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The effects of reaction parameters on the non-enzymatic browning reaction between L-ascorbic acid and glycine

Abstract: The non-enzymatic browning (NEB) reaction between L-ascorbic acid (ASA) and glycine (Gly), including the effects of temperature (110–150 °C), time (10–150 min) and pH (4.5, 6.8, 8.0 and 9.5) on the formation of un-colored intermediate products (UIPs), browning products (BPs) and volatile products (VPs), were investigated. The results showed that pH had a remarkable effect on the reaction. The characteristics of zero-order kinetics for the formation of UIPs and BPs were discussed, and the corresponding activation energy ($E_a$) was also calculated. When the pH was 4.5, the $E_a$ for the formation of UIPs was approximate 53.76 kJ/mol and less than that at other pH values; while the $E_a$ for BPs formation was approximate 94.06 kJ/mol and much higher than that at other pH values. The results suggested that an acidic environment facilitated the generation of UIPs, but did not remarkably promote the formation of BPs. The possible reaction pathway between ASA and Gly was proposed according to the experimental results.

Keywords: glycine; kinetic; L-ascorbic acid; non-enzymatic browning reaction.

1 Introduction

The non-enzymatic browning (NEB) reaction is one a very important reaction in the food industry that significantly affects the color, flavor, taste and nutritional quality of food [1–3]. The NEB reaction is easily influenced by some reaction parameters, such as temperature, time, solution pH, buffer solution type and reactant concentration [4–6]. According to previous reports [7–9], pH had a significant impact on the reaction pathway, especially for the formation of different volatile products (VPs). In recent years, the NEB reaction has attracted widespread attention, and extensive related researches have been conducted in the heat treatment of food. L-ascorbic acid (ASA) is a polyhydroxy compound and can generate carbonyl radicals when it is heated [10–12]. ASA is widely found in fresh fruits, vegetables and many other organisms. This compound has a potential carbonyl structure and is usually used as a food additive and an antioxidant to protect against oxidative damage in the food industry. At the same time, ASA has high biological activity and participates in many metabolic processes [13–15]. Glycine (Gly) is one of the components of reduced glutathione, a semiessential amino acid and is used in medicine, feed and food industries [16–19]. The Maillard reaction behavior between ASA and Gly has been studied in the past few decades [5, 20, 21]. A number of kinetic studies have also been carried out on the Maillard reaction. However, the effect of different pH values on the NEB reaction between ASA and Gly and their kinetic characteristics have not been fully studied. A kinetic study of the NEB reaction would help us understand the formation mechanism of food coloring and volatile substances during food processing. However, the kinetics of the formation of un-colored intermediate products (UIPs) and browning products (BPs) are unclear. The NEB reaction is indeed very complicated. Even through the well-known initial reaction step method, the kinetic description of the NEB reaction is still very difficult [22–24]. In the manuscript, the influences of parameters, such as temperature (range of 110–150 °C), time (range of 10–150 min) and pH (ranging from acidic to alkaline conditions, 4.5, 6.8, 8.0 and 9.5) on the formation of VPs, UIPs and BPs were investigated. At the same time, the formation kinetic characteristics of UIPs and BPs were investigated and the activation energy ($E_a$) was calculated. According to the experimental results, a possible mechanism of the NEB reaction between ASA and Gly under different pH values was proposed.
2 Materials and methods

2.1 Materials

All experimental reagents were purchased from Sinopharm Chemical Reagent Co., Ltd. (Shanghai, China). ASA, Gly, NaCl, HPO₃, NaH₂PO₄, Na₂HPO₄, NaOH, and 2,4-dinitrofluorobenzem was of AR grade. Other reagents were of HPLC grade. The standard reagents and C5-C22 n-alkanes for gas chromatography (GC) were purchased from Pure Chemical Analysis Co., Ltd (Borney, Belgium). Water was redistilled before use.

