizes in the data being compared.

Assumptions for some of the statistical analyses were homoscedasticity and normality of the data. Homoscedasticity was determined by the Levene's test. Determination of whether the data followed a Gaussian distribution was performed using the Kolmogorov-Smirnov test.

Individual olive oil sample data sets were analyzed for significant differences among the means for various response variables. For homoscedastic data that followed a Gaussian distribution, the one-way analysis of variance (ANOVA) was used with pair-wise comparisons using the conservative Tukey's honestly significant difference (HSD) test to help minimize inflation of Type I errors due to performing

all pair-wise comparisons; for heteroscedastic data or if the data were not normally distributed, the Games-Howell test was used to analyze pair-wise comparisons as it neither requires homoscedasticity nor normality of the data.

Simple linear regression analysis and correlation analysis were used to analyze relationships between two variables for individual olive oil samples. For homoscedastic data that followed a Gaussian distribution, simple linear regression analysis was used where each individual olive oil sample's mean explanatory variable value was paired with all of the response variable values and the Pearson's correlation coefficient (r) and the coefficient of determination (R^2) were calculated; for heteroscedastic data, weighted least squares linear regression was used where the weights were based on the reciprocals of the variance in the response variable; if the data were not normally distributed, they were not analyzed by linear regression, however the non-parametric Kendall's correlation coefficient (τ_b) was calculated as it does not require normality of the data.

Extreme outliers, defined as any data points greater than 2.5 standard deviations away from the upper or lower quartile values, were excluded from all data and noted when their exclusion allowed for more appropriate analyses and results. Statistical significance was generally declared at $P < 0.05$ unless noted otherwise.

Results and Discussion

Extraction of each olive oil sample was performed to remove phytochemicals from the oil matrix so that they could be analyzed in hydrophilic environments. Most

antioxidant assays require such extractions and thus determination of a superior extraction method is crucial. Extraction of antioxidant compounds from olive oil samples yields greater concentrations of these phenolic compounds when absolute methanol is used as the extraction solvent as compared to an 80 % (v/v) methanol solution (Owen et al., 2000c). Additionally, performing any number of extractions greater than two resulted in little to no additional phenolic compounds being extracted (Owen et al., 2000c).

The TPC of oil extracts quantify their total concentration of phenolic compounds which include: simple phenolic compounds, secoiridoid compounds, flavonoids, lignans, and tocopherols (Singleton et al., 1998). As compounds containing phenolic structures will react with the FC reagent, it is hypothesized that the molybdenum-containing FC reagent can be reduced by an electron transfer which in turn converts the rather yellow FC reagent to a blue species which can be measured spectroscopically (Huang et al., 2005). Some of the TPC of the individual olive oil samples significantly differed from one another (Figure 1). The observed values ranged from 553.7 to 810.4 μg GAE/g oil with mean and median of 688.4 and 671.5 μg GAE/g oil, respectively. Thus the consumer can never be fully assured that one brand of olive oil has higher concentrations of phenolic compounds. Such variability was also reported by others who used the same solvent system and standard compound (Montedoro et al., 1992; Del Carlo et al., 2004). The use of various extraction solvents and standard compounds results in much variation in the TPC of olive oil samples (Gutfinger, 1981; Bacchiocca et al., 2001; Ninfali et al., 2002a; Psomiadou & Tsimidou, 2002a; Psomiadou & Tsimidou, 2002b; Patumi et al., 2003;

Romero et al., 2003; Salvador et al., 2003; Ninfali et al., 2005). Overall, the absolute methanol extraction was superior to the other extraction solvents used.

Although the TPC correlates well with many free radical scavenging capacities, this is not always the case. The FC reagent does react with tyrosine side-chains even though these groups do not have free radical scavenging capacities (Huang et al., 2005). Specifically, TPC measures the total concentration of compounds that contain aromatic rings that can dissociate a proton at ca. pH 10 (Huang et al., 2005) and thus appropriately approximates the phenolic fraction of sample extractions. Yet this procedure also usually overestimates the antioxidant activity of the extraction due to the lack of antioxidant capacity of some phenolic compounds (Huang et al., 2005). Thus, it is essential to verify that the phenolic compounds found in olive oil and olive oil extracts actually possess antioxidant activity.

The $DPPH^{\bullet}$ scavenging ability, as % $DPPH^{\bullet}$ remaining over time, was determined for every olive oil sample to help gather a full picture of the free radical scavenging abilities of the olive oil extracts. Most of the $DPPH^{\bullet}$ scavenging abilities significantly differed among the individual olive oil samples. However, there were too many interrelationships to illustrate them on the figure itself (Figure 2; statistical analysis not shown for clarity). Measured at 10 minutes antioxidant-radical reaction and for 50 mg olive oil equivalents (OOE)/mL reaction mixture, olive oil extracts quenched 16.03 to 70.12 % $DPPH^{\bullet}$ in the radical-antioxidant reaction mixture with mean and median of 37.43 and 36.39 % $DPPH^{\bullet}$ quenched, respectively. These $DPPH^{\bullet}$ scavenging capacity values differed from those of other studies, however the

other studies either used different concentrations of extract and different times for the antioxidant-radical reaction (Gorinstein et al., 2003) or used different measuring methods.

