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ARTICLE

Study of Chemical Changes Produced in Virgin Olive Oils with Different Phenolic Contents during an Accelerated Storage Treatment

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Chemical changes produced in an extra virgin olive oil sample in the presence (EVOO) and absence (EVOOP) of its phenolic fraction during an accelerated storage treatment at 60 °C up to 7 weeks were studied. Modifications in phenol content, as well as changes in several quality parameters (free acidity, peroxide value, UV absorbance, fatty acid composition, oxidative stability index, and tocopherol content) were also evaluated under the same storage conditions and compared to those of the same sample deprived of phenolic compounds. When the phenolic extract of the EVOO was studied, a decrease of the antioxidants first present in the sample and an increase of the oxidized products were observed. In addition, oxidation seemed to produce the transformation of such phenolic compounds as secoiridoids and the appearance of oxidized forms of them. These latter compounds could be used as molecular markers of the lack of extra virgin olive oil freshness.

KEYWORDS: Aging; HPLC; oxidation; phenolic compounds; virgin olive oil

INTRODUCTION

Several studies regarding natural antioxidants from vegetable matrices have shown that olives and olive derivatives play an important role in the Mediterranean diet and, along these lines, are now considered as a source of natural phenolic antioxidants (*1*, *2*). These compounds are supposed to have chemoprotective properties in human beings (anticancer, antioxidant, and anti-inflammatory properties) (*3*, *4*) and also to contribute to the sensorial properties of virgin olive oils (VOO) by conferring bitterness, pungency, and astringency (*5*−*7*). Moreover, the high oxidative stability of VOO is related not only to the high monounsatured/polyunsatured ratio but also to the presence of phenolic compounds with antioxidant action.

During storage, the phenolic compounds present in VOO could undergo oxidative degradation; for this reason, the molecules that appeared after oxidation are being investigated. Rovellini and Cortesi have proposed several oxidized forms derived from phenolic compounds of VOO exposed to light for 2 years (*8*). More recently, Ríos et al. (*9*) have collected individual oxidation products from an oxidized VOO sample (at 100 °C for 8 h under an air flow) by preparative high-performance liquid chromatography (HPLC) and determined the structures of these oxidized forms by gas chromatography (GC)−mass spectrometry (MS) after their conversion to trimethylsilyl ethers. However, oxidation conditions applied in these studies do not often reflect the real storage conditions of VOO. In addition, the results of the analytical methods used to evaluate natural antioxidants must be carefully interpreted depending on the conditions of oxidation (i.e., temperature or oxygen availability), as suggested by Frankel (*10*).

The extraction procedure of the phenolic fraction of VOO may offer several problems in the presence of their oxidation forms. For this reason, Armaforte et al. (*11*), comparing different methods usually employed to extract the phenolic fraction of VOO, proved that the solid phase extraction procedure (by means of diol phase) was not appropriate when VOOs contain significant amounts of polar oxidation products from phenols or lipids; in fact, these polar products could interfere with the retention mechanism of phenols during their extraction.

As shown by several authors (*11*−*15*), modifications due to hydrolysis/oxidation reactions during VOO storage or as consequence of heating treatments produce changes of the total antioxidant power of the phenolic fraction and, thus, of the oxidative stability of VOO. Five different kinds of reactions that involve phenols are generally described (*12*−*15*): (1) lysis of complex phenols, which increase the content of low molecular weight (MW) phenolics such as hydroxytyrosol (HYTY) and tyrosol (TY); (2) increase of the dialdehydic forms of decarboxymethyl oleuropein aglycon (DOA) and decarboxymethyl ligstroside aglycon (DLA); (3) hydrolysis of the acetic ester

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occurring for HYTY; (4) cleavage of elenolic acid (EA) with loss of the carboxymethyl moiety and conversion of the monoaldehyde form to its dialdehyde form; and (5) appearance of oxidation products of phenolics (especially the oxidized derivatives of dialdehyde forms of DOA and DLA).

In particular, several authors have studied the presence of the closed aldehydic form of oleuropein aglycon (OA) and the open dialdehydic form of DOA by NMR (*16*, *17*), LC-MS (*8*, *9*, *18*), and GC-MS (*9*, *19*, *20*). The analogous forms for ligstroside aglycon (LA) have been also suggested (*8*, *9*, *20*). Rovellini et al. (*8*) have confirmed the presence of the dialdehydic open structures of the OA and LA without the loss of the carboxymethyl group by exposing a monovarietal Coratina extra VOO to light for 2 years and evaluating its phenolic extract by means of HPLC-MS equipped with both electrospray ionization (ESI) and atmospheric pressure chemical ionization (APCI) sources.

These authors (*8*) proposed that the ratio between aldehydic (due to enzymatic natural reaction) and dialdehydic (due to hydrolytic chemical reaction) forms of secoiridoids and their oxidized derivatives could be used to evaluate the incidence of technological/storage processes. Moreover, they identified the major oxidized derivatives of secoiridoid molecules, underscoring that, for this type of analysis, the ESI source in the positive-ion mode was better than the APCI, due to the capacity of ESI to form adduct ions, which gave more diagnostic information. However, and as far as we are concerned, no research has monitored the evolution of oxidized phenolic compounds during storage treatment.