2.2 Experimental procedure

The NEB reaction between ASA and Gly was prepared according to previous reports [7, 9]. The reaction solution of ASA and Gly was adjusted to 4.5, 6.8, 8.0 and 9.5 (PB-21; Sartorius AG Inc., Beijing, China) by using a buffer solution of Na₂HPO₄, Na₂HPO₄-NaH₂PO₄, adjusted to 4.5, 6.8, 8.0 and 9.5 (PB-21; Sartorius AG Inc., Beijing, China) by using a buffer solution of Na₂HPO₄, Na₂HPO₄-NaH₂PO₄, NaHCO₃, and NaOH. The NEB reaction between ASA and Gly was prepared according to previous reports [7, 9]. The reaction solution of ASA and Gly was adjusted to 4.5, 6.8, 8.0 and 9.5 (PB-21; Sartorius AG Inc., Beijing, China) by using a buffer solution of Na₂HPO₄, Na₂HPO₄-NaH₂PO₄, NaHCO₃, and NaOH. The reaction was carried out in a 15 mL Synthware pressure glass vial (Beijing Synthware Glass, Inc., China) and heated in an oil bath with stirring (DF-101S; Shanghai Qiangqiang Equipment Co., Ltd., Shanghai, China). The experimental errors were eliminated by repeating the experiment under the identical experimental conditions.

All experiments were performed at least in triplicate and the relative standard deviation (RSD) of the results was controlled less than 4.5%.

2.3 Measurement methods

2.3.1 Determination of the concentration of ASA and Gly: The concentration of ASA and Gly left in solution was determined by reverse-phase (RP) high-performance liquid chromatography (HPLC) [7, 8], which was an Agilent Technology model 1260 apparatus (Agilent, Santa Clara, CA, USA), equipped with a UV diode array detector and a C18 chromatographic column (3.5 μm, 4.6 mm i.d. × 100 mm). ASA and Gly were quantified by using the external standard curves of ASA and Gly (correlation coefficient, R², close to 0.9998), respectively. The wavelengths used to detect ASA and Gly were 234 and 360 nm, respectively.

ASA was detected with gradient elution mode with a mobile phase composed of 0.1 wt percent (wt. %) water/methanol/meta-phosphate [7]. The derivatization method was used to detect Gly [9]. The mobile phase used for Gly detection, which was composed of water/acetonitrile/phosphate buffer solution (0.01 M). Gradient elution was adopted, the flow rate was 1.0 mL/min, the column temperature was 30 °C, and the injection volume was 5 μL. Before detection, the production samples were derivatized with 2,4-dinitrofluorobenzem (FDNB) under alkaline conditions, and the alkaline solution was 1 mL of NaHCO₃ (0.05 M). Approximately 2 wt. % FDNB solution was dissolved in 1 mL of acetonitrile. The above solution was mixed and reacted in a water bath at 60 °C without light for 60 min. The samples were immersed in an ice-water bath to cool and diluted with phosphate buffer solution (0.01 M) to 10 mL. The samples were filtered before detection.

2.3.2 Measurement of UIPs and BPs

UIPs and BPs were successively formed as the NEB reaction proceed. The UV absorbance and browning intensity of the model reaction solutions were measured at ambient temperature at 294 and 420 nm by using a Cary 300 spectrophotometer (Shimadzu (China) Co., Ltd., China), and these wavelengths represented UIPs and BPs, respectively. To obtain the highest optical density, the product solution was diluted appropriately 25-, 50-, and 100-fold. The final absorbance results shown in the figures at 294 and 420 nm were actual absorbance values multiplied by dilution factors of 25-, -50, or -100.

2.4 Kinetic modeling

The reaction rate constants (k) for the formation of UIPs and BPs were calculated from the linear regression of A_{294 nm} and A_{420 nm} respectively. The relationship between ln(A_{294 nm})/ln(A_{420 nm}) and reaction time t based on the model of zero-, first- and second order reaction kinetics was determined [25, 26]. The kinetics formula was as follows,

\[ \frac{d[A]}{dt} = k[A]^n \quad (n = 0, 1, 2) \]  \tag{1}

The effects of temperature on k were calculated from the Arrhenius equation, Eq. (2), and E_a was calculated in different situations.

\[ k = A \exp(-E_a/RT) \]  \tag{2}

2.5 Statistical analysis

The experiments were repeated at least three times, and RSD was controlled within 4.5% to assure that R² was close to 1.000. All data were processed using Microsoft Office 2010 and Origin 8.5.

