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New Investigation of the Isothermal Oxidation of Extra Virgin Olive Oil: Determination of Free Radicals, Total Polyphenols, Total Antioxidant Capacity, and Kinetic Data

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As a follow-up of the research programs carried out by our group concerning the artificial isothermal rancidification process in extra virgin olive oil (EVOO), in the present work the trends of both the total antioxidant capacity and the total polyphenols concentration as well as the main kinetic parameters of the process during the thermal oxidation of EVOO were studied and compared. In addition, the possibility of evaluating the increase in radicals concentration during the thermal oxidation process using a superoxide dismutase biosensor was also studied. The present investigation concerning this important food product is highly topical as it refers to the state of alteration of the EVOO used for cooking or frying, as a function of the temperature reached.

KEYWORDS: Extra virgin olive oil; forced rancidification; free radicals; polyphenols; antioxidant capacity; kinetic analysis

INTRODUCTION

Extra virgin olive oil (EVOO) is a widely used product in the food field, in both the domestic and industrial sectors; it is used as a condiment as well as a medium for cooking foods, for example, in the Mediterranean countries for frying many food products. The process of cooking food is necessary to produce a more desirable product and also of course to make the food more easily digestible.

Moreover, there are several methods of cooking that, unfortunately, in many cases, produce significant changes in the healthfulness and salubriousness of food, such as cooking over charcoal.

An unsuitable cooking process occurs when an altered oil is used, that is, an oil used for many fryings. Unfortunately, only rather generic rules exist concerning the precautions that have to be taken to maintain a satisfactory quality level for the oil used for cooking and frying. Moreover, in view of the wide variety and diversity of the oils produced and their widespread use, as well as their multiple uses in industrial, commercial, and food production, the study of the most valuable among all the commercial olive oil products, namely, EVOO, is highly topical, especially with reference to its state of conservation and its alteration.

In the past few years, our group has devoted a large number of studies to these problems. We have recently shown that a very easy method of biological control (basically a respirometric system based on yeast cells) can be very useful for checking a toxicity index (*1*) and the maximum time during which a peanut oil or an EVOO can be subjected to thermal stress produced under different well-controlled conditions of temperature, aeration, holder type, and so on. In general, our study attempts to demonstrate how it is possible to lay the foundations for a true evaluation of the degree of oil alteration using different specific biosensors, which account, for instance, for the effect of toxicity on a simple monocellular organism (*1*), or, alternatively, the level of hydroperoxide concentration (*2*), or, in the case of EVOO, the degradation kinetics of total polyphenols (*3*). More recently, our group undertook a thorough investigation of the kinetic aspects of the thermal oxidative degradation of EVOO (*4*). In the present paper, starting from the degradation kinetics of total polyphenols, which are the main natural antioxidants contained in significant amounts in the EVOO, the question asked was whether it was possible to investigate the forced rancidification process under isothermal conditions at fixed temperatures. We therefore decided to monitor the changes in total antioxidant capacity that occur in the EVOO because of the oxidative degradation process caused by forced rancidification using a well-known spectrofluorimetric method previously

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applied to other foodstuffs (*5*) that we recently utilized in several studies (*6, 7*).

At the same time, because the occurrence of the rancidification processes in fatty acids determines the formation of hydroperoxides, produced essentially in the free radical reactions, we have also examined the possibility to detect the likely increase in free radical content in the EVOO matrix undergoing artificial rancidification using a superoxide dismutase based enzyme biosensor recently developed in our laboratory for other purposes (*8*). Lastly, it is evident that, as clearly stated in previous papers (*3, 4*), the measurements provided by the tyrosinase biosensor and the superoxide dismutase biosensor, as well as those concerning the total antioxidant capacity, represent not so much an analytical measurement in the strict sense, but rather several indices (*4*), the variation of which, which is relatively easy to measure, is found to be extremely useful in making determinations of both the stability of EVOO and the kinetic parameters of the process.

MATERIALS AND METHODS

Chemicals. Superoxide dismutase (SOD) (EC 1.15.1.1) from bovine erythrocytes, 7000 units mg^{-1}, dimethyl sulfoxide, potassium superoxide, dialysis membrane (art. D-9777), silicon oil 350 Cps Crual srl, *n*-hexane RPE, phenol RPE, β-phycoerythrin, and the inner solution of the Clark electrode, that is, phosphate buffer solution 0.067 mol L^{-1}, pH 7.5, were supplied by Sigma-Aldrich; κ-carrageenan and tyrosinase (EC 1.14.18.1) from mushroom 3216 units mg^{-1} were supplied by Fluka. Gas permeable membrane in PTFE (YSI model 5775) was supplied by Yellow Springs, and 0.8 μm filters by Sartorius AG Germany. Monobasic, dibasic potassium phosphate RPE, and chloroform RPE by were supplied by Carlo Erba Reagents, potassium chloride was supplied by Riedel-de Haën (Seelze, Germany), and 2,2′-azobis(2-amidinopropane) dihydrochloride (ABAP) was supplied by Wako Chemicals.

Samples. All of the commercial EVOO samples analyzed, stored in sealed dark glass bottles, were purchased from a local shop and were commercialized by a single supplier operating in the center of Italy, whereas the other samples were EVOO products supplied directly by a farmer from an area in northern Rome (Italy). Practically, all of the samples were produced from the same kind of polycultivar sited in the center of Italy. All of the samples, both commercial and those supplied by the farmer, were produced just under one year before and obtained by means of cold pressing using mills of the same type.

Analytical Instruments. The spectrofluorimetric measurements performed to determine the total antioxidant capacity were carried out using an Perkin-Elmer spectrofluorimeter (LS-5 model) equipped with a Perkin-Elmer recorder (model 561).

The transducer employed for the evaluation of free radicals in EVOO samples using a biosensor was an Orion model 97-08-99 electrode, suitable for measuring O_2 concentration, connected to an Orion pH-meter model SA720 and an Amen (Milan, Italy) model 868 recorder.

The transducer employed for the biosensor determination of the total polyphenols concentration in the EVOO samples was a modified amperometric electrode for oxygen supplied by Universal Sensor Inc. (New Orleans, LA; model 4000-1). Measurements were performed using a potentiostat supplied by Radelkis (model OP 960). The current was converted into potential and read off on a Mitek multimeter (model MK 5001).

The oxidative thermal degradation of the EVOO samples examined in this study took place in a silicon oil-based thermostatic bath (from 0 to 250 °C, ± 0.1) in an air stream of 140 mL min^{-1} generated by a Unistar model AIR 1000-1 pump (*3*).

