Variations of Phenols and Squalene in Evoos under Different Conditions

Abstract
Analysis by HPLC indicates that the variation of most of phenols in EVOOs is very complicated according to different oil matrixes. Most of them are in decreases with the increase of oxidation degree under different conditions. While a few of phenols show increase under darkness at room temperature for 14 weeks, vanillin increasing by 52%, quercetin increasing by 21% and chrysin increasing by 42% in EVOO3, which happened to Compound 1 in EVOO2 and EVOO3 under similar circumstances. These substances, including Compounds 1, 2, 3 and 4, are inferred to be phenols or their derivatives. Rancimat and oven tests have proved that hydroxytyrosol has strong antioxidant activity but squalene and tyrosol do not have. The results however show squalene can retard oxidation of EVOOs under light radiation. HPLC-MS analysis indicates that during the experiments squalene monohydroperoxides are formed and increased to 5-10 times as the beginning, which is about 4 times as much as that in EVOOs stored in darkness for the same period of time.

Keywords: Light radiation; Air exposure; Extra virgin olive oil; Phenols; Squalene

Xinchu Weng1,2,* , Sheng Luo2 and Yu Song2
1 School of Food Engineering, Qinzhou University, 12 Binghai Avenue, Qinzhou 535011, Guangxi, China
2 School of Life Sciences, Shanghai University, Shanghai 200444, China

*Corresponding author: Xinchu Weng
E-mail: wxch@staff.shu.edu.cn (X.W)
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Introduction

Known as the nutritional vegetable oil, olive oil has been entitled as one of the four woody plant oils together with tea oil, coconut oil and palm oil. Its famous reputation could date back to almost 6000 years ago around the Mediterranean area where there is a lower prevalence for cardiovascular disease. It turns out that olive oil possesses high nutritional value and beneficial therapeutic properties including antioxidant and anti-inflammatory ability with the existence of a high level of monounsaturated fatty acids and various minor components [1-4]. Moreover, olive oil has been involved in the bioactivities of anti-cancer and anti-aging [1,5].

In the Asian region, most people consume animal oils and vegetable oils, like lard, beef tallow, rapeseed oil, soybean oil and peanut oil. Animal oils containing high amount of saturated fatty acids but little antioxidant substances are conducive to blood vessel diseases and other diseases [6-8]. In comparison to soybean oil and peanut oil and other regular table oils, olive oil has stronger oxidation stability because of two main reasons: its manufacturing technique and its composition. Regardless of the optimum ratio of monounsaturated fatty acids to polyunsaturated fatty acids, the minor components in olive oil, only accounting for 1-2% of all, also play an unexceptional role in protection of it from oxidation [4,9-14].

There are lots of researches about phenols in olive oil, whose content can be strongly influenced by olive variety, location, environmental conditions, maturation degree and production procedure [5,9]. Tocopherols, with around 95% of α-tocopherol (αT) and 5% of other tocopherols found in olive oil, offer up certain antioxidant properties for virgin olive oil (VOO) [1,6,9,10,15]. However, the amounts of other antioxidant compounds are relatively high in olive oil [6,7,9,10]. For instance, there are various phenols, including phenolic acids and their derivatives, phenolic alcohols, flavones, secoiridoids, hydroxyl-isocromans and lignans, found in a high level in olive oil [5,8,11,14]. They can not only extend the shelf life of olive oil and prevent oxidation reactions but contribute to the satisfactory organoleptic characteristics of olive oil as well [3,4,7]. So these phenols also have been considered as an important factor when evaluating the quality of olive oil [3].

The phenols in olive oil are a very complex group and only some of them have been determined and reported in olive oil, they are: hydroxybenzoic acid, vanillic acid (VA), caffeic acid (CA), p-coumaric acid (PCA), m-coumaric acid, syringic acid, gallic acid (GA), sinapic acid, ferulic acid, cinnamic acid (CNA), protocatechuic acid, rutin, hesperidin, quercetin, luteolin (LT), apigenin (Apig), taxifolin, tyrosol (TY), hydroxytyrosol (HTTY), oleuropein, 3,4-dihydroxyphenylglycol, oleuropein aglycon, ligstroside, ligstroside aglycone, 1-acetoxypinoresinol, pinosanol, dialdehydic aglycone of oleuropein glucoside lacking a carboxymethyl group, dialdehydic aglycone of ligstroside lacking a carboxymethyl group [2,6,11-21]. HTTY, TY and oleuropein and their derivatives are the most major antioxidants in VOO, and oleuropein is the main donor to the unique flavor of olive oil [6].

