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Original Article

Long-Term Preservation of Total Phenolic Content and Antioxidant Activity in Extra Virgin Olive Oil: A Physico-biochemical Approach

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ABSTRACT

Objectives: This work, on low-cost controlled freeze methodology for stabilizing the phenolic content in Extra Virgin Olive Oil (EVOO), is evaluated by studying four Quality Indexes (QIs): free acidity, as % of oleic acid (1st-QI); UV-absorption values; K_{272}, K_{232} and ΔK (2nd-QI); total phenolic content (TPC), as Gallic acid equivalence (3rd-QI); and lipid peroxidation products, as free malondialdehyde (MDA) (4th-QI). **Methods:** The study of QIs for EVOO under storage, absence of light, at 25, 4, -20 and -80°C, with or without N_2-purge showed that the phenolic charge is stabilized for > 12 months, at refrigeration conditions storage (~ 4°C) in the absence of oxygen, whilst the peroxidation of lipids is being kept at a low level. **Results:** The results showed that the high concentration of natural hydroxylphenols in the EVOO in combination with its degassing (via N_2-purge) and storage at ~ 4°C, constitute the optimum conditions for preservation of olive oil as a long-lasting EVOO (LL [legal limit]-EVOO) with high added value. **Conclusion:** The main scientific contribution of this study is: (i) development and evaluation of a methodology (freeze controlled treatment) for the long-term preservation of EVOO quality, (ii) understanding the physicochemical mechanism and factors determining EVOO quality and (iii) ready-to-use technology for the local and international market.

Key words: Freeze Stabilization, Natural Polyphenols, Free radicals, Lipid Peroxidation, Antioxidant activity.

INTRODUCTION

The preservation of Extra Virgin Olive Oil (EVOO) quality is of uttermost economic and health importance, as EVOO is prone to oxidation both during production and storage. In particular, the storage conditions of EVOO are very important for avoiding or reducing the negative effects by autoxidation on the qualitative characteristics of the packaged product.[1] The level of oxidation degradation is greatly affected by the EVOO storage conditions; temperature; exposure to light and oxygen, mainly singlet oxygen[1] O_2, free fatty acids; chemical composition; saturated to unsaturated fatty ratio; total phenol content (TPC), etc.[2] Moreover, the existence of unsaturated fatty acids in oils and fats is the intrinsic reason for lipids oxidation. As reported by Di Giovacchino *et al.* the TPC of EVOO decreases during storage because its oxidation protects the oils from autoxidation, especially where the initial content of total phenols is higher than 150 mg/L.[3] This phenolic antioxidant mechanism has been confirmed.[4] According to Bendini *et al.* the phenols in EVOO are very sensitive to interfacial physicochemical reactions (e.g., at air-oil or oil-water interfaces), which significantly affect their relative activities in different lipidic systems.[5] Moreover, the EVOO remains in good state for a long time when stored in a fully filled bottle at room temperature (commercial conditions 20-25°C), where tyrosol derivatives are more stable than LH-ROH compounds.[6,7] Moreover, the storage of EVOO is sensitive to temperature changes. Previous research showed that the degradation of secoiridoid phenolic during storage displayed pseudo-first-order kinetics and depended on the initial phenolic content.[7] In particular, the initial degradation rate was similar at 5 and 15°C but increased considerably at 25°C and was even faster at 50°C. Within this context, as reported by Krichene *et al.* the increase in the content of simple phenolics, the decrease of their secoiridoid derivatives, or the ratio of simple to secoiridoid phenolics could be used as indices of the oxidative and hydrolytic degradation of phenolics.[7] Additionally, when EVOOs were stored in the light had significantly lower tocopherol, carotenoid and chlorophyll content, while EVOO colour values changed from green to yellow versus in comparison to a 12-month storage in the dark.[8] As reported by Caponio *et al.* oils stored in the dark contained

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mainly primary oxidation products, whilst oils stored in the light contained secondary oxidation products as confirmed by the K_{270}, K_{232} and ΔK UV-absorption values.[9]