The DPPH$^{\bullet}$ scavenging ability, expressed as % DPPH$^{\bullet}$ remaining, of olive oil extracts was both time- and dose-dependent from 45 seconds to 60 minutes and over the doses of 10 to 50 mg OOE/mL reaction mixture, respectively (Figure 3). This illustrates that olive oil extracts affect the kinetics and equilibrium of the free radical-quenching reaction. Kinetically, as the concentration of antioxidant substrates increases, the rate of the forward reaction (i.e. from free radicals to quenched free radicals) increases while the rate of the reverse reaction does not change. Thus, based on the rate law, an increase in olive oil extracts results in the net overall forward reaction proceeding at a faster rate. More DPPH$^{\bullet}$ are being quenched in a shorter time period. In addition, as the reaction tends toward equilibrium (i.e. at the farther time points) the net reaction rate slows and the overall rate of quenching DPPH$^{\bullet}$ lessens. As most of the data points differed significantly, these results verify that the olive oil extracts possess both time- and dose-dependent free radical scavenging abilities, at least against the stable DPPH$^{\bullet}$.

The oxygen radical absorbance capacity assay using fluorescein ($ORAC_{FL}$) as the fluorescent probe measures antioxidant scavenging activity against peroxyl radicals induced by AAPH at a physiological pH (Cao & Prior, 1998; Ou et al., 2001; Ou et al., 2002; Huang et al., 2005). Further support for the effectiveness of the ORAC method is that the ORAC of individual phenolic compounds is in reasonable agreement with each of their structures (Roginsky & Lissi, 2005), and thus its use on

olive oil samples that contain high concentrations of phenolic compounds is appropriate. As was the case for the $DPPH^{\bullet}$ scavenging ability results, many of the individual $ORAC_{FL}$ values for each of the olive oil samples significantly differed from one another although there were too many interrelationships to illustrate them on the figure itself (Figure 4; statistical analysis not shown for clarity). Collectively the values found in this research ranged from 1.39 to 11.67 μmol TE/g oil with mean and median of 4.82 and 4.74 μmol TE/g oil, respectively.

The ORAC assay has been conducted on olive oil samples from Italy and Spain by only Ninfali, Bacchiocca, and colleagues (Bacchiocca et al., 2001; Ninfali et al., 2001; Ninfali et al., 2002a; Ninfali et al., 2002b; Bacchiocca et al., 2003; Ninfali et al., 2005). They have measured the ORAC values for olive oil samples by using B-phycoerythrin (B-PE) as the fluorescent probe until recently when for one EVOO sample they used fluorescein (Ninfali et al., 2005). B-PE is an inferior detector of ORAC as compared to the fluorescein probe utilized in this research as it tends to result in inconsistent data (Cao & Prior, 1999, Ou et al., 2001). Based on the one result from the $ORAC_{FL}$ assay and the other data from the $ORAC_{B\text{-}PE}$ assay, the present results are similar (Bacchiocca et al., 2001; Ninfali et al., 2001; Ninfali et al., 2002a; Ninfali et al., 2005). Based on the critical analysis of the probe used in the assay (Ou et al., 2001), the results from this research (where fluorescein was used as the fluorescent probe) seem more reliable than those obtained using B-PE. To put these values in perspective, Ou and colleagues (2002) found $ORAC_{FL}$ values, for 50 % acetone extracted common vegetables, ranging from 12 to 300 μmol TE/g oil, dry weight (lowest was peas and highest was green peppers) which are all greater than

those found for all of the EVOO samples studied in this research. However, using peanut oil, whose predominant fatty acid is also oleic acid, Ninfali and colleagues (2005) found the $ORAC_{FL}$ value, for an 80 % methanol extraction, to be 1.06 μmol TE/g oil. This is lower than the values found for all of the EVOO samples studied in this research.

Free radical scavenging assays such as $DPPH^{\bullet}$ scavenging ability and $ORAC_{FL}$ do not necessarily correlate with TPC values. Every antioxidant/phenolic compound differs in their free radical scavenging ability. In this study, the TPC of olive oil extracts did not significantly correlate to their $DPPH^{\bullet}$ scavenging abilities. However, the TPC of each olive oil extract did significantly correlate with each sample's $ORAC_{FL}$ value, although rather weakly with Kendall's correlation coefficient of $\tau_b = 0.30$ and a coefficient of determination of $R^2 = 0.09$ ($P < 0.01$), due to presence of an extreme outlier in the data. This finding suggests that the $ORAC_{FL}$ assay is a better measure of free radical scavenging ability of olive oil extracts.

For the second batch of olive oils that were purchased for this research (sample numbers 12 – 28) the price of each was noted, as another objective of this research was to explore any affects the price of olive oils commercially available in the United States had on their total phenolic contents and/or antioxidant capacities. Calculation of the price per volume (PPV) of an olive oil normalizes the original price of each olive oil to the amount of oil actually in the bottle. Although some olive oils were sold at reduced sale prices, the original prices were used for all analyses as they seemed like better predictors of the usual price of the oils. There was a good amount of variation in the PPVs of the individual olive oil samples (Table 1). Their values

ranged from 0.72 to 1.92 cent/mL oil with mean and median of 1.27 and 1.20 cent/mL oil, respectively. Thus as was the case for many of the analyses, the consumer cannot be completely guaranteed that one olive oil is better than another on a price per volume basis.

The price of the olive oil samples, per unit volume, in this research were significantly correlated to some of the antioxidant parameters studied. Of these, the PPV was moderately correlated to the $DPPH^{\bullet}$ scavenging ability, measured as the % $DPPH^{\bullet}$ remaining after a given amount of reaction time, and inversely, as expected, with Kendall's correlation coefficient of $\tau_b = -0.41$ and a coefficient of determination of $R^2 = 0.17$ ($P < 0.01$), due to lack of normality of the data. PPV was directly correlated to TPC yet not to the same extent as $DPPH^{\bullet}$ scavenging, but still significantly at $P < 0.05$, with weighted Pearson's correlation coefficient of $r = 0.35$ and a coefficient of determination of $R^2 = 0.12$, due to heteroscedasticity of the data. This shows that most likely as the PPV of an olive oil sample increases, so do the expected findings for two of the three antioxidant-related parameters studied in this research. However, as all significant associations had $R^2 < 0.20$ and thus only less than 20 % of the variation in each of the response variables was explained by PPV, the strength and relevance of these associations is in question.