Except for phenols, the rich abundance of squalene (SQ) is also the reason why olive oil is more and more favored. The health benefits of SQ have attracted a great deal of attention. VOO is a major source of SQ, with a content ranging from 100 to 1200 mg/100 g oil depending on the olive cultivar [22,23]. More and more people believe that SQ is partially responsible for the protective effects of olive oil against certain cancers [23]. It has been reported that SQ might induce the regeneration of αT in photo-oxidation process, and it shows a synergistic influence on αT and β-sitosterol, reducing the oxidation rate [24].

The research interest of phenols and SQ and other minor compounds [6] in olive oil was mostly concentrated on detecting their content, variety and health function. Some researchers notice the effects of the changes of storage conditions on olive oil quality during storage. Méndez et al. [25] studied the effect of five types of containers on the deterioration in quality of EVOO. Pristouri et al. [26] studied the effects of packaging material headspace, oxygen and light transmission, temperature, and storage time on the quality of EVOO. The quality of EVOO was assessed by the determination of acietyd, peroxide value (PV), absorption coefficients K_270 and K_230, Percentage of humidity, impurity content and other regular indexes mostly. Some researchers have further studied the effect of storage temperature or light transmission on some minor compounds in VOO, regardless of the determination of regular indexes, for example, Kalua et al. [27] studied the changes of volatile substances and oleuropein in VOO during low temperature storage for 3 weeks for fruit and they found that oleuropein declined during the experiment. They also studied the changes of a few phenols (hydroxytyrosol, tyrosol, ligstroside dialdehyde, oleuropein aglycon and (+)-acetoxypinoresinol) in VOO stored in different conditions (darkness and direct sunlight) during 12 months [28]. Cicerale et al. [29] studied the effects of light and oxygen transmission on oleocanthal content in EVOO stored in 10 months, and they come to a conclusion that light and oxygen transmission seriously cause the decline of oleocanthal in EVOO, but increase the content of phenolic alcohols. Babola et al. [30] also studied the effect of temperature on the quality of VOO during storage for 12 months. They detected the regular indexes of free fatty acids, PV, K_270 and K_230. In addition, total phenols, total ketones and volatile substances have been studied. Zeb et al. [31] did some work on the effect of temperature, UV, sun and white lights on the stability of olive oil. Except for some regular indexes, they found the carotene value showed a considerable variation in olive oil. Makni et al. [32] made a comparison between olive oil and soybean oil on the changes of physico-chemical properties, composition and oxidative stability under the storage with natural light or not. They added chlorophylls content, DPPH scavenging ability and total antioxidant activity into the assessment of oil quality. Moreover, the addition of vitamin E as an extra antioxidant was also regarded as a variable to assess its effect on the quality of oils. The variation of αT in sunflower oil under different storage conditions has been studied [33], which indicates light transmission other than temperature has much higher effects on tocopherols, especially for αT. Rigane et al. [34] put chlorophylls and SQ in refined olive oil and determined the effect of temperature on its quality, including the changes of chlorophylls, SQ and total phenols and found SQ could inhibit refined olive oil from thermal deterioration by improving its hydrolytic stability, inhibiting double bond conjugation and reducing the formation of second products.
of oxidation. Rastrelli et al. [35] found SQ was not degraded until \( \alpha_T \) was oxidized to certain content so that SQ could not be protected by \( \alpha_T \). However, few researches referred to the variation process of SQ during the storage.

This article mainly studies under different conditions (exposure to light, thermal treatment and continuous air flow), the changes of SQ and phenols in EVOO, providing a certainty of guidance to assess the quality of EVOO. A second objective was to determine the antioxidant activity of HYTY, TY and SQ on oil under different conditions.

**Materials and Methods**

**Chemicals and materials**

Commercial standard of SQ and \( \alpha_T \) were purchased from Sigma-Aldrich Corporation (USA). The phenols standard of GA, TY, CA, Pca, FA, VA, CNA, LT, Apig, quercetin (Qu), kaempferol (Ka), chrysin (Chr) and 4-methylcetehol were all purchased from Aladdin Industrial Inc. (Shanghai, China).