Artificial Rancidification Process. The artificial oxidation of the EVOO was obtained by placing the sample in a Pyrex glass tube (containing 25 mL of sample under a constant stream of air of 140 mL min^{-1}) (*3*) immersed in a silicon oil thermostatic bath either at 98 °C, according to the method proposed by the AOM (*9*), or at the selected temperatures of 140, 160, and 180 °C, using the above apparatus, which was previously illustrated in detail (*3, 4*). During the artificial rancidification process under isothermal conditions at the above-mentioned selected temperatures, fixed amounts of the sample contained in the glass test tube were collected at regular time intervals and immediately analyzed. The determination of the total polyphenols concentration and free radicals content was carried out using an enzymatic tyrosinase biosensor and an enzymatic SOD biosensor, respectively, both applied to the crude EVOO samples and to the thermally degraded samples, whereas the antioxidant capacity was determined on the same samples using the spectrofluorimetric ORAC method.

Total Polyphenols Concentration Measured by the Tyrosinase Organic Phase Enzyme Electrode (OPEE). The biosensor method adopted was based on the oxidation of phenols to quinone catalyzed by the tyrosinase enzyme. The recorded signal was proportional to the variation of the partial vapor pressure of the oxygen dissolved in solution, which was consumed in the course of the enzymatic reaction (1):

$$\text{phenol} + O_2 \xrightarrow{\text{tyrosinase}} o\text{-quinone} + H_2O \qquad (1)$$

For this purpose, a tyrosinase OPEE was used as described in detail in previous works (*3, 10*). It was made of PTFE and capable of measuring the decrease in dissolved oxygen concentration due to the oxidation of a phenolic compound to *o*-quinone, operating in *n*-hexane. The variation of the signal recorded was proportional to the species reduced at the cathode (O_2), the amount of which was found to be dependent on the concentration of the oxidized phenol in the sample. The decision to carry out the determination of the total polyphenols in an organic solvent (*n*-hexane) was based on the very high solubility of the oil matrix in this solvent and on its very low solubility in aqueous solution (*10*). Each sample after heating was placed in an Eppendorf vial, which was cooled very rapidly by dipping it in cold water. Before measurements were performed both to reduce the viscosity of the oily sample and to facilitate its immediate dissolution in the organic solvent (*n*-hexane solution contained in the measurement cell) in which the test was performed, the sample was prediluted using the same solvent (*n*-hexane) in the ratio of 1:4 (v/v).

The biosensor measurement was carried out under constant magnetic stirring in a glass cell thermostated at 23.0 ± 0.2 °C and containing 10 mL of *n*-hexane, in which the bionsensor was immersed for the time necessary to achieve a stable signal. When the signal was constant, 25 μL of a 10^{-3} mol L^{-1} standard phenol solution in *n*-hexane was added and the corresponding signal variation was recorded. When the new stationary state was reached, a second addition of 25 μL of the standard phenol solution in *n*-hexane was made and the new corresponding variation of the signal was recorded. Finally, an addition of 50 μL of the oil sample (diluted 1:4, v/v) with the *n*-hexane solution was made, and the corresponding variation of the signal was measured. The total polyphenols concentration index (C_{EVOO}) in the sample examined was obtained using the algorithm

$$C_{EVOO} = \frac{\Delta S_{EVOO} / \mu L_{EVOO}}{\Delta S_{std} / \mu L_{std}} \times C_{std} \times 4 \qquad (2)$$

where ΔS_{EVOO} and ΔS_{std} are the recorded signal variations (in parts per million of oxygen, i.e., $\Delta ppmO_2$) after the addition of EVOO and the second addition of the standard (std) solution, respectively, whereas 4 is the multiplier factor due to dilution.

Total Antioxidant Capacity Measured by the ORAC Spectrofluorimetric Method. As far as the measurements in the EVOO samples are concerned, a small variation, already applied in a previous study (*7*), was introduced in the classical ORAC spectrofluorimetric method (*5*)): a small volume (20 μL) of acetone was added to the aqueous solution of reagents to facilitate the dissolution of the oily sample in the aqueous solution.

The main features of the described method are well-known; the protein β-phycoerythrin (β-PE) loses >90% of its fluorescence within 30 min when the free radicals or oxidizing species are present in solution. The addition of oxidizing species that react with the free radicals inhibits the decrease of the fluorescence of the protein. The

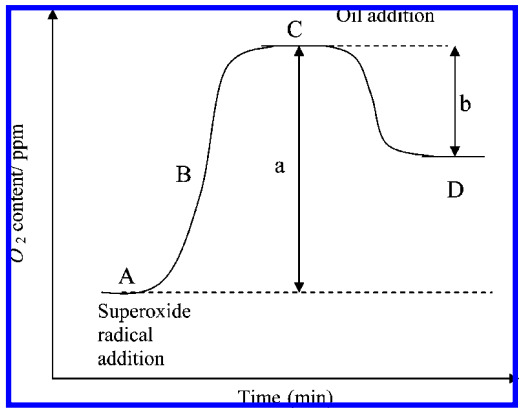

Figure 1. Trend of the experimental curve for the determination of the index *b*/*a*.

inhibition of the action of the free radicals is linked to the antioxidant capacity of the sample (*5*). The 2,2′-azobis(2-amidinopropane) dihydrochloride (ABAP) is used as a generator of peroxide radicals ($ROO^{\bullet}$). The method including the small solvent modification for the application to the oily matrices was previously described in detail (*7*).

Indirect Evaluation of Free Radicals Content Using the Superoxide Dismutase (SOD) Biosensor. As already extensively reported in the literature (*1, 11, 12*), the heating process in the EVOO samples determines chemical changes leading to the formation of different compounds; of these, the radical species are those that cause an appreciable increase in oil toxicity. In this study, the presence of these species in the overheated oil, which was subjected to a forced rancidification process, was detected using an indirect method based on a SOD biosensor obtained by coupling an amperometric Clark-type electrode with the immobilized SOD enzyme. The electrode used was suitably assembled and built entirely of PTFE so as to be able to operate in dimethyl sulfoxide instead of in aqueous solution. SOD is an enzyme that catalyzes the following reaction:

$$O_2^{\bullet -} + O_2^{\bullet -} + 2H^+ \xrightarrow{\text{SOD}} O_2 + H_2O_2 \quad (3)$$

Therefore, when the superoxide anion was present in solution, the Clark electrode, which represented the biosensor transducer, measured the increase in O_2 concentration caused by the enzymatic reaction. The method consisted of adding an oil sample (previously heated under isothermal conditions at a selected time and temperature) to a dimethyl sulfoxide solution containing the superoxide anion. To obtain the superoxide radical in solution, a dimethyl sulfoxide solution of KO_2 was prepared. When this solution containing the superoxide radical was added to the dimethyl sulfoxide, an increase in the signal was recorded until a second steady state was reached as the enzyme catalyzes the disproportion of the superoxide radical into oxygen and hydrogen peroxide. When the oil sample containing the radical species formed during the heating process was added, this steady state was perturbed due to the reactions of the radicals contained in the rancid oil with the superoxide radical previously generated in solution. These reactions, which remove the superoxide radical from the solution, can be represented as follows:

$$R^{\bullet} + O_2 \rightarrow ROO^- \quad (4)$$

$$RC^{\bullet}HOH + O_2^{\bullet -} \rightarrow RC^{\bullet}HOO^- + HO^{\bullet} \quad (5)$$

As a consequence, the concentration of the superoxide radical in solution decreased, and, therefore, O_2 concentration, which was the product of the reaction catalyzed by the SOD enzyme in solution, also decreased. This decrease was linked to the radicals concentration in the oil subjected to the heating process. The trend of the experimental changes in the signal detected in the course of the measurement is shown in **Figure 1**. The ratio of the changes in the O_2 concentration, detected, respectively, before and after the addition of the oil, was related to the concentration of the radicals formed in the oil. This ratio has been denoted *b*/*a* and represents an index of the formation of radicals.