In another treatment approach, N_2 has been utilized as a conditioner gas to study the possibility of improving the stability of EVOO. Studies showed that the presence of N_2 in the bottle headspace can increase the EVOO shelf-life, preserving its organoleptic and functional features; whilst another study showed that free radical production was markedly reduced by N_2 bubbling.[10,11] Moreover, the effects of a storage up to 18 months after bottling of Tuscan EVOOs filtered and frozen at −23°C in comparison to same samples maintained at room temperature in the dark, were evaluated by monitoring the evolution of their phenolic composition and aromatic profile.[12] The results showed that an increase of tyrosol, hydroxytyrosol (LH-ROH) and % of hydrolysis was observed in EVOOs stored at room temperatures starting from 3-months storage and increased thereafter, whilst all frozen EVOOs showed negligible differences in aromatic profile up to a 12-month storage. In addition, the filtering step allows the suspended particles to be separated, thus avoiding the formation of deposits and mucilage. This significantly improves the preservation of the product.[13] In fact, if the storage temperature decreases, for instance to -6°C, the oil will change its physical state. That might imply a loss of the total availability by the antioxidant compounds, reducing its natural protection against lipid oxidation. In conclusion, the effect of storage temperature, especially below 0°C, requires much more attention from a technological point of view because, in some way, it appears to affect the oil shelf life.[14] Thus, **c**ontrolled freezing provides a promising method for longer life than any other preservation technique used for olive oil. At present, this method has to be optimized and parameterised with regard to the preservation of phenols in olive oil and mostly in the higher quality grades, such as EVOO.

This study aims to evaluate a novel methodology for the stabilisation of TPC in EVOO. The goal is to determine the optimal cost-effective freezing-temperature zone where the phenolic content will be preserved. The proposed research is investigated by studying four Quality **Indexes** (QIs) of olive oil under controlled O_2 and light at various storage temperatures (25, 4, -20 and -80°C). These QIs are: free acidity, as % of oleic acid (1st-QI); UV-absorption values; K_{272}, K_{232} and ΔK (2nd-QI); TPC as **g**allic acid equivalence (GAE) (3rd-QI); and lipid peroxidation, as free malondialdehyde (MDA) (4th-QI). These QIs are monitored by analytical and spectroscopic methodologies. *Expected outcomes of this study's aims are:* (i) development and evaluation of a methodology (freeze controlled treatment) for the long-term preservation of EVOO quality, (ii) understanding the physicochemical mechanism and factors determining EVOO quality and (iii) ready-to-use technology for the local and international market.

MATERIALS AND METHODS

The olive oil evaluated in this work represents an autochthonous olive (*koroneiki* variety) cultivar grown in Western Greece, where the peculiar microclimate has a significant impact on cultivar features, conferring upon the final product (mono-varietal olive oil) organoleptic and nutritional characteristics which are much appreciated in Greece and abroad.[15]

Samples

For the production of olive oil, olive fruit of the *koroneiki* variety (*Olea europaea var. microcarpa alba*) were used. The olive fruit was harvested by hand from five non-irrigated trees during the harvest period and their skin colours were green and purple. The infected olives were removed and the rest were washed with tap water. The olive oil was produced at optimum conditions within two hours after harvest by an olive oil factory. After production, the olive oil was filled in 50 ml Teflon bottles and two treatments followed with and without degassing/purging of olive oil. The degassing was carried out for 1 hr by purge N_2 (99,999%) and all the samples were immediately analysed. The samples were stored at 25, 4, -20 and -80°C and they were analysed after 12 months from the date of storage.

Chemical Reagents

All solvents and chemical standards used were of a high purity grade and were procured from Fluka and Sigma–Aldrich.

