Effects of pH, Temperature, and Reactant Molar Ratio on L-Leucine and D-Glucose Maillard Browning Reaction in an Aqueous System

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The effects of pH, temperature, and reactant molar ratios on L-leucine and D-glucose Maillard browning reaction in an aqueous system were studied. At pH 9 and 10, glucose consumption rate increased with time for all temperatures and molar ratios. At 100 °C and pH 9 and 10, samples with excess leucine had higher mean values for color than the mean color values for samples using glucose/leucine ratios of 1:1 or 2:1. At 122.5 °C and pH 9 and 10, samples with glucose/leucine ratios of 1:2 had a significantly higher rate of glucose loss than samples containing glucose/leucine ratios of 2:1 and 1:1 at the corresponding pH. Temperature and the initial pH of the sample were positively correlated with glucose loss ($r = 1.0$ and 0.85, respectively). Typically, sample browning rate was greater at 122.5 °C than at 100 °C. Browning rate was positively correlated with pH ($r = 0.94$).

Keywords: *Maillard browning; kinetics; L-leucine; D-glucose*

INTRODUCTION

Maillard browning is partly responsible for the color and aroma changes of the roasted cocoa beans during cocoa processing. During this process carbonyl compounds formed from the Maillard reaction (van Praag et al., 1968; van der Wal et al., 1971) react with amino acids (Strecker degradation) to form volatile aldehydes (Nyhammar et al., 1983). Darsley and Quesnel (1972) used radioactive tracer techniques to show that these compounds are derived from amino acids such as leucine, threonine, and phenylalanine.

Temperature, pH, water activity, and the concentration and/or molar ratios of sugar/amino acid can affect the rate and extent of Maillard browning. Roasting temperatures for cocoa beans are usually in the range of 112−140 °C (Arnoldi et al., 1987). The optimum pH for Maillard browning is between pH 6 and 10 (Ashoor and Zent, 1984; Wolfrom et al., 1974). Wolfrom et al. (1953) showed the browning rate between D-xylose and alanine and glycine was optimal between pH 6.5 and 8.5 at 100 °C. Ashoor and Zent (1984) reported no browning in any amino acid−glucose mixtures below pH 6.0 (including leucine) and that the maximum browning occurred at pH ∼10. Maillard browning occurs mostly when water activities are in the range of 0.3−0.7 (Karel, 1960; Heiss, 1968). Some studies have shown that as water activity increases reaction rate decreases (Eichner and Karel, 1972). Other studies have shown an increase in browning rate with increasing (Jones, 1954, 1956) as well as decreasing (Rosen et al., 1953; Loncin et al., 1965) water content. Typically, an excess of reducing sugar over amino acid has been reported to promote the Maillard browning (Lea and Hannan, 1951; Wolfrom et al., 1974; Warmbier et al., 1976; O'Brian and Morissey, 1989).

The reaction of L-leucine with reducing sugars, such as D-glucose, is important in the formation of some of the volatile compounds responsible for the flavor of cocoa. Leucine reacts with 3-methylbutanal from the Maillard reaction to form 2-isopropyl-5-methyl-2-hexenal, which has a sharp cocoa-like aroma (Hartman, 1983). The reaction of leucine and reducing sugars has been studied in water with methanol (Eichener and Karel, 1972; Lee et al., 1984), ethanol (Ledl, 1982), diethylene glycol (Koehler and Odell, 1970), and octanol (Westphal and Cieslik, 1983). The reaction rate of L-leucine and D-glucose in a purely aqueous system, however, has not yet been examined. We therefore investigated the effects of temperature, pH, and molar ratios of glucose/leucine on the kinetics of the Maillard browning reaction between L-leucine and D-glucose in an aqueous system.

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MATERIALS AND METHODS

L-Leucine, D-glucose, sodium hydroxide, phenol, and sulfuric acid were obtained from Fisher Scientific Co., Orlando, FL. Trinitrobenzenesulfonic acid was obtained from Sigma Chemical Co., St. Louis, MO.