3 Results and discussion

3.1 The effects of pH on correlations between the concentration of ASA/Gly and absorbance values of UIPs/BPs

The correlation between the concentration of ASA/Gly and the absorbance of UIPs/BPs at 110 and 150 °C, from 20 to 150 min, and at pH values of 4.5, 6.8, 8.0 and 9.5, is shown in Figure 1. The results clearly showed that the concentration of ASA/Gly decreased, and the absorbance of UIPs/BPs increased, which meant these starting reaction materials responded similarly to changes in reaction parameters. However, the decrease in concentrations of ASA/Gly and the increase in the absorbance of UIPs/BPs were slow at relatively low temperatures (110 °C); while these changes occurred rapidly at higher temperature (150 °C). Furthermore, these reaction parameters had different effects on the changes of ASA and Gly concentration. The concentration of ASA decreased dramatically as the temperature increased to 150 °C, while the decreased concentration of Gly was not as obvious as the temperature increased. The concentration of Gly ranged from 3.29 to 2.80 mg/mL at 150 °C and a pH of 4.5, as shown in Figure 1 (A₂). Gly was...
much more stable than ASA under different reaction conditions due to the structural characteristics of Gly.

The observed changes also implied that pH had different effects on the production of UIPs and BPs, which is shown in Figure 1. When the pH was 4.5, the absorbance of UIPs, $A_{294\ \text{nm}}$, was increased from 4.10 to 16.94 and the absorbance of BPs, $A_{420\ \text{nm}}$, was increased from 0.08 to 1.04 at 110 °C, as shown in Figure 1 (A1). However, the absorbance of UIPs, $A_{294\ \text{nm}}$, was increased from 39.80 to 89.25, and the absorbance of BPs, $A_{420\ \text{nm}}$, increased from 4.13 to 17.50 at 150 °C, as shown in Figure 1 (A2). When the pH was 9.5, the absorbance of UIPs, $A_{294\ \text{nm}}$, was increased from 3.03 to 12.64, and the absorbance of BPs, $A_{420\ \text{nm}}$, was increased from 0.37 to 1.12 within the reaction time at 110 °C and is shown in Figure 1 (D1). The absorbance of UIPs, $A_{294\ \text{nm}}$, was increased from 23.54 to 87.45, and the absorbance of BPs, $A_{420\ \text{nm}}$, increased from 2.36 to 10.90 at 150 °C and is shown in Figure 1 (D2). These results implied that a relatively high temperature (150 °C) and low pH (4.5) would intensify the consumption of ASA and the formation of UIPs.

### 3.2 The effects of pH on the formation UIPs and BPs

The effects of pH on the formation of UIPs and BPs at different temperatures and times are shown in Figure 2. According to the results, the temperature at different pH values had a remarkable effect on the formation of UIPs.
and BPs. The absorbance value at 294 nm ($A_{294 \text{ nm}}$) was increased from 4.10 to 16.94 at 110 °C and pH 4.5 and is shown in Figure 2 (A1). At all temperatures, the values of $A_{294 \text{ nm}}$ at pH values of 6.8, 8.0 and 9.5 were less than that value at 4.5, which was shown in Figure 2 (B1–D2). These results indicated that increasing the temperature and the acidity of the solution would promote the formation of UIPs. When the reaction time was greater than 90 min, the increase in $A_{294 \text{ nm}}$ plateaued at a temperature of 150 °C.

The change in BPs absorbance, $A_{420 \text{ nm}}$, is shown in Figure 2 (A1–D1). The result suggested that the value of $A_{420 \text{ nm}}$ at a pH of 4.5 was much higher than those at other pH values, which implied that the effects of temperature on the formation of BPs at pH 4.5 were more remarkable than those at other pH values. At the higher temperature (150 °C), the value of $A_{420 \text{ nm}}$ was not changed when the reaction time was greater than 90 min, which is shown in Figure 2 (A1). At the lower temperatures (110, 120 °C), the values of $A_{420 \text{ nm}}$ were not greater than 3.50. However, the maximum $A_{420 \text{ nm}}$ was only close to 17.5 at 150 °C and 150 min when the pH was 4.5. During the experiment, as the temperature increased and the reaction time increased, the color solution became darker brown.

Figure 2: The effects of pHs on formation of UIPs and BPs, $A_{294 \text{ nm}}$ and $A_{420 \text{ nm}}$
(A: pH = 4.5; B: pH = 6.8; C: pH = 8.0; D: pH = 9.5, 1: $A_{294 \text{ nm}}$; 2: $A_{420 \text{ nm}}$).
Comparing the values of $A_{294\text{ nm}}$ and $A_{420\text{ nm}}$ at different temperatures in Figure 2, it was found that the value of $A_{294\text{ nm}}$ was much larger than the value of $A_{420\text{ nm}}$ under the corresponding reaction conditions. The results indicated that UIPs were the precursor for the formation of BPs; BPs was the final product of the NEB reaction. According to the results, it was again verified that when ASA and Gly were consumed, UIPs formed first, which has been reported in our previous papers [7, 9]. When the UIPs accumulated to a certain extent, BPs began to be generated.

