



# The browning kinetics of the non-enzymatic browning reaction in L-ascorbic acid/basic amino acid systems

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## Abstract

Under the conditions of weak basis and the reaction temperature range of 110-150 °C, lysine, arginine and histidine were reacted with L-ascorbic acid at equal amount for 30-150 min, respectively and the formation of browning products was monitored with UV-vis spectrometry. The kinetic characteristics of their non-enzymatic browning reaction were investigated. The study results indicated that the non-enzymatic browning reaction of these three amino acids with L-ascorbic acid to form browning products was zero-order reaction. The apparent activation energies for the formation of browning products from L-ascorbic acid/lysine, L-ascorbic acid/arginine and L-ascorbic acid/histidine systems were 54.94, 50.08 and 35.31kJ/mol. The activation energy data indicated the degree of effects of reaction temperature on non-enzymatic browning reaction. Within the temperature range of 110-150 °C, the reaction rate of L-ascorbic acid/lysine system was the fastest one, followed by that of the L-ascorbic acid/arginine system. The reaction rate of L-ascorbic acid/histidine system was the slowest one. Based on the observed kinetic data, the formation mechanisms of browning products were proposed.

**Keywords:** kinetics; non-enzymatic browning reaction; ascorbic acid; Maillard reaction; amino acid.

**Practical Application:** These findings can improve our understanding of the formation mechanisms of food colors for the more effective control of the colors of final products in the foods rich in L-ascorbic acid and basic amino acids.

## 1 Introduction

Non-enzymatic browning reactions include Maillard reaction, caramelization and ascorbic acid browning reaction. Their products can be divided into small molecule products, which are the important sources of food flavor (Adams & Kimpe, 2006), colorless intermediate products and the finally formed browning macromolecular products (Kim & Lee, 2008), i.e. the browning products, which confer the food colors. Lysine, arginine and histidine are the basic amino acids for the protein synthesis. They are widely present in the nature. In food industry, lysine is commonly used as the food fortifier and feed additive. L-ascorbic acid is an important nutrient for human beings and is also widely present in the nature. In food processing, L-ascorbic acid is widely used as food additive and as the anti-oxidant. Thus, to study the non-enzymatic browning reaction of L-ascorbic acid/basic amino acid system is of important significance for food industry.

As mentioned above, the products formed by the non-enzymatic browning reaction of L-ascorbic acid/amino acid system can be divided into two categories: one category is the small molecule aroma substances, which confer the food flavors; and the other category is the brown macromolecule substances, i.e. the browning products. In the recent years, the studies on the non-enzymatic browning reaction of L-ascorbic acid/amino acid system have been mainly focused on the formation of small

molecule aroma substances (Adams & Kimpe, 2009; Li et al., 2016; Obretenov et al., 2002; Pischetsrieder, 1996; Tan & Yu, 2012; Yu & Deng, 2009; Yu et al., 2012a, b, 2013; Yu & Zhang, 2010a, b) while much fewer studies on the macromolecule products of non-enzymatic browning reactions have been reported. The previous studies including those conducted by our research group (Adams & Kimpe, 2009; Obretenov et al., 2002; Pischetsrieder, 1996; Yu et al., 2012a) reported the studies on the formation of aroma substances from non-enzymatic browning reaction of L-ascorbic acid/basic amino acid system. The aim of the present study was to report the kinetics of formation of browning reaction products of three basic amino acids and L-ascorbic acid and propose the mechanism underlying the formation of the browning products.

During the food processing, to study on the browning kinetics of non-enzymatic browning reaction can help to us to understand the formation mechanisms underlying the formation of food colors, which, in turn, can help us to further control the food colors. The non-enzymatic browning reaction is a very complicated process. Sometime, it is hard to clearly describe its kinetic characteristics. However, for the formation of brown macromolecule substances, i.e. the browning products, we can use the so-called "browning index" to monitor the reaction

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process of the entire non-enzymatic browning reaction and study its reaction kinetics (Martins et al., 2000; Ling et al., 2015).