Extraction and quantitation of total phenols

The olive oil polyphenol fraction was isolated by liquid-liquid extraction, according to Montedoro *et al.* forty ml of methanol/water mixture (80:20 v/v) was added directly, twice to 100 gr of each olive oil sample.[16] The mixture was suspended with a vortex at 5000 *g* for 15 min and centrifuged at 5000 *g* for 10 min. The supernatants were then collected and concentrated by a rotary evaporator at room temperature until a syrupy consistency was obtained. The Folin and Ciocalteu method was used for determining the phenols and the results were expressed in terms of mg GAE/100 g of olive oil.[17]

Quality and Oxidation Indices

FFA (as oleic acid %), as well as conjugated trienes (K_{270}) and dienes (K_{232}) and ΔK were estimated according to the analytical methods described by the ECR No. 2568/91 and subsequent amendments.[18] UV absorbance was collected in 232, 266, 270 and 274 nm by using UV Spectrophotometer (Shimadzu-UV-1601, Japan) and ΔK values were calculated with the following equation (1):

$$\Delta K = K_{270} - \frac{K_{266} - K_{274}}{2} \quad (1)$$

Additionally, the late stage of lipid peroxidation in olive oil was determined by measuring its MDA, as described in previously reported methodology.[19,20] MDA is a product of lipid peroxidation and has been used as a bio-marker of oxidative stress.[21] The main endogenous production of MDA arises from the oxidation of polyunsaturated fatty acids with more than two methylene-interrupted double bonds.[22] In order to determine the MDA in olive oil, 0.5 ml samples were vortexed with an equal volume of double distilled water, followed by centrifugation at 4000 *g* for 5 min at room temperature. MDA was fluorometrically measured in 0-150 μl of the resulting aqueous supernatant.

Statistical analysis

Statistical analysis was performed with SPSS statistical software and used the One-way Anova method. All results were expressed as mean ± standard deviation (SD) of five independent experiments and differences among all groups were determined by *F*- test ($p<0.05$).

RESULTS

Chemical analysis

The determination of QIs: FFA, K_{270}, K_{232} and ΔK UV-absorption values were conducted according to ECR No. No. 2568/91 and the results are summarised in Table 1 and Table 2 for fresh olive oil and long-time storage olive oil respectively.[18]

In particular, Table 1 indicates that the fresh olive oil has a very low concentration in FFA (namely; 0.22 ± 0.12 < 0.80), low UV-absorption values; K_{270}, K_{232} and ΔK (namely, K_{270}=0.110 ± 0.002 < 0.220, K_{232}=0.150 ± 0.003 < 2.500 and ΔK=0.001 ± 0.001< 0.010) and according to ECR No. 2568/91 the above olive oil is classified as EVOO.[18]

In accordance with Table 2, it was observed 12 months after the storage of the products that all the QIs in both the degassed and non-degassed samples that are stored at -80°C (Deep Freeze), maintain very low values that are almost identical to those for fresh olive oil. However, upon increasing the temperature in the non-degassed samples of the olive oil all of the aforementioned QIs exhibit an increasing trend in relation to their corresponding values in the degassed samples of the olive oil. We have furthermore noted that in the non-degassed samples of the olive oil, stored at 25°C (room temperature), all of the aforementioned QIs exceed the limits set by ECR No. 2568/91, where by the olive oil under these storage conditions is not classified as EVOO.[18] On the other hand, in all the degassed samples that are stored at 25°C, all of the QIs exhibit a minor increase within the limits set by ECR No. 2568/91 and the olive oil continues to be classified as EVOO.[18] These results are in agreement with a recent study of Smeriglio *et al*. that demonstrated that the nitrogen headspace improves the EVOO shelf-life, by preserving its functional properties.[17]

Concentration of the MDA as a product of lipid peroxidation of olive oil.