It is interesting to note that some of the olive oils used in the analyses were bottled by the same company and only differed in one being of the extra virgin quality while the other was of the pure quality. Three pairs of olive oils fit this description and interestingly, in one pair the EVOO had a larger PPV than its POO counterpart, in another pair the EVOO and its POO counterpart had the same PPV,

while in the last pair the EVOO had a smaller PPV than its POO counterpart (Appendix – Table 2). For these pairs, there was no consistent pattern to which type of olive oil had better results from each analysis performed. Only one EVOO and one POO sample were analyzed within each company and thus, in order to draw more conclusive results, the study should be repeated using multiple bottles of EVOO and POO from each company and should be purchased on separate days. This would increase the statistical significance of the findings and allow the date of purchase to be analyzed as for its associations.

Conclusions

These results demonstrate that olive oils commercially available in the United States differ based on their total phenolic contents and antioxidant capacities. As quality of life and human health may be significantly affected by diet (Dreher, 1997), the desire for better health and less occurrence of disease in consumers leads them to make more educated decisions when purchasing food products. In order to make these informed decisions when purchasing foods, it is vital to be aware that some food products have more variability in their quality than other food products, in terms of antioxidant capacities and free radical scavenging abilities, than other food products. Additionally, a significant trend exists where as the PPV of an olive oil sample increases, so does its $DPPH^{\bullet}$ scavenging ability and TPC, as well as $ORAC_{FL}$ when analyzed at a $P < 0.06$. Thus, the PPV may be associated with the quality of most olive oils based on those studied in this research.

Overall, these findings demonstrate that olive oils commercially available in the United States have variable levels of quality as assessed by antioxidant-related assays. The decision to purchase a certain product should not be based solely on what is presented on the label. Detailed and recent phytochemical analyses are far superior predictors of a product's quality and may differ from the quality inferred by the label.

Figures and Tables

Table 1: Price – price per unit volume (PPV) of olive oil samples*

Sample Num	PPV (cent/mL)
12	1.68
13	1.92
14	1.52
15	1.40
16	1.20
17	1.00
18	1.47
19	0.72
20	0.72
21	1.05
22	1.40
23	1.24
24	1.00
25	1.92
26	1.20
27	1.19
28	1.05

*: The data for individual samples are each expressed as one value as PPV was calculated from two given values (i.e. none were determined experimentally) and thus was only determined once per sample. The PPV is a measure of the cost of each oil sample, normalized to its volume and is expressed as cent/mL oil.

Figure 1 – Total phenolic contents (TPC) of methanolic extracts of olive oil samples. The clear and hashed bars represent the extra virgin olive oil samples (n = 25) and pure olive oil samples (n = 5), respectively. The TPC value is a representation of the total concentration of phenolic compounds in an absolute methanol oil sample extract and is expressed as μg of gallic acid equivalents (GAE) per g of oil. The TPC procedure was conducted in triplicate for each sample. Data are expressed as means ± standard deviations (n = 3). Bars labeled with the same letter have means that are not significantly different from one another ($P < 0.05$) based on the ANOVA Games-Howell pair-wise comparisons test due to heteroscedasticity, as this test does not require homoscedasticity of the data. All pair-wise comparisons were analyzed at $P < 0.01$ significance level in order to retain legibility of the figure that was lost at $P < 0.05$.