For this analysis a superoxide dismutase biosensor operating in dimethyl sulfoxide, described in detail in a previous paper (*7, 13*) and already applied in our laboratory, although to measure another quantity, was utilized. This biosensor was prepared by sandwiching a gel disk of κ-carrageenan (0.5 cm diameter), on which the SOD enzyme was adsorbed (*13*), between an external PTFE membrane and an internal 0.1 cm thick membrane of cellulose acetate, both fixed to the Clark electrode. It is important to stress that the membrane separating the organic solvent from the gel disk was made of PTFE in order to prevent the organic solvent from spreading over the κ-carrageenan disk in which the enzyme was immobilized so as not to denature the latter. The experiments were performed in a thermostated cell at room temperature under continuous magnetic stirring (200 rpm). In practice, in a 40 mL glass cell containing 15 mL of dimethyl sulfoxide the biosensor was introduced and immersed at a level that was maintained rigorously constant in all tests.

After the signal had stabilized for about 10 min (part A of the curve in **Figure 1**) and a value of 8.4 ppm of O_2 was reached, 100 μL of a 0.03 mol L^{-1} superoxide radical in a dimethyl sulfoxide solution was added. In this solution the superoxide radical was obtained by the following reaction:

$$KO_2 \rightarrow K^+ + O_2^{\bullet -} \quad (6)$$

As a result, after passing through the PTFE membrane (*13*), the superoxide radicals were readily decomposed by the SOD enzyme catalyst, producing O_2, which was detected by the sensor (part B of the curve in **Figure 1**). After a stationary state was reached (part C of the curve in **Figure 1**), 100 μL of EVOO was added and the corresponding decrease in the signal was recorded until a new steady state was reached (part D of the curve in **Figure 1**). From the measurement of the current variations related to the three stationary states of the curve in **Figure 1**, the value of the *b*/*a* index, which is proportional to the amount of radicals contained in the EVOO samples subjected to the rancidification process, was determined.

Determination of Kinetic Parameters. The MacCallum method (*14*) enables the activation energy related to the process to be determined by carrying out isothermal experiments at different prefixed temperatures using the equation

$$\ln(t_{iso})_\alpha = a + b/(T_{iso})_\alpha \quad (7)$$

where t_{iso} is the time necessary to achieve a given degree of conversion α [$\alpha = (V_i - V_t)/(V_i - V_f)$, where V_i and V_f are the initial and final value of the antioxidant capacity and V_t is the value at the time t] under isothermal conditions at a given temperature T_{iso}. The kinetic parameters (pre-exponential factor A and activation energy E_a) are calculated from the regression parameters of eq 7 via the following relations: $a = \ln[f(1 - \alpha) - \ln A]$ and $b = E_a/R$, where $f(1 - \alpha)$ is a function representing the fraction of the sample not converted during the isothermal degradation process and R is the gas constant. From the slope b of eq 7, calculated at each degree of conversion α, the corresponding activation energy value is derived for the process considered. The time dependence of the degree of conversion at each isothermal temperature enables the $\ln(t_{iso})_\alpha$ values corresponding to given $(T_{iso})_\alpha$ values to be derived with the values of α ranging from 0 to 0.20, in accordance with the recommended literature interval for the application of the method (*14*). Moreover, this method requires the construction of interpolated α_i versus t_i curves based on the experimental data at each temperature (T_{iso}) for the purpose of determining the time values necessary to achieve the prefixed α values.

To confirm the reliability of the kinetic results obtained from the MacCallum method using a spectrofluorimeter, the so-called "model-fitting" method (*15*), recently applied to investigate the polyphenol thermal oxidation process using the tyrosinase biosensor (*4*), was also considered in this study. Details of this procedure are given in a previous paper (*4*); however, the selection of the most suitable kinetic models, described by appropriate integral $g(\alpha)$ model functions $f(\alpha)$ and respectively reported in the literature, is based on the best linear fit of the experimental data to the following relationship (*15*):

$$g(\alpha) = \int_0^{\alpha} d\alpha / f(\alpha) = k_{iso} t \quad (8)$$

Once the best $g(\alpha)$ function was selected, the specific rate constant k_{iso} values were obtained at each isothermal temperature for the selected model from the slopes of the corresponding "best-fitting" $g(\alpha)$ versus t regression line. Lastly, the Arrhenius parameters, pre-exponential factor ln A, and activation energy E_a were determined respectively from the intercept and the slope of the linear regression

$$\ln k_{iso} = c - b/T_{iso} \quad (9)$$

where $c = \ln A$ and $b = E_a/R$.

RESULTS AND DISCUSSION

Variation of the Total Polyphenols Concentration in the EVOO As Determined by the Tyrosinase OPEE. The decrease of the total polyphenols concentration was monitored by analyzing several samples of EVOO taken during the forced isothermal rancidification process carried out at 180 °C and the corresponding results have been reported partly in this work and partly in a previous paper (*4*). First of all, it is interesting to note that the initial total polyphenols concentration could change even substantially depending on whether the EVOO was homemade, and thus could be stored in conditions that were not completely suitable (in which the total polyphenols concentration was found to be about 0.5 mmol L^{-1}), or a good commercial product, which could thus be stored in sealed dark glass bottles (in which the total polyphenols concentration was found to be over 1 mmol L^{-1}, for a superior quality product) (*3, 16*).

The thermal degradation trend of polyphenols at 180 °C for a typical homemade EVOO sample (from the same Italian region and produced by the same polycultivar as the commercial one) is reported in **Figure 2**, whereas the investigation of polyphenol degradation made on a well-preserved commercial EVOO sample was carried out under isothermal conditions at four different temperatures ranging from 98 to 180 °C (**Figure 3**). A very detailed study of the latter case was reported in a previous paper (*4*), in which we were able to calculate the kinetic data related to the process investigated, namely, activation energy $E_a \approx 38$ kJ mol^{-1} and rate constant k, which lies between 4.2×10^{-2} and 7.6×10^{-2} min^{-1}.

