Enzymatic Assay for the Determination of Olive Oil Polyphenol Content: Assay Conditions and Validation of the Method

Luciana Mosca,*,† Carlo De Marco,† Francesco Visioli,‡ and Carlo Cannella§

Department of Biochemical Sciences and Institute of Food Science and Nutrition, University of Rome "La Sapienza", p.le Aldo Moro 5, 00185 Rome, Italy, and Institute of Pharmacological Sciences, University of Milan, via Balzaretti 9, 20133 Milan, Italy

A new spectrophotometric assay for the determination of the polyphenolic content of olive oil is presented. It is a substrate-recycling assay for phenolic compounds that employs tyrosinase in the presence of excess NADH. The reaction of various phenols with the enzyme produces an *o*-quinone, which is detected by recycling between reactions with the enzyme and NADH. The method offers some advantages over the classical methods employed to determine the polyphenolic content of olive oil, that is, ease and reproducibility of the analysis, highly increased sensitivity, and selectivity toward phenolic compounds. The amount of total polyphenols was determined in virgin olive oils both with the Folin−Ciocalteu reagent and with the proposed enzymatic method. The results suggest a better estimation of the polyphenol content, as compared with the colorimetric method. This has to be attributed to the different reactivities of the two methods toward phenols and catechols. Finally, the enzymatic method demonstrates that there is a linear relationship between the olive oil phenolic content and the antioxidative capacity of oil extracts.

Keywords: *Tyrosinase; polyphenols; olive oil; antioxidants; hydroxytyrosol*

INTRODUCTION

Polyphenols are secondary metabolites occurring widely in plant foods (Harborne, 1989). They exhibit antioxidant and free radical-scavenging properties that render them potential protecting agents against cardiovascular diseases [reviewed by Visioli and Galli (1998a)].

As opposed to refined olive oil, virgin olive oil contains a considerable amount of polyphenols that are responsible for its pungent flavor and for its stability to peroxidation. Olive oils are lower in tocopherols than seed oils (Boskou, 2000); therefore, the presence of phenolic compounds with antioxidant activity is of particular importance because it correlates with the resistance of oil to the development of rancidity (Gutfinger, 1981; Papadopoulos and Boskou, 1991; Tsimidou et al., 1992). Olive oil is the fat of choice in the Mediterranean area, where the diet has been associated with a lower incidence of coronary heart disease (Keys, 1995; Willet et al., 1995) and cancer (Trichopoulou, 1995; Lipworth et al., 1997). The major phenolic compounds thus far identified in olive oil are caffeic, vanillic, *p*-coumaric, *o*-coumaric, syringic, gallic, and *p*-hydroxybenzoic acids; tyrosol, hydroxytyrosol, oleuropein and its aglycon, and ligstroside and its aglycons, together with some nonphenolic compounds such as cinnamic and elenolic acids (Boskou, 1996).

The most widely employed methods for evaluating the polyphenolic content of olive oil are the Folin−Ciocalteu colorimetric assay and high-performance liquid chromatography (HPLC). The former is quite simple and requires few reagents, but it is limited by the low specificity of the reagent toward polyphenols as any reducing substance may interfere in the assay. The method provides only quantitative information on the phenolic content of the oil. An intrinsic limitation of the method lies in the large number of phenolic compounds present in olive oil. At present, at least 30 different compounds have been reported to be present in virgin olive oil (Boskou, 1996). As a consequence, the polyphenol content of biological samples has to be referred to a standard compound. On the other hand, HPLC is a very sensitive and specific method, from both qualitative and quantitative points of view, but it is time-consuming because of the length of the chromatographic run. In addition, as not all of the peaks revealed in the HPLC chromatograms have been identified, it is impossible to quantify the single phenols due to the absence of suitable standard compounds.

