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Enzymatic Method with Polyphenol Oxidase for the Determination of Cysteine and *N*-Acetylcysteine

F. GARCÍA-MOLINA, M. J. PEÑALVER, J. N. RODRÍGUEZ-LÓPEZ, F. GARCÍA-CÁNOVAS,* AND J. TUDELA

GENZ: Grupo de Investigación de Enzimología, Departamento de Bioquímica y Biología Molecular-A, Facultad de Biología, Universidad de Murcia, E-30100 Espinardo, Murcia, Spain

Thiols, such as cysteine and *N*-acetylcysteine, are included in many pharmaceutical products for their mucolytic properties. The method described here uses mushroom polyphenol oxidase (PPO) to determine two thiols and consists of measuring the lag period in the formation of the product generated as PPO acts on *o*-diphenol in the presence of a thiol. In the experimental conditions, *o*-quinone is formed enzymatically and then reacts stoichiometrically with the thiol, originating the corresponding thiol−diphenol adduct, which does not absorb visible light. Once the thiol has been used up, the *o*-quinone can be observed in the medium. It must be borne in mind that the inhibition of PPO is practically null at low concentrations of thiol, and the only effect observed is the formation of the thiol−diphenol adduct. In the following, an exact kinetic method capable of rapidly and accurately assaying thiols with PPO and *o*-diphenol is optimized and is shown to be a straightforward way of calculating thiol concentration. The method has been successfully applied to the determination of cysteine in model solutions and of *N*-acetylcysteine in pharmaceutical products.

KEYWORDS: Cysteine; determination; diphenols; enzyme; kinetics, enzymatic method; functional foods; mushroom; *N*-acetylcysteine; pharmaceuticals; polyphenol oxidase; thiols; tyrosinase

INTRODUCTION

Tyrosinase or polyphenol oxidase (PPO; monophenol, *o*-diphenol: O_2 oxidoreductase, EC 1.14.18.1) is an enzyme that catalyzes two different reactions, in both of which it uses O_2. These reactions are orthohydroxylation of monophenols to *o*-diphenols (monophenolase activity) and oxidation of *o*-diphenols to their *o*-quinones (diphenolase activity) (*1*). The structure of PPO contains a type III bicupric active site and a characteristic thioether bond (*2*, *3*). In animals, PPO triggers the biosynthesis of melanins in skin, hair, and eyes and of the ink in cephalopods. These melanins protect the animal from solar radiation and from predators because they help in camouflage (*4*). In plants (*5*), fungi (*6*), and bacteria (*3*), PPO sets off enzymatic browning reactions. This phenomenon is also a defense against predators and is based on the toxicity of melanges or intermediates of melanogenesis, such as *o*-quinones. It is for this reason that PPO inhibitors are used as antitumoral agents against malignant melanoma (*7*) or as depigmenting agents in the case of skin stains (*8*). PPO inhibitors are also used as antibrowning agents in the processing of fruits and vegetables (*5*). The enzymes can be used, too, for depurating effluents contaminated with phenols and for the stereospecific synthesis of phenols (*9*). Of special interest is its use in the analysis of phenols (*9*). However, the satisfactory application of these methods depends on a thorough knowledge of the catalytic activity of PPO. In this respect, one aspect of prime importance is the high nonenzymatic reactivity of the quinones generated by the enzyme (*10*, *11*). Another important property is the suicide inactivation of PPO originated by its own *o*-diphenolic substrates (*12*). Understanding both phenomena has made it possible to undertake the kinetic characterization of the PPO reaction mechanism on monophenols and diphenols (*13*−*16*).

The amino acid L-cysteine (Cys), or (*R*)-2-amino-3-mercapto-propanoic acid, participates in a complex series of metabolic reactions and is incorporated in most proteins and glutathione. It is also a precursor of coenzyme A and can easily be oxidized to cystine; however, the intercellular cysteine/cystine ratio is very high (*17*, *18*). Melanogenesis in the presence of Cys and or glutathione leads to the formation of pheomelanins, pigments with a yellowish red color (*4*). Cys analysis is important for establishing the nutritive value of foods and dietetic supplements destined for human consumption (*19*). *N*-Acetyl-L-cysteine (NACys) is a therapeutic medicine frequently used as a mucolytic agent and for treating acetaminophen hepatoxicity (*20*). It increases the cell reserves of free radicals and acts as an antioxidant (*20*). It also inhibits the replication of HIV (*21*) and prevents apoptosis in neurones (*22*).

* Corresponding author (fax +34 968 363963; e-mail canovasf@um.es; URL http://www.um.es/genz).