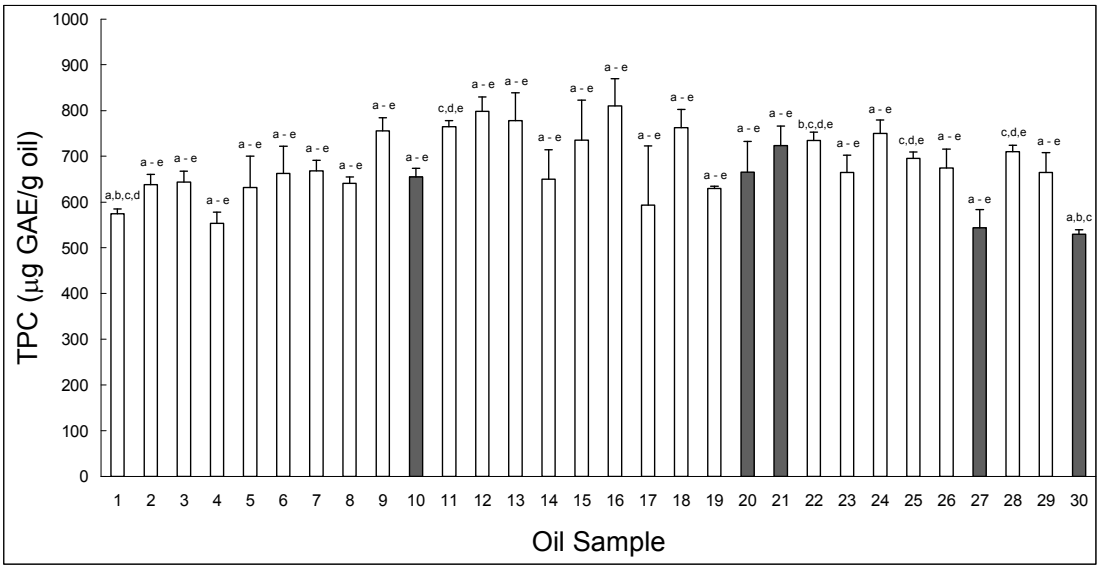


Figure 1

Figure 2 – DPPH free radical (DPPH•) scavenging ability of methanolic extracts of olive oil samples. The dotted, clear, and hashed bars represent the control, extra virgin olive oil samples (n = 23), and pure olive oil samples (n = 3), respectively. The DPPH• scavenging ability is a representation of the radical scavenging capacity against the stable DPPH• in an absolute methanol oil sample extract and is expressed as % DPPH• remaining. The initial concentration of DPPH• was 100 μM and the concentration of olive oil extracts was 50 mg olive oil equivalents (OOE) per mL of the radical-antioxidant reaction mixture for all antioxidant-radical reactions. The DPPH• scavenging capacity was measured at 10 min of antioxidant-radical reaction. The DPPH• scavenging assay was conducted in triplicate for each sample. Data are expressed as means ± standard deviations (n = 3). All pair-wise comparisons were not shown to retain legibility of the figure as even at a $P < 0.01$ significance level there were too many unique differences between the means to report.

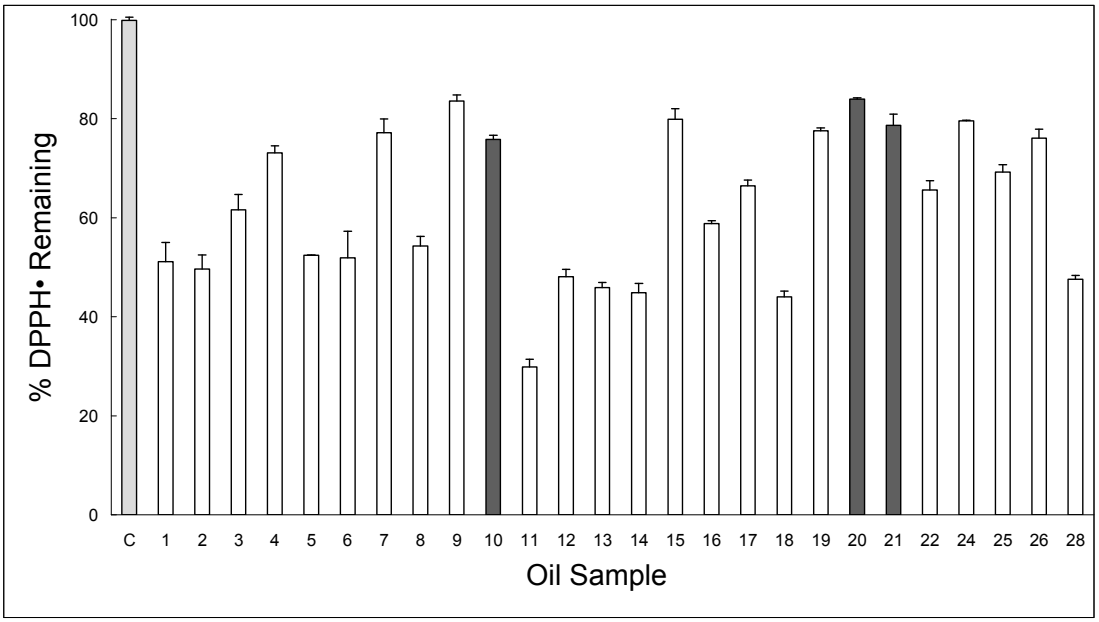


Figure 2

Figure 3 – DPPH free radical (DPPH•) scavenging ability of methanolic extracts of an olive oil sample over time at varying concentrations. The initial concentration of DPPH• was 100 μM for all antioxidant-radical reactions. The concentration of absolute methanol oil sample extract was analyzed at 10, 18, 25, 33, 40, 45, and 50 mg/mL reaction mixture and is expressed as mg of olive oil equivalents (OOE) per mL of the radical-antioxidant reaction mixture. The DPPH• scavenging capacity was measured at 0.75, 4, 10, 20, 40, and 60 min of antioxidant-radical reaction. The DPPH• scavenging procedure was conducted in triplicate for each sample. Data are expressed as means ± standard deviations (n = 3).