The objective of this work is to study the chemical changes produced in an extra VOO sample in the presence and absence of its phenolic fraction during storage. For this purpose, an accelerated storage treatment at 60 °C for up to 7 weeks was performed. Modifications in phenol content, as well as changes in several quality parameters [free acidity, peroxide value (PV), UV absorbance, fatty acid (FA) composition, oxidative stability index (OSI), and tocopherol content] were studied. In addition, phenol transformation during the accelerated storage treatment in extra VOO samples with phenolic fraction was also studied.

MATERIALS AND METHODS

Reagents. The following reagents were used: sodium hydroxide (NaOH), sodium chloride (NaCl), potassium hydroxide (Carlo Erba, Milan, Italy); *n*-hexane, methanol, diethyl ether, α-tocopherol, apigenin (API), luteolin (LUT) (Sigma-Aldrich, St. Louis, MO); chloroform, acetonitrile (ACN), hydrochloric acid (HCl), anhydrous sodium sulfate, formic acid, ethanol, phenolphthalein, sodium thiosulfate, starch indicator (Merck, Darmstadt, Germany); iso-octane, potassium iodide, 3,4-dihydroxyphenylacetic acid (3,4-DHPAA), and acetic acid (Fluka, Buchs, Switzerland).

Instruments. HPLC analyses were performed with an 1100 series liquid chromatograph (Agilent Technologies, Palo Alto, CA) provided with a binary pump delivery system, a degasser, an autosampler, and a diode array UV−Vis detector (DAD). The liquid chromatograph was also coupled (in series with the DAD) to the ESI source of an HP 1100 series quadrupole mass spectrometer (MS) (Agilent). Phenol separation was carried out with a reverse phase C18 Luna column (5 μm, 25 cm × 3 mm i.d., Phenomenex, Torrance, CA), with a C18 precolumn (Phenomenex), whereas tocopherol separation was performed using a CN Luna 100A column (5 μm, 15 cm × 4.6 mm i.d., Phenomenex).

Fatty acid (FA) composition of samples was established by capillary GC employing a fused silica capillary column BPX70 (50 m × 0.22 mm i.d., 0.25 μm film thickness) from SGE Forte (Palo Alto, CA) that was fitted on a Clarus 500 gas chromatograph from Perkin-Elmer (Waltham, MA) equipped with a flame ionization detector (FID).

The oxidative stability of samples was evaluated by the OSI, using an eight-channel oxidative stability instrument (Omnion, Decatur, IL).

Sample Preparation. An extra VOO sample from the olive fruit variety Brugnola (picked on October 2008 at San Marino) was used. The olives were processed using an Oliomio 150 extraction machine (Tem, Tavernelle Val di Pesa, Florence, Italy) to obtain extra VOO sample. Samples oxidation was evaluated on two aliquots of the oil sample: extra VOO with phenols (EVOO) and extra VOO without phenols (EVOOP). Phenolic compounds were removed from EVOO according to the procedure described by Bonoli-Carbognin et al. (*21*). Briefly, 35 g of EVOO was washed with several aliquots of 0.5 M NaOH (4 × 15 mL). To eliminate the aqueous phase, the mixture was centrifuged (1000*g*, 5 min) after each washing. Combined olive oil fractions were then washed with 0.5 M HCl (2 × 10 mL) and saturated NaCl solution (5 × 10 mL), centrifuged at 1000*g* for 5 min, dried with anhydrous sodium sulfate, and finally filtered under vacuum. Dried EVOOP was then obtained.

Storage Treatment. According to several authors (*21*−*23*), both samples, EVOO and EVOOP, were divided in eight aliquots each (250 mL, 228.8 g) and kept in the dark at 60 °C for up to 7 weeks. Each aliquot was stored in an individual open glass bottle of 300 mL (i.d. = 6 cm; surface area exposed to the air = 28.3 cm^2). Two bottles, one of EVOO and the other of EVOOP, were removed every week from the oven and then analyzed. Triplicate analyses were carried out for each analytical determination at each storage time on both EVOO and EVOOP samples.

Quality Parameters. The chemical parameters measured were free acidity (free fatty acid content of the oil expressed as the percentage of oleic acid), PV (amount of hydroperoxides expressed as mequiv of O_2 kg^{-1}), and UV absorbance at 232 and 270 nm (k_{232} and k_{270}, which provide a measurement of the state of oxidation of the oils). These analyses were performed according to the official methods of the European Commission (*24*).

FA composition has been also established according to the method of Bendini et al. (*22*). The methyl esters of FAs were obtained after a cold basic transmethylation procedure and then analyzed by GC-FID. The results were expressed as percentage of saturated, monounsaturated, and polyunsaturated. The ratio of oleic/linoleic acids was also calculated.

Oxidative Stability. A stream of purified air (120 mL min^{-1} air flow rate) was passed through a 5 g oil sample, and the effluent air for the oil sample was then bubbled through a vessel containing deionized water. The effluent air contains especially volatile organic acids as formic acid and other volatile compounds formed during thermal oxidation of the oil, which increased the conductivity of the water. The temperature at which this test was carried out was 110 °C. The OSI (or OSI time) was expressed in hours.

