Kinetics of Interaction of Vanillin with Amino Acids and Peptides in Model Systems

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Model systems were used to study the reaction kinetics of vanillin and pentalysine, lysine, glutathione, cysteine, aspartame, or phenylalanine (molar ratio 1:1) in phosphate buffer. The buffer pH was adjusted to the pK_{a2} of the available α-amino group of each amino acid or peptide. Reductions of vanillin followed first-order kinetics at 55, 65, and 75 °C in the presence of each of the amino acids or peptides used. The reaction rates were accelerated as the temperature increased. The rate constants were highest for pentalysine followed by lysine, phenylalanine, glutathione/cysteine, and aspartame. The reduction of phenylalanine followed first-order kinetics, whereas the formation of its reaction product followed zero-order kinetics. The activation energy (E_a) for the reaction ranged from 5.6 to 14.5 kcal/mol.

Keywords: *Vanillin; kinetics order; amino acids; peptides; flavor*

INTRODUCTION

Vanillin, an aromatic aldehyde (4-hydroxy-3-methoxybenzaldehyde), is the major compound in vanilla extract that is responsible for its characteristic flavor. However, the intensity of the vanillin flavor in a food system is affected greatly by food components, especially proteins. Ng et al. (1989) reported that binding of vanillin increased when the concentration of faba bean protein micellar mass in water suspension increased. The authors also observed that heat treatment of faba bean protein increased its binding capacity with vanillin. Hansen and Heinis (1991) reported that the intensity of vanillin flavor decreased in a system containing whey protein concentrate and sodium caseinate. McNeill and Schmidt (1993) reported that sodium caseinate interacted more with vanillin than whey protein isolate in sweetened drinks. Hansen and Booker (1996) found that whey protein concentrate bound flavor more in an ice cream mix than sodium caseinate. In addition, they found that binding of β-lactoglobulin with benzaldehyde was higher at 70 °C than at room temperature.

The literature suggests that the interaction of vanillin and proteins may occur through the formation of covalent bonding via the Schiff base arrangement. Cha and Ho (1988) reported that the reduction of vanillin followed second-order kinetics in a methanol system containing aspartame and attributed it to Schiff base formation. Kim and Min (1989) suggested that volatile aldehydes can react with free amino acids or with the free amino group of proteins and form reversible Schiff bases. However, the process by which volatile compounds bind to proteins through covalent linkages can be irreversible (Feeney et al., 1975). Graf and de Roos (1996) suggested that the Schiff base formation between vanillin and proteins can be accelerated in ice cream by lowering the fat content.

Many studies have shown that the Schiff base formation is temperature dependent. Ho et al. (1988) studied the interaction of aspartame and glucose (0.1 M) at 80, 90, and 100 °C using a model system of 20% methanol in water and observed acceleration of their reactivity at higher temperatures. On the other hand, Ge and Lee (1997) observed that the rate of Schiff base formation was slow at lower temperature (40 and 54 °C) with phenylalanine and glucose in an aqueous model system.

Although a number of kinetics studies have been reported on the Maillard reaction between saccharides and amino acids (Ashoor and Zent, 1984) or glutathione (Zheng et al., 1997), little information is available on the role of different amino acids or peptides in the protein interaction with vanillin. The objectives of this study were to determine the reaction kinetics of vanillin with different types of amino acids or peptides in buffer systems utilizing elevated reaction temperatures and to study the kinetics of the formation of possible reaction products.

MATERIALS AND METHODS

Materials. All chemicals used in this study were of ACS analytical reagent grade. Vanillin, L-cysteine, L-lysine, L-phenylalanine, aspartame, glutathione, pentalysine, and sodium phosphate (monobasic, dibasic, and tribasic) were obtained from Sigma Chemical Co. (St. Louis, MO), and methanol (HPLC grade) was obtained from Fisher Scientific (Pittsburgh, PA).

Preparation of Buffer and Standard Solution. All reaction mixtures, vanillin standard solution, and amino acid or peptide solutions were prepared using 0.05 M phosphate buffer. The buffer pH values were adjusted to the pK_{a2} values of the available α-amino groups of amino acids or peptides: 10.70 for cysteine; 9.06 for lysine and pentalysine; 9.31 for phenylalanine; 9.90 for aspartame; and 9.47 for glutathione (Voet and Voet, 1995). The vanillin standard solutions contained 3.228 g/L of phosphate buffer (0.021 M vanillin) at the pK_{a2} values of the amino acids or peptides.