Preparation of Reaction Mixture. Stock L-leucine and D-glucose solutions (0.01 or 0.02 M) were prepared by dissolving the amino acid or the sugar in distilled deionized water (final solution volume was 1000 mL in each case). Four 250 mL aliquots of the sugar−amino acid solution were adjusted to pH 7.0, 8.0, 9.0, or 10.0 with 0.1, 1.0, or 10 N sodium hydroxide. From each pH solution a 5 mL aliquot was taken and pipetted into a test tube for heating. The test tubes were stoppered with wooden corks to help control water evaporation and then heated at 100 °C (dry heat) for the following time intervals: 15, 30, 45, 60, 75, 90, 105, 120, 180, 240, 360, 480, and 720 min. At each particular interval the test tube was placed aside to cool and then stored at 4 °C until analysis. All samples were done in triplicate. The variations of this reaction were as follows: pH 7.0, 8.0, 9.0, and 10.0 heated at 100 °C with a 1:1 glucose/leucine ratio, 2:1 glucose/leucine ratio, and 2:1 leucine/glucose ratio; and pH 7.0, 8.0, 9.0, and 10.0 heated to 122.5 °C under autoclave with a 1:1 glucose/leucine ratio, 2:1 glucose/leucine ratio, and 2:1 leucine/glucose ratio.

Analytical Procedures. All samples were observed for any color and aroma changes over the time intervals indicated above. Color changes (browning) were determined objectively by measuring the absorbance of the mixture at 420 nm using a Perkin-Elmer Lambda 3 UV−visible spectrophotometer.

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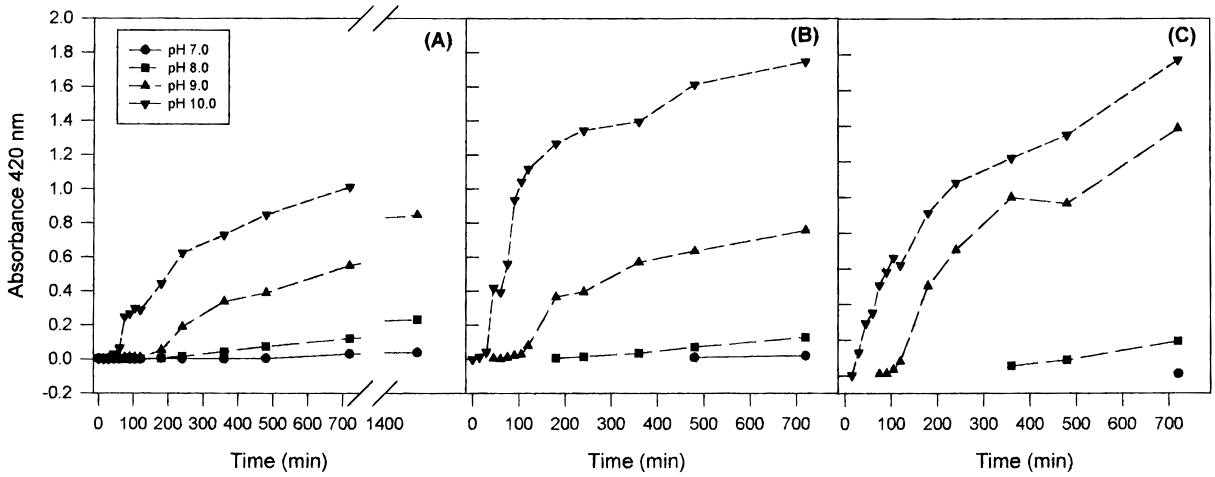

Figure 1. Sample browning vs time, 100 °C: (A) 1:1 glucose/leucine; (B) 2:1 glucose/leucine; (C) 2:1 leucine/glucose.

