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Catalytic Spectrophotometric Determination of Ascorbic Acid in Tea Drink with 1,5-bis(*p*-Hydroxybenzaldene)thiocarbohydrazone as the Substrate for Horseradish Peroxidase

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1,5-bis(p-Hydroxybenzaldene)thiocarbohydrazone (BHBTZ) had been synthesized and used as a new kind of substrate for horseradish peroxidase (HRP). The experiment results showed that ascorbic acid (AsA) had strongly inhibiting effect on the BHBTZ−H_2O_2−HRP reaction system, so AsA had been spectrophotomericly determined as the inhibitor by enzymatic inhibition method. Under experimental conditions, the linear relationship between ΔA_{386} and AsA concentration was in the range of 0.82−18.0 μg ml^{-1} with a detection limit of 0.245 μg ml^{-1}. The effect of interferences on AsA determination was investigated. The proposed method was successfully applied to the determination of AsA in tea drink.

KEYWORDS: Catalytic spectrophotometry; enzymatic inhibition method; ascorbic acid determination; BHBTZ; HRP

INTRODUCTION

Ascorbic acid (AsA) is one of the most important vitamins that can maintain normal functions of the human body and participates in many metabolizing process. It cannot be synthesized in the human body; it can only obtained from foodstuffs. Fruits and vegetables are important sources of ascorbic acid. Researchers have found that many kinds of sickness such as scurvy and hemophthisis are caused by deficiency of ascorbic acid in the human body, they also found that the content of ascorbic acid can be used in sickness indication and nutrient assessment. Therefore, it is quite necessary for us to detect the content of ascorbic acid in many foodstuff samples.

The common spectral analysis methods for AsA determination are 2,6-dichlorophenol indophenol (2,6-DIP) method (*1*) and 2,4-dinitrophenylhydrazine (2,4-DNP) method (*2*). However, these methods have many disadvantages. For example, the 2,6-DIP method is not very sensitive, and many substances such as glutathione in foodstuff samples can interfere with the AsA determination by this method. Although the 2,4-DNP method is more sensitive, complicated operation and long operation time are needed by this method. The better method for AsA determination is enzymatic assay of AsA, which is based on the oxidation reaction of AsA with H_2O_2 under the catalysis of HRP (*3*−*5*). The concentration of AsA is spectrometricly determined at 265 nm, because AsA has a maximum absorbance at 265 nm. However, many substances including many biomolecular in foodstuff samples have 265 nm absorbance, so this method is highly sensitive, but it is not free from interferences.

The enzymatic inhibition method has been widely used in the quantitative determination of enzymatic inhibitors, including inorganic ions and organic compounds (*6*−*8*). This method has a low detection limit and a high selectivity.

As a kind of Schiff base, 1,5-bis(*p*-hydroxybenzaldene)-thiocarbohydrazone(BHBTZ) can be oxidized by H_2O_2 to form a quinoid product. We have found that AsA has a serious inhibition effect on the oxidation reaction of BHBTZ with H_2O_2 under the catalysis of HRP. Therefore, AsA can be spectrophotometricly determined as an inhibitor by the enzymatic inhibition method. In this method, the 368 nm absorbance wavelength of BHBTZ has been selected as the determination wavelength, because coexisting substances in most biological samples have no interference at 368 nm. Therefore, the advantage of this method is its high selectivity and sensitivity. The proposed method has been applied to the determination of AsA in tea drink with satisfactory results.

MATERIALS AND METHODS

Apparatus. All absorbance measurements were carried out on a Shimadzu UV-265 spectrophotometer equipped with 1.0 cm quartz cells. All pH measurements were made with a pHs-3C digital pH-meter (Shanghai Lei Ci Device Works, Shanghai China) with a combined glass-calomel electrode. The elemental analysis was carried out on a Yanaco (Japan) model MF-3 elemental analyzer.

Regents. All chemicals used were of analytical-reagent or higher grade. Deionized water was used throughout. An AsA stock solution (10 mg ml^{-1}) was prepared every week. The working solution (0.1 mg ml^{-1}) was prepared daily by diluting a stock solution kept in a refrigerator. H_2O_2 solution (1.5×10^{-4}mol l^{-1}) was prepared by dilution of a 30% solution with distilled deionized water (standardized by titration with potassium permanganate). The solution of HRP (Shanghai Dongfeng Biochemical Technology, China) was applied at 10 μg ml^{-1}. BHBTZ solution (5.0×10^{-4}mol l^{-1}) was prepared by dissolving an

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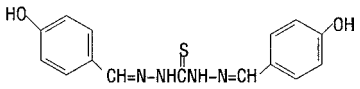
Scheme 1. The Structure of BHBTZ.



appropriate amount of the reagent (synthesized in-house) in dimethylformamide. An H_3BO_3−NaOH buffer (0.05mol l^{-1}, pH =9.540) was used.