Here we present a simple enzymatic assay aimed at obtaining a selective and sensitive evaluation of phenolic content of olive oil. It adopts a substrate-recycling procedure that employs tyrosinase together with NADH as a reducing agent. Tyrosinase (polyphenol oxidase, EC 1.14.18.1) is a multifunctional copper-containing enzyme that catalyzes both the hydroxylation of phenols and the oxidation of catechols to quinones (Figure 1). In the presence of excess NADH, the resulting quinone is reduced to catechol (Brown et al., 1994); the rate of NADH oxidation is directly related to the phenol/catechol content, thus allowing the estimation of the total substrate amount. The recycling of the products provides an amplification of the signal originally derived from the enzymatic reaction (Brown et al., 1994). Reduction of the quinones by excess NADH also increases the stability of tyrosinase and prevents the subsequent quinone polymerization that occurs in nonrecycling assays.

* Author to whom correspondence should be addressed (telephone +39-06-49910856; fax +39-06-4440062; e-mail lmosca@uniroma1.it).

† Department of Biochemical Sciences.

‡ Insitute of Pharmacological Sciences.

§ Institute of Food Science and Nutrition.

10.1021/jf990556+ CCC: $19.00 © 2000 American Chemical Society
Published on Web 02/02/2000

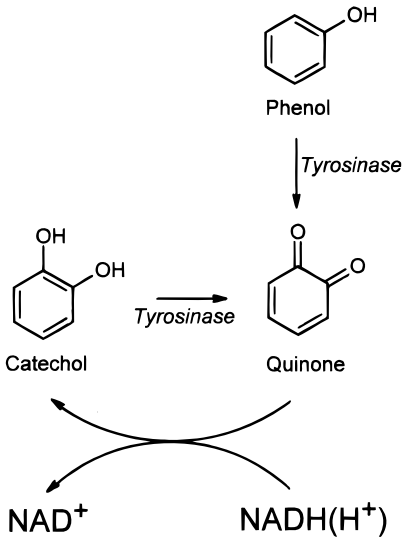

Figure 1. Schematic pathway of the reactions catalyzed by tyrosinase and of the recycling reaction between *o*-quinone and NADH.

EXPERIMENTAL PROCEDURES

Materials. Tyrosinase (3400 units/mg) and β-NADH were from Sigma-Aldrich Co. (St. Louis, MO). Standard compounds known to be present in olive oil (gallic acid, vanillic acid, cinnamic acid, tyrosol, protocatechuic acid, *p*-hydroxybenzoic acid, *p*-coumaric acid, and caffeic acid) were purchased from Fluka (Buchs, Switzerland). All other reagents were analytical grade products from Merck (Darmstadt, Germany). All of the buffers and solutions used in the analyses were prepared by the use of ultrapure water of the highest electrical resistance (>18.2 MΩ·cm^2, obtained through a Milli-Q Millipore filter system) to minimize the presence of metals that could catalyze autoxidation of polyphenols.

Stock solutions of the standard phenolic compounds (1 mM) were freshly prepared when needed by dissolving the compounds in 7% acetic acid. NADH and tyrosinase solutions were prepared in 50 mM phosphate buffer, pH 7.4. NADH concentration was estimated by the molar extinction coefficient of 6220 at 340 nm.

Six different virgin olive oil samples were obtained from various locations in Italy. An additional sample was a seed oil (mixture of various seeds).

Polyphenol Extraction. Polyphenols were extracted from oil according to the method of Montedoro et al. (1993). The residues were kept at −20 °C until use. Immediately before analyses, the extracts were dissolved in 1 mL of 7% acetic acid (stock extract solutions) and kept in the dark in an ice bath. Extracts labeled A−F were obtained from extra virgin olive oils, whereas sample G is an extract of a seed oil devoid of phenolic compounds that was used as negative control.

Tyrosinase-Catalyzed Oxidation of Phenolic Compounds. The enzymatic oxidation of various standard phenolic compounds by tyrosinase was monitored spectrophotometrically with a Hitachi U-2000 doublebeam spectrophotometer, with the scanning mode set between 200 and 600 nm. Incubation mixtures contained 20 μM phenolic compound and 20 EU of tyrosinase in 1 mL of 50 mM phosphate buffer, pH 7.4. The quinone products were estimated in the 380−420 nm range. Kinetics of quinone production was followed continuously at 400 nm. In the recycling assay, incubation mixtures contained 20 μM phenolic compound, 150 μM NADH, and 20 EU of tyrosinase in 1 mL of 50 mM phosphate buffer, pH 7.4. Kinetics of NADH oxidation was followed continuously either spectrophotometrically at 340 nm or fluorometrically, with excitation wavelength set at 345 nm/5 nm slit width and emission wavelength set at 450 nm/5 nm slit width, in a Jasco spectrofluorometer, model FP 770.

