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Faculty of Graduate Studies

Effect of Storage Containers on Selected Quality Parameters of Palestinian Olive Oil

تأثير عبوات التخزين والتجميد على بعض مؤشرات جودة زيت الزيتون الفلسطيني

This Thesis was submitted in partial fulfillment of the requirements for the Master's Degree in Environmental Biology from the Faculty of Graduate Studies at Birzeit University, Palestine.

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By

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The work provided with this thesis, unless otherwise referenced, is the researcher's own work, and has not been submitted elsewhere for any other degree or qualification.

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Date: 3/6/2020

Dedication

This thesis is dedicated to Allah who provided me with all what I needed to complete this study. Also, I dedicate this work to my beloved family, Birzeit University, Deir Dibwan, and Palestine.

Acknowledgements

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List of Abbreviations

Abbreviation	Full Name
FFA	Free Fatty Acid
ANOVA	Analysis-of-Variance
DG	Dark Glass
EC	European Commission
EEC	European Economic Community
FAP %	Fatty Acid Profile percentage
FDG	Frozen Dark Glass (container stored at -20 °C)
GC	Gas Chromatography
HDPE	High density polyethylene
HPLC	High Performance Liquid Chromatography
IOOC	International Olive Oil Council
IOC	International Olive Council
ISO	International Standardization Organization
K_{232}	Extinction coefficient at 232 nm
K_{270}	Extinction coefficient at 270 nm
mEq	Milli Equivalent
MOA	Ministry of Agriculture
MUFA	Monounsaturated fatty acids
PE	Polyethylene
PET	Polyethylene terephthalate
PCBS	Palestinian Central Bureau of Statistics
PP	Palestinian pottery
PSI	Palestinian Standards Institution
PUFA	Polyunsaturated fatty acids
PV	Peroxide Value
RT	Room temperature
TAGs	Triacylglycerol molecules
UV	Ultra violet

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**Effect of Storage Containers on Selected Quality Parameters
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Abstract

The olive tree (*Olea europaea* L.) and olive oil play important roles in the heritage of Palestinians. As an agriculture country, Palestine and its economy are dependent on olive harvesting and the production of olive oil. The quality of Palestinian extra virgin olive oil (EVOO) is excellent as it contains a plethora of nutritious molecules that contribute to its remarkable health benefits. However, olive oil can be considered labile to some extent, since its distinctive chemicals start to deteriorate from the moment of production if the environment is not feasible for storage.

It is a Palestinian societal habit to purchase (or produce) excessive amounts of olive oil at the beginning of the harvest season in October, and store it for long periods (at least one year) in various types of storage containers. It is therefore paramount to correctly characterize the best type of container to use for the long-term storage of olive oil that may significantly contribute to maintaining its EVOO designation. Hence, the overarching goal of this research was to study the effects of storing freshly-produced EVOO from Palestine (Deir Dibwan, Ramallah) in different storage containers on its

characteristics. Storage methods similar to the way many Palestinians store their oil were followed, in terms of duration and containers type. We systematically studied some selected key values that measure the quality of olive oil according to international and local standards. To complete this study, EVOO was continuously checked from the moment of production, and every four months for one year, at room temperature (RT) in the dark in three different types of containers: high density polyethylene (HDPE), dark glass (DG) and Palestinian pottery (PP). A commonly-used container in the households of most Palestinians' to store EVOO is HDPE. DG is also a common container found in major supermarkets and points of sale. The use of PP, albeit not commonly-used, is an ancient method (that has been used for thousands of years) for storage of Palestinians olive oil. After careful analysis, we show that storing EVOO in DG maintained its current established designation for 8 months while failing at 12 months, whereas EVOO stored in the other containers failed the established EVOO designation at 8 months. Therefore, we conclude that DG is the best packaging material for the long-term storage of EVOO. We can also suggest that storage of EVOO in PP is more favored to storage in HDPE due to its advantages in quality testing results and its environmental superiority.

In addition, the effects of freezing freshly-produced EVOO for 12 months were compared to RT storage. Indeed, freezing Palestinian EVOO at -20 °C in DG maintained current EVOO established designation while oil stored for 12 months at RT failed such designation. Moreover, for the first time, we show that storing EVOO at -80 °C maintained its designation and slightly preserved its vitamin E content better with a 40% decrease in content after 12 months of storage. In contrast, vitamin E content decreased

by 45% and 48% when stored at -20 °C and RT, respectively. Therefore, freezing olive oil could be a method of extending EVOO designation to 12 months and beyond, while keeping its nutritional advantage.

1.   Introduction

The health benefits of olive oil are well established in the literature; for instance, consumption of chemical components within EVOO reported to be a contributing factor to the reduction of the incidence of oxidative and inflammatory-related pathologies, such as cardiovascular diseases and cancer [1]. Upon the analysis of clinical and preclinical studies conducted on EVOO components, many scientists are currently urging for further examination of EVOO in order to understand its overall nutritional value [1, 2]. Olive oil presents considerable amounts of natural antioxidants and is therefore considered important for the prevention of several diseases such as myocardial infraction [2-5]. The flavonoid polyphenols in olive oil are natural antioxidants. They have been shown to have a host of beneficial effects from healing sunburn to lowering cholesterol, blood pressure, and risk of coronary disease [6]. Several clinical studies have shown that consumption of olive oil can provide heart health benefits, such as favorable effects on cholesterol regulation and LDL cholesterol oxidation, exerting anti-inflammatory, antithrombotic and antihypertensive effects [7].

Olive oil is extracted from the fruits of the olive trees (*Olea europaea* L.). Olive fruit production is a major contributor to the Palestinian national income [8]. Olives are a major agricultural crop, covering 57 % of cultivated land [9]. The average annual production of olive oil reached 19,532 tons in the 2017 season [10]. Palestinian areas cultivated with olive trees account for more than 80% of the fruit tree areas, about 76,000 hectares [11]. Olive harvest accounts for about 12% of the total national agricultural

output, reaching 20% in the West Bank, and represents about 4.6% of the Palestinian gross domestic product [12]. Olive cultivation is therefore an essential contributor to the social and economic wellbeing of Palestinians. Not only does the olive tree serve as an economic boost to Palestinians, olives have deep cultural significance as symbols of the traditional society with strong ties to the land [12-14]. The olive tree is also a symbol for peace. The emblem of our university carries the sketch of an olive tree. In fact, the Arabic name "Birzeit" (well of oil) is an embodiment of the importance of olive oil wells in the Palestinian culture [15].

Several cultivars of olives are found in Palestine such as Nabali Mohassan, Nabali Baladi, Chemlali, Jebbah, K 18, Manzolino, Shami, and Souri [9, 16]. As an essential household item used for food and non-food usage such as cosmetics, olive oil must hold its characteristic qualities since they decrease during storage and the benefits deteriorate with time [9]. There are several factors that affect the quantity and quality of the extracted olive oil: cultivar, cultural practices, harvesting methods, processing, harvesting time, handling and storage [12-14, 17].

In order to define the "quality" of oil, the International Olive Council (IOC) divided virgin olive oils fit for human consumption into: (i) EVOO, with free acidity, expressed as oleic acid, of 0.8 grams per 100 grams or less. (ii) Virgin olive oil (VOO), with a free acidity of not more than 2 grams per 100 grams. (iii) Ordinary virgin olive oil (OVOO), with a free acidity of not more than 3.3 grams per 100 grams. Other types of virgin olive oil which are not fit for human consumption are designated as lampante virgin olive oil (a

free acidity of more than 3.3 grams per 100 grams). This type of oil is intended for refining or industrial uses [18]. In addition, several other quality parameters are tested including sensorial evaluation to prove oil is EVOO (Table 1).

Table 1: Some of the quality tests and their maximum accepted values for each olive oil category [19].

Category	Acidity, %	PV, mEq O_2/kg	Waxes, mg/kg	Stigmasta-dienes, mg/kg	K_{232}	K_{270}	Organoleptic assessment, Median of defects (Md)	Organoleptic assessment, Median of fruity (Mf)
EVOO	≤ 0.8	≤ 20	≤ 250	≤ 0.15	≤ 2.50	≤ 0.22	Md = 0	Mf > 0
VOO	≤ 2.0	≤ 20	≤ 250	≤ 0.15	≤ 2.60	≤ 0.25	Md ≤ 2.5	Mf > 0
Lampante olive oil	> 2.0	_	≤ 300	≤ 0.50	_	_	Md > 2.5	_

In this study we aimed to uncover the best type of containers for the long-term storage of freshly-produced Palestinian EVOO that will keep the quality of the oil longer. Long-term storage of olive oil lowers the nutritional values of the oils and increases its negative characteristics. Rancidity which is due to lipid peroxidation is a major undesirable outcome of storage. Not only does it have implications on the bad taste of the oil, it may also cause DNA damage, Parkinsonism, carcinogenesis, and coronary heart diseases in humans [17, 20]. Additionally, we evaluated freezing EVOO at two temperatures as an option for preserving the oil. Vitamin E content was assayed as a means to assess the oil quality after 12-month storage at RT, -20 °C and -80 °C. This experiment was performed for the first time as we have no knowledge of reports on freezing EVOO at -80 °C.

1.1. Major components of olive oil

1.1.1. Fatty acids

Fatty acids are key components of olive oil. They are simple long chain hydrocarbons ending with a carboxylic group at one end. Fatty acids can be divided into 'saturated' or 'unsaturated' (Fig. 1) depending on the absence or presence of double bonds, respectively. Whereas a saturated fatty acid does not contain double bonds, unsaturated fatty acids may contain one or more. Monounsaturated fatty acids have one double bond and polyunsaturated fatty acids contain two or more double bonds. The number of double bonds is written in the fatty acid abbreviation, for example 'C18:1Δ^9' represents 18 carbons and one double bond at C-9. Most naturally-occurring unsaturated fatty acids have *cis* double bonds. The molecular structure of fatty acids can be bent (*cis* form) or straight (*trans* form) [21].
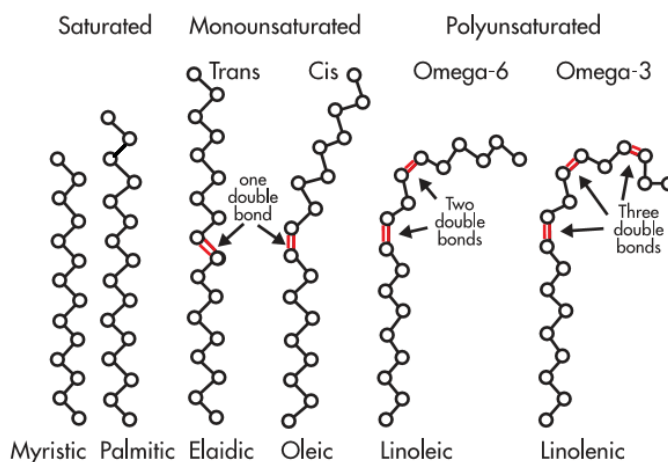
Figure 1. Cartoon sketches of various types of fatty acids found in olive oil [21]. Saturated fatty acids (C14:0, myristic and C16:0, palmitic) are linear, and so is the *trans* molecule elaidic acid (C18:1Δ^{9trans}). Naturally occurring unsaturated fatty acids have mostly *cis* double bonds and the molecules are bent. Oleic acid (C18:1Δ^9) is a monounsaturated fatty acid, whereas linoleic (C18:2$\Delta^{9,12}$) and linolenic (C18:2$\Delta^{9,12,15}$) are polyunsaturated. The top circle in each of the fatty acids presented represent the terminal methyl carbon and the lowermost circle represents the carboxylic carbon number 1.

1.1.2. Triacylglycerols

Fatty acids are connected in groups of three together with a molecule of the trialcohol glycerol in ester bonds forming triacylglycerols (TAGs) in esterification reactions (Fig. 2). About 95–98% of olive oil consists of TAGs. A good quality oil has intact TAGs. A TAG molecule may lose one or two fatty acids due to hydrolysis or oxidation, yielding free fatty acids in the oil sample. This contributes to the acidity of the oil sample and the loss of quality of the oil.
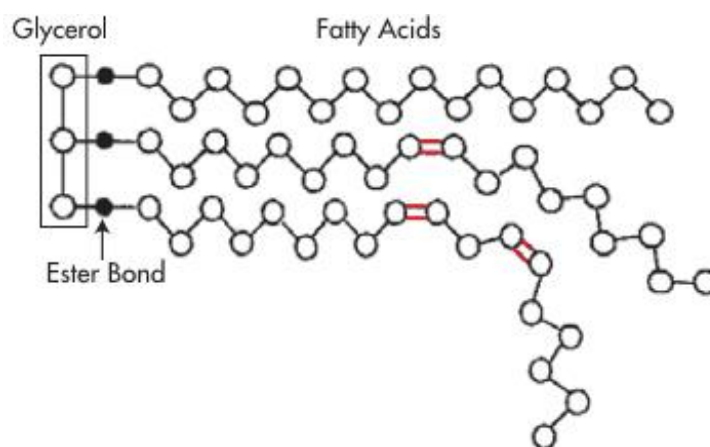
Figure 2. A cartoon of a TAG molecule made of three different fatty acids: palmitic, oleic and linoleic acids [21]. The ester bonds between the glycerol molecule (rectangle) and the fatty acids are shown by the arrow.

1.2. Minor components of olive oil

Olive oil contains a variety of components in minute amounts such as sterols, hydrocarbons (e.g., squalene) and ß-carotene, tocopherols (including vitamin E), fatty alcohols, and waxes. In addition, oil have pigments such as chlorophyll and carotenoids. Chlorophyll pigments give a characteristic and sometimes desirable green color to olive

oil. However, it is a photosensitizer and contributes toward photooxidation of the oil. Moreover, volatile components such as aldehydes, ketones, thiols, alcohols, and acids give the oil its unique odor and contribute to its flavor [21].

1.3. Quality tests for olive oil

Quality tests performed in this study were the free fatty acid content (acidity), peroxide value (PV), ultra-violet UV) absorbance, and fatty acid profile (FAP) per international and local requirements [18, 19, 22].

The acidity is a measurement of the release of fatty acid chains from TAGs indicating their breakdown. It gives an indication of oil quality based on fruit initial condition and handling. It is usually given as a percentage of free fatty acids on the basis of oleic acid, which is the prominent fatty acid in olive oil [23].

