Photometric Assay for Polyphenol Oxidase Activity in Olives, Olive Pastes, and Virgin Olive Oils

Luca Valgimigli[a], Enrico Sanjust[b], Nicoletta Curreli[b], Augusto Rinaldi[b], Gian F. Pedulli[a], and Antonio Rescigno[b,*]

[a]Dipartimento di Chimica Organica "A. Mangini," Universita' di Bologna, 40127 Bologna, Italy, and [b]Dipartimento di Scienze Mediche Internistiche, Universita' di Cagliari, 09042 Monserrato (CA), Italy

ABSTRACT: A photometric method is proposed that allows the determination of phenolase activity in olive fruits, olive pastes, and virgin olive oil. The method can also be used to quantify partially purified phenolase from olives, and is based on the coupling between 4-methyl-*o*-benzoquinone, the reaction product of phenolase toward its substrate 4-methylcatechol, and the aromatic amine 4-amino-*N,N*-diethylaniline. The deep-blue adduct arising from this reaction has been characterized by means of nuclear magnetic resonance and mass spectrometric techniques and identified as 4-(4′-diethylaminophenylimino)-2-hydroxy-5-methyl-cyclohexa-2,5-dienone. This compound shows an absorption band, centered (in dichloromethane) at 617 nm, with an ε of 11,080 $M^{-1}cm^{-1}$. The main advantage of the proposed method resides in the high absorption coefficient of the adduct and its ultraviolet/visible absorption pattern, with a λ_{max} in a spectral region void of significant interferences by the pigments that ultimately will probably be present in the extracts to be tested by this proposed method. The method has proven to be sensitive, specific, and reliable.

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Polyphenol oxidases (PPO; EC 1.14.18.1) are widespread copper-containing monooxygenases (1). They carry out a number of functions by catalyzing the *o*-hydroxylation of phenols (cresolase activity), and the oxidation of *o*-diphenols to the corresponding *o*-quinones (catecholase activity) at the expense of molecular oxygen. PPO are typically product-sensitive enzymes, being irreversibly inactivated by the quinones they form. As a consequence, a number of different assays have been proposed to overcome this problem (2–6). However, these improved assays have the disadvantage of being carried out in aqueous solution, where major interferences arising from sample turbidity and pigmentation strongly affect the reproducibility and reliability of the analysis when crude materials have to be assayed. This is the case of PPO estimation in olives and olive pastes, where the enzyme is profoundly involved in the determination of the overall features of the final product, the virgin oil.

This paper is dedicated to the memory of Professor Francesco Corongiu, Dean of the Faculty of Sciences, University of Cagliari, prematurely deceased one year ago.

*To whom correspondence should be addressed at Cattedra di Chimica Biologica, Universita' di Cagliari, Cittadella Universitaria, 09042 Monserrato (CA), Italy. E-mail: rescigno@unica.it

More recently, a spectrophotometric method for the detection of PPO activity in aqueous solution (7) and also on polyacrylamide gel electrophoresis (PAGE) slabs (8) was described. This assay is based on the formation of an adduct between the enzymically produced 4-*tert*-butyl-1,2-benzoquinone and 4-amino-*N,N*-diethylaniline sulfate (ADA). The blue adduct formation can be continuously monitored as no extraction is required.

Unfortunately, 4-*tert*-butyl-catechol has proven to be a very poor substrate for olive PPO. Therefore, a modification of the method is described here that uses a good enzyme substrate, 4-methylcatechol (MC), whose oxidation product 4-methyl-*o*-benzoquinone (MQ) is capable of forming a blue adduct in the presence of ADA.

EXPERIMENTAL PROCEDURES

Reagents and materials. MC and ADA were from Fluka (Buchs, Switzerland). All other reagents were of the highest grade commercially available and were used without further purification. Stock solutions of ADA (20 mM) and MC (100 mM) were freshly prepared in 5 mM HCl to avoid autoxidation.

Olives were from the same batch composed of the Italian cultivar 'Frantoio.' Fully developed fruits, whose color was just turning from green to purple-violet, were considered in this work.

Ground and kneaded pastes were from a local olive milling plant. Kneaded pastes were drawn after 30 min of kneading (malaxation). PPO was partially purified from the olive batch above mentioned by strictly following a published procedure (9). All preparations were stored at −20°C until use.

MQ. MQ was prepared from MC by stirring in the dark for 1 h at room temperature a concentrated solution in freshly distilled tetrahydrofuran with an excess of Ag_2O. The inorganic material was then removed by filtering and the organic solution was deoxygenated by bubbling with nitrogen and evaporated *in vacuo* at room temperature. No further purification of the dark orange microcrystalline material was necessary. The yield was almost quantitative.