Colorimetric Evaluation of Total Phenols. The total phenols were determined at 725 nm using the Folin−Ciocalteu reagent. Five microliters of the stock extract solution was diluted to 100 μL with 7% acetic acid. Then 50 μL of Folin−Ciocalteu reagent was added. After 3 min, 100 μL of saturated (~35%) Na_2CO_3 solution was added. The solution was diluted to 2.5 mL with water and vortex-mixed. Solutions were kept in the dark for 90 min, and then the absorbance was measured at 725 nm against a suitable blank. Caffeic acid, in the 0−50 μM range, was employed as a standard for the preparation of the calibration curve. Results are expressed as milligrams of caffeic acid equivalents per kilogram of olive oil (parts per million).

Table 1. Substrates for Tyrosinase

compound	reactivity[a]	compound	reactivity[a]
caffeic acid	+	*p*-hydroxybenzoic acid	−
vanillic acid	−	*p*-coumaric acid	+
tyrosol	+	protocatechuic acid	+
hydroxytyrosol	+	gallic acid	+
cinnamic acid	−		

[a] +, substrate; −, not substrate.

Tyrosinase−NADH Recycling Assay. An aliquot of the stock extract solution was diluted 1:20 with 7% acetic acid. Forty microliters of this diluted solution was treated with 20 EU of tyrosinase and 150 μM NADH in 1 mL of 50 mM phosphate buffer, pH 7.4. Reaction was followed by measuring the rate of NADH oxidation to NAD^+ at 340 nm. A calibration curve of caffeic acid was prepared as described above. Results are expressed as milligrams of caffeic acid equivalents per kilogram of olive oil (parts per million).

DPPH Quenching Test. The radical-scavenging properties of olive oil polyphenols were assayed by the 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical quenching test. Two microliters of the stock extract solution was added to 1 mL of a 15 μM ethanolic solution of DPPH and vortex-mixed. After 15 min of incubation, the absorbance was measured at 517 nm (Visioli and Galli, 1998b).

LDL Oxidation. LDLs were isolated by sequential ultracentrifugation from plasma obtained from healthy, normolipidemic human volunteers. Prior to their use, LDL samples were desalted by size-exclusion chromatography. Protein content was measured according to the method of Lowry et al. (1951).

LDLs were diluted with PBS and incubated at 37 °C in a shaking bath throughout the experiments. Olive oil extracts were added 30 min before the addition of 5 μM $CuSO_4$ (final concentration). Control samples were added with the vehicle (ethanol) alone. Aliquots were withdrawn at 30 min, 1 h, 3 h, and 6 h for the evaluation of oxidation markers.

The formation of thiobarbituric acid-reacting substances (TBARS) and of lipid hydroperoxides (LOOH) was assessed according to the method of Balla et al. (1991).

Statistics. Values are the mean of at least three determinations ± SD. Linear regression analyses were performed with appropriate software (Axum 6.0, Mathsoft).

RESULTS

Tyrosinase-Catalyzed Oxidation of Standard Phenolic Compounds. Mushroom tyrosinase is well-known for exhibiting a wide substrate specificity. Accordingly, it oxidizes a number of *o*-diphenols and phenols such as catechol, 4-methylcatechol, phenol, tyrosine, 3,4-dihydroxyphenylalanine, 2,4-dihydroxyphenylalanine, dopamine, noradrenaline, adrenaline (Sugumaran et al., 1996; Prota, 1992), tyrosine amino-terminal peptides, and tetrahydroisoquinolines (Rosei et al., 1992, 1995). Given the broad tyrosinase specificity, it was of interest to test tyrosinase activity on phenolic compounds that are reported to be present in virgin olive oil. Tyrosinase oxidation of standard phenolic compounds was followed spectrophotomerically in the range 200−600 nm. A list of the compounds tested is reported in Table 1. The majority of the polyphenols tested were found to be good substrates for tyrosinase. Tyrosinase action on phenolic compounds gives rise to the corresponding *o*-quinones, which exhibit a maximum