As the reaction proceeded, the microenvironment of the solution changed, and the pH value of the solution also changed. Martins [19] once reported that the pH value would be changed when the primary solution pH was over 7.0. In the present study, when the initial pH of the solution was 8.0 and 9.5, the pH of the solution decreased to approximately 7.1 as the reaction proceeded. However, when the initial pH was 6.8, the change in pH was small [7]. According to the above results, the concentration of ASA decreased rapidly under acidic conditions. The absorbance of BPs, $A_{420\text{ nm}}$, was mainly due to the degradation of ASA because of the stability of Gly. In the NEB reaction system, the pH has a different influence on the formation of UIPs and BPs.

### 3.3 The effects of pH on total products from ASA/Gly

The total products generated, including UIPs, BPs, and VPs [8], from the NEB reaction between ASA and Gly at 150 °C, 120 min and different pH values, are shown in Figure 3. There was no significant difference in the yield of UIPs and BPs at different pH values. However, the generated amount of VPs was different at different pH values. When the pH of the solution was 8.0, the amount of VPs increased rapidly. According to the above results, when the initial pH of the solution was 8.0, the pH decreased to approximately 7.1 as the reaction proceeded for 120 min [7, 8]. This greatly promoted the nucleophilic substitution reaction between ASA and Gly, thereby making it easier to generate pyrazine or derivatives of pyrazine, the main component of VPs, at this pH value.

### 3.4 The kinetics of UIPs and BPs formation

Based on the relationship between $k$ and $R^2$, it was found that the formation of UIPs and BPs conformed to the pseudo-zero-order kinetics model by calculating $k$ and $R^2$ according to Eq. (1). Considering the influence of variable parameters and integrating different equations into appropriate sequences and equations related to time, Eq. (1) would be transformed into Eq. (3).

$$[A] - [A]_0 = -kt$$

According to the formation kinetics model of UIPs and BPs, the relationship between $k$ for the formation of UIPs ($A_{294\text{ nm}}$) and BPs ($A_{420\text{ nm}}$) versus time was calculated in terms of Eq. (3) and is shown in Tables 1(A), 1(B). When the temperature ranged from 110 to 150 °C, $k$ was simulated at different pH values (4.5, 6.8, 8.0, 9.5). According to the classic Arrhenius formula [20, 21], the function of 1/T versus ln $k$ at different pH values was calculated based on the zero-order model [9].

$$E_a = \frac{RT}{k} + \ln A$$

The results suggested that the formation $E_a$ of UIPs was $53.76 \pm 2.62$ kJ/mol at pH of 4.5 and shown in Table 1(A); this value was less than those at other pH values. However, the formation $E_a$ of BPs was $79.03 \pm 2.76$ kJ/mol at pH 6.8, which was less than those at other pH values, as shown in Table 1(B). These data once again confirmed the previous results of Figures 1 and 2.

### 3.5 The mechanism for the NEB reaction between ASA and Gly

According to the experimental results, there are two possible reaction mechanisms for the NEB reaction.
The results indicated that UIPs would be formed by the aggregation of small substances in the initial stage of the NEB reaction. In an acidic solution, furan and furfural derivatives were the main products from the degradation of ASA without Gly participation and are shown in Scheme 1 [30]. Under these conditions, the alkenyl alcohol structure of ASA was mutually converted and then dehydrated to form the Havus structure of the multicomponent compound. Then, the Havus ring was opened to form the derivative of 2-carboxyl acetic acid from the internal ester. Finally, VPs, such as furan and furfural derivatives, were generated due to the decomposition of the 2-carboxyl acetic acid derivatives. Under neutral and alkaline solutions, the NEB reaction pathway between ASA and Gly was different from that under the acidic condition [31, 32]. The aldehyde and ketones produced in these solutions are different from that under the acidic condition [31, 32]. The aldehyde and ketones produced in these solutions are different from that under the acidic condition [31, 32].