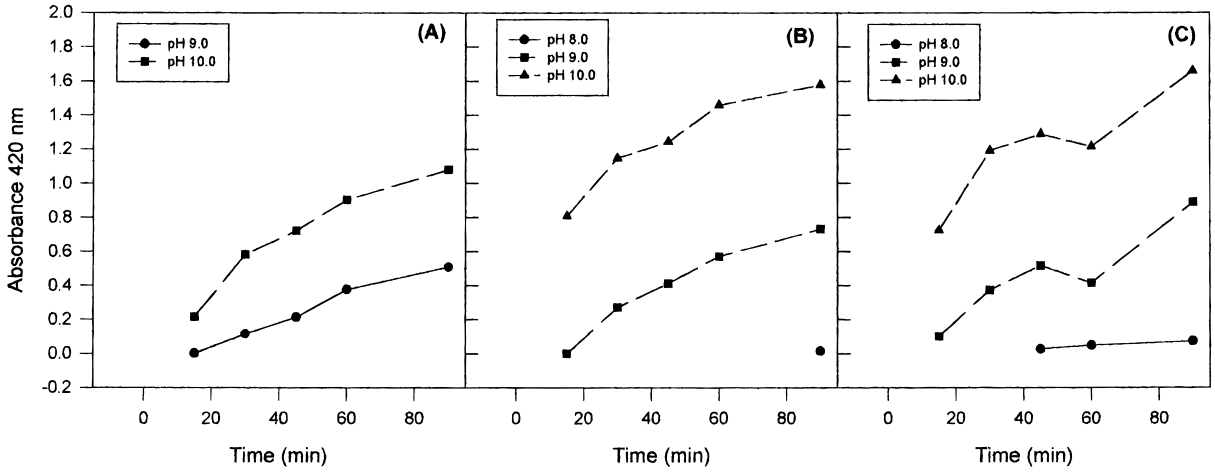

Figure 2. Sample browning vs time, 122.5 °C: (A) 1:1 glucose/leucine; (B) 2:1 glucose/leucine; (C) 2:1 leucine/glucose.

After heating, the tubes were analyzed at each time interval for glucose and leucine consumption. Glucose determination was done according to the method described by Dubois et al. (1956). A 2 mL sample containing between 10 and 100 μg of glucose was used for the determination. To the 2 mL sample was added 50 μL of 80% (w/v) aqueous phenol. The tube was vortexed, and 5 mL of 95% (v/v) aqueous sulfuric acid was then added. The tubes were allowed to cool to room temperature (25 °C), and the absorbance of the mixture was read at 490 nm against a water−reagent blank in a Perkin-Elmer Lambda 3 UV−visible spectrophotometer. A 0−100 μg glucose standard curve was constructed and used to calculate the sample sugar content.

Leucine concentration was measured by 2,4,6-trinitrobenzenesulfonic acid (Adler-Nissen, 1979). A 250 μL aliquot of 0.15−1.5 mM leucine was added to 2 mL of 0.2125 M phosphate buffer (0.2125 M NaH_2PO_4 and 0.2125 M Na_2HPO_4, pH 8.2). Two milliliters of 0.1% (w/v) aqueous 2,4,6-trinitrobenzenesulfonic acid solution was then added and vortexed immediately. The tubes were covered with aluminum foil and incubated at 50 °C (controlled-temperature water bath) for 60 min. At the end of the incubation period, 4.0 mL of 0.1 N HCl was added to terminate the reaction. The absorbance was read against a water−reagent blank at 340 nm in a Perkin-Elmer Lambda 3 UV−visible spectrophotometer. A 0−1.5 mM leucine standard curve was prepared simultaneously. When appropriate, absorbance values for a particular set were avaraged over the entire time period and at all pH levels to enable comparisons between two sets. For example, to compare browning at 100 °C and 2:1 glucose/leucine ratio, absorbance values from all time intervals and pH levels for this ratio were averaged and this average value was then compared with the average value determined for another set (such as glucose/leucine ratio of 1:1 or 1:2, etc., at 100 °C).

The pH of the solutions was monitored over all time intervals to determine any changes in the pH of the solution. The pH was measured using an Orion pH meter.

Data Analysis. Data were plotted and analyzed using SigmaPlot scientific graphing software, San Rafael, CA. A 2 × 3 × 4 (temperature, molar ratio, pH, respectively) or a 2 × 3 × 4 × 3 (temperature, molar ratio, pH, phase, respectively) factorial treatment structure analysis of variance (ANOVA) was used to describe the significance of the effects of pH, temperature, molar ratio, and phase on glucose reaction rate constants. Dependent variables were reaction rates, and the independent variables were pH, molar ratio, and temperature. All factors were repeated measures. Each value was an average of three determinations. The statistical significance was determined by Fisher's least significant difference test ($\alpha = 0.05$), using SAS version 6.10 (SAS, 1995−1996). Linear regression analysis by Sigma Plot scientific graphing software was used on all standard curves, and correlations were established between reaction rate constants and browning rate and the independent variables.