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Thiolic substances such as Cys and NACys take part in numerous redox addition and complexation reactions. DTNB was used some years ago to determine thiols in general (*23*−*25*). NACys and cysteine can be measured by high-performance liquid chromatography or gas chromatography (*26*, *27*), although both methods require specialized equipment. A simple colorimetric method for the simultaneous determination of NACys and cysteine has also been described (*28*). In general, nonenzymatic methods show high sensitivity as seen from their low detection limit values (LOD): $LOD^{Cys} = 0.15-8.25\ \mu M$ and $LOD^{NACys} = 0.11-0.40\ \mu M$.

The enzyme PPO shows affinity toward thiolytic reagents, which is described by its dissociation constant, K_I^{thiol}. At values of $[thiol]_0 < K_I^{thiol}$, the thiol cannot reach the active site of PPO in any great quantity and only provokes the nonenzymatic addition to the *o*-quinones generated by PPO, producing colorless thiol−diphenol adducts (*1*). However, when $[thiol]_0 \geq K_I^{thiol}$, the thiol can also inactivate PPO, irreversibly binding to the copper cations of the active site of PPO (*1*, *5*). A method has been proposed for determining $[thiol]_0$, based on its inhibition of PPO during its action on catechol (*29*). The limitations of this method will be discussed below.

The aim of this work is to propose an enzymatic method for determining $[thiol]_0$ in concentrations below those that provoke PPO inhibition. The method is based on previous studies on the first stages of pheomelanogenesis (*30*) and the chromophoric detection of PPO activity (*31*). The kinetic method will attempt to overcome the limitations inherent in the above-described method involving PPO inhibition (*29*) while reaching the sensitivity described for the determination of Cys and NACys by other authors (*19*, *20*). The enzymatic method will also be applied to the determination of NACys in commercial pharmaceuticals.

MATERIALS AND METHODS

Reagents. 4-*tert*-Butylcatechol (TBC) was from Fluka, whereas 3,4-dihydroxymandelic acid (DOMA), Cys, and NACys were from Sigma. The chromophoric products were 4-*tert*-butylcatechol quinone (TBCQ), $\epsilon_{400}^{TBCQ} = 1150\ M^{-1}\ cm^{-1}$, and 3,4-dihydroxybenzaldehyde (DOBA), $\epsilon_{350}^{DOBA} = 11800\ M^{-1}\ cm^{-1}$ (*31*). Stock solutions of phenolic substrates were prepared in 0.15 mM phosphoric acid to prevent autoxidation. The thiol solutions were prepared immediately before using. The total thiol content was measured using 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB) reagent and a slight modification of the method described by Habeeb (*32*). All other chemicals were of analytical grade and were supplied by Merck. Milli-Q system (Millipore Corp.) ultrapure water was used throughout this research. The pharmaceutical products, Fluimucil oral and Fluimucil oral forte, were obtained from a local pharmacy.

Mushroom tyrosinase (5350 units/mg) was purchased from Sigma. We define the International Unit (IU) of the monophenolase and diphenolase activities of PPO as the quantity of enzyme that produces 1 μmol of product per minute working in optimal assay conditions [i.e., substrate saturation (L-tyrosine or L-dopa), optimum pH, and 25 °C] (*33*). For this reason, there must be an equivalence between the International Units of mono- and diphenolase activities, which would be the ratio between V_{max}^D and V_{max}^D. Thus, according to ref *33*, the equivalence of 1 unit of Sigma is 0.165 mIU of monophenolase activity and 2.24 mIU of diphenolase activity, using L-tyrosine and L-dopa as substrates. Accordingly, the enzyme used was 882.75 mU/mg of monophenolase activity. Protein concentrations were determined according to the method described by Bradford (*34*) using bovine serum albumin as standard.

Spectrophotometric Assays. A Perkin-Elmer Lambda-35 spectrophotometer interfaced on-line with a PC-compatible computer with an Intel-Pentium processor, running with the UV-Winlab software, was used. The temperature was controlled at 25 °C with a Haake D1G circulating water bath with a heater/cooler and checked using a Cole-Parmer digital thermometer with a precision of ±0.1 °C.

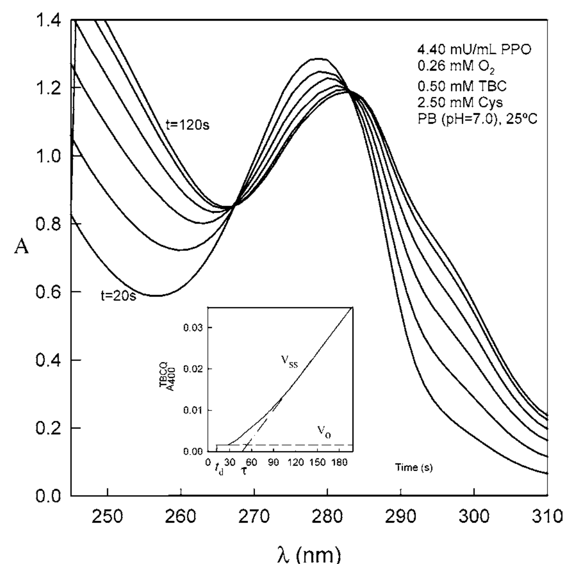


Figure 1. Spectra obtained during the oxidation of TBC by tyrosinase in the presence of Cys. (Inset) (—) 0.3 mM TBC, 10 μM Cys, and 0.88 mU/mL PPO; (- - -) same conditions without PPO.

