JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Protective Effects of Extra Virgin Olive Oil Phenolics on Oxidative Stability in the Presence or Absence of Copper Ions

ALESSANDRA BENDINI,*,† LORENZO CERRETANI,*,† SAMUELE VECCHI,† ALEGRIA CARRASCO-PANCORBO,‡ AND GIOVANNI LERCKER†

Dipartimento di Scienze degli Alimenti, Università di Bologna, Piazza Goidanich 60, I-47023 Cesena (FC), Italy, and Departamento de Química Analítica, Universidad de Granada, C/Fuentenueva s/n, E-18071 Granada, Spain

The antioxidant activity of the phenolic fraction of extra virgin olive oil was assessed in samples that had a decreasing content of antioxidants in the presence and absence of copper ions as a catalyst of autoxidation. The oxidation process was evaluated by measuring primary and secondary oxidation products. Changes in phenols and tocopherols were investigated by high-performance liquid chromatography. Both the total phenol content and their antioxidant activity were monitored by spectrophotometric assays (with Folin−Ciocalteu and ABTS$^{\bullet+}$ reagents). The important role of phenolic compounds (particularly the *o*-diphenols) in protection from autoxidation was confirmed. However, the tocopherols were more quickly consumed in oils that had the lowest content of *o*-diphenols, which also showed evidence of an ability to chelate copper. In particular, a dramatic decrease was observed in the isomeric form of decarboxymethyl-oleuropein aglycone after addition of the metal, despite its significant increase in samples stored in the absence of copper.

KEYWORDS: Virgin olive oil; phenolic compounds; lipid oxidation; antioxidant activity; metal-chelating ability

INTRODUCTION

During storage, fatty acids contained in extra virgin olive oil undergo oxidative degradation with a progressive accumulation of odorless molecules, such as hydroxyperoxides and secondary products. Lipid oxidation occurs by the interaction of lipids with molecular oxygen by a self-catalyzed mechanism. However, because the activation energy of the reaction is high, the initiation of lipid oxidation is due mostly to the decomposition of hydroperoxides using metal catalysts such as copper and iron or by exposure to light. The high oxidative stability of extra virgin olive oil is mainly due to its fatty acid composition, in particular to the monounsaturated-to-polyunsaturated ratio, and to the presence of minor compounds that also have a major role in preventing oxidation. This means that extra virgin olive oil has a longer shelf life as compared to other edible vegetable oils, with long-term preservation of its intrinsic nutritive and hedonistic properties (*1*−*6*). In addition to lipophilic antioxidants such as tocopherols, extra virgin olive oil also contains various polar phenolic compounds that contribute significantly to its taste (bitter and pungency) (*1*, *2*), prolonged shelf life, and its beneficial effects on human health (anticancer, antioxidant, and antiinflammatory properties) (*3*, *4*). Several classes of phenolic compounds have been identified in extra virgin olive oil: phenolic acids (hydroxybenzoic and hydroxycinnamic derivatives), phenylethyl alcohols (tyrosol and hydroxytyrosol), flavonoids (apigenin and luteolin), lignans [(+)-pinoresinol and (+)-1-acetoxypinoresinol], and secoiridoids (*5*). The secoiridoids are aglyconic derivatives of oleuropein and ligstroside, the most important and abundant constituents of extra virgin olive oil. Phenolic compounds belonging to *o*-diphenolic category, such as oleuropein aglycon, decarboxymethyl-oleuropein aglycon, and hydroxytyrosol, are mainly responsible for the oxidative resistance of extra virgin olive oil (*6*). Phenolic compounds can inhibit oxidation by a variety of mechanisms based on radical scavenging, hydrogen atom transfer, and metal-chelating attributes (*7*−*10*). The presence of metals in extra virgin olive oil is due to endogenous factors linked to plant metabolism, exogenous factors such as olive contamination by agricultural practices (fertilizers and pesticide use), or during oil extraction (by contact between olive paste and metallic surfaces in crushing and malaxation steps) or oil storage (depending on the type of container used) (*11*, *12*). Endogenous and exogenous metals can be dissolved in oils as fatty acid salts (*13*). Transition metals, such as iron and copper, can catalyze the decomposition of hydroperoxides according to their oxidation−reduction potential to yield lipid peroxyl and alkoxyl radicals that initiate free radical chain oxidation (*14*). Phenolic compounds in extra virgin olive oil normally act as antioxidants but, in particular conditions, become pro-oxidant agents and increase the oxidative reaction rate. In fact, their metal-reducing properties take metal

* To whom correspondence should be addressed. (A.B.) Tel: +390547338121. Fax: +390547382348. E-mail: bendini@alma.unibo.it. (L.C.) Tel: +390547338121. Fax: +390547382348. E-mail: lorenzo.cerretani2@unibo.it.
† Università di Bologna.
‡ Universidad de Granada.

10.1021/jf060481r CCC: $33.50
Published on Web 06/07/2006

ions to their lowest oxidation state (for example, $Fe^{3+} \rightarrow Fe^{2+}$ or $Cu^{2+} \rightarrow Cu^{+}$) where they are more active to catalyze hydroperoxide decomposition. As suggested by Yamamoto and Niki (*15*), it is mainly the metal in the lowest oxidation state that induces lipid peroxidation, since the reaction is much faster. Previous investigations have examined the effect of iron on the antioxidant activity of extra virgin olive oil phenolic extracts in bulk oil and in oil-in-water emulsions with emphasis on the interactions between iron or copper ions and phenolic compounds (*16*−*19*). The aim of the present study was to investigate the protective effects on the oxidative stability of virgin olive oil samples of the phenolic fraction in the presence and absence of copper ions. For this purpose, we have determined how copper ions or heat affect olive oil composition, including changes in the content of phenolic compounds, tocopherols, fatty acid methyl esters (FAMEs) analysis, primary and secondary autoxidation products, oxidative stability, bitterness index, and antioxidant activity. The depletion in polar phenolic compounds in extra virgin olive oil is our major goal in studying their protective effects on oxidative stability in the presence of copper, which has not been previously investigated.

MATERIALS AND METHODS

Apparatus. All high-performance liquid chromatography (HPLC) analyses were performed using a HP 1100 Series instrument (Agilent Technologies, Palo Alto, CA) equipped with a binary pump delivery system, degasser, autosampler, diode array UV−vis detector (DAD), mass spectrometer detector (MSD), and fluorescence detector (FLD). All solvents were HPLC grade and filtered through a 0.45 μm nylon filter disk (Lida Manufacturing Corp., Kenosha, WI) prior to use. All analyses were carried out at room temperature. Spectrophotometric determinations were carried out using an UV−vis 1610 instrument (Shimadzu Co., Kyoto, Japan), which had a six slot shuttle and a system for temperature control of working conditions. FAMEs analysis was performed using a GC Clarus 500 Perkin-Elmer (Wellesley, MA) equipped with a flame ionization detector (FID).