Preparation of Reaction Mixtures. Each reaction mixture was prepared in 0.05 M phosphate buffer to an equivalent molar ratio (1:1) of vanillin and each of the six amino acids or peptides. This was done for each model system by dissolving an appropriate amount of the amino acid or peptide in 10 mL of the phosphate buffer in a 25-mL volumetric flask, adding 5

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mL of the vanillin standard solution, and making it up to the volume. All stock solutions for amino acids or peptides were completely dissolved before the aliquot transfers were made. The final volume contained 1.06×10^{-4} mol of vanillin and an amino acid (or peptide) per 25 mL of the buffer. The mixture was shaken for 1 min and transferred to 3.5-mL serum vials (1.5 mL each), which were sealed with Teflon-lined aluminum caps. Controls were prepared for each model system following the same procedure, but no amino acid or peptide was added.

Sample Treatment. The vials of reaction mixtures were placed in water baths at 55, 65, and 75 °C. On the basis of preliminary work, these temperatures were chosen to represent milk pasteurization temperatures as well as to accelerate the chemical reactions. The samples were removed from the water bath at 2-h intervals immediately following the induction period at each temperature. Upon removal, samples were cooled immediately in ice water to stop further reactions.

High-Performance Liquid Chromatography (HPLC). All reaction mixtures were analyzed for free vanillin by HPLC. Phenylalanine and its reaction product also were determined for the vanillin−phenylalanine model system. The reaction mixtures were diluted with additional phosphate buffer (1:15, v/v) before being injected into the HPLC.

Vanillin for all model systems and other chemical components for the vanillin−phenylalanine model system were determined with a Hewlett-Packard 1090A series II HPLC equipped with an HP 1040 photodiode array UV−visible detector and an HP 9000 series 300 ChemStation (Palo Alto, CA). The separation of chemical components was achieved using a Spherisorb WP 10-μm C18 reversed-phase column (250 mm × 4.6 mm) with a C18 guard column (10 mm × 4.6 mm), which were obtained from Alltech Associates, Inc. (Deerfield, IL). The mobile phase consisted of solvent A (methanol) and solvent B (0.05 M phosphate buffer at pH 3.5). The gradient program was 0−8 min, 70−40% B in A, and 8−15 min, 40−35% B in A. The flow rate was 1.0 mL/min at 25 °C, and the injection volume was 20 μL. Retention times and on-line UV spectra were used for peak identification. Quantitation was done by comparing peak areas of vanillin, phenylalanine, and the reaction product in control and reaction mixtures.

Determination of Kinetics Constant. Least-squares linear regression was used to determine the best fit of kinetic reaction orders for each model system. The rate constant (k) at each given temperature (55, 65, and 75 °C) was calculated from the best fit regression. The activation energy (E_a) for the disappearance of vanillin was computed from the slope of the Arrhenius plot by using the two-step regression method.

Statistical Analysis. The experiment was duplicated, and a split plot design was used with amino acids or peptides as whole plots and temperatures as subplots. Least-squares linear regression, analysis of variance (ANOVA), and a paired t test were done using Statistical Analysis System software programs (SAS, 1997). Significance of differences among means was defined at $p < 0.05$.

RESULTS AND DISCUSSION

The vanillin and amino acid or peptide in model systems showed good separation on a C18 column (Figure 1). The diode array UV spectra were typical for vanillin and phenylalanine in the vanillin−phenylalanine model system (Figure 2). However, the spectrum for the reaction product was not verified, because no standard compound was available for comparison. The reaction product appeared to be unstable when its chromatographic fraction was collected and injected into an HPLC−MS equipped with a thermospray interface. Ammonium acetate buffer (0.05 M) at pH 3.5 was used as a mobile phase instead of phosphate buffer because of its high volatility for thermospray MS. The mass spectrum of the compound was not seen with the MS detector. Some evidence indicated that the compound in the fraction had reversed by itself to its original