## 2 Materials and methods

### 2.1 Materials

L-ascorbic acid ( $\geq 99.7\%$ ), lysine ( $\geq 98.5\%$ ), arginine ( $\geq 98.5\%$ ), and histidine ( $\geq 98.5\%$ ) were purchased from Sinopharm Chemical Reagent Co., Ltd. (Shanghai, China). 2,4-Dinitrofluorobenzene ( $\geq 99.0\%$ ) was purchased from TCI Development Co., Ltd. (Shanghai, China). Double-distilled water was used in all the experiments. The other chemical reagents with analytical purity were all produced in China.

### 2.2 Preparation of reaction solutions

The reaction conditions were set according to those previously reported by our research group (Yu et al., 2012a). The reaction components were listed in Table 1.

The reaction reagents listed in Table 1 (System No. 1-7) were dissolved in 15 mL of 0.2 mol/L phosphate buffer (pH 8.0) and pH was adjusted to 8.0 (25 °C) with sodium hydroxide. The prepared solution was then sealed in Synthware® pressure glass vials (Beijing Synthware Glass, Inc, China), stirred well and sealed tightly. The solution was incubated at 110, 120, 130, 140 and 150 °C oil bath for 30, 60, 90, 120 and 150 min with magnetic stirring, respectively. Each experimental condition was repeated three times. When the reaction finished, the reactions were immediately stopped by cooling under a stream of cold water and then stored in refrigerator (4 °C) for subsequent analysis.

### 2.3 Measurement of UV-vis absorption

The UV-vis absorption of reaction solution was measured according to our previously described method (Zhou et al., 2016).

### 2.4 Determination of the concentration of residual amino acids

According to the methods described previously (Lü et al., 2009), the reaction solutions were pre-column derivated with 2,4-dinitrofluorobenzene and analyzed with reverse-phase high-performance liquid chromatography (HPLC). The HPLC analyses were performed on a 1260 HPLC system equipped with a UV diode-array detector (Agilent Technologies, Waldbronn,

Germany) and an Agilent C18 column at 30 °C (3.5  $\mu\text{m}$ , 4.6 $\times$ 100 mm; Agilent Technologies, Santa Clara, CA) using water/acetonitrile/0.01 mol/L phosphate buffer gradient to elute all fractions. The flow rate of the mobile phase was set to 1.0 mL/min and the injection volume was 5  $\mu\text{L}$ .

### 2.5 Kinetic calculation

The reaction rate constants ( $k$ ) for the formation of browning products were calculated from linear regression of absorbance ( $Abs$ ),  $\ln Abs$ , and  $1/Abs$  versus reaction time  $t$  (min) for zero, first, and second order reaction kinetics, respectively. The effects of temperature on  $k$  were calculated from the Arrhenius equation ( $\ln k = \ln k_0 - E_a/RT$ ), to determine the activation energy  $E_a$ .  $E_a$  for the formation of browning products was estimated from the linear regression of  $\ln k$  versus  $1/T$ .

### 2.6 Statistical analysis

All the experiments were carried out in triplicate and the mean  $\pm$  standard deviation was reported. The curve fitting, kinetics calculation and correlation analysis were performed using OriginPro 8.0 software (OriginLab Co., Northampton, MA). All statistical analyses were carried out at a 95% level of confidence.

## 3 Results and discussion

The entire process of the formation of browning products by the non-enzymatic browning reaction usually experiences three phases, i.e. the early, middle and late phases (Kim & Lee, 2008). With UV-vis spectroscopy assay, the formation of colorless intermediate products can be indicated by measuring the ultraviolet absorbance at 294 nm (Ajandouz et al., 2001; Benjakul et al., 2005; Liang et al., 2014; Yu et al., 2012c) while the browning products formed in the final phase of the browning reaction can be usually monitored by measuring the absorbance at 420 nm (Hong et al., 2015; O'Charoen et al., 2015; Yu et al., 2012c). It has been indicated (Ajandouz et al., 2001) that the colorless intermediate products are the precursors of the browning products. Therefore, the absorbance of the reaction system at 294 nm can be used to predict the color changes of the system. The absorbance at 420 nm is usually used to measure the browning degree of non-enzymatic browning reaction, i.e. the status of the formation of the browning products (Lertittikul et al., 2007; O'Charoen et al., 2015).