Liquid−Liquid Extraction (LLE) of Phenolic Compounds from EVOO and EVOOP. The LLE procedure was adapted from that of Carrasco-Pancorbo et al. (*14*). Briefly, 50 g of oil containing 200 μL of 3,4-DHPAA (1000 mg L^{-1}, used as internal standard to evaluate the extraction recovery) were dissolved in 50 mL of *n*-hexane, and the solution was extracted successively with four 20 mL portions of methanol/water (60:40, v/v). The combined extracts of the hydrophilic layer were brought to dryness in a rotary evaporator under pressure at 40 °C. Finally, the residue was redissolved in 1 mL of methanol/water (50:50, v/v) and filtered through a 0.45 μm filter.

Tocopherol Extraction. One gram of oil sample was dissolved in 10 mL of *n*-hexane, these extracts being filtered through a 0.45 μm nylon filter.

HPLC Analysis. *Phenolic Compounds*. Mobile phases were prepared by mixing water containing 0.5% formic acid and ACN. A gradient elution was performed according to the conditions described by Carrasco-Pancorbo et al. (*14*). UV−Vis detection was set at 240, 280, and 330 nm. In all cases, 10 μL was injected, the flow rate being 0.5 mL min^{-1}. The MS working conditions were as follows: ESI interface; nebulizer gas pressure, 50 psi; drying gas flow, 9 L min^{-1} at 350 °C; capillary voltage, 3 kV. Nitrogen was used as nebulizer and drying gas. The MS scanned within the m/z 50−800 range in the positive-ion mode. The calibration curves were constructed with standard solutions of 3,4-DHPAA to quantify compounds detected at 280 (r^2 = 0.999) and 240 nm (r^2 = 0.998) and API (r^2 = 0.995) and LUT (r^2 = 0.988) to quantify these compounds at 330 nm. Results are given in milligrams per kilogram of oil.

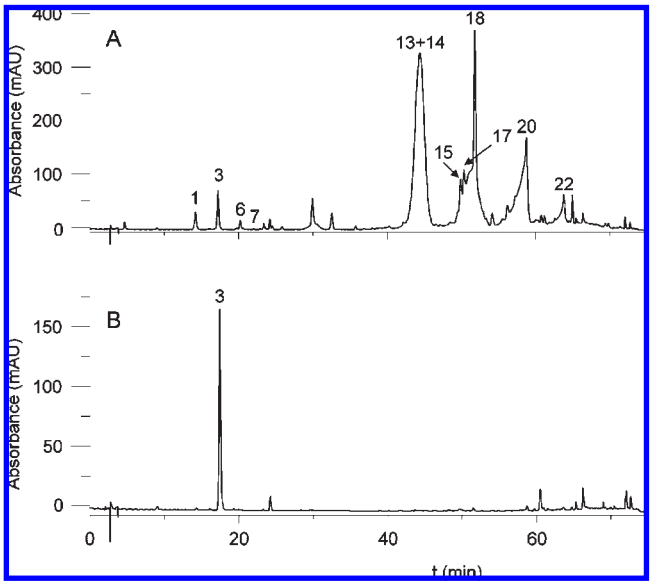

Figure 1. UV chromatograms of (**A**) EVOO and (**B**) EVOOP samples at t_0. Peaks: 1, HYTY; 3, 3,4-DHPAA; 6, unknown; 7, unknown; 13, OxDOA; 14, DOA; 15, OxDLA; 17, DLA; 18, AcPIN; 20, OA; 22, LA. Detection wavelength was 280 nm.

Tocopherols. α-, β-, and γ-tocopherols were determined using isocratic conditions with *n*-hexane/dichloromethane (95:5, v/v). UV−Vis detection was performed at 295 nm. In all cases, 10 μL was injected, the flow rate being 1.0 mL min^{-1}. Analyses were carried out at room temperature. The total run time was 10 min. The calibration curves were constructed with standard solutions of α-tocopherol ($r^2 = 0.999$) and used for quantification. Results are given in milligrams of α-tocopherol per kilogram of oil.

Statistical Analysis. Means and standard deviations were calculated with SPSS (version 15.0, SPSS Inc., Chicago, IL) statistical software. SPSS was used to perform one-way analysis of variance and Tukey's honest significant difference test at a 95% confidence level ($p < 0.05$) to identify differences in the samples at different storage times. A Student *t* test ($p < 0.05$) was also used to identify differences between samples for the same parameter at each storage time.

RESULTS AND DISCUSSION

Evaluation of the Differences Observed between EVOO and EVOOP Samples during the Accelerated Storage Treatment. Phenolic fractions of EVOO and EVOOP were first analyzed to verify the efficiency of phenolic compound stripping. Considering the sum of all quantified phenolic compound (22 individual phenols), a concentration of 164 mg kg^{-1} oil is obtained in EVOO sample at storage time zero (t_0), being the concentration of EVOOP sample of 0.70 mg kg^{-1} oil at the same storage time. Then, a decrease of 99.6% in phenol content was obtained (see **Figure 1**). A similar effect has been previously observed by Bonoli-Carbognin et al. (*21*).