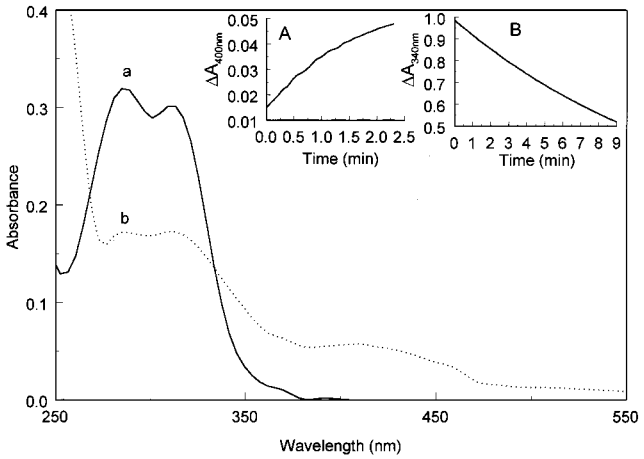

Figure 2. UV−vis spectral changes of caffeic acid oxidized by tyrosinase: (a) 20 μM caffeic acid in 50 mM phosphate buffer, pH 7.4; (b) caffeic acid spectrum recorded after 3 min of oxidation by tyrosinase. Incubation mixtures contained 20 μM caffeic acid and 20 EU of tyrosinase in 1 mL of 50 mM phosphate buffer, pH 7.4. (Inset A) Kinetics of caffeic acid oxidation at 400 nm. Incubation mixture was as above. (Inset B) Kinetics of caffeic acid oxidation at 340 nm. Incubation mixture contained 20 μM caffeic acid, 150 μM NADH, and 20 EU of tyrosinase in 1 mL of 50 mM phosphate buffer, pH 7.4.

absorbance at ~400 nm. Typical spectral modifications for the oxidation of caffeic acid are reported in Figure 2. The reaction rate can be monitored either by measuring the rate of *o*-quinone production around 400 nm (inset A) or, in the presence of excess NADH, by measuring the rate of NADH oxidation to NAD^+ at 340 nm (inset B). The rate of NADH oxidation is directly related to the amount of phenolic compound present in the incubation mixture. Also, the kinetics of the recycling reaction is linear for a longer period of time and the ΔA per minute is much higher, as compared to the kinetics of the nonrecycling reaction. This phenomenon makes it possible to calculate with great precision and ease the reaction rate. Absorbance measurements of the NADH oxidation rate enhance the assay sensitivity by ~100-fold, as compared to the nonrecycling *o*-quinone detection, yielding a detection limit of 0.20 μM for caffeic acid. Fluorescence monitoring of the recycling reaction achieved a further 10-fold enhancement of the detection limit, being 0.019 μM for caffeic acid.

It is known that nonsubstrate compounds having a structure similar to that of substrates may exert a competitive inhibition on enzyme activity. A question may arise as to whether nonsubstrates such as vanillic or cinnamic acids may exert an inhibitory effect on the enzyme activity. This hypothesis was investigated by running experiments aimed at testing the effect of nonsubstrates on the enzyme activity. No inhibitory effect of these compounds on the kinetics of the enzyme was observed (data not shown).

Evaluation of Phenolic Content of Olive Oil by Enzymatic Assay. The polyphenol content of the oil samples is given in Table 2. The results are obtained both by the classic Folin−Ciocalteu colorimetric method and by the enzymatic recycling assay. The values seem to indicate a different phenolic content, as compared with the colorimetric methods. However, a linear regression analysis indicates that the two detection methods are perfectly correlated, having an R^2 value of 0.99 (Figure 3).

Antioxidant Capacity of Olive Oil Polyphenols. In a first series of experiments, 5 μL of the stock extract

Table 2. Polyphenol Content of Various Oil Samples[a]

oil sample	colorimetric assay	enzymatic assay
A	66.0 ± 3.4	287.1 ± 5.6
B	40.6 ± 2.8	89.2 ± 3.4
C	34.6 ± 1.8	47.5 ± 0.8
D	114.3 ± 5.1	566.0 ± 6.2
E	68.3 ± 2.1	287.1 ± 3.1
F	62.4 ± 3.4	222.7 ± 2.8
G	0 ± 0	0 ± 0

[a] Values are means ± SD. Results are expressed as mg of caffeic acid/kg of oil (ppm).

