

SPECTROPHOTOMETRIC DETERMINATION OF ASCORBIC ACID BY HORSERADISH PEROXIDASE

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ABSTRACT

L-ascorbic acid is one of the essential nutrients and most common food supplements, fortificants, and preservatives. It is commercially available as solutions, drops, tablets, capsules, crystal powder, beverage mixtures, multivitamin formulations, and multi antioxidant formulations. The usual daily dose is from 25 mg to 1.5 g. Ascorbic acid is a distinctly reducing agent with low redox potential (0.18 and 0.08 V at pH 4.5 and 6.4, respectively). Based on ascorbate property, numerous methods for its quantitative determination are developed, from titrimetric, electrochemical, and chromatographic methods, to fluorometric and kinetic ones. Enzyme peroxidase is interfered with by ascorbic acid, which decreases the oxidation speed of its co-substrates during hydrogen peroxide decomposition by peroxidase. Absorbance changes at the wavelength of corresponding reagents are in correlation with ascorbate concentration. During this study, benzidine and o-tolidine have been used as chromogenic reagents. Reaction conditions were optimized for various buffer systems, calibration curves were constructed, and limits of detection (0.04 $\mu\text{mol/L}$) and quantification (0.12 $\mu\text{mol/L}$) were calculated. Using calibration charts, it was possible to detect ascorbic acid within limits from 0.4 to 10 $\mu\text{mol/L}$. The optimized method was applied for the determination of ascorbic acid in pharmaceutical products. The method was characterized by exceptional sensitivity and accuracy, but only for preparations not containing substances that affect enzyme peroxidase.

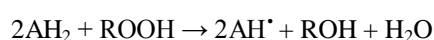
Keywords: L-ascorbic acid, Peroxidase, Spectrophotometry.

INTRODUCTION

Enzyme peroxidase (E.C.1.11.1.7) plays an essential role in various physiological processes of microorganisms, plants, and animals (Wen et al., 2011). Peroxidase of some biological species mostly represents a mixture of different isoenzymes. Thus from horseradish (*Armoracia rusticana* G.Gaertn., B.Mey & Scherb.), more than 40 peroxidase isoenzymes have been isolated by now, with isoenzyme C as the most abundant one (Krieg et al., 2010).

Primary substrates and the only molecules reacting directly with the enzyme are peroxides, namely both forms of them, hydrogen peroxide free form, and its organic complexes or salts.

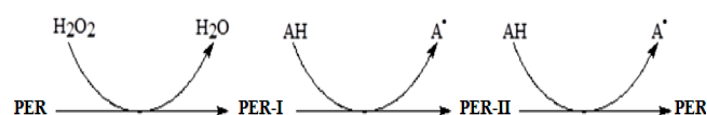
The role of peroxidase in the cell is primarily discussed regarding the regulation of intracellular H_2O_2 level (Mittler, 2002). Peroxidase transforms peroxide into water and oxygen radicals. In the presence of co-substrate – hydrogen donor (AH_2), it further transforms oxygen radical to another water molecule while co-substrate is transformed into corresponding free radical (AH^\bullet), as presented in the following chemical equation:



That way, electrons from activated oxygen are being transferred to molecules of co-substrates that stabilize them by inherent resonant structures (Bagirova et al., 2001).

The co-substrate role can be played by many phenols, aminophenols, aromatic amines and acids, indophenols, leuco dyes, ascorbic acid, some amino acids nitrites, as well as a series of other compounds.

The general mechanism of peroxidase (PER) catalytic activity is presented in scheme 1.



Scheme 1. AH and A[•] represent a reduced and oxidized form of co-substrate (hydrogen donor).

The first step of the catalytic cycle is the reaction between H_2O_2 and Fe(III)-center of a relaxed enzyme (PER) and the formation of the so-called compound I (PER I). It is an intermediary with a higher oxidation state, which involves oxyferril center and porphyrin cation radical. The formation of so-called compound II (PER II) demands the presence of co-substrate – the donor of hydrogen. Both Fe(IV) oxyferril species, as well as compound I and compound II, are powerful oxidants with redox potential close to +1V. The next reduction step with co-substrate turns compound II back to the enzyme's original state (PER).