Differences produced in the chemical parameters of the EVOO and EVOOP samples at t_0 have been also studied. As reported in **Table 1**, the free acidity percentage of EVOO and EVOOP ranged from 0.24 to 0.18%, respectively, whereas the PV varied from 11.96 to 12.44 mequiv O_2 kg^{-1} of oil. All of these values were below the limits set by EC regulation for extra VOO (*24*).

The FA composition of both samples at t_0 is also reported in **Table 1**. EVOO and EVOOP showed very similar values in terms of FA composition, which demonstrated that the phenol-removing procedure did not affect this fraction. Both samples were also characterized by high values of MUFA and oleic/linoleic acid ratio, as expected. The high oleic content of both samples was reported to give a large contribution to the oxidative stability of this oil (*25*).

Table 1. Chemical Parameters of the EVOO and EVOOP Samples at Storage Time Zero

parameter	EVOO	EVOOP
free acidity (%)	0.24	0.18
PV (mequiv of O_2 kg^{-1})	11.96	12.44
oleic/linoleic acid	7.87	7.85
MUFA[a] (%)	73.80	73.75
PUFA[b] (%)	9.67	9.68
SFA[c] (%)	16.53	16.57

[a] Monounsaturated fatty acids. [b] Polyunsaturated fatty acids. [c] Saturated fatty acids.

Changes in the oxidative status of EVOO and EVOOP are shown in **Table 2** as conjugated dienes (k_{232}), trienes (k_{270}), and OSI time. The two oil samples at t_0 showed k_{232} and k_{270} values lower than the legal limit values established by EC regulation for the extra VOO category (*24*) (2.50 and 0.22, respectively). However, after 1 week of storage (t_1), both samples exceeded the limit for k_{232}, reaching values of 7.71 and 9.74 for EVOO and EVOOP, respectively, after 7 weeks (t_7) of storage. After 1 week of storage (t_1), only the EVOO sample exceeded the EC limit for k_{270}, but 7 days later (t_2), EVOOP also surpassed the legal value, reaching both after 7 weeks (t_7) of storage the final value of ~0.7. Similar trends were also found by Bendini et al. under comparable experimental conditions (*22*). With regard to OSI time, the EVOOP sample at t_0 showed a lower value than the EVOO sample, probably related to the different amounts of phenolic compounds. EVOOP exhibited a OSI value of about 10 h that could only be related to the FA composition of the oil (high oleic acid content, low amounts of polyunsaturated FA, and the high oleic/linoleic acid ratio), as previously reported (*25*). Both EVOO and EVOOP samples showed a significant decrease of oxidative stability (OSI time value) during the storage process, being more evident for EVOOP, confirming the role of the phenolic fraction in the oxidative stability of EVOO.

The tocopherol content for both EVOO and EVOOP samples is also shown in **Table 2**. At t_0, the two oil samples did not show significant differences in tocopherol content. Thus, the alkaline procedure used to wash polar phenols did not affect this lipophilic antioxidant fraction. Tocopherol content remained substantially unvaried for EVOO from t_0 to t_3; then, a strong decrease was observed until the end of the storage time. The constant loss of oxidative stability (**Table 2**) is probably related to the decrease of polar phenols, which during the first 3 weeks, may act as antioxidant molecules also protecting tocopherols against oxidation (*22*, *26*). On the other hand, tocopherols started to decrease after 2 weeks of storage for EVOOP, but at higher storage times an oscillating trend was evidenced. This trend could be explained by taking into account a synergic effect between α-tocopherol and phospholipids (*27*) and the formation of tocopherol oxidized derivatives. These last compounds could overlap with tocopherol peaks during HPLC elution, interfering in tocopherol determination and not allowing their correct quantification.

Phenolic Compound Transformation in EVOO Samples during the Accelerated Storage Treatment. Figures 2, **3**, and **4** report the UV chromatograms detected at 280, 240, and 330 nm, respectively, showing the 22 phenolic compounds at three times of the storage process (t_0, t_3, and t_7). In particular, at 280 nm (**Figure 2**), the decrease of DOA (peak 14), DLA (peak 17), and LA (peak 22), the disappearance of OA (peak 20), and the formation of their possible oxidized derivatives (peaks 13, 15, and 21 and traces of OxOA) are evident. **Figure 3** shows the trend of EA (peak 12) and the appearance of several hypothetical oxidized compounds

Table 2. Chemical Parameters for EVOO and EVOOP Samples at Different Storage Times[a]

	k_{232}		k_{270}		OSI time (h)		tocopherols (mg kg^{-1})	
storage time (weeks)	EVOO	EVOOP	EVOO	EVOOP	EVOO	EVOOP	EVOO	EVOOP
t_0	2.24 f	2.45 d	0.19 g*	0.16 f	33.65 a*	10.8 a	181.9 a	148.4 a
t_1	3.51 e	3.67 cd	0.24 f*	0.17 f	22.23 b*	9.03 b	191.1 a	170.6 a
t_2	4.52 d	5.43 bc	0.26 f	0.23 ef	19.20 c*	6.2 c	195.5 a*	140.8 a
t_3	5.42 c	6.43 b*	0.34 e	0.29 e	15.78 d*	3.8 d	181.9 a*	90.6 b
t_4	7.48 b	8.41 a	0.40 d	0.39 d	10.55 e*	1.3 e	139.6 b*	42.0 c
t_5	9.69 a*	8.48 a	0.51 c	0.47 c	7.288 f	0 f	85.48 c	46.9 c
t_6	7.48 b	8.93 a	0.59 b	0.59 b	5.83 g*	0 f	55.3 d	70.3 bc*
t_7	7.71 b	9.74 a*	0.68 a	0.67 a	3.438 h	0 f	25.9 e	97.2 b*

[a] Mean values (n = 3). Means followed by different letters in the same column are significantly different (p < 0.05). Means with an asterisk for the same parameter at each storage time are significantly different (p < 0.05).