RESULTS AND DISCUSSION

Browning Rate. Results of the effects of pH, reactant molar ratios, temperature, and time on color (browning) reaction are summarized in Figures 1 and 2. At 100 °C sample browning increased with increasing pH for all molar ratios (Figure 1). At pH 9 and 10, samples with excess glucose and excess leucine both had higher mean values for color (browning) than the mean values for color (browning) for equal concentrations of leucine and glucose. Samples with excess leucine at pH

Table 1. Mean Comparisons of First-Order Glucose Reaction Rate Constants (k_R) for Temperature, Molar Ratio, and pH Individually and for pH and Temperature Collectively (min^{-1} M^{-1})[a]

temp, °C	rate	ratio[b]	rate	pH	rate	temp, °C; pH	rate
100	868.8[a]	1:1	2100.3[a]	7.0	44.1[a]	100; 7.0	61[a]
122.5	3951.6[b]	2:1	1876.9[b]	8.0	324.2[b]	100; 8.0	130.5[a]
		1:2	3253.5[c]	9.0	1455.9[c]	100; 9.0	480.4[b]
				10.0	7816.6[d]	100; 10.0	2803.48[d]
						122.5; 7.0	27.3[a]
						122.5; 8.0	518[b]
						122.5; 9.0	2431.3[c]
						122.5; 10.0	12829.8[e]
LSD	174.46		213.66		246.72		348.9

[a] Mean rates with the same superscript in the same column are not significantly different. [b]Glucose/leucine.

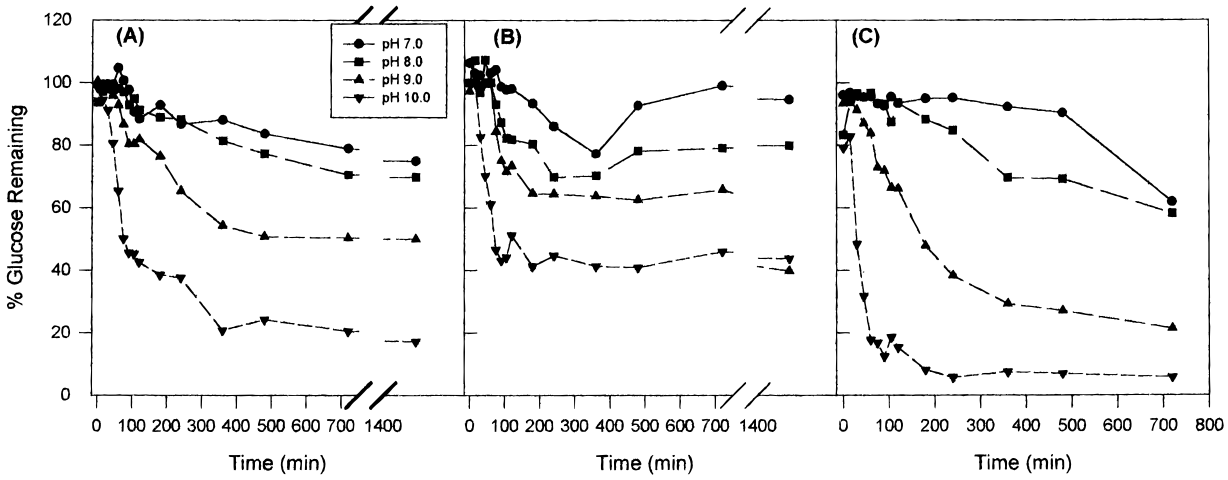

Figure 3. Percent glucose remaining vs time, 100 °C: (A) 1:1 glucose/leucine; (B) 2:1 glucose/leucine; (C) 2:1 leucine/glucose.

9 browned more rapidly than samples with excess glucose at pH 9 by approximately 100%. The opposite effect was seen in samples with solution pH 10, where browning in samples with excess glucose was 16% higher than in samples with excess leucine. These data indicate that the excess of one reactant for increased browning rate may partly depend on the pH of the reaction mixture.