Acetonitrile, isopropanol, dichloromethane, methanol, n-hexane (HPLC grade) used for extraction and mobile phase preparation and other organic solvents (Analytical grade), such as alcohol, petroleum ether, anhydrous diethyl ether, glacial acetic acid, chloroform were purchased from Sinopharm Group Co. LTD. (Shanghai, China). Ultrapure water was obtained from a Milli-Q Gradient water system (Millipore, Bedford, MA, USA).

**Preparation of stock solution and EVOO samples**

Stock standard solutions of SQ and \( \alpha_T \) were respectively prepared by exact weighing of the appropriate amounts and then dissolved in ethanol. The stock solution of each of phenolic compounds was respectively prepared by exact weighing of the appropriate powder and dissolved in methanol. All of these solutions were prepared before using and stored at -20°C in darkness.

EVOO1, EVOO2 and EVOO3 were purchased from Walmart supermarket located in Shanghai, China in 1 L package, and they were subpackaged into several clean, dry, transparent 15 mL plastic bottles sealed with 5% headspace and were stored at room temperature (25 ± 3°C), the bottles covered with aluminum foil. To study the variation of phenols and SQ in EVOOs under different conditions, part of EVOO1, EVOO2 and EVOO3 were exposed to natural light and were extracted for phenols and SQ to analyze at 1, 3, 6, 10, 14 weeks, part of EVOO1, EVOO2 were heated at 100°C under continuous air flow (20 L/h) in a Rancimat 617 apparatus (Metrohm, Herisau, Switzerland), and EVOO3 were heated at 100°C under continuous air flow (20 L/h) in a Rancimat 617 apparatus (Metrohm, Herisau, Switzerland), and were extracted for phenols and SQ to analyze every 7 hours until they were oxidized to induction period (IP). The phenols and SQ were extracted from EVOOs according to methods of Reboredo-Rodriguez et al. [8] and Lu et al. [36].

**Determination of total phenol and peroxide value (PV)**

Total phenol was determined according to the Folin-Ciocalteu colorimetric method of Alu’datt et al. [17]. The result of the total phenol was expressed as mg of GA per kg of oil. And PV was determined according to the official EU method 2568/91.

**Oven tests**

To study the antioxidant activity of HYTY, TY and SQ on oil, 0.02% of these three compounds were added into lard samples, respectively, and kept at 65 ± 1°C in an oven. PV of TY and SQ were determined every day and PV of HYTY was determined every five days. HYTY was replaced by 4-methylcetehol.

**Rancimat test**

HYTY, TY and SQ were also tested on the Rancimat at 100°C under continuous air flow (20 L/h). HYTY was replaced by 4-methylcetehol.

**High performance chromatography (HPLC)**

The methods used for preparation and analysis of phenols and SQ in EVOOs were referred by Brabcova et al. (36) and Lu et al. [37].

The liquid chromatographic system used for determination of phenols and SQ in EVOOs was an Agilent 1100 Technologies system. Chromatographic separations were performed with a ZORBAX Eclipse XDB-C18 analytical column, 4.6 x 250 mm ID, 5 μm particle size, purchased from Agilent Technologies corporation, LTD. The temperature of the HPLC column was kept at 30°C. A mixture of acetonitrile-ultrapure water (92:8) was performed in gradient elution to detect the phenols in EVOOs, at a flow rate of 1 mL/min. For the detection of SQ, acetonitrile was used as the mobile phase of isocratic elution at a flow rate of 1.5 mL/min. The injection volume was set to 5 μL. The phenols and SQ extracted from EVOOs were monitored with a VWD detector at a wavelength of 278 and 210 nm, respectively.

**HPLC-MS analysis**

The determination of the molecular mass of SQ and its co-elution substance was performed by HPLC coupled with mass spectrometer Agilent 1200 using analytic column: ZORBAX Eclipse SB-C18 4.6 x 250 mm ID, 5 μm particle size (Agilent Technologies corporation, LTD). The injection volume was 10 μL. Acetonitrile was used as the mobile phase of isocratic elution at a flow rate of 1 mL/min. The experiment was analyzed by direct infusion electrospray ionization-mass spectrometry (ESI-MS) and flow injection atmospheric pressure chemical ionization-MS (APCI-MS) after dilution with ACN.