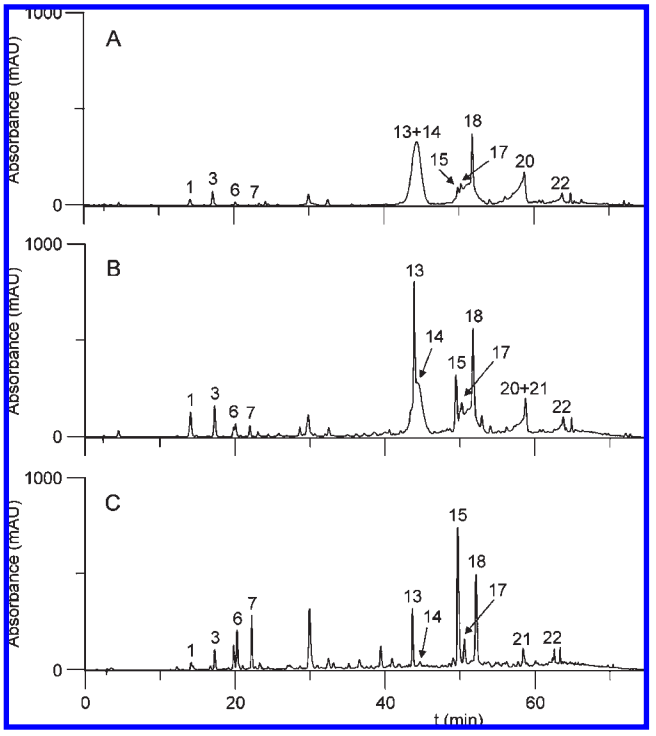

Figure 2. UV chromatograms showing the evolution of the EVOO phenolic profile after storage treatment at 60 °C: (**A**) t_0; (**B**) t_3; (**C**) t_7. Peaks: 21, OxLA; other peaks as in **Figure 1**. Detection wavelength was 280 nm.

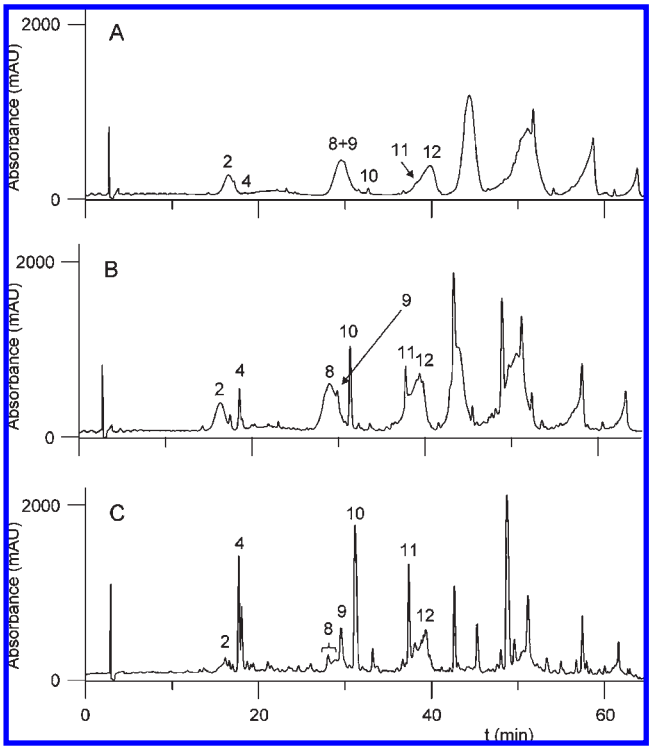

Figure 3. UV chromatograms showing the evolution of the EVOO phenolic profile after storage treatment at 60 °C: (**A**) t_0, (**B**) t_3; (**C**) t_7. Peaks: 2, DEA; 4, OxDEA; 8, unknown; 9, unknown; 10, unknown; 11, OxEA; 12, EA. Detection wavelength was 240 nm.

that absorb only at 240 nm (4, 9, 10, and 11). Finally, **Figure 4** shows the trend at 330 nm of the loss of LUT (peak 16) and the slightly decreased of API (peak 19) during storage.