Reagents and Standards. The standards used for spectrophotometric phenol quantification (gallic acid), for HPLC quantification (3,4-dihydroxyphenylacetic acid), for evaluation of antioxidant capacity of phenolic extracts (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid, Trolox), and for oxidized fatty acid (OFA) quantification (tricaproin and triheptadecanoin) were obtained from Sigma-Aldrich (St. Louis, MO) as were ABTS [2,2′-azinobis(3-ethylbenzothiazoline)-6-sulfonic acid, diammonium salt] and sodium benzyloxyde in benzyl alcohol. All solvents used were analytical or HPLC grade (Merck & Co. Inc., Darmstadt, Germany).

Samples. A blend of extra virgin olive oils P.O.D "Chianti Classico" (unfiltered), obtained from the Tuscany region (cvs. Frantoio, Moraiolo, and Leccino), was used for this study. This sample was termed VOOA.

Reduction of Phenols Concentration in the Oil. Phenols were partially removed from VOOA by one cycle (VOOB) and two cycles (VOOC) of methanol washing according to the following protocol: Three hundred milliliters of VOOA was filtered through a paper filter filled with anhydrous sodium sulfate and added to 150 mL of methanol in a separation funnel. After agitation for 1 min, the alcoholic phase was separated and the washed oil was evaporated under vacuum at 40 °C to eliminate the solvent. Thirty grams of anhydrous sodium sulfate was then added to the resulting oil, mixed for 10 min, and filtered through a paper filter.

Sample Storage. Oil samples (45 mL) were poured in 100 mL polypropylene open bottles to ensure constant oxygen availability having 16.6 cm^2 of free surface area. A set of open bottles for each sample was placed in an oven and kept at 60 °C during the experimental period (42 days). After 3 weeks, copper standard in Certipur oil (Merck) was added to three oil matrices to obtain a sample concentration of 1 mg kg^{-1} of oil. Three bottles were periodically analyzed. All analyses were performed in triplicate.

Fatty Acid Composition. The fatty acid composition of oil samples at the beginning was determined as FAMEs by capillary gas chromatography (GC) analysis after alkaline treatment; this was obtained by mixing 0.05 g of oil dissolved in 2 mL of *n*-hexane with 1 mL of 2 N potassium hydroxide in methanol according to Christie (*20*). One microliter of the upper phase was injected into a split (split ratio 1:20) GC port set at 240 °C; a fused silica capillary column (50 m length, 0.25 mm i.d.), coated with CPSil-88 (0.25 μm film thickness, Varian, Palo Alto, CA), was utilized. A flow rate of 1.25 mL min^{-1} of helium as a carrier gas was used. The FID detector was at 240 °C. The initial oven temperature was kept at 120 °C for 1 min and raised to 240 °C at a rate of 4.0 °C/min and maintained for 4 min.

Table 1. Absorption Maxima (UV) and Fragmentation Patterns (ES Interface in Positive Polarity) of Benzyl-Ester Derivatives Analyzed by HPLC-DAD/MSD

			major fragments ESI positive		
peak no.	benzyl-ester derivatives	λ_{max} (nm)	*m/z* $[M + 1]^+$	*m/z* $[M + Na]^+$	*m/z* $[M + K]^+$
1	keto-linolenic acid	279	383	405	421
2	hydroxy-linoleic acid	225	387	409	425
3	keto-linoleic acid	277	385	407	423
3a	keto-linoleic acid	277			
3b	keto-linoleic acid	277			
3c	keto-linoleic acid	279			
4	hydroxy-oleic acid	256	389	411	427
5	keto-oleic acid	210–224	387	409	425
5a	keto-oleic acid				

Primary Autoxidation Products. Evaluation of primary autoxidation products was carried out by determination of the peroxide value (PV) and conjugated dienes or K_{232} (conjugated dienes calculated from absorbance reading at 232 nm) according to the official methods described in European Regulation EEC 2568/91 and the following amendments (*21*). PV was expressed as mequiv O_2 kg^{-1} of oil. The K_{232} value was calculated from absorbance readings of oil samples after they were diluted in isooctane resulting in a 1% solution of the sample, placed into a 1 cm cuvette, and analyzed at the corresponding wavelength.

Evaluation of Oxidative Stability under Forced Conditions. An eight-channel oxidative stability instrument (OSI) (Omnion, Decatur, IL) was used. A 5.0 ± 0.1 g amount of oil sample was heated at 110 °C under atmospheric pressure, and air (150 mL min^{-1} of flow rate) was allowed to bubble through the oil. Under these conditions, the lipoperoxidative process reached its final steps, and the short chain volatile acids that were produced were recovered and measured conductometrically in distilled water. The time required to produce a sudden increase of the conductivity (due to volatile acid formation) determined an induction period (OSI time), expressed in hours and hundredths of hours, which was defined as a measure of the stability of an oil.

Secondary Autoxidation Products. Determination of OFA was carried out according to Rovellini and Cortesi (*22*) by HPLC-DAD analyses after transesterification with 1.0 M sodium benzyloxyde in benzyl alcohol; the chromatograms were recorded at 255 nm. The results were expressed as percentage considering benzyl heptadecanoate as an internal standard. These compounds were tentatively identified based on their UV−vis and mass spectra (**Table 1**) obtained by HPLC-DAD/ESI-MSD and literature data (*23*).

Extraction of Polar Phenolic Extracts. The phenolic fraction was extracted from the oil by a liquid/liquid extraction method according to Pirisi et al. (*24*). The dry extracts were redissolved in 0.5 mL of methanol/water (50:50, vol/vol) solution and filtered through a 0.2 μm nylon filter (Whatman Inc., Clinton, NJ) before HPLC. To carry out the antioxidant test and quantify the total phenol (TP) content by spectrophotometric analysis, the dry extract was kept in a 5 mL flask with the same solvent mixture. The extracts were frozen and stored at −43 °C.