Table 1(A): The dynamic date. The dynamic date of the UIPs based on zero models.

| pH | 110 °C | 120 °C | 130 °C | 140 °C | 150 °C | $E_a$ (kJ/mol) |
|----|--------|--------|--------|--------|--------|----------------|
|    | $k$ ($R^*$) | $k$ ($R$) | $k$ ($R$) | $k$ ($R$) | $k$ ($R$) | $E_a$ ($kJ/mol$) |
| 4.5 | 0.1012 (0.9960) | 0.2200 (0.9981) | 0.4615 (0.9955) | 0.4823 (0.9466) | 0.4896 (0.9076) | 53.76 ± 2.62 |
| 6.8 | 0.0601 (0.9931) | 0.1342 (0.9976) | 0.2562 (0.9903) | 0.4458 (0.9836) | 0.5357 (0.9651) | 75.52 ± 2.25 |
| 8.0 | 0.0609 (0.9858) | 0.1171 (0.9935) | 0.2510 (0.9930) | 0.4147 (0.9921) | 0.5043 (0.9602) | 74.35 ± 2.08 |
| 9.5 | 0.0729 (0.9890) | 0.1333 (0.9902) | 0.2183 (0.9975) | 0.3656 (0.9866) | 0.4819 (0.9628) | 64.63 ± 3.23 |

Table 1(B): The dynamic date. The dynamic date of the BPs based on zero models.

| pH | 110 °C | 120 °C | 130 °C | 140 °C | 150 °C | $E_a$ (kJ/mol) |
|----|--------|--------|--------|--------|--------|----------------|
|    | $k$ ($R^*$) | $k$ ($R$) | $k$ ($R$) | $k$ ($R$) | $k$ ($R$) | $E_a$ ($kJ/mol$) |
| 4.5 | 0.0077 (0.9976) | 0.0271 (0.9937) | 0.0713 (0.9920) | 0.1017 (0.9725) | 0.1264 (0.9432) | 94.06 ± 1.93 |
| 6.8 | 0.0078 (0.9890) | 0.0195 (0.9990) | 0.0423 (0.9952) | 0.0662 (0.9889) | 0.0781 (0.9633) | 79.03 ± 2.76 |
| 8.0 | 0.0064 (0.9701) | 0.0134 (0.9967) | 0.0342 (0.9942) | 0.0549 (0.9934) | 0.0668 (0.9692) | 85.59 ± 2.43 |
| 9.5 | 0.0056 (0.9904) | 0.0113 (0.9978) | 0.0256 (0.9997) | 0.0506 (0.9956) | 0.0650 (0.9687) | 86.64 ± 3.03 |

The correlation coefficient.

between ASA and Gly [27–29] under different pH and anaerobic conditions, which are shown in Schemes 1, 2.

The alkenyl alcohol structure of ASA was mutually converted and then dehydrated to form the Havus structure of the multicomponent compound. Then, the Havus ring was opened to form the derivative of 2-carboxyl acetic acid from the internal ester. Finally, VPs, such as furan and furfural derivatives, were generated due to the decomposition of the 2-carboxyl acetic acid derivatives. Under neutral and alkaline solutions, the NEB reaction pathway between ASA and Gly was different from that under the acidic condition [31, 32]. The aldehyde and ketones produced in these solutions are different from that under the acidic condition [31, 32]. The aldehyde and ketones produced in these solutions are different from that under the acidic condition [31, 32]. The aldehyde and ketones produced in these solutions are different from that under the acidic condition [31, 32].

Scheme 1: NEB reaction at acidic solution (pH: 4.5).

Scheme 2: NEB reaction at neutral and alkaline solution (pH: 6.8, 8.0, 9.5).

4 Conclusions

In the present work, the influence of reaction parameters on the NEB reaction between ASA and Gly was investigated, including its effect on the consumption of ASA and Gly, influence on the formation of UIPs and BPs, and effects on the mechanism of NEB. The results revealed that pH had a significant effect on the NEB reaction, which would vary the reaction pathway of NEB. When the pH was 4.5, it was conducive to the formation of UIPs, while the formation of BPs was promoted when the pH was 6.8. The formation
kinetic characteristics of UIPs and BPs were discussed, and the formation $E_a$ of UIPs and BPs were calculated under different pH values. The mechanism of the NEB reaction between ASA and Gly has been discussed. However, the reaction mechanism under different pH values requires further discussion.

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