Table 1, (see 4th QI) shows that the fresh olive oil has a low concentration of MDA, namely 126 ± 9 nmoles/ 1 L olive oil. After 12 months of storing the samples (Table 2, 4th-QI), the MDA concentration in the degassed olive oil samples that were at a temperature of -80°C (deep freeze) the MDA concentration retained approximately the same value as that for the fresh olive oil, whilst the MDA concentration is slightly increased in the non-degassed samples that were stored at the hereinabove temperature. This may be attributed to the oxidisation of the lipids during the transfer of the samples from the -80°C under measurement conditions. Upon increasing the temperature to 4°C, the MDA concentration in the degassed and non-degassed samples exhibits almost the same increasing trend. Conversely, at 25°C the production of MDA in the non-degassed samples (220 ± 15) is much higher in relation to the corresponding production in the degassed samples (196 ± 16). In accordance with the bibliographic citations, this result is attributed to the reactive oxygen species (ROS) and mainly O_2 in the aquatic phase causing lipid peroxidation.[1,2]

Total phenolic content of olive oil

According to Table 1 (3rd OI), fresh olive oil has a high concentration of TPC, namely; 85.15 ± 0.12 mg GAE/100 g of produced olive oil. This result is in accordance with the research by Vekiari *et al*. who calculated the TPC for olive oil of the Koroneiki variety from the same region as being between 62 and 120 mg GAE/100g of olive oil, according to the period of harvesting.[15] After 12 months of storage at a temperature of -80°C (deep freeze), in both the degassed as well as the non-degassed olive oil, the concentration of TPC maintained the same value as that for fresh olive oil. On the other hand, upon increasing the temperature to 25°C, it was observed that in the non-degassed samples of olive oil the concentration of TPC exhibits a higher trend of reduction (73.78 ± 0.09 mg GAE/100 g of olive oil) as opposed to the degassed samples. This outcome is in accordance with other bibliographic references and is attributed to the presence of ROS in the solution.[1,2] In particular, this result is attributed to the presence of O_2 in the aquatic phase of the olive oil, which appears to significantly contribute to the rate of reduction of the TPC in the olive oil, which is 13% at a temperature of 25°C in the

Table 1: Quality Indexes (QIs) values of fresh olive oil. The values are expressed as mean ± SD of five determinations (*n* = 5).

Storage conditions		Physicochemical Indexes (UV-absorption values)					Biochemical Index
		1st	2nd			3rd	4th
Treatment (with a purge N_2)	Temperature (°C)	FFA (% of oleic acid)	K_{270} Conjugated trienes	K_{232} Conjugated dienes	ΔK	TPC (mg GAE/100 g Olive oil)	MDA (nmoles MDA/ 1 L Olive oil)
No	Room Temperature	0.22 ± 0.12	0.110 ± 0.002	0.150 ± 0.003	0.001 ± 0.001	85.15 ± 0.12	126 ± 9
limits by ECR No. 2568/91 (Ref.18)		0.8	0.22	2.5	0.010	-	-

Table 2: Variations of QIs values of storage olive oil with and without a nitrogen purge at deferent storage conditions for 12 months. Data are expressed as mean value ± SD of five determinations (*n* = 5).

Storage conditions		Physicochemical Indexes (UV-absorption values)					Biochemical Index
		1st	2nd			3rd	4th
Treatment	Temperature (°C)	FFA (% of oleic acid)	K_{270} Conjugated trienes	K_{232} Conjugated dienes	ΔK	TPC (mg GAE / 100 g Olive oil)	MDA (nmoles MDA/ 1 L Olive oil)
without a purge N_2	25	1.22 ± 0.09c	0.245 ± 0.002a	2.477 ± 0.003c	0.018 ± 0.002c	73.78 ± 0.09a	220 ± 15
	4	0.71 ± 0.12b	0.198 ± 0.002a	2.355 ± 0.001b	0.014 ± 0.003b	79.34 ± 0.15a	158 ± 13
	-20	0.45 ± 0.13a	0.150 ± 0.001b	1.816 ± 0.002c	0.004 ± 0.001a	81.22 ± 0.07b	138 ± 9
	-80	0.25 ± 0.10a	0.117 ± 0.001b	1.602 ± 0.001b	0.001 ± 0.001c	84.48 ± 0.10c	134 ± 12
with a purge N_2	25	0.42 ± 0.11a	0.156 ± 0.003a	1.834 ± 0.002a	0.001 ± 0.001b	81.61 ± 0.11a	196 ± 16
	4	0.37 ± 0.05b	0.143 ± 0.001c	1.753 ± 0.002a	0.002 ± 0.001a	83.34 ± 0.12b	154 ± 12
	-20	0.29 ± 0.17a	0.128 ± 0.002a	1.703 ± 0.001c	0.002 ± 0.001c	83.98 ± 0.08c	128 ± 11
	-80	0.23±0.13c	0.116 ± 0.001c	1.601 ± 0.001c	0.003 ± 0.001b	84.08 ± 0.13a	127 ± 12