4-(4′-Diethylamino-phenylimino)-2-hydroxy-5-methyl-cyclohexa-2,5-dienone. This was synthesized as follows: a concentrated solution of MQ (2 mmol) in dichloromethane (Aldrich Chemie, Steinheim, Germany) was dropwise added to 2.2 mmol of ADA sulfate (Fluka) dissolved in water. After

addition, the biphasic system was vigorously stirred at room temperature for 1 h, and the blue adduct was extracted with dichloromethane. The organic solution was washed with water, dried over Na_2SO_4, and evaporated *in vacuo* to yield the blue product, which was purified by preparative thin-layer chromatography on silica gel (20 cm × 30 cm × 1 mm; Merck, Darmstadt, Germany) by eluting with dichloromethane/methanol (95:5). The purified product was extracted from the plate with isopropanol, which was evaporated *in vacuo* at room temperature. Yield = 75% after purification.

^1H and ^{13}C nuclear magnetic resonance spectra were recorded on a Varian Gemini 300 spectrometer by using a 20-mg sample of purified adduct dissolved in 0.75 mL of $CDCl_3$ (Sigma, Diesenhofen, Germany). Fourier transform infrared spectra of the purified adduct were recorded with a Nicolet Protégé 460 spectrometer (Madison, WI) from a solution of the sample in chloroform (Aldrich) by using a KBr cell with an optical path of 0.1 mm. Mass spectroscopy was performed on a double-analyzer VG 7070E spectrometer (EI+ 70 EV), with direct probe-introduction of the sample. Ultraviolet-visible (UV-Vis) spectra were recorded on a double-beam Jasco V-550 spectrophotometer.

Sample preparation. Ground and kneaded olive pastes were treated with liquid nitrogen immediately after being drawn from the olive mill and were kept at −80°C until use. To 1 g of the paste, 0.1 g of crosslinked polystyrene (Bio-Beads SM-2, Bio-Rad, Hercules, CA) and 0.25 g of crosslinked poly(vinylpyrrolidone) (Aldrich Chemie) beads were added, immediately followed by 8 mL of 10 mM sodium citrate buffer, pH 5.5. The suspension was gently stirred for 10 min, and subsequently centrifuged at 4°C for 20 min at 15,000 × *g*. The supernatant was finally filtered through Whatman 1 paper (Maidstone, England).

Fresh olives were lyophilized. The fruitstones were then removed, and stoned fruits (10 g) were suspended in 100 mL of 10 mM sodium citrate buffer, pH 5.5, containing 1 g of SM-2 Bio-Beads and 2.5 g of crosslinked poly(vinylpyrrolidone) beads. The mixture was homogenized for 2 min at 16,000 rpm in an Ultraturrax (Ika-Werhe GmbH, Staufen, Germany). The homogenate was centrifuged at 4°C and 22,000 × *g*, and the supernatant was filtered through Whatman 1 paper. Virgin oil samples were extracted as described elsewhere (10).

Enzyme assay. All the enzyme assays were performed at 30°C, with the aid of a thermostated water bath and a thermostatic device mounted within the cuvette chamber.

Standard activity measurements. PPO activity was determined photometrically by measuring the initial rate of oxidation of MC at 400 nm by using a Pharmacia Ultrospec 4000 spectrophotometer. The assay mixture contained, in a final volume of 2 mL, 400 µL of enzyme solution and 10 mM MC in 50 mM sodium citrate buffer, pH 5.5. An ε value of 1300 $M^{-1}cm^{-1}$ at λ = 400 nm was used for calculations (5).

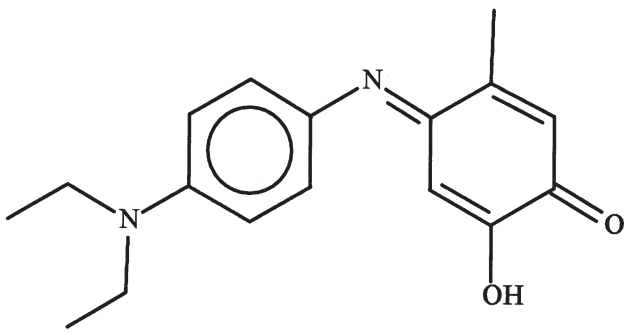
ADA-based activity measurements. PPO activity was determined photometrically by measuring the overall formation of the blue adduct of the enzyme with its substrate MC in the presence of ADA. The assay mixture contained, in a final volume of 2 mL, 400 µL of enzyme solution, 10 mM MC, and 2mM ADA in 50 mM sodium citrate buffer, pH 5.5. After 5 min, dichloromethane (2 mL) was added and the mixture vortexed for 15 s in a stoppered test tube, to avoid dichloromethane evaporation. The upper aqueous layer was drawn off, the organic solution transferred to a spectrophotometer cuvette, and the absorbance read at 617 nm. An ε value of 11,080 $M^{-1}cm^{-1}$ (in dichloromethane) was used for calculations (discussed in the Results and Discussion section).

