Changes Produced in Extra-Virgin Olive Oils from cv. Coratina during a Prolonged Storage Treatment

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Abstract

Baiano A., Terracone C., Viggiani I., Del Nobile M.A. (2014): **Changes produced in extra-virgin olive oils from cv. Coratina during a prolonged storage treatment**. Czech J. Food Sci., **32**: 1–9.

Extra-virgin oil is obtained from olive fruits only by mechanical means. The quality of extra-virgin olive oils is affected mainly by hydrolytic and oxidative reactions. For this reason, the commercial shelf-life is usually no longer than 18 months. In order to investigate the effects of a prolonged storage, olives from cv. Coratina were crushed using a three phase system to produce extra virgin olive oil analysed for sensory and chemical-physical indices, phenolic profile, tocopherol content, and antioxidant activity during a 8-years storage. The oil lost its characteristics of extra-virgin after 6 years of storage, time at which the median of the defects was higher than 0 and free acidity exceeded the limit fixed for this category by the European Regulation whereas the stability against oxidation persisted for a longer period due to the high concentration of oleuropein derivatives. A strong positive linear correlation was observed between the phenolic content and antioxidant activity measured according to the ABTS$^{+\cdot}$ to indicate a noticeable radical scavenging ability of phenolic compounds.

Keywords: aging; antioxidant activity; olive oil; hydrolysis; oxidation; phenolic compounds

Extra virgin olive oil is obtained only from the olive, the fruit of the olive tree, using solely mechanical or other physical means, in conditions, particularly thermal conditions, which do not alter the oil in any way. It has not undergone any treatment other than washing, decanting, centrifuging, and filtering (International Olive Oil Council 2003).

It is appreciated for the presence of phytochemicals such as phenolic compounds, known for their anticancer, antioxidant, anti-inflammatory properties (Bendini *et al.* 2007), influence on the sensory characteristics of bitterness and pungency (Andrewes *et al.* 2003; Gutiérrez-Rosales *et al.* 2003), and contribution, together with monounsaturated fatty acids, to the stability to oxidation (Lerma-García 2009a). Nevertheless, some minor compounds of virgin olive oils such as free fatty acids and photosensitisers, which are not present in other oils since they are eliminated during refining, are pro-oxidant agents and consequently reduce the stability of virgin olive oils (Velasco 2002).

Lipid oxidation is the main cause of the reduction of extra-virgin olive oil quality and seriously affects its shelf-life (Gómez-Alonso *et al.* 2007). Autoxidation and photosensitised oxidation of lipids derive from the interaction of the fatty acids of the triacylglycerols with triplet and singlet oxygens, respectively, although the initiation of lipid oxidation is due mostly to the decomposition of hydroperoxides catalysed by the presence of traces of transition metals or by exposure to light and high temperature (Choe & Min 2006). The result is that a series of compounds are formed causing rancidity and off-flavours, and finally consumer rejection (Lerma-García 2009b).

During storage or heating treatments of oils, the phenolic compounds act as antioxidants and thus undergo a series of reactions that determine substantial changes of the phenolic profile and of the total antioxidant power. According to several authors (Carrasco-Pancorbo *et al.* 2007; Boselli *et al.* 2009), the most important reactions include:

appearance of oxidation phenolics; lysis of complex phenols with the consequent increase of low molecular weight phenolics; increase of the dialdehydic forms of decarboxymethyl oleuropein aglycone and decarboxymethyl ligstroside aglycone. In the course of the oxidation reaction, minor components are degraded with a loss of nutritional value.