ESI-MS analysis was run in positive ion mode with data collected between 200 and 1000 amu. Optimized MS condition was: Gas Temperature: 340°C; Gas flow: 11 L/min; Nebulizer: 36 psi; Sheath Gas Temperature: 350°C; Sheath Gas flow: 11 L/min; Capillary Voltage: 3500 V; Nozzle Voltage: 500 V.

APCI-MS analysis was run in positive ion mode. Optimized MS condition was: Nebulizer: 60 psi; Gas Temperature: 350°C; Gas flow: 4 L/min; Vaporization Temperature: 350°C; Capillary Voltage: 3500 V; Corona Current: 20 μA (neg).

**Results and Discussion**

**Variation of total phenols in EVOOs under different conditions**

Reactions resulting in an increase of PV are autoxidation and photo-oxidation that are coherent. Pристори et al. [26] have proved that light radiation, temperature and oxygen availability...
are three major reasons to speed up the oxidation of EVOOs. Light radiation has the biggest effect on olive oil [32], which has been further confirmed in this article again. The tendency in PV changes of EVOO samples stored in darkness and EVOO samples exposed to natural light for different periods was observed (Figure 1), the former increasing very gently with slight increases of 3.0 ± 0.3 meq/kg, 3.2 ± 0.3 meq/kg and 5.0 ± 0.3 meq/kg and the latter presenting a significant rise of 52.7 ± 0.3 meq/kg, 58.5 ± 0.3 meq/kg and 53.7 ± 0.6 meq/kg in EVOO1, EVOO2 and EVOO3, respectively, after 14 week. During this process, EVOOs have antioxidant compounds to protect themselves from oxidation. According to Mohamed Makni et al. [32], the contribution of phenols in Virgin olive oil (VOO) to confer an effective defense system against free radicals attack is about 30%, better than 11% by αT.

The changes of total phenols extracted from the EVOO samples under darkness (Figure S2 (II)) and exposure to natural light (Figure S2 (III)) have been described. The initial contents of total phenols were 376.4 ± 23.3, 450.3 ± 21.7 and 382.6 ± 36.7 mg/kg in EVOO1, EVOO2 and EVOO3, respectively. After 3 weeks, obvious decreases appeared to EVOO1, EVOO2 and EVOO3, which were 314.8 ± 43.7, 333.3 ± 37.5, 314.8 ± 37.5 mg/kg in these three EVOO samples stored in darkness and were 314.8 ± 31.4, 321.0 ± 25.2, 277.8 ± 37.5 mg/kg in EVOOs exposed to light, respectively. Since then, the total phenols kept very slightly decreasing in EVOO2 and EVOO3 no matter if they were exposed to light or not for the last 11 weeks. For EVOO1, the decrease was a little bit more obvious, which decreased to 259.3 ± 25.2 mg/kg in EVOOs stored in darkness and 200.0 ± 23.7 mg/kg in EVOOs exposed to light after 6 weeks in storage. It can be observed that total phenols decreased in almost the same way and at the same speed in different conditions, which was obtained the same results in the study by Méndez et al. [25]. These changes may be associated with the composition of phenols in EVOOs. The initial decreasing is caused from the oxidation of some phenols with good antioxidant activity, like αT and some phenolic acids. Phenolic alcohols in EVOO take a big proportion of phenols so that they have strong influences on the changes of total phenol and quality in EVOOs.

**Variation of phenols in EVOOs under different conditions**

TY and HYTY occupy a large proportion of phenols in EVOOs. The results from the study of Kalua et al. [28] show the changes of HYTY and TY under exposure to light during 1 year for storage. During their experiments, HYTY in VOO which was stored in darkness and exposed to light both decreased from 25 ± 2 to 21.2 ± 0.9 mg/g, TY in VOO decreased from 35 ± 3 to 29 ± 2 mg/g and to 31 ± 2 mg/g when the condition of VOO were light storage and dark storage, respectively. It costs 14 weeks to study the changes of phenols in this article and the similar results have been obtained. The content of TY had an upper and lower variation in three EVOO samples no matter they were exposed to light or were stored in darkness (Figure S5(II) in A, B and C), which shows that TY was not disposed of anti-oxidation. In EVOO1, HYTY decreased by 18% in the condition of darkness and decreased by 20% in the exposure of light. In EVOO2, HYTY decreased by 20% in the condition of darkness and decreased by 27% in the exposure of light. These changes indicate that HYTY takes responsibility to inhibit the oxidation of EVOO, including against autooxidation under the darkness and the oxidation catalyzed by light radiation. In EVOO3 under the condition of darkness, it came to a slight increase of 6% (Figure S5C (I)). This is possibly caused by hydrolysis of ligstroside and oleuropein compounds, which in turn act as a substrate for the formation of such phenolic alcohols [17].