Different superscript letters in the same column indicate significant difference between mean values. All values were determined by F-test (p<0.05).

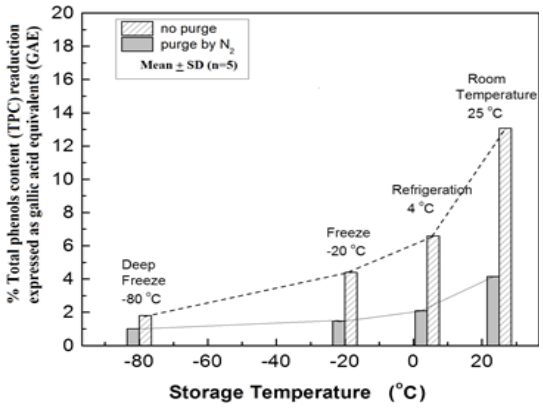

Figure 1: Total phenols reduction vs temperature and O_2 storage conditions.

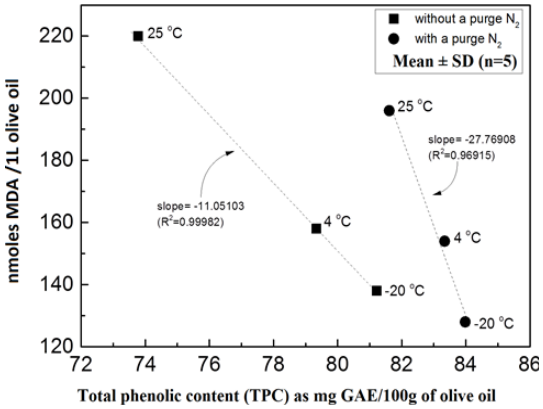

Figure 2: Production of MDA (as lipid peroxidation products) against the TPC reduction at different storage temperatures, (■) without a purge N_2 and (●) with a purge N_2.Where, TPC as mg GAE/100 g of olive oil and MDA at nmoles per 1 ml olive oil.

non-degassed samples, in accordance with Figure 1. Conversely, in the degassed sample of olive oil that have been stored at 25°C, the reduction in the TPC is very low, namely 4.5% (Figure 1).

Thermodynamic stabilisation of the EVOO quality

Figure 2 shows the production of MDA against the TPC reduction at different storage temperatures.

According to Figure 2 the production of MDA (lipid peroxidation product) against the TPC decrease at temperatures between -20 and 25°C exhibits a different linear regression. Specifically, as the temperature is increased from -20 to 25°C, the rate of MDA production versus TPC decrease, as a slope $\Delta C_{MDA}/\Delta C_{TPC}$ of the regression line, is -11.05103 (R^2=0.99982) in the non-degassed and -27.76908 (R^2=0.96915) in the degassed olive oil samples (Figure 2). More specifically, this result indicates that as the temperature is increased approximately double the quantity of TPC is expended in the non-degassed samples as opposed to the degassed samples for the same MDA production. This is an indication that the O_2 in the aqueous phase has a greater affinity to the functional groups of the total phenols in relation to those for the lipids. According to this hypothesis the, LH-ROH phenols create a diffuse double layer in interface between the aqueous phase containing the oxidizing agent and the hydrophobic phase protecting lipids from their oxidation (Figure 3).