The oxidative evolution of virgin olive oils has been object of a number of studies but, as a consequence of the relatively long shelf-life of these products, the induction period of the oxidation reaction has been generally investigated by accelerated methods (Gallina-Toschi *et al.* 2005; Carrasco-Pancorbo *et al.* 2007; Lerma-García 2009a; Issaoui *et al.* 2011) that allow to made comparative studies, to investigate the effects of specified variables on the oxidative stability of oil, or to identify the oxidised products. However, the oxidation process that occurs under drastic conditions follows the kinetics different from those typically occurring at room temperature and demonstrates no satisfactory correlation with extra-virgin olive oil shelf-life. Very few researches concerned the evolution of the quality parameters and shelf-life during prolonged time (beyond 24 months) even though it is well known that, in the traditional producer countries, in the better vintages, people usually purchase great amounts of olive oils which are then stored for more years in glass mainly in steel tanks, but sometimes in glass demijohns located in a cellar (dark conditions and temperatures always below 20°C). Degradative phenomena can happen in olive oil during prolonged storage but their trends depend on the keeping conditions, being slower in dark conditions, at temperatures bellow 25°C, and in the absence of or under a reduced headspace.

The present study investigated the changes of oxidation indices, phenolic profile, and antioxidant activity of extra-virgin olive oils from cv. Coratina an important Italian cultivar, during 8 years of storage.

MATERIAL AND METHODS

Oil samples. Extra-virgin olive oil samples were obtained from healthy olive fruits (*Olea europaea* L.) of Coratina cultivar grown in a grove located in the countryside near Cerignola (Apulia, Italy), by a three phases system. The production was repeated three times and the obtained extra-virgin oils were filled in 1-l dark glass bottles that were sealed leaving a headspace of no more than 2.5 mm^3 and stored in a basement at a temperature ranging from 15 to 20°C and in the dark. In the production, the oils were

characterised by the following values: carotenoids 6.20 ± 0.15 mg/kg, chlorophyll 10.10 ± 0.10 mg/kg, fatty acid composition: C16:0 – 12.0 ± 1.1; C16:1 – 0.9 ± 0.3; C18:1 – 74.5 ± 1.5; C18:2 – 5.1 ± 2.3; C18:3 – 0.5 ± 0.1. The analyses were performed over the course of 8-year storage. In particular, 3 bottles deriving from each extraction were withdrawn and analysed after 0, 4, 6, 7, and 8 years.

Organoleptic assessment of the oils. The sensory analysis was performed by a group of eight tasters selected and trained as a panel according to the International Olive Oil Council (1996).

Quality indices. Acidity, peroxide value, and spectrophotometric indices (K_{232}, K_{270}, and ΔK) were determined according to the AOCS methods (2003).

Extraction of phenolic compounds. Phenols were recovered from extra-virgin olive oils by liquid-liquid extraction using methanol as the solvent and following the procedure reported in Montedoro *et al.* (1992), opportunely modified. 2 ml of methanol/water (70:30, v/v) and 2 ml of hexane were added to 5 g of virgin olive oil and mixed with a Vortex (Velp Scientifica, Usmate, Italy) for 10 minutes. The hydroalcoholic phase containing phenolics was separated from the oily phase by centrifugation (4032 *g*, 4°C, 10 min). The hydroalcoholic phase was collected and submitted to another centrifugation (30 474 *g*, 4°C, 5 min). Finally, hydroalcoholic extract was recovered with a syringe and then filtered though a 0.45-µm nylon filter (DISMIC-13NP, Advantec; Toyo Roshi Kaisha, Ltd., Tokyo, Japan) before analysis.

Total phenolic content. The total phenolic content was measured at 765 nm through an UV-visible spectrophotometer (Varian Cary 50 SCAN; Varian, Palo Alto, USA) according to the Folin-Ciocalteu method as reported by Singleton and Rossi (1965). Quantification was based on a standard curve constructed with 50-100-200-400-600-800-1000 mg/l gallic acid (ExtraSynthese, Genay, France) prepared in an aqueous solution of methanol (70%). The total phenolic content was expressed as mg gallic acid equivalents/kg oil.