At 122.5 °C browning did not occur for pH 7 samples at any of the molar ratios (Figure 2). Samples with solution pH 8 and 10 browned more rapidly with excess glucose or leucine than samples with equal concentrations. Samples with pH 9 and 10 containing excess glucose browned at a faster rate than those containing excess leucine under the same reaction conditions. In samples with solution pH 9 and 10 with excess leucine, browning increased steadily up to 45 min, dropped slightly until 60 min, and then rapidly increased up to 90 min. Samples with pH 9 and 10 had a ~4-fold increase in glucose consumption rate at 122.5 °C compared to 100 °C (Table 1, columns 4 and 8). For the time frames compared, the effects of temperature on pH 7 samples were inconclusive. Overall, browning rate was positively correlated with pH ($r = 0.935$) and not correlated with molar ratios of reactants ($r = 0.024$).

We found that pH and temperature both had an effect on which molar ratio provided a faster browning rate. This may partly explain the conflicting results in the literature as to which reactant in excess produces a faster rate (O'Brian and Morissey, 1989; Wolfrom et al., 1974; Warmbier et al., 1976; Baisier and Labuza, 1992).

Glucose Analysis. Glucose loss appeared to occur in three stages. In the first stage, a linear decrease in glucose concentration occurred. The second stage began when glucose consumption plateaued or an increase in reducing power occurred, as shown by an increase in percent glucose left (possibly due to the increased concentration of intermediate reducing compounds in the solution). The third phase began when the glucose concentration began to decrease again until the reaction was terminated.

First-order (phase 1) reaction rate constants (k_R) for glucose loss are listed in Table 1. To ensure that the reaction rates were first order, the values used in calculating the rate constants were cut off at the point of 50% loss. The rate of glucose consumption increased with increasing pH for all temperatures and molar ratio (leucine/glucose) levels. At 100 °C the mean k_R values for glucose loss over all four pH values and for all glucose/leucine ratios were statistically significantly different (Table 1, column 4). Samples with 1:2 glucose/leucine ratio had a mean k_R significantly greater than the 1:1 and 2:1 glucose/leucine samples when all temperature and pH values for each molar ratio were averaged. Total glucose loss was the lowest in samples with excess glucose (Figure 3).

Glucose loss at pH 7 was negligible for all molar ratios. Glucose loss at pH 8 was steady early in the reaction and then leveled off. In samples with 1:1 glucose/leucine and excess glucose at pH 9, glucose loss was rapid and then leveled off. In solutions with excess leucine at pH 9, glucose loss was rapid in the early stages of the reaction, and glucose concentration continued to steadily decrease. In samples with excess glucose at pH 10, rapid glucose consumption in the initial stage of the reaction was followed by a no-loss period. At pH 10, samples with glucose/leucine ratios 1:1 and 1:2 had almost total loss of glucose, with the excess leucine samples having approximately 95% loss of glucose after 12 h.

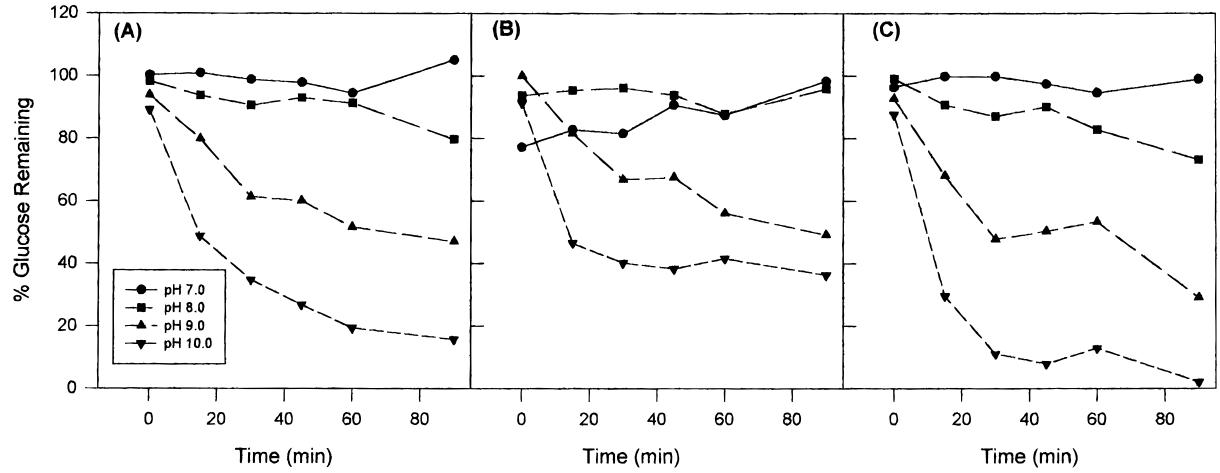

Figure 4. Percent glucose remaining vs time, 122.5 °C: (A) 1:1 glucose/leucine; (B) 2:1 glucose/leucine; (C) 2:1 leucine/glucose.