Determination of TPs. The TP content in the polar fraction extracted from olive oil samples was measured by colorimetric (TP) and chromatographic (TPH) methods. First, TPs were determined using Folin−Ciocalteu reagent and determining the absorbance at 750 nm

Table 2. Absorption Maxima (UV) and Fragmentation Patterns (ES Interface in Positive Polarity) of Phenolic Compounds Analyzed by HPLC-DAD/MSD

peak no.	phenolic compound	λ_{max} (nm)	major fragments ESI positive m/z $[M+1]^+$	m/z $[M+Na]^+$	m/z $[M+K]^+$	other fragments
I	hydroxytyrosol	232/280				137.1
II	tyrosol	230/276				121.0
III	vanillic acid	228/260/294	169.1	191.1		
IV	isomeric forms of	232/282		343.1	359.1	137.1
V	decarboxymethyl-oleuropein aglycon					
VI	isomeric form of oleuropein aglycon	232/280	379.1	401.1	417.1	137.1
VII	decarboxymethyl-ligstroside aglycon	234/280		327.1	343.1	121.0
VIII	isomeric form of oleuropein aglycon	232/280	379.1	401.1	417.1	137.1
IX	ligstroside aglycon	230/276	363.1	385.1	401.1	121.0

(*25*); the results were expressed as mg gallic acid kg^{-1} oil (calibration curve, $r^2 = 0.998$). Second, TPH determination was carried out by HPLC-DAD/ESI-MSD equipped with a reverse phase C_{18} Luna column (5 μm, 25 cm × 3.00 mm i.d.; Phenomenex, Torrence, CA) according to Rotondi et al. (*26*). Phenolic compounds detected at 280 nm were quantified using a calibration curve constructed with 3,4-dihydroxyphenylacetic acid (Sigma-Aldrich Inc.) ($r^2 = 0.999$). Phenolic compounds were tentatively identified based on their UV−vis and mass spectra (**Table 2**) obtained by HPLC-DAD/ESI-MSD.

Determination of Bitterness Index. Evaluation of the index of bitterness (IB) in polar extracts was carried out spectrophotometrically at 225 nm according to Gutièrrez Rosales et al. (*27*).

Chromatographic Analysis of Tocopherols. One gram of oil sample was dissolved in 10 mL of *n*-hexane. α-, *β*-, and *γ*-tocopherols (α-toc, *β*-toc, and *γ*-toc, respectively) were determined by HPLC/FLD analysis using a HP Hypersil column (3 μm, 100 mm × 3.00 mm, Agilent Technologies) in isocratic conditions with *n*-hexane/2-propanol (95.5:0.5 v/v) as the mobile phase at a flow rate of 1.2 mL/min. The injection volume was 5 μL, and fluorimetric detection was used (λ_{exc}, excitation length, 290 nm; λ_{em}, emission wavelength, 330 nm). Three calibration curves were constructed with standard solutions of each compound (α-, *β*-, and *γ*-toc, $r^2 = 0.991$, $r^2 = 0.986$, and $r^2 = 0.997$, respectively) and used for quantification.

Measurement of Antioxidant Activity. The radical-scavenging capability of phenolic extracts was evaluated by $ABTS^{\bullet+}$ radical cation assay according to Re et al. (*28*) method with detection at 734 nm. Results were expressed in Trolox equivalent using its calibration curve ($r^2 = 0.981$).

RESULTS AND DISCUSSION

Phenolic Content and Composition. The methanol-washing procedure was very effective in decreasing the phenolic fraction of samples; the total phenolic (TP and TPH) and *o*-diphenol (*o*-DPH) contents decreased about 50 and 85% for VOOB and VOOC, respectively (**Table 3**). The TP contents as determined by the Folin−Ciocalteu at the beginning of the analyses were 368.0, 176.4, and 41.4 mg kg^{-1} for VOOA, VOOB, and VOOC, respectively.

TP content determination by spectrophotometric (TP), chromatographic analysis (TPH), and the other determinations concerning the phenolic fraction (IB and ABTS) were effected in VOOC only at the beginning of the experiment. In fact, heat treatment strongly decreased the phenol content, and consequently, it was not possible to determine these compounds at later times. The phenolic fraction is very important for the quality of virgin olive oil (*6*, *29*), and its contribution to the oxidative stability is widely accepted; TPs and particularly the oleuropein derivatives, which belong to the secoiridoid family, were highly correlated with the oxidative stability of oil samples ($r = 0.87$, $p < 0.001$), whereas tocopherols showed a positive lower correlation ($r = 0.82$, $p < 0.001$). Previous reports (*30*) have suggested that hydroxytyrosol, oleuropein aglycon, and decarboxymethyl-oleuropein aglycon have stronger antioxidant activities than α-toc when testing in bulk oil. Molecules with an *ortho*-dihydroxyl structure, are characterized by high antioxidant activities (*6*). In the current study, nine phenolic compounds representing different classes of simple phenols (SPs) and secoiridoid derivatives (SIDs) were identified in chromatographic data based on retention times and UV and mass spectra. In particular, hydroxytyrosol (HYTY), tyrosol (TY), and vanillic acid (VA), two isomeric forms of decarboxymethyl-oleuropein aglycon (DOA1 and DOA2), and oleuropein aglycon (OLA1 and OLA2), decarboxymethyl-ligstroside aglycon (DLA), and ligstroside aglycon (LA) were identified. The oleuropein and ligstroside aglycon derivatives differ in their elenolic acid ring structure (open or closed), in the number of aldehydes, and by the presence or absence of a carboxy-methyl group. The sum of components with *o*-diphenolic structure (HYTY, DOA1, DOA2, OLA1, and OLA2) was indicated as *o*-DPH. This latter class showed a good correlation ($r = 0.91$, $p < 0.001$) with OSI (*31*). Among the SPs identified, tyrosol showed a noticeable increase during the 6 week storage period. In the presence of copper, its content was higher than in the same samples without the metal. Tyrosol, as previously reported (*6*, *18*), shows a lack of radical scavenger activity. In this case, as expected, significant correlations were not found with regards to its content and the oxidative stability of oil samples and the antioxidant power of their phenolic fraction (tyrosol vs OSI and tyrosol vs ABTS, respectively). Hydroxytyrosol showed an increase from start time to 2 weeks of storage, followed by a decrease at 6 weeks of storage and a tendency to degrade when copper was added. This increase in SPs could result from hydrolytic activities on the secoiridoid derivatives with more complex molecular structures, a behavior that has already been observed by other authors (*32*−*34*). Storage did not seem to have any significant effects on the content of vanillic acid content, which was present at low concentrations. However, there was a significant decrease after storage in the content of DOA2, OLA1, DLA, OLA2, and LA. This reduction was more noticeable among the most representative phenolic compounds (DOA2, DLA, and OLA2) indicating their participation in the oxidative process. The significant increase in DOA1 concentrations during storage suggested that DOA2 was isomerized to DOA1; however, when copper was added, DOA1 was consumed, perhaps due to its metal-chelating ability and a structure having two adjacent hydroxyl groups on a benzene ring, which adds support to this hypothesis. This mechanism could justify the lower quantity of primary and secondary oxidation products in VOOA as compared to VOOB in the presence of copper. In fact, after 6 weeks