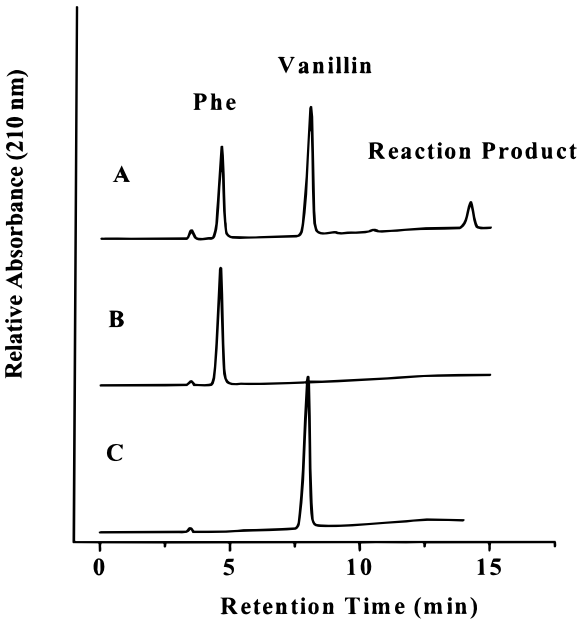

Figure 1. Typical HPLC chromatograms obtained from samples of (A) vanillin−phenylalanine reacted at 75 °C for 8 h, (B) phenylalanine (933 ng), and (C) vanillin (861 ng).

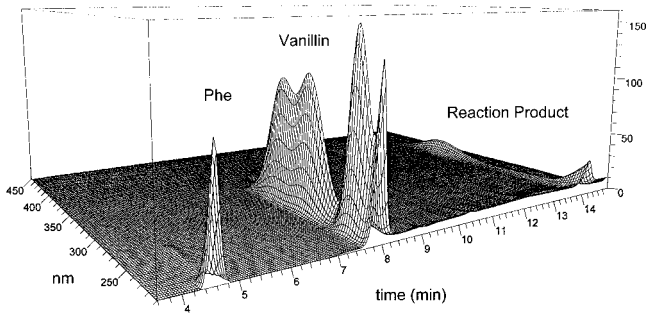

Figure 2. UV absorption spectra of vanillin, phenylalanine, and vanillin−phenylalanine reaction product obtained with a diode array detector during HPLC separation.

Table 1. Vanillin Concentration (Percent) Remaining in Vanillin−Amino Acid or − Peptide Model Systems after 16 h at 55, 65, and 75 °C

	reaction temp		
amino acid or peptide	55 °C	65 °C	75 °C
pentalysine	$_x89.30^a$	$_x44.90^a$	$_y15.20^a$
lysine	$_x89.20^a$	$_y74.40^c$	$_y22.60^b$
phenylalanine	$_x95.80^b$	$_y73.20^b$	$_z56.20^c$
glutathione	$_x98.80^b$	$_y71.90^b$	$_z69.00^e$
cysteine	$_x98.00^b$	$_y76.00^d$	$_z62.40^d$
aspartame	$_x99.56^b$	$_y92.93^e$	$_z87.23^f$

$^{a-f}$ Means within each column with different letters are significantly different ($p < 0.05$). $_{x-z}$ Means within each row with different letters are significantly different ($p < 0.05$).

compounds (vanillin and amino acids) during its collection. The literature suggests that the interaction of aldehydes and amine groups may be reversible or nonreversible (Feeney et al., 1975; Kim and Min, 1989). Therefore, further studies are needed to verify the reversibility.

Effect of Reaction Temperature. The reactivities of vanillin with one amino acid or peptide or with different amino acids and peptides were significantly different at 55, 65, and 75 °C (Table 1). For example, reduction of the vanillin was <11% after 16 h in the vanillin−lysine model system at 55 °C, whereas it was

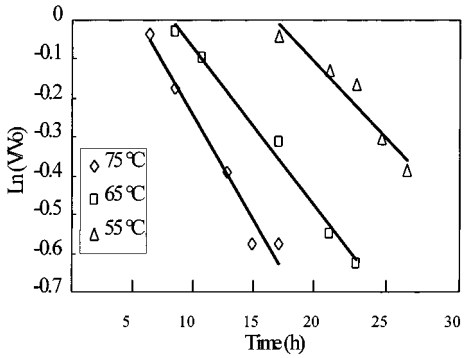

Figure 3. First-order plot obtained from reductions of vanillin in the vanillin−phenylalanine model system at 55, 65, and 75 °C: [V] = vanillin concentration at time *t*; [V_0] = vanillin concentration at time 0.