PV is a measure of peroxide compounds arising from primary oxidation. A high PV is usually indicative of poor processing, and that the oil might not keep well for longer periods of time. The final stage in oxidation is peroxide breakage, resulting in the formation of new compounds that can be perceived as rancid smelling. Understanding this chemical value is quite easy and useful when measured in freshly-made virgin oils, but later in the life of the oil it cycles up and down [23].

UV absorbance of olive oil is an indicator of oxidation using the UV spectrum. For the quality of olive oil, two values are normally measured. K_{232} and K_{270} are the tests used to measure the quantity of secondary oxidative compounds at wavelengths of 232 and 270

nm. K_{232} is considered a critical marker for good quality EVOO. Oxidation can be the result of natural aging or indicative of poor handling or heating during the refining process [23].

FAP is a measure of the proportions of individual fatty acids in the oil and thus an important part of the oil quality. The proportions of the different fatty acids can impact the nutritional value of the oil and affect its stability [24].

1.4.   Deterioration of olive oil

Freshly-pressed EVOO normally passes all quality control if the handling and cultivation were up to the standards. However, as the oil is stored for longer periods, its quality becomes reduced due to several factors, primarily oxidation. Deterioration of olive oil by oxidation requires the availability of oxygen, light and heat. Olive oil can undergo two types of oxidation, which are responsible for its oxidative rancidity and a change in taste [3, 25]: *(1) Autoxidation,* which occurs in the absence of light in a free radical-induced mechanism, whereby the oil decomposes producing a mixture of compounds such as ketones, aldehydes, alcohols, and esters. *(2) Light oxidation,* which is a direct outcome of olive oil exposure to light. Light exposure initiates the oxidative reactions. This type of oxidation is 30,000 times faster than autoxidation [6]. Therefore, packaging can directly influence olive oil quality by protecting the product from both oxygen and light [3]. Hence, the selection of the correct type of storage container is preferable to keep the product quality at the maximum level for the longest period [26].

1.5. Environmental factors affecting olive oil

Environmental factors such as temperature, exposure to light and contact with oxygen are the most important elements affecting the quality of olive oil after processing and during storage. Hence, storage containers are key components that affect the quality of the oil. Each type of container has advantages such as impermeability to gases (glass and tin) and disadvantages such as favoring light oxidation (glass and plastic containers) [3, 27, 28]. Globally, studies were conducted to assess the quality of olive oil from different countries, cultivars, conditions, and containers.

1.5.1. Effect of temperature

The effect of temperature on EVOO was evaluated in different countries. For example, Spanish EVOO samples from Picual and Hojiblanca cultivars were stored in transparent glass containers at different temperatures for six months. The acidity quality standard values were maintained in the samples stored at 2 °C but increased significantly in the samples stored at 30 °C [29]. Moreover, Greek EVOO from "Koroneiki variety" was used to test temperature effect and storage time on the oil quality. Olive oil stored at 13 °C retained its EVOO designation and showed no change in stability and quality, whereas oil stored at 22 °C and 35 °C failed EVOO designation after three months [27]. On the other hand, Italian EVOO from Tuscany cultivar was stored at 20 °C for 21 months in DG bottles which were shipped to different locations to imitate real life commercial situations. The study concluded that EVOO stability was not significantly changed at this temperature [28]. These studies concur that different temperatures affect different

cultivars of olives differentially, indicating a need to study each geographically distinct EVOO separately.

1.5.2. Effect of light

The effect of light on EVOO quality was evaluated for Tunisian and Spanish olive oils. Tunisian EVOO samples were studied under diffused light for 12 months in different types of containers. In all types of containers, after 9 months of light exposure, the oil lost its EVOO designation [30]. In contrast, another study showed that the oil from three Spanish cultivars (Picual, Arbequina, and Hojiblanca) kept their EVOO designation for 12 months after storage in DG or transparent containers at controlled RT [26]. It is evident that light exposure expedites the oxidation process and hence affects the quality of the oil negatively.

1.5.3. Effect of storage containers

The effect of different types of containers on EVOO was evaluated in different countries. For example, Spanish EVOO purchased from a supermarket was tested in various containers: clear polyethylene terephthalate (PET) bottle, PET bottle (covered with Aluminum foil), transparent glass bottle, tin, and Tetra-brik Aseptic bottles. The study was performed at RT at the same surface area of exposition to air and light to simulate the real conditions in a market place. The olive oils were analyzed at zero time (time of purchase) and after 3 and 6 months of storage. All oil samples lost their EVOO designation after three months [31] indicating that all of the used containers had negative effects on EVOO. In addition, the stability of Tunisians Chemlali EVOO in different

containers such as clear and DG bottles, polyethylene (PE) and tin containers was studied for six months. The different oil samples were stored under light at RT. This study showed that the best packaging materials for the commercial packing of Chemlali EVOO are tin containers and DG bottles. The oil stayed within EVOO limits throughout the duration of the study [3].

1.5.4. The effects of temperature and containers

The combined effect of temperature and containers on EVOO was evaluated in Palestine. Olive fruits of the Palestinian cultivar 'Nabali Baladi' were used to produce EVOO. The EVOO was distributed in different packaging materials (amber glass bottles, PET, HDPE, tin plate cans hermetically sealed, and pottery jars with covers, maintaining 2% headspace in each bottle. Bottled oil was stored under different storage temperatures ($18 \pm 1°C$ and $37 \pm 1°C$) for six months. This study concluded that for ambient storage temperature, the best container to maintain the quality of stored oil is glass followed by HDPE, followed by both cans and PET, and the worst was pottery. At elevated temperatures, glass was found to be the best primary packaging material, followed by PET, followed by cans, followed by HDPE, and the worst container was again pottery [20].

1.5.5. Effect of freezing

The effect of freezing EVOO on its quality parameters was evaluated using different olive cultivars and storage periods. For example, the effect of long storage period (up to 18 months) on Tuscan EVOO filtered and frozen at -23 °C in comparison with same sample at RT in the dark were evaluated. Frozen samples at -23 °C showed lower PV compared

to EVOO stored in DG at RT in the dark. The decrease was not statistically significant. No change in acidity for frozen and unfrozen samples after 9 months of storage [32]. In addition, comparison of lower storage temperatures (4 °C and -20 °C) to RT storage using Buza, Crna, and Rosinjola Croatian cultivars for 12 months was performed. EVOO stored at lower temperatures showed better quality parameters than EVOO stored at RT. It is concluded that lower storage temperature prolonged shelf-life of EVOO [33].

1.5.6. Effect of storage time, headspace, and temperature on vitamin E content

Different olive cultivars in different countries were used to evaluate the effect of storage time and temperature on vitamin E. For example, two different EVOO samples named "Scalicelle" and "Terzera" from Italy were stored at RT under diffused light for 1 year using clear and dark bottles. Half the bottles were filled with ~ 3% headspace and the rest of the bottles were filled with ~ 50% headspace. After 12 months of storage at RT and diffused light the vitamin E content of the Scalicelle sample with 3% headspace was reduced by 20.8% in comparison with a reduction of 91.5% in the half-empty bottles. The Terzera sample showed very close results to Scalicelle sample [34].

In another study, Lianolia variety olives grown in Preveza, Greece were collected at various stages of ripeness and stored under diffused light for 6 months and in the dark for 12 months, and their vitamin E contents were compared. About 50% reduction in vitamin E was reported at 12 months of storage in the dark at RT using DG compared to approximately 100% reduction under diffused light [35]. Moreover, the effect of two

different growing areas and four harvest periods on EVOO vitamin E content was evaluated at RT, 4 °C, and -20 °C using Oblica and Leccino cultivars from Croatia. After 12 months of storage, vitamin E content for each cultivar was reduced by a different percentage for each storage temperature and for each cultivar. The study concluded that lower temperatures did not always contribute to the higher stability of vitamin E content compared to RT [36]. Finally, EVOO from Arbequina cultivar collected from three different mills from Catalonia, Spain at different harvesting periods was evaluated. The collected EVOO was stored in Amber glass at RT in the dark for 12 months. A 100% loss of vitamin E content was reported [37].

1.5.7. Effect of storage time and containers on FAP %

FAP % content of four commercial samples of EVOO from a Spanish supermarket (unreported cultivar) was evaluated at 0, 3, and 6 months storage periods using different types of containers at RT. No statistically significant variation in FAP % content observed after 6 months of storage compared to 0 and 3 months of storage periods [31]. FAP % was also not significantly changed in Tunisian olive oil during storage time (0 to 12 months) under diffused light and RT, indicating that there is no effect of storage time or containers on FAP % [30].

2.   Materials and Methods

2.1.   EVOO and containers

Twenty-five L of EVOO were purchased from an olive oil mill from the town of Deir Dibwan, Ramallah, Palestine. Deir Dibwan is located 6.4 kilometer east of Ramallah city at an altitude of 739 meter above sea level [38]. Its mean of minimum air temperature during the year of 2018 was 14.6 °C and mean maximum of 21.5 °C with annual rainfall quantity of 804 mm [39]. Its mean relative humidity in the year 2018 was 76% [39].

The EVOO was freshly produced from a mixture of local Deir Dibwan olive cultivars (to assimilate normal Palestinian household practice) produced during the 2018 crop season, in October. Olives were crushed in an olive crusher to obtain a semi-paste. Delivered to malaxator chambers by pumps, the semi-paste was kneaded and pumped to the decanter by the paste pump and water was added at 27 °C. Olive paste was disintegrated into phases in the decanter and was separated as olive oil and black water. Olive oil's fine particles were removed in the separator centrifuge (7000 round per minute) and delivered to the customer.

The purchased EVOO was transported to campus in HDPE containers. Fresh EVOO was mixed and homogenized and multiple samples were aliquoted into pre-labeled amber dropper containers for the initial (control) quality assessment (T = 0 month). Immediately after transportation into the laboratory, fresh EVOO was distributed into three different types of containers. The containers were 1 L dark glass (DG), 1 L Palestinian light-colored pottery with a cap (PP), and 1 L high density polyethylene (HDPE). Five brand

new bottles of each type were thoroughly washed with MilliQ water for three times followed by a fourth rinse with fresh EVOO prior to filling each container up to ~ zero headspace with the purchased EVOO. All containers were subsequently properly sealed to minimize air mixing with EVOO.

2.1.1. Containers used for freezing EVOO

One hundred mL of EVOO were aliquoted into pre-labeled five DG containers and five polytetrafluoroethylene (Teflon) bottles. DG containers were rinsed with MilliQ water for three times followed by one rinse with fresh EVOO prior to aliquoting. Teflon containers were rinsed three times with deionized water followed by a rinse with fresh EVOO prior to aliquoting.

2.2. Storage conditions

DG, PP, and HDPE containers were stored at RT (minimum 10 °C to maximum 27 °C during the year) in a closed dark wooden cabinet in the laboratory with occasional exposure to artificial lighting (to simulate normal household storage conditions of freshly produced EVOO). The location of each container in the cabinet was rearranged approximately every two weeks to eliminate any potential variations in exposure to air and light inside the cabinet. Quality testing on EVOO stored in all containers was performed every four months for a period of one year.

2.2.1. Storage conditions for containers used for freezing EVOO

The 5 DG bottles were stored in a dark -20 °C freezer with occasional exposure to artificial light. The 5 Teflon bottles were stored in a dark -80 °C freezer. Both frozen bottles were stored for 1 year and were not thawed during their storage.

2.3. Thawing of frozen EVOO

One year after storage at -80 °C in Teflon containers and -20 °C in DG containers, the containers were transferred to a 4 °C (freezing point for olive oil) refrigerator in order to slow the process of phase changing from solid to liquid as a precautionary step to lower the propensity of oil damage. This was performed to reduce the potential negative effects of rapid changes in the physical form of EVOO. The bottles were kept for 4 days at 4 °C refrigerator before moving them to a dark cabinet at RT for complete thawing.

2.4. EVOO stability and quality tests

The effects of packaging materials on the quality of EVOO were monitored every 4 months by using indicators that include acidity, peroxide value, ultraviolet extinction (K_{232} and K_{270}). In addition, frozen and thawed EVOO for DG containers were evaluated at T = 12 months for acidity, peroxide value, ultraviolet extinction coefficients, FAP %, and vitamin E content. FAP was used to check the integrity of EVOO after 12 months of storage in DG container at RT and -20 °C. Vitamin E content was used as an indicator of the nutritional status of EVOO after 12 months of storage in DG containers at RT and at

-20 °C compared to the control. Frozen EVOO at -80 °C in Teflon bottles was also evaluated by the same quality indicators as previously mentioned.

2.5. Determination of EVOO quality indicators

2.5.1. Acidity

According to the international standard ISO 660: 2009 (E) [40], 10 g of each tested sample were weighed into a 150 mL glass Erlenmeyer flask. The guidelines in Table 2 were followed according to the color and expected acid value. Testing was performed at Birzeit University Quality Control laboratory.

Table 2: Test portion masses and alkali concentration

Product group	Acid value approx.	Mass of test portion, g	Concentration of NaOH, M	Accuracy of weighing, g
Crude vegetable oils	1 to 4	10	0.1	0.02

Briefly, 50 mL of 99% ethanol containing 0.5 mL of phenolphthalein indicator were heated until boiling. While the temperature of ethanol was still above 70 °C, it was neutralized with a solution of 0.1 M NaOH. The neutralized ethanol was subsequently added to the 10 g of the oil sample and the ensuing solution was mixed thoroughly. The solution was boiled and titrated with the NaOH until the end point was reached. The acid content was determined as shown in Appendix I.

2.5.2. Spectrophotometric assays

EVOO samples were assayed for their characteristic absorbance using Shimadzu UV 1800, following Commission Regulation EC 2568/91 [19]. Beer-Lambert law was

applied to measure the extinction coefficients at 270 nm and at 232 nm of EVOO samples dissolved in iso-octane or cyclohexane. In order to get the extinction of 1% w/v solution of EVOO in the specified solvent, 0.25 g of olive oil was weighed in a 25 mL volumetric flask. The flask was filled to the 25 mL mark with the sample diluent and homogenized to obtain a perfectly clear solution. The extinction at 270 nm was measured against the solvent as a reference. For K_{232} measurements, 0.05 g of EVOO was weighed in a 25 mL volumetric flask. The flask was filled to the 25 mL mark with cyclohexane and homogenized to obtain a perfectly clear solution. The extinction at 232 nm was measured against a solvent blank. Triplicate measurements were obtained for each sample using a rectangular Quartz cuvette having a 1 cm optical path length. The calculations of extinction coefficients are presented in Appendices II and III.