Table 3. Bitterness Index (IB), Antioxidant Activity (ABTS), and Phenol Content on Three Oil Samples Stored for 6 Weeks at 60 °C in the Presence and Absence of Copper [Mean Values ($n = 3$)][a]

samples	weeks	IB	TP	ABTS	HYTY	TY	VA	DOA1	DOA2	OLA1	DLA	OLA2	LA	SPs	SIDs	*o*-DPH	TPH
VOOA	0	0.37 ab	368 a	1.17 ab	5.93 bcde	3.48 def	1.95 b	1.50 g	72.4 a	5.25 a	52.5 a	20.8 a	7.52 a	11.4 cde	160 a	106 a	171 a
VOOA	1	0.34 a	332 ab	1.19 a	6.22 bc	3.73 de	2.01 ab	8.87 c	41.7 b	3.42 cd	31.7 b	16.9 b	7.08 ab	12.0 cd	110 b	77.1 b	122 b
VOOA	2	0.35 a	316 b	1.05 ab	7.51 ab	4.83 cd	2.05 a	11.81 b	31.2 c	3.90 bc	32.4 b	15.0 b	6.34 b	14.4 bc	101 c	69.4 c	115 b
VOOA	6	0.18 bc	223 c	0.93 abc	6.37 abcd	8.02 b	2.06 a	13.92 a	8.12 ef	4.28 b	30.1 bc	7.97 cd	4.69 c	16.4 ab	69.1 de	40.7 e	85.6 c
VOOA + Cu	6	0.15 bc	176 cd	0.89 bc	4.43 cdef	12.43 a	2.05 a	5.36 de	4.40 ef	3.99 bc	26.5 cd	5.06 cde	4.71 c	18.9 a	50.0 fg	23.2 fg	68.9 d
VOOB	0	0.18 bc	176 cd	0.70 cd	3.44 def	2.65 ef	1.51 d	1.51 g	33.0 c	2.76 ef	23.6 d	9.07 c	5.02 c	7.60 ef	74.9 d	49.8 d	82.5 c
VOOB	1	0.18 b	174 d	0.67 cd	5.83 bcd	3.28 ef	1.80 c	4.16 ef	21.7 d	2.41 ef	18.7 e	7.44 cd	4.71 c	10.9 de	59.2 ef	41.6 e	70.1 d
VOOB	2	0.15 c	133 e	0.43 de	8.66 a	8.78 b	1.78 c	5.20 de	7.80 e	2.15 fg	14.0 f	6.41 cd	3.26 d	19.2 a	38.8 h	30.2 f	58.0 e
VOOB	6	0.08 d	90.3 f	0.40 e	3.01 ef	5.18 c	1.53 d	6.81 d	5.03 ef	2.98 de	15.7 ef	3.89 de	3.32 d	9.72 def	37.7 gh	21.7 g	47.4 e
VOOB + Cu	6	0.06 de	28.3 g	0.26 ef	1.53 f	8.31 b	1.53 d	3.42 fg	1.90 f	2.40 ef	8.17 g	2.43 e	2.05 e	11.4 cde	20.4 i	11.7 h	31.7 f
VOOC[b]	0	0.03 e	41.4 g	0.11 f	1.97 f	2.11 ef	1.52 d	1.52 g	3.61 ef	1.52 g	2.18 h	2.00 e	2.03 e	5.59 f	12.9 i	10.6 h	18.5 g

[a] +Cu, copper addition. Different letters (a–e) in the same column indicate significantly different values ($p < 0.05$). [b] These determinations were carried out for VOOC only at time 0, since during heating the decrease of phenolic molecules was so drastic that it was not possible to determine them at later times.

Figure 1. TT content of the three oil samples (VOOA, VOOB, and VOOC) as a function of storage time at 60 °C.

of storage, VOOA contained about twice the amount of DOA1 with respect to VOOB.

After 6 weeks, only VOOA and VOOB to which copper had been added showed a brown precipitate, which was more abundant in the former. To explain this observation, we propose two types of interactions: phenol-copper or pectin/protein-copper. In 1994, Lercker et al. (*35*) reported the presence of dispersed and suspended particles, probably protein substances, that constitute the "veiling" of extra virgin olive oils. The methanol-washing step carried out on VOOB may reduce both the phenols and the pectins/proteins present in the dispersion−suspension of VOOA, leading to a precipitate that is quantitatively less abundant.

Tocopherol Content and Composition. Following 6 weeks of storage, the total tocopherol (TT) and particularly the α-toc content (**Table 4**) decreased about 90, 80, and 75% in VOOC, VOOB, and VOOA, respectively. In VOOC, both β-toc and γ-toc disappeared completely after 6 weeks of storage, whereas after this period their levels fell 100% in both VOOB and VOOA in the presence of copper. The consumption of TTs was more consistent in samples that contained lesser amounts of polar phenolic compounds (**Figure 1**), suggesting their role as antioxidants through donation of a phenolic hydrogen atom to lipid free radicals, by a chain-breaking electron donor mechanism. As shown in **Figure 1**, after 1 week, the TT content decreased by about 30% in all samples, although there may have been a lag phase from 1 to 2 weeks of storage that was more evident in VOOA. To explain this behavior, it can be hypothesized that polar phenols with an *ortho*-dihydroxyl structure, particularly DOA2, are able to reduce the oxidized forms of tocopherols (tocopheryl radicals and quinones).

Primary and Secondary Autoxidation Products. As stated in the Materials and Methods, the fatty acid composition was determined only for oil samples at time 0 as FAMEs. The fatty acid composition of VOOA (extra virgin olive oil used to obtain the other two samples VOOB and VOOC after phenolic reduction treatment) was not significantly different from VOOB and VOOC and showed a high oleic/linoleic ratio (14.2), suggesting a good oxidative stability of the lipid matrix (data not shown).

It is well-known that the primary derivatives of fatty acids oxidation can be evaluated by the PV test and the absorbance measurement at 232 nm, which is relative to the conjugated double bonds present in hydroperoxides (K_{232}) (see **Table 4**). During autoxidation, hydroperoxides undergo decomposition reactions that give rise to hydroxy, keto, epoxy, and epidioxy fatty acids (*36*). On the basis of literature data (*22*, *23*) and UV and MSD spectra obtained by HPLC-DAD/ESI-MSD, we were able to identify and quantify nine species of OFAs present in triacylglycerols (**Tables 1** and **4**). The keto derivatives of the