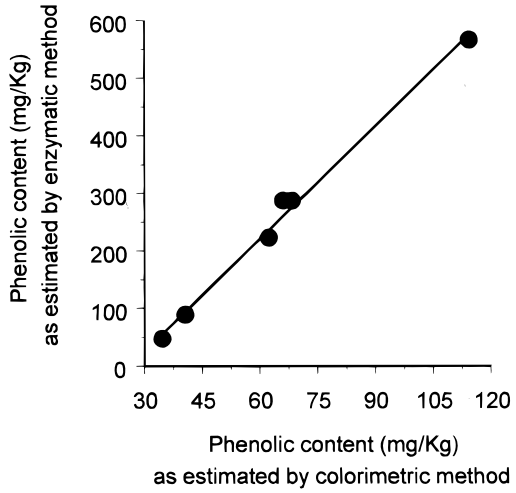

Figure 3. Linear regression between polyphenol content estimated by the colorimetric method and by the enzymatic method.

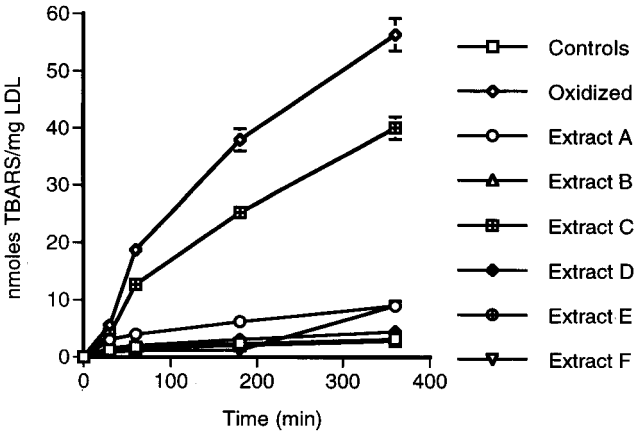

Figure 4. TBARS levels in oxidized LDL. Samples were incubated with olive oil extracts as indicated under Materials and Methods.

solution was added to the LDL samples that were to be oxidized. As shown in Figure 4 and Figure 5, olive oil extracts inhibited LDL oxidation in a manner that is proportional to their phenolics concentration, as estimated by the enzymatic assay. In particular, the degree of potency of the extracts is D > E = A > F > B > C.

To confirm the accuracy of the methodology, 30 ppm of polyphenols (as evaluated by the enzymatic method) from each extract was added to the LDL samples before the addition of $CuSO_4$. As shown by both TBARS and LOOH evaluations, the inhibition curves of each extract are superimposable, confirming that the enzymatic method estimated the phenolic content of each extract in a comparable manner (Figures 6 and 7).

Another series of experiments was made to assess the radical-scavenging capacity of polyphenol extracts. A

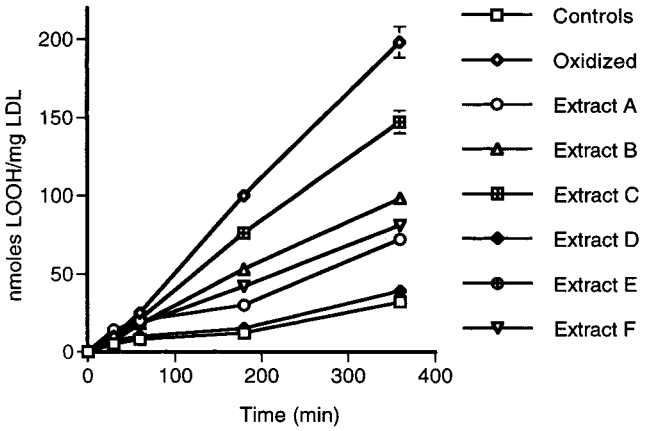

Figure 5. LOOH levels in oxidized LDL. Samples were incubated with olive oil extracts as indicated under Materials and Methods.

