Antioxidant Capacity of Extra-Virgin Olive Oils

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ABSTRACT. In this study, the oxygen radical absorbance capacity (ORAC) of vegetable oils was investigated using a spectrofluorometric method, which measures the protection of the phenolic substances of the oil on the β-phycoerythrin fluorescence decay in comparison with Trolox. More than 97% of the phenolic substances was extracted from the oil using methanol, and the methanolic extract was then used for the ORAC and the total phenolics assay. We found a significant correlation between ORAC values of different olive oils and the total amount of phenolics. For extra-virgin olive oils, maximal ORAC values reached 6.20 ± 0.31 μmol Trolox equivalent/g, while refined and seed oils showed values in the 1–1.5 μmol Trolox equivalent/g range. Our method is useful to assess the quality of olive oils and to predict, in combination with the rancidity tests, their stability against oxidation.

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KEY WORDS: Extra-virgin olive oil, oxygen radical absorbance capacity (ORAC), peroxyl radicals, phenolic compounds.

Vegetable oils contain various antioxidants that can scavenge oxygen radicals in biological systems (1,2) and keep both the quality and the stability of the oil during prolonged storage (3,4). Among vegetable oils, extra-virgin olive oils are very rich in phenolic compounds. The most important groups of dietary phenolics are polyphenols such as flavonoids and phenolic acids. The concentrations of these substances in the olive fruits vary a great deal according to the area of production, the cultivar, the climate, the harvest time, and the storage time before milling (4,5). Other sources of variability stem from the method of production of the oil, which may be by pressure or continuous centrifugation. A lower amount of water is present in the olive pulp with the former system than the latter. Because the phenolics are found both in the oil and in the waste water, if there is a large percentage of water in the olive pulp, many phenolics are lost. Duration of the production process and the method of storing the oil also play a role in stabilizing the phenolic concentration, because light, temperature, dissolved oxygen, and free radicals are all factors that may increase the oxidation rate of phenolic compounds and fatty acids. In order to define the damage that all the above parameters cause in the oil, it is necessary to check the product for its stability against oxidation.

The analytical evaluation of the antioxidant capacity of foodstuffs may be performed by detecting the concentration of the main antioxidant molecules contained (6,7) or, alternatively, by evaluating the whole antioxidant capacity of an extract. Owing to its greater simplicity, the second approach is receiving increased attention (8,9). The oxygen radical absorbance capacity (ORAC) assay, one of the methods that falls within this category, is widely used to determine the antioxidant capacity of pure compounds or mixtures obtained from fruit juices or beverages (8). These data have interesting applications in the field of human nutrition (10).

Owing to their hydrophobic moiety, oils cannot be used in aqueous mixture, which is peculiar to this method. Turbidity of the solution hampers the spectrofluorometric measurements and does not yield reproducible results. However, since the phenolics are amphipathic molecules, it is possible to find the conditions that permit their maximal transfer in the aqueous phase.

In this study, we investigated the ORAC of vegetable oils toward the peroxyl radicals, using the method of Cao *et al.* (11). This method, found to be sensitive and accurate, allowed us to compare the oils produced by major companies with locally produced oils. Using the method of Montedoro *et al.* (4), we obtained a methanolic extract containing most of the phenolic substances of the oil. The presence of triacylglycerols or free fatty acids was excluded by the total lipid assay performed on this extract. The ORAC value was proposed as a new parameter to assess the quality and stability against oxidation of extra-virgin olive oils.

MATERIALS AND METHODS

Chemicals. Porphyridium cruentum β-phycoerythrin (B-PE) and test-combination kits for free fatty acids and total lipids were obtained from Boehringer (Mannhein, Germany). 2,2′-Azobis (2-amidinopropane) dihydrochloride (AAPH) was obtained from Polyscience (Warrington, PA). 6-Hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox) was obtained from Aldrich Chemical Co. (Milwaukee, WI). All other analytical grade chemicals were from Sigma (Milan, Italy).