2.5.3. Determination of FAP % content

Fatty acid methyl esters (FAMEs) were prepared and profiled by gas chromatography (GC) at the Birzeit University Quality Control Laboratory. Into a 50 mL round-necked volumetric flask, 9.2 mL of 2.2 N HCl were added and the 50 ml mark was filled with methanol. From this dilution, 300 μL were mixed with 100 μL of EVOO sample dissolved in 200 μL toluene. To the ensuing mixture, 1.5 mL methanol was then added. The mixture was vortexed and boiled for 90 minutes. A certified standard was also prepared in an identical procedure. After cooling to RT, 3 mL of n-hexane and 2 mL of MilliQ water were added to extract FAMEs. The mixture formed two layers. The hexane (upper) layer was used to inject (0.2 μL) into Agilent 6890 GC (USA). The GC was fitted with 60-m

capillary column (SGE-BPX 70) with a 0.25 mm I.D., 0.25 μm film thickness and equipped with split injection and flame ionization detector. Nitrogen was the carrier gas, and injector and detector temperatures were set to 240 °C and 280 °C, respectively. Oven temperature was programmed at 120 °C for 1 min, increased from 120 °C to 230 °C at 3 °C min^{-1}, and then was increased to 245 °C at a rate of 10 °C min^{-1}. Individual fatty acids were determined by comparison with retention times of known standards. The calculation of fatty acids is presented in Appendix IV.

2.5.4. Peroxide Value - Iodometric (visual) endpoint determination

Peroxide value (PV) is the quantity of those substances in the sample, expressed in terms of active oxygen, that oxidize potassium iodide under the conditions specified in the International Standard ISO 3960 [41]. The PV is usually expressed in milliequivalents (mEq) of active oxygen per kilogram of oil. PV was determined in the Birzeit University Quality Control Laboratory.

Briefly, 50 mL of acetic acid/iso-octane (60:40, v/v) solution (degassed by purging with Nitrogen gas) were added to 5 g of test sample in a conical flask and the sample was dissolved by gentle swirling. To the mixture, 0.5 mL of saturated potassium iodide solution was added and with occasional mixing. After exactly 60 seconds, 30 mL of demineralized water were added. The following solution was titrated with 0.01 N sodium thiosulfate, adding it gradually and with constant, vigorous agitation, until the yellow iodine color has almost disappeared. 0.5 ml of 1% starch solution was added and titration

continued until the dark color of the solution became colorless. The calculations of results are presented in Appendix V.

2.5.5. Determination of vitamin E

The tocopherol content was determined by high performance liquid chromatography (HPLC) at the Birzeit University Quality Control Laboratory. Into a 10 mL volumetric flask 0.5 g of EVOO was weighed and the flask was filled to the mark with 100% isopropanol. The mixture was vortexed and filtered through a 0.45 µL pore size filter. Separation by HPLC was performed using HPLC Waters 2690 Liquid chromatographic system (Milford, MA, USA). The instrument was equipped with a photodiode-array detection system 996 which was used as the detector. Empower software by Waters was used to store and process data. The used column was Phenomenex, Luna 5 µm ODS-C18 (100 X 4.6 mm I.D., 5 µm particle size). The injection volume was 10 µL. The mobile phase was methanol-acetonitrile (75:25, v/v) and the elution was carried out at a flow-rate of 1.2 mL/min. The analytical column was kept at 30 °C. To determine the compounds in the samples, a reference standard of alpha-tochopherol was used. Duration of each run was 6.5 minutes and detection carried out at 300 nm. Results were given in mg of tocopherols per kg of oil.

2.6. Statistical analysis

All results presented in this study were expressed as means ± standard deviation (SD). Five independent biological replicates were used for each container type ($n = 5$). Triplicate (K_{232} and K_{270}) and duplicate (peroxide value, acidity, FAP, vitamin E) testing

were performed for the determination of each measurement. A linear regression analysis was performed using Microsoft Excel 2013. Significant differences between the values of all parameters were determined at $p < 0.05$ using one-way ANOVA followed by Bonferroni post hoc analysis. The data analysis software Statistix 9 (FL, USA) was used for statistical analysis. All analyses are presented in Appendix IX.

3. Results and Discussion

In order to study the effects of storage containers on the quality of olive oil, a fresh sample was obtained from a local oil mill which had previously been awarded for the quality of their produce. All containers used in this study were newly purchased and thoroughly cleaned prior to use. Quality controls of the oil were performed in accordance to international and local standards.

3.1. Composition and quality indices of the initial EVOO sample

The initial analyzed EVOO sample (control) at T= 0 month passed the quality control values as established by IOC [18], EC [19], and PSI [22] (Table 3). For FAP, the focus was on the major fatty acids found in EVOO (oleic, palmitic, linoleic, and stearic acids) which comprises more than 95 % of the total fatty acid content of EVOO sample.

Table 3: Quality of initial (T = 0 month) EVOO sample

Quality characteristic	Value	Unit	IOC[18], EC[19], PSI[22]
Acidity	0.62	g oleic acid per 100 g EVOO (%)	≤ 0.80
Peroxide value	9.79	equivalent O_2 per kg oil	≤ 20
K_{232}	1.78	absorbance	≤ 2.5
K_{270}	0.16	absorbance	≤ 0.22

Vitamin E	340.30	mg / kg oil	-
Oleic	70.90	% content	$\leq$ 55 - 83
Palmitic	11.44	% content	$\leq$ 7.5 - 20
Linoleic	10.65	% content	$\leq$ 3.5 - 21
Stearic	4.28	% content	$\leq$ 0.5 - 5

3.2. EVOO deterioration upon long-term storage due to oxidation

Autoxidation and photosensitized oxidation, through different chemical mechanisms (appendix VI and VII), are accountable for the oxidation of edible oils during processing and storage. Edible oils can react with two types of oxygen: *Atmospheric triplet oxygen*, $^3O_2$ that reacts with lipid radicals and causes autoxidation (a free radical chain reaction). *Singlet oxygen*, $^1O_2$ which is produced from atmospheric oxygen in the presence of light and sensitizers (such as chlorophyll) and is responsible for photosensitized oxidation of edible oils [42].

3.2.1. Peroxide Value is maintained in all containers for 12 months at RT

PV is defined as the amount of peroxide oxygen per 1 kg of fat or oil. The PV represents the total hydroperoxide content and is one of the most common quality indicators of fats and oils during production and storage [43-45]. It is an indicator of the initial stages of oxidative change [43, 46].

HDPE containers had significantly lower PV at 12 months compared to both PP and DG and significantly higher PV than DG container at 4 months. As expected, the PV increased for EVOO stored in all three types of containers as time passed (Figure 3A). This increase has been observed in other reports [30]. In that report, EVOO in glass containers failed

the permitted limit at 9 and 12 months; while oil stored in jar container failed at 6, 9, and 12 months [30]. Whereas our study was conducted in the dark, the aforementioned study was under diffused light. Hence, the failure of PV could be due to exposing the bottles to light which increases PV due to increased oxidation of oil [42].

After 12 months of storage in the dark at RT, EVOO stored in all three types of containers did not exceed the established limit (PV ≤ 20) contrary to some previous reports that showed a failed EVOO stored in plastic containers for 12 months [27]. In our study, DG container maintained a lower PV at 4 and 8 months compared to PP and HDPE containers. However, at 12 months DG was the highest (appendix VIII, Table 1).

PP and DG containers increased their EVOO PV from 4 to 12 months in contrary to HDPE containers. The PV value of HDPE container was the highest compared to DG and PP for up to 7 months, then became less than PP at 8- and 12-months testing period (Figure 3A). This fluctuation in PV for EVOO stored in HDPE container may be due to the permeability of plastic containers for oxygen. This initiates the primary step of oil oxidation giving a maximum PV. However, the oil gets further oxidized into aldehyde and ketones (secondary oxidation) which causes a reduction in PV [30]. Since PV represents the total hydroperoxide content and hydroperoxides are intermediates in the autoxidation reaction and they decompose to yield secondary oxidation products [43]. When hydroperoxides decompose to secondary compounds, PV will decline. Such fluctuations in PV have been observed elsewhere [20, 29, 31]. In this case, a low PV is therefore not an indication that the oil is of good quality [47, 48].

3.2.2. Extinction values at 232 nm indicate the suitability of DG as long-term storage containers

The absorbency at 232 nm is caused by hydroperoxides (primary stage of oxidation) and conjugated dienes (intermediate stage of oxidation) [43]. Hydroperoxides increase in oils due to oxygen fixation, which occurs through a free radical chain reaction, in the double bonds of linolenic and linoleic acids. This kind of conjugate systems presents a maximum absorption at 232 nm [43]. EVOO stored in DG containers was the sample that did not exceed the established maximum limit of 2.5 for K_{232} during the testing period (Figure 3B). EVOO in DG containers showed statistically significant lower values compared to oil in PP or HDPE containers ($p < 0.05$) (Appendix VIII, Table 2).

EVOO stored in both PP and HDPE containers failed to pass the established maximum limit at 8 months. It has been previously shown that EVOO in DG passed the established limit at 6 months, in concurrence with our study. However, the same report demonstrated that EVOO in HDPE and pottery passed at 6 months [20]. Failure at 6 months in plastic and glass containers was also reported [3, 27]. Others reported failure of the EVOO stored at RT and diffused light after 6 months in DG and after 3 months in jars [30] while others reported failure after 28 days of storage in greenish glass at 6 °C and at 26 °C [49]. The failure is probably due to exposing the oil to diffused light which accelerates oxidation and the formation of conjugated diene. The greater the value of K_{232}, the greater the concentration of conjugated dienes [47]. This increases the absorption at 232 nm and consequently leads to failing established maximum limits. Since K_{232} seems to be the

most useful index for analysis and monitoring to determine the commercial category of olive oil [50], our results confirm that storing EVOO for a year requires DG containers to keep its quality.

3.2.3. Extinction values at 270 nm show similar deterioration of EVOO upon long-term storage

Linolenic acid is the most susceptible VOO fatty acid to autoxidation and its hydroperoxides undergo rapid decomposition, yielding compounds that absorb UV radiation at 270 nm [50]. Absorbance of EVOO at 270 nm is caused by carbonylic compounds (secondary stage of oxidation) and conjugated trienes [43]. K_{270} value is proportional to the concentration of conjugated trienes [47].

Olive oil stored in all three container types in our study showed no statistically significant ($p < 0.05$) distinction in K_{270} and passed the maximum limit established up to 8 months of storage, in agreement with previous reports [20]. However, all samples failed the EVOO distinction at 12 months (Figure 3C). Other investigators demonstrated failure of K_{270} distinction at 9 months for oil stored in glass and plastic containers [27] while others reported passing that distinction in DG and plastic at 12 months, and failing in jars at 6 months [30]. However, others reported failure after 28 days of storage at 26 °C [49].
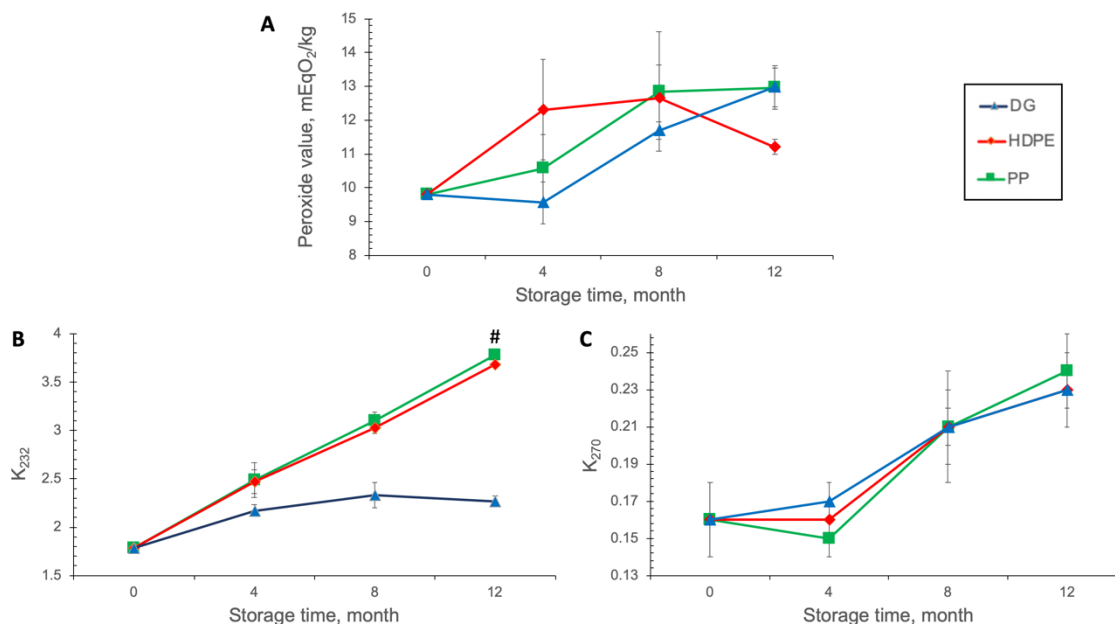
Figure 3. Changes in EVOO quality parameters (A) PV (B) K_{232} (C) K_{270} after storage in the dark for one year at RT in PP, HDPE, and DG containers. The results are means and standard deviations of five independent experiments. # indicates extrapolation (Appendix III.1, III.2, VIII. Table 1, 2, 3).

3.2.4. The acidity values of EVOO are maintained upon storage in PP containers for 12 months

Free fatty acid formation can be considered the most indicator of oil deterioration. Free acidity levels increase by TAG hydrolysis early in the production process, from harvest through milling, while water and plant enzymes (lipases) are still in contact with the oil. Free acidity values provide an indication of how the fruit was handled prior to processing and the length of time from harvest to milling. Free acidity is also an early marker of the potential longevity of the oil. Freshly-produced higher quality oils will show very low acidity. Acidity level is a measure of the free fatty acids in the oil expressed as a

percentage of oleic acid by mass [43]. Acidity of more than 3.3 percent makes olive oil not fit for human consumption [18].