Variation in the Total Antioxidant Capacity of EVOO Determined by the ORAC Method. One of the characteristics of olive oil is its greater resistance to oxidation stress with respect to other commercial vegetable oils. This peculiarity is known to be due to the high percentage of natural antioxidant molecules, especially polyphenols, contained in its matrix, as well as to other compounds (tocopherols, chlorophyll, etc.) known to possess high antioxidant capacity, although contained in very low concentrations in the oil. It was considered useful to evaluate the total antioxidant capacity of EVOO as a function of degradation time during the forced rancidification process at different selected temperatures (in particular at 180 °C) in an oxidizing atmosphere, that is, in the previously described conditions, and to evaluate the total antioxidant capacity of EVOO, as a function of degradation time as determined by the well-known ORAC spectrofluorimetric method, comparing it with the variation in the total polyphenols concentration obtained under identical operating conditions. The percentage decrease trends in antioxidant capacity in a commercial EVOO at the four isothermal temperatures selected during the first 60 min of its thermal degradation are shown in **Figure 3**. In the same figure the trends are compared with those of the decrease in

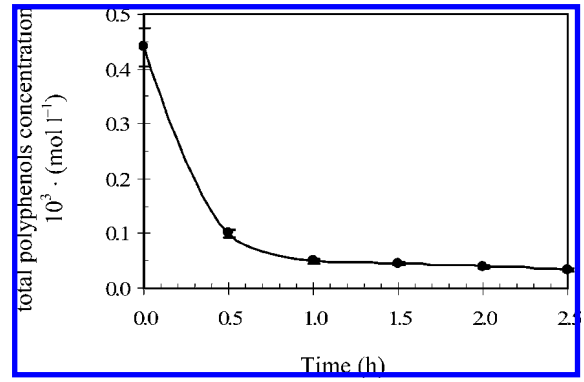

Figure 2. Decreasing trend of the total polyphenols concentration index (mol L^{-1}) for the homemade EVOO submitted to isothermal heating at 180 °C. Error bars represent the standard deviations (SD $\leq$ 0.03).

polyphenols subjected to the same treatment. By analogy with what was done in a previous paper (*4*) for the degradation process of polyphenols, in the present case it was attempted to estimate the kinetic parameters related to the trends of the total antioxidant capacity variation during the forced rancidification process at four different temperatures, on the assumption that in any case the kinetic data thus obtained would prove to be useful in characterizing the rancidification process itself. By applying the MacCallum method (*14*), relatively constant values of the activation energy (E_a) were obtained from the slope of the corresponding regression lines (eq 7 and **Figure 4**) at several degrees of conversion α in the range $0.10 \leq \alpha \leq 0.20$ and were found to be of the order of 30 kJ mol^{-1} (**Figure 5**). These E_a values were found to be slightly lower than those obtained by applying the same method to the total polyphenols forced degradation process monitored by the tyrosinase biosensor, which was found to be in the range of 37.4−39.1 kJ mol^{-1} (*4*). However, using the model-fitting method the rate constant and the Arrhenius parameters for the most suitable methods (D2, F1, and F2) (see **Figure 6**) were determined and are shown in **Tables 1** and **2**, respectively. For comparison purposes the corresponding values obtained from the study of the thermal degradation process of the total polyphenols obtained using the same kinetic method and the same models (*4*) are also reported in **Tables 1** and **2**. For this commercial EVOO sample the times after which half of the original total antioxidant capacity value of the EVOO remained ($TACV_{1/2}$) obtained for each of the four selected degradation temperatures were finally compared in **Figure 7A** with the residual percentage of the total polyphenols in the sample, measured at the time corresponding to the $TACV_{1/2}$ (see **Figure 7B**) and the half-life values for the total polyphenols concentration (**Figure 7C**). Above all, it is interesting to observe how the values obtained for the activation energy using the "model-fitting" method coincide almost exactly with those obtained from the polyphenols degradation process ($E_a \approx 38$ kJ mol^{-1}) and those obtained from the total antioxidant capacity trend ($E_a \approx 37$ kJ mol^{-1}) at the four different temperatures considered. Moreover, as clearly shown by the data contained in **Table 1**, also the kinetic constant values obtained using each of the most suitable methods (D2, F1, and F2), whether computed at the same temperatures on the basis of the degradation trend of polyphenols or on that of the variation in total antioxidant capacity, are very similar (*4*). In addition, by observing the graphs contained in **Figure 7**, it was found that the $TACV_{1/2}$ at 98 °C is 12.0 min, whereas it decreases to only 6.8 min at 180 °C. It was interesting to note that the $TACV_{1/2}$ at 180 °C (i.e., 7 min) is almost the same as the half-life of the total polyphenols concentration (see **Figures 3D** and **7C**); it is

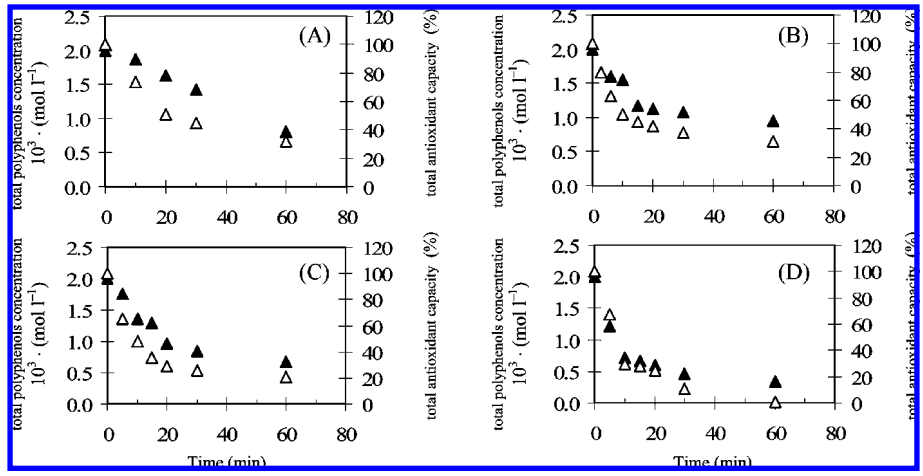

Figure 3. Comparison of decreasing trends in total antioxidant capacity (△) in the first 60 min (RSD ≤ 0.05) ($n \geq 5$) and of the total polyphenols concentration (▲) in the EVOO (RSD ≤ 0.04_7) ($n \geq 5$) during the isothermal degradation processes at four different isothermal temperatures: (**A**) 98 °C; (**B**) 140 °C; (**C**) 160 °C; (**D**) 180 °C.

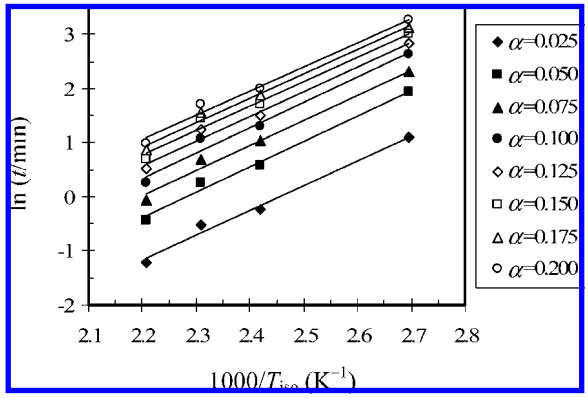

Figure 4. Linear trend of the $\ln(t_{iso})_\alpha$ values as a function of T_{iso}^{-1} at the given values of α for the thermal oxidation processes of EVOO, monitored on the basis of the total antioxidant capacity variation trend. (t_{iso}) is the time necessary to achieve a given degree of conversion (α), where $\alpha = (V_i - V_t)/(V_i - V_f)$ is the degree of conversion; (T_{iso}) is the isothermal temperature selected.