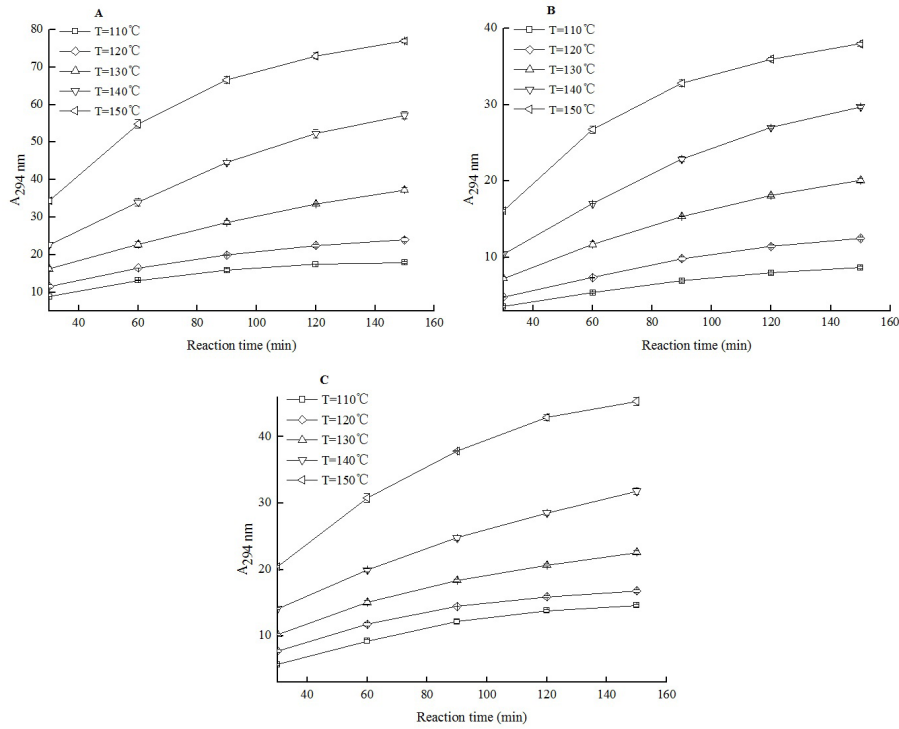
Figure 1 showed the changing curves of the absorbance at 294 nm against reaction time at 110, 120, 130, 140 and 150 °C, respectively, i.e. the changing curves of the colorless intermediate products with time. It can be seen from Figure 1 that the changing trends of the formation of colorless intermediate products of three basis amino acids at various temperatures are quite similar.

Figure 2 showed the changing curves of absorbance at 420 nm of the reaction solutions at the reactions temperatures of 110, 120, 130, 140 and 150 °C, respectively, i.e. the changing curves of the formation of browning products with reaction time. It can be seen from Figure 2 that the amounts of the browning products formed were increased with prolonging the reaction time and were also increased with increasing reaction