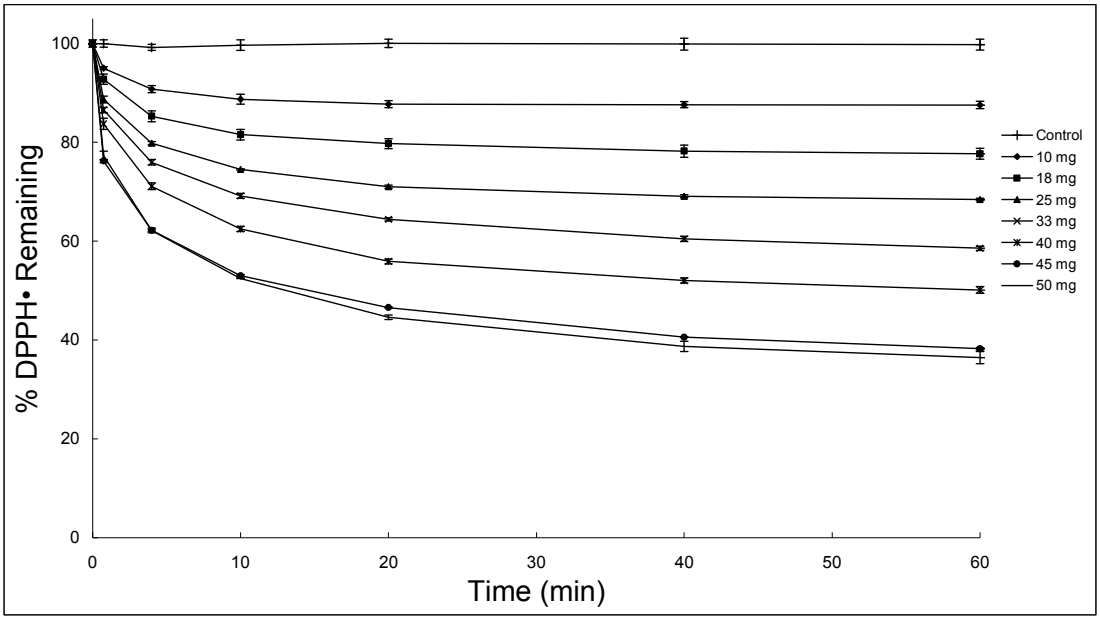


Figure 3

Figure 4 – Oxygen radical absorbance capacity ($ORAC_{FL}$) of methanolic extracts of olive oil samples. The clear and hashed bars represent the extra virgin olive oil samples (n = 24) and pure olive oil samples (n = 4), respectively. The $ORAC_{FL}$ value is a representation of the radical scavenging capacity against the peroxyl free radical in an absolute methanol oil sample extract and is expressed as μmoles of Trolox equivalents (TE) per g of oil. The $ORAC_{FL}$ procedure was conducted in triplicate for each sample except in a few cases where it was only conducted in duplicate due to very similar results. Data are expressed as means ± standard errors of the means (n = 3 or n = 2). All pair-wise comparisons were not shown to retain legibility of the figure as even at a $P < 0.01$ significance level there were too many unique differences between the means to report.

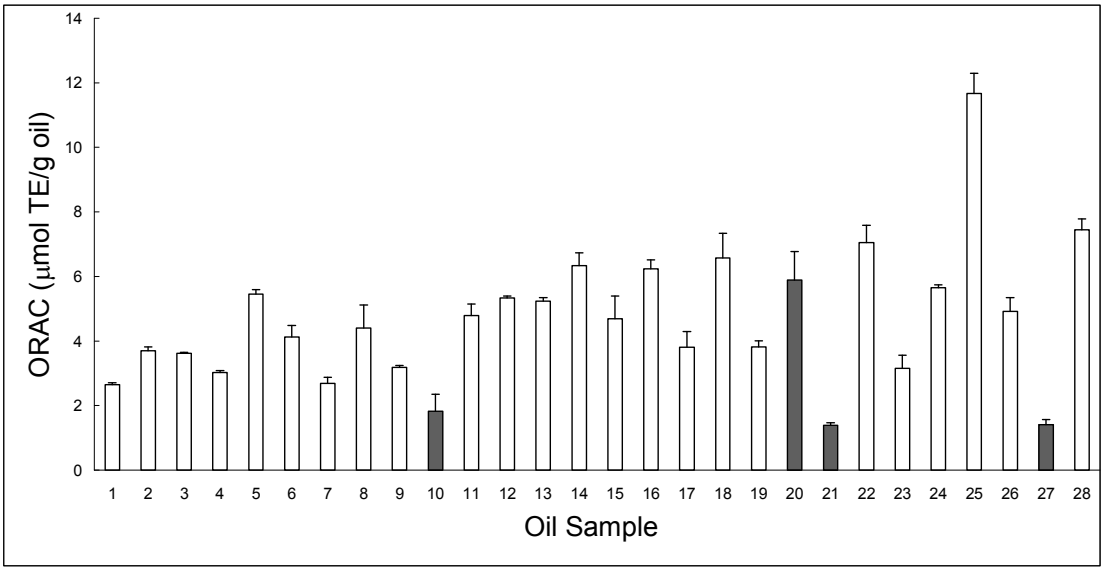


Figure 4

Appendix

Table 1: Fatty acid composition of olive oil samples*

	Fatty acid composition (g/100 g fatty acids)			
	16:0	**16:1n-7**	**18:0**	**18:1n-9**
1	15.97 ± 0.01	1.82 ± 0.00	1.70 ± 0.01	67.96 ± 0.04
2	12.66 ± 0.01	0.91 ± 0.00	2.44 ± 0.01	72.34 ± 0.03
3	10.87 ± 0.01	0.61 ± 0.00	2.93 ± 0.02	78.48 ± 0.05
4	11.31 ± 0.01	0.63 ± 0.00	2.77 ± 0.01	76.68 ± 0.02
5	11.51 ± 0.01	0.70 ± 0.01	2.27 ± 0.01	78.11 ± 0.01
6	11.30 ± 0.01	0.63 ± 0.00	1.77 ± 0.01	78.79 ± 0.02
7	9.49 ± 0.02	0.49 ± 0.00	2.42 ± 0.02	79.82 ± 0.05
8	11.15 ± 0.00	0.73 ± 0.00	2.92 ± 0.01	80.12 ± 0.02
9	12.35 ± 0.02	0.85 ± 0.01	1.57 ± 0.01	76.77 ± 0.02
10	10.17 ± 0.01	0.66 ± 0.01	3.30 ± 0.01	78.18 ± 0.05
11	12.64 ± 0.02	0.97 ± 0.00	2.54 ± 0.01	73.32 ± 0.03
12	14.84 ± 0.01	1.60 ± 0.01	2.47 ± 0.01	64.06 ± 0.01
13	13.51 ± 0.01	1.14 ± 0.00	2.68 ± 0.01	70.61 ± 0.03
14	9.32 ± 0.01	0.53 ± 0.01	3.19 ± 0.02	79.89 ± 0.03
15	11.62 ± 0.01	0.59 ± 0.01	2.42 ± 0.01	74.88 ± 0.02
16	14.11 ± 0.00	1.00 ± 0.01	2.76 ± 0.01	71.39 ± 0.02
17	13.44 ± 0.01	1.23 ± 0.01	3.01 ± 0.01	71.21 ± 0.03
18	8.94 ± 0.01	0.47 ± 0.01	2.56 ± 0.01	77.28 ± 0.03
19	10.41 ± 0.01	0.70 ± 0.00	3.30 ± 0.01	78.39 ± 0.03
20	12.32 ± 0.01	0.76 ± 0.01	2.40 ± 0.01	73.30 ± 0.02
21	10.80 ± 0.02	0.60 ± 0.01	3.08 ± 0.02	75.12 ± 0.05
22	10.65 ± 0.01	0.65 ± 0.01	2.71 ± 0.02	78.06 ± 0.01
23	12.05 ± 0.01	0.66 ± 0.00	2.75 ± 0.01	74.16 ± 0.02
24	10.89 ± 0.01	0.59 ± 0.00	2.90 ± 0.01	76.67 ± 0.01
25	10.53 ± 0.01	0.60 ± 0.00	2.58 ± 0.01	78.93 ± 0.02
26	11.36 ± 0.00	0.67 ± 0.01	2.59 ± 0.02	77.93 ± 0.05
27	10.66 ± 0.01	0.68 ± 0.00	3.23 ± 0.01	78.05 ± 0.03
28	10.11 ± 0.01	0.59 ± 0.00	2.87 ± 0.01	77.68 ± 0.02
29	15.64 ± 0.01	1.73 ± 0.00	1.98 ± 0.01	66.04 ± 0.01
30	11.14 ± 0.01	0.74 ± 0.01	2.90 ± 0.01	77.25 ± 0.03