EVOOs were pre-treated by Rancimat to study the effect of air exposure on the changes in oil. EVOO1 and EVOO2 were heated at 100°C under a continuous air flow (20 L/h) in the Rancimat. 2 Grams of oil sample was collected and was extracted for phenols and SQ every 7 hours, the last one done after 1 hour of the induction period (IP). IPs of EVOO1 and EVOO2 were 56 and 49 hours, respectively. The changes of HYTY and TY were given in Figure 5 and the results show that TY declined a lot suddenly when it exceeded IPs (56 hours in EVOO1 and 49 hours in EVOO2), indicating that TY is not oxidized until other antioxidant compounds being consumed entirely. However, HYTY in EVOO1 and EVOO2 decreased continuously and got lost before IP ended (14 hours). This change of HYTY shows its antioxidant activity, but it is not very strong. The antioxidant activities of HYTY and TY have been investigated by Rancimat tests and oven tests in section 3.4.

For other phenols in EVOO, most kinds of phenols were in continuous decreases as the extension of oxidation degree. The decrease rate of these phenols in EVOO exposed to light was faster than that in EVOOs stored in darkness. However, VA increased by 52% (Figure S5C (IV)), Qu increased by 21% (Figure S5C (VII)) and Chr increased by 42% (Figure S5C (VIII)) during the experiment when EVOO3 was stored in darkness for how long? The results can infer that VA, Qu and Chr were possibly the products hydrolyzed from ligstroside and oleuropein compounds.

Plenty of substances were extracted together with the phenols from EVOOs, and four peaks attracted our attention, which were marked as Compounds 1, 2, 3, and 4 (Figure S4). These four compounds had a medium chemical polarity based on their retention time in the HPLC chromatograph. Compound 1 in EVOO2 and EVOO3 had an initial surge of 106% and 53%, respectively, followed by a continuous decrease until the end of experiment (Figure S5(X) in B and C). The content of Compound 1 decreased faster in EVOO3 (58.85%) than that in...
EVOO2 (48.2%). However, a continuous increase of Compound 1 occurred when EVOOs were stored in darkness with 113% of increase in EVOO2 and 25% of increase in EVOO3.

Compound 3 in EVOO1 had the similar circumstance to Compound 1. Under the exposure to natural light, the content of Compound 3 increased to 66% at the first week of storage and then decreased slowly during the following 13 weeks. Under the condition of darkness, the content of Compound 3 increased by 66% (Figure S5A (XII)). The content of Compound 1 and Compound 3 increased from the beginning of oxidation, so in a lot of chances these two substances are the consequences of photo-oxidation. The reaction rates were different due to different EVOO matrixes. The increase rate (under dark storage) of Compound 1 was as fast as its decrease rate (under exposure to light) in EVOO2, while this comparison was bias to the latter in EVOO3. This shows that the formation rate and oxidation rate of Compound 1 are almost identical in EVOO2, but the formation rate of Compound 1 is slower than its oxidation rate in EVOO3, which indicates that light radiation has serious effects of oxidation on Compound 1. For Compound 3 in EVOO1, it has a higher increase rate (under dark storage) than decrease rate (under exposure to light), showing that its formation is quicker than its oxidation. This demonstrates that light radiation can only cause the oxidation and degradation of Compound 3 but even promote its formation since its oxidation rate is slow under the exposure to light.