Synthesis and Properties of BHBTZ. The procedure was as follows: Thiocarbohydrazine (0.01mol) was dissolved in 10 mL absolute ethanol, and 0.025mol of *p*-hydroxybenzaldehyde was dissolved in 15 mL of absolute ethanol. After the *p*-hydroxybenzaldehyde was added dropwise to the thiocarbohydrazine solution by stirring, the mixture was refluxed in 80−85 °C water for 4 h. Drops of 25% hydrochloric acid were added, and the mixture was maintained at room temperature for a night. Then, the solution was filtered and the precipitate was washed with absolute ethanol. After infrared desiccation, a light-yellow crystal was obtained (0.8 g, yield 80%) The structure of BHBTZ was shown in **Scheme 1**.

The melting point of BHBTZ is 280 °C. Elemental analysis gave a composition of C, 57.46; H, 4.65; N, 18.12; which is in good agreement with the theoretical composition of BHBTZ (C, 57.31; H, 4.48; N, 17.82).

The infrared spectrum of BHBTZ (KBr disks) was obtained, and the bands were assigned as follows: $\nu_{N-H}\nu_{O-H}$ (3200−3550 cm^{-1}), ν_{C-H} (1620 cm^{-1}), σ_{NH} and ν_{C-H} (1560 cm^{-1}), $\nu_{C=S}$ (1275 cm^{-1}).

Determination of AsA. In a 10-mL comparison tube were placed 2.0 mL of buffer (pH 9.54), 2.0 mL of 5.0×10^{-4} mol l^{-1} BHBTZ solution, 0.6 mL HRP of 10 μg ml^{-1}, and 0.3 mL of 1.5×10^{-4} mol l^{-1} H_2O_2 solution. After dilution to volume with deionized water, the solution (3.0 mL) was put in a 1.0 cm quartz cell. The initial absorbance (A_i) at 368 nm was recorded immediately. After addition of different amounts of AsA standard solutions, the mixture was equilibrated at room temperature for 6min, then the final absorbance (A_f) was recorded at 368 nm. The absorbance difference was defined as $\Delta A_{368} = A_f - A_i$.

RESULTS AND DISCUSSION

Discussion of Reaction Mechanism. In BHBTZ, the pare group of the phenolic group is a schiff base group, which strengthens the dispersity of the electron cloud in the benzene cyclic group. Therefore, the phenolic group in BHBTZ can be easily oxidized by H_2O_2 for its enhanced reducibility. The light-yellow BHBTZ was oxidized to form a blue product, which showed that the unstauration level of the compound was increased. BHBTZ had a maximum absorbance at 368 nm. When it was oxidized by H_2O_2 to form a blue quinoid product under the catalysis of HRP, a 628 nm absorbance peak appeared, which showed that the oxidation product had a maximum absorbance at 628 nm. At the same time, the 368 nm absorbance of BHBTZ decreased for BHBTZ concentration was decreased by the oxidation reaction. When AsA was added into the BHBTZ $-H_2O_2$ reaction system, it suppressed the oxidization reaction. As a result, the 368 nm absorbance increased, and the 628 nm peak height decreased. The possible reason was as follows: AsA and phenolic compounds such as BHBTZ can all react with H_2O_2 as the substrates for HRP, but the rate constant for HRP− H_2O_2 compound with BHBTZ is much higher than that with AsA (*9*). Therefore, AsA cannot suppress the activity of HRP because it cannot compete against BHBTZ in combining with HRP. However, the primary oxidation product of BHBTZ is a phenoxyl radical, which can rapidly react with AsA to form BHBTZ again. Therefore, AsA addition has an inhibition effect on the H_2O_2−BHBTZ reaction system, which led up to the amount of the oxidation product of BHBTZ (A = 628 nm) decrease and content of BHBTZ (A = 368 nm) increase. Because the change of the 628 nm peak height was unsensitive, the 368 nm was selected as the determination wavelength. On the basis of the linear relationship between A_{368} and AsA concentration, AsA can be determined as an inhibitor of the BHBTZ $-H_2O_2$ reaction by the enzymatic inhibition method.