The volume of the reaction medium was 1 mL. The standard mixture contained saturating oxygen (0.26 mM) in 0.1 M phosphate buffer (pH 7.0) at 25 °C. The reactions were monitored at 350 nm (5 mM DOMA) and at 400 nm (0.3 mM TBC), depending on the diphenolic substrate used. The thiol, Cys or NACys, was varied from 0 to 17 μM. As general methods, Cys and NACys were determined in the presence of DOMA and TBC, respectively. The reaction was started by injecting the enzyme (0.17−4.40 mU/mL). One unit of enzyme is the quantity of enzyme that yields 1 μmol of chromophoric product within 1 min of assay time. In the figure legends are detailed some reagent concentrations required for specific experimental assays.

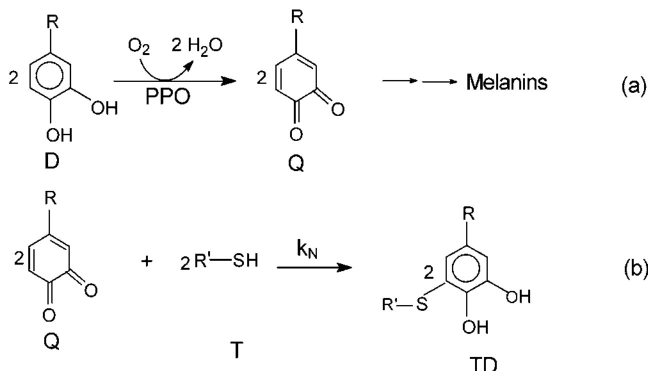
Data Analysis. The value of τ (lag period) (**Figure 1**, inset) is determined by extrapolation of the straight line of the slope V_{ss} until it intersects a line parallel to the abscissa axis localized at the initial absorbance of each time recording. This compensates for the small fluctuations in initial absorbance between experimental recordings. The reliability of the value of the enzymatic reaction rate (V_{ss}) is thus favored, establishing experimental conditions with a negligible contribution from the fluctuations (σ_0) to the nonenzymatic reaction rate (V_0), that is, $V_{ss} \geq (V_0 + 10\sigma_0)$ (**Figure 1**, inset). The reliability of the lag period (τ) is favored by choosing assay conditions that give τ values significantly far from the fluctuations (σ_d) in the dead time (t_d) of the spectrophotometric recordings (**Figure 1**, inset), that is, $\tau \geq (t_d + 10\sigma_d)$.

The sensitivity of the method was tested from 10 replicates of the dead time of the spectrophotometer and further determination of the limits of detection and quantitation, LOD and LOQ, respectively (*35*). The precision of the method was checked by 10 replicates each of the minimum, midrange, and maximum values of the initial concentration of thiol, the coefficients of variation of which were calculated (*35*). The experimental data of the lag time versus initial concentration of thiol were fitted to a straight line equation using Marquardt's algorithm (*36*), implemented in the Sigma Plot software (*37*).

NMR Assays. ^{13}C NMR spectra of the different thiols considered here were obtained in a Varian Unity spectrometer of 300 MHz. The spectra were obtained at the optimum pH for mushroom tyrosinase (6.8) and using ^2H_2O as solvent for the substrates. δ values were measured relative to those for 3-(trimethylsilyl)propionic acid-d_4 sodium salt ($\delta = 0$). The maximum line width accepted in the NMR spectra was 0.06 Hz. Therefore, the maximum accepted error for each peak was ±0.03 ppm.

The dependence of the δ values of a carbon atom on its electron density is known (*38*, *39*). It has been previously demonstrated that

Scheme 1. Enzymatic (**a**) and Nonenzymatic (**b**) Reactions Related with the Oxidation of *o*-Diphenols by O_2 Catalyzed by PPO in the Presence of Thiol Compounds



there is a linear correlation between δ in ^{13}C and the constant σ, and such a correlation has been used in both reaction rate and mechanistic studies. δ values in ^{13}C have been used to corroborate or to better calculate σ values (*40*).