Our findings showed that the EVOO in all three types of containers did not exceed the established limit for acidity quality parameter of 0.80 for 12 months of storage in the dark at RT (Figure 4) in agreement with other reports [3, 20, 27]. This proofs that the initial quality of the oil was excellent and it was processed with high standards. However, clear variations among storage containers were evident. In our study, oil stored in PP maintained significantly lower acidity values ($p < 0.05$) at all time points compared to the other two containers, in concurrence with other reports [20] at 6 months. Monitoring the level of acidity for 12 months shows that EVOO in PP maintained a stable level compared to the control. Surprisingly, no significant differences were found between HDPE and DG containers on the acidity of EVOO at any time point. In agreement with our results, others [30] reported that storage of olive oil in jars gave lower acidity values than both DG and plastic for up to 12 months of storage. From these results, PP is the best container for the long-term storage of EVOO in terms of the acidity test. This can be explained by the high thermal conductivity of glass compared to plastics and for the cooling effect of pottery on stored oil [20].
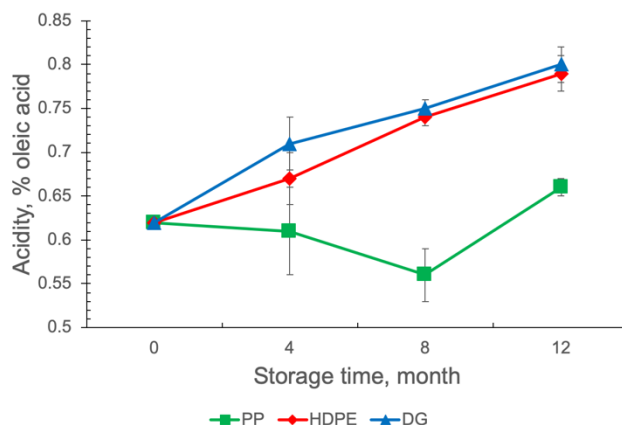
Figure 4. Changes in EVOO acidity quality parameter in different containers after storage in the dark at RT for one year. The results represent means and standard deviations of five independent experiments (Appendix VIII. Table 4).

From the aforementioned results, EVOO stored in DG passed all quality standard tests for PV, K_{232}, K_{270}, and acidity up to 8 months, while failing at 12 months in one category only (K_{270}). Storage of EVOO in PP and HDPE showed failed K_{232} at 8 month (Appendix VIII, Table 5). Therefore, it is safe to conclude that DG is a superior material for the long-term storage of EVOO compared to PP and HDPE.

To differentiate between PP and HDPE, no significant differences were found for PV quality standard test at up to 8 months. However, PV of EVOO from HDPE was significantly lower (*p < 0.05*) than that from PP at 12 months. Given the fact that PV normally fluctuates and that PV for oil in HDPE in this study was found to be low, the lower PV may not be an indication that the HDPE is superior to PP and the oil is of a better quality [47, 48]. As for the acidity values, EVOO in PP was significantly lower than that in HDPE up to 12 months in acidity (*p < 0.05*) which clearly demonstrates the superiority of PP over HDPE as a storage container.

In Palestine, the majority of containers used for storing olive oil are HDPE which are not recycled and usually used for only one season. The time frame for the complete degradation of HDPE could, in some situations, be several hundred years [51]. This means that such choice of containers is very harmful to the environment and to the health of consumers, because the chemical ingredients in 50% of plastics listed as hazardous (United Nations' Globally Harmonized System of Classification and Labelling of Chemicals) [51, 52] and such material is possibly "just the start of long term ecological and health problems associated with waste plastics in the environment to the environment and to our health" [52]. In comparison, using PP container is environmentally safe because it is usually made of clay and it is an ancient container used for storing olive oil for thousands of years [53]. So, our findings indicate that DG is the best material for storing EVOO followed by PP, followed by HDPE.

3.3.   The effects of freezing at -20 °C on the quality of EVOO

In the second part of this study, we set out to uncover the effects of freezing on the quality of long-term stored EVOO. Different quality parameters were measured after 12 months of storage at RT or -20 °C in DG containers. The results are summarized below.

3.3.1.   Freezing of EVOO did not enhance its FAP % content compared to RT storage in DG containers

FAP is a measure of the proportions of individual fatty acids in the oil and is consequently an important part of the oil quality. The proportions of the different fatty acids can influence the stability of the oil as well as determining its nutritional value, since some

fatty acids are better than others. Oleic acid, a monounsaturated acid (MUFA), is the desirable from a nutritional standpoint, whereas, a polyunsaturated acid (PUFA) such as linolenic acid, with three double bonds, is the most chemically reactive and therefore undesirable in terms of stability, despite its nutritional benefits. The fatty acid profile is influenced by cultivar and the environment [8, 24]. Fatty acid composition is vital in terms of detecting frauds and assuring authenticity [54]. In our EVOO sample, the main fatty acids were oleic acid, palmitic acid, linoleic acid, and stearic acid. In order to study the effect of freezing on EVOO quality, EVOO samples were stored in DG at RT and at -20 °C for 12 months. The FAP for the initial and final samples was determined (Figure 5). All tested samples were within the maximum limit established for FAP % content. A slight, nonsignificant decrease was noticed in oleic acid after storage for 12 months at both temperatures. Similar reduction was also reported by others [30, 31] due to oxidation of the samples [55].

Comparing EVOO initial sample (Figure 5A) to EVOO stored in DG for 12 months at RT (Figure 5B) and at -20 °C (Figure 5C) showed no detectable changes in oleic, palmitic, stearic, and linoleic acid indicating that the FAP of the initial sample held for the storage duration in DG regardless of the temperature. Therefore, storing EVOO at -20 °C for 12 months did not demonstrate superiority in terms of FAP compared to RT storage, which may be an indication of the quality of DG as a storage material.
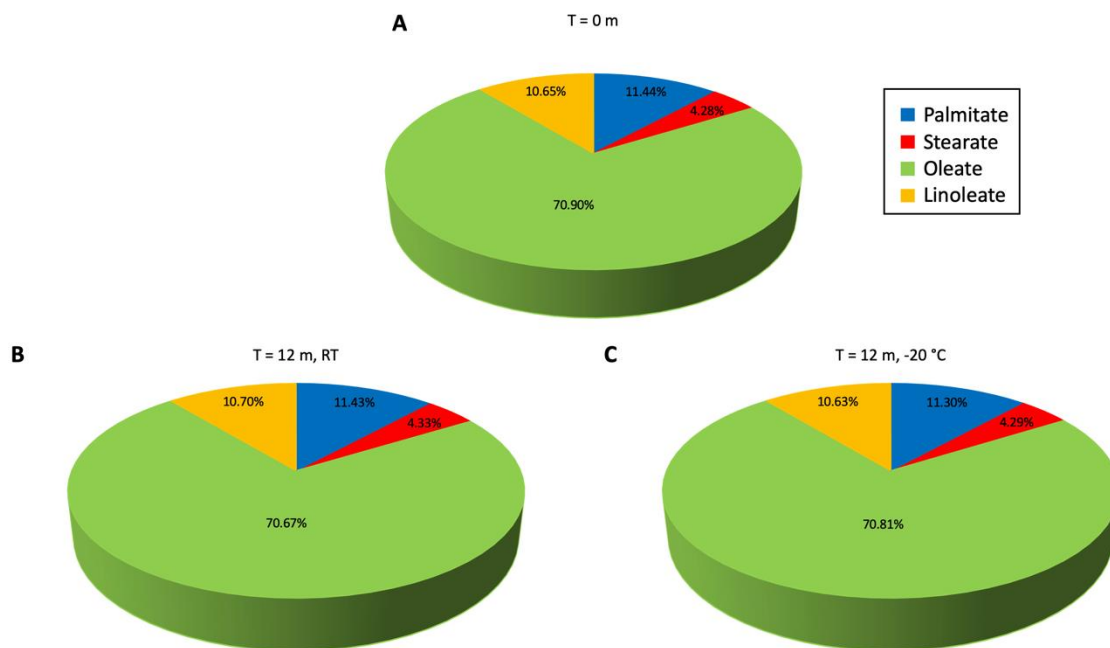
Figure 5. No significant change in FAP % for palmitic, stearic, oleic, and linoleic fatty acid compared to the initial sample composition (A) after 1 year storage in DG containers at RT (B) or at -20 °C (C).

3.3.2   Freezing EVOO at -20 °C maintained its quality values similar to the fresh sample

Freezing EVOO in DG for 12 months at -20 °C resulted in a slightly lower PV compared to that stored at RT. However, this decrease was not statistically significant (Figure 6A). These findings are in agreement with other reports [32]. In contrast, statistically significant lower PV for samples stored for 12 months at -20 °C compared to samples stored in tapped dark bottles filled with nitrogen at RT was reported [33] indicating some variability in the results. Lower PV upon freezing is expected because freezing decreases the oxidation process [32, 33]. Since the PV of the EVOO used (10-12 mEq/kg) was well under the allowed limit ($\leq$ 20 mEq/kg), it did not increase effectively even after long-term

storage. This is evident by the lack of significance for freezing on PV. This is an excellent indication of the quality of the original sample.

The effect of freezing of EVOO was more pronounced when considering the extinction coefficients at 232 nm (Figure 6B) and 270 nm (Figure 6C), and the acidity (Figure 6D). Remarkably, freezing the sample retained the original values for these parameters, indicating the efficacy of the lower temperature in preventing the deterioration of the oil.

Storing EVOO in DG for 12 months at -20 °C resulted in a significantly lower K_{232} compared to storage at RT (Figure 6B). The same was reported by others for two of the three different cultivars tested [33].

Similarly, the extinction coefficient at 270 nm was retained by freezing as compared to RT storage (Figure 6C). This was in accordance to earlier reports on one of three different cultivars tested [33]. Thus, freezing EVOO at -20 °C for 12 months kept both K_{232} and K_{270} below the established maximum limit. It is worth noting that while EVOO stored in DG at RT in the dark for 12 months exceeded the established maximum limit standard for K_{270}, the freezing process maintained it. Therefore, frozen samples are better in terms of quality than RT-stored oil, due to the decrease of the oxidation process at lower temperatures [32, 33].

Moreover, the acidity of frozen EVOO was almost identical to the initial sample indicating total prevention of time-induced hydrolysis (Figure 6D). Our findings are in agreement with other reports [33]. Freezing leads to slowing or stopping the activity of lipases which are responsible for breaking fatty acids from TAGs [24]. This, however,

contradicts previous reports showing no change in acidity between frozen and RT samples [32].

Hence, our results indicate that freezing freshly-milled EVOO could be a good practice for its long-term preservation, since all quality parameters tested in this study were significantly below the established maximum limit for 12 months of storage for frozen samples.
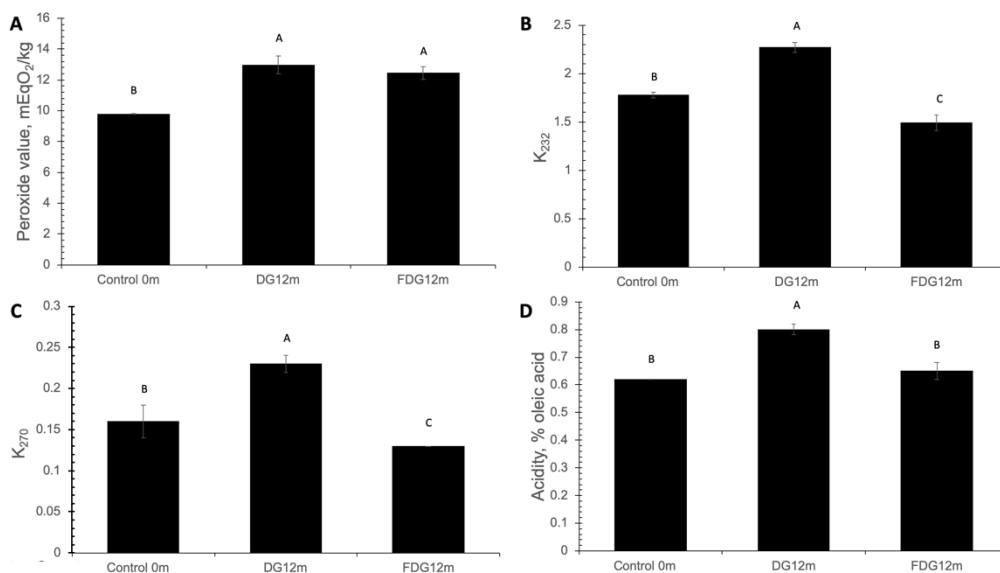
Figure 6. Quality control parameters are maintained by long-term freezing. EVOO stored in DG containers for 12 months at RT (DG12m) and at -20 °C (FDG12m) were compared to the initial EVOO control sample according to (A) PV, (B) K_{232}, (C) K_{270}, and (D) acidity. Different capital letter indicates significant difference ($p < 0.05$, n = 5).

3.3.3. Freezing EVOO at -20 °C slightly salvages its vitamin E content

Vitamin E is one of the most widely distributed antioxidants in nature [56-58]. The generic term vitamin E refers to at least eight structural isomers of tocopherols or tocotrienols [56, 57, 59]. α-tocopherol is the most active form of vitamin E and the major

membrane-bound antioxidant in the cell. Vitamin E triggers apoptosis of cancer cells and protects cells from oxidative damage by inhibiting free radical formation [56, 57, 60, 61].