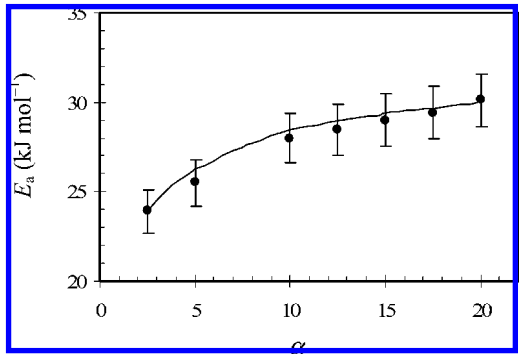

Figure 5. Activation energy (E_a) trend found for the thermal oxidation processes of EVOO as a function of the degree of conversion α. Error bars represent the standard deviations.

clear, therefore, that at this temperature the decreasing trend of the antioxidant capacity of the EVOO with time is certainly determined by the forced consumption of polyphenols. By contrast, the $TACV_{1/2}$ at 98 °C is somewhat shorter than the half-life of total polyphenols: about 12 min (**Figure 7A**) compared with about 30 min (**Figure 7C**). These results suggest that, at this temperature, even the other antioxidant compounds contained in the EVOO significantly affect the trend of the antioxidant capacity, which therefore decreases more rapidly than expected on the basis of the decreasing rate of the total polyphenols only. This is supported by the scant information found in the literature concerning the degradation kinetics of molecules such as chlorophyll and tocopherols (*17*) also contained in the EVOO, the activation energy values of which during degradation are 24 and 21 kJ mol^{-1} (*17*), respectively, that is, much lower than that of total polyphenols degradation, which is of the order of 38 kJ mol^{-1} (*3*). As a result, the degradation of the other antioxidants is therefore faster than that of the polyphenols. However, their contribution to the kinetic trend is still clear-cut at around 100 °C but no longer detectable at 180 °C, because at this high temperature both chlorophyll and tocopherols decompose too quickly with respect to polyphenols.

Evaluation of the Variation in the Free Radicals Index Using a Superoxide Dismutase Biosensor. The sample of EVOO was also analyzed during the thermal oxidation process carried out under the operating conditions described under Materials and Methods for the specific purpose of analyzing the presence of free radicals. The results of the trend in the *b*/*a* index related to the formation of free radicals are reported in **Figure 8A**. These data show that the initial value of *b*/*a*, obtained from the analysis of the raw, that is, yet to be heated, oil, seems to be much higher than all of the values obtained during the thermal oxidation process. These results cannot be explained in terms of the formation of free radicals in the raw EVOO, initially in very small amounts due to the fact that the EVOO is not yet artificially rancidified. The only reasonable explanation is that the EVOO initially utilized contains a large amount of antioxidants, such as polyphenolic compounds. The latter react with the superoxide radicals, which are introduced into the solution as described in the method, and decrease the superoxide concentration in solution, which explains why the *b*/*a* index seems to be so high at the beginning. The fact that the first value, that is, that of the oil still to be subjected to the thermooxidative process and thus still containing all of the polyphenols, attains a value of >1 must be assigned to the complex oxidative reactions involving the polyphenols in the reaction environment as a result of which not only the whole superoxide radical is consumed in the reaction with the polyphenols but at the same time a small consumption of the dissolved oxygen in the solution is also observed. This accounts for the fact that the value of the *b*/*a* index can have values of >1.

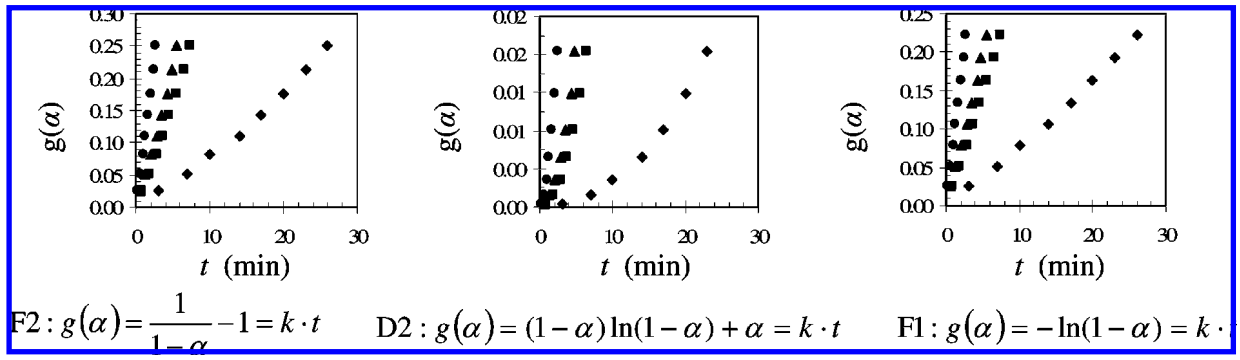

Figure 6. Trend of the values of the integral function g(α) as a function of time *t* for the most suitable selected model functions (F2, D2 and F1) at the different selected temperatures for the variation of the antioxidant capacity of EVOO.

Table 1. Comparison of the Rate Constant Values Obtained Using the Model-Fitting Method for the Most Suitable Models at the Selected Temperatures Following both the Variation of the Total Antioxidant Capacity Variation (TACV) and the Thermal Degradation of the Total Polyphenols (TDTP)[a]

	$k \times 10^{-3}$/min^{-1}											
	98 °C			140 °C			160 °C			180 °C		
model function	TACV	TDTP[b]	Δ[c]	TACV	TDTP[b]	Δ[c]	TACV	TDTP[b]	Δ[c]	TACV	TDTP[b]	Δ[c]
D2	0.9	1.0	0.12	3.2	3.8	0.14	4.2	5.2	0.19	8.7	10.3	0.15
F1	8.6	8.0	0.03	30.4	32.0	0.05	40.0	44.5	0.10	81.5	86.8	0.06
F2	9.8	10.2	0.04	34.4	36.8	0.06	45.2	51.1	0.11	92.2	99.8	0.08

[a] The relative deviation of the two values at each temperature for each model function (Δ) is also given. The associated errors (standard deviations of the slopes related to eq 8) for $n = 8$ data points) are always less than 8%. [b] Values calculated using the kinetic parameters of the TDTP reported in ref *4*. [c] $\Delta = [(k_{TDTP} - k_{TACV})/k_{TDTP}]$.