HPLC phenolic profile. The HPLC analysis of the phenolic extracts was carried out according to Previtali *et al.* (2003), using a HPLC binary system consisting of a degasser mod. G1322A, a binary pump mod. G1312A, an autosampler mod. G1329A equipped with a 20-µl loop, and a diode array detector mod. G1315D (Agilent, Santa Clara, USA). The stationary phase was a Nova-Pack C18 analytical column (150 × 3.9 mm *i.d.*) with a particle size of 4 µm (Waters, Milford, USA). Diode array detection

of phenolic compounds was carried out at 280 nm. Spectra were recorded at wavelengths between 240 and 380 nm. The mobile phases for chromatographic analysis were (A) 2% acetic acid in water and (B) methanol:acetonitrile (1:1, v/v) at a constant flow rate of 1 ml/minute. The gradient program of the solvent was as follows: 100–70% A in 30 min, 70–50% A in 15 min, 50–40% A in 10 min, 40–0% A in 10 min, 0% for 5 min, and 0–100% A in 5 minutes. The identification of some phenolic components was carried out comparing the peak retention times with those obtained by injection of pure standards (hydroxytyrosol and tyrosol, purchased at Extrasynthese, Genay Cedex, France; vanillin, purchased at Sigma-Aldrich, Milan, Italy) and analysing the obtained spectra. The other phenolics were tentatively identified on the basis of the studies reported in the literature (Lee *et al.* 1995; Brenes *et al.* 2000; Gómez-Alonso *et al.* 2002, 2007; Morello *et al.* 2004; Gómez-Rico *et al.* 2008). Quantification of phenolic compounds was performed according to the method of the external standard (gallic acid, Extrasynthese) and on the basis of the response factors, which were determined taking into account the recovery percentages of the phenolic compounds and the external standard. The lower recovery percentages were the following: *p*-HPEA-EDA (decarboxymethyl ligstroside aglycone) 76.9%, pinoresinol 77.5%, 3,4-DHPEA-EDA (decarboxymethyl oleuropein aglycone) 78.5%, hydroxytyrosol 78.0%, tyrosol 80.0%, and gallic acid 82.0%. The other compounds revealed recovery percentages within the range of 85–95%. The response factor was calculated as the ratio between the peak area in the sample and the peak area of the standard multiplied by the response factor of the standard.

Tocopherol content. Tocopherol content was analysed according to the IUPAC 2432 method (IUPAC 1992); 1.5 g of oil were dissolved in 0.5% isopropanol in *n*-hexane to 10 ml. The chromatographic separation was performed using the already described HPLC binary system. The stationary phase was a Lichrosphere Si60 column (250 mm length, 4.6 mm *i.d.*, 5 mm particle size; Merck, Darmstadt, Germany). The mobile phase was 0.5% isopropanol in *n*-hexane at a constant flow rate of 1 ml/minute. The absorbance was measured at 295 mm. Tocopherols were identified comparing the retention time with those of pure standards. Quantification was performed by external standard calibration curves. The results were expressed as mg of tocopherol per kg of oil.

Evaluation of the antioxidant activity. The antioxidant activity of the oil phenolic extracts was evaluated according to 3 tests: the β-carotene-linoleate model system (Baiano *et al.* 2009), in which the antioxidant activity was measured by the ability of a compound to minimise the loss of β-carotene in an emulsified aqueous system in the presence of oxygen at a high temperature (50°C), the ABTS$^{+\cdot}$ (Re *et al.* 1999) and DPPH (Brand-Williams *et al.* 1995) assays based on the abilities of the antioxidants present in the extracts to scavenge the radical in comparison with that of Trolox, a standard antioxidant whose chemical name is 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (a water-soluble derivative of a-tocopherol).