Oil samples and phenolic extraction. The present study was performed on name brand olive and seed oils obtained from the supermarket and on locally produced oils supplied by small producers. The locally produced oils, named in the text as "minor brand oils," were obtained as follows. We selected, in different Italian regions, five farmers who had olives *(Olea europoea)* of different cultivars. The olives were picked and the indicated mixtures were obtained. The olives were then brought to a mill equipped with continuous cen-

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trifugation modulated systems (Pieralisi, Italy), and the oil was drawn. This whole process was concluded within 24 h after the harvest, except in the case of minor brand oil 3. That oil was produced from the olives stored for 2 wk before the milling. The oils were drawn immediately after the production and stored 24 h at 15°C in the dark before the assays. To perform the ORAC assay, 10 g of oil was diluted (1:1 wt/vol) with 80% methanol in H_2O (4), then vortexed 2 min at room temperature. The mixture was centrifuged 10 min at 5000 × *g*, and the supernatant was drawn with a Pasteur pipette. The extraction was repeated once again, the supernatants were combined, and the volume was measured. The phenolic extract was diluted with 0.075 M Na-K phosphate buffer with pH 7.0 (1:20 vol/vol), filtered through a 0.22 µm Millipore (Bedford, MA) filter when necessary, then used for the assays.

ORAC assay. The method of Cao *et al.* (11) was used with slight modifications (12). Peroxyl radicals were generated with AAPH, and B-PE was used as a detector of radical activity. When the sample had exhausted its capacity to trap peroxyl radicals, B-PE became the target of the radicals and rapidly lost its fluorescence. The area under the curve of fluorescence decay was proportional to the antioxidant capacity of the sample, and a comparative evaluation with Trolox was performed. The final reaction mixture for the assay was prepared in 10-mm-wide quartz cuvettes as follows: 1600 µL of 0.04 µM B-PE in 0.075 M Na-K phosphate buffer pH 7.0, 200 µL of diluted phenolic extract, or 200 µL of 50 µM Trolox. A blank with 200 µL of methanol, diluted 1:20 (vol/vol) with 0.075 M Na-K phosphate buffer, pH 7.0, was also used; but, this blank did not differ from the one performed with the phosphate buffer alone. The mixture was kept at 37°C in the dark, and the fluorescence was read every 5 min at 565 nm emission and 540 nm excitation, using a PerkinElmer (Norwalk, CT) LS-5 spectrophotometer. When stability was obtained, the reaction was started with the addition of 200 µL of 40 mM AAPH and the fluorescence was measured at 37°C every 5 min until zero fluorescence was detected.

The ORAC value refers to the net protection area under the curve of B-PE in the presence of the oil phenolic extract or Trolox, minus the blank. The peroxyl radical absorbing ability of the sample was determined as µmol of Trolox equivalent/g of oil. The results were calculated with the following equation:

$$\text{ORAC (µmol Trolox equivalent/g)} = kah[(S_{\text{sample}} - S_{\text{blank}})/(S_{\text{Trolox}} - S_{\text{blank}}) \quad [1]$$

where k is the final dilution factor of the methanolic extract, a is the ratio between the volume (mL) of the methanolic extract and the grams of oil, S is the area under the curve of B-PE in the presence of the sample or Trolox or the buffer solution, and h is the final concentration of Trolox expressed as nmol/mL. In each experiment, the starting fluorescence of B-PE was normalized to 100% arbitrary units to make up for the decay of the stock solution of B-PE or variations in the sensitivity of the fluorometer. The statistical analysis was carried out using analysis of variance. The statistical significance level was set at $P < 0.05$ in comparison with the control.

Other assays. Phenolic compounds were assayed by the Folin-Ciocalteu method (13) and total lipids by the Boehringer test-combination kit. Free acidity and peroxide number were determined according to the Italian Official Methods of Analysis of Oils and Fats (14). Dissolved oxygen was measured with an O_2 transmitter 4100 (Mettler-Toledo, Milan, Italy).

