

Kinetics of Non-Enzymatic Browning Reaction from the L-Ascorbic Acid/L-Cysteine Model System

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Abstract

YU A.-N., TANG L.-P. (2016): **Kinetics of non-enzymatic browning reaction from the L-ascorbic acid/L-cysteine model system.** Czech J. Food Sci., 34: 503–510.

The kinetics of the non-enzymatic browning reaction from L-ascorbic acid/L-cysteine model systems was investigated at 125–155°C for 10–120 min by measuring the loss of reactants and monitoring the brown colour development. The result showed that the browning products were produced at the first order reaction kinetics requirement, with the E_a being 114.33 kJ/mol. The mechanism for the browning development was proposed. The correlation coefficient between browning products and uncoloured intermediate products increased with the rising temperature. At temperatures below 110°C, the non-enzymatic browning reaction did not occur at all or the reaction was slow. Browning products could not be produced solely by self-degradation of L-cysteine. The amount of browning products negatively correlated with the L-ascorbic acid concentration.

Keywords: Maillard; mechanism; amino acid; first order reaction; activation energy

The non-enzymatic browning reaction plays an important role in colour and flavour generation during food processing (OBRETENOV *et al.* 2002). L-Cysteine (Cys) is known to be one of the most important precursors of meat-like flavour compounds. L-Ascorbic acid (ASA) is a common ingredient of the human diet, especially in fruit and vegetables, herbs, and to a lesser extent in meat (liver). In addition, ascorbic acid is frequently used as food additive, antioxidant, and flour improver in bakeries. In addition to reducing carbohydrates, ASA may also react via the Maillard browning reaction (CORZO-MARTÍNEZ *et al.* 2012). ASA is a reductone and will form brown pigments in the presence of amino compounds and will accelerate Maillard browning (WROLSTAD 2012).

With regard to the ASA non-enzymatic browning reaction, there are many reports related to it (CORZO-MARTÍNEZ *et al.* 2012; WROLSTAD 2012). However, as regards the ASA/Cys system, there is a lack of

research findings on the non-enzymatic browning reaction in the model reactions of ASA with Cys. ADAMS and DE KIMPE (2009) reported the formation of furan derivatives and thiophenes produced by heating a model reaction of ASA with Cys under dry roasting conditions in the presence of K_2CO_3 , but data were not attached. YU *et al.* (1974) studied the colour formation of the browning reactions between ASA and Cys at 44 and 72°C for 4 h–12 days by measurement of the absorbance at 400 nm. The results showed that the progress and the extent of the colour development in unbuffered systems were dependent on temperature, incubation period, etc. There are several papers published by our laboratory. In one of these papers, we reported the effect of pH on the formation of aroma compounds from ASA and Cys during the non-enzymatic browning reaction and discovered that the reaction between ASA and Cys led mainly to the formation of alicy-

Supported by the National Natural Science Foundation of China, Grant No. 31360408, and by the Dominant and Characteristic Discipline Group for Hubei Provincial Universities (Hubei degree 2015-5).

clic sulphur compounds, thiophenes, thiazoles, and pyrazines (YU & ZHANG 2010). In another paper (YU *et al.* 2012a), we reported the mechanism of the formation of sulphur compounds produced by ASA and Cys during the non-enzymatic browning reaction, and elucidated the formation of chemical pathways for sulphur compounds formed from ASA and Cys during the non-enzymatic browning reaction by using isotope-labelled reagents to elucidate the origin of carbons in the sulphur compounds. However, the browning kinetics of the non-enzymatic browning reaction from an ASA/Cys model has not been reported.

A kinetic description would improve the understanding of the mechanism leading to colour and flavour, which could further help to develop methods to control colour and flavour formation in food. The non-enzymatic browning reactions are indeed very complex, and even the well-studied initial reaction steps have been difficult to describe kinetically. However, the degree of browning, as a monitoring index of the reaction process, was often used analytically to assess the extent to which the non-enzymatic browning reaction had taken place in foods (MARTINS *et al.* 2001; LING *et al.* 2015). As mentioned above, we reported the mechanism of formation of aroma compounds produced by ASA and Cys during the non-enzymatic browning reaction (YU *et al.* 2012a). The objective of this study is to elucidate the kinetic characteristics of the browning process, and also to reveal the underlying mechanism of the browning process. We also aim to discuss the relationship between the browning products and the uncoloured intermediate products (UIPs), as well as the relationship of the browning products with the reactants. An improved understanding of the kinetics of the colour and consumption rate of ASA and Cys will lead to the more effective control of colour formation in food.