The β-carotene-linoleate assay is based on the thermal (50°C) autoxidisation of linoleic acid and the consequent formation of the peroxidic radical that is able to scavenge hydrogen atoms from the β-carotene molecule, determining its bleaching. The β-carotene bleaching is detectable through the absorbance decrease, which is greater when the antioxidant content is low. Synthetic β-carotene type II (5 mg) (Sigma, St. Louis, USA) were dissolved in 50 ml of chloroform (J.T. Baker, Mallinckrodt Baker, Milan, Italy). 3 ml of this solution were pipetted into a round-bottom flask containing 40 mg of linoleic acid and 400 mg of Tween 40 (both Sigma, St. Louis, USA). After evaporation of chloroform to dryness under vacuum at 40°C, 100 ml of distilled water enriched with oxygen (produced by bubbling oxygen into the water for at least 1 h) were added to the flask and the mixture was shaken to form a liposome solution. 1.5 ml aliquots of this solution were pipetted into test tubes containing 20 μl of phenolic extracts which were immediately put into a water bath at 50°C. The absorbances at 470 nm of the samples and of a control containing an aqueous solution of methanol (70%) were monitored at regular intervals (15 min) on a Varian Cary 50 Scan UV-Visible spectrophotometer (Varian, Palo Alto, USA) until the complete β-carotene bleaching (after about 2 h). The absorbance decreased rapidly in the absence of antioxidants and slowly in the sample extracts. The results were expressed as mmol of the Trolox equivalent per kg of oil. The Trolox calibration curve of antioxidant power was produced by analysing methanolic solutions in the range of 2–200 μmol/l.

The 1,1-diphenyl-2-picrylhydrazyl (DPPH) method was used to measure the free radical scavenging capacity of the extracts. To 0.25 ml aliquots of phenolic extracts opportunely diluted, 1.75 ml of a $6 \times \cdot 10^{-5}$M methanolic solution of the stable organic radical DPPH (DPPH$^{\cdot}$) were added. The absorbance

at 515 nm was read at regular intervals of time until the end of the reaction (after about 2 h). The results were expressed as mmol of Trolox equivalent per kg of oil. The Trolox calibration curve of antioxidant power assay was constructed by analysing methanolic solutions in the range 20–200 μmol/l.

The 2,2-azinobis-(3-ethylbenzothiazoline)-6-sulfonic acid (ABTS$^{+\cdot}$) method measures the absorbance of the chromophore ABTS$^{+\cdot}$ radical cation at 734 nm. Briefly, ABTS$^{+\cdot}$ was dissolved in water at a 7 mmol/l concentration. ABTS$^{+\cdot}$ was produced by reacting ABTS$^{+\cdot}$ stock solution with 2.45 mmol/l potassium persulphate (final concentration) and allowing the mixture to stand in the dark at room temperature for 12–16 h before use. The ABTS$^{+\cdot}$solution was diluted with ethanol to an absorbance of 0.70 (± 0.02) at 734 nm and equilibrated at 30°C. After the addition of 1.0 ml of diluted ABTS$^{+\cdot}$ solution to 30 μl of phenolic extract opportunely diluted with ethanol, the absorbance reading was taken at 30°C exactly 1 min after the initial mixing and further up to 6 minutes. Appropriate solvent blanks were run in each assay. The results were expressed as the mmol of Trolox equivalent per kg of oil. The Trolox calibration curve of antioxidant power assay was constructed by analysing ethanolic solutions in the range 25–700 μmol/l.

Statistical analysis. From each bottle, the extractions of the phenolic compounds were carried out at least three times and the analyses were performed at least in triplicate. All other analyses were performed at least in triplicate. The averages and the standard deviations were calculated using Excel software version 11.5.1 (Microsoft, Redmond, USA). The analysis of variance (ANOVA) at $P < 0.05$ followed by the Fisher test was applied to highlight the significant differences between the samples. All the statistical analyses were made through the software STATISTICA Version 7 (Statsoft, Tulsa, USA).