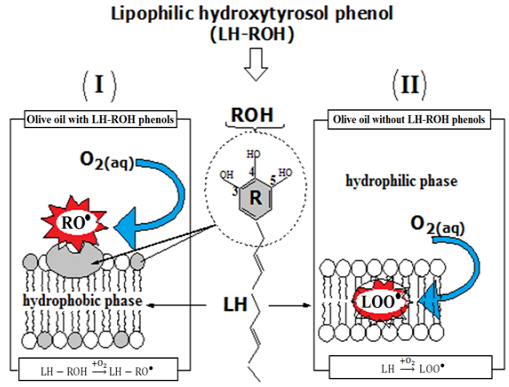

Figure 3: Schematic mechanism of the LH-POH free radical scavenging activity as a lipid-protective agent in EVOO.

DISCUSSION

The phenolic charge in EVOO is very sensitive to interfacial physicochemical reaction, e.g. at [air-oil] and [oil-water] interfaces, whilst its storage conditions are very important for avoiding or reducing the negative effects of autoxidation on the qualitative characteristics of the packaged product. According to the above presented results, the storage of olive oil for 12 months at a temperature of 25, 4, -20 and -80°C indicated that a well degassed olive oil by N_2 purging retains the quality indicators FFA, UV-absorption values; K_{272}, K_{232} and ΔK for the EVOO, according to the EEC Regulation, even at a storage temperature of 25°C (room temperature).[18] Conversely, the presence of ROS and mainly O_2 in the aquatic phase of the olive oil increased the values of the QIs (Table 2) and at a storage temperature of 25°C all of the aforementioned indicators (QIs) exceed the limits imposed by ECR No. 2568/9112 that classified the olive oil as EVOO.[18]

Recently, Giannnakopoulos *et al.* through a combination spectroscopic study of EPR and UV/Vis spectra demonstrated that the polyphenols bearing hydroxyl groups –OH at positions 3, 4 and 5 of the benzene ring (gallic acid type ring) which are known as LH-ROH phenolic molecules, have a high free radical activity that is activated by the presence of O_2 in the solution.[23] This leads to the creation, in accordance with the herein below mechanism (equation 2), of a free radical RO•, type gallic acid, with the unpaired electron being delocalized on the aromatic ring of the LH-ROH phenols.

$$\text{LH-ROH} \xrightarrow{+O_2} \text{LH-RO}^{\bullet} \qquad (2)$$

This mechanism is spectroscopically confirmed by the reduction in the absorption at 280 nm.[23] Furthermore, according to Figure 1, we have observed that at a temperature of 4°C the phenolic load in the non-degassed olive oil was decreased to one-half, namely 6.5%, by exhibiting a further reduction trend as the storage temperature is decreased. On the other hand, it appears in the degassed olive oil samples at temperatures less than 4°C that the decrease in the TPC is insignificant (within the limits of statistical error) and the TPC appears the thermodynamically as being stabilised. According to Giannakopoulos *et al.* this thermodynamic stabilisation in the phenolic load at temperatures less than 12°C may be attributed to the strong adsorption of phenolic molecules of the Gallic acid type, with hydrophobic surfaces through an enthalpy adsorption mechanism.[24] Moreover, the De convolution analysis of the aforementioned absorption indicated that only molecules that have hydroxyl groups –OH at position 3, 4 and 5 in the benzene ring (type LH-ROH phenols) can bond strongly with the hydrophobic surface by

directing the level of the ring perpendicular to the hydrophobic surface and to the aqueous phase.[24] On the other hand, as the temperature increases, the interaction of the phenolic molecules with the hydrophobic surface weakens and is effected via an entropy adsorption mechanism, where by the equilibrium of the reaction shifts to the hydrophilic phase.