MATERIAL AND METHODS

ASA ($\geq 99.7\%$), Cys ($\geq 99.5\%$), acetonitrile (HPLC grade), metaphosphoric acid (analytical grade), Na_2HPO_4 (analytical grade), NaH_2PO_4 (analytical grade), and NaOH (analytical grade) were purchased from Sinopharm Chemical Reagent Co., Ltd. (Beijing, China). 2,4-Dinitrofluorobenzene ($\geq 99.0\%$) was purchased from TCI Development Co., Ltd. (Shanghai, China). Double-distilled water was used in all experiments.

Preparation of model reaction solutions. The model reaction system and the pH value were set according to our previous research (YU & ZHANG 2010). ASA (1.0 mmol) and Cys (1.0 mmol) were dissolved in 10 ml of phosphate buffer (0.2 M, pH 8), and the pH of the solutions was adjusted to 8.0 using NaOH with pH meter (Sartorius AG, Shanghai, China). The mixtures were then sealed in 15-ml Synthware[®] pressure glass vials (Beijing Synthware Glass, Inc., China) and heated in an oil bath while stirring at 110, 125, 135, 140, 145, and 155°C for 10, 20, 30, 40, 60, 90, and 120 min, respectively. The progresses of the reaction were immediately stopped by cooling under a stream of cold water. Each experiment was done at least in triplicate, and each data point was collected from three samples.

Measurement of UV absorbance. In a non-enzymatic browning reaction, reactants formed complex browning products through three major stages, namely early, intermediate, and final stage. The UV absorbance at 294 nm was often used to indicate the UIPs of browning products while the final stage was monitored by the absorbance at 420 nm (YU *et al.* 2012b; HONG *et al.* 2015; O'CHAROEN *et al.* 2015). In the final stage, it leads to the formation of brown nitrogenous polymers and co-polymers, known as melanoidins (MARTINS *et al.* 2001). The melanoidins have various kinds of biological activities (TAYLOR *et al.* 2004; KITRYTÉ *et al.* 2012). The absorbance at 420 nm, as a common measurement for the formation of browning products, indicates the browning degree of non-enzymatic browning reactions (LERTITTIKUL *et al.* 2007; O'CHAROEN *et al.* 2015). Previous kinetics studies on browning index reactions were also based on absorbance at 420 nm measurement in citrus juices, apple juices (BURDURLU & KARADENIZ 2003), and pineapple puree (LING *et al.* 2015). The UV absorbance of model reaction solutions was measured according to the method of AJANDOUZ *et al.* (2001). The UV absorbance of model reaction solutions was measured at room temperature at 294 and 420 nm, using an UV2550 spectrophotometer (Shimadzu (China) Co., Ltd., China). When necessary, appropriate dilutions (0–25-fold) were made in order to obtain an appropriate optical density. All the experiments were carried out in triplicate and the mean values were used to draw up the kinetic plots. The relative standard deviations (RSD) were lower than 14.6 and 14.5%, and average RSD were 4.7 and 6.0% at 294 and 420 nm, respectively.

doi: 10.17221/86/2016-CJFS

Determination of ASA concentration. ASA was determined by UV spectrophotometry according to our previous paper (TANG *et al.* 2014). 50 µl of model reaction solutions were diluted to 50 ml with double-distilled water. Absorbance was measured at 243 nm with an UV2550 spectrophotometer (Shimadzu (China) Co., Ltd., China). ASA was quantified by an external standard procedure with L-ascorbic acid (0~40 µg/ml; Sigma-Aldrich, Germany), using a calibration curve ($R^2 = 0.9998$). All the experiments were carried out in triplicate with RSD lower than 5.4% and average RSD of 1.5%.

Determination of Cys concentration. Cys concentration of model reaction solutions was determined by performing reverse phase high-performance liquid chromatography (HPLC) after pre-column derivatisation with 2,4-dinitrofluorobenzene as described by Lü *et al.* (2009). HPLC analyses were performed at 30°C on an Agilent Technologies model 1260 liquid chromatograph equipped with a UV diode array detector (Agilent Technologies, Wallbronn, Germany). Samples were fractionated in an Agilent C18 column (3.5 µm, 4.6 × 100 mm; Agilent Technologies, Inc., Santa Clara, USA) using the water/acetonitrile/phosphate buffer (0.02 M, pH 7) mixture as mobile phase eluting and taking the gradient elution mode. The flow rate was set to 1.0 ml/minute. The injection volume was 5 µl. The eluted Cys ramification was detected at 360 nm, which was the maximum absorbing wavelength of Cys ramification. Cys was quantified by an external standard procedure with L-cysteine (Sigma-Aldrich, Germany), using a calibration curve ($R^2 = 0.9998$). Each data point was collected from three samples. All the experiments were carried out in triplicate with RSD lower than 11.9% and average RSD of 3.3%.