RESULTS AND DISCUSSION

Diphenolase Activity of PPO and Presence of Thiols. *o*-Diphenols (D) are oxidized nonenzymatically by O_2 to *o*-quinones (Q) at low velocity (V_0). However, when catalyzed by the enzyme PPO, the same reaction occurs rapidly with a velocity of V_{ss} (**Scheme 1a**). Some *o*-quinones, such as *o*-benzoquinone, an oxidation product of catechol, evolve rapidly toward melanin (**Scheme 1a**). For this reason, catechol (*13*) and other *o*-diphenols that generate unstable *o*-quinones are not suitable substrates for measuring the enzymatic activity of PPO. However, some *o*-diphenols, such as TBC, generate chromophoric *o*-quinones such as TBCQ that remain stable for several minutes (*13*). In another (**Scheme 2a**), DOMA is oxidized by O_2 to a quinone (DOMAQ), which undergoes rapid oxidative decarboxylation to a methide quinone, which originates a stable chromophoric product, 3,4-dihydroxybenzaldehyde (DOBA) (*31*). The good stability of the chromophoric products, TBCQ and DOBA, led us to choose TBC and DOMA as the substrates for assaying the diphenolase activity of PPO, as described in this paper.

Thiols (T) react with *o*-quinones (Q) through nucleophilic addition to generate colorless thiol−diphenol adducts (TD) (**Schemes 1b** and **2b**). The simultaneous nature of the oxidation and addition reactions follow a well-defined stoichiometry (**Schemes 1** and **2**), as confirmed by the appearance of isosbestic points in iterative spectra such as those obtained for the PPO-catalyzed oxidation of TBC in the presence of an excess of L-cysteine (**Figure 1**). In the presence of a low concentration of thiol (**Figure 1**, inset), this reaction reaches the steady state corresponding to the enzymatic reaction, V_{ss} (**Schemes 1a** and **2a**) after a lag stage, τ (**Figure 1**, inset), which is related with the time necessary for the thiol in the nonenzymatic reaction to be used up (**Schemes 1b** and **2b**).

Kinetic Method for Thiol Determination. Kinetic analysis of the diphenolase activity of PPO in the presence of a low thiol concentration (*30*) provides the following expression for τ (eq 1) (**Figure 1**, inset) (see Appendix):

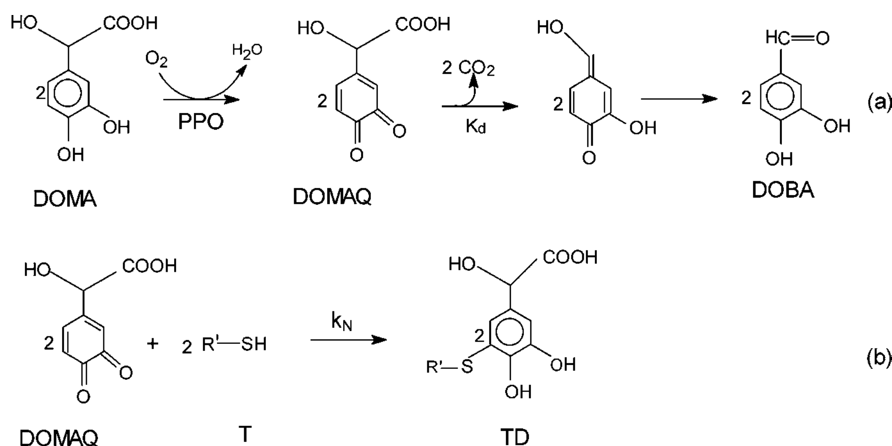
$$\tau = [\text{thiol}]_0/V_{ss} = [\text{thiol}]_0/\frac{k_{cat}^{D}[E]_0[D]_0}{K_m^{D} + [D]_0} \quad (1)$$

Hence, the lag time (τ) is directly proportional to the initial concentration of the thiol that is consumed in the nonenzymatic reaction (**Schemes 1b** and **2b**). The proportionality constant (eq 1) is the inverse of the steady-state rate (V_{ss}) of the enzymatic oxidation reaction (**Schemes 1a** and **2a**).

The low thiol concentrations considered in this kinetic analysis mean that V_{ss} does not depend on $[\text{thiol}]_0$ (eq 1). Rather, $[\text{thiol}]_0 \ll K_I^{\text{thiol}}$, and the thiol does not inhibit the enzymatic oxidation (**Schemes 1a** and **2a**).This has been proved experimentally (**Figure 2**) by carrying out assays in the absence (control) and presence of different concentrations of $[\text{Cys}]_0$, in PPO-catalyzed DOMA oxidation reactions. In the absence of Cys there is no lag period, while τ values increase as $[\text{Cys}]_0$ increases (**Figure 2**). Furthermore, the fact that the recordings evolve toward a group of lines parallel to the lines obtained in the control without Cys (**Figure 2**) indicates that the values of $[\text{Cys}]_0$ used do not inhibit the enzymatic reaction (**Scheme 1a**) and that the kinetic analysis proposed is valid (eq 1).