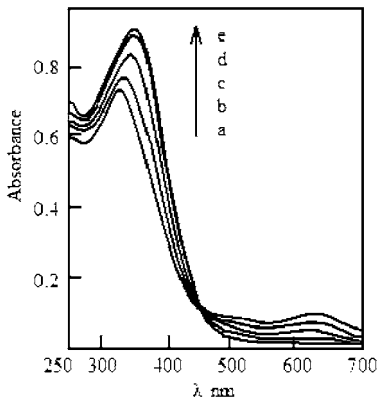


Figure 1. Effect of AsA on the oxidization reaction of BHBTZ with H_2O_2. BHBTZ, 2.5×10^{-6} mol l^{-1}; H_2O_2, 4.5×10^{-5} mol l^{-1}; HRP, 0.6μg ml^{-1}; H_3BO_3–NaOH buffer (pH 9.54). (a) AsA, 0μg ml^{-1}; (b) AsA, 3μg ml^{-1}; (c) AsA, 8μg ml^{-1}; (d) AsA, 10μg ml^{-1}; (e) AsA, 12μg ml^{-1}.

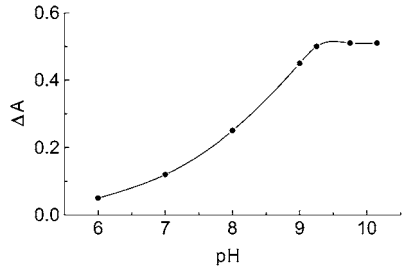


Figure 2. Effect of pH on A_{368}. BHBTZ, 2.5×10^{-6} mol l^{-1}; H_2O_2, 4.5×10^{-5} mol l^{-1}; HRP, 0.6μg ml^{-1}; AsA, 8μg ml^{-1}.

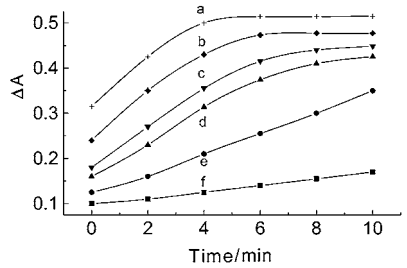


Figure 3. Effect of HRP concentration on A_{368}. BHBTZ, 2.5×10^{-6} mol l^{-1}; H_2O_2, 4.5×10^{-5} mol l^{-1}; AsA, 8μg ml^{-1}. HRP (a) 0.6μg ml^{-1}; (b) 0.5μg ml^{-1}; (c) 0.4μg ml^{-1}; (d) 0.3μg ml^{-1}; (e) 0.2μg ml^{-1}; (f) 0.0μg ml^{-1}.

Effect of pH. The experimental variables were optimized by applying the univariate method. The pH dependence of the system was studied over the range of 6.0−10.0. The experimental results (**Figure 2**) showed that the optimum pH range was between 9.0 and 10.0. Therefore, a pH of 9.54 was fixed with the use of H_3BO_3−NaOH buffer.

Influence of HRP Concentration. The effect of HRP concentration on the catalytic system is shown in **Figure 3**. The result demonstrated that the initial A_{368} increased with the increase of the HRP concentration, which meant that the catalytic reaction rate quickened with the increasing HRP concentration. When 0.6μg ml^{-1} of HRP was added, the catalytic reaction quickly completed within 6 min. Therefore, the optimum HRP concentration and the reaction time were chosen as 0.6 μg ml^{-1} and 6 min, respectively.

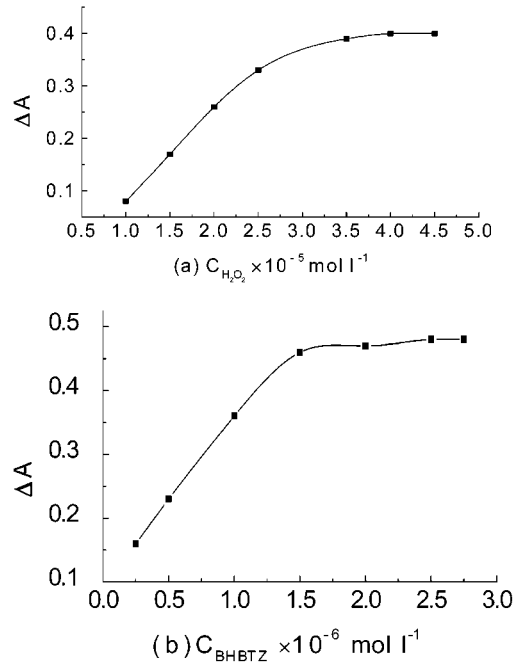


Figure 4. Effect of H_2O_2 (a) and BHBTZ (b) concentrations. HRP, 0.6μg ml^{-1}; BHBTZ, 2.5×10^{-6} mol l^{-1}; AsA, 8μg ml^{-1}. (b) H_2O_2, 4.5×10^{-5}mol l^{-1}; HRP, 0.6μg ml^{-1}; AsA, 8μg ml^{-1}.