Compound 2 also had an increase at the first week of storage before it decreased, which occurred mostly in the condition of darkness (Figure S5(XI) in A, B, C). It was increased by 28% and 25% in EVOO2 and EVOO3, respectively, during the storage for one week. Then the content of Compound 2 kept decreasing, by 38% in EVOO2 and 34% in EVOO3. Compound 2 had a little change (data not given) at the first week in EVOO1 stored in darkness, while it is very interesting that there was an almost entire consumption before 6th week and it was consumed even faster in 3 weeks when it came to the exposure to light (Figures S4B and S5A (XI)). Compound 2 in EVOO2 and EVOO3 exposed to light decreased by 29% and 31%, respectively (Figure S5(XI) in B and C). Just like most of phenols, Compound 4 in EVOO1 decreased by 47% in the condition of darkness and 39% in the exposure of light (Figure S5A, XIII)).

These four kinds of substances are waiting for certain identification. It can be speculated that these four substances either belong to phenols or the degradation products formed by phenols or other substances. The contents of these four substances dropped off as the oxidation degree increased, indicating they should be under some reactions, like anti-oxidation.

**Variation of SQ in EVOOs under different conditions**

SQ is a typical substance in olive oil. However, few research studies the variation of SQ during the storage. The description of SQ in EVOOs was given in Figure 1. The results show that high content of SQ, together with tiny amount of unknown compound, was extracted from EVOOs in low degree of PV, while high content of unknown compound and tiny SQ were

![Figure 2: Changes of SQ and unknown compounds extracted from EVOO samples in different storage conditions. A, B and C were the EVOOs stored in darkness at room temperature; D, E and F were the EVOOs exposed to natural light at room temperature.](image-url)
Figure 3: HPLC-MS chromatograms of SQ (A and C) and unknown compounds (B and D). A and B used ESI+ as ionization source: 449.30 = [SQ + K]+; 443.10 = SQ mono-OOH; 465.40 = [SQ mono-OOH + Na]+; 481.40 = [SQ mono-OOH + K]+; 675.40 = [GDO mono-OOH + Na]+; C and D used APCI+ as ionization source.
extracted from EVOOs in high PVs. EVOO samples were accelerated to oxidize by exposure to natural light at room temperature and thermal treatment at 100°C under continuous air flow.

The results in Figure 2 show that under the condition of exposure to light, as the oxidation degree became more serious the content of SQ in EVOO decreased by 42% in EVOO1, 43% in EVOO2 and 40% in EVOO3. Meanwhile, the content of this unknown compound increased by about 10 times, 5.5 times and 10 times in EVOO1, EVOO2 and EVOO3, respectively (Figure 2D, E, F). The decrease of SQ and the increase of the unknown compound also happened in the EVOO samples stored in darkness owing to the autoxidation of EVOOs (Figure 2A, B, C). However, when EVOO samples were under a continuous air flow (20 L/h) at 100°C, the content of SQ in EVOO1 did not change much until its IP finished. Similar circumstance occurred in EVOO2 except for a decrease in the initial 21 hours. The ratio of the content of SQ and the unknown compound kept stable during the experiment. SQ is an oxidation inhibitor in mild UVA-mediated oxidation of polyunsaturated fatty acid, acting as a peroxy radical scavenger [38], but it does not work on the oxidation caused by air exposure.

Antioxidant activity of HYTY, TY and SQ by Rancimat tests and oven tests

HYTY, TY and SQ are three kinds of most representative substances in olive oil. HYTY and TY are always accepted as good antioxidant compounds in olive oil together with other kinds of phenols [6,7,16,20,21], which are good donors for hydrogen atom and could form stable radicals themselves to fight against the lipid oxidation [24]. Theoretically, TY is much weaker than HYTY since HYTY contained one more hydroxyl on the α-position of hydroxyl in TY, which can donate electrons to decline the bond dissociation energy and improve its antioxidant activity. It has been also reported that SQ is a potent oxidation inhibitor and is contributed to oil stability [23,34,36]. According to the results in this article, HYTY has some antioxidant activity while TY hardly has antioxidant activity, and SQ may only inhibit mild UVA-mediated oxidation. Oven tests and Rancimat tests were used to prove the results in this article.