A list of the main phenolic compounds studied in this work as well as their retention times, UV absorbance maxima, molecular weights, and MS fragmentation patterns is summarized in **Table 3**. From the information shown in this table it is possible to summarize some general concepts:

(1) The absorbing band near 240 nm is typical of a carboxymethyl enol-ether group. Thus, for example, EA (peak 12) is characterized by this band. On the other hand, the bands at 277 and 282 nm are due to a monohydroxyphenyl group and to an *o*-hydroxyphenyl group, respectively; so, for example, HYTY (peak 1) and secoiridoid derivatives containing HYTY [OxDOA (peak 13), DOA (peak 14), OxOA (tr), OA (peak 20), and the unknown peaks 3, 6, and 7] exhibit the second UV maximum near 280 nm, whereas the molecules having a monohydroxyphenyl group such as TY [OxDLA (peak 15), DLA (peak 17), OxLA (peak 21), and LA (peak 22)] show the second maximum near 277 nm.

(2) HYTY exhibits only the ion derived by the neutral loss of water, because the presence of a high initial percentage of water inhibits a good electrospray ionization of molecules such as HYTY and TY having acidic properties. As suggested by Rovellini et al. (*8*), it is not possible to reveal the pseudomolecular ion for HYTY due to its difficulty in giving protonated adducts.

(3) Some authors (*8*, *9*) have indicated that the oxidation of secoiridoid structures involves the acidic portion (EA) and not the aromatic alcoholic moiety (HYTY and TY); for this reason, the oxidized forms shown in **Table 3** maintain the UV specific absorbance of their nonoxidized forms.

(4) The oxidation involves the conversion of the aldehydic group of EA to carboxylic group (according to the scheme reported in **Figure 5**).

(5) The oxidized forms of secoiridoids, which are more polar than their respective nonoxidized derivatives, elute before them. In the case of the couple DEA−OxDEA (peaks 2 and 4 , respectively), the presence of a second carboxylic group in the molecule does not cause the anticipated elution.

(6) Under the ESI conditions applied in this study, both the oxidized and nonoxidized forms of secoiridoids are characterized by the presence of the sodium adduct $[M + Na]^+$, the loss of the phenolic group (*m*/*z* 241 and 225 for the oxidized and nonoxidized forms of secoiridoids, respectively; *m*/*z* 183 and 167 for the oxidized and nonoxidized forms of the decarboxymethyl structures of secoiridoids, respectively), and the loss of the acidic group (*m*/*z* 137 and *m*/*z* 121 for molecules having HYTY and TY, respectively), according to the scheme reported in **Figure 5**.

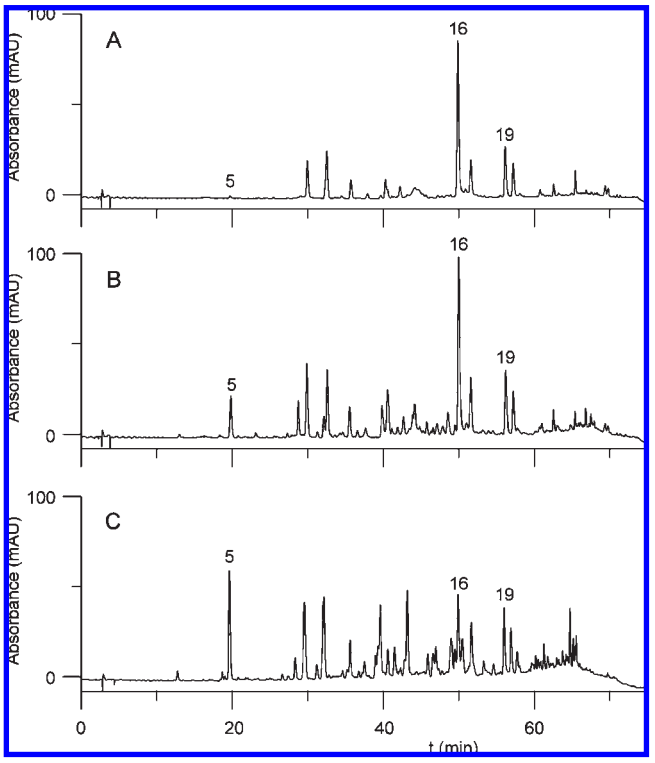

Figure 4. UV chromatograms showing the evolution of the EVOO phenolic profile after storage treatment at 60 °C: (**A**) t_0; (**B**) t_3; (**C**) t_7. Peaks: 5, unknown; 16, LUT; 19, API. Detection wavelength was 330 nm.

(7) The peaks related to the oxidized forms of secoiridoids are characterized by a narrower profile than for the molecules having one or two aldehydic groups.

Figure 6A shows the trend of the phenolic compounds of EVOO during the storage treatment. Area values were divided by 3,4-DHPAA area (to estimate the extraction recovery) and expressed as natural logarithm for a better evaluation of the different trends of disappearance of the phenolic compounds. Generally, a decrease of the more abundant compounds (secoiridoids) is observed. Peaks 21 and 22 were jointly evaluated: peak 21 appeared overlapped with peak 22 from t_3 to t_7. At t_7, only peak 21 was present. As previously observed at room temperature (*12*, *28*), transformations of secoiridoids to simpler compounds (for example, decarboxymethyl structures) followed by a further conversion to phenylethyl alcohols such as TY and HYTY occurred. In this work, TY was not found in EVOO sample at t_0, whereas HYTY, which was initially found at low concentrations, increased from t_0 to t_4. This tendency was also observed for oils stored at room temperature (*12*, *28*). Among lignans, in particular AcPIN (peak 18 of **Figure 2C**) slightly decreased, exhibiting a high content also at the end of storage process (t_7). This tendency for lignans has been also observed when oils were heated in a conventional or microwave oven (*14*, *29*).