Tocopherols compete with unsaturated fats and oils for lipid peroxy radicals which are lipids attached to peroxide radical (OO·). Lipid peroxy radicals react with tocopherols much faster at a rate of 10^4 to 10^9 $M^{-1}s^{-1}$ than with lipids (10 to 60 $M^{-1}s^{-1}$)[42].Tocopherol donates hydrogen to lipid peroxy radicals (ROO·), and produces lipid hydroperoxide (ROOH) and tocopheroxy radicals. Tocopheroxy radicals are more stable than lipid peroxy radicals due to resonance stabilization (Figure 7). This ultimately slows down the oil oxidation rate in the propagation stage of autoxidation [42]. The α-tocopherol content of EVOO is therefore important to protect its lipids against autoxidation and thereby increases the storage life and nutritious value of EVOO [36]. Cultivar, environmental conditions (growing area) and time of harvesting (fruit ripening stage) affect the α-tocopherol content in EVOO [36, 62, 63].
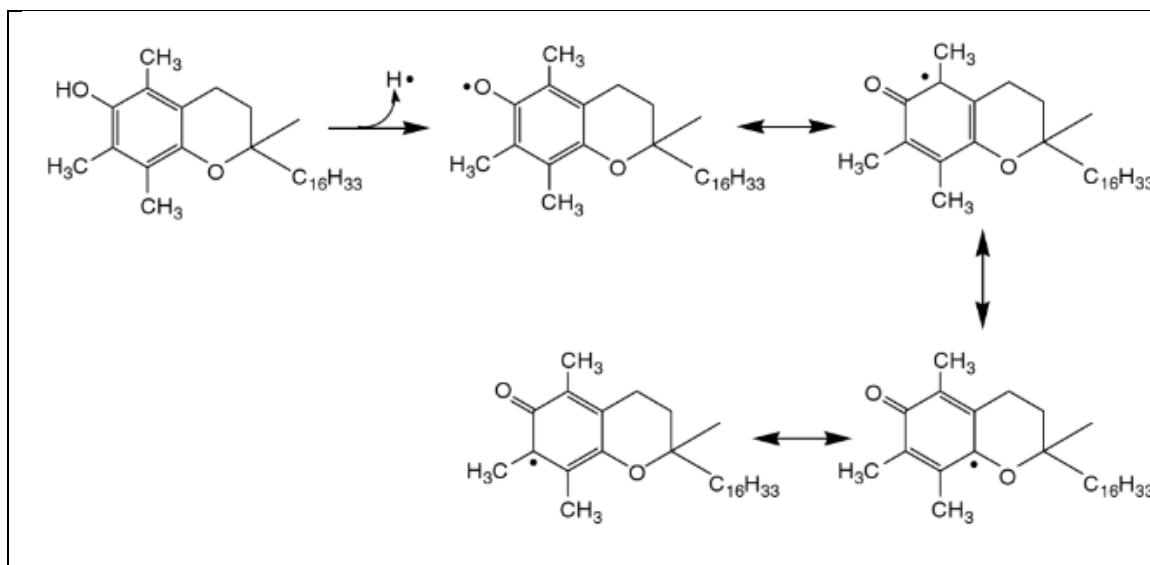
Figure 7. Resonance stabilization of α-tocopheroxy radical. Adapted from [64].

As storage time increases, vitamin E content of the EVOO decreases due to its consumption in the aforementioned protective oxidative reactions. Vitamin E in our samples was reduced by 48% after 12 months of storage in the dark at RT in DG compared to the initial fresh sample. Others reported vitamin E content reduction of 20.8% to 24.0% [34], and 50% [35] during 12 months storage of EVOO in DG container with 3% headspace at RT and in darkness, meaning that our results are consistent with earlier explorations.

The decrease in vitamin E content seems to be cultivar-specific. Oblica cultivar at 05/11 harvest period stored in DG for 12 months at -20 °C with zero headspace showed a 21% decrease in α-tocopherol. Leccino cultivar showed a significant decrease (28%) in α-tocopherol at -20 °C storage for 12 months at zero headspace compared to 16% reduction at RT at 22/10 harvest period [36]. A 100% loss of α-tocopherol in Arbequina cultivar after storage in DG for 12 months at RT was reported [37].

In order to analyze whether freezing slows down the inevitable decrease in vitamin E content upon long-term storage, EVOO samples were stored at -20 °C for 12 months in DG containers prior to vitamin E testing. Vitamin E content in frozen EVOO was reduced by 45% in comparison with the initial fresh sample. Freezing EVOO had a slight statistically significant increase in vitamin E content compared to RT-stored samples (185.92 mg/kg, versus 175.66 mg/kg, respectively) (Figure 8). Thus, in our samples, there is a slight advantage of storing EVOO in the freezer. It might be more significant using other cultivars due to the cultivar-specificity effects of vitamin E.
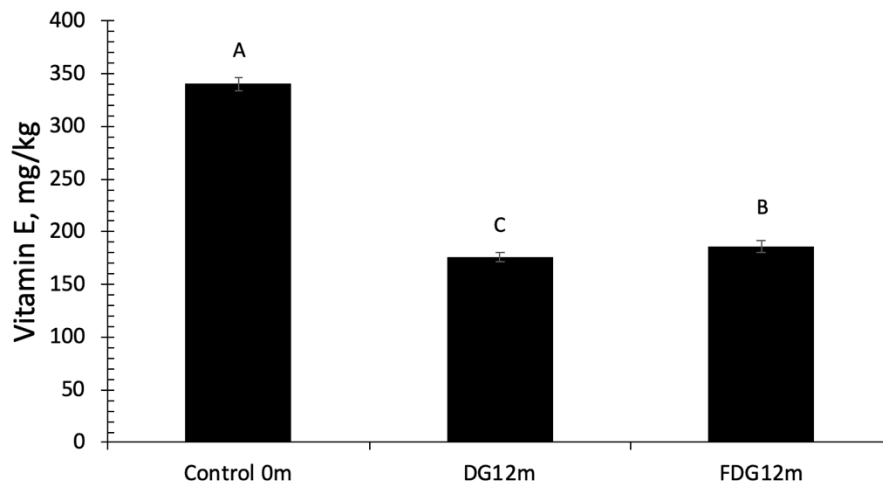
Figure 8. Vitamin E content (mg/kg) in EVOO is reduced with long-term storage in DG containers at RT (DG12m) or at -20 °C (FDG12m) for 12 months. Different capital letters indicate significant difference ($p < 0.05$, n = 5).

3.4   EVOO freezing at -80 °C

To the best of the authors' knowledge, no article has been published to date on evaluating the changes of a long-term freezing at -80 °C on EVOO quality parameters. Therefore, we set out to determine these effects. Since DG will not tolerate these temperatures. EVOO samples were stored in Teflon (polytetrafluoroethylene) containers at -80 °C in the dark for 12 months. These samples did not exceed the maximum limit established for EVOO after 12 months of storage (Table 4).

Additionally, vitamin E content of this sample was better than storage at -20 °C (202.56 mg/kg) which corresponds to a reduction of 40% of the original vitamin E content. These results confirm that lower temperatures of storage have beneficial effects on the quality of long-term stored EVOO.

Table 4: Quality of frozen EVOO stored in Teflon container at -80 °C for 12 months

Quality characteristic	-80 °C	IOC[18], EC[19], PSI[22]
Acidity (% as oleic acid)	0.67	≤ 0.80
PV (as milliequivalent $O_{2/}$ kg oil)	11.91	≤ 20
K_{232} (absorbance)	1.67	≤ 2.5
K_{270} (absorbance)	0.14	≤ 0.22
Vitamin E content (mg/kg)	202.56	-
Oleic (% content)	70.70	≤ 55 - 83
Palmitic (% content)	11.32	≤ 7.5 - 20
Linoleic (% content)	10.68	≤ 3.5 - 21
Stearic (% content)	4.32	≤ 0.5 - 5

4.   Conclusions

The data and results presented in this study propose that the best container for storing Palestinian EVOO is DG followed by PP, and the worst in terms of environmental and chemical tests is HDPE. Palestinian EVOO stored in DG at RT in the dark failed EVOO designation at 12 months by one parameter. However, Palestinian EVOO failed EVOO designation at 8 months in both PP and HDPE containers. Frozen EVOO for 12 months at -20 °C in DG passed all standard tests including FAP % content, but with a 45% reduction in vitamin E content.

We also report that PV is not the best test for assessing the stability of EVOO for long periods due to fluctuations in the readings. However, acidity, K_{232} and K_{270} are the most valuable tests for evaluating stability for long storage periods.

To the best of our knowledge, this is the first study which evaluated freezing EVOO at -80 °C. We determined that Palestinian EVOO stayed within EVOO designation after 12 months of storage at -80 °C including FAP % content with 40% reduction in vitamin E content.

This study provides a scientific approach to guide Palestinians on the best methods in handling EVOO after processing at the mill. It also gives the consumer an option to freeze unused EVOO in order to extend its benefits for longer periods.

5.   Appendices

Appendix I.   Calculation of acidity

The acidity or free fatty acid content expressed as a percentage mass fraction, and according to fat type (see Table 2), is equal to:

Acidity = V x C x M x 100 / (1000 x m)

Where:

V = the volume, in milliliters, of the standard volumetric sodium hydroxide solution used

C = the concentration, in moles per liter, of the standard volumetric sodium hydroxide solution used

M = the molar mass, in grams per mole, of the oleic acid (see Table I)

m = the mass, in grams, of the test portion

Table I: Choice of fatty acid for expression of acidity

Type of fat	Expressed as	Molar mass (g/mol)
All other fats	Oleic acid	282

Appendix II. Calculation of extinction coefficient

Extinction coefficient (K) at the various wavelengths calculated as follows:

$$K_\lambda = E_\lambda /(C \times S)$$

where:

λ = wavelength

K_λ = specific extinction at wavelength λ;

E_λ = extinction measured at wavelength λ;

C = concentration of the solution in g/100 mL;

S = path length of the Quartz cell in cm

Appendix III. K_{232} extrapolation

Both PP and HDPE containers failed EVOO at 8 months testing period. The values for K_{232} for both PP and HDPE containers at 0, 4, and 8 months provided a perfect straight line when K_{232} absorbance values plotted against time. Thus, the K_{232} absorbance for 12

months for both PP and HDPE were extrapolated for each container for the 12 months testing period using the equation of the linear line (Figure III.1, III.2).
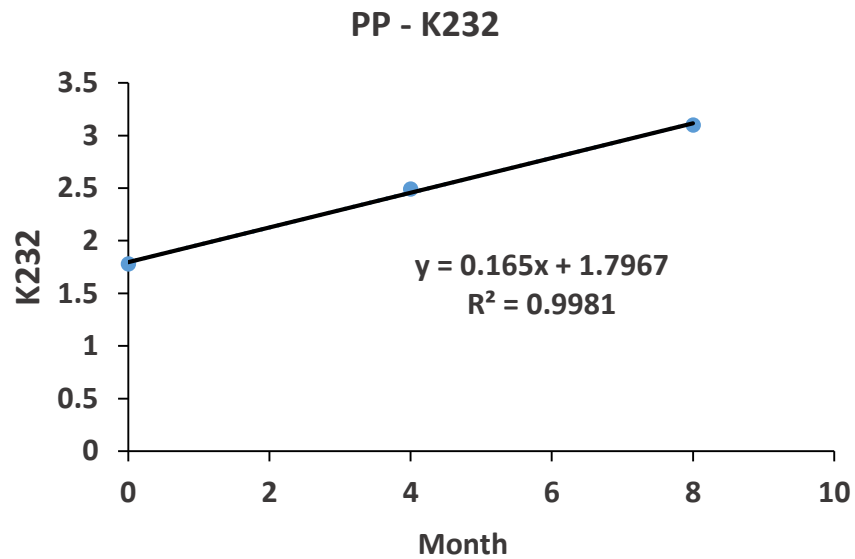
Figure III.1. Regression line and equation for straight line for PP container for K_{232} absorbance vs. time at 0, 4, and 8 months
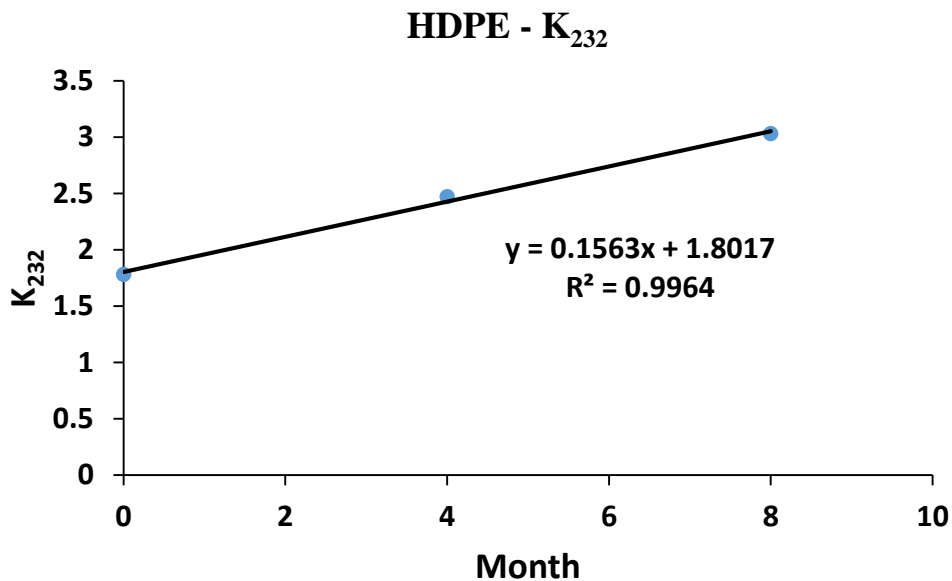
Figure III.2. Regression line and equation for straight line for HDPE container for K_{232} absorbance vs. time at 0, 4, and 8 months

Appendix IV. FAP % content and expression of results

Each fatty acid was expressed as a percentage by mass of methyl esters, by determining the percentage represented by the area of the corresponding peak relative to the sum of the areas of all the peaks using the formula:

Wi = Ai/ ΣA ↔100

Where:

Ai is the area under the peak of the individual fatty acid methyl ester i;

ΣA is the sum of the areas under all the peaks of all the individual fatty acid methyl esters

Appendix V. PV calculation and expression of results

PV (in mEq of active oxygen per kilogram) = $(V – V_0)$ x C (thio) x 1000/m

where:

V = the volume of sodium thiosulfate solution used for the determination, in milliliters;

V_0 = the volume of the sodium thiosulfate standard solution used for the blank test, in milliliters

C (thio) = the concentration of the sodium thiosulfate solution, in moles per liter

m = the mass of the test portion, in grams

Appendix VI.   Autoxidation mechanism in olive oil

Autoxidation of oils, free radical chain reaction (Figure VI.1), includes initiation, propagation, and termination steps:

Initiation	RH	→ R· + H·
Propagation	R· + $^3O_2$	→ ROO·
	ROO· + RH	→ ROOH + R·
Termination	ROO· + R·	→ ROOR
	R· + R·	→ RR
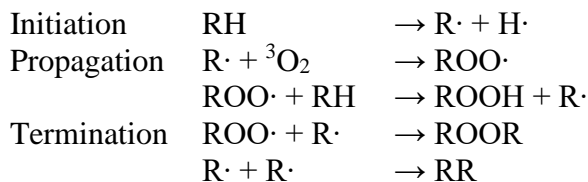
Figure VI.1. Free radical chain reaction steps. R: lipid alkyl group; · indicates a radical [42].