At 122.5 °C glucose k_R followed the trend pH 7 < pH 8 < pH 9 < pH 10 (Table 1, column 8). The consumption of glucose at pH 7 was negligible for all molar ratios (Figure 4). At pH 8 glucose concentration decreased slightly up to 30 min, increased by approximately 50% of the initial concentration, and then decreased steadily up to 90 min for samples with 1:1 glucose/leucine and excess leucine. Glucose loss in samples with excess glucose at pH 8 was negligible when compared with glucose loss in samples with glucose/leucine ratios 1:1 and 1:2. At 122.5 °C and pH 9, glucose loss profiles had similar shapes for all glucose/leucine ratios. The fluctuations in glucose concentration were more pronounced in samples with excess leucine. Samples at pH 10 showed minor fluctuations in glucose concentration in solutions with excess glucose and excess leucine. In general, the overall trend for glucose loss at 122.5 °C was 1:2 glucose/leucine > 1:1 glucose/leucine > 2:1 glucose/leucine.

A 4 × 2 × 3 factorial treatment structure analysis of variance of the treatment groups pH, temperature, and molar ratios showed that the overall difference in treatment means was significant ($Pr > F_{\alpha.05} = 0.0001$). Temperature, molar ratio, pH, and the temperature × pH and molar ratio × pH interactions all significantly affected the glucose reaction rates ($Pr > F_{\alpha.05} = 0.0001$, 0.0001, 0.0001, 0.0001, and 0.0001, respectively). Temperature and solution pH were positively correlated with glucose k_R ($r = 1.000$ and 0.847, respectively), and the glucose/leucine molar ratio was negatively correlated with k_R ($r = -0.716$). Glucose k_R was positively correlated with browning rate ($r = 0.857$).

Glucose reaction rate constants were calculated individually for each temperature, molar ratio, pH, and reaction phase. The overall mean comparisons for temperature, molar ratio, pH, and reaction phase individually are summarized in Table 2. Glucose reaction rates increased with increasing temperature and pH. The glucose reaction rate was significantly higher at 122.5 °C than at 100 °C. Samples with 1:2 glucose/leucine had a significantly higher reaction rate than samples with either 1:1 or 2:1 glucose/leucine. Reaction rates were statistically significantly different for all pH values. Overall, phase 1 showed significantly higher reaction rates than either phase 2 or 3.

The combined effects of pH, temperature, and molar ratio on the glucose mean reaction rate constants are summarized in Table 3. Overall, reaction rates increased as the temperature, pH, and leucine/glucose ratio increased. The combination of factors that led to the highest mean reaction rate was 122.5 °C, pH 10.0, and 1:2 glucose/leucine. This mean rate was 1.4 times the next highest mean rate. It can be seen from this table that the molar ratio of the reactants can help to increase the mean reaction rate without increasing pH. Data from Table 3 support the findings of Baisier and Labuza (1992) that increasing the amount of amine relative to reducing sugar increases the reaction rate under certain conditions. This suggests that the browning reaction rates can be manipulated at a given pH

Table 2. Mean Comparisons of Glucose Reaction Rate Constants for Temperature, Molar Ratio, pH, and Phase Individually (min^{-1} M^{-1})[a]

temp, °C	rate	ratio[b]	rate	pH	rate	phase	rate
100	628.0[a]	1:1	1518.5[a]	7.0	73.1[a]	1	3141.3[a]
122.5	3176.5[b]	2:1	1278.1[a]	8.0	600.3[a]	2	1002.9[b]
		1:2	2910.0[b]	9.0	2233.7[b]	3	1562.4[b]
				10.0	4701.7[c]		
LSD	782.23		958.03		1106.2		958.03

[a] Mean rates with the same superscript in the same column are not significantly different. [b] Glucose/leucine.