Table 4. Primary (K_{232} and PV) and Secondary (OFA) Autoxidation Products, OSI, and Tocopherol Content on Three Oil Samples Stored for 6 Weeks at 60 °C in the Presence and Absence of Copper [Mean Value (n = 3)][a]

samples	weeks	K_{232}	PV (mequiv O_2 kg^{-1} oil)	OFA (%)	OSI (h)	mg kg^{-1} oil			
						α-toc	β-toc	γ-toc	TT
VOOA	0	1.75 i	5.43 n	0.88 j	40.6 a	210 a	7.89 ab	6.10 a	224 a
VOOA	1	2.46 h	13.0 l	1.80 ij	32.2 b	147 bc	6.21 bc	4.21 cd	157 bc
VOOA	2	2.63 fgh	14.7 k	2.90 ghi	30.7 c	167 b	7.28 abc	4.90 bc	179 b
VOOA	3		23.1 g	3.93 fg					
VOOA	6	7.59 d	31.3 e	5.76 de	13.2 g	47.1 e	6.45 abc	3.21 efg	56.8 e
VOOA + Cu	6	6.72 e	28.1 f	5.14 def	6.73 j	45.6 e	<LOD	<LOD	45.6 f
VOOB	0	1.70 i	7.76 m	0.90 j	28.8 d	198 a	8.18 a	5.21 ab	211 a
VOOB	1	2.49 h	16.0 j	2.12 ij	20.6 e	150 bc	5.90 c	3.95 def	160 bc
VOOB	2	2.77 fg	18.3 i	3.46 gh	17.4 f	142 c	6.71 abc	4.09 de	153 c
VOOB	3		27.9 f	4.10 efg					
VOOB	6	7.72 d	37.9 d	6.16 d	6.38 j	34.5 e	6.01 bc	3.13 fg	43.6 e
VOOB + Cu	6	17.7 ba	154 b	15.5 b	0.00 k	19.9 f	<LOD	<LOD	19.9 f
VOOC	0	1.38 i	7.84 m	0.91 j	17.6 f	196 a	8.12 a	5.21 ab	209 a
VOOC	1	2.54 gh	16.4 j	2.29 hij	11.1 h	132 cd	6.41 abc	4.23 cd	142 cd
VOOC	2	2.83 f	20.3 h	3.99 fg	7.85 i	110 d	6.09 bc	3.93 def	120 d
VOOC	3		32.0 e	4.33 efg					
VOOC	6	11.1 c	81.4 c	10.4 c	0.20 k	20.0 e	<LOD	<LOD	20.0 e
VOOC + Cu	6	19.5 a	171 a	17.3 a	0.00 k	19.8 e	<LOD	<LOD	19.8 e

[a] Different letters (a–n) in the same column indicate significantly different values ($p < 0.05$). +Cu, copper addition; α-toc, LOD = 0.07 μg mL^{-1}; β-toc, LOD = 0.06 μg mL^{-1}; and γ-toc, LOD = 0.03 μg mL^{-1} [mean values ($n = 3$)].

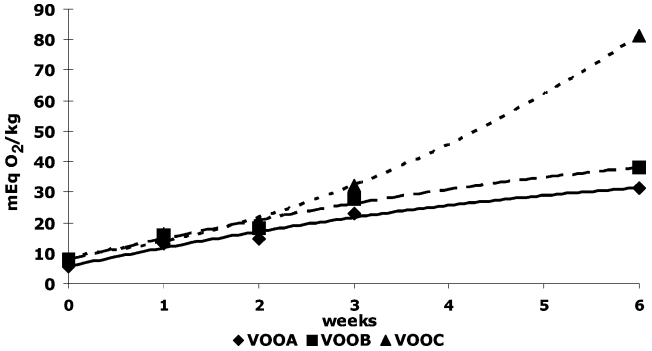

Figure 2. PV values of the three oil samples (VOOA, VOOB, and VOOC) as a function of storage at 60 °C.

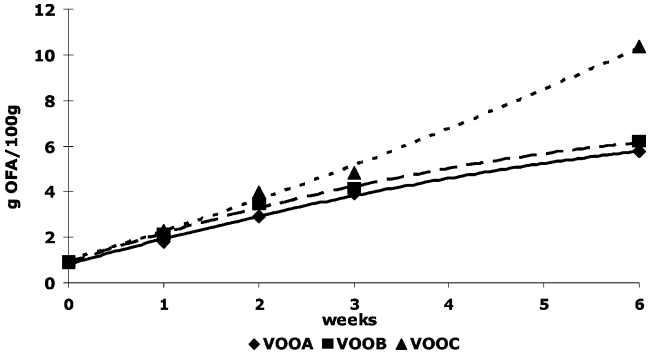

Figure 3. OFA values of the three oil samples (VOOA, VOOB, and VOOC) as a function of storage at 60 °C.

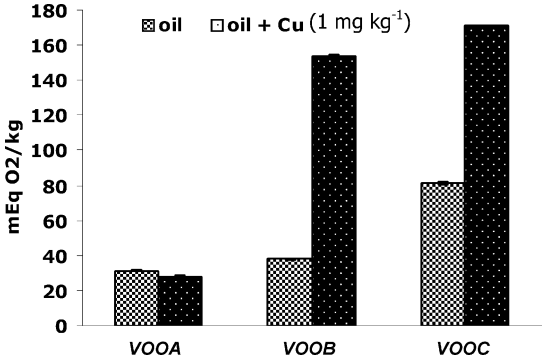

Figure 4. Histogram showing PV data of samples in the presence and absence of copper after 6 weeks of storage at 60 °C.

fatty acids were the most abundant OFAs in all oil samples, suggesting that the hydroperoxides tend to stabilize themselves (*36*). As shown in **Table 1**, the 1, 3, 3a, 3b, 3c, 5, and 5a peaks were relative to keto-linolenic acid, four isomeric forms of keto-linoleic acid and two isomers of keto-oleic acid, respectively; the isomers vary according to the position of the oxy-group on the carbon chain. Moreover, the hydroxy-linoleic (peak 2) and hydroxy-oleic acid (peak 4) were also found in low quantities. The overlapping of chromatographic traces (see **Figure 2** available in the Supporting Information) demonstrates the large increase of isomeric forms of keto-linoleic acid during oil storage, particularly in the presence of copper ions (**Table 4**). **Figures 2** and **3** show the continuous increment of PV and OFA values plotted against time, particularly in the traces corresponding to VOOC. After 2 weeks of storage, the differences for PV and OFA values among the three oils were statistically significant (**Table 4**). The result was clearly related to the different polar phenolic contents; in fact, the low amounts of phenols in VOOC were not able to impede the oxidative process, and the radical generation rate was too high for scavenging by antioxidants. Copper addition to the oils highlighted these different behaviors (see **Figures 4** and **5**): Drastic increases of PV and OFA after 6 weeks of storage in the presence of copper were observed for VOOB and VOOC samples without copper (**Table 4**). However, VOOA did not show significant differences in the presence and absence of copper. The trend of K_{232} values for the oil samples closely followed those of PV and OFA, whereas it was inverse with respect to tocopherols content, according to Deiana et al. (*37*). It was clear that copper promoted autoxidation, but polar phenolic components, intrinsic to extra virgin olive oil, acted as antioxidants. According to Rovellini and Cortesi (*22*), OFA percentages ranging from 2 to 4% are typical for extra virgin olive oil stored from 2 to 18 months at room temperature, while oil samples characterized by a total OFA higher than 4% must be considered "expired". After 1 week at 60 °C, the maximum OFA content determined was 2%. However, when the storage period exceeded 3 weeks, the OFA