RESULTS AND DISCUSSION

ORAC assay. Figure 1 shows the typical fluorescence decay curves obtained with the methanolic extracts of three "minor brand" extra-virgin olive oils from The Marches region. In comparison with the Trolox control, the methanolic extracts had a rapid initial decrease of fluorescence, which was followed by a slower decay in the final stage. This pattern was commonly found both in solutions with a single antioxidant and in solutions containing a mixture of antioxidants (11,12). The ORAC method showed good reproducibility with olive oils. By repeating the measurement on the same oil, we observed a coefficient of variation of less than 10%. Moreover, by using dilutions of the extracts in the range of 200 to 2000 times, a linear plot was obtained ($R = 0.99223$, $P = 0.00082$, $n = 5$).

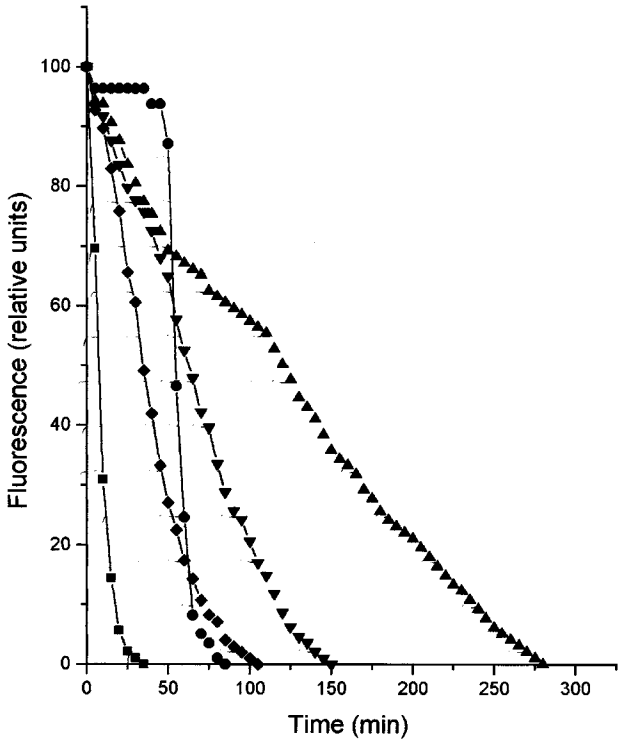

FIG. 1. Fluorescence decay curves of methanolic extracts of three minor brand oils from The Marches region (oils 1, 2, and 3, in Table 2). (■) Blank; (●) 5 µM Trolox; (▲) minor brand oil 1; (▼) minor brand oil 2; (◆) minor brand oil 3.

TABLE 1
Oxygen Radical Absorbance Capacity (ORAC) and Phenolic Values of Olive Oil Methanolic Extracts[a]

Extraction step	ORAC (μmol Trolox equivalent/g)	Total phenols mg/kg	Total phenols %[b]
1 + 2	4.83 ± 0.28	254 ± 21	98
3	0.54 ± 0.12	3.1 ± 0.6	1.2
4	0.48 ± 0.11	1.7 ± 0.5	0.6

[a]The first and second methanolic extracts of the Apulia oil were combined and assayed for the ORAC and total phenol values. Third and fourth methanolic extractions were performed and assayed for the same parameters. Results are mean ± SD of four different determinations.
[b]Percentage of compounds in the four extracts.