“Transfer” of electrons from peroxide to co-substrate can involve ascorbic acid (vitamin C), which itself can become a co-substrate of peroxidase. However, its affinity for forming a

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ternary complex with the enzyme and substrate is markedly lower concerning competitive co-substrates (aryl phenols and arylamines). By coupled non-enzymatic reaction, ascorbic acid instantly reduces oxidized co-substrate molecules, while itself being oxidized to dehydroascorbic acid and immobilizing peroxide electrons (White-Stevens, 1982):



This reaction is quantitative, so in *in vitro* trials, with a right choice of co-substrates (chromogens – indicators), it can be used for determination of ascorbic acid (Thompson, 1987;

Shekhovtsova et al., 2006; Martinello et al., 2006; Arnao et al., 1996), but also for determination of the other components involved in the reaction – hydrogen peroxide, co-substrate and the peroxidase enzyme itself.

In order to measure ascorbic acid content in various samples, numerous spectrophotometric methods have been developed. Some of them, with their essential characteristics, are given in table 1.

Table 1. Review of spectrophotometric methods for the determination of ascorbic acid.

Spectrophotometric method		LOD (μmol/L)	LOQ (μmol/L)	Determination range (μmol/L)	Sample	Ref.
Peanut peroxidases	o-dianisidine			0.8–10	Fruit juices, milk, and sour-milk products	Shekhovtsova et al., 2006
	3,3',5,5'-tetramethylbenzidine			0.1-10		
Horseradish peroxidases	o-dianisidine			2–10		
	3,3',5,5'-tetramethylbenzidine			0.1-10		
Horseradish peroxidases with a quinoid dye produced				2.3-68.1	Pharmaceutical formulations	Zhu et al., 1997
Withiron(III) complex (ferritin, [Fe(phen)3] 3 ⁺) in the presence of 1,10-phenanthroline				4.3–74.1	In serum and urine samples	Moghadam et al., 2011
With Cu(II) phosphate		0.3		5-40	Pharmaceutical formulations	Pereira et al., 1997
Modified CUPRAC method				8-80	Fruit juices and pharmaceutical preparations	Ozyurek et al., 2007
With 2,4-dinitrophenylhydrazine		5.68	9.65	10-550	Fruits and vegetables	Kapur et al., 2012
With Fast Red AL salt				28.4-142	Pharmaceutical preparations and fresh fruit juices	Backheet et al., 1991
With 4-chloro-7-nitrobenzofurazan				28.4-113.6	Fresh fruit juices, some vegetables, and infant milk product	Abdelmageed et al., 1995

EXPERIMENTAL

The all essential solutions have been prepared using deionized water characterized by the electrical conductivity of 0.5 μS/cm². The all used chemicals were of p.a. purity grade.

Preparation of horseradish peroxidase of purity grade 200 kU/g was supplied from Merck KGaA (Darmstadt, Germany). Solutions of the enzyme were prepared immediately before executing the trial, within concentration range from 1 to 30 U/L, by basic dilution solution obtained by dissolving 50 mg (10000 U) of peroxidase in 1000 ml of deionized water.

Solutions of hydrogen peroxide (Merck KGaA, Darmstadt, Germany), within concentration range from 0.04 to 2 mmol/L, were prepared by basic dilution solution obtained by dissolving 95 ml of 33.3% hydrogen peroxide in 1000 ml of deionized water, and their exact concentration was controlled by the standard permanganometric method (Vajgand, 1986).

Solutions of benzidine (Reanal Laborvegyszer Kft., Budapest, Hungary) and o-tolidine (Centrohém DOO, Stara Pazova, Serbia) were prepared within the concentration range from 0.1 to 5 mmol/L, by basic dilution solution with a concentration of 50 mmol/L, obtained by dissolving 0.9210 g of benzidine in 5ml of 1% CH₃COOH, i.e., 1.0615 g of o-tolidine in 5 ml of 1% HCl, and then filled up to 100 ml by deionized water.

The basic solution (0.1 mol/L) of L-ascorbic acid (Galenika AD, Belgrade, Serbia) was prepared by dissolving 17.613 g of the substance in 1000 ml of deionized water, while working solutions were within the concentration range from 0.1 $\mu\text{mol/L}$ to 10 mmol/L, with or without various stabilizing components in different concentrations. Ascorbic acid concentration of these solutions was controlled by the standard method using 2,6-dichlorophenolindophenol (Official Methods, 1995).

The following buffer systems were used as media with defined pH value: acetate buffer of pH 4 (prepared in the laboratory, 180 ml of 0.2 mol/L sodium acetate solution and 820 ml of 0.2 mol/L acetic acid solution; citrate buffers pH 5 and 6 (Farmitalia Carlo Erba Ltd., Milan, Italy); phosphate buffer of pH 7 (Fisher Scientific UK Ltd., Loughborough, UK); and borate buffer of pH 8 (Farmitalia Carlo Erba Ltd., Milan, Italy). Buffer pH values were controlled by pH-meter Hanna HI-207 (Hanna Instruments Inc., Woonsocket, RI, USA).