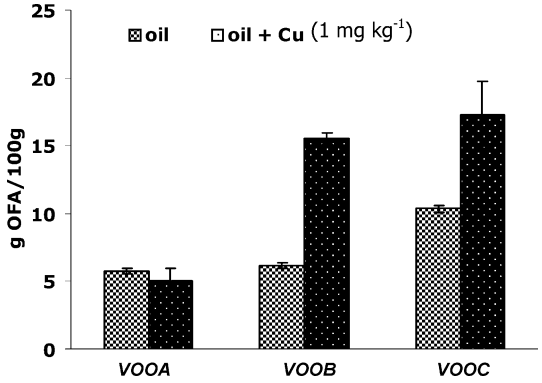

Figure 5. Histogram showing the OFA of samples in the presence and absence of copper after 6 weeks of storage at 60 °C.

values of oils increased above 4% and this was accepted as expired. These data were in good agreement with PV and K_{232} values, which were also higher than the limits established by EEC Regulation 2568/91 (*21*) for extra virgin olive oil ($\leq$20 mequiv of active oxygen per kg of oil and $\leq$2.50 K_{232}, respectively). As expected, a very good correlation was found between OFA and PV ($r = 0.97$, $p < 0.001$) as well as between OFA and K_{232} ($r = 0.98$, $p < 0.001$). Furthermore, a significant negative correlation was found between OFA and OSI ($r = -0.71$, $p < 0.001$), and the increase of OFAs caused a lower stability of oil under accelerated conditions of oxidation.

Oxidative Stability. At the beginning of the experiment, notable decreases in the OSI time were seen among the three samples, which were about 30 and 60% lower for VOOB and VOOC, respectively, as compared to VOOA; this is in good agreement with their phenolic contents (**Table 3**). Some authors (*38*) have estimated that the contribution of phenolic compounds to virgin olive oil stability is at least 30%. Considering these data, it is worthwhile underlining that the phenolic fraction also showed a significant antioxidant capacity at high temperatures (110 °C) (*39*). The behavior of oil samples with respect to the oxidative process during thermal storage (60 °C) was monitored by both the determination of the oxidation products and the antioxidant molecule trends. The stability of virgin olive oil can exceed 18 months; hence, accelerated methods are generally employed to estimate the trends of the oxidative process in a relatively short period of time. Significant differences (in hours) among the OSI time values of the three samples (VOOA, VOOB, and VOOC) were observed, but fewer differences considering the trend for this parameter during storage were seen (**Table 4**). At the starting time, the OSI time values for the three samples were rather different: 40.6, 28.8, and 17.6 h for VOOA, VOOB, and VOOC, respectively. Taking into account that the qualitative and quantitative compositions in fatty acids and tocopherols were very similar, the differences in oxidative stability can be linked to the phenol content of oils. As expected, good correlations were found between DOA2, DLA, and OLA2 phenolic compounds and the oxidative stability of oil samples expressed as OSI time ($r = 0.91$, $r = 0.87$, and $r = 0.89$, respectively; $p < 0.001$). The rate of linear decrements of OSI time values was similar (−4.5, −3.6, and −2.3 for VOOA, VOOB, and VOOC, respectively). For VOOC, at 6 weeks of storage, the OSI time was close to zero, while for VOOB this value was achieved only after copper had been added. The VOOA sample did not reach this OSI time value even after 6 weeks in the presence of copper.

Antioxidant Activity. The antioxidant activity of the phenolic fraction of virgin olive oils is mainly due to radical scavenging; this was confirmed by investigating the ability of the polar extracts to scavenge $ABTS^{\bullet+}$ radicals. Many authors have frequently studied the ability of antioxidant molecules or extracts to scavenge some free radicals, and in this regard, several stable, colored, free radicals ($DPPH^{\bullet}$ and $ABTS^{\bullet}$) are widely used due to their intensive absorbance in the visible region. In this case, the H-donating activity is determined. The trolox (hydrophilic analogue of vitamin E) equivalent antioxidant capacity (TEAC) assay (*40*) is frequently applied to assess the amount of radicals that can be scavenged by an antioxidant, i.e., the antioxidant capacity (*41*). This approach enabled the comparison with values derived from other assays (*42*, *43*). In the most recent version of this assay, an antioxidant is added to a preformed ABTS radical ($ABTS^{\bullet+}$) solution, and after a fixed time period, the remaining $ABTS^{\bullet+}$ is quantified spectrophotometrically (*28*, *44*, *45*). The reduction in $ABTS^{\bullet+}$ concentration, induced by a certain concentration of antioxidant, is related to that of trolox and gives the TEAC value of the antioxidant. The assay is rapid and easy and correlates with the total content of phenols and *o*-diphenols (*46*). As shown in **Table 3**, the antiradical power of samples at the start time decreased in the following order: VOOA > VOOB > VOOC according to their phenolic content. During storage, significant differences in terms of ABTS, TP, and TPH values were observed between VOOA and VOOB. The antioxidant activity of extracts was highly correlated with TP, TPH, and SIDs ($r = 0.96$, $r = 0.94$, and $r = 0.93$, respectively; $p < 0.001$.) When copper was added to the oil samples, the antiradical capacity was significantly reduced for VOOB to a TP content of about 70%. Despite this, the phenolic fraction of VOOA in the presence of metal did not show significant reduction in terms of its antioxidant power and TP content, whereas in the presence or absence of copper the TPH and SIDs contents were significantly different. The antioxidant activity of DOA2, DLA, and OLA2 phenolic compounds has already been evaluated in previous investigations in which they were able to prolong the shelf life of virgin olive oil (*30*, *32*).

IB. IB values followed the same pattern as TP content ($r = 0.97$; $p < 0.001$), and it is generally accepted that the phenolic fraction of virgin olive oil is mainly responsible for its bitter attributes. In particular, some authors (*1*, *2*) suggest that the main contributors to virgin olive oil bitterness are decarboxymethyl-oleuropein aglycon, decarboxymethyl-ligstroside aglycon, and oleuropein aglycon. Statistical analysis showed good correlations between the IB values and the content of DOA2, DLA, and OLA2 ($r = 0.86$, $r = 0.93$, and $r = 0.96$, respectively; $p < 0.001$), respectively.