RESULTS AND DISCUSSION

Concerning the results of the sensory assessment (Table 1), the median of the defects was equal to 0 and the median of the fruity attribute was above 0 in the first 6 years of storage. This means that, according to the International Olive Oil Council (1996), the oils could be classified as extra-virgin. The oils were also judged as slightly bitter (median value equal to 2). After 7 years, the tasters perceived mainly two defects, oxidation and cucumber flavour (usually perceived when an oil is hermetically packed for too long). The median of the defects was referred to the oxidation, which was the defect perceived with the strongest intensity. The oils were classified as ordinary virgin. After 8 years, the main defect was that of cucumber flavour and the oils were downgraded to the lampante virgin grade.

The evolution of acidity, peroxide value, and spectrophotometric indices during prolonged storage are illustrated in Table 1. An increase of free acidity was observed with its value exceeding the maximum limit (0.8 g/100 g) established by the EU Regulation 1989 (2003) for extra virgin olive oil at 6 years of storage. Then free fatty acids decreased. This behaviour is difficult to explain although, according to Di Giovacchino *et al.* (2002), the oxidation of the compounds formed from the decomposition of fatty acids hydroperoxides, such as aldehydes, ketones, and alcohols, that can be partially oxidised to the corresponding acids, will contribute to the increased acid levels. Concerning the oxidation status, changes in peroxide values versus storage time were observed, although the amount of 20 meq/kg was never exceeded, and observer were also increases of spectrophotometric indices, which remained within the respective limits ($K_{232} \leq 2.50$, $K_{270} \leq 0.22$, $\Delta K \leq 0.01$) up to 7 years of storage. These parameters

Table 1. Sensory results, acidity, peroxide value, spectrophotometric indices, and total phenolic content of Coratina olive oils during storage

Storage (years)	Median		Acidity (g oleic acid/100 g oil)	Peroxide value (meq O$_2$/kg oil)	K_{232}	K_{270}	ΔK	Total phenolic content (mg gallic acid eq/kg oil)
	fruity	defects						
0	5.0	0	0.6 ± 0.0[a]	11.3 ± 0.7[a]	1.80 ± 0.07[a]	0.15 ± 0.00[a]	0.001 ± 0.000[a]	250.5 ± 9.1[d]
4	3.5	0	0.7 ± 0.1[a]	12.0 ± 0.8[a]	1.85 ± 0.05[a]	0.15 ± 0.02[a]	0.001 ± 0.001[a]	190.5 ± 9.1[c]
6	1.5	0	1.2 ± 0.1[c]	12.8 ± 1.3[a]	2.00 ± 0.23[a]	0.19 ± 0.02[ab]	0.001 ± 0.001[a]	180.6 ± 17.0[c]
7	0	2	0.9 ± 0.1[b]	9.8 ± 1.6[a]	1.86 ± 0.10[a]	0.21 ± 0.01[b]	0.006 ± 0.001[b]	115.7 ± 1.6[b]
8	0	8	0.9 ± 0.1[b]	19.1 ± 1.0[b]	2.71 ± 0.40[b]	0.31 ± 0.04[c]	0.013 ± 0.001[c]	93.9 ± 11.0[a]

In columns, different letters indicate significant differences ($P < 0.05$) between the different times of storage

indicated a substantial increase of primary oxidation, measured by the peroxide value (formation of hydroperoxides) and K_{232} (formation of conjugated dienes), and of secondary oxidation, represented by K_{270} and ΔK (formation of conjugated trienes) starting from the seventh years of storage.