Effect of Enzyme Concentration. Kinetic behavior similar to that observed for the oxidation of TBC (data not shown) was observed for the PPO-catalyzed oxidation of DOMA with different $[\text{Cys}]_0$ (**Figure 2**). A lineal dependence of τ versus $[\text{Cys}]_0$ was observed in the enzymatic oxidation of both diphenols, as can be seen from the DOMA assays (**Figure 3**). In agreement with the kinetic analysis (eq 1), if $[D]_0$ and $[\text{thiol}]_0$ are kept constant, the decrease in $[E]_0$ increases τ (**Figure 3**). This indicates that using a low concentration of $[E]_0$ increases the sensitivity for determining $[\text{thiol}]_0$ (**Figure 3**). The minimum

Scheme 2. Enzymatic (**a**) and Nonenzymatic (**b**) Reactions Related with the Oxidation of DOMA by O_2 Catalyzed by PPO in the Presence of Thiol Compounds



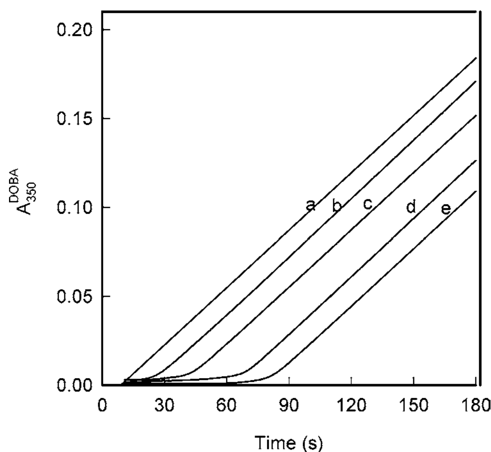


Figure 2. Time course of DOBA production by tyrosinase in the presence DOMA and Cys: (**a**–**e**) time courses of kinetic assays with the indicated concentrations of Cys and 0.53 mU/mL of PPO.

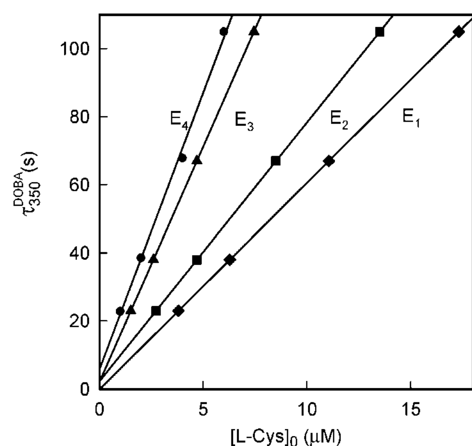


Figure 3. Effect of enzyme concentration on the lag time (τ) versus Cys. Experiments were performed under the same conditions as in **Figure 2** using the indicated concentrations of PPO.

value of $[E]_0$ is limited by the reliability of V_{ss} with respect to V_0 (**Figure 1**, inset), as explained under Materials and Methods. Obviously, low $[E]_0$ values are preferable to keep the cost of enzymatic assays as low as possible (eq 1).

Effect of *o*-Diphenol. The choice of *o*-diphenol must take into account the need for a stable chromophoric product, such as TBC (**Scheme 1**) or DOMA (**Scheme 2**). In addition, in the presence of thiol (**Schemes 1** and **2**) a specific stoichiometry must exist because of the presence of an isosbestic point (**Figure 1**). This does not occur in the case of the PPO-catalyzed oxidation of DOMA in the presence of NACys (data not shown) because NACys is less nucleophilic than Cys, as shown by the δ values for the thiol-linked carbon atoms ($\delta^{Cys} = 24.86$ and $\delta^{NACys} = 25.36$) because a higher δ indicates lower nucleophilicity, meaning that TD formation does not exceed DOBA generation (**Scheme 2**). Therefore, NACys can be determined only by using TBC as substrate for PPO (**Scheme 1**). The linearity of τ_{400}^{TBCQ} versus $[NACys]_0$ was satisfactory (**Figure 4**). However, the higher value of $\epsilon_{350}^{DOBA} = 11800$ M^{-1} cm^{-1} (*31*) compared to $\epsilon_{400}^{TBCQ} = 1150$ M^{-1} cm^{-1} (*13*) demonstrates the greater sensitivity of using DOMA as PPO substrate when Cys is determined (**Figure 4**).