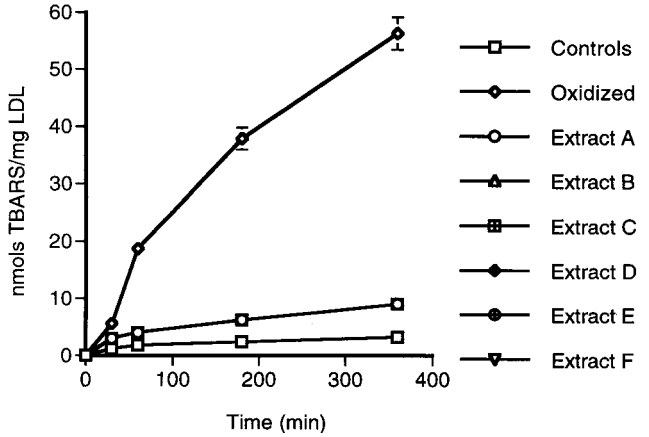

Figure 6. TBARS levels in LDL samples incubated with olive oil extracts at the concentration of 30 ppm.

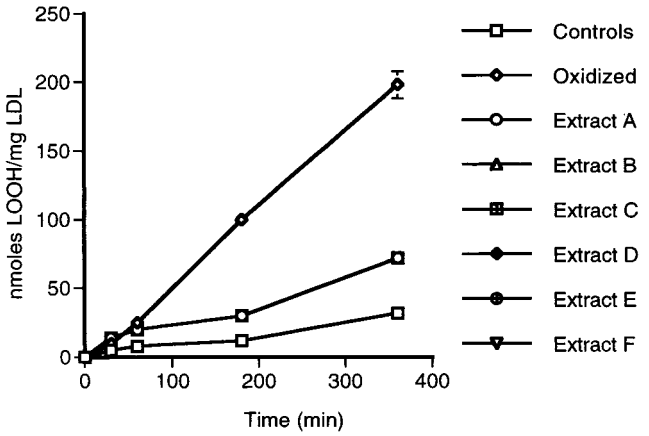

Figure 7. LOOH levels in LDL samples incubated with olive oil extracts at the concentration of 30 ppm.

stock extract solution (2 μL) was incubated in the presence of 15 μM DPPH. The radical-scavenging capacity reveals that the activity is directly related to the polyphenol content as assessed by the enzymatic method (see Figure 8). The IC_{50} values for the polyphenol extract are reported in Table 3.

DISCUSSION

In this paper, we propose a novel spectrophotometric assay for the evaluation of the polyphenolic content of olive oil. It appears from this study that estimating

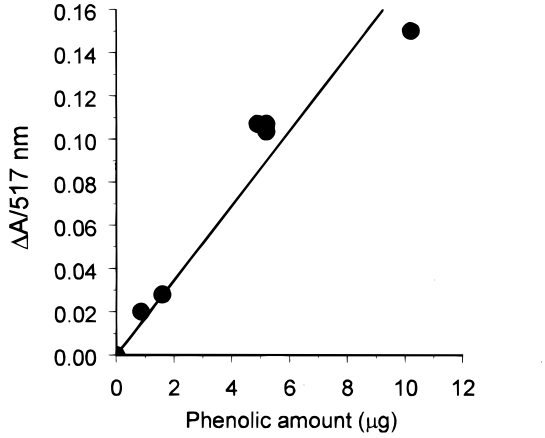

Figure 8. Linear regression between radical quenching effect of the oil extracts and phenolic content, as estimated by the enzymatic method.

Table 3. DPPH Quenching Test

extract	IC_{50} (μg)	extract	IC_{50} (μg)
A	6.9	D	3.32
B	21.8	E	6.7
C	43.12	F	9.86

[a] The scavenging effect of olive oil extracts on DPPH was evaluated as under Experimental Procedures, and IC_{50} values were calculated by employing MacALLFIT as software.

polyphenol content in olive oil by the enzymatic method may offer several advantages over the classical methods. These advantages are as follows:

- The enzymatic method is rapid and easy, in contrast to the Folin−Ciocalteu and the chromatographic methods, which need very long times.
- The enzymatic method had increased specificity and sensitivity compared with the colorimetric method, as the enzyme is able to specifically catalyze the oxidation of phenolic or catecholic comopunds; also, the absorbance measurements of the NADH consumption rate enhance the detection limit by 100-fold.
- In the case of weak activities or substrates giving very unstable and reactive quinones, the recycling assay allows a more accurate determination of the initial rates. The duration of the linear period of kinetics is increased, as compared to nonrecycling assay, allowing a better estimation of kinetic values.
- The assay may be quickly and routinely performed with standard laboratory spectrophotometers.