Spectrophotometric measurements were carried out using the Beckman DU-650 spectrophotometer (Beckman Coulter, Inc., Brea, CA, USA).

Relative activity of peroxidase was observed by spectrophotometry, measuring the change of absorbance during the emergence of the oxidized form of co-substrate, benzidine, and o-tolidine, within the corresponding time interval. Depending on the applied buffer system, absorbance change for benzidine was observed at 410 nm (in acetate and citrate buffers), at 362 nm (in phosphate buffer), and 425 nm (in borate buffer), while for o-tolidine, it was observed at 630 nm (in acetate and citrate buffers), at 411 nm (in phosphate buffer) and 436 nm (in borate buffer).

Before measurements were carried out, the all used solutions had been thermostated at the temperature of 20°C.

Measurements were carried out in glass cuvettes 1ml of volume. The reaction mixture was composed of 0.5 ml of the corresponding buffer, 0.1 ml of peroxidase, 0.1 ml of co-substrate – indicator (o-tolidine or benzidine), and 0.2 ml H₂O or ascorbic acid. Cuvettes with the reaction mixture then were thermostated at 25°C for five minutes, and after that, absorbance change was observed from the moment when 0.1 ml of H₂O₂ had been injected into the mixture.

To evaluate the proposed method, test solutions were prepared by grinding tablets and dissolving powder of different preparations in 1 L of deionized water. For that purpose, depending on ascorbic acid content, 20 to 100 tablets or bags were used. Diluted preparations firstly were filtered, and then they were used to prepare working solutions for the analyses.

The following preparations were used to make samples for evaluation of the proposed method:

- Preparation 1 – Vitamin C, tablets, 500 mg (Galenika AD, Belgrade, Serbia). Excipients: microcrystalline cellulose, croscarmellose sodium, magnesium stearate, lactose anhydrous, and corn starch.

- Preparation 2 – Vitamin C, powder, 1000 mg (BG Pharm DOO, Belgrade, Serbia), no excipients.
- Preparation 3 – Easy soluble vitamin C, powder, 50 mg (Ivančić i sinovi DOO, Belgrade, Serbia). Excipients: 450 mg of glucose.

RESULTS AND DISCUSSION

The addition of hydrogen peroxide to the analytic mixture, composed by peroxidase enzyme and one of co-substrates/chromogens, leads to the rapid oxidation of co-substrate, and that can be detected by spectrophotometry where rapid development of absorption maximum can be observed at corresponding wavelength.

The rate of oxidized co-substrate form development can serve as a measure for the relative activity of peroxidase. The starting speed (v_0) can be presented graphically by the slope coefficient of the initial part of the curve, which is by the tangent of slope angle – ($\text{tg}\alpha$) (figure 1). This speed depends on concentrations in the reaction mixture of all components of ternary complex enzyme-substrate-cosubstrate.

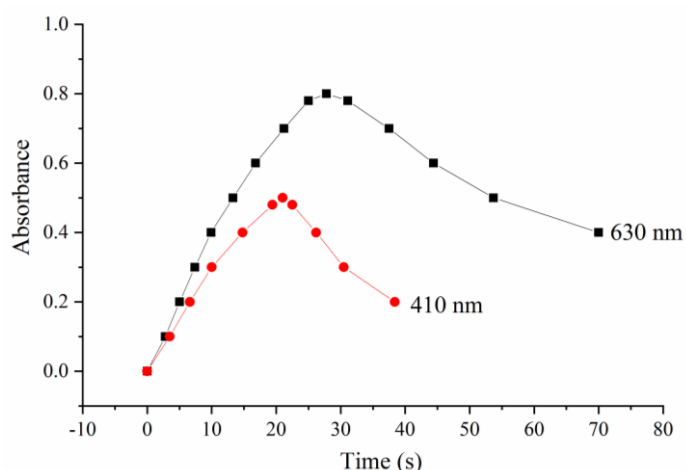


Figure 1. Formation of o-tolidine (630 nm) oxidation products and benzidine (410 nm), at equimolar concentrations, during breaking down of hydrogen peroxide by horseradish peroxidase.

During this study, two co-substrates have been used, each of them having absorption maximum within the visible part of the specter, benzidine, and its derivative o-tolidine with absorption maximums that depended on pH value.

The dependence of the initial reaction speed on the enzyme concentration was directly proportional.