The total phenolic content and antioxidant activity are strongly interrelated parameters. The contribution of phenols to the virgin olive oil stability and antioxidant activity was estimated to be higher than that of other compounds, since it was quantified to approximately 50% by Gutiérrez *et al.* (2001). Therefore, a change in one of these parameters has a direct influence on the others. Tables 1, 2, and 4 show that phenolic content and antioxidant activity decreased with the storage time, whereas, during the same period, changes occurred in the concentration of each phenolic compound. It is well known that storage affects the phenolic profile as a consequence of the oxidative stress. In fact, due to their chemical properties, phenolic compounds inhibit lipid oxidation and change to their oxidised forms. According to Gómez-Alonso *et al.* (2007), the reduction of total phenolic compounds of several samples of extra-virgin olive oil during 21 months of storage ranged from 43% to 73%, being higher in the samples having higher initial phenolic contents. In the present work, since the phenolic content decrease was of about 24, 28, 54, and 65% after 4, 6, 7, and 8 years, it can be said that only little changes occurred in the antioxidant fraction during a period of storage longer than that corresponding to the labelled shelf-life (generally 18 months). The data from Tables 1 and 2 highlight noticeable differences between the total phenolic content determined through the Folin-Ciocalteau method, which was higher, and the sum of the concentrations of the individual phenolic compounds. These differences can be explained by the lack of specificity of the Folin-Ciocalteau method. Therefore, an overestimation can not be excluded of the phenolic contents because other readily oxidisable substances interfere even though the phenolic fraction was previously extracted and separated from the oils.

The phenolic compounds were tentatively identified. At the production, the most abundant ones were 3,4-DHPEA (hydroxytyrosol), *p*-HPEA-EDA (decarboxymethyl ligstroside aglycone), an unknown compound that eluted at 32 min, 3,4-DHPEA-EDA (decarboxymethyl oleuropein aglycone), 1-acetoxypinoresinol + trans-cinnamic acid, 3,4-DHPEA-EA (an isomer of the oleuropein aglycone also known as oleuropein-aglycone mono-aldehyde), and *p*-HPEA-EA

Table 2. Concentration of several phenolic compounds (in mg/kg) of Coratina olive oils during storage

Phenolic compounds identified	Assignation	Time of storage (years)				
		0	4	6	7	8
3,4-DHPEA	T.A.	17.2 ± 0.9[b]	16.7 ± 0.8[b]	9.7 ± 1.2[a]	18.3 ± 2.7[b]	8.5 ± 1.0[a]
p-HPEA	T.A.	0.5[c]	0.5[c]	0.4[b]	0.7 ± 0.1[d]	0.3[a]
Vanillin	S.	0.2[a]	0.2[a]	0.3[b]	0.1[a]	0.4[c]
p-Coumaric acid	S.	0.9 ± 0.1[b]	0.7 ± 0.3[b]	0.5 ± 0.1[a]	0.6[a]	0.6[a]
3,4-DHPEA-AC	T.A.	0.4 ± 0.1[c]	0.3[b]	0.3[b]	0.5[d]	0.2[a]
Ferulic acid	S.	0.2 ± 0.0[c]	0.1[b]	0.1[b]	0.0[a]	0.1[b]
3,4-DHPEA-EDA	S.	6.2 ± 0.3[d]	5.5 ± 0.4[c]	5.3 ± 0.7[c]	0.5 [a]	1.7[b]
Unknown peak 1 (26 min)	–	1.5 ± 0.2[b]	1.5[b]	1.5 ± 0.1[b]	0.4[a]	0.6 ± 0.1[a]
p-HPEA-AC	T.A.	0.2[c]	0.2[c]	0.1[b]	0.1[a]	0.2[c]
p-HPEA-EDA	S.	10.7 ± 1.0[c]	10.7 ± 0.8[c]	10.9 ± 2.0[c]	0.2[a]	3.4 ± 0.6[b]
1-Acetoxipinoresinol + *trans*-cinnamic acid	T.A.	2.6 ± 0.3[a]	2.5 ± 0.2[a]	6.9 ± 0.9[c]	1.8 ± 0.2[a]	5.1 ± 0.8[b]
Unknown peak 2 (32 min)	–	10.0 ± 1.3[c]	10.5 ± 0.6[c]	11.8 ± 2.2[c]	6.3 ± 0.3[a]	7.6 ± 1.4[ab]
3,4-DHPEA-EA	T.A.	2.3 ± 0.3[c]	1.4 ± 0.2[b]	1.2 ± 0.1[b]	0.3[a]	0.3[a]
p-HPEA-EA	T.A.	1.7 ± 0.1[c]	1.2 ± 0.2[b]	1.1 ± 0.2[b]	0.3[a]	1.0 ± 0.1[b]
Total		54.6	52.0	50.1	30.1	30.0