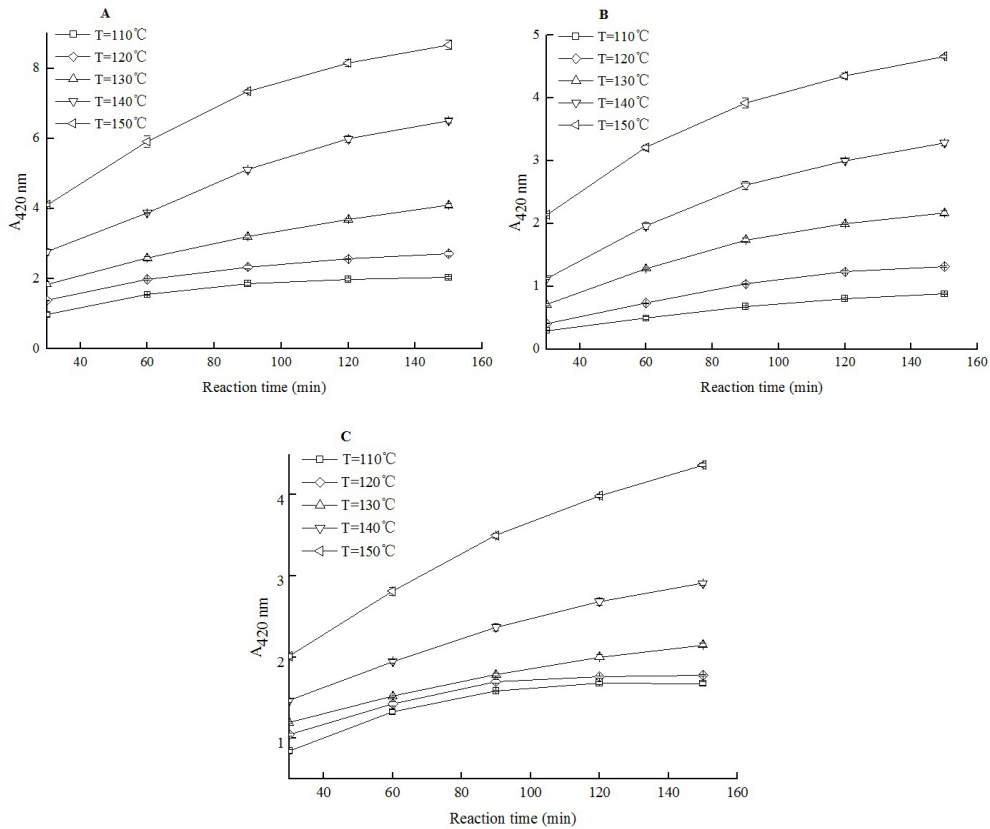
**Table 1.** The compositions of model reaction systems for non-enzymatic browning reactions.

System No.	L-ascorbic acid	Lysine	Arginine	Histidine
1	1.5 <sup>a</sup>			
2		1.5		
3			1.5	
4				1.5
5	1.5	1.5		
6	1.5		1.5	
7	1.5			1.5

<sup>a</sup>Amount (mmol).



**Figure 1.** The changing trends of the absorbance at 294 nm of the reaction solutions at different temperatures in (A) L-ascorbic acid/lysine system; (B) L-ascorbic acid/arginine system, and (C) L-ascorbic acid/histidine system.



**Figure 2.** The changing trends of the absorbance at 420 nm of the reaction solutions with reaction time at different temperatures in (A) L-ascorbic acid/lysine system; (B) L-ascorbic acid/arginine system, and (C) L-ascorbic acid/histidine system.

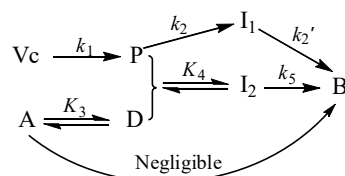
temperature. The trends of absorbance at 420 nm of the three amino acids were quite similar to those of absorbance at 294 nm of the corresponding amino acids, indicating that the formation of the browning products and the formation of the colorless intermediate products are correlated.

The absorbance at 420 nm of lysine/L-ascorbic acid system, arginine/L-ascorbic acid system and histidine/L-ascorbic acid system were linearly regressed against reaction time according to the method described in Kinetic calculation, they all met the kinetic characteristics of zero-order reaction and were concordant with those of glucose/glycine and galactose/glycine systems (Chen et al., 2005), i.e. the formation of the browning products of basic amino acids/L-ascorbic acid system met the kinetic characteristics of the zero-order reaction. The values of their rate constants  $k$  at various temperatures were presented in Table 2. It can be seen from the mean rate constants in Table 2 that within the temperature range of 110-150 °C, the browning reaction rate of L-ascorbic acid/lysine system was the rapidest one among three amino acids/L-ascorbic acid systems, followed by that of L-ascorbic acid/arginine system. The reaction rate of L-ascorbic acid/histidine system was the slowest one.