Kinetics calculations. The rate constants k for the formation of browning products were calculated by linear regression of [absorbances], \ln [absorbances], and $1/[\text{absorbances}]$ versus reaction time t (min) for zero, first, and second order reaction kinetics, respectively. The effect of temperature on the reaction rate was parameterised according to the Arrhenius equation using the rate constants: $\ln k = \ln k_0 - E_a/(RT)$ to yield the activation energy (E_a). The E_a for the formation of browning products was estimated numerically by linear regression of a plot of $\ln k$ versus $1/T$ according to the method of ROZYCKI *et al.* (2010) and LIU *et al.* (2011).

Statistical analyses. All experiments were carried out in triplicate and expressed as mean value ±

standard deviation. The data analysis (curve fitting) and the calculation of kinetic rate constants were performed using the OriginPro 8 software (OriginLab Co., Northampton, USA). Correlation analysis was performed using the Pearson correlation coefficient (r). All statistical analyses were carried out at a 95% level of confidence.

RESULTS AND DISCUSSION

Kinetics of the formation of browning products.

Figure 1A summarises how the absorbance of the reaction solution at 420 nm varied with reaction time at different temperatures. As shown in Figure 1A, absorbance at 420 nm increased with the increase of reaction temperature and reaction time, and the higher the temperature, the faster the rate of the absorbance increased. In the method described above, fitting the changes of absorbance at 420 nm versus reaction time at different temperatures proved that the changes met the first order reaction kinetics requirement. Table 1 shows the reaction rate constants of browning development at 420 nm at different temperatures. The reaction rate constant at 110°C was discarded for not meeting the first order reaction kinetics requirement. The kinetic data obtained showed that the reaction temperature had a major influence on the formation of browning products.

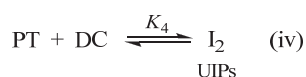
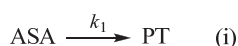
The ASA may undergo decarboxylation and dehydration to form decarboxylated ASA, i.e. pentoses (PT), and may also be browning (DAVIES & WEZICHA 1994). The PTs were the key intermediate products in the non-enzymatic browning reaction of ASA (DAVIES & WEZICHA 1994). The melanoidins (browning products) were made up from two residues of decarboxylated ASA and amino acid (DAVIES & WEZICHA 1994), and ASA itself browning. As reported previously (DING *et al.* 2003), amino acids could self-degrade when heated, which could be divided

Table 1. The rate constants of browning development at 420 nm at different temperatures

Temperatures (°C)	Rate constants (k)	R^2
110	–	–
125	0.0466	0.9299
135	0.126	0.9889
140	0.170	0.9695
145	0.260	0.9319
155	0.539	0.9185

into 3 stages: the amino acid would lose the water of crystallisation during the first stage, then lose the amine group and carboxyl group during the second stage, and finally thermal oxidation reaction. In this work, we used a sealed aqueous solution as the non-enzymatic browning reaction model for blocking off oxygen. Therefore, the Cys would firstly undertake thermal self-degradation instead of the non-enzymatic browning reaction to form degradation products of Cys (DC). Another experiment in a similar system without ASA also showed that the heating of Cys for 2 h at 140°C only resulted in a light yellow solution (absorbance at 420 nm was 0.08). As can be seen, the absorbance is a very small value, indicating that the self-degradations of Cys to browning products are very limited. Therefore, the contribution to the colour change (browning) from the Cys self-browning becomes approximatively negligible.