Autoxidation of linoleic and linolenic acids produces only conjugated products (Figure VI.2). Primary oxidation products of this reaction are lipid hydroperoxides, which are relatively stable at RT in the absence of metals. However, in the presence of metals or at high temperature they are readily decomposed to alkoxy radicals and then form

secondary oxidation products such as aldehydes, ketones, acids, esters, alcohols, and short chain hydrocarbons (Figure VI.3). The off-flavor in the oxidized oil is caused by the decomposition products of hydroperoxides [42].
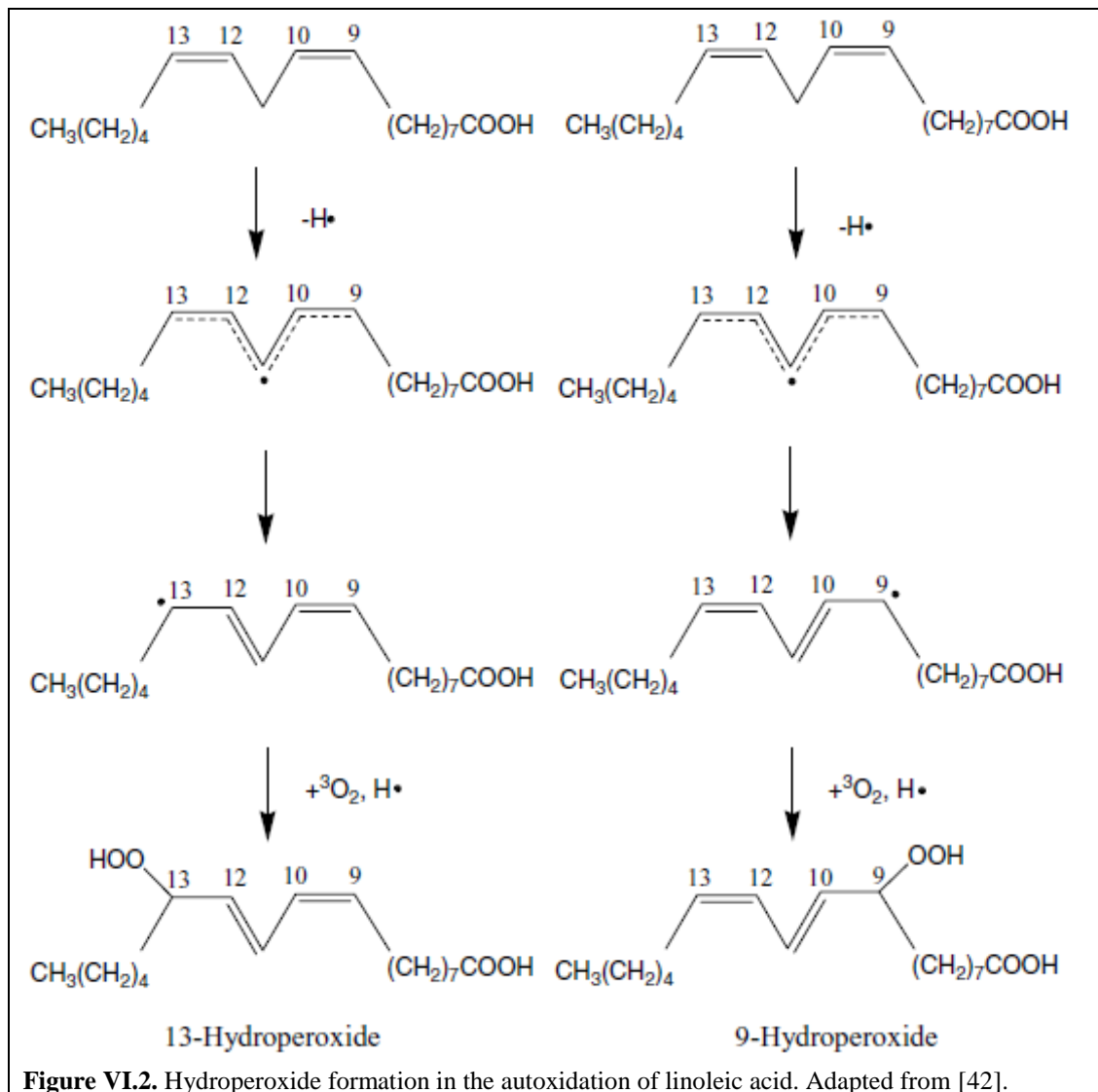
Figure VI.2. Hydroperoxide formation in the autoxidation of linoleic acid. Adapted from [42].

Figure VI.3. Mechanisms of hydroperoxide decomposition to form secondary oxidation products. Adapted from [42].
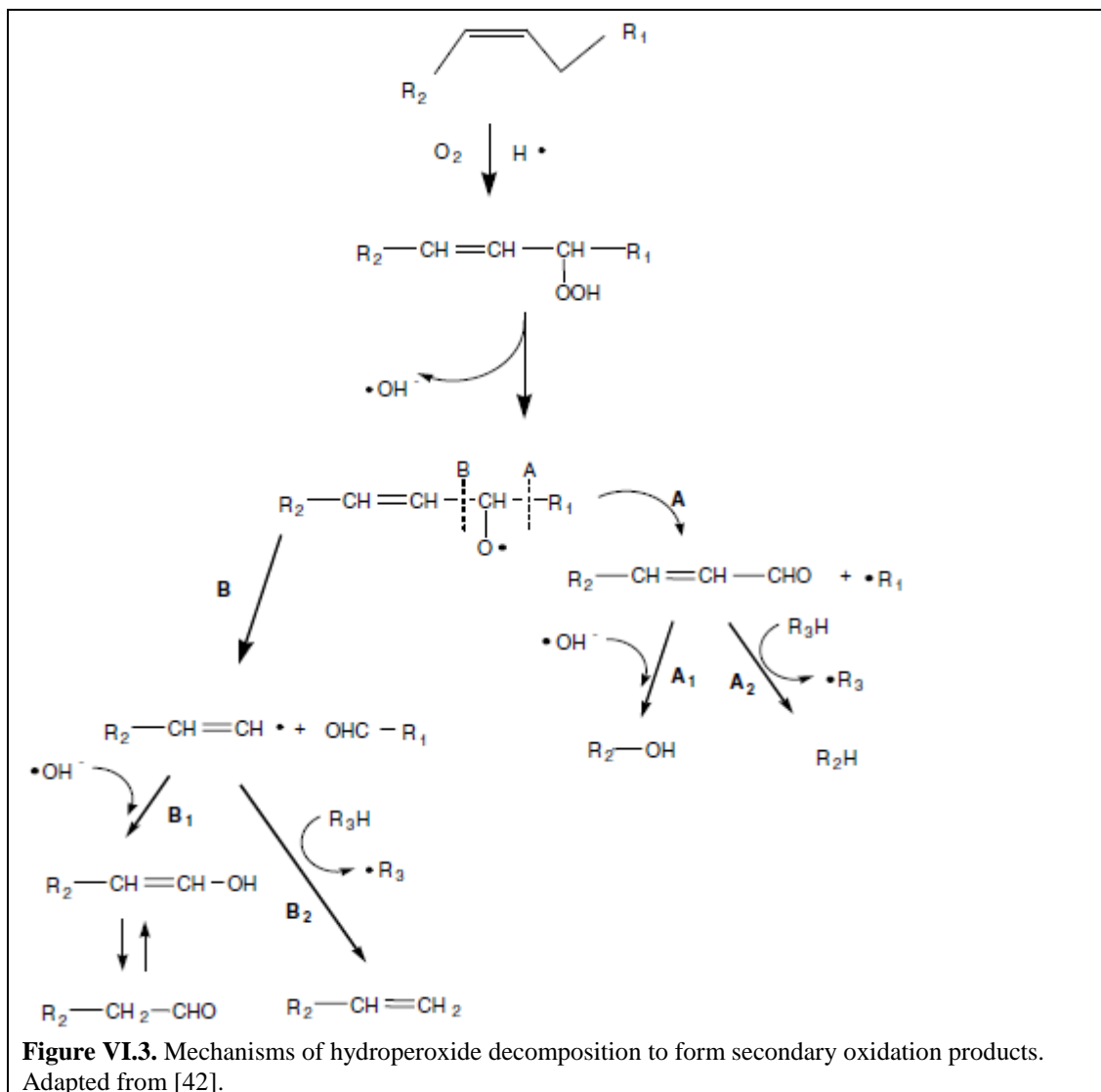
Appendix VII.   Photosensitized oxidation mechanism in olive oil

Oil oxidation is accelerated by light, particularly in the presence of sensitizers such as chlorophylls. The excitation energy from the excited sensitizers can be moved onto adjacent $^3O_2$ to form $^1O_2$ and the sensitizers revert to their ground state. Photosensitized oxidation of olive oil follows the singlet oxygen oxidation pathway (Figure VII). Electrophilic $^1O_2$ can directly react with high-electron-density double bonds without the formation of alkyl radical, and form hydroperoxides at the double bonds. When hydroperoxide is formed, double bond migration and *trans* fatty acid occur, making both conjugated and nonconjugated hydroperoxides. Production of nonconjugated hydroperoxides is not observed in autoxidation. The reaction rate between lipid and oxygen is dependent on the type of oxygen; the reaction rate of $^1O_2$ with lipids is greater

than that of $^3O_2$ because $^1O_2$ can directly react with lipids. $^3O_2$ reacts with the radical state of lipids [42].

Figure VII. Hydroperoxide formation in linoleic acid oxidation by singlet oxygen. Adapted from [42].
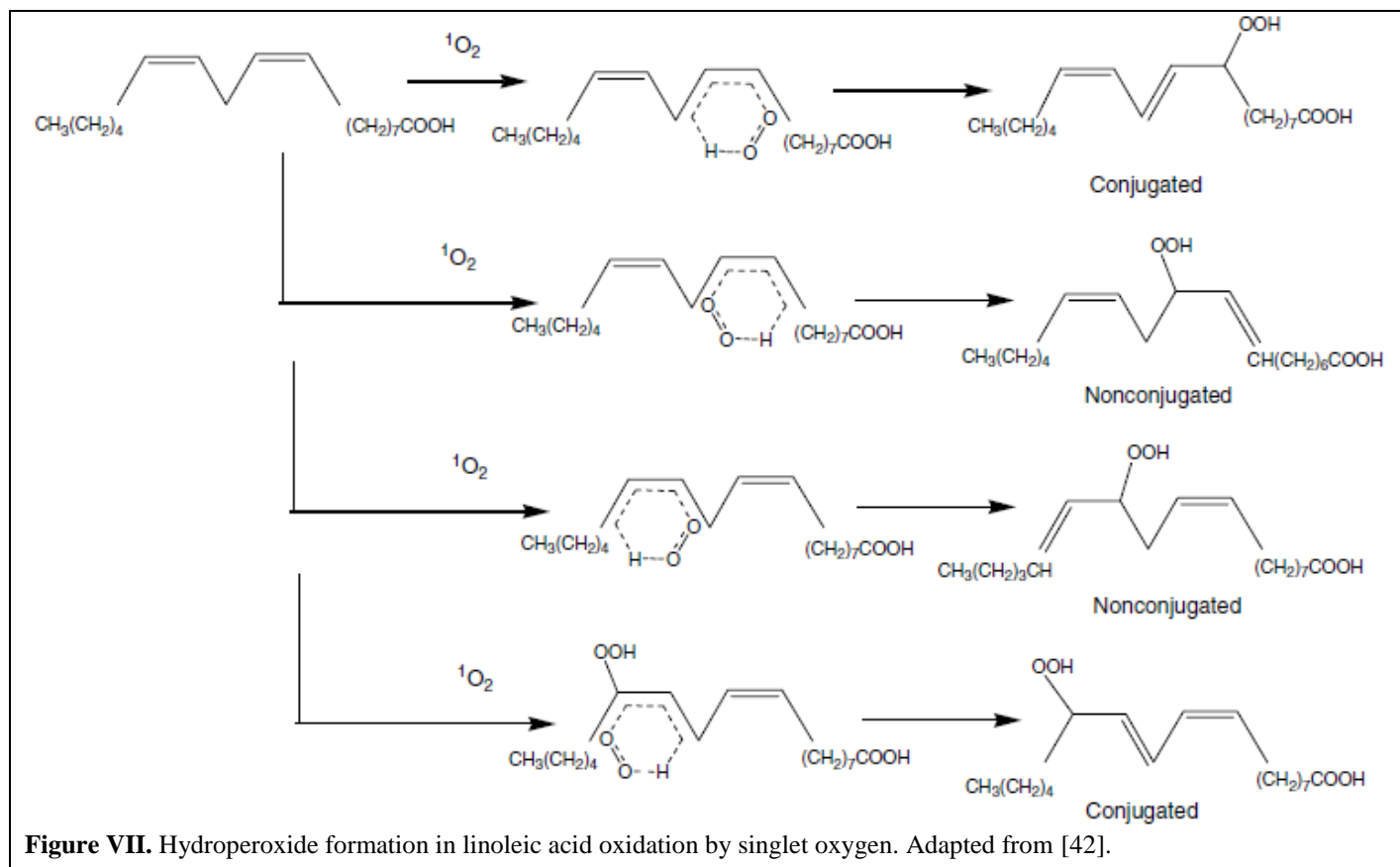
Appendix VIII.   Data tables

Table 1: Peroxide value (as milliequivalent O_2 /kg oil) for DG, HDPE, and control at 0, 4, 8, and 12 months

Month	PP	HDPE	DG
0	9.79 ± 0.01A	9.79 ± 0.01A	9.79 ± 0.01A
4	10.56 ± 1.01AB	12.31 ± 1.48A	9.55 ± 0.63B
8	12.85 ± 1.76A	12.65 ± 0.98A	11.69 ± 0.25A
12	12.96 ± 0.64A	11.21 ± 0.22B	12.97 ± 0.58A

Values are mean ± SD. Maximum allowed for EVOO is ≤ 20. Different capital letter within each line indicate significant difference $(p < 0.05, n = 5)$, one-way ANOVA followed by Bonferroni post hoc analysis.

Table 2: K_{232} for DG, HDPE, PP, and control at 0, 4, 8, and 12 months

Month	PP	HDPE	DG
0	1.78 ± 0.03A	1.78 ± 0.03A	1.78 ± 0.03A
4	2.49 ± 0.18A	2.47 ± 0.12A	2.17 ± 0.06B
8	3.10 ± 0.09A	3.03 ± 0.06A	2.33 ± 0.13B
12	3.78 ± 0.00[#]A	3.68 ± 0.00[#]A	2.27 ± 0.05B

Values are mean ± SD. Maximum allowed for EVOO is ≤ 2.5. Different capital letter within each line indicate significant difference *(p < 0.05, n = 5)*, one-way ANOVA followed by Bonferroni post hoc analysis. [#] Indicates extrapolation (Figure III.1, III.2).