All of the above data confirmed the important role that polar phenolic compounds play in extra virgin olive oil, and among these, the secoiridoid derivatives show a large capacity to protect extra virgin olive oil from oxidative degradation. A continuous increment of primary and secondary oxidation products particularly in the olive oil samples with lower phenolic content was observed during storage. The great increase of isomeric forms of ketolinoleic acid was also observed, especially in the presence of copper. While it is clear that copper promoted autoxidation, the polar phenolic components, characteristic of extra virgin olive oil, acted as antioxidants. During storage, the secoiridoids underwent considerable degradation, while a significant increase of the structural isomer (DOA1) of decarboxymethyl-oleuropein aglycon was seen; moreover, the content of simple phenols, especially tyrosol, increased depending on the hydrolysis reactions of the secoiridoids. The tocopherols were more quickly consumed in the oils that had the lowest amounts of *o*-DPHs (VOOB and VOOC); in VOOA, where the contents of *o*-DPHs, and particularly decarboxymethyl-oleu-

ropein aglycon (DOA2), were higher, it can be hypothesized that these compounds are able to reduce the oxidized forms of tocopherols. It appears that DOA1 may possess a copper-chelating ability as its levels decreased only after the metal was added, an aspect that merits further investigation. From the above, it can be concluded that more chemical study of the brown precipitate formed during virgin olive oil storage in the presence of copper is necessary for a better evaluation of potential phenol−metal interactions.

Supporting Information Available: Three figures of experimental design (**1**) and chromatograms of OFA (**2**) and phenolic fraction (**3**) of several samples. This material is available free of charge via the Internet at http://pubs.acs.org.

LITERATURE CITED

(1) Andrewes, P.; Busch, J. L. H. C.; De Joode, T.; Groenewegen, A.; Alexandre, H. Sensory properties of virgin olive oil polyphenols: Identification of deacetoxy-ligstroside aglycon as a key contributor to pungency. *J. Agric. Food Chem.* **2003**, *51*, 1415−1420.

(2) Gutiérrez-Rosales, F.; Ríos, J. J.; Gómez-Rey, M. L. Main polyphenols in the bitter taste of virgin olive oil. Structural confirmation by on-line high-performance liquid chromatography electrospray ionization mass spectrometry. *J. Agric. Food Chem.* **2003**, *51*, 6021−6025.

(3) Hamdi, H. K.; Castellon, R. Oleuropein, a nontoxic olive iridoid, is an antitumor agent and cytoskeleton disruptor. *Biochem. Biophys. Res. Commun.* **2005**, *334*, 769−778.

(4) Beauchamp, G. K.; Keast, R. S. J.; Morel, D.; Lin, J.; Pika, J.; Han, Q.; Lee, C.; Smith, A. B.; Breslin, P. A. S. Ibuprofen-like activity in extra-virgin olive oil. *Nature* **2005**, *437*, 45−46.

(5) Carrasco-Pancorbo, A.; Cerretani, L.; Bendini, A.; Segura-Carretero, A.; Gallina-Toschi, T.; Fernandez-Gutiérrez, A. Analytical determination of polyphenols in olive oils. *J. Sep. Sci.* **2005**, *28*, 837−858.

(6) Carrasco-Pancorbo, A.; Cerretani, L.; Bendini, A.; Segura-Carretero, A.; Del Carlo, M.; Gallina Toschi, T.; Lercker, G.; Compagnone, D.; Fernandez-Gutierrez, A. Evaluation of the antioxidant capacity of individual phenolic compounds in virgin olive oil. *J. Agric. Food Chem.* **2005**, *53*, 8918−8925.

(7) Roginsky, V.; Lissi, E. A. Review of methods to determine chain-breaking antioxidant activity in food. *Food Chem.* **2005**, *92*, 235−254.

(8) Huang, D.; Ou, B.; Prior, R. L. The chemistry behind antioxidant capacity assays. *J. Agric. Food Chem.* **2005**, *53*, 1841−1856.

(9) Prior, R.; Wu, X.; Schich, K. Standardized methods for the determination of antioxidant capacity and phenolics in foods and dietary supplements. *J. Agric. Food Chem.* **2005**, *53*, 4290−4302.

(10) Decker, E. A.; Warner, K.; Richards, M. P.; Shahidi, F. Measuring antioxidant effectiveness in food. *J. Agric. Food Chem.* **2005**, *53*, 4303−4310.

(11) La Pera, L.; Lo Curto, S.; Visco, A.; La Torre, L.; Dugo, G. Derivative potentiometric stripping analysis (dPSA) used for the determination of cadmium, copper, lead and zinc in sicilian olive oils. *J. Agric. Food Chem.* **2002**, *50*, 3090−3093.

(12) Lo Coco, F.; Ceccon, L.; Ciraolo, L.; Novelli, V. Determination of cadmium(II) and zinc(II) in olive oils by derivative potentiometric stripping analysis. *Food Control* **2003**, *14*, 55−59.

(13) Angerosa, F.; Di Giacinto, L. Oxidation des huiles d'olive vierges par les metaux: Manganese et nickel. Note I. *Rev. Fran. Corps Gras* **1993**, *40*, 41−48.

(14) Benjelloun, B.; Talou, T.; Delmas, M.; Gaset, A. Oxidation of rapeseed oil: Effect of metal traces. *J. Am. Oil Chem Soc.* **1991**, *68*, 210−211.

(15) Yamamoto, K.; Niki, E. Interaction of α-tocopherol with iron: Antioxidant and prooxidant effects of α-tocopherol in the oxidation of lipids in aqueous dispersions in the presence of iron. *Biochim. Biophys. Acta* **1988**, *958*, 19−23.

(16) Keceli, T.; Gordon, M. H. The antioxidant activity and stability of the phenolic fraction of green olives and extra virgin olive oil. *J. Sci. Food Agric.* **2001**, *81*, 1391−1396.

(17) Keceli, T.; Gordon, M. H. Ferric ions reduce the antioxidant activity of the phenolic fraction of virgin olive oil. *J. Food Sci.* **2002**, *67*, 943−947.

(18) Paiva-Martins, F.; Gordon, M. H. Interactions of ferric ions with olive oil phenolic compounds. *J. Agric. Food Chem.* **2005**, *53*, 2704−2709.

(19) Briante, R.; Febbraio, F.; Nucci, R. Antioxidant properties of low molecular weight phenols present in the mediterranean diet. *J. Agric. Food Chem.* **2003**, *51*, 6975−6981.

(20) Christie, W. W. The preparation of derivatives of fatty acids. In *Gas Chromatography and Lipids*; The Oily Press: Ayr, Scotland, 1998; pp 64−84.

(21) Commission Regulation 2568/91, July 11. *Off. J. Eur. Commun.* **1991**, *L248*, 1−82.