In these two experiments, 4-methylcatechol was used to replace HYTY to evaluate the antioxidant activity of HYTY due to the similarity of their structures, an hydroxyethyl group in HYTY and a methyl group in 4-methylcatechol owing similar functional characteristics. Generally considering, animal oils become rancid if PV exceeds 20 meq/kg. The results in Figure 4 show TY and SQ had almost the same oxidation tendencies as control. PVs of lard samples in addition of 0.02% of TY and SQ increased from 1.8 ± 0.0 to 17.8 ± 0.0 and 19.1 ± 0.0 meq/kg, respectively, after 11 days when control group had achieved 19.8 ± 0.0 meq/kg, and then they reached 21.1 ± 0.0 and 22.6 ± 0.0 meq/kg after one day. In the case of 4-methylcatechol, it spent a longer time of 31 days reaching 19.5 ± 0.0 mmol/kg. The results obtained from oven tests indicate that TY and SQ have not shown any antioxidant activity to oxidation caused by thermal treatment, however, 4-methylcatechol has quite strong antioxidant activity (Figure 4).

In Rancimat tests, TY, SQ and 4-methylcatechol were tested on the Rancimat at 100°C, under a continuous air flow (20 L/h). To interpret the effects of these three compounds on the antioxidative activities, the protection factors (P) were calculated according to Huang et al. [39]. The higher the P is, the stronger antioxidative activity the compound has. If P < 1, the compound has pro-oxidative activity; if P ≠ 1, the compound has no antioxidative activity; if 2 > P > 1, the compound has some antioxidative activity; if 3 > P > 2, the compound has an obvious antioxidative activity; if P > 3, the compound has a strong antioxidative activity [39,40]. The results in Tab. 1 show that 4-methylcatechol has strong antioxidative activities in different concentrations. For TY, it shows pro-oxidative activity if it is in lower concentrations (<0.04%), and it shows some antioxidant activity when its concentration reached 0.04%. In the case of SQ, it showed pro-oxidative activity since the P was between 0.75 ± 0.01 and 0.80 ± 0.08. According to Belitz, et al. [41] acyl lipid constituents, such as oleic, linoleic and linolenic acids, have one or more allyl groups within the fatty acid molecule and thus are readily oxidized to hydroperoxides. There are six allyl groups within SQ molecule, which makes it easier to be oxidized.

Analysis of SQ and co-extracts using HPLC-MS

HPLC-UV analysis show lots of substances co-extracted with SQ including 6T (Figure 1). The SQ extracted from EVOO has been identified based on the ion at m/z 449.30 and 411.30 by HPLC-MS/ESI and HPLC-MS/APCI, respectively (Figure 3A and 3C). According to the HPLC results, the unknown compound appeared under different conditions, while only after the storage under the exposure to natural light did it appear to a continuous decrease on SQ and a continuous increase on the unknown compounds (Figure 2D, E, F). The unknown compounds was determined roughly by method 2.7, indicating that the unknown compound belonged to hydroperoxides, which was confirmed by ESI-MS analysis (Figure 3B). The ion at m/z 443.10, 465.40 and 481.40 indicated that the unknown compound includes SQ monohydroperoxide (SQ-OOH), and the ion at m/z 675.40 should refer to the existence of glyceryl dioleate monohydroperoxide.

Since ESI is generally considered a better ionization technique for polar substances than APCI, APCI-MS was used to further identify the unknown compound because it is generally favored as an ionization technique for lipophilic and poorly functionalized molecules, for instance SQ [42]. The ion at m/z 425.30 and 407.30 in APCI-MS chromatograph showed signs of

![Figure 4](image-url)
SQ-OOH (Figure 3D). The most abundant ion was m/z 425.30 assigned as \([\text{M} + \text{H}] - \text{H}_2\text{O}\)^+, followed in decreasing order of abundance by m/z 407.30 \([\text{M} + \text{H}] - (\text{H}_2\text{O})_2\)^+, which was as similar as what Mountfort et al. [42] has obtained. The ion at m/z 407.30 could be the fragment from the ion at m/z 425.30 losing two molecules of H\(_2\)O. Synthesizing the result of HPLC-MS and the determination of peroxidation, the unknown compound was mainly composed of SQ-OOH (Table 1).