In line, different letters indicate significant differences ($P < 0.05$) between the different times of storage; T.A. – tentatively assigned; S. – standard available or produced

Table 3. Tocopherol contents (in mg/kg) of Coratina olive oils during storage

	Time of storage (years)				
	0	4	6	7	8
α-Tocopherol	240 ± 23[b]	235 ± 5[b]	201 ± 12[a]	202 ± 3[a]	202[a]
β-Tocopherol	2.2 ± 0.5[c]	1.8 ± 0.1[b]	1.7 ± 0.3[ab]	1.5 ± 0.2[a]	1.5 ± 0.1[a]
δ-Tocopherol	3.0 ± 1.6[b]	2.5 ± 1.3[ab]	2.3 ± 1.0[a]	2.3 ± 0.8[a]	2.3 ± 0.5[a]

In line, different letters indicate significant differences ($P < 0.05$) between the different times of storage

(an isomer of the ligstroside aglycone also known as ligstroside-aglycone mono-aldehyde). Oleuropein derivatives were present in higher concentrations than ligstroside derivatives during all the storage time. The compounds such as *p*-HPEA (tyrosol), vanillin, *p*-coumaric acid, 3,4-DHPEA-AC (4-(acetoxyethyl)-1,2-dihydroxybenzene), ferulic acid, and *p*-HPEA-AC (4-(acetoxyethyl)-1-hydroxybenzene) occurred in very low concentrations. According to Lavelli *et al.* (2006), degradation occurred in a similar pattern in all the olive oils although oleuropein derivatives are less stable than the corresponding ligstroside derivatives, which are the first to be oxidised thus providing oil oxidative stability (Nissiotis & Tasioula-Margari 2001). According to Carrasco-Pancorbo *et al.* (2005), hydroxytyrosol, deacetoxy oleuropein aglycone, and oleuropein aglycone are the strongest in the classification in terms of the antioxidant power, whereas (+)-pinoresinol, tyrosol, ligstroside aglycone, (+)-1-acetoxypinoresinol, and elenolic acid acted as pro-oxidants. In the present work, during the 8-year storage, the concentrations of 3,4-DHPEA, 3,4-DHPEA-AC, 3,4-DHPEA-EDA, and 3,4-DHPEA-EA decreased by about 50, 50, 72, and 87%, respectively, whereas the mean reduction of *p*-HPEA, *p*-HPEA-AC, *p*-HPEA-EDA, and *p*-HPEA-EA was equal to 40, 0, 68, and 41%, respectively. These data confirmed the results obtained in the previous studies

performed on the thermal oxidation. In the present work, both hydroxytyrosol and tyrosol decreased during a prolonged storage as a consequence of oxidation reactions, whereas it has been demonstrated that, in the first phase of storage that coincides with the usual shelf-life, they increase due to the partial non-oxidative hydrolysis which oleuropein and ligstroside undergo (Brenes *et al.* 2001). Table 3 shows the trends of the tocopherol contents during storage. About 98% of the total tocopherol content consisted of α-tocopherol. The tocopherols contents of the oils in this study showed only a slight decline during storage. In particular, at the end of the storage, the decrease of α-, β-, and δ-tocopherol was 16, 32, and 23%, respectively. These data were in agreement with the finding of Blekas *et al.* (1995). According to their work, in the presence of strong antioxidants naturally occurring in olive oil such as *o*-diphenols, α-tocopherol did not show any significant additional antioxidant effect during the period of low peroxide accumulation. Furthermore, tocopherols are able to transfer hydrogen atoms to lipids during the oxidation process resulting in tocopheroxy radicals, which are more stable than lipid peroxy-radicals and ultimately slow down the oxidation rate (Choe & Min 2006).