The trend of the neoformation compounds during the storage treatment is shown in **Figure 6B** (area values were also divided by the 3,4-DHPAA area and expressed as natural logarithm). The most important neoformation compounds are peaks 4 (oxidation form of decarboxymethyl elenolic acid, OxDEA) and 15 (oxidized form of decarboxymethyl ligstroside aglycon, OxDLA),

Table 3. Retention Times, UV Absorbance Maxima, Molecular Weights (MW), and MS Fragmentation Patterns of the Phenolic Compounds

					major fragments ESI positive					
analyte	peak	t_r (min)	λ_{max} (nm)	MW	$[M + H]^+$	$[M + Na]^+$	$[M - H_2O + H]^+$	loss of phenolic group	loss of acidic group	other fragments
HYTY	1	11.6	232/280	154			137.1			
DEA	2	16.8	230	184	185.1	207.1				
unknown	3	18.0	232/280	260		283.2				299.0 $[M + K]^+$
OxDEA	4	18.5	236	200		223.1				123.1/165.0
unknown	5	20.0	290/310							338.4/321.8/191.1/185.8
unknown	6	20.1	232/280							177.0/235.1/668.1
unknown	7	22.0	232/280							113.1/157.1/349.2
unknown	8	29.5	234							297.1/239.1/221.1/181.1/165.1
unknown	9	30.4	234	336		359.0				375.1 $[M + K]^+$
unknown	10	36.1	240							237.1/197.1/165.1
OxEA	11	38.9	240	258	259.1	281.1				185.1/227.1/241.1 $[M - OH]^+$
EA	12	39.9	240	242	243.1	265.1				211.1 $[M - OCH_3]^+$
OxDOA	13	44.0	234/282	336	337.1	359.1		183.1	137.1	375.1 $[M + K]^+$
DOA	14	45.0	234/282	320		343.1			137.1	361.1
OxDLA	15	49.8	242/276	320		343.1		183.1	121.1	359.1 $[M + K]^+$
LUT	16	50.0	254/348	286	287.1	309.1				
DLA	17	51.5	236/276	304		327.1			121.1	
AcPIN	18	53.8	236/280	416	417.1	439.1				455.1 $[M + K]^+$ /357 $[M - CH_3COOH + H]^+$ /233 $[M - CH_3COOH - phenylOCH_3 + H]^+$
API	19	56.1	268/338	270	271.1					
OxOA	tr[a]	56.1	236/280	394	395.1	417.1		241.1	137.1	439.1
OA	20	58.7	236/282	378	379.1	401.1		225.1	137.1	419.1
OxLA	21	60.1	232/276	378	379.1	401.1		241.1	121.1	
LA	22	63.9	230/276	362	363.1	385.1		225.1	121.1	

[a] tr, trace.

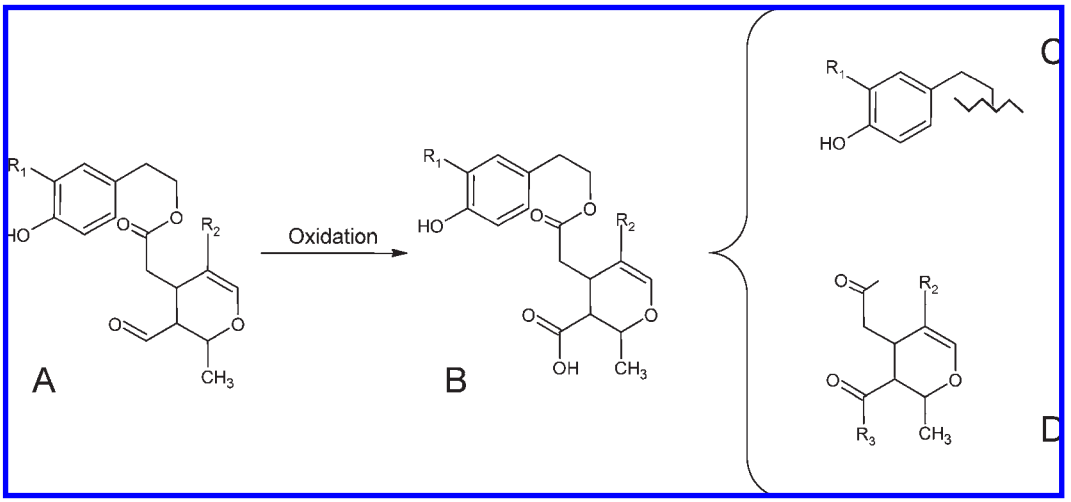

Figure 5. General scheme of storage treatment of secoiridoids: **A**, structure of secoiridoids [LA (R_1 = H and R_2 = $-COOCH_3$); OA (R_1 = OH and R_2 = $-COOCH_3$); DLA (R_1 = H and R_2 = $-H$); DOA (R_1 = OH and R_2 = $-H$)]; **B**, oxidized forms of secoiridoids [OxLA (R_1 = H and R_2 = $-COOCH_3$); OxOA (R_1 = OH and R_2 = $-COOCH_3$); OxDLA (R_1 = H and R_2 = $-H$); OxDOA (R_1 = OH and R_2 = $-H$)]; **C**, loss of acidic group during mass fragmentation (R_1 = H fragment with m/z = 121; R_1 = OH fragment with m/z = 137); **D**, loss of phenolic group during mass fragmentation (R_2 = $-COOCH_3$ and R_3 = OH fragment with m/z = 241; R_2 = $-COOCH_3$ and R_3 = H fragment with m/z = 225; R_2 = H and R_3 = OH fragment with m/z = 183; R_2 = H and R_3 = H fragment with m/z = 167).