Table 1. Effect of Interferents on the Determination of AsA (8.0μg ml^{-1})

interferent	tolerance ratio (m/m)	interferent	tolerance ratio (m/m)
D-fructose	2950	tyrosine	100
Ca^{2+}	2000	glycine	100
Mg^{2+}	2000	tryptophane	100
F^-	1000	Ca^{2+}	100
I^-	1000	Ni^{2+}	100
$H_2PO_4^-$	1000	Cd^{2+}	100
NO_3^-	1000	Zn^{2+}	100
SO_4^{2-}	1000	lysine	50
CO_3^{2-}	1000	Zr^{4+}	40
D-galactose	250	Al^{3+}	20
malate	250	Fe^{3+}	20
sucrose	250	Cu^{2+}	20
glucose	150	Pb^{2+}	20
phenylalanine	150	citric acid	15
V_{B1}	130	tartrate	15
V_{B6}	130	Mn^{2+}	11

Effect of H_2O_2 and BHBTZ Concentrations. The effect of H_2O_2 concentration is shown in **Figure 4a**, which demonstrates that the A_{368} value increased with an increase in H_2O_2 concentration from 1.0×10^{-5}mol l^{-1} to 3.3×10^{-5} mol l^{-1}, then it remained unchanged when H_2O_2 concentration continually increased; hence, the optimum concentration of H_2O_2 was chosen as 4.5×10^{-5}mol l^{-1}.

The influence of BHBTZ concentration was studied (**Figure 4b**). The result showed that the A_{368} value increased in direct ratio with BHBTZ concentration. *A* reached a maximum when 2.0×10^{-6}mol l^{-1} of BHBTZ was added, then it remained constant. Therefore, the optimum substrate concentration was chosen as $2.5 \times 10\text{-}^{6}$mol l^{-1}.

Analytical Characteristics. Under the optimum experimental conditions, there was a linear relationship between A_{368} and AsA concentration in the range of 0.82−18.0μg ml^{-1}, with a correlation coefficient of 0.9995. The regression equation was $A_{368} = 0.028c$ (μg ml^{-1}) − 0.0073. The RSD was 1.35%, obtained from a series of 10 standard solutions, each containing 8.0μg ml^{-1} of AsA. The SD was 0.00229, obtained from a series of 10 blank solutions. The limits of detection and determination of the method were established according to the IUPAC definitions (*10*), and the values found were 0.245 and 0.820 μg ml^{-1}, respectively.

Table 2. AsA Determination in Tea Drink ($P = 0.95$, $n = 6$)

AsA added μg ml^{-1}	mean found μg ml^{-1}	recovery value μg ml^{-1}	mean recovery (%)
0.00	3.05 ± 0.01		
2.00	5.14 ± 0.04	2.09 ± 0.03	105
4.00	7.00 ± 0.03	3.95 ± 0.02	99
0.00	4.10 ± 0.01		
2.00	6.02 ± 0.02	1.92 ± 0.01	96
4.00	8.19 ± 0.03	4.09 ± 0.02	102
0.00	5.23 ± 0.01		
2.00	7.27 ± 0.02	2.04 ± 0.01	102
4.00	9.15 ± 0.01	3.92 ± 0.01	98

Effect of Interference. To examine the possible interference by various constituents in biological samples, the concentration of AsA was fixed at 8.0μg ml^{-1}, and the tolerable error was ≤±5%. The result is given in **Table 1**, which shows that most of the coexisting substances in the foodstuff samples had no interference on the determination of AsA.

Sample Analysis. The proposed method was applied to the determination of AsA in three batches of Bazheng tea drink (Shandong Longxi Ltd.). To 1.00 mL of sample solution were directly added various amounts of standard AsA solution, without sample treatment process. AsA concentration could be determined by the standard addition method (*11*). The result is given in **Table 2**.

Conclusions. In this work, BHBTZ (synthesized in-house) has been successfully used as the new kind of substrate for HRP in highly sensitive and selective determination of AsA by the enzymatic inhibition method. The proposed method was applied to the determination of AsA in tea drink with satisfactory results.

ABBREVIATIONS USED

AsA, ascorbic acid; HRP, horseradish peroxidase; BHBTZ, 1,5-bis(*p*-hydroxybenzaldene)thiocarbohydrazone.

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