On the basis of experimental results and above facts, the mechanism for the browning development may be suggested as follows:



On the basis of the above scheme, the rate of colour development may be given as:

$$\frac{d[\text{browning}]}{dt} = k_2'[\text{I}_1] + k_5[\text{I}_2]$$

According to steady-state conditions, the rate of colour development may also be given as:

$$\frac{d[\text{browning}]}{dt} = k_2[\text{PT}] + k_5[\text{I}_2] \quad (1)$$

The [PT] may be obtained by applying steady-state conditions with respect to steps (i) and (ii). The [PT] is given as:

$$[\text{PT}] = \frac{k_1[\text{ASA}]}{k_2} \quad (2)$$

Further, considering the equilibrium step (iii) we can get:

$$[\text{DC}] = K_3[\text{Cys}] \quad (3)$$

Similarly from equilibrium steps (iv) and (v) by applying steady-state conditions, we get:

$$[\text{I}_2] = \frac{K_4[\text{PT}][\text{DC}]}{k_5} \quad (4)$$

On substituting the values of [PT] and [DC] from Eqs (2) and (3) into Eq. (4), respectively, we obtain:

$$[\text{I}_2] = \frac{k_1 K_3 K_4 [\text{ASA}][\text{Cys}]}{k_2 k_5} \quad (5)$$

On substituting the values of [PT] and [I₂] (Eq. 5) in Eq. (1), the rate law is obtained as follows:

$$\frac{d[\text{browning}]}{dt} = k_1[\text{ASA}] \left\{ 1 + \frac{K_3 K_4 [\text{Cys}]}{k_2} \right\} \quad (6)$$

On the basis of Figure 2B, the Cys residue concentration dropped significantly ($P < 0.05$), and a complete degradation could be observed after 30 min at above 135°C, i.e. Cys is in low concentration during the reaction. Taking suitable approximation as $K_3 K_4 [\text{Cys}]/k_2 \ll 1$, the rate law (Eq. 6) reduces to:

$$\frac{d[\text{browning}]}{dt} = k_1[\text{ASA}] \quad (7)$$

This suggested that the rate of brown colour development depended on ASA and met the first order reaction kinetics requirement. But, the Cys residue concentration was not negligible at 110°C (Figure 2B). Perhaps, it was a reason why the reaction rate at 110°C did not meet the first order reaction kinetics requirement. The proposed rate laws were in agreement with the experimental results and were also in agreement with the self-degradation kinetics of ASA (Li *et al.* 2016).

The temperature sensitivity of rate constant was analysed using the Arrhenius equation according to the method described above. Linear fitting of $\ln k - 1/T$ using the Arrhenius equation gave the results of $y = -13751.79x + 31.54$ ($R^2 = 0.9948$) for 420 nm. The E_a successfully described the relationship of the non-enzymatic browning reaction to temperature. The E_a of the ASA/Cys model amounted to 114.33 kJ/mol at 420 nm. The browning product formation (measured as absorption at 420 nm) was related to the melanoidin concentration (MARTINS

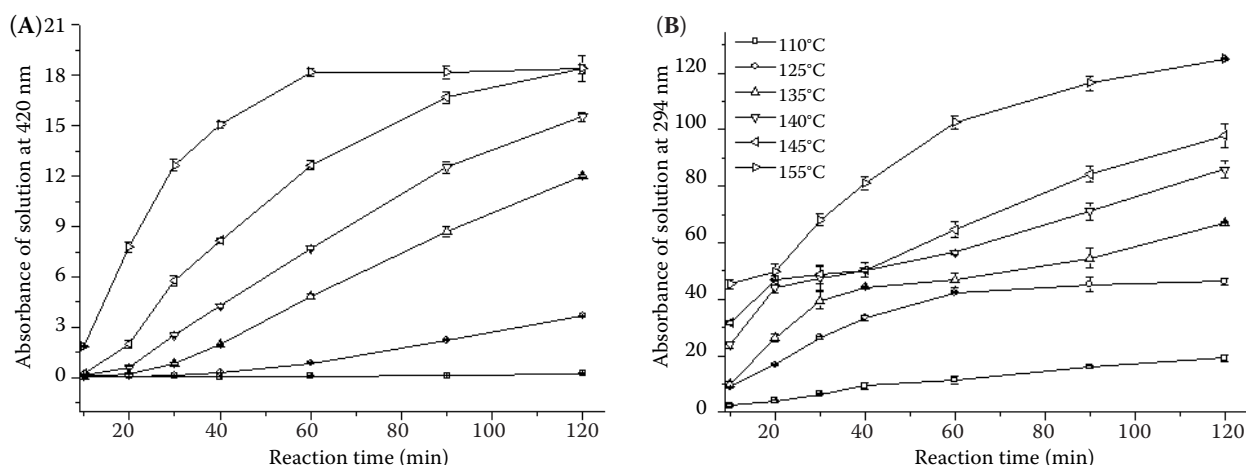


Figure 1. The absorbance of the reaction solution varied with reaction time at different temperatures; (A) at 420 nm; (B) at 294 nm

et al. 2001). ROGACHEVA *et al.* (1999) investigated the E_a of the melanoidin formation of three amino acids with ASA. The authors showed that the E_a of ASA with L-glutamic acid, glycine and L-lysine model amounted to 136.96, 106.07, and 99.79 kJ/mol, respectively. The comparison of the E_a of ASA reaction with Cys and ASA reactions with L-glutamic acid, glycine, and L-lysine shows that the E_a of the four models indicates that acidic L-glutamic acid has a higher E_a value, basic L-lysine has a lower E_a value, and neutral Cys and glycine are between them. The similar E_a of ASA/Cys and ASA/glycine indicates a similar temperature dependence of the reaction rate.