Similarly, the speed of co-substrate oxidation was also directly proportional to the concentration of the substrate, i.e., hydrogen peroxide.

The introduction of ascorbic acid into the reaction mixture caused occurring a parallel non-enzymatic reaction, in which oxidized co-substrate molecules were instantly reduced to the starting point, and that resulted in delaying (lag time) appearance

of analytical signal, by the moment when ascorbic acid was quantitatively transferred to dehydroascorbic acid.

Lag time duration depended on the available amount of ascorbic acid (figure 2).

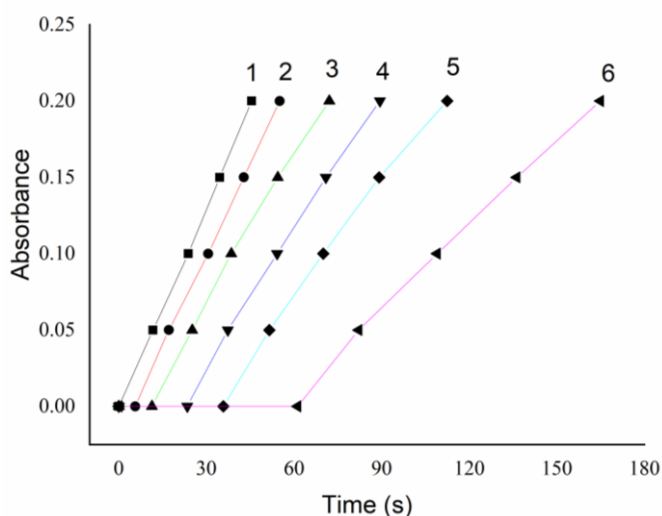


Figure 2. The effect of ascorbic acid on kinetics of peroxidase reaction. Conditions of the reaction: peroxidase 10 U/L; H_2O_2 0.1 mmol/L; *o*-tolidine 1 mmol/L; citrate buffer pH 6. Curve 1 represents blank, curves 2–6 AscH_2 concentration 5; 10; 20; 30 and 50 $\mu\text{mol/L}$, respectively. Slope coefficients of the curves were the following: 0.0041 (1); 0.0040 (2); 0.0037 (3); 0.0032 (4); 0.0029 (5) and 0.0021 (6).

Lag time at a defined concentration of ascorbic acid represented the period during which the corresponding amount of the enzyme broke down a certain quantity of hydrogen peroxide equivalent to ascorbic acid's quantity in the reaction mixture. Having in mind that co-substrate oxidation speed depends on peroxide concentration, in the moment of measurement, it was lowered proportionally to the applied amount of ascorbic acid, which was visible from the values of the slope coefficient.

This lag time proved itself a useful and accurate kinetic parameter for observing the mechanism of the studied reaction, and its values reflected reciprocal of initial reaction speed, $1/v_0$ i.e., $1/tga$.

During the lag time, the absorbance value change at the chosen wavelength was equal to zero ($\Delta[\text{Abs}] = 0$). The second part of the curve after lag time, described as $\Delta[\text{Abs}]/\Delta t$, represented co-substrate oxidation speed, and it also could be exploited as a parameter of the reaction system because that parameter reflected the altered state of the system after oxidation of ascorbic acid.

Calibration curves for the determination of ascorbic acid in the presence of benzidine and *o*-tolidine as chromogenic co-substrates are presented in figure 3.

The calibration line constructed by the use of benzidine as co-substrate of peroxidase had a more significant slope

coefficient regarding the calibration line constructed by the use of *o*-tolidine, which pointed out to a higher sensitivity of benzidine as an indicator. However, water solutions of benzidine are unstable, and after a few hours, they convert to a colloid state, which negatively affects solution transparency during measurement and calls measurement reproducibility in question.

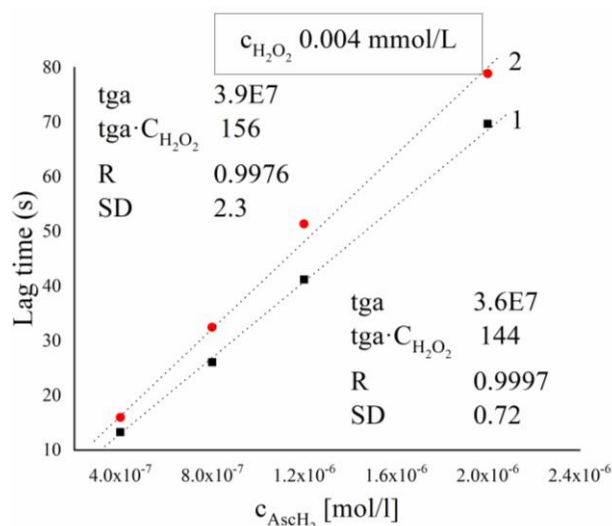


Figure 3. Calibration curves for determination of ascorbic acid, at H_2O_2 concentration of 40 $\mu\text{mol/L}$, peroxidase concentration of 10 U/L; equimolar amounts of *o*-tolidine (1) and benzidine (2) 0.5 mmol/L and citrate buffer pH 6.