However, this method also presents some disadvantages that can be summarized as follows:

- The assay provides only quantitative information in contrast to the chromatographic method, which allows both the identification of several compounds—those for which authentic standards are available—and their subsequent quantitation.
- The enzyme does not catalyze the oxidation of compounds such as cinnamic acid (not bearing a phenolic group on the benzene ring), vanillic acid (bearing an *o*-methoxyl group that does not allow the formation of the *o*-quinone), and *p*-hydroxybenzoic acid [in which the carboxylic function may deactivate the *m*-position, thus preventing the oxidation to *o*-quinone (Prota, 1992)].

It is well-known that polyphenols have outstanding antioxidant and free radical-scavenging properties that render olive oils resistant to peroxidation. Previous work by Gutfinger (1981) revealed that the stability to oxidation is directly proportional to phenolic content, as

estimated by the colorimetric method. Hence, the antioxidant activity is proportional to the phenolic content. To validate the phenolic values obtained by the novel methodology topic of this paper, a series of tests aimed at evaluating the antioxidant capacity of the extracts were performed. In particular, Figures 4 and 5 show that the antioxidant capacity of the extracts, assessed in a model of chemically oxidized LDL, was proportional to their phenolic content, estimated according to the enzymatic method. Similar results are obtained by the DPPH radical quenching test (Figure 8). A second series of experiments, in which the extracts were added at the same concentration, confirmed that the values obtained with the enzymatic method were reproducible regardless of the extract employed. In fact, when the extracts were added at the concentration of 30 ppm, the inhibitory effects of LDL oxidation were almost identical.

The colorimetric and enzymatic methods are thus perfectly correlated, in terms of estimation of the antioxidant activity of the phenolic extracts evaluated with the two methods. Given this perfect correlation between the two methods, the difference in the evaluation of the polyphenol content may lie in the different response of phenols and catechols to the two quantitation methods. Actually, when equimolar amounts of either phenolic or catecholic compounds are treated with the Folin−Ciocalteu reagent, the colorimetric responses are different: catechols yield higher responses. Conversely, when a phenol or a catechol is treated with the enzymatic system, the ΔA per minute values are similar for the two compounds, the only difference being an initial lag-phase for the phenolic substrate (data not shown). The different responses of the substrates may therefore explain why the enzymatic assay seems to overestimate the polyphenolic levels as compared with the colorimetric method. Actually, in light of the abovementioned results, we conclude that the enzymatic assay allows a more precise estimation of the phenolic content because the response is similar regardless of the substrates. It is noteworthy, though, that most phenolic compounds are present in various aglycon forms, at the moment unavailable as true standards. Future availability of pure compounds will allow the estimation of complex mixtures.

In conclusion, the enzymatic method presented in this paper is a valid and quick method to measure the total amout of phenolics in olive oil. However, despite the many advantages of the recycling assay, this method cannot be proposed as the best way to identify and quantify single phenolics. In fact, depending on the purpose of the research, the chromatographic method is to be employed as the only suitable method for a simultaneous quali-quantitative determination of various phenolic compounds. Finally, it will be very interesting to test the enzymatic method for the detection and measurement of polyphenols in biological fluids such as human plasma, given the great importance of phenolics in human nutrition.

ABBREVIATIONS USED

HPLC, high-performance liquid chromatography; NADH, reduced nicotinamide adenine dinucleotide; DPPH, 1,1-diphenyl-2-picrylhydrazyl; PBS, phosphate-buffered saline; TBARS, thiobarbituric acid-reacting substances; LOOH, lipid hydroperoxides; LDL, low-density lipoproteins; UV−vis, ultraviolet−visible.

ACKNOWLEDGMENT

We thank Dr. Daniela Di Sciullo for technical assistance.