The concentration of *o*-diphenol used in the experiments must be such that the steady-state rate V_{ss} is easily reached and so $[D]_0 \cong$ constant. If the *o*-diphenol concentration is high, the lag times (τ) will be very short, although this can be countered

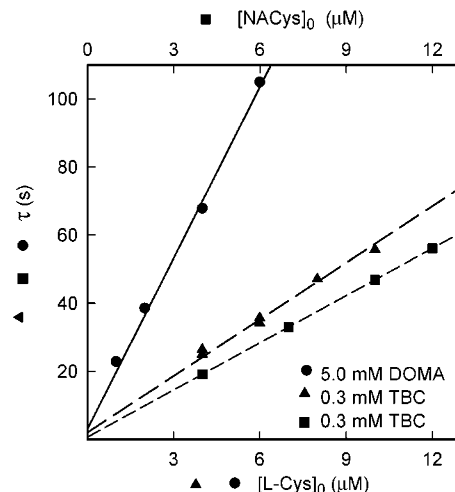


Figure 4. Effect of varying the substrate: (●) τ versus $[Cys]_0$ data with 5 mM DOMA and 0.17 mU/mL of PPO; (▲) τ versus $[Cys]_0$ data with 0.3 mM TBC and 0.88 mU/mL of PPO; (■) τ versus $[NACys]_0$ data with 0.3 mM TBC and 0.88 mU/mL of PPO.

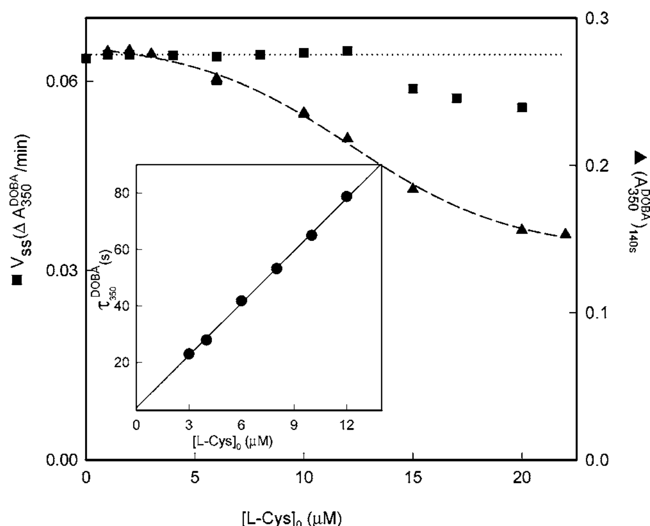


Figure 5. Influence of Cys concentration on PPO activity. The reaction medium contained 5 mM DOMA and 0.53 mU/mL of PPO: (■) steady-state rate V_{ss} measured as ΔA_{350}^{DOBA}/min versus $[Cys]_0$; (▲) value of A_{350}^{DOBA} at $t = 140$ s versus $[Cys]_0$. (Inset) (●) Dependence of the lag period, τ, on $[Cys]_0$.

by diminishing the concentration of enzyme. However, V_{ss} (the enzymatic reaction rate) must reach a significantly higher value than the nonenzymatic rate, V_0, as mentioned under Materials and Methods. For this reason, the *o*-diphenol concentration chosen was around K_m [5 mM DOMA, $K_m^{DOMA} = 5.1 \pm 0.2$ mM (*31*); 0.3 mM TBC, $K_m^{TBC} = 1.41 \pm 0.20$ mM (*14*)].

Effect of Thiol Concentration. The use of low concentrations of thiol demonstrates the suitability of the proposed kinetic method (eq 1) for determining $[thiol]_0$ (**Figures 2–4**). When high values of $[thiol]_0$ are used, an additional effect, the direct inhibition of PPO by thiol, appears (**Schemes 1a** and **2a**), as seen from the less pronounced slopes obtained for V_{ss} (**Figure 5**). Whatever the case, the method of measuring the absorbance at a fixed time (*29*) does not seem to be the most suitable because it may originate nonlineal dependencies with respect to $[thiol]_0$ (**Figure 5**). Therefore, the suitable concentration range of thiol requires $[thiol]_0$ values corresponding to a constant value of V_{ss} (**Figure 5**).

Table 1. Accuracy and Precision of the Kinetic Method with PPO for the Determination of L-Cysteine and *N*-Acetylcysteine Concentrations

thiol	LOD (μM)	LOQ (μM)	CV_{min} (%)	CV_{mid} (%)	CV_{max} (%)
Cys[a]	2.12	2.50	10	7	2
NAcCys[b]	4.94	5.77	9	5	3

[a] Assay conditions: 0.53 mU/mL of PPO. [b] Assay conditions: 0.44 mU/mL of PPO.