One study (Davies & Wedzicha, 1994) reported that during the non-enzymatic browning reaction, L-ascorbic acid was firstly de-carboxylated and dehydrated to form a pentose ( $P$ ) substance. This pentose substance is the key intermediate product of the non-enzymatic browning reaction and can react with the products of amino acid degradation to form the browning products. Another study (Ding et al., 2003) reported that the thermal reaction took place, thermal degradation of amino acids themselves could take places and form the auto-thermal degradation products ( $D$ ). According to the research scheme listed in Table 1, each of three amino acids and L-ascorbic acid were thermally degraded alone at 150 °C for 150 min, i.e. under the conditions of the highest reaction temperature, the longest reaction time and the highest browning degree, their absorbance at 420 nm were as follows: lysine, 0.8024; arginine, 0.1210; histidine, 0.9196 and L-ascorbic acid, 4.2333 while under such conditions, the minimal value of the absorbance of the reaction of L-ascorbic acid with three amino acids, respectively, were 4.3687. Thus, the contribution of the individual auto-degradation of three amino acids to the total absorbance is very small and can be almost negligible. However, the individual auto-degradation of L-ascorbic acid cannot be negligible.

Based on the above analysis and the experimental results, the mechanism for the formation of browning products of the

non-enzymatic browning reaction of three amino acids with L-ascorbic acid was proposed as follows:



where  $Vc$  is L-ascorbic acid;  $P$  is a pentose formed by decarboxylation and dehydration of L-ascorbic acid;  $I$  is the colorless intermediate products (the products indicated by the absorbance at 294 nm);  $B$  is the browning products (the products indicated by the absorbance at 420 nm);  $A$  is basic amino acids; and  $D$  is the products of auto-thermal degradation of basic amino acids.

According to the proposed reaction mechanism described above, by referring literature (Leong & Wedzicha 2000; Martins & Van Boekel, 2005) and by applying the law of mass action, the formation rate of the browning reaction product can be expressed by the following Equation 1:

$$\frac{d[B]}{dt} = k_2' [I_1] + k_5 [I_2] \quad (1)$$

According to the mechanism,  $[D]$  can be expressed as follows (Equation 2):

$$[D] = K_3 [A] \quad (2)$$

According to the mechanism, by applying the steady state conditions and by properly operating, we can get the following expressions (Equations 3-7):

$$[P] = \frac{k_1 [Vc]}{k_2} \quad (3)$$

$$[I_1] = \frac{k_1}{k_2} [Vc] \quad (4)$$

$$[I_2] = \frac{K_4 [P] [D]}{k_5} \quad (5)$$

$$[I_2] = \frac{k_1 K_3 K_4 [Vc] [A]}{k_2 k_5} \quad (6)$$

**Table 2.** The rate constants  $k$  of browning reaction of L-ascorbic acid/lysine, L-ascorbic acid/arginine and L-ascorbic acid/histidine system at different temperatures.

Temperature(°C)	L-ascorbic acid/lysine		L-ascorbic acid/arginine		L-ascorbic acid/histidine	
	k	r <sup>2</sup>	k	r <sup>2</sup>	k	r <sup>2</sup>
110	0.0085	0.9248	0.0049	0.9850	0.0074	0.9393
120	0.0109	0.9646	0.0077	0.9764	0.0058	0.9413
130	0.0187	0.9929	0.0121	0.9730	0.0080	0.9905
140	0.0320	0.9893	0.0179	0.9773	0.0121	0.9896
150	0.0379	0.9726	0.0206	0.9707	0.0194	0.9877
Average value	0.0216	-	0.0126	-	0.0105	-

$$\frac{d[B]}{dt} = k_1[Vc] \left\{ 1 + \frac{K_3 K_4 [A]}{k_2} \right\} \quad (7)$$

In this study, during non-enzymatic browning reaction, the total concentration of L-ascorbic acid  $[Vc]_T$  was as follows:

$$[Vc]_T = [Vc] + [P] + [I_1] + [I_2] + [B] \quad (8)$$

The browning products are usually some macromolecule polymers and their water solubility is low (Corzo-Martínez et al., 2012). In this study, we found in the experiments were all the clean reaction solutions and no turbid solution were formed, indicating that very limited amounts of browning products were formed in the non-enzymatic browning reaction. Therefore, only a small amount of L-ascorbic acid was consumed to form browning products as compared to those of the other items included in Equation 8. Thus,  $[B]$  can be almost negligible and Equation 8 can be simplified as follows:

$$[Vc]_T = [Vc] + [P] + [I_1] + [I_2] \quad (9)$$

Based on Equations 7 and 9, by properly operating, we can get the following expressions (Equations 10 and 11):