Table 3: K_{270} for DG, HDPE, PP, and control at 0, 4, 8, and 12 months

Month	PP	HDPE	DG
0	0.16 ± 0.02A	0.16 ± 0.02A	0.16 ± 0.02A
4	0.15 ± 0.01A	0.16 ± 0.00A	0.17 ± 0.01A
8	0.21 ± 0.02A	0.21 ± 0.01A	0.21 ± 0.03A
12	0.24 ± 0.02A	0.23 ± 0.02A	0.23 ± 0.01A

Values are mean ± SD. Maximum allowed for EVOO is ≤ 0.22. Different capital letter within each line indicate significant difference *(p < 0.05, n = 5)*, one-way ANOVA followed by Bonferroni post hoc analysis

Table 4: Acidity (% as oleic acid) for DG, HDPE, PP, and control at 0, 4, 8, and 12 months

Month	PP	HDPE	DG
0	0.62 ± 0.00A	0.62 ± 0.00A	0.62 ± 0.00A
4	0.61 ± 0.05B	0.67 ± 0.03AB	0.71 ± 0.03A
8	0.56 ± 0.03B	0.74 ± 0.01A	0.75 ± 0.01A
12	0.66 ± 0.01B	0.79 ± 0.02A	0.80 ± 0.02A

Values are mean ± SD. Maximum allowed for EVOO is ≤ 0.80. Different capital letter within each line indicate significant difference *(p < 0.05, n = 5)*, one-way ANOVA followed by Bonferroni post hoc analysis.

Appendix IX. Statistical analyses

1.   Statistical analysis results using the software Statistix 9.0 for PV – (Appendix VIII. Table 1)

1.1.   Results for 4-month testing period (m=month)

DG4m   HDPE4m   PP4m

10.16   11.76   12.22

8.52   11   9.58

9.92	13.06	10.05
9.54	11.19	10.63
9.62	14.52	10.31

1.1.1 One Way Analysis of Variance results

```
One-Way AOV for: PP4m HDPE4m DG4m

Source    DF        SS        MS        F         P
Between    2    19.4201    9.71005    8.13    0.0059
Within    12    14.3379    1.19482
Total     14    33.7580

Grand Mean 10.805    CV 10.12

Homogeneity of Variances          F         P
Levene's Test                   1.59    0.2434
O'Brien's Test                  1.17    0.3432
Brown and Forsythe Test         0.94    0.4180

Welch's Test for Mean Differences
Source         DF      F        P
Between        2.0    7.33    0.0184
Within         7.2

Component of variance for between groups    1.70304
Effective cell size                             5.0

Variable     Mean
PP4m       10.558
HDPE4m     12.306
DG4m        9.552
Observations per Mean             5
Standard Error of a Mean      0.4888
Std Error (Diff of 2 Means) 0.6913
```

1.1.2. Significant difference determination at 4 months

```
Bonferroni All-Pairwise Comparisons Test

Variable     Mean  Homogeneous Groups
HDPE4m     12.306  A
PP4m       10.558  AB
DG4m        9.5520  B

Alpha             0.05     Standard Error for Comparison  0.6913
Critical T Value  2.779    Critical Value for Comparison  1.9215
There are 2 groups (A and B) in which the means
are not significantly different from one another.
```

1.2 Results for 8 month testing period (m=4m)

DG8m	HDPE8m	PP8m
11.7	12.02	13.14
11.56	13.58	15.55
11.98	13.35	11.07
11.85	13.06	12.96
11.35	11.26	11.52

1.2.1. One Way Analysis of Variance results

One-Way AOV for: PP8m DG8m HDPE8m

```
Source    DF        SS        MS        F         P
Between    2      3.8607   1.93033    1.41    0.2816
Within    12     16.4177   1.36814
Total     14     20.2783
```

Grand Mean 12.397   CV 9.44

```
Homogeneity of Variances          F         P
Levene's Test                    2.43    0.1302
O'Brien's Test                   1.78    0.2098
Brown and Forsythe Test          2.29    0.1435
```

```
Welch's Test for Mean Differences
Source         DF        F         P
Between        2.0     2.91    0.1340
Within         5.7
```

```
Component of variance for between groups    0.11244
Effective cell size                             5.0
```

```
Variable    Mean
PP8m       12.848
DG8m       11.688
HDPE8m     12.654
Observations per Mean              5
Standard Error of a Mean     0.5231
Std Error (Diff of 2 Means) 0.7398
```

1.2.2. Significant difference determination at 8 months

There are no significant pairwise differences among the means

1.3. Results for 12 months testing period (m=month)

DG12m	HDPE12m	PP12m
13.05	11.48	12.38
12.92	11.08	12.16
12.61	10.96	13.54
12.36	11.4	13.39
13.89	11.13	13.33

1.3.1. One Way Analysis of Variance results

One-Way AOV for: PP12m HDPE12m DG12m

```
Source    DF       SS        MS       F        P
Between    2   10.2435   5.12173   19.29   0.0002
Within    12    3.1863   0.26553
Total     14   13.4298

Grand Mean 12.379    CV 4.16
```

```
Homogeneity of Variances        F        P
Levene's Test                  2.09   0.1662
O'Brien's Test                 1.54   0.2545
Brown and Forsythe Test        0.83   0.4588
```

```
Welch's Test for Mean Differences
Source         DF       F        P
Between        2.0   29.77   0.0005
Within         6.5
```

```
Component of variance for between groups   0.97124
Effective cell size                            5.0
```

```
Variable     Mean
PP12m     12.960
HDPE12m   11.210
DG12m     12.966
Observations per Mean            5
Standard Error of a Mean     0.2304
Std Error (Diff of 2 Means) 0.3259
```

1.3.2. Significant difference determination at 12 months

Bonferroni All-Pairwise Comparisons Test

```
Variable     Mean  Homogeneous Groups
DG12m     12.966  A
PP12m     12.960  A
HDPE12m   11.210   B

Alpha              0.05    Standard Error for Comparison  0.3259
```

```
Critical T Value  2.779    Critical Value for Comparison  0.9058
There are 2 groups (A and B) in which the means
are not significantly different from one another.
```

2. Statistical analysis results using the software Statistix 9.0 for K$_{232}$ – (Appendix VIII. Table 2)

2.1   Results for 4 month testing period (m=month)

DG4m	HDPE4m	PP4m
2.237	2.62	2.452
2.22	2.573	2.407
2.18	2.407	2.43
2.135	2.353	2.367
2.06	2.41	2.808

2.1.1. One Way Analysis of Variance results

```
One-Way AOV for: DG4m HDPE4m PP4m

Source     DF       SS        MS       F        P
Between     2    0.33451   0.16725   9.90    0.0029
Within     12    0.20283   0.01690
Total      14    0.53733

Grand Mean 2.3773    CV 5.47

Homogeneity of Variances        F        P
Levene's Test                  1.01    0.3929
O'Brien's Test                 0.74    0.4965
Brown and Forsythe Test        0.23    0.7999

Welch's Test for Mean Differences
Source         DF      F        P
Between        2.0   15.26    0.0027
Within         7.1

Component of variance for between groups   0.03007
Effective cell size                           5.0

Variable    Mean
DG4m       2.1664
HDPE4m     2.4726
PP4m       2.4928
Observations per Mean           5
Standard Error of a Mean    0.0581
Std Error (Diff of 2 Means) 0.0822
```

2.1.2. Significant difference determination at 4 months

```
Bonferroni All-Pairwise Comparisons Test

Variable    Mean   Homogeneous Groups
PP4m       2.4928  A
HDPE4m     2.4726  A
DG4m       2.1664   B

Alpha             0.05     Standard Error for Comparison  0.0822
Critical T Value  2.779    Critical Value for Comparison  0.2285
There are 2 groups (A and B) in which the means
are not significantly different from one another.
```

2.2.   Results for 8 month testing period (m=month)

DG8m	PP8m	HDPE8m
2.137	3.223	3.123
2.3	3.018	3.043
2.525	3.028	3.033
2.413	3.06	2.993
2.262	3.16	2.955

2.2.1. One Way Analysis of Variance results

```
One-Way AOV for: PP8m HDPE8m DG8m

Source    DF       SS        MS        F         P
Between    2    1.81833   0.90917   80.41    0.0000
Within    12    0.13569   0.01131
Total     14    1.95402

Grand Mean 2.8182    CV 3.77


Homogeneity of Variances          F         P
Levene's Test                    2.19    0.1547
O'Brien's Test                   1.61    0.2405
Brown and Forsythe Test          1.12    0.3575


Welch's Test for Mean Differences
Source         DF      F        P
Between       2.0    49.86    0.0001
Within        7.3

Component of variance for between groups    0.17957
Effective cell size                             5.0


Variable    Mean
PP8m       3.0978
```

```
HDPE8m    3.0294
DG8m      2.3274
Observations per Mean            5
Standard Error of a Mean    0.0476
Std Error (Diff of 2 Means) 0.0673
```

2.2.2. Significant difference determination at 8 months

Bonferroni All-Pairwise Comparisons Test

```
Variable    Mean  Homogeneous Groups
PP8m      3.0978  A
HDPE8m    3.0294  A
DG8m      2.3274   B

Alpha               0.05     Standard Error for Comparison  0.0673
Critical T Value  2.779     Critical Value for Comparison  0.1869
There are 2 groups (A and B) in which the means
are not significantly different from one another.
```

2.3.   Results for 12 month testing period (m=month)

PP12m	HDPE12m	DG12m
3.78	3.68	2.272
		2.207
		2.327
		2.247
		2.32

2.3.1. One Way Analysis of Variance results

One-Way AOV for: PP12m HDPE12m DG12m

```
Source    DF       SS        MS       F        P
Between   2   3.03098   1.51549  597.52   0.0000
Within    4   0.01015   0.00254
Total     6   3.04113

Grand Mean 2.6904    CV 1.87

Homogeneity of Variances         F        P
Levene's Test                  0.93   0.4663
O'Brien's Test                    M        M
Brown and Forsythe Test        1.56   0.3157

Welch's Test for Mean Differences
Source         DF      F        P
Between        2.0      M   0.0000
Within          M
```

```
Component of variance for between groups    0.88256
Effective cell size                              1.7
```

```
Variable  N    Mean       SE
PP12m     1   3.7800   0.0504
HDPE12m   1   3.6800   0.0504
DG12m     5   2.2746   0.0225
```

2.3.2. Significant difference determination at 12 months

```
Bonferroni All-Pairwise Comparisons Test
```

```
Variable     Mean  Homogeneous Groups
PP12m       3.7800  A
HDPE12m     3.6800  A
DG12m       2.2746   B
```

```
Alpha              0.05    Standard Error for Comparison  0.0552 TO
0.0712
Critical T Value  3.961 Critical Value for Comparison  0.2185 TO
0.2821
There are 2 groups (A and B) in which the means
are not significantly different from one another.
```

3. Statistical analysis results using the software Statistix 9.0 for K_{270} – (Appendix VIII. Table 3)

3.1. Results for 4 month testing period (m=month)

DG4m	HDPE4m	PP4m
0.145	0.166	0.132
0.168	0.158	0.144
0.174	0.162	0.152
0.165	0.163	0.164
0.179	0.17	0.151

3.1.1. One Way Analysis of Variance results

```
One-Way AOV for: PP4m HDPE4m DG4m
```

```
Source     DF      SS          MS         F         P
Between    2    0.00091    4.555E-04    4.17    0.0422
Within    12    0.00131    1.092E-04
Total     14    0.00222
```

```
Grand Mean 0.1595    CV 6.55
```

```
Homogeneity of Variances          F        P
Levene's Test                   1.11   0.3600
O'Brien's Test                  0.82   0.4644
Brown and Forsythe Test         0.85   0.4514


Welch's Test for Mean Differences
Source          DF        F        P
Between        2.0     3.54   0.0913
Within         6.5


Component of variance for between groups 6.925E-05
Effective cell size                          5.0


Variable     Mean
PP4m        0.1486
HDPE4m      0.1638
DG4m        0.1662
Observations per Mean             5
Standard Error of a Mean    4.674E-03
Std Error (Diff of 2 Means) 6.610E-03
```

3.1.2. Significant difference determination at 4 months

```
Bonferroni All-Pairwise Comparisons Test


Variable     Mean  Homogeneous Groups
DG4m        0.1662  A
HDPE4m      0.1638  A
PP4m        0.1486  A


Alpha              0.05     Standard Error for Comparison  6.610E-03
Critical T Value  2.779     Critical Value for Comparison  0.0184
There are no significant pairwise differences among the means.
```

3.2.   Results for 8 month testing period (m=month)

DG8m	PP8m	HDPE8m
0.195	0.182	0.227
0.203	0.235	0.214
0.187	0.2	0.198
0.19	0.189	0.209
0.255	0.228	0.191

3.2.1. One Way Analysis of Variance results

```
One-Way AOV for: PP8m HDPE8m DG8m


Source     DF         SS         MS        F         P
```

```
Between    2   0.00001   4.067E-06    0.01   0.9921
Within    12   0.00616   5.131E-04
Total     14   0.00617
```

```
Grand Mean 0.2069    CV 10.95
```

Homogeneity of Variances   F   P
```
Levene's Test                  0.77   0.4854
O'Brien's Test                 0.56   0.5831
Brown and Forsythe Test        0.30   0.7486
```

Welch's Test for Mean Differences
Source   DF   F   P
```
Between     2.0    0.01   0.9912
Within      7.3
```

```
Component of variance for between groups -1.018E-04
Effective cell size                           5.0
```

Variable   Mean
```
PP8m      0.2068
HDPE8m    0.2078
DG8m      0.2060
Observations per Mean           5
Standard Error of a Mean     0.0101
Std Error (Diff of 2 Means) 0.0143
```

3.2.2. Significant difference determination at 8 months

```
There are no significant pairwise differences among the means.
```

3.3.   Results for 12 month testing period (m=month)

DG12m	HDPE12m	PP12m
0.219	0.236	0.221
0.223	0.252	0.235
0.218	0.231	0.24
0.233	0.22	0.242
0.24	0.209	0.263