(22) Rovellini, P.; Cortesi, N. Oxidative status of extra virgin olive oils: HPLC evaluation. *Ital. J. Food Sci.* **2004**, *16*, 335−344.

(23) Rovellini, P.; Cortesi, N.; Fedeli, E. Profilo ossidativo e struttura chimica dei prodotti di ossidazione dei trigliceridi mediante HPLC-ES-MS. *Riv. Ital. Sost. Grasse* **1998**, *75*, 57−70.

(24) Pirisi, F. M.; Cabras, P.; Falqui Cao, C.; Migliorini, M.; Mugelli, M. Phenolic compounds in virgin oliv oil. 2. Reappraisal of the extraction, HPLC separation, and quantification procedures. *J. Agric. Food Chem.* **2000**, *48*, 1191−1196.

(25) Singleton, V. L.; Rossi, J. A. Colorimetry of total phenolics with phosphomolybdic-phosphotungstic acid reagents. *Am. J. Enol. Vitic.* **1965**, *16*, 144−158.

(26) Rotondi, A.; Bendini, A.; Cerretani, L.; Mari, M.; Lercker, G.; Gallina Toschi, T. Effect of olive ripening degree on the oxidative stability and organoleptic properties of cv. Nostrana di Brisighella extra virgin olive oil. *J. Agric. Food Chem.* **2004**, *52*, 3649−3654.

(27) Gutiérrez Rosales, F.; Perdiguero, S.; Gutiérrez, R.; Olias, J. M. Evaluation of the bitter taste in virgin olive oil. *J. Am. Oil Chem. Soc.* **1992**, *69*, 394−395.

(28) Re, E.; Pellegrini, N.; Proteggente, N.; Pannala, A.; Yang, M.; Rice-Evans, C. Antioxidant activity applyng an improved ABTS radical cation decolorization assay. *Free Radical Biol. Med.* **1999**, *26*, 1231−1237.

(29) Gallina Toschi, T.; Cerretani, L.; Bendini, A.; Bonoli-Carbognin, M.; Lercker, G. Oxidative stability and phenolic content of virgin olive oil: An analytical approach by traditional and high-resolution techniques. *J. Sep. Sci.* **2005**, *28*, 859−870.

(30) Baldioli, M.; Servili, M.; Perretti, G.; Montedoro, G. F. Antioxidant activity of tocopherols and phenolic compounds of virgin olive oil. *J. Am. Oil. Chem. Soc.* **1996**, *73*, 1589−1593.

(31) Cerretani, L.; Bendini, A.; Rotondi, A.; Mari, M.; Lercker, G.; Gallina Toschi, T. Evaluation of the oxidative stability and organoleptic properties of extra-virgin olive oils in relation to olive ripening degree. *Prog. Nutr.* **2004**, *6*, 50−56.

(32) Morelló, J. R.; Motilva, M. J.; Tovar, M. J.; Romero, M. P. Changes in commercial virgin olive oil (cv. Arbequina) during storage, with special emphasis on the phenolic fraction. *Food Chem.* **2004**, *85*, 357−364.

(33) Pagliarini, E.; Zanoni, B.; Giovanelli, G. Predictive study on Tuscany extra virgin olive oil stability under several commercial condition. *J. Agric. Food Chem.* **2000**, *48*, 1345−1351.

(34) Brenes, M.; Garcia, A.; Garcia, P.; Garrido, A. Acid hydrolysis of secoiridoid aglycons during storage of virgin olive oil. *J. Agric. Food Chem.* **2001**, *49*, 5609−5614.

(35) Lercker, G.; Frega, N.; Bocci, F.; Servidio, G. Veiled extra virgin olive oils—Dispersion response related to oil quality. *J. Am. Oil. Chem. Soc.* **1994**, *71*, 657−658.

(36) Frankel, E. N. Lipid oxidation: Mechanism, products and biological significance. *J. Am. Oil. Chem. Soc.* **1984**, *61*, 1908−1916.

(37) Deiana, M.; Rosa, A.; Cao, C. F.; Pirisi, F. M.; Bandino, G.; Dessi, M. A. Novel approach to study oxidative stability of extra virgin olive oils: Importance of α-tocopherol concentration. *J. Agric. Food Chem.* **2002**, *50*, 4342−4346.
(38) Aparicio, R.; Roda, L.; Albi, M. A.; Gutiérrez, F. Effect of various compounds on virgin olive oil stability measured by Rancimat. *J. Agric. Food Chem*. **1999**, *47*, 4150−4155.
(39) Mateos, R.; Uceda, M.; Aguilera, M. P.; Escuderos, M. E.; Maza, G. B. Relationship of Rancimat method values at varying temperatures for virgin olive oils. *Eur. Food Res. Technol.* In press.
(40) Miller, N. J.; Diplock, A. T.; Rice-Evans, C. A. Evaluation of the total antioxidant activity as a marker of the deterioration of apple juice on storage. *J. Agric. Food Chem.* **1995**, *43*, 1794−1801.
(41) Rice-Evans, C. A.; Miller, N. J.; Paganga, G. Structure-antioxidant activity relationships of flavonoids and phenolic acids. *Free Radical Biol. Med.* **1996**, *20*, 933−956.
(42) Gil, M. I.; Tomás-Barberán, F. A.; Hesse-Pierce, B.; Holcroft, D. M.; Kader, A. A. Antioxidant activity of pomegranate juice and its relationship with phenolic composition and processing. *J. Agric. Food Chem.* **2000**, *48*, 4581−4589.
(43) Chinnici, F.; Bendini, A.; Gaiani, A.; Riponi, C. Radical scavenging activities of peels and pulps from cv. Golden Delicious apples as related to their phenolic composition. *J. Agric. Food Chem.* **2004**, *52*, 4684−4689.
(44) van den Berg, R.; Haenen, G. R. M. M.; van den Berg, H.; Bast, A. Applicability of an improved Trolox equivalent antioxidant capacity (TEAC) assay for evaluation of antioxidant capacity measurements of mixtures. *Food Chem.* **1999**, *66*, 511−517.
(45) Leong, L. P.; Shui, G. An investigation of antioxidant capacity of fruits in Singapore markets. *Food Chem.* **2002**, *76*, 69−75.
(46) Bendini, A.; Cerretani, L.; Pizzolante, L.; Gallina Toschi, T.; Guzzo, F.; Ceoldo, S.; Marconi, A. M.; Andreetta, F.; Levi, M. Phenol content related to antioxidant and antimicrobial activities of Passiflora spp. extracts. *Eur. Food Res. Technol.* **2006**, *223*, 102−109.

Received for review February 17, 2006. Revised manuscript received May 3, 2006. Accepted May 8, 2006.

JF060481R