	Fatty acid composition (g/100 g fatty acids)			
	18:2n-6	**18:3n-3**	**20:0**	**20:1n-9**
1	11.31 ± 0.07	0.60 ± 0.01	0.39 ± 0.01	0.25 ± 0.00
2	10.32 ± 0.01	0.63 ± 0.00	0.40 ± 0.01	0.31 ± 0.00
3	5.67 ± 0.01	0.68 ± 0.01	0.45 ± 0.01	0.30 ± 0.00
4	7.06 ± 0.01	0.75 ± 0.01	0.48 ± 0.01	0.32 ± 0.01
5	5.86 ± 0.01	0.69 ± 0.00	0.43 ± 0.00	0.42 ± 0.01
6	6.04 ± 0.02	0.71 ± 0.00	0.36 ± 0.01	0.39 ± 0.01
7	6.43 ± 0.02	0.62 ± 0.01	0.40 ± 0.01	0.33 ± 0.01
8	3.81 ± 0.02	0.62 ± 0.00	0.38 ± 0.00	0.27 ± 0.01
9	7.05 ± 0.02	0.73 ± 0.01	0.32 ± 0.00	0.37 ± 0.01
10	6.42 ± 0.02	0.59 ± 0.00	0.40 ± 0.01	0.27 ± 0.01
11	9.06 ± 0.02	0.78 ± 0.01	0.40 ± 0.00	0.29 ± 0.01
12	15.61 ± 0.01	0.70 ± 0.01	0.43 ± 0.00	0.27 ± 0.01
13	10.60 ± 0.01	0.71 ± 0.01	0.45 ± 0.01	0.29 ± 0.00
14	5.72 ± 0.05	0.64 ± 0.00	0.40 ± 0.00	0.31 ± 0.01
15	9.07 ± 0.01	0.62 ± 0.00	0.44 ± 0.01	0.37 ± 0.00
16	9.23 ± 0.02	0.72 ± 0.01	0.48 ± 0.01	0.31 ± 0.01
17	9.72 ± 0.02	0.68 ± 0.00	0.41 ± 0.00	0.30 ± 0.00
18	9.07 ± 0.01	0.98 ± 0.01	0.33 ± 0.00	0.36 ± 0.01
19	5.92 ± 0.02	0.62 ± 0.00	0.40 ± 0.00	0.26 ± 0.00
20	9.58 ± 0.02	0.67 ± 0.00	0.44 ± 0.00	0.53 ± 0.01
21	9.01 ± 0.02	0.63 ± 0.01	0.44 ± 0.01	0.31 ± 0.01
22	6.54 ± 0.02	0.65 ± 0.01	0.41 ± 0.00	0.33 ± 0.00
23	8.94 ± 0.01	0.59 ± 0.01	0.46 ± 0.01	0.38 ± 0.01
24	7.50 ± 0.02	0.66 ± 0.01	0.48 ± 0.01	0.30 ± 0.01
25	5.97 ± 0.02	0.65 ± 0.00	0.42 ± 0.00	0.32 ± 0.00
26	5.95 ± 0.02	0.69 ± 0.01	0.47 ± 0.01	0.32 ± 0.01
27	6.13 ± 0.02	0.59 ± 0.01	0.40 ± 0.00	0.26 ± 0.00
28	7.42 ± 0.02	0.67 ± 0.00	0.37 ± 0.00	0.28 ± 0.00
29	13.17 ± 0.00	0.79 ± 0.01	0.36 ± 0.00	0.27 ± 0.01
30	6.62 ± 0.02	0.65 ± 0.01	0.41 ± 0.00	0.29 ± 0.01