Table 2. Comparison of the Arrhenius Parameters Obtained from the Slopes and Intercepts of Equation 9 ($n = 5$ Data Points) Using the Model-Fitting Method for the Most Suitable Models at the Selected Temperatures Following both the Variation of the Total Antioxidant Capacity Variation (TACV) and the Thermal Degradation of the Total Polyphenols (TDTP)[a]

	E/kJ mol^{-1}		ln(*A*/min^{-1})	
model function	TACV	TDTP[b]	TACV	TDTP[b]
D2	36.9 ± 3.3	37.8 ± 0.3	0.9 ± 0.1	1.29 ± 0.01
F1	36.8 ± 3.2	37.5 ± 0.3	3.1 ± 0.3	3.35 ± 0.03
F2	36.8 ± 3.2	37.6 ± 0.3	3.2 ± 0.3	3.50 ± 0.03

[a] The associated errors are standard deviations. [b] Values calculated using the kinetic parameters of the TDTP reported in ref *4*.

It is interesting to note that the value of *b*/*a* then decreases drastically and, after only 0.5 h of isothermal heating at 180 °C, becomes significantly lower than that obtained for the raw oil; this behavior is related to the degradation process of the polyphenolic antioxidants during the isothermal heating at 180 °C, the concentration of which is close to zero after about 1 h at this temperature, as can be seen in **Figure 3**.

Conversely, the results obtained for the *b*/*a* radical index after the first 0.5 h display an increasing trend, which can effectively be accounted for by an increase in the radicals produced in the sample during the artificial rancidification process. To confirm that this is the correct interpretation of the experimental trend found, a sample of the same EVOO was again subjected to thermal oxidation, but after being previously pretreated with active carbon before the isothermal rancidification process occurred at 180 °C in order to remove practically all of the polyphenolic compounds and other antioxidants contained in the EVOO. Indeed, after the treatment with active carbon, the final concentration of total polyphenols in the EVOO measured with the enzymatic tyrosinase biosensor was found to be about 10^{-6} mol L^{-1}, whereas before treatment with active carbon it was of the order of 10^{-3} mol L^{-1}. The EVOO thus treated was then subjected to the isothermal heating process at 180 °C, using

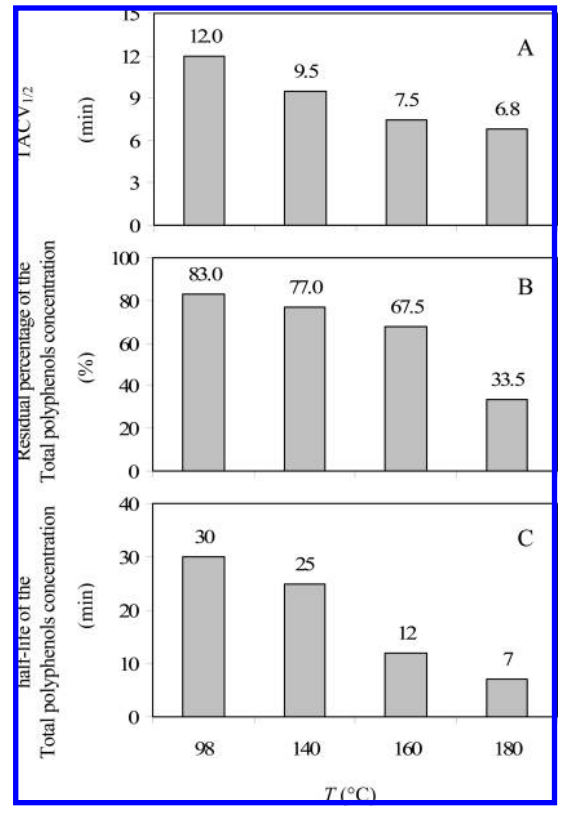

Figure 7. Comparison among (**A**) the $TACV_{1/2}$ (the time after which half of the original total antioxidant capacity value of the EVOO remained), (**B**) the simultaneous residual percentage of total polyphenols concentration, and (**C**) the half-life values for the total polyphenols concentration at the four selected isothermal temperatures ranging from 98 to 180 °C.

the same operating conditions as adopted in the previous experiments, and the resulting *b*/*a* values, which are due to the formation of radicals, are plotted in **Figure 8B**. As can be seen, the value of the index obtained using the EVOO, but after

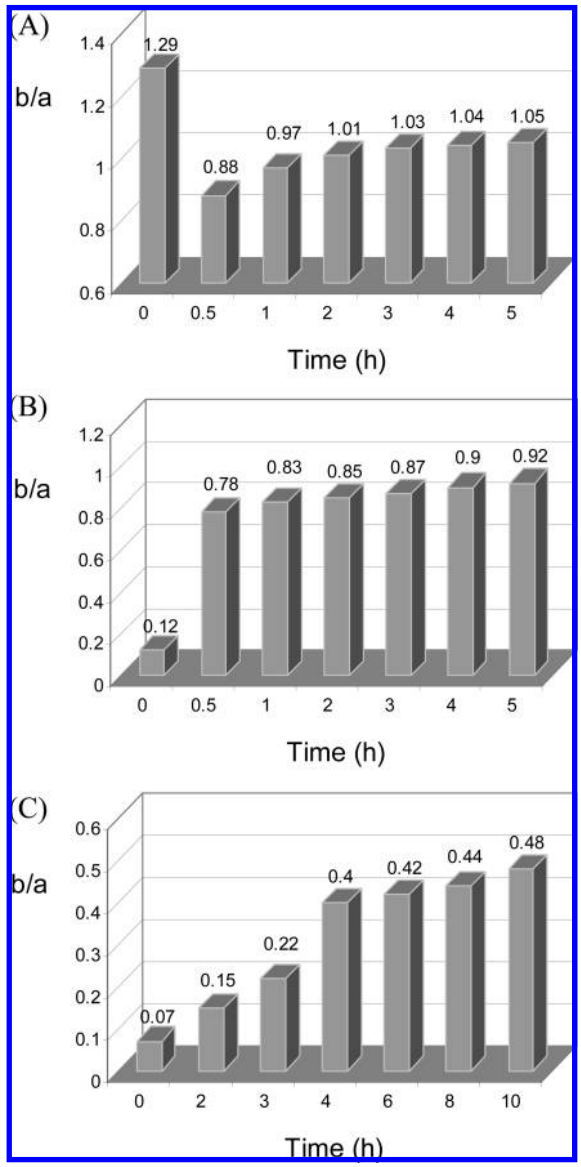

Figure 8. Trend of the values for the index of radical formation *b*/*a* for the EVOO (**A**) heated at 180 °C (RSD ≤ 0.04) ($n \geq 5$), (**B**) pretreated to remove all polyphenols and then submitted to a controlled heating at 180 °C (RSD ≤ 0.03) ($n \geq 5$), and (**C**) pretreated to remove all polyphenols before being subjected to controlled heating at 98 °C according to the recommended AOM method (RSD ≤ 0.05) ($n \geq 5$).