Tests for phenolic extraction. Table 1 shows the concentrations of phenolic compounds of consecutive methanolic extracts performed on the same oil sample. It was evident that 98% of the phenolics were recovered by the first and second extractions, whereas the third and fourth treatments had negligible phenolic content. Thus, the true ORAC value was referred to as the first + second, although a significant antioxidant capacity, on the order of 0.5 μmol Trolox equivalent/g, still remained in the third and fourth extracts. This baseline value may be accounted for by carotenoids or short-chain unsaturated aldehydes. It is very likely that small amounts of these compounds, slightly soluble in methanol, continue to be progressively extracted in each step. Because the composition and concentration of these substances in extra-virgin olive oils are very low and quite similar, their capacity to scavenge peroxyl radicals is not a variable in the measurement of the ORAC value. On the other hand, phenolics are an important variable. The same reasoning may be applied to other antioxidant molecules, which remained in the fat moiety of the oil after the extraction. Tocopherols are, in fact, strong antioxidants and are able to scavenge peroxyl radicals; however, their concentration does not change significantly in different extra-virgin olive oils, since for most of them it is in the range 190–200 mg/kg (15). The phenolic type and composition, instead, vary in olive oils. Therefore, the particular antioxidant capacity of an olive oil may be determined by the type and concentration of phenolics.

Comparison of oil samples. Table 2 shows some charac-

TABLE 2
Characteristics, Phenolics, and ORAC Values of the Extra-Virgin Olive Oils Considered in This Study

Oil type[a]	Region of production	Cultivar	Date of harvest[b]	Acidity (% of oleic acid)	Peroxides (meq peroxides/kg)	Dissolved O_2 (ppm)	Phenolics (mg/kg)	ORAC (μmol Trolox equivalent/g)	ORAC/phenolics ratio
Extra-virgin olive oils									
Minor brand oil 1	The Marches	1/3 Moraiolo, 1/3 Maurino, 1/3 Pendolino	October 10, 1999 (early)	0.42	7.2	5.9	236 ± 20	5.08 ± 0.26	0.021
Minor brand oil 2	The Marches	1/2 Raggiola, 1/2 Frantoio	December 4, 1999 (mid)	0.45	7.8	7.7	168 ± 5.9	3.60 ± 0.15[c]	0.021
Minor brand oil 3	The Marches	1/3 Raggiola, 1/3 Frantoio, 1/3 Leccino	November 2, 1999 (mid)	0.39	16.0	2.1	50 ± 6.4	1.78 ± 0.16[c]	0.035
Minor brand oil 4	Apulia	Coratina	December 10, 1999 (mid)	0.39	16.0	4.5	254 ± 21	4.83 ± 0.35	0.019
Minor brand oil 5	Umbria	Frantoio	December 2, 1999 (mid)	0.35	8.0	5.2	135 ± 11	4.12 ± 0.35	0.030
Name brand oil "fructate"	Umbria	Unknown	Unknown	0.78	5.6	6.8	210 ± 23	6.20 ± 0.31	0.029
Name brand oil 1	Tuscany	Unknown	Unknown	1.00	8.8	6.9	99 ± 6.6	4.15 ± 0.21[d]	0.041
Name brand oil 2	Tuscany	Unknown	Unknown	1.00	8.2	6.4	130 ± 13	4.55 ± 0.19[d]	0.035
Other name brand oils									
Peanut oil							1.3 ± 1	1.06 ± 0.09	0.810
Refined olive oil							10 ± 3	1.55 ± 0.08	0.155

[a]Minor brand oils were produced by small-scale local producers equipped with continuous centrifugation modulated systems. The locally produced oils were obtained from olives picked by five different farmers and brought to a mill where oil samples were drawn. The whole process was concluded within 24 h of the harvest, except the minor brand oil 3, which was produced from a mixture of olives stored for 2 wk in plastic containers before the milling. Name brand oils were obtained from the local supermarket and were produced and distributed nationally by major producers. Results are mean ± standard deviation of four different determinations.
[b]"Early" and "mid" refer to stage of maturation.
[c]Significantly different from minor brand oil 1.