Table 2. Linear Regression (R^2) for Various Amino Acids or Peptides on First-Order Reaction Plots

	reaction temp		
amino acid or peptide	55 °C	65 °C	75 °C
pentalysine	0.9984	0.9851	0.9985
lysine	0.9968	0.9851	0.9824
phenylalanine	0.9304	0.9892	0.9688
glutathione	0.9693	0.9992	0.9917
cysteine	0.9480	0.9875	0.9918
aspartame	0.9424	0.9426	0.9642

77% at 75 °C. At 55 °C, reductions of vanillin in the vanillin−pentalysine and lysine model systems were not significantly different but were higher ($p < 0.05$) than those in the cysteine, glutathione, phenylalanine, or aspartame model systems. At 65 °C, the reductions of vanillin were significantly different for most model systems, but not glutathione and phenylalanine, whereas at 75 °C, the reductions were significantly different for all model systems.

The first-order plot (natural log V/V_0 versus time) indicated that the disappearance of vanillin, after an induction period, was linear for each model system (Figure 3). However, the induction period was long but dependent on the reaction temperature. The linear fits of the model systems had correlations of determination (R^2) from 0.93 to 0.99 (Table 2). Cha and Ho (1988) reported that the Schiff base formation between vanillin and aspartame in a methanol system ($a_w = 0$) followed second-order kinetics at 3.3, 25, and 37.8 °C and had a higher rate of reaction than that observed in this study. Hussein et al. (1984) reported that the reaction rate between vanillin and aspartame or benzaldehyde decreased significantly when more water was present in the methanol system. This suggests that reactants in different solvents may lead to different pathways affecting the rates of reaction as well as the equilibrium process involved with different water activity. Likewise, Cha and Ho (1988) also observed that the reaction rate was lower with the increasing of water content in vanillin and aspartame model systems. They hypothesized that the presence of water in the model system inhibited the aldehyde−amine condensation reaction and prevented Schiff base formation.

The *k* values derived from the first-order equations suggested that the reactions accelerated as temperature increased. The reaction rates for all model systems were higher ($p < 0.05$) at 75 °C than at 65 or 55 °C (Table 3). When the temperature increased from 55 to 65 °C, the rate increases ranged from about 25 to 97%. However, the rate showed increases from 65 to 260% at 75 °C. In addition, each system seemed to exhibit different rates at the various temperatures. For example, the cysteine system showed the greatest rate increase (97%) at 65 °C, whereas the aspartame system had the greatest increase (260%) at 75 °C.

Table 3. Rate Constant (k, h^{-1}) and Activation Energy (E_a) of Vanillin Reduction in the Presence of Amino Acids or Peptides at Various Temperatures

	reaction temp			E_a
amino acid or peptide	55 °C	65 °C	75 °C	(kcal/mol)
pentalysine	$_x0.0769^a$	$_y0.1163^a$	$_z0.1726^a$	9.18
lysine	$_x0.0496^b$	$_y0.0702^b$	$_z0.1238^b$	10.40
phenylalanine	$_x0.0347^c$	$_y0.0432^c$	$_z0.0572^c$	5.67
glutathione	$_x0.0238^d$	$_y0.0337^d$	$_z0.0407^d$	6.10
cysteine	$_x0.0187^d$	$_y0.0368^d$	$_y0.0412^d$	9.03
aspartame	$_x0.0036^e$	$_x0.0051^e$	$_y0.0130^e$	14.50

$^{a-e}$ Means within each column with different letters are significantly different ($p < 0.05$). $_{x-z}$ Means within each row with different letters are significantly different ($p < 0.05$).

Effect of Type of Amino Acid or Peptide. The rate constants for vanillin reduction indicated that reaction rates were affected significantly by the type of amino acids or peptides involved (Table 3). The reaction rate of vanillin was highest at all three temperatures with pentalysine, followed by lysine, phenylalanine, glutathione/cysteine, and aspartame. These results are similar to those for the Maillard reaction between reducing sugars and amino acids (Ashoor and Zent, 1984). In the Maillard reaction, L-lysine is known to be most reactive followed by L-phenylalanine and L-cysteine.