Table 2. Determination of *N*-Acetylcysteine in Pharmaceutical Formulations Using the DTNB Method and the Kinetic Method with PPO Described in This Paper

sample (Fluimucil)	NACys (mg/L)		relative error (%)
	DTNB method	PPO method	
(A) Fluimucil Forte 600 mg of NACys	1.58 ± 0.005	1.55 ± 0.04	+1.5
(B) Fluimucil 200 mg of NACys	1.47 ± 0.02	1.42 ± 0.02	+2.5

[a] Assay conditions: 10 μM sample A or B (1.63 mg/L NACys) was prepared in two dissolutions of ultrapure water. The reactions were monitored at 412 nm with 0.1 mM DTNB. In PPO method 0.3 mM TBC as substrate of 0.88 mU/mL of PPO.

The optimal assay conditions imply a low $[E]_0$, whereas $[D]_0$ should be near K_m^D, which would give rise to τ and V_{ss} values that are significantly higher than t_d and V_0, as mentioned under Materials and Methods. The accuracy of the kinetic method here proposed was defined by determining the LOD and the LOQ (**Table 1**). The precision of the method was assessed by determining the coefficients of variation in the minimum (CV_{min}), midrange (CV_{mid}), and maximum (CV_{max}) values of $[\text{thiol}]_0$ used (**Figure 4**; **Table 1**). As expected, reproducibility increased or the values of CV decreased as $[\text{thiol}]_0$ increased (**Table 1**). The values of LOQ^{Cys} (**Table 1**) were lower than values that were determined by HPLC with fluorometric detection (*41*). The LOD values obtained (**Table 1**) were similar to those obtained with some fluorometric methods in the case of Cys ($LOD^{Cys} = 2.6\ \mu$M), whereas for NACys the LOD is 1 order of magnitude greater ($LOD^{NACys} = 0.4\ \mu$M) (*42*).

The above determinations were made in model aqueous solutions of Cys and NACys (**Table 1**). The use of both thiols as active ingredients in medicines implies that they will be mixed with excipients and additives such as polyethylene glycol, magnesium stearate, or aspartame. These substances did not interfere to any great extent when the method was applied to determining NACys in two medicines (**Table 2**), each containing a concentration of 1.63 mg L^{-1} of NACys in the medium of reaction. The same medicines were also analyzed according to the nonenzymatic method that uses the chromogenic reagent DTNB (*43*). Our method shows good accuracy and precision (**Table 2**).

Comparison with Another Enzymatic Method. The proposed kinetic method using PPO to determine $[\text{thiol}]_0$ is an improvement over the other enzymatic method proposed (*29*), in several ways.

Enzyme Source and Supply. The other method (*29*) involves the time-consuming extraction of PPO from potato tubers and its continuous supply to the carrier current of a flow injection system, whereas our method uses discrete assays with small concentrations of mushroom PPO prepared from lyophilized commercial extracts. The proposed method is therefore more rapid, cheaper, and more reproducible than the flow injection enzymatic method (*29*).

Monitoring Substrate. The ortho-diphenolic substrate proposed in ref *29* for potato PPO is catechol, the *o*-quinone of which is unstable at pH 7.0, which makes it unsuitable for measuring PPO activity in these assay conditions. Instead, we use mushroom PPO acting on TBC or DOMA, monitoring substrates that generate stable and sensitive products, TBCQ ($\epsilon_{400}^{TBCQ} = 1150\ M^{-1}\ cm^{-1}$) (*13*) and DOBA ($\epsilon_{350}^{DOBA} = 11800\ M^{-1}\ cm^{-1}$) (*31*), respectively.

Parameter verus $[\text{thiol}]_0$. Absorbance graphs of the product versus initial thiol concentration (*29*) may not be lineal (**Figure 5**) and, therefore, inappropriate for determining $[\text{thiol}]_0$. Our kinetic method measures the lag period (τ) in recordings that evolve toward the same steady-state rate (V_{ss}) with different initial concentrations of thiol (**Figures 2** and **5**). This kinetic behavior (eq 1) ensures the linearity of τ versus $[\text{thiol}]_0$ (**Figures 2**−*5*).

Sensitivity. The methods based on the inhibitory action of thiol on PPO (*29*) need higher thiol concentrations than those methods based on nonenzymatic additions to *o*-quinones generated by PPO (**Schemes 1b** and **2b**; **Figure 5**), which is why they are less sensitive than our proposed kinetic method (**Table 1**).

This analysis method using an enzyme of agrofood origin is applicable to only the determination of pure or total thiols, in the absence of other nucleophiles that react with quinones. The method could be used in quality control assays in the preparation of medicines, nutrition supplements, functional foods, or other food products, which use thiols as active principles, but which have no other nucleophiles that would react with quinones. In short, the kinetic method with PPO proposed for determining Cys and NACys satisfactorily fulfills the objectives set out in the introduction of this paper.