The experiment revealed that peroxidase activity was highest in citrate buffer pH 6, and lowest in borate buffer pH 8. Previous reports pointed out the optimal pH value for peroxidase lying between 4.5 and 6.5, while enzyme activity weakened by increasing alkalinity of the environment (Shekhovtsova et al., 2006; Arnao et al., 1996).

Slope coefficients of the curves obtained using benzidine were greater than the curves obtained using *o*-tolidine with no exception, which suggested that transport of hydrogen from benzidine to peroxidase was slower, compared with the speed of hydrogen transport from *o*-tolidine to peroxidase.

The limit of detection (LOD) and limit of quantification (LOQ) amounted 0.04 and 0.12 $\mu\text{mol/L}$, respectively. Linear dependence between lag time and ascorbic acid concentration was within the determination range of 0.4–10 $\mu\text{mol/L}$.

This study included testing the effect of numerous substances that might be present in samples containing ascorbic acid, such as solution stabilizers and extraction aids, and various components of pharmaceutical preparations (excipients) found in combination with vitamin C the accuracy of its determination. We established that the kinetic method results were comparable with results obtained by the standard DCP/IP method only when ascorbic acid was determined in preparations containing no other active components.

The described method has been applied to determine ascorbic acid in several samples different from each other. The

results of the measurements are presented in table 2.

Table 2. Content of ascorbic acid (mg) in various preparations, determined using the enzymatic method, with co-substrates o-tolidine and benzidine at pH 6.

Sample	c(AscH ₂) mg/one dosage	Found c(AscH ₂) with o-tolidine	RSD (%)	Recovery (%)	Found c(AscH ₂) with benzidine	RSD (%)	Recovery (%)
Preparation 1	500	511.8±18.4	3.60	102.36	510.3±12.7	2.49	102.06
Preparation 2	1000	1014.4±19.1	1.88	101.44	1009.8±11.0	1.09	100.98
Preparation 3	50	54.2±6.8	12.56	108.40	55.3±6.9	12.48	110.60

Based on the presented results, one can say that the proposed method has its advantages and disadvantages. The advantage of the method is its exceptional sensitivity, clearly visible by comparing the obtained results with the ones from table 1 and accuracy, which can be concluded based on the results from table 2. Furthermore, this method uses a simple apparatus and does not demand expensive reagents.

The disadvantage of the method, above all, is an excellent susceptibility of peroxidase enzyme because numerous foreign substances affect its relative activity (Hernandez-Ruiz, 2001; Pandey et al., 2011; Rodriguez-Lopez et al., 1997; Wen et al. 2011; Krieg et al., 2010; Bagirova et al., 2001), which shows an effect on reproducibility and accuracy of the method. To avoid this, preliminary elimination of such substances would be necessary or their transformation to an inactive state, creating a problem having in mind complete application of the method. On the other hand, a deviation from the expected values during a routine control of samples by this method could point out possible impurities present in the sample with such deviation.

CONCLUSION

Ascorbic acid is a potent antioxidant thanks to very low redox potential, preventing oxidizing other organic molecules in the environment or turning them instantaneously back to their reduced form. Thus, there are numerous co-substrates of peroxidase enzyme that facilitate, as proton donors, its transforming hydrogen peroxide to water, but being reduced themselves in ascorbic acid's presence to the moment when it is quantitatively transferred to dehydroascorbic acid. Proper choice of co-substrate, as chromogen, whose one of the redox forms absorbs in the visible part of the specter, has enabled developing a kinetic spectrophotometric method for successful determination of ascorbic acid.

The procedure, which included the presence of co-substrates benzidine and o-tolidine at various pH values, has been exploited for the determination of ascorbic acid in pharmaceutical products. The presented results point to the method as one of the most sensitive and most accurate spectrophotometric kinetic methods, demanding standard

laboratory equipment, and acceptable price reagents. Flaws of the method are caused by the over susceptibility of peroxidase molecule to the presence of impurities and foreign substances that interfere with measurement accuracy.

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