Although vanillin was most reactive with pentalysine, the reaction rate was only ~39−66% higher than that with lysine. This is interesting because pentalysine has four more available ϵ-amino groups than lysine. The bulky structure of pentalysine may give different molecular arrangements that hinder some of the ϵ-amino groups from interacting with vanillin. The *k* values for the glutathione and cysteine systems were not different ($p < 0.05$) but significantly lower than that for lysine. The lower rate may be related to the availability of amino groups. Because the amino group in the cysteine residue is used for the peptide bond formation in glutathione, the only available amino group is in the glutamate residue. However, our results showed that cysteine had a reactivity similar to that of glutathione. This suggests that vanillin might react with other side-chain groups such as a sulfhydryl group (−SH).

Activation Energy. The E_a was calculated for each model system using the Arrhenius plots (Figure 4), and values for vanillin reduction in different amino acid or peptide systems ranged from 5.6 to 14.5 kcal/mol (Table 3). Note that the phenylalanine model system required the lowest E_a (5.6 kcal/mol), whereas the aspartame system had the highest (14.5 kcal/mol). This indicates that the interaction of vanillin with aspartame was more sensitive to temperature changes and was more favorable at higher temperatures. Cha and Ho (1988) reported that the E_a value for vanillin−aspartame (molar ratio, 1:1) in a methanol model system was 11.67 kcal/mol. Our E_a value of the vanillin−aspartame model system was higher than that of the methanol system. This implies that the aqueous system requires higher activation energy than the methanol system.

Reduction of Amino Acid and Formation of Reaction Product. The reduction of amino acid and the formation of reaction product were determined using

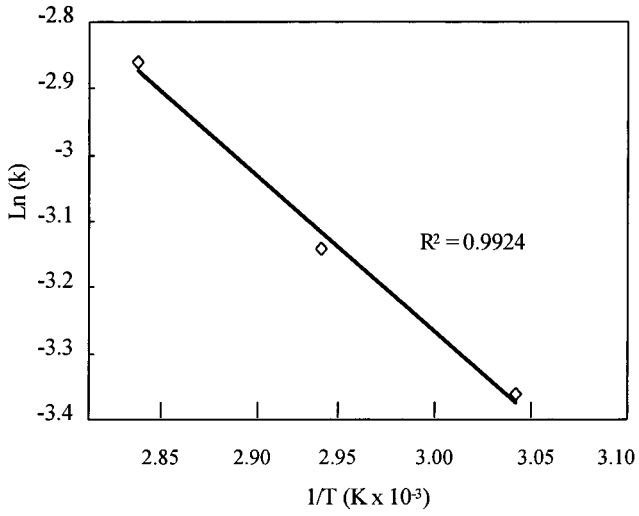

Figure 4. Arrhenius plot for vanillin reduction in the vanillin−phenylalanine model system.

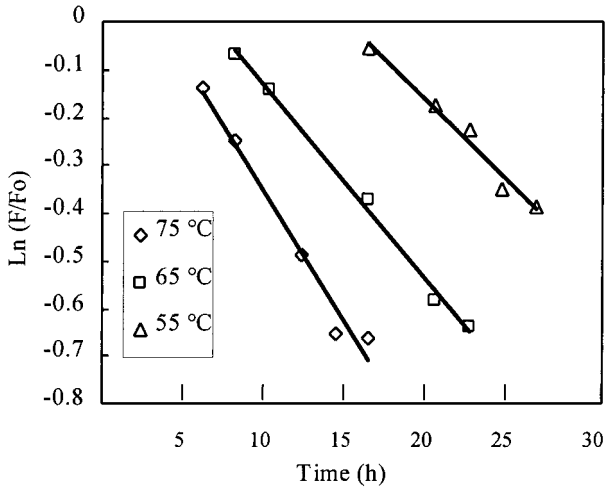

Figure 5. First-order plot obtained from reductions of phenylalanine in the vanillin−phenylalanine model system at 55, 65, and 75 °C: [F] = phenylalanine concentration at time *t*; [F_0] = phenylalanine concentration at time 0.