The antioxidant activity of oils has been measured according to three methods, ABTS⁺˙, DPPH, and β-carotene (Table 4). DPPH measures the overall

Table 4. Antioxidant activity measured using ABTS⁺˙, DPPH, and β-carotene assays of Coratina olive oils during storage (in mmol Trolox eq/kg oil)

Time of storage (years)	ABTS		DPPH		β-carotene	
0	46 421 ± 6 746[d]		35.3 ± 3.4[d]		1.1 ± 0.1[c]	
4	31 155 ± 3 402[c]	(−33%)	16.5 ± 1.6[c]	(−54%)	0.4 ± 0.1[b]	(−55%)
6	31 044 ± 5 791[c]	(−33%)	16.0 ± 2.2[c]	(−55%)	0.4 ± 0.0[b]	(−55%)
7	16 754 ± 2 700[a]	(−64%)	12.3 ± 1.7[b]	(−65%)	0.2 ± 0.0[a]	(−82%)
8	20 534 ± 3 478[b]	(−56%)	3.5 ± 0.4[a]	(−90%)	0.2 ± 0.0[a]	(−82%)

In columns, different letters indicate significant differences ($P < 0.05$) between the different times of storage; the reduction percentage of the antioxidant activity is reported between brackets

radical-scavenging activity of a sample since is not specific to any particular antioxidant component whereas ABTS⁺˙ is generally used to screen the relative radical-scavenging abilities of flavonoids and phenolics. The results deriving from the application of these methods are generally different due to the specific free radical used as a reactant. The β-carotene assay quantifies the resistance of lipid or lipid emulsions to oxidation in the presence of the antioxidant tested and, consequently, gives further different results. As expected, the antioxidant activity decreased during the prolonged storage. After 4–6 years, the antioxidant activity decreased by 1/3 according to the ABTS⁺˙ assay, and by more than 50% according to the other 2 assays. The final total phenolic content and antioxidant activity value measured through the ABTS⁺˙ assay were about 37.5% and 44%, respectively, of the initial values, due to the high presence of oleuropein derivatives.

A strong positive linear correlation was observed between phenolic content and antioxidant activity measured according to the ABTS⁺˙ assay (R = 0.998) whereas weaker linear correlations were found between total phenolics and DPPH or β-carotene assays (R = 0.873 and 0.848, respectively), and between α-tocopherol content and ABTS⁺˙, DPPH, or β-carotene assays (R = 0.787, 0.761, and 0.764, respectively). These results indicate that the contribution of phenolics to the radical scavenging activity was predominant with respect to tocopherols and carotenoids, which are another antioxidants contained in extra-virgin olive oil, and are consistent with the finding of Aparicio *et al.* (1999), who observed that the contribution of phenolic and orthodiphenolic compounds to the oil oxidative stability was around 51%, the composition of fatty acids contributed 24%, and in lower percentages α-tocopherol, carotenoids, and chlorophylls whereas none or very small effects were shown by β- and γ-tocopherols.

CONCLUSIONS

The oil maintained its characteristics of extra-virgin for a time of storage no longer than 6 years, which is anyway, a period 4 times longer than the shelf-life fixed by the European Regulation, due to the perception of defects (oxidation and cucumber flavour) and the increase in acidity over the legal limit. The oxidative stability was guaranteed by the high phenolic contents, typical of Coratina extra-virgin olive oils, and, in particular, by the high concentration of oleuropein derivatives, which were effective as radical scavengers and further prolonged the oil shelf-life. The consequence was a strong decrease of the oil antioxidant activity values, which were reduced to between 33% (ABTS⁺˙) and 55% (DPPH and β-carotene) after 6 years, and fell below 40% (ABTS⁺˙), 20% (β-carotene), and 10% (DPPH) of the initial values at the end of the considered storage time.

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