### Conclusion

Since HYTY and TY occupy big proportions of total phenols in EVOOs, the changes of total phenols in EVOOs are predominately affected by these two phenolic alcohols. During the experiment under the exposure to natural light, phenols possessing strong antioxidant activities like \(\alpha\)T, were declined fast at the beginning, which leading to total phenols in EVOOs decreasing obviously in the initial 3 weeks, and the change of total phenols slowed down when these strong antioxidant compounds tailed off to a certain concentration but left behind TY and some other phenols which contain weak antioxidant activities. Under the conditions of darkness and exposure to natural light at room temperature, HYTY was in continuous decreases of 18% and 20% in EVOO1, 20% and 27% in EVOO2, and a discontinuous decrease and a slight increase of 6% in EVOO3, respectively. The increasing change of HYTY is possibly caused by the hydrolysis from ligstroside and oleuropein compounds. These changes of HYTY and TY are consistent with those under the condition of a continuous air flow. As the increase of oxidation in EVOOs, HYTY gradually declined and were lost before EVOOs got oxidized while TY kept almost unchangeable and were decreased as EVOOs started to oxidize. HYTY has been proved to have strong antioxidant activity and TY very weak antioxidant activity by both of Rancimat tests and oven tests.

Most kinds of phenols, in low content in EVOOs, decreased with the increase of oxidative degree. VA, Qu, Chr, Compound 1 and Compound 3 in EVOO3 had increases under the darkness. Hence, they are possibly caused by hydrolysis of ligstroside and oleuropein compounds. According to the changes of Compound 1, 2, 3 and 4 in this article, it has big opportunities that these compounds belong to phenols or oxidation products.

Under the condition of exposure to light, SQ in EVOOs decreased significantly. While in EVOOs oxidized by a continuous air flow at 100°C it showed an unchangeable status until EVOOs started to oxidize. These results indicate that SQ only has certain antioxidant activity on the defence against mild UVA-mediated oxidation, however, it shows very weak antioxidant activity or pro-oxidant activity to EVOOs under other conditions. In contrast, with the increase of oxidation degree by photo-oxidation, the unknown compounds increased as SQ decreased and it increased to 5-10 times as that at the beginning and was about 4 times as that in EVOOs in darkness. The analysis of HPLC-MS/ESI and HPLC-MS/APCI has primarily proved that the unknown compound is SQ-OOH.

It is not an easy job to assess the quality of EVOOs only using one technique or one parameter. The variation process and SQ-OOH formed during the storage could be also regarded as an important indicator which refers to the quality and freshness of EVOO.

### Acknowledgment

The authors thank Dr. Zhang in Wilmar International for providing HPLC-MS to determine the samples.

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Table 1: IP and Pf values of TY, SQ and 4-methylcatechol in different concentrations.

<table>
<thead>
<tr>
<th>Compounds</th>
<th>IP (h)</th>
<th>Pf *</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.01% TY</td>
<td>1.66 ± 0.03</td>
<td>0.74 ± 0.02</td>
</tr>
<tr>
<td>0.01% SQ</td>
<td>1.65 ± 0.03</td>
<td>0.75 ± 0.01</td>
</tr>
<tr>
<td>0.01% 4-methylcatechol</td>
<td>11.08 ± 0.06</td>
<td>4.95 ± 0.04</td>
</tr>
<tr>
<td>0.02% TY</td>
<td>2.04 ± 0.03</td>
<td>0.91 ± 0.02</td>
</tr>
<tr>
<td>0.02% SQ</td>
<td>1.71 ± 0.01</td>
<td>0.76 ± 0.01</td>
</tr>
<tr>
<td>0.02% 4-methylcatechol</td>
<td>13.77 ± 0.01</td>
<td>6.15 ± 0.03</td>
</tr>
<tr>
<td>0.04% TY</td>
<td>2.65 ± 0.06</td>
<td>1.17 ± 0.02</td>
</tr>
<tr>
<td>0.04% SQ</td>
<td>1.79 ± 0.05</td>
<td>0.80 ± 0.08</td>
</tr>
<tr>
<td>0.04% 4-methylcatechol</td>
<td>17.05 ± 0.08</td>
<td>7.61 ± 0.06</td>
</tr>
</tbody>
</table>

Figure 5: Changes of HYTY, TY and SQ extracted from EVOOs which were in accelerated oxidation using Rancimat every 7 hours. A and B represented for EVOO1 and EVOO2.
Citation: Weng X, Luo S, Song Y. Variations of phenols and squalene in EVOOs under different conditions. J Res Anal. 2017; 3(1): 24-33

References