the phenylalanine model system because of its UV absorption characteristics. The pattern of phenylalanine reduction was similar to that of vanillin reduction. The disappearance of phenylalanine followed first-order kinetics (Figure 5), and the calculated E_a was 7.0 kcal/mol according to the Arrhenius plot. Note that the E_a for the reduction of vanillin in the phenylalanine system was 5.67 kcal/mol. The formation of reaction product between vanillin and phenylalanine followed zero-order kinetics (Figure 6), and the rate was time and temperature dependent. These results are similar to the formation of brown color in the Maillard reaction between reducing sugars and amino acids (Pilar-Buera et al., 1987; Peterson et al., 1994). This suggests that intermediates formed from the vanillin−phenylalanine reaction are similar to those of the Maillard reaction.

Significance of Reaction Rate. The Schiff base formation is reported in the literature as one of the major reactions that cause the loss of vanillin in food systems (Cha and Ho, 1988; Graf and de Roos, 1996). However, this study suggests that a significant reduction of vanillin with the reaction takes a considerable amount of time. Extrapolation of the Arrhenius plots indicates that a 1% reduction of vanillin at 20 °C may require 0.69−43.86 h depending on the amino acids or peptides involved (Table 4). In addition, the induction times were surprisingly long. For example, measurable reductions of vanillin occurred after 6, 8, and 16 h at 75, 65, and 55 °C, respectively, in a vanillin−phenylalanine model system (Figure 3). Similar results were observed with the reduction of phenylalanine (Figure 5). These observations suggest that the loss of vanillin via Schiff base formation may not be the major reaction causing the instant reduction of vanilla flavor intensity in nonfat food systems that contain amino acids, peptides, and proteins. However, other components in food systems may impact the vanillin interaction because our food systems are complex. The thermodynamic component is indeed one of the important factors on flavor loss. Flavor compounds could simultaneously interact with other components in food system. This may also contribute to the flavor loss. However, we believe that kinetic components, especially protein, still are important to the flavor loss. Proteins are abundant in food, and the increased use of proteins in nonfat food systems, particularly as protein-based fat replacers, will bring about more challenges on this flavor issue.

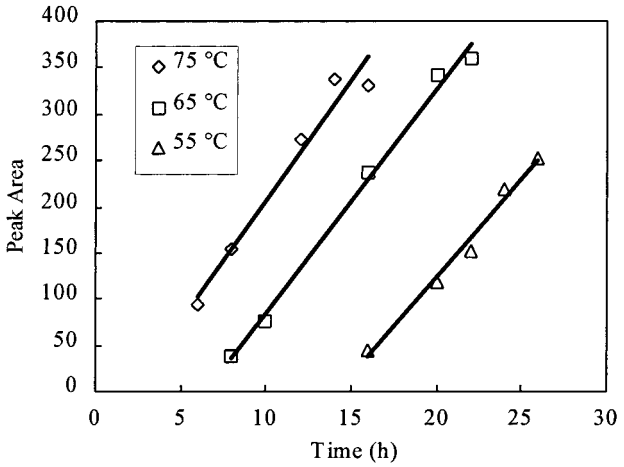

Figure 6. Kinetics of the formation of vanillin−phenylalanine reaction product at 55, 65, and 75 °C.

Table 4. Calculated Rate Constants (*k*) and $t_{1\%}$ at 20 °C at pK_{a2} of Available Amino Group of Each Model System by Extrapolation

amino acid or peptide	*k* at 20 °C ($\times 10^{-3}$, h^{-1})	$t_{1\%}$ (h)
pentalysine	14.45	0.69
lysine	7.16	1.40
phenylalanine	12.1	0.82
glutathione	7.96	1.26
cysteine	8.09	1.24
aspartame	0.228	43.86

Conclusion. The interactions of vanillin and amino acids or peptides in a buffer system followed first-order kinetics. The reaction rates were significantly higher at elevated temperatures and different with each type of amino acid and peptide. However, the reaction required a considerable amount of time to produce a significant reduction of vanillin in the system. Further studies are underway to better understand the reactions that are primarily responsible for the instant reduction of vanillin flavor intensity in nonfat food systems. Therefore, preventing the reduction of vanillin flavor in food systems, particularly ice cream mix, may need more investigation beyond the chemical reaction involving the Schiff base formation.