Table 3. Glucose Reaction Rate Constant Means Comparisons for All Combinations of Temperature, pH, and Molar Ratio (min^{-1} M^{-1})[a]

temp, °C	pH	ratio[b]	mean rate	± SD
122.5	10.0	1:2	15875[a]	± 732
122.5	10.0	2:1	11752[b]	± 795
122.5	10.0	1:1	10861[c]	± 638
100	10.0	1:2	5000[d]	± 602
122.5	9.0	1:2	2955[e]	± 777
100	10.0	1:1	2359[ef]	± 169
122.5	9.0	1:1	2325[f]	± 393
122.5	9.0	2:1	2013[f]	± 546
100	10.0	2:1	1052[g]	± 386
100	9.0	1:2	993[g]	± 23
122.5	8.0	1:2	923[g]	± 167
122.5	8.0	1:1	631[gh]	± 151
100	9.0	1:1	316[hi]	± 73
100	8.0	1:2	207[hi]	± 15
100	8.0	1:1	139[hi]	± 95
100	9.0	2:1	132[hi]	± 30
100	7.0	1:1	89[hi]	± 33
122.5	7.0	1:1	82[hi]	± 75
100	7.0	1:2	74[hi]	± 54
100	8.0	2:1	45[hi]	± 10
100	7.0	2:1	20[i]	± 5
LSD			604.3	

[a] Mean rates with the same supercript are not significantly different. [b] Glucose/leucine.

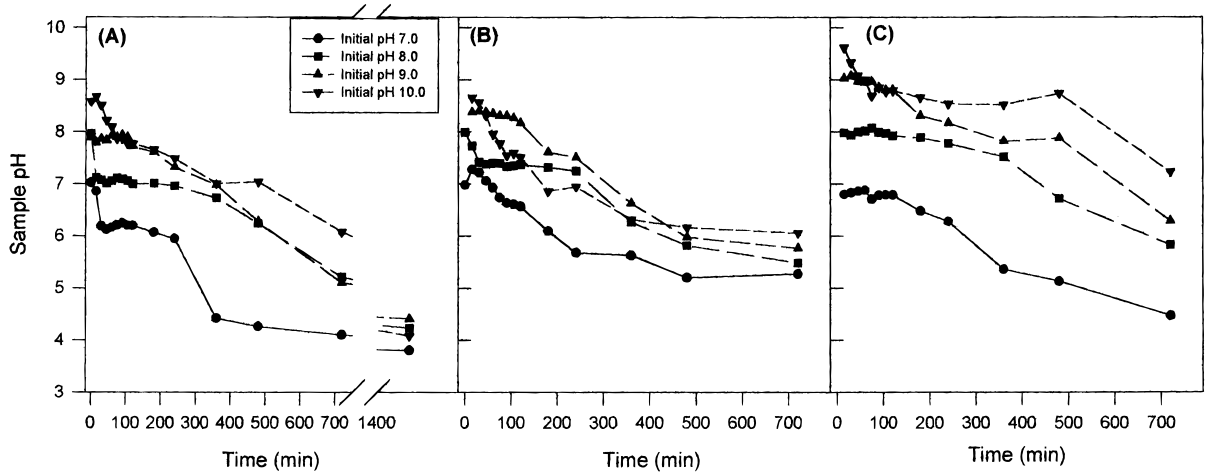

Figure 5. Change in sample pH vs time, 100 °C: (A) 1:1 glucose/leucine; (B) 2:1 glucose/leucine; (C) 2:1 leucine/glucose.