Relationship between browning products and UIPs. As shown in Figure 1, the UIPs (absorbance at 294 nm) exhibited a similar trend with the browning products (absorbance at 420 nm), i.e. the absorbance at 294 and 420 nm significantly ($P < 0.05$) increased with rising temperature and increasing reaction time, and the reaction rate increased at higher temperatures. Generally, the UIPs formed at intermediate stages contributed to the browning product formation (AJANDOUZ *et al.* 2001; BENJAKUL *et al.* 2005a, b; LIANG *et al.* 2014). It is usually considered that the absorbance at 294 nm wavelength reflects the amount change of the UIPs in a non-enzymatic browning reaction (AJANDOUZ *et al.* 2001; BENJAKUL *et al.* 2005a; YU *et al.* 2012b; LIANG *et al.* 2014), and there are suggestions in literature that these compounds could be the UIPs in the glycosylation process, such as aldehydes, low-molecular-weight ketones and so on. AJANDOUZ *et al.* (2001) believed that these compounds could be the precursors for the browning products in non-enzymatic browning

reaction or caramelisation reaction, and therefore the absorbance at 294 nm could indicate the formation of UIPs and thus the precursors of browning products. The change of absorbance at 294 nm was correlated with the change of browning products to some extent, which can reveal their relationship and predict the colour change of the reaction solution.

Comparison between absorbances at 420 nm and 294 nm of reaction solutions could also reveal the correlation between browning products and the UIPs at different temperatures (Figure 1). The Pearson r

Table 2. The correlation analysis

Absorbances	Reaction temperature (°C)	Pearson r
Between absorbance at 294 and 420 nm	110	0.67
	125	0.76
	135	0.87
	140	0.95
	145	0.96
	155	0.92
Between absorbance at 420 nm and residue ASA concentration	110	−0.72
	125	−0.90
	135	−0.97
	140	−0.99
	145	−0.99
	155	−0.95

The negative Pearson r indicates a negative correlation and the positive value indicates a positive correlation; the absolute value of r : 0.8–1.0: very strong positive correlation; 0.6–0.8: a strong correlation; 0.4–0.6: a moderate correlation; 0.2–0.4: a weak correlation; 0.0–0.2: a little discernible relationship between fluctuations of the variables

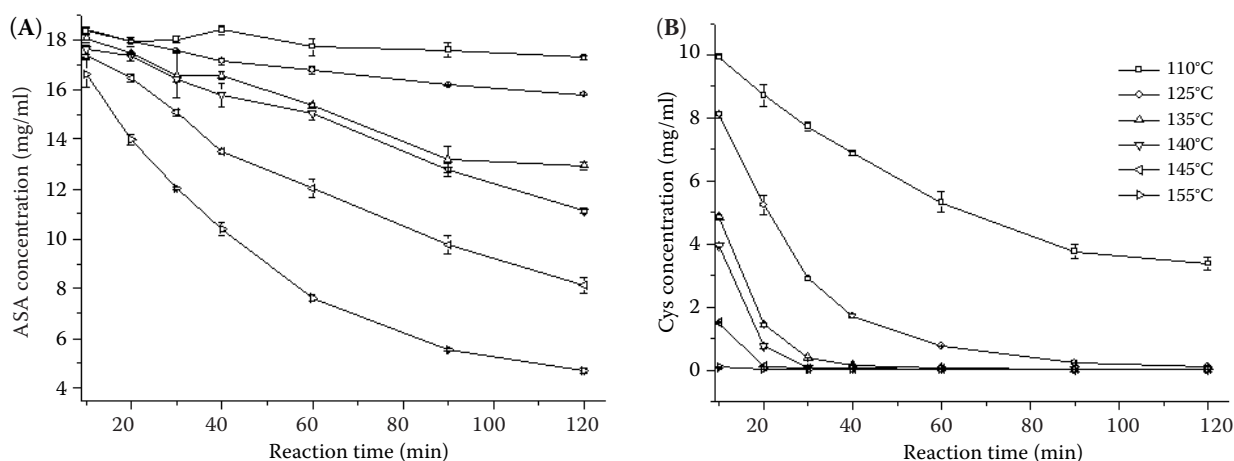


Figure 2. Relationship between reactant concentration and reaction time at different temperatures (A) ASA and (B) Cys