RESULTS AND DISCUSSION

ADA and MQ rapidly and quantitatively react with a 1:1 stoichiometry to give the intensely blue product, 4-(4′-diethylamino-phenylimino)-2-hydroxy-5-methyl-cyclohexa-2,5-dienone. The structure of this has been determined (Scheme 1), in accordance to the following analytical data: η_{max} (cm^{-1}) 3386, 3040, 2964, 2929, 1720, 1630, 1600. δ_H (300 MHz, $CDCl_3$) 1.21 (*t*, 6H, *J* = 7.5), 1.60 (OH, broad *s*, 1H), 2.18 (*s*, 3H), 3.42 (*q*, 4H, *J* = 7.5), 5.31 (*s*, 1H), 5.32 (*s*, 1H), 6.62 (*d*, 2H, *J* = 6.0), 7.01 (*d*, 2H, *J* = 6.0) ppm. δ_C (75 MHz, $CDCl_3$) 13.7, 30.6, 45.6, 103.5, 112.5, 125.7, 126.1, 139.2, 147.7, 151.0, 153.7, 163.1, 183.4 ppm. *m/z* 284 (M^+, 20%), 269 (32%), 255 (37%), 163 (14%), 119 (30%), 97 (25%), 83 (35%), 69 (38%), 55 (40%), 43 (100%). Anal. calcd. for $C_{17}H_{20}N_2O_2$: C 71.81%, H 7.09%, N 9.85%. Found: C 71.83%, H 7.08%, N 9.81%. The UV/Vis spectrum (Fig. 1) of the compound showed a λ_{max} of 617 nm, ε 11,080 $M^{-1}cm^{-1}$ (in dichloromethane).

The substance is immediately and completely extracted from aqueous solutions by dichloromethane, where it shows a remarkable stability. As MC is among the best substrates for olive PPO, which oxidizes it to the corresponding MQ, the described method is quite suitable for PPO determination.

To check and validate the assay, olives, ground and kneaded olive pastes, and virgin oil were all tested for PPO activity by using both the standard method and that described above. Generally speaking, these results, obtained by using the proposed colorimetric method, parallel those achieved with the standard one (Table 1), although higher activity was found with the use of the ADA-based method (see below).



SCHEME 1

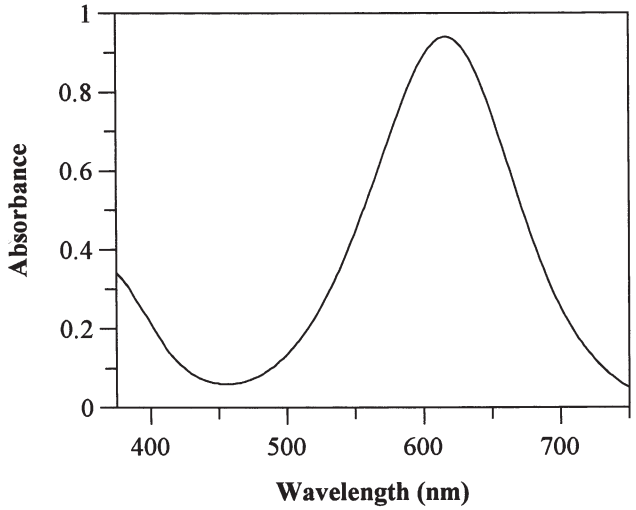


FIG. 1. The ultraviolet-visible absorption spectrum of the blue adduct, 4-(4′-diethylaminophenylimino)-2-hydroxy-5-methyl-cyclohexa-2,5-dienone.

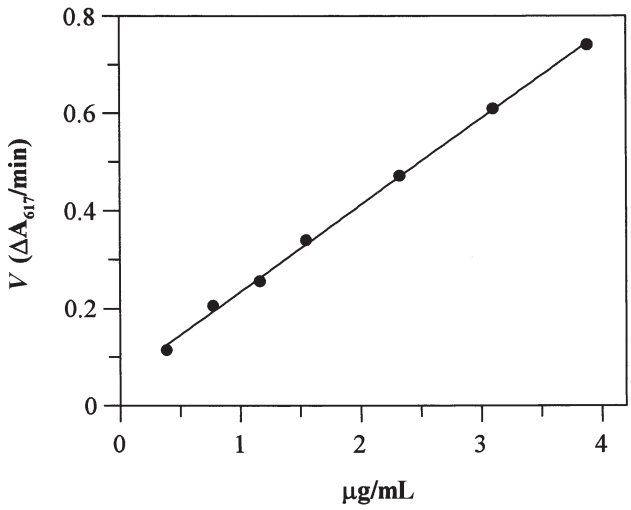


FIG. 2. Dependence of enzyme activity on enzyme concentration.