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LITERATURE CITED

Ashoor, S. H.; Zent, J. B. Maillard browning of common amino acids and sugars. *J. Food Sci.* **1984**, *49*, 1206−1207.

Cha, A. S.; Ho, C.-T. Studies of the interaction between aspartame and flavor vanillin by high performance liquid chromatography. *J. Food Sci.* **1988**, *53*, 562−564.

Feeney, R. E.; Blankenhorn, G.; Dixon, H. B. F. Carbonyl-amine reactions in protein chemistry. *Adv. Protein Chem.* **1975**, *29*, 136−203.

Ge, S.-J.; Lee, T.-C. Kinetics significance of the Schiff base reversion in the early-stage Maillard reaction of a phenyl-alanine−glucose aqueous model system. *J. Agric. Food Chem.* **1997**, *45*, 1619−1623.

Graf, E.; de Roos, K. B. Performance of vanilla flavor in low-fat ice cream. In *Flavor−Food Interaction*; McGorrin, R. J., Leland, J. V., Eds.; American Chemical Society: Washing-ton, DC, 1996; pp 24−35.

Hansen, A. P.; Booker, D. C. Flavor interaction with casein and whey protein. In *Flavor−Food Interaction*; McGorrin, R. J., Leland, J. V., Eds.; American Chemical Society: Washington, DC, 1996; pp 75−89.

Hansen, A. P.; Heinis, J. J. Decrease of vanillin flavor perception in the presence of casein and whey proteins. *J. Dairy Sci.* **1991**, *74*, 2936−2939.

Ho, C. T.; Huang, T. C.; Cha, A. S.; Sotirhos, N. Studies on the stability of aspartame in the presence of glucose and vanillin. In *Frontiers of Flavor, Proceedings of the 5th International Flavor Conference*; Charalambous, G., Ed.; Elsevier Science Publishers: Amsterdam, The Netherlands, 1988; pp 233−240.

Hussein, M. M.; D'Amelia, R. P.; Manz, A. L.; Jacin, H.; Chen, W.-T. C. Determination of reactivity of aspartame with flavor aldehydes by gas chromatography, HPLC and GPC. *J. Food Sci.* **1984**, *49*, 520−524.

Kim, H.; Min, D. B. Interaction of flavor components with protein. In *Flavor Chemistry of Lipid Foods*; Min, D. B., Smouse, T. H., Eds.; American Oil Chemists' Society: Champaign, IL, 1989; pp 404−420.

McNeill, V. L.; Schmidt, K. A. Vanillin interaction with milk protein isolates in sweetened drinks. *J. Food Sci.* **1993**, *58*, 1142−1147.

Ng, P. K. W.; Hoehn, E.; Bushuk, W. Binding of vanillin by fababean proteins. *J. Food Sci.* **1989**, *54*, 105−107.

Peterson, B. I.; Tong, C.-H.; Ho, C.-T.; Welt, B. A. Effect of moisture content on Maillard browning kinetics of a model system during microwave heating. *J. Agric. Food Chem.* **1994**, *42*, 1884−1887.

Pilar-Buera, M. D.; Chirife, J.; Resnik, S. L.; Wetzler, G. Nonenzymatic browning in liquid model systems of high water activity: kinetics of color changed due to Maillard's reaction between different single sugars and glycine and comparison with caramelization browning. *J. Food Sci.* **1987**, *52*, 1063−1067.

SAS Institute, Inc. *SAS/STAT® Software: Change and En-hancements*, release 6.12; SAS Institute Inc.: Cary, NC, 1997.

Voet, D.; Voet, J. G. Amino Acids. In *Biochemistry*, 2nd ed.; Wiley: New York, 1995; pp 58−59.

Zheng, Y.; Brown, S.; Ledig, W. O.; Mussinan, C.; Ho, C.-T. Formation of sulfur-containing flavor compounds from reac-tions of furaneol and cysteine, glutathione, hydrogen sulfide, and alanine/hydrogen sulfide. *J. Agric. Food Chem.* **1997**, *45*, 894−897.

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