may quantitatively reflect the correlation. Table 2 lists the Pearson r within 10–120 min between browning products (420 nm) and the UIPs (294 nm) at the same temperature, calculated by the OriginPro 8 software (OriginLab Co., USA). As shown in Table 2, with the reaction temperature increasing, the correlation coefficient between the browning products and UIPs increased, and was gradually synchronised above 140°C. As a whole, from the above results, the browning products (Figure 1A) of all samples increased with the concomitant increase in UIPs (Figure 1B). The result was in agreement with LERTITIKUL *et al.* (2007), who also found similar results in the porcine plasma protein-glucose model system.

Relationship between browning products and reactants. Figure 2 illustrate the relationship between residue concentration and reaction time for the reactants ASA and Cys at different temperatures. The investigation of changes in ASA and Cys residue concentrations with reaction time at 110°C revealed that, as the reaction proceeded, the ASA residue concentration showed minor changes, which indicated that the non-enzymatic browning reaction was not pronounced at this temperature. In contrast, the Cys residue concentration decreased pronouncedly at this temperature. A comparison between the two reactant concentrations at higher temperatures (above 110°C) showed that the concentration of Cys residues decreased significantly ($P < 0.05$), and almost complete degradation could be observed after 30 min at a temperature above 135°C. Meanwhile, the ASA residue concentration decreased rather slowly at 110°C, but it decreased quickly with the increase of temperature above 110°C, indicating a pronounced non-enzymatic browning reaction above 110°C.

It could be found out by comparing concentrations of browning products (Figure 1A) with substrates (Figure 2) at different temperatures that at low temperatures (110°C) the concentrations of browning products and ASA showed only small changes, but the Cys residue concentration dropped significantly ($P < 0.05$). This indicated that the browning effect was not solely due to the self-degradation of Cys, which was consistent with previous results by Yu and ZHANG (2010). Our previous work regarding flavour substances in the ASA/Cys system also proved that the flavour substances would not be produced at 110°C (TANG *et al.* 2015). Therefore we concluded that when the temperature reached 110°C and above, the ASA would then be consumed and considered as the starting point of a non-enzymatic browning reaction.

Correlations between browning products and ASA residue concentrations could be extracted by comparing their concentration changes at different temperatures. From Figures 1A and 2A we discovered that there was a very similar tendency of changes between the browning products and the ASA. This correlation indicates that the reaction temperature affected the formation rates of browning products, and the amount of the browning products was associated with the reaction concentration of ASA residues. A further study of Figures 1A and 2A showed that the browning products and ASA residue concentration had a quantitative correlation. Table 2 lists the Pearson r within 10–120 min between browning products (420 nm) and ASA residue concentrations at the same temperature, calculated by OriginPro 8. High correlations were observed between the browning products and ASA residue concentration. It was shown that the amount

doi: 10.17221/86/2016-CJFS

of browning products negatively correlated with the ASA residue concentration, proving an association between the increases of browning products and the decrease of ASA residue concentration. The formation of browning products was possibly a result of the integration of non-enzymatic browning reactions and ASA browning reaction. However, Figures 1A and 2B did not show any obvious correlation between the browning product concentration and the Cys residue concentration. This was in agreement with the above-mentioned kinetics analyses.

CONCLUSIONS

The presented results showed that: (1) In the ASA/Cys non-enzymatic browning reaction system, at 125–155°C and within 10–120 min reaction time, the browning products were produced at the first order reaction kinetics requirement, with the E_a being 114.33 kJ/mol. The formation of browning products went through UIPs from the reaction between ASA with Cys, and ASA self-degradation. (2) The correlation coefficient between browning products and the UIPs increased with the rising temperature. At temperatures below 110°C, the non-enzymatic browning reaction did not occur at all or the reaction was slow. (3) Browning products could not be produced solely by self-degradation of Cys. The amount of browning products negatively correlated with the reactant ASA concentration, indicating an association between the increases of browning products with the decrease of ASA concentration. These findings can optimise the manufacturing conditions for the more effective control of the colour of final products in the foods rich in ASA and Cys.

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Received: 2016–03–12

Accepted after corrections: 2016–11–16

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