removal of its phenolic compounds, is found to be much lower than that of the raw oil untreated with active carbon; these results confirm the finding that the abnormally high value of the *b*/*a* index obtained at the beginning for the untreated raw EVOO could be due to the presence of the polyphenolic antioxidant compounds contained in it. Furthermore, it was observed that the value of the index after only 0.5 h of heating had become much higher and was already highly comparable with that of non-dephenolized oil; after practically 1 h, the index value was close to the higher values, that is, almost the same as that found in the untreated EVOO heated for 1 h at the same temperature. These results show that after 1 h of heating the concentration of the antioxidants present is already highly reduced, which is in agreement with the concentration of the total polyphenols, the value of which is close to zero in the EVOO heated at 180 °C shown in **Figure 3**. Lastly, the radical index and total polyphenols content trends are in perfect agreement with the trend in the increased toxicity index for EVOO heated at 180 °C, which we recorded in a previous work (*1*) using a biosensor based on a yeast cell. Finally, the results obtained for the subsequent measurements show a slightly increasing trend in *b*/*a* values with time, somewhat similar to what was found in the experiment carried out with the EVOO untreated with active carbon. These results confirm that the trend of the index observed after the first hour of treatment is effectively ascribable to the formation of free radicals during the artificial rancidification process. Another isothermal heating experiment similar to that of the EVOO heated at 180 °C but using the EVOO pretreated with active carbon was also carried out, nevertheless, at a much lower temperature, namely, 98 °C [which is the temperature recommended by the Active Oxygen Method (AOM) of the AOCS (*9*)]. The trend found in this experiment is also shown in **Figure 8C**. As expected, even when results derived from previous papers describing polyphenols decomposition (*3, 4*) are taken into account, at this temperature the rancidification process is noticeably slower than that at 180 °C. As a consequence, the formation of radicals, as evidenced by the increase in the *b*/*a* index, is much slower than that at 180 °C; for instance, after about 5 h of heating at 98 °C, the radicals index reaches a value of 0.4, which is about half of that observed after 5 h of heating at 180 °C.

Evaluation of the Methods and Discussion of the Most Significant Results. As described above, to control the forced rancidification process of EVOO under isothermal conditions, both the decrease in total polyphenols concentration using a tyrosinase biosensor and the total antioxidant capacity using the ORAC spectrofluorimetric method were first determined at fixed time intervals. The two trends were in quite good agreement because also the decreasing trend of the antioxidant capacity showed that at 180 °C it falls to practically zero in about 60 min, whereas it decreases drastically within the first 30 min, thus confirming the observations made for the decreasing trend of the total polyphenols concentration at 180 °C. Moreover, also the kinetic data values were in good correlation (see **Tables 1** and **2**). Indeed, as evidenced in **Figure 4**, the activation energy value was found to increase, at the beginning of the reaction, from 23 to about 30 kJ mol^{-1}, up to $\alpha = 0.20$, and then to remain practically constant. This result is in good agreement with the conclusion stated in the previous paragraph: the first compounds subjected to degradation during the thermal oxidation process are the nonphenolic antioxidants, because the initial E_a value found is of the same order of magnitude as that reported in the literature for these compounds (21−24 kJ mol^{-1}); the degradation of the total polyphenols occurs subsequently, as confirmed by the E_a values ranging from 30 to 37 kJ mol^{-1} (**Figure 4** and **Table 2**), in good agreement with the values calculated in a previous paper (*3*).

The tyrosinase-based OPEE is found to be particularly suitable for total polyphenols determination, as it allows a relatively short time analysis (a few minutes) compared with the time taken by other chemical methods, such as the spectrophotometric method (*18*). Moreover, the good solubility of the oily sample in *n*-hexane, the solvent used for the total polyphenols concentration measurements, enabled the problem of inaccuracy encountered in the determination of oily matrices carried out with other methods (*18*) to be overcome brilliantly, particularly as a result of the very low solubility of the oil in the aqueous medium. Lastly, the solvent selected to perform the biosensor measurement, *n*-hexane, is also, from a theoretical point of view, particularly suitable for carrying out these experiments, because a sufficiently high enzymatic activity, as well as a good tyrosinase biosensor response, is obtained in

this medium (*19*). Once again, as in other circumstances (*6, 7*), the spectrofluorimetric method with the little variation introduced was found to be extremely valid and reliable also for hydrophobic matrices, even if rather expensive and time-consuming.

Finally, the indirect method for estimating the increase in radicals concentration during artificial rancidification has shown that it does not provide correct results when, as in the case of raw EVOO, a high concentration of polyphenols is present; in this case, instead, it gives the total antioxidant capacity of the sample, as shown in previous papers (*6*−*8*), whereas when the polyphenols are no longer present, or have been radically removed, the method actually seems to give the expected results and can be, on the contrary, effectively useful.

Conclusions. The thermal oxidation process of EVOO at 180 °C was investigated by monitoring both the trends of the antioxidant capacity and the total polyphenols concentrations. The main kinetic parameters (E_a values between 30 and 37 kJ mol^{-1}; k_{iso} values between about 9×10^{-3} and 9×10^{-2} min^{-1} depending on the selected model function) derived for the process described were compared with each other and critically discussed on the basis of the corresponding results found independently in previous research (*4*). Moreover, the possibility of employing a SOD biosensor working in dimethyl sulfoxide to check the free radical concentrations during the rancidification process was evaluated and the limits of application were discussed. Also, the possible applications of this method were found to be interesting provided that it was used after the polyphenols and other antioxidants contained in the sample had been removed. Lastly, the experimental results obtained in this study confirm the findings of previous papers (*1*−*4*): time and temperature values used in cooking processes in which the important foodstuff, EVOO, is used are crucial factors in establishing the possible, formation of radicals and thus the best cooking procedures to be adopted to ensure EVOO can be safely utilized for cooking foods.

APPENDIX A

The superoxide dismutase biosensor was originally developed by us precisely to measure free radicals in aqueous solutions (or aqueous samples) (*8*). It was later found to be very useful and highly valid (using a suitable measuring procedure) in determining the total antioxidant capacity of a certain number of beverages such as wine (*6*), tea (*20*), fruit juices (*21*), and many vegetal species (*22*), fresh fruit (*23*), and spices (*24*), of course always operating in aqueous solution.

Only more recently (*13*), after transformation into an organic phase enzyme electrode (OPEE), using a different transducer, that is, the Clark electrode, instead of the amperometric hydrogen peroxide sensor, was it possible to propose its use to determine the antioxidant capacity of oily drug integrator specialties (*7*). In the present research, on the other hand, it was attempted for the first time to apply the OPEE thus developed for the purpose for which the biosensor had been originally constructed, namely, to measure free radicals not present in aqueous solutions but in artificially rancidified oily solutions. We consider the present findings of great interest as this OPEE may be correctly used for this purpose, although only after a simple dephenolization treatment of the oily sample, which can be carried out by means of simple carbon treatment as illustrated here.

APPENDIX B

All of the values in all trends considered (both that of the total polyphenols concentration and that of the antioxidant capacity) are the average of at least two experiments in which EVOO samples of the same type were subjected to a thermooxidative process under identical operative conditions and analyzed at identical prefixed time intervals. In all cases, for each of the time intervals selected, at least five measurements were made from the two (or more) experiments carried out. Results of typical repeatability tests carried out on the degradation trend performed at 98 °C and at different time intervals are displayed in **Table 3**. Seven measurements were made at each of the following time intervals: 0, 30, and 50 h. The corresponding total polyphenols concentration values, their means, and standard deviations are reported in **Table 3**. The values selected show a limited scatter around their means, and the standard deviations were between 2 and 5%.