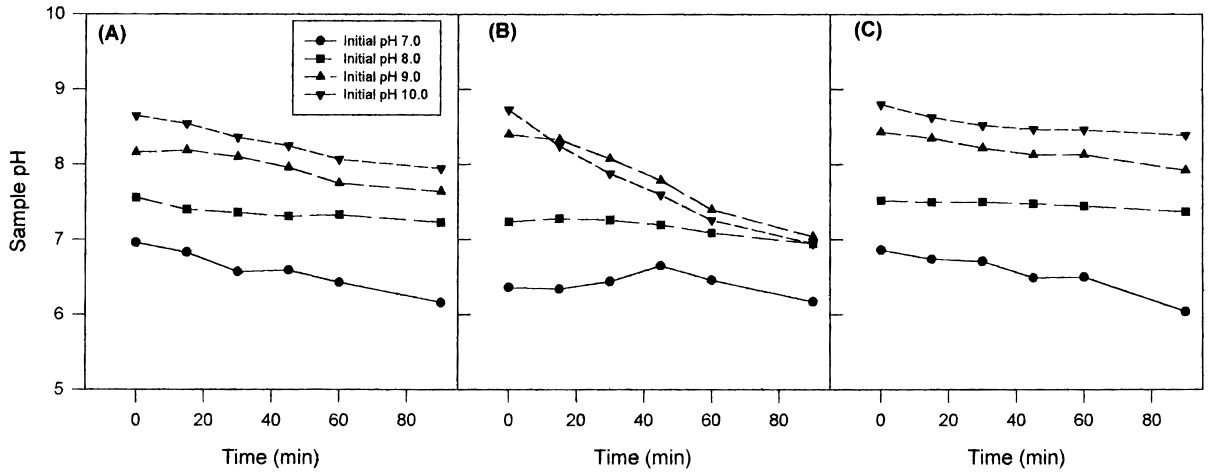

Figure 6. Change in sample pH vs time, 122.5 °C: (A) 1:1 glucose/leucine; (B) 2:1 glucose/leucine; (C) 2:1 leucine/glucose.

and temperature by controlling the molar ratio of the reactants.

The reaction occurring in three different stages may be due to the production of intermediate reducing compounds (Hodge, 1953, 1967). During the first stage of the reaction glucose and leucine may mostly react exclusively with each other (glucose is the only source of reducing power). The length of phase 1 varied with reaction conditions (Figures 3 and 4). Late in phase 1, Maillard reaction products and Amadori rearrangement products may be increasing in concentration, but the reaction between glucose and leucine is still occurring at a fast enough rate to keep the overall concentration of reducing compounds decreasing (isomerizations may be occurring between the Maillard reaction products at this point, and there may not be much Strecker degradation). Toward the end of phase 1 the concentration of isomerized Amadori rearrangement products begins to increase, thereby increasing the reducing power of the reaction mixture. This can be seen by either the plateau or increase in glucose concentration, as seen in Figures 3 and 4. The method of Dubois et al. (1956) used in this study to detect the presence of glucose will detect all reducing compounds, including Amadori rearrangement and Maillard reaction products. The amount of reducing compounds may have exceeded the available amino acid present, and hence an increase in reducing power was seen (phase 2). Eventually, the amount of available amine increased, either through recycling (where the amine groups are cleaved during enolization in the intermediate stages of Maillard browning; Hodge, 1967) or through production of NH_3 during Strecker degredation, and a depletion of reducing power occurred again (phase 3).

Leucine Analysis. The trinitrobenzenesulfonic acid test (Adler-Nissen, 1979) for amines was used to determine if the test could monitor the amount of free amino acids in the reaction mixture. The factors affecting the reaction rate constants for the disappearance of leucine were inconclusive. There was no statistical significance between any of the treatment groups for leucine disappearance. Leucine reaction rate constants were not correlated with browning rate.

Reaction Mixture pH. The pH of the reaction mixture was monitored over the time intervals used. At 100 °C the solution pH decreased for all initial pH levels over the time interval of the reaction (Figure 5). The magnitudes of the change in pH of the reaction mixture were similar for all initial pH levels and molar ratios. At 122.5 °C there was no substantial decrease from the initial solution pH for any of the molar ratios except for the samples with excess glucose, where only the samples with initial pH of 9 or 10 decreased substantially (Figure 6). The decrease in reaction mixture pH tended to follow the same general trend as glucose loss. However, there was no correlation between the two occurrences ($r = -0.3875$). The decrease in reaction mixture pH followed a trend similar to browning rate. There was no correlation between the two occurrences ($r = -0.3628$).

Conclusions. As the initial reaction mixture pH increased, browning rate and glucose loss increased

significantly. Excess leucine was more effective in increasing the browning rate than the excess glucose under same conditions. As expected, higher temperature resulted in higher browning rates. There was a measurable and continuous decrease in the reaction mixture pH.

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