	Fatty acid composition (g/100 g fatty acids)					
	SAT	**MUFA**	**PUFA**	**MUFA:SAT**	**SAT:PUFA**	**MUFA:PUFA**
1	18.06 ± 0.02	70.03 ± 0.04	11.91 ± 0.07	3.88 ± 0.00	1.52 ± 0.01	5.88 ± 0.04
2	15.49 ± 0.02	73.56 ± 0.03	10.95 ± 0.01	4.75 ± 0.01	1.41 ± 0.00	6.72 ± 0.01
3	14.26 ± 0.03	79.39 ± 0.05	6.38 ± 0.01	5.57 ± 0.02	2.25 ± 0.00	12.51 ± 0.03
4	14.56 ± 0.01	77.63 ± 0.01	7.88 ± 0.01	5.33 ± 0.00	1.86 ± 0.00	9.93 ± 0.02
5	14.21 ± 0.01	79.23 ± 0.02	6.55 ± 0.01	5.57 ± 0.00	2.17 ± 0.00	12.10 ± 0.02
6	13.43 ± 0.01	79.82 ± 0.02	6.75 ± 0.02	5.94 ± 0.00	1.99 ± 0.00	11.82 ± 0.03
7	12.31 ± 0.03	80.64 ± 0.05	7.05 ± 0.02	6.55 ± 0.02	1.75 ± 0.00	11.44 ± 0.05
8	14.45 ± 0.01	81.12 ± 0.02	4.43 ± 0.02	5.61 ± 0.00	3.27 ± 0.01	18.33 ± 0.07
9	14.24 ± 0.02	77.99 ± 0.02	7.77 ± 0.01	5.48 ± 0.01	1.83 ± 0.00	10.03 ± 0.02
10	13.87 ± 0.03	79.11 ± 0.04	7.01 ± 0.02	5.70 ± 0.01	1.98 ± 0.00	11.29 ± 0.04
11	15.58 ± 0.01	74.58 ± 0.02	9.85 ± 0.02	4.79 ± 0.00	1.58 ± 0.01	7.57 ± 0.02
12	17.74 ± 0.00	65.94 ± 0.01	16.32 ± 0.02	3.72 ± 0.00	1.09 ± 0.00	4.04 ± 0.00
13	16.64 ± 0.02	72.04 ± 0.03	11.32 ± 0.01	4.33 ± 0.01	1.47 ± 0.00	6.37 ± 0.01
14	12.91 ± 0.03	80.73 ± 0.04	6.36 ± 0.05	6.25 ± 0.01	2.03 ± 0.02	12.69 ± 0.11
15	14.48 ± 0.01	75.84 ± 0.01	9.69 ± 0.01	5.24 ± 0.00	1.49 ± 0.00	7.83 ± 0.01
16	17.35 ± 0.00	72.70 ± 0.02	9.96 ± 0.02	4.19 ± 0.00	1.74 ± 0.00	7.30 ± 0.02
17	16.86 ± 0.01	72.74 ± 0.02	10.40 ± 0.02	4.32 ± 0.00	1.62 ± 0.00	7.00 ± 0.01
18	11.83 ± 0.01	78.11 ± 0.02	10.06 ± 0.01	6.60 ± 0.01	1.18 ± 0.00	7.77 ± 0.01
19	14.11 ± 0.02	79.35 ± 0.03	6.54 ± 0.02	5.62 ± 0.01	2.16 ± 0.00	12.13 ± 0.04
20	15.17 ± 0.02	74.58 ± 0.02	10.25 ± 0.02	4.92 ± 0.01	1.48 ± 0.00	7.27 ± 0.02
21	14.32 ± 0.04	76.04 ± 0.04	9.64 ± 0.01	5.31 ± 0.01	1.49 ± 0.00	7.89 ± 0.01
22	13.77 ± 0.01	79.04 ± 0.01	7.18 ± 0.02	5.74 ± 0.00	1.92 ± 0.01	11.00 ± 0.03
23	15.26 ± 0.03	75.20 ± 0.02	9.54 ± 0.02	4.93 ± 0.01	1.60 ± 0.00	7.89 ± 0.01
24	14.27 ± 0.02	77.56 ± 0.02	8.16 ± 0.02	5.43 ± 0.01	1.75 ± 0.01	9.50 ± 0.02
25	13.52 ± 0.01	79.85 ± 0.02	6.62 ± 0.02	5.90 ± 0.00	2.04 ± 0.01	12.06 ± 0.04
26	14.43 ± 0.02	78.93 ± 0.03	6.65 ± 0.02	5.47 ± 0.01	2.17 ± 0.00	11.88 ± 0.04
27	14.29 ± 0.02	78.99 ± 0.03	6.72 ± 0.02	5.53 ± 0.01	2.13 ± 0.00	11.75 ± 0.04
28	13.36 ± 0.01	78.55 ± 0.02	8.09 ± 0.02	5.88 ± 0.00	1.65 ± 0.00	9.71 ± 0.03
29	17.99 ± 0.01	68.04 ± 0.01	13.96 ± 0.01	3.78 ± 0.00	1.29 ± 0.00	4.87 ± 0.00
30	14.45 ± 0.02	78.28 ± 0.03	7.27 ± 0.02	5.42 ± 0.01	1.99 ± 0.00	10.77 ± 0.03