The most significant difference was the excellent reliability of the data, obtained with the ADA-based assay, whereas a huge variability was seen with the standard method. Overestimation of PPO activity, arising from nonspecific oxidation of endogenous phenolics, and leading to products absorbing at 400 nm, is prevented by the specific reaction between MQ and ADA. On the other hand, MQ is not very stable and tends to undergo a rather complex autoxidative process, leading to 2-hydroxy-5-methyl-1,4-benzoquinone (11) and several other unidentified melanin-like products. Therefore, a noticeable underestimation of PPO could arise. Moreover, MQ can covalently react with PPO itself, leading to enzyme inactivation. On the contrary, in the presence of ADA, MQ is continuously removed from the reaction medium in the form of the quite stable and unreactive blue adduct, thereby avoiding further chemical changes. Not even traces of PPO in filtered virgin oil samples were found, regardless of the method used, contrary to that found by others (10). This observation suggests that PPO content of virgin oils is notably affected by the particular technology adopted in different olive milling plants. Most probably PPO, when present, is dissolved within aqueous microdrops, which are eventually present in nondecanted and/or nonfiltered virgin oils. Our samples were perfectly clear and virtually anhydrous, implying that PPO could have been eliminated at the filtration step in the oil plant.

The development of a blue color as a result of any enzymic activity is in principle very useful in order to minimize interferences by pigments present in the sample to be analyzed. This is because only seldom do blue pigments occur in natural products. Moreover, when present, those blue pigments are usually anthocyanins and/or related compounds, whose highly polar character prevents their extraction into a dichloromethane phase.

Therefore, the reaction between some *o*-quinones (potentially arising from PPO action toward their catechol counterparts) and suitable aromatic amines has been explored in some detail (12). ADA has no significant action toward PPO activity or other enzyme activities present in olives. However, ADA is potentially useful when checking for the presence of laccase and peroxidase (13), because it is an excellent substrate for both these enzymes. Fortunately, those activities could not be detected in olives (and in olive pastes and olive oil) under the described analytical conditions. Therefore, ADA could be added to the samples without any inconvenience. The method is well suited for accurate and

TABLE 1
PPO Activities, Calculated with Standard and ADA-Based Methods[a].

	Standard activities				ADA-based activities			
Samples (n = 10)	m	s	s/m	μ	m	s	s/m	μ
Olives	0.114	0.049	0.434	0.114 ± 0.053	0.248	0.006	0.025	0.248 ± 0.006
Olive pastes	0.170	0.063	0.369	0.170 ± 0.068	0.295	0.009	0.030	0.295 ± 0.010
Kneaded pastes (10 min)	0.249	0.080	0.321	0.249 ± 0.087	0.351	0.012	0.034	0.351 ± 0.013
Kneaded pastes (20 min)	0.219	0.058	0.267	0.219 ± 0.063	0.318	0.004	0.012	0.318 ± 0.004
Kneaded pastes (30 min)	0.202	0.066	0.328	0.202 ± 0.072	0.310	0.004	0.013	0.310 ± 0.004
Kneaded pastes (60 min)	0.176	0.040	0.227	0.176 ± 0.043	0.302	0.003	0.010	0.003 ± 0.003

[a]The Student function t_{975} = 3.250 was used to estimate μ, assuming P = 0.99 (double-tailed). PPO, polyphenol oxidase; ADA, 4-amino-*N*,*N*-diethylaniline sulfate; m, mean value of samples; s, standard deviation; s/m, variation coefficient; μ mean value of population.

reproducible measurements when working at the same time on many samples, or when kinetic measurements are required. Therefore, K_m and V_{max} determinations, performed with the aid of the described method, gave results that were in full agreement with those obtained following an already described procedure (9). Highly turbid and/or cloudy olive extracts could be tested for their PPO activity and showed no significant differences when compared with perfectly clear samples. Besides, linearity was obtained between enzyme activity and PPO concentration. The method was applied in triplicate and a linear regression fitting was obtained (Fig. 2) within the considered range.

In conclusion, the method is fast, simple, reliable, and well suited for both routine measurements and kinetic studies.

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