On the basis of the hereinabove and in light of the findings by this research, the stabilisation of the TPC in the olive oil at a low temperature of ~4°C is due to the thermodynamic stabilisation of the LH-ROH macro-molecules in the olive oil that direct their hydrophobic chain (LH) to the hydrophobic phase of the lipids with which they bind strongly protecting the lipids from the oxidation (Figure 3, I).[25]

In the case of the presence of O_2 and a low concentration of LH-ROH in the olive oil, the oxidation of the lipids proceeds in two steps according to the following mechanism (equations 3, 4), producing free radical lipid peroxidation LOO• according to equation (4) (see Figure 3, II).

$$1^{st}\ \text{step:}\quad \text{LH-ROH:} \xrightarrow{+O_2} \text{LH-RO}^{\bullet} \qquad (3)$$

$$2^{nd}\ \text{step:}\quad \text{L'H:} \xrightarrow{+O_2} \text{L'OO}^{\bullet} \qquad (4)$$

Additionally, where the storage temperature of the EVOO is low (e.g. refrigeration conditions) and the LH-ROH phenols have a large LH, then the molecules with the hydrophobic surface develop a stronger bond (Figure 3), effectively protecting the lipids from the ROS. In conclusion, at a storage temperature of ~4°C (refrigeration conditions) an interface composed of LH-ROH phenol molecules with strong radical activity (of the type of free radical scavenging activity of gallic acid) binds around the hydrophobic phase of the lipids, which can be preserved for a long period in excess of 12 months, protecting the lipids from oxidation, in accordance with the proposed mechanism that is described in Figure 3.

CONCLUSION

Today more than ever, EVOO is increasingly considered to be the main ingredient of a well-balanced diet, specifically the Mediterranean diet. It has been demonstrated that EVOO has antioxidant effects which relate to protecting blood lipids due to its fatty acid composition and the presence of bioactive compounds, mainly type LH-ROH phenols with radical scavenging activity. These molecules are identified in the interface between the hydrophobic and hydrophilic phase of the EVOO, [oil-H_2O], by directing the hydrophilic section of the hydroxyl groups –OH in relation to the hydrophilic phase and the lipid chain to the hydrophobic phase. The present study demonstrated that the storage of olive oil in the absence of O_2 at temperatures of ~4°C (refrigeration conditions) thermodynamically stabilizes the TPC and simultaneously protects the EVOO lipids against peroxidation from the ROS for a period greater than 1 year. Finally, the optimum long-term storage conditions for EVOO as a long-life EVOO product with high added value is ensured by the high concentration of LH-ROH phenols in the olive oil in combination with its degassing (by an inert gas) and its storage at refrigeration conditions of ~ 4°C.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

ABBREVIATIONS

EVOO: Extra Virgin Olive Oil; **ECR:** European Community Regulation; **LH-ROH: hydroxytyrosol**; **QIs: Quality Indexes; MDA:** free malondialdehyde; **GAE:** Gallic acid equivalent; **ROS:** reactive oxygen species; **TPC:** total phenolic content; **RO•:** free radicals; **LH:** hydrophobic chain.

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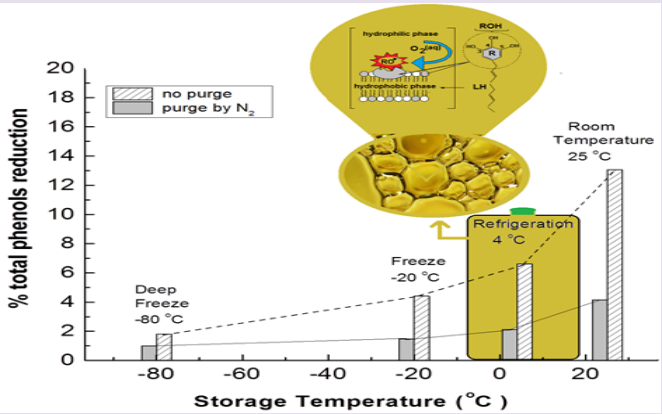
GRAPHICAL ABSTRACT



SUMMARY

- Development and evaluation a freeze controlled treatment for the long-term preservation of EVOO quality (total phenolic content and antioxidant activity)
- Understanding of physicochemical mechanism and factors determining EVOO quality by free radical mechanism.
- Lipophilic hydroxytyrosol phenols protect EVOO lipids against peroxidation.

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