Table 3. Repeatability of Measurements for the Determination of the Total Polyphenols Concentration Heated at 98 °C for Different Times

mg of phenol/kg of EVOO		
0 h	30 h	50 h
115.5	49.4	28.6
114.4	46.3	28.2
113.2	48.7	29.5
113.9	50.1	28.9
110.4	51.0	30.6
114.4	45.8	28.9
117.6	47.4	32.3
114.2 ± 2.2[a]	48.4 ± 2.0[a]	29.6 ± 1.4[a]

[a] Mean ± SD.

LITERATURE CITED

(1) Campanella, L.; Dragone, R.; Fisco, L.; Tomassetti, M. Study of toxicity and variation in polyphenol and hydroperoxide content of food oils subjected to thermal stress or used for frying foodstuffs. *Anal. Lett.* **2004**, *37*, 3047–3061.

(2) Campanella, L.; Giancola, D.; Gregori, E.; Tomassetti, M. Determination of hydroperoxides in nonaqueous solvents or mixed solvents, using a biosensor with two antagonist enzymes operatine in parallel. *Sens. Actuators B* **2003**, *95*, 321–327.

(3) Campanella, L.; Favero, G.; Pastorino, M.; Tomassetti, M. Monitoring the rancidification process in olive oils using a biosensor operating in organic solvents. *Biosens. Bioelectron.* **1999**, *14*, 179–186.

(4) Campanella, L.; Nuccilli, A.; Tomassetti, M.; Vecchio, S. Biosensor analysis for the kinetic study of polyphenols deterioration during the forced thermal oxidation of extra-virgin olive oil. *Talanta* **2008**, *74*, 1287–1298.

(5) Cao, G.; Verdon, C. P.; Wu, A. H. B.; Wang, H.; Prior, R. L. Automated assay of oxygen radical absorbance capacity with the COBAS FARA II. *Clin. Chem* **1995**, *41*, 1738–1744.

(6) Campanella, L.; Bonanni, A.; Finotti, E.; Tomassetti, M. Biosensor for determination of total and natural antioxidant capacity of red and white wines: comparison with other spectrophotometric and fluorimetric methods. *Biosens. Bioelectron.* **2004**, *19*, 641–651.

(7) Campanella, L.; Bonanni, A.; Bellantoni, D.; Tomassetti, M. Biosensor for determination of total antioxidant capacity of phytotherapeutic integrators: comparison with other spectrophotometric, fluorimetric and voltammetric methods. *J. Pharm. Biomed. Anal.* **2004**, *35*, 303–320.

(8) Campanella, L.; Favero, G.; Persi, L.; Tomassetti, M. New biosensor for superoxide radical used to evidence molecules of biomedical and pharmaceutical interest having radical scavenging properties. *J. Pharm. Biomed. Anal.* **2000**, *23*, 69–76.

(9) AOCS. Method Cd 12-57. *Official and Tentative Methods of the American Oil Chemist's Society*, 3rd ed.; AOCS: Champaign, IL, 1964; Vol. 1, pp 1−4.

(10) Campanella, L.; Favero, G.; Sammartino, M. P.; Tomassetti, M. The effect of organic solvent properties on the response of a tyrosinase enzyme sensor. *Talanta* **1994**, *41*, 1015–1023.

(11) Biffoli, R. *Chimica degli Alimenti*; USES: Firenze, Italy, 1984; Chapters 9−10, pp 121−161.
(12) Kiritsakis, A.; Markakis, P. Olive oil: a review. *Adv. Food Res.* **1987**, *31*, 453–482.
(13) Campanella, L.; De Luca, S.; Favero, G.; Persi, L.; Tomassetti, M. superoxide dismutase biosensors working in non-aqueous solvent. *Fresenius' J. Anal. Chem.* **2001**, *369*, 594–600.
(14) Abate, L.; Blanco, I.; Motta, O.; Pollicino, A.; Recca, A. The isothermal degradation of some polyetherketones: a comparative kinetic study between long-term and short-term eperiments. *Polym. Degrad. Stab.* **2002**, *75*, 465–471.
(15) Gao, X.; Chen, D.; Dollimore, D. The correlation between the value of α at the maximum reaction rate and the reaction mechanisms. A theoretical study. *Thermochim. Acta* **1993**, *223*, 75–82.
(16) Campanella, L.; Favero, G.; Sammartino, M. P.; Tomassetti, M. An organic-phase enzyme sensor for polyphenol determination in olive oil. *Polyphenols Actualites* **1996**, *14*, 9–12.
(17) Montiel, G. M.; Avanza, J. R. Kinetic study of color degradation of commercial olive oils using UV-C radiation. *Inf. Tecnol.* **2001**, *12*, 43–46.
(18) Vasquez-Roncero, A.; Janer Del Valle, C.; Janer Del Valle, M. L. Determinacion de los polifenoles totales del aceite de oliva (Determination of total polyphenols in olive oils). *Grasas Aceites (Seville)* **1973**, *24*, 350–357.
(19) Campanella, L.; Favero, G.; Sammartino, M. P.; Tomassetti, M. Further development of catalase, tyrosinase and glucose oxidase based organic phase enzyme electrode response as a function of organic solvent properties. *Talanta* **1998**, *46*, 595–606.
(20) Campanella, L.; Bonanni, A.; Tomassetti, M. Determination of the antioxidant capacity of samples of different types of tea, or of beverages based on tea or other herbal products, using a superoxide dismutase biosensor. *J. Pharm. Biomed. Anal.* **2003**, *32*, 725–736.
(21) Campanella, L.; Bonanni, A.; Furlani, G.; Tomassetti, M. Determinazione della capacità antiossidante totale di matrici alimentari. V Congresso Nazionale di Chimica degli Alimenti. Qualità e sicurezza degli alimenti. Parma (Italy) 2003, *Atti* pp 58−63, Morgan Ed., Milan, Italy, 2004.
(22) Campanella, L.; Favero, G.; Persi, L.; Tomassetti, M. Evaluation of radical scavenging properties of several plants, fresh or from a herbalist's, using a superoxide dismutase biosensor. *J. Pharm. Biomed. Anal.* **2001**, *24*, 1055–1064.
(23) Campanella, L.; Bonanni, A.; Favero, G.; Tomassetti, M. Determination of antioxidant properties of aromatic herbs, olives and fresh fruit using an enzymatic sensor. *Anal. Bioanal. Chem.* **2003**, *375*, 1011–1016.
(24) Bonanni, A.; Campanella, L.; Gatta, T.; Gregori, E.; Tomassetti, M. Evaluation of the antioxidant and prooxidant properties of several commercial dry spices by different analytical methods. *Food Chem.* **2007**, *102*, 751–758.

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