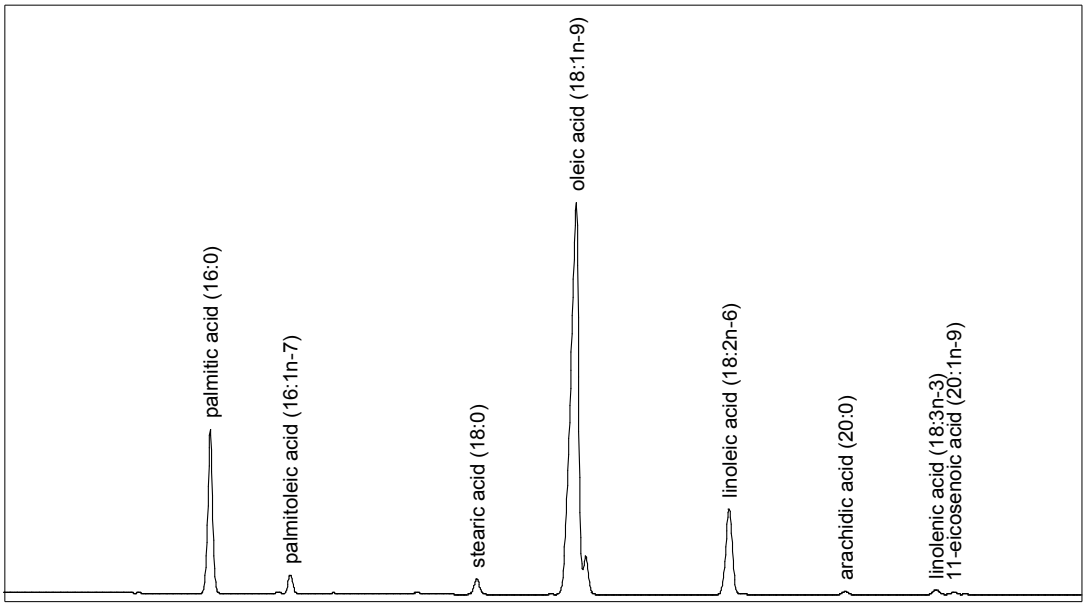
*: The data for individual samples are expressed as means ± standard deviations (n = 3, as each sample was analyzed in triplicate). SAT, MUFA, and PUFA, stand for total saturated fatty acids, total monounsaturated fatty acids, and total polyunsaturated fatty acids, respectively. Only fatty acids that were present at greater than 0.05 % were included in this analysis.

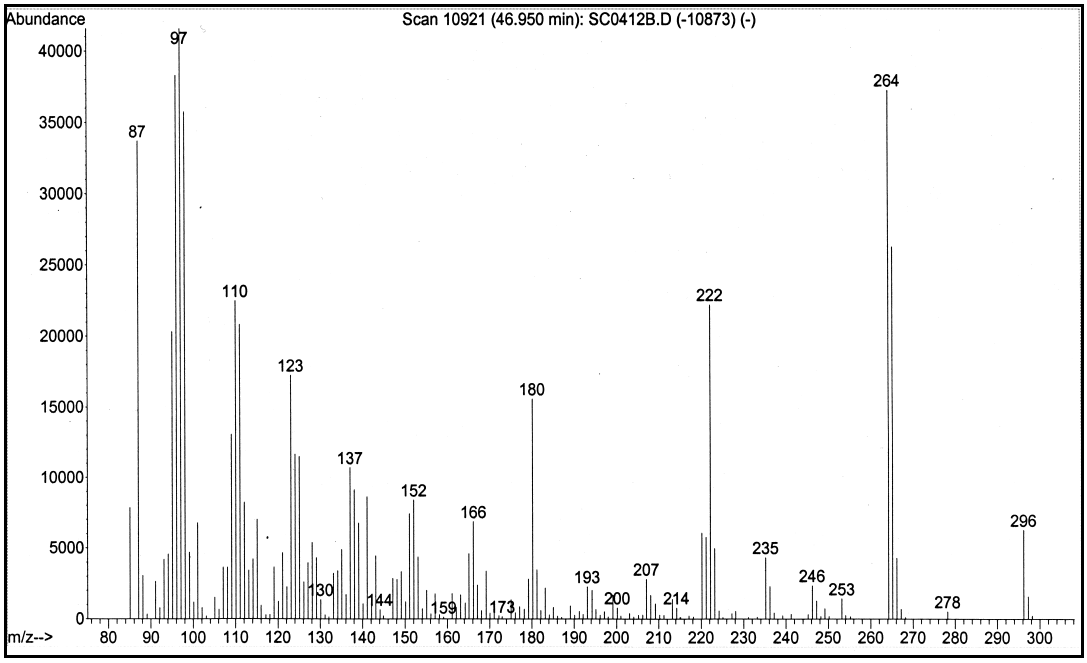
Table 2: Intracompany comparison – comparison of olive oil samples bottled and sold by the same company*

Analysis	18 vs. 21	19 vs. 20	28 vs. 27
PPV (cent/mL)	+ + +	*	–
FFA (g oleic acid/100 g oil)	+ + +	+ + +	+ + +
Smoke Point (°C)	*	*	–
***p*-Anisidine Value**	–	–	+ + +
PV-FOX (μmol tBPE/g oil)	*	*	*
OSI (hours)	+ + +	*	+ + +
Density (25 °C/water)	*	*	–
Refractive Index (η^{25}_{D})	*	–	*
TPC (μg GAE/g oil)	*	*	+ + +
ORAC (μmol TE/g oil)	+ + +	*	*
% DPPH Remaining	– – –	– – –	N/A

*: Each pair of samples was bottled and sold by the same company. The first sample number listed is the extra virgin olive oil (EVOO) while the second listed is the pure olive oil (POO). All entries are relative to the EVOO as a minus sign, a plus sign, or an asterisk indicates if the value for the EVOO sample is significantly less than, significantly greater than, or not significantly different from that of the POO sample ($P < 0.05$). An entry that contains three of the specific symbol indicates that the result was "expected" based on the assumption that EVOO samples most likely retain more phytochemicals due to their method of extraction from the olive fruits (Visioli & Galli, 1998a). N/A means that the samples were not analyzed using that procedure.

Figure 1 – GC-FID chromatogram and GC-MS mass spectrum. The chromatogram is from a representative olive oil sample and the mass spectrum is of the predominant fatty acid found in olive oil, oleic acid (18:1n-9).





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