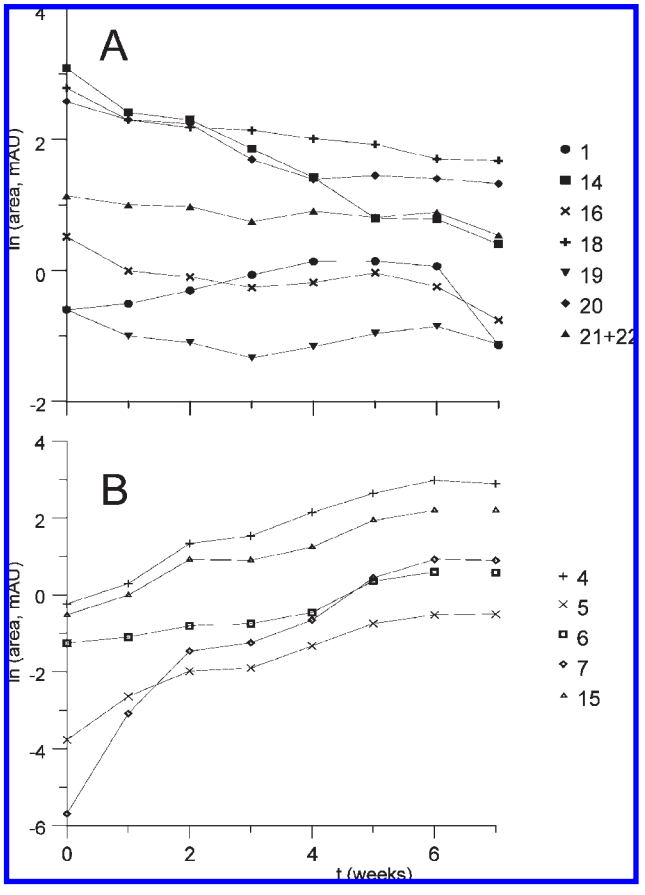

Figure 6. Plots showing the trends of phenolic (**A**) and neoformation compounds (**B**) during the storage treatment (from t_0 to t_7) of EVOO. Area values were divided by 3,4-DHPAA area (internal standard). Peak identification is as reported in **Table 3**.

respectively, followed by peaks 5, 6, and 7 (unknown peaks). Peak 13 (data not reported in **Figure 6B**) probably represents an oxidized secoiridoid compound, being tentatively assigned as an oxidized form of DOA (OxDOA).

In this work, the importance of the role of phenolic compounds in oil stability against oxidation has been demonstrated. The differences observed between EVOO and EVOOP samples enforced this agreement due to the different OSI values obtained that demonstrated a high contribution of polar phenolic compounds to oil shelf life.

In EVOO samples, a decrease of the major secoiridoids and the formation of some of their oxidized forms were observed during the storage treatment. For this reason, these latter compounds could be considered as potential markers of the loss of extra VOO freshness.

A next step of this work will be the investigation of the residual antioxidant activity (in vitro) of these oxidized derivatives, in particular OxDOA and OxOA, which could be of great interest due to the fact that these molecules appeared to maintain unchanged the *o*-hydroxyphenyl part of the original molecular structure. Another challenge will be the identification of other peaks observed but still not identified using other techniques such as TOF-MS or NMR.

ABBREVIATIONS USED

3,4-DHPAA, 3,4-dihydroxyphenylacetic acid; AcPIN, (+)-1-acetoxypinoresinol; API, apigenin; APCI, atmospheric pressure chemical ionization; DEA, decarboxymethylated form of elenolic acid; DLA, decarboxymethyl ligstroside aglycon; DOA, decarboxymethyl oleuropein aglycon; EA, elenolic acid; ESI, electrospray ionization; EVOO, extra virgin olive oil sample with phenols; EVOOP, extra virgin olive oil sample without phenols; FA, fatty acid; FID, flame ionization detector; HYTY, hydroxytyrosol; LA, ligstroside aglycon; LUT, luteolin; MUFA, monounsaturated fatty acid; MW, molecular weight; OA, oleuropein aglycon; OxDEA, oxidized form of decarboxymethyl elenolic acid; OxDLA, oxidized form of decarboxymethyl ligstroside aglycon; OxDOA, oxidized form of decarboxymethyl oleuropein aglycon; OxLA, oxidized form of ligstroside aglycon; OxOA, oxidized form of oleuropein aglycon; OSI, oxidative stability index; OxEA, oxidized form of elenolic acid; PUFA, polyunsaturated fatty acid; PV, peroxide value; SFA, saturated fatty acid; TY, tyrosol; VOO, virgin olive oil.

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