3.3.1. One Way Analysis of Variance results

One-Way AOV for: PP12m HDPE12m DG12

Source   DF   SS   MS   F   P
```
Between    2   0.00051   2.553E-04    1.31   0.3067
Within    12   0.00235   1.954E-04
Total     14   0.00286
```

```
Grand Mean 0.2321    CV 6.02
```

Homogeneity of Variances   F   P
```
Levene's Test                 0.69   0.5210
O'Brien's Test                0.51   0.6152
Brown and Forsythe Test       0.32   0.7292
```

Welch's Test for Mean Differences
Source   DF   F   P
```
Between     2.0    1.33   0.3207
Within      7.5
```

```
Component of variance for between groups 1.196E-05
Effective cell size                          5.0
```

Variable   Mean
```
PP12m    0.2402
HDPE12m  0.2296
DG12     0.2266
Observations per Mean           5
Standard Error of a Mean    6.252E-03
Std Error (Diff of 2 Means) 8.842E-03
```

3.3.2. Significant difference determination at 12 months

```
There are no significant pairwise differences among the means.
```

4.   Statistical analysis results using Statistix 9.0 for Acidity – (Appendix VIII. Table 4)

4.1.   Results for 4 month testing period (m=month)

DG4m	HDPE4m	PP4m
0.68	0.62	0.55
0.66	0.69	0.62
0.73	0.67	0.58
0.73	0.67	0.61
0.73	0.68	0.67

4.1.1   One Way Analysis of Variance results

One-Way AOV for: PP4m HDPE4m DG4m

Source   DF   SS   MS   F   P
```
Between   2    0.02533   0.01267   9.77   0.0030
Within   12    0.01556   0.00130
```

```
Total     14    0.04089
```

Grand Mean 0.6593   CV 5.46

Homogeneity of Variances   F   P
```
Levene's Test                    0.90   0.4322
O'Brien's Test                   0.66   0.5339
Brown and Forsythe Test          0.41   0.6713
```

Welch's Test for Mean Differences
Source   DF   F   P
```
Between     2.0    7.31   0.0166
Within      7.7
```

```
Component of variance for between groups   0.00227
Effective cell size                            5.0
```

Variable   Mean
```
PP4m       0.6060
HDPE4m     0.6660
DG4m       0.7060
Observations per Mean            5
Standard Error of a Mean     0.0161
Std Error (Diff of 2 Means) 0.0228
```

4.1.2. Significant difference determination at 4 months

Bonferroni All-Pairwise Comparisons Test

Variable   Mean   Homogeneous Groups
```
DG4m       0.7060   A
HDPE4m     0.6660   AB
PP4m       0.6060    B
```

```
Alpha              0.05     Standard Error for Comparison  0.0228
Critical T Value  2.779     Critical Value for Comparison  0.0633
There are 2 groups (A and B) in which the means
are not significantly different from one another.
```

4.2.   Results for 8 month testing period (m=month)

DG8m	HDPE8m	PP8m
0.74	0.75	0.54
0.76	0.72	0.55
0.74	0.74	0.54
0.76	0.76	0.56
0.76	0.74	0.61

4.2.1. One Way Analysis of Variance results

One-Way AOV for: DG8m HDPE8m PP8m

```
Source    DF        SS        MS        F        P
Between    2    0.11681   0.05841  147.24   0.0000
Within    12    0.00476   0.00040
Total     14    0.12157
```

```
Grand Mean 0.6847    CV 2.91
```

Homogeneity of Variances   F   P
```
Levene's Test                     1.35   0.2966
O'Brien's Test                    0.99   0.4000
Brown and Forsythe Test           0.53   0.6009
```

Welch's Test for Mean Differences
Source   DF   F   P
```
Between      2.0   88.38   0.0000
Within       7.3
```

```
Component of variance for between groups   0.01160
Effective cell size                            5.0
```

Variable   Mean
```
DG8m       0.7520
HDPE8m     0.7420
PP8m       0.5600
Observations per Mean               5
Standard Error of a Mean    8.907E-03
Std Error (Diff of 2 Means) 0.0126
```

4.2.2. Significant difference determination at 8 months

Bonferroni All-Pairwise Comparisons Test

Variable   Mean Homogeneous Groups
```
DG8m       0.7520  A
HDPE8m     0.7420  A
PP8m       0.5600   B
```

```
Alpha              0.05     Standard Error for Comparison  0.0126
Critical T Value  2.779     Critical Value for Comparison  0.0350
There are 2 groups (A and B) in which the means
are not significantly different from one another.
```

4.3.   Results for 12 month testing period (m=month)

```
DG12m        HDPE12m      PP12m

0.84         0.81         0.67
```

0.79	0.77	0.64
0.8	0.79	0.66
0.78	0.77	0.66
0.8	0.81	0.67

4.3.1. One Way Analysis of Variance results

One-Way AOV for: PP12m HDPE12m DG12m

```
Source    DF       SS        MS        F         P
Between    2    0.06201   0.03101   86.93    0.0000
Within    12    0.00428   0.00036
Total     14    0.06629

Grand Mean 0.7507    CV 2.52
```

```
Homogeneity of Variances          F        P
Levene's Test                    0.80   0.4720
O'Brien's Test                   0.59   0.5709
Brown and Forsythe Test          0.60   0.5621
```

Welch's Test for Mean Differences
```
Source         DF       F        P
Between        2.0   111.85   0.0000
Within         7.4
```

```
Component of variance for between groups    0.00613
Effective cell size                            5.0
```

```
Variable     Mean
PP12m       0.6600
HDPE12m     0.7900
DG12m       0.8020
Observations per Mean            5
Standard Error of a Mean    8.446E-03
Std Error (Diff of 2 Means) 0.0119
```

4.3.2. Significant difference determination at 12 months

Bonferroni All-Pairwise Comparisons Test

```
Variable     Mean   Homogeneous Groups
DG12m       0.8020   A
HDPE12m     0.7900   A
PP12m       0.6600    B
```

```
Alpha              0.05    Standard Error for Comparison  0.0119
Critical T Value  2.779    Critical Value for Comparison  0.0332
There are 2 groups (A and B) in which the means
are not significantly different from one another.
```

5.   Quality test for EVOO frozen at -20 °C for 12 months compare to control and RT sample at 12 months using One Way Analysis of Variance

5.1.   PV for DG12m and FDG12m compare to control (m=month, DG12m= DG stored at RT, FDG= frozen at -20 °C in DG) – Figure 6A

PeroxCont	DG12m	FDG12m
9.79	13.05	12.6
9.78	12.92	12.9
	12.61	12.46
	12.36	12.5
	13.89	11.76

5.1.1.   One Way Analysis of Variance results using Statistix 9.0

```
One-Way AOV for: DG12m FDG12m PeroxCont

Source    DF        SS        MS       F         P
Between    2    14.8919   7.44594   32.52    0.0001
Within     9     2.0605   0.22894
Total     11    16.9524

Grand Mean 12.218    CV 3.92

Homogeneity of Variances          F         P
Levene's Test                   0.76    0.4973
O'Brien's Test                     M         M
Brown and Forsythe Test         1.00    0.4055

Welch's Test for Mean Differences
Source         DF       F        P
Between       2.0   155.47   0.0000
Within        5.3

Component of variance for between groups    1.92453
Effective cell size                             3.8

Variable    N    Mean      SE
DG12m       5  12.966  0.2140
FDG12m      5  12.444  0.2140
PeroxCont   2   9.785  0.3383
```

5.1.2.   Significant difference determination

```
Bonferroni All-Pairwise Comparisons Test

Variable     Mean  Homogeneous Groups
DG12m       12.966  A
FDG12m      12.444  A
PeroxCont   9.7850   B


Alpha             0.05     Standard Error for Comparison  0.3026 TO
0.4003
Critical T Value  2.933    Critical Value for Comparison  0.8877 TO
1.1743
There are 2 groups (A and B) in which the means
are not significantly different from one another.
```

5.2. K_{232} for DG12m and FDG12m compare to control (m=month, DG12m= DG stored at RT, FDG= frozen at -20 °C in DG) – Figure 6B

K232Cont	FDG12m	DG12m
1.819	1.387	2.272
1.772	1.437	2.207
1.759	1.542	2.327
	1.528	2.247
	1.577	2.32

5.2.1. One Way Analysis of Variance results using Statistix 9.0

```
One-Way AOV for: K232Cont DG12m FDG12m

Source    DF       SS        MS       F        P
Between    2   1.54613  0.77307  207.90   0.0000
Within    10   0.03718  0.00372
Total     12   1.58332

Grand Mean 1.8611    CV 3.28

Homogeneity of Variances        F        P
Levene's Test                  2.44   0.1374
O'Brien's Test                 1.70   0.2319
Brown and Forsythe Test        0.86   0.4538

Welch's Test for Mean Differences
Source        DF      F        P
Between      2.0   203.41   0.0000
Within       6.5

Component of variance for between groups   0.18185
```

```
Effective cell size                                    4.2
```

```
Variable   N    Mean      SE
K232Cont   3   1.7833   0.0352
DG12m      5   2.2746   0.0273
FDG12m     5   1.4942   0.0273
```

5.2.2. Significant difference determination

Bonferroni All-Pairwise Comparisons Test

```
Variable     Mean  Homogeneous Groups
DG12m      2.2746  A
K232Cont   1.7833   B
FDG12m     1.4942    C
```

```
Alpha              0.05     Standard Error for Comparison  0.0386 TO
0.0445
Critical T Value  2.870     Critical Value for Comparison  0.1107 TO
0.1278
All 3 means are significantly different from one another.
```

5.3. K_{270} for DG12m and FDG12m compare to control (m=month, DG12m= DG stored at RT, FDG= frozen at -20 °C in DG) – Figure 6C

K270Cont	FDG12m	DG12m
0.185	0.1297	0.219
0.155	0.1326	0.223
0.149	0.1342	0.218
	0.1292	0.233
	0.1306	0.24

5.3.1. One Way Analysis of Variance results using Statistix 9.0

One-Way AOV for: DG12m FDG12m K270Cont

```
Source      DF        SS        MS        F          P
Between      2    0.02331   0.01165   103.44   0.0000
Within      10    0.00113   0.00011
Total       12    0.02444
```

```
Grand Mean 0.1753    CV 6.06
```

```
Homogeneity of Variances          F          P
Levene's Test                   5.23    0.0278
O'Brien's Test                  2.61    0.1228
```

```
Brown and Forsythe Test      1.56    0.2575
```

Welch's Test for Mean Differences
```
Source          DF        F        P
Between       2.0   203.72    0.0002
Within        3.7
```

```
Component of variance for between groups    0.00273
Effective cell size                             4.2
```

Variable N   Mean   SE
```
DG12m     5  0.2266  4.75E-03
FDG12m    5  0.1313  4.75E-03
K270Cont  3  0.1630  6.13E-03
```

5.3.2. Significant difference determination

Bonferroni All-Pairwise Comparisons Test

Variable   Mean   Homogeneous Groups
```
DG12m     0.2266  A
K270Cont  0.1630   B
FDG12m    0.1313    C
```

```
Alpha              0.05      Standard Error for Comparison  6.713E-03
TO 7.752E-03
Critical T Value  2.870     Critical Value for Comparison  0.0193 TO
0.0222
All 3 means are significantly different from one another.
```

5.4. Acidity for DG12m and FDG12m compare to control (m=month, DG12m= DG stored at RT, FDG= frozen at -20 °C in DG) - Figure 6D

AcidCont	FDG12m	DG12m
0.62	0.64	0.84
0.62	0.62	0.79
	0.62	0.8
	0.69	0.78
	0.66	0.8

5.4.1. One Way Analysis of Variance results using Statistix 9.0

One-Way AOV for: DG12m FDG12m AcidCont

Source   DF   SS   MS   F   P

```
Between    2   0.07887   0.03943   63.38   0.0000
Within     9   0.00560   0.00062
Total     11   0.08447

Grand Mean 0.7067    CV 3.53
```

Homogeneity of Variances   F   P
```
Levene's Test                    0.89    0.4446
O'Brien's Test                      M        M
Brown and Forsythe Test          1.31    0.3161
```

Welch's Test for Mean Differences
Source   DF   F   P
```
Between     2.0        M   0.0000
Within       M
```

```
Component of variance for between groups   0.01035
Effective cell size                            3.8
```

Variable N   Mean   SE
```
DG12m     5   0.8020   0.0112
FDG12m    5   0.6460   0.0112
AcidCont  2   0.6200   0.0176
```

5.4.2. Significant difference determination

Bonferroni All-Pairwise Comparisons Test

Variable   Mean   Homogeneous Groups
```
DG12m      0.8020   A
FDG12m     0.6460    B
AcidCont   0.6200    B
```

```
Alpha              0.05    Standard Error for Comparison  0.0158 TO
0.0209
Critical T Value  2.933    Critical Value for Comparison  0.0463 TO
0.0612
There are 2 groups (A and B) in which the means
are not significantly different from one another.
```

6.   Vitamin E content - Figure 8

6.1.   Vitamin E Results for DG12m and FDG12m compare to control, (m=month, DG12m= DG stored at RT, FDG= frozen at -20 °C in DG)

VitECont	DG12m	FDG12m
344.5	174.7	184.7
336.1	180.7	189.9
	177.7	193.8

```
     176.7          179.6

     168.5          181.6
```

6.1.1. One Way Analysis of Variance results using Statistix 9.0

```
One-Way AOV for: DG12m FDG12m VitECont


Source     DF        SS         MS        F         P
Between     2    42668.9    21334.5   749.63    0.0000
Within      9      256.1       28.5
Total      11    42925.0


Grand Mean 207.38    CV 2.57


Homogeneity of Variances          F         P
Levene's Test                    0.36    0.7040
O'Brien's Test                     M         M
Brown and Forsythe Test          0.29    0.7521


Welch's Test for Mean Differences
Source          DF        F         P
Between         2.0   522.86    0.0002
Within          2.9


Component of variance for between groups    5681.60
Effective cell size                             3.8


Variable   N     Mean       SE
DG12m      5   175.66   2.3858
FDG12m     5   185.92   2.3858
VitECont   2   340.30   3.7723
```

6.1.2. Significant difference determination

```
Bonferroni All-Pairwise Comparisons Test


Variable     Mean   Homogeneous Groups
VitECont   340.30   A
FDG12m     185.92    B
DG12m      175.66     C


Alpha            0.05    Standard Error for Comparison  3.3740 TO
4.4634
Critical T Value  2.933   Critical Value for Comparison  9.8971 TO
13.093
All 3 means are significantly different from one another.
```

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