LITERATURE CITED

Balla, G.; Jacob, H. S.; Eaton, J. W.; Belcher, J. D.; Vercelloti, G. M. Hemin a possible physiological mediator of low-density lipoprotein oxidation and endothelial injury. *Arterioscler. Thromb.* **1991**, *11*, 1700−1711.

Boskou, D. *Olive Oil Chemistry and Technology*; AOCS Press: Champaign, IL, 1996.

Boskou, D. Olive oil. In *Characteristics of the Mediterranean Diets*; Simopoulos, A., Visioli, F., Eds.; World Review of Nutrition and Dietetics Vol. 87; Karger Press: Basel, Switzerland, 2000; in press.

Brown, R. S.; Male, K. B.; Luong, J. H. T. A substrate recycling assay for phenolic compounds using tyrosinase and NADH. *Anal. Biochem.* **1994**, *222*, 131−139.

Gutfinger, T. Phenols in olive oil. *J. Am. Oil Chem. Soc.* **1981**, *68*, 966−968.

Harborne, J. B. General procedures and measurement of total phenolics. In *Methods in Plant Biochemistry 1, Plant Phenolics*; Harborne, J. B., Ed.; Academic Press: New York, 1989; pp 1−28.

Keys, A. Mediterranean diet and public health personal reflections. *Am. J. Clin. Nutr.* **1995**, *61*, 1321S−1323S.

Lipworth, L.; Martinez, M. E.; Angell, J.; Hsien, C. C.; Trichopoulos, D. Olive oil and human cancer an assessment of the evidence. *Prev. Med.* **1997**, *26*, 181−190.

Lowry, O. S.; Rosebrough, N. J.; Farr, A. L.; Randall, R. J. Protein meausurement with the Folin phenol reagent. *J. Biol. Chem.* **1951**, *193*, 265−272.

Montedoro, G. F.; Servili, M.; Baldioli, M.; Miniati, E. Simple and hydrolyzable compounds in virgin olive oil. 1. Their extraction, separation, and quantitative and semiquantitative evaluation by HPLC. *J. Agric. Food Chem.* **1993**, *41*, 1571−1576.

Papadopoulos, G.; Boskou, D. Antioxidant effect of natural phenols on olive oil. *J. Am. Oil Chem. Soc.* **1991**, *68*, 669−671.

Prota, G. *Melanins and Melanogenesis*; Academic Press: New York, 1992.

Rosei, M. A.; Mosca, L. Production of melanin pigments by chemical and enzymatic oxidation of tetrahydroisoquinolines. *Biochem. Mol. Biol. Int.* **1995**, *35*, 1253−1259.

Rosei, M. A.; Mosca, L.; De Marco, C. Melanins production from enkephalins by tyrosinase. *Biochem. Biophys. Res. Commun.* **1992**, *184*, 1190−1196.

Sugumaran, M.; Tan, S.; Sun, H. Tyrosinase-catalyzed oxidation of 3,4-dihydroxyphenylglycine. *Arch. Biochem. Biophys.* **1996**, *329*, 175−180.

Trichopouolu, A. Olive oil and breast cancer. *Cancer Causes Control* **1995**, *6*, 475−476.

Tsimidou, M.; Papadopoulos, G.; Boskou, D. Phenolic compounds and stability of virgin olive oil—Part 1. *Food Chem.* **1992**, *45*, 141−144.

Visioli, F.; Galli, C. Olive oil polyphenols and their potential effects on human health. *J. Agric. Food Chem.* **1998a**, *46*, 4292−4296.

Visioli, F.; Galli, C. The effect of minor constituents of olive oil on cardiovascular disease: new findings. *Nutr. Rev.* **1998b**, *56*, 142−147.

Willet, W. C.; Sacks, S.; Trichopoulou, A.; Drescher, G.; Ferro-Luzzi, A.; Helsing, E.; Trichopoulos, D. Mediterranean diet pyramid a cultural model for healthy eating. *Am. J. Clin. Nutr.* **1995**, *61*, 1402S−1406S.

Received for review May 24, 1999. Revised manuscript received November 2, 1999. Accepted November 9, 1999. This work was supported by grants from MURST.

JF990556+