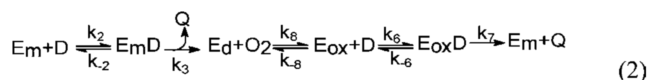
APPENDIX: RELATIONSHIP BETWEEN THIOL CONCENTRATION AND LAG PERIOD GENERATED BY THIS AGENT IN THE DIPHENOLASE ACTIVITY OF TYROSINASE

When tyrosinase acts on *o*-diphenols, there is no lag period when the absorbance of the product generated is measured. However, there is a lag period when thiol is used, the length of which depends on the thiol concentration. Analyzing the material balance of the steady-state of the pathway gives

$$V_{ss}t - [\text{thiol}] = [Q] \quad (1)$$

where $V_{ss} = d[Q]/dt$ is the rate of *o*-quinone production at time t and $[Q]$ is the *o*-quinone concentration.

Tyrosinase acts on *o*-diphenols according to the following mechanism:



(2)

Thiols (I) inhibit tyrosinase by binding to the E_{ox} form of the enzyme as follows (*44*):

$$E_{ox} + I \overset{K_I}{\rightleftharpoons} E_{ox}I \quad (3)$$

The expression of the initial rate is given by

$$V_{ss} = \frac{V_{max}[D]_0[O_2]_0}{K_s^{O_2}K_{m_2}^D + K_m^{O_2}[D]_0 + [K_{m_1}^D + K_{m_2}^D][O_2]_0 + [D]_0[O_2]_0} \quad (4)$$

where

$$V_{max} = k_{cat}[E]_0 = k_3k_7/(k_3 + k_7) \quad (5)$$

$$K_s^{O_2} = k_{-8}/k_8 \quad (6)$$

$$K_{m_1}^{D} = k_{cat}/k_2 \quad (7)$$

$$K_{m_2}^{D} = k_{cat}/k_6 \quad (8)$$

In the presence of thiols, eq 4 becomes

$$V_{ss} = \frac{V_{max}[D]_0[O_2]_0}{K_s^{O_2} K_{m_2}^{D} + K_m^{O_2}[D]_0 + \left[K_{m_1}^{D} + K_{m_2}^{D}\left(1 + \frac{[I]_0}{K_I}\right)\right][O_2]_0 + [D]_0[O_2]_0} \quad (9)$$

The oxygen concentration is saturating, and so eq 9 becomes

$$V_{ss} = \frac{V_{max}[D]_0}{K_{m_1}^{D} + K_{m_2}^{D}\left(1 + \frac{[I]_0}{K_I}\right) + [D]_0} \quad (10)$$

$K_{m_1}^{D} < K_{m_2}^{D}$ because $k_2 \gg k_6$ (*14*), and so

$$V_{ss} = \frac{V_{max}[D]_0}{K_{m_2}^{D}\left(1 + \frac{[I]_0}{K_I}\right) + [D]_0} \quad (11)$$

If $[I]_0 \ll K_I$, then

$$V_{ss} = \frac{V_{max}[D]_0}{K_{m_2}^{D} + [D]_0} \quad (12)$$

According to eq 1, when $t = \tau$ (lag period), $[Q] = 0$, and so

$$V_{ss}\tau = [\text{thiol}] \quad (13)$$

and

$$\tau = \frac{[\text{thiol}]}{v_0} = \frac{[\text{thiol}]}{V_{max}\,[D]_0/K_{m_2}^{D} + [D]_0} \quad (14)$$

ABBREVIATIONS USED

A_λ, absorbance at the wavelength $\tilde{\lambda}$; CV, coefficient of variation or relative standard deviation (%); Cys, L-cysteine; D, *o*-diphenol; DOBA, 3,4-dihydroxybenzaldehyde; DOMA, 3,4-dihydroxymandelic acid; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); E, enzyme; E_m, mettyrosinase; E_d, deoxytyrosinase; E_{ox}, oxytyrosinase; I, inhibitor (thiol); IU, International Unit of enzyme activity; K_m^x, Michaelis constant of PPO toward the species *X*; K_I, dissociation constant of $E_{ox}I$ complex; LOD, limit of detection; LOQ, limit of quantitation; NACys, *N*-acetyl-L-cysteine; PB, phosphate buffer; PPO, polyphenol oxidase or tyrosinase (EC 1.14.18.1); Q, *o*-quinone; T, thiol; TBC, 4-*tert*-butylcatechol; TBCQ, 4-*tert*-butylcatechol quinone; TD, thiol−diphenol adduct; t_d, dead time of the spectrophotometic recordings; V_0, initial rate of the nonenzymatic oxidation of D; V_{ss}, steady-state rate of the enzymatic oxidation of D, catalyzed by PPO; $[X]_0$, initial concentration of the species *X*; λ, wavelength; σ_x, standard deviation of the parameter *X*; δ_3, chemical displacement value at C-3; ϵ_λ^x, molar absorptivity of the species *X* at the wavelength λ.

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