$$[Vc] = \frac{k_2 k_2' k_5 [Vc]_T}{k_2 k_2' k_5 + k_1 k_2' k_5 + k_1 k_2 k_5 + k_1 k_2' K_3 K_4 [A]} \quad (10)$$

$$\frac{d[B]}{dt} = \frac{k_1 k_2 k_2' k_5 [Vc]_T}{k_2 k_2' k_5 + k_1 k_2' k_5 + k_1 \{k_2 k_5 + k_2' K_3 K_4 [A]\}} \left\{ 1 + \frac{K_3 K_4 [A]}{k_2} \right\} \quad (11)$$

With the method described in Determination of the concentration of residual amino acids and by applying HPLC analysis, the reaction systems at 110 °C for 30 min, i.e. the highest concentration of three amino acids in the reaction systems under lowest reaction temperature and the shortest reaction times was 0.093 mol/L, thus,  $[A] \ll 1$ , and is suitable for approximate treatment.  $\frac{K_3 K_4 [A]}{k_2} \ll 1$ ,  $k_2' K_3 K_4 [A] \ll k_2 k_5$ , Equation 11 could be simplified as follows:

$$\frac{d[B]}{dt} = \frac{k_1 k_2 k_2' k_5 [Vc]_T}{k_2 k_2' k_5 + k_1 k_2' k_5 + k_1 k_2 k_5} \quad (12)$$

For a specified reaction system, the total concentration  $[Vc]_T$  has constant value, thus, Equation 12 has constant value at specified reaction temperature. These are the kinetic characteristics of the zero-order reaction. This is consistent with those calculated based on experimental results, indicating that the above proposed mechanism for the formation of browning products are reasonable. Thus, in the non-enzymatic browning reaction of the L-ascorbic acid/basic amino acid systems, the browning products are derived from two pathways: one pathway is that degradation of L-ascorbic acid lead to formation of the pentose substance, from which. the colorless intermediate products are then formed and the browning products are finally formed; the other pathway is the auto-thermal degradation of amino acids.

The thermally degraded products react with pentose substance to form the colorless intermediate products, from which, the browning products were finally formed.

It can be seen from Table 2 that the reaction temperature caused relatively large impacts on the rate constants of the formation of browning products. By applying kinetic calculation method described in Kinetic calculation, with Arrhenius equation, linear regression with  $\ln k$  against  $1/T$  was calculated and the apparent activation energies for L-ascorbic acid/lysine, L-ascorbic acid/arginine and L-ascorbic acid/histidine systems were 54.94 ( $r^2=0.9869$ ), 50.08 ( $r^2=0.9901$ ) and 35.31 ( $r^2=0.8721$ ) kJ/mol, respectively, indicating that L-ascorbic acid/lysine and L-ascorbic acid/arginine systems are more sensitive to the reaction temperature than L-ascorbic acid/histidine system was.

## 4 Conclusions

Under the conditions of weak basis and the reaction temperature range of 110-150 °C, three basic amino acids, i.e. lysine, arginine and histidine, were reacted with L-ascorbic acid at equal amount for 30-150 min, respectively, the kinetic characteristics of non-enzymatic browning reaction were investigated. The study results indicated that non-enzymatic browning reaction of three basic amino acids with L-ascorbic acid leading to the formation of the browning products was zero-order reaction. The browning degree of the reaction systems was only related to the total amount of L-ascorbic acid in the reaction system. We also proposed the mechanisms for the formation of browning products. Within the reaction temperature range of 110-150 °C, the reaction rate of L-ascorbic acid/lysine system was the fastest one, followed by that of the L-ascorbic acid/arginine system. The reaction rate of L-ascorbic acid/histidine system was the slowest one. The apparent activation energies for the formation of browning products from these three systems were 54.94, 50.08 and 35.31 kJ/mol, respectively. The L-ascorbic acid/lysine and L-ascorbic acid/arginine systems were more sensitive to reaction temperature than L-ascorbic acid/histidine was. These results are of important significance for guiding the processing of those foods rich in L-ascorbic acid and basic amino acids.

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