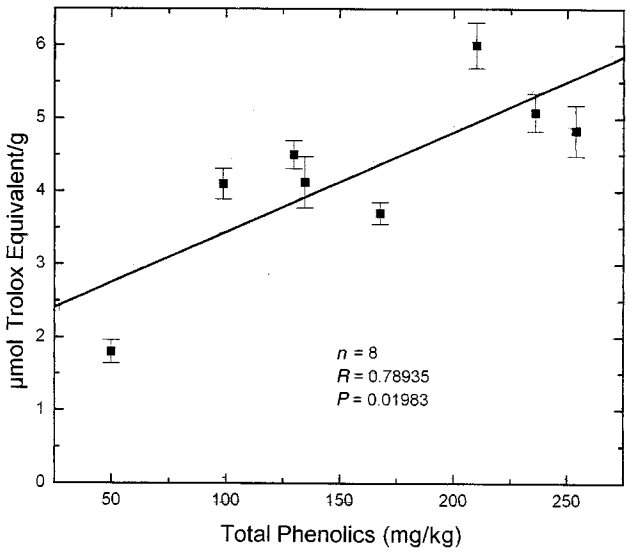

FIG. 2. Relationship between the ORAC values and the total phenolics for the minor brand and the name brand extra-virgin olive oils. The plot was drawn from the data of Table 2.

teristics of the oils used in this study together with the phenolic and ORAC values. Among the minor brand oils, the highest ORAC value was obtained with oil 1, an oil produced with equal amounts of three varieties of olives, picked in the early period of maturation and milled within 24 h. Minor brand oil 3 was produced from three olive cultivars, one of which (Leccino) was picked in the late period of maturation; then, the whole mixture of olives was stored in plastic containers 2 wk before the milling. The ORAC value and the phenolic content of this oil was low, near the value obtained with a refined oil. Three commercial oils showed significant differences in their ORAC values: the "fructate" oil showed a value 30% higher than the other two brands. By mixing (1:1 vol/vol) minor brand oil 1 and minor brand oil 3, the ORAC value obtained was 3.40 ± 0.24 µmol Trolox equivalent/g. This shows that by mixing two oils with different antioxidant capacities, the resulting ORAC value is exactly the sum of the respective antioxidant values divided by the dilution factor. Therefore, the antioxidant capacity of one oil can be increased by mixing it with oils endowed with higher ORAC values.

Concerning the phenolic compounds, we found a significant correlation ($P < 0.05$) between the total phenolics and the ORAC values. Figure 2 shows the regression line obtained with the extra-virgin olive oils of Table 2. This result, however, does not exclude the possibility that oils with phenolic contents lower than those of Table 2 could show higher ORAC values due to the particular composition of the phenolic pool. It has been demonstrated that in some cases the predominance of one phenolic compound leads to the expression of a very high ORAC value (16). In these cases, the ratio ORAC/phenolics reported in Table 2 does not seem a good indicator of the method.

The commercial seed or refined oils had very low phenolic substances and ORAC values 4–5 times lower than the "fructate" olive oil.

No significant correlation was found between the ORAC value and the peroxide number or the dissolved oxygen. It is very likely that these parameters influence the ORAC value after prolonged storage of the oil but not in the period immediately following its production.

ORAC is a reliable method for determining the total antioxidant capacity of phenolic compounds of olive oils. Among the minor brand oils, we found an apparent predominance of those produced from the drupes harvested during the early period of maturation and milled 24 h later. It is very likely that these oils represent the top level for stability during prolonged storage, and studies are in progress in our laboratory to confirm this suggestion. An olive oil made from olives harvested in the mid-period of maturation but stored for 2 wk before milling had a value significantly lower than the top level minor brand oil. Among the commercial oils, the "fructate" was characterized by the comparable ORAC value as the top level oil, while the other two brands of olive oil had lower values. Refined olive oils have values three times lower than the top level oil. The concentrations of phenolic compounds significantly correlate ($P < 0.05$) with ORAC values. Our method is fast, can be readily reproduced, and can be used for the comparative evaluation of the quality and stability against oxidation of olive oils produced from different cultivars, methods of milling, and conditions of storage.

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