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Effects of Water Content on Volatile Generation and Peptide Degradation in the Maillard Reaction of Glycine, Diglycine, and Triglycine

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Peptides abundant in food and protein hydrolysates are known to be important to process flavors. The present study reports the volatile profile of the Maillard reactions of glycine, diglycine, and triglycine. The reaction with glucose was conducted at 0−100% water content in glycerol medium at 160 °C for 1 h. Volatile compounds were quantified by stir bar sorptive extraction−gas chromatography−mass spectrometry, and nonvolatile compounds were quantified by high-performance liquid chromatography−tandem mass spectrometry. The major volatiles produced from each of the reaction systems were trimethylpyrazine and 2,5-dimethylpyrazine. Volatile generation increased as water decreased, and the overall reactivity of the glycine and glycine peptides in volatile formation was glycine ≈ triglycine > diglycine. Triglycine was very unstable and mainly degraded into cyclic Gly-Gly and glycine, whereas diglycine had a higher stability than triglycine toward hydrolytic cleavage of the peptide bond. The amounts of glycine, diglycine, cyclic (Gly-Gly), and triglycine in the peptide−glucose reaction mixtures at different water content were reported.

KEYWORDS: Peptide; volatiles; SBSE; glycine; water content; diketopiperazine; LC−tandem MS

INTRODUCTION

Oligopeptides have been extensively isolated and identified in both natural and artificial protein hydrolysates of foods such as seafood, coffee beans, soy, and wheat gluten (*1*−*4*). They have been recognized as important flavor potentiators and precursors of the Maillard reaction, which leads to the pleasant aroma and color of processed foods (*2*, *5*).

Water is one of the most important parameters that affects the aroma characteristics in foods; the overall aroma profile from the free amino acids reaction system is known to be influenced by the presence or absence of water (*6*, *7*, *13*). However, only a few studies investigated the volatile profile from the Maillard reaction of oligopeptides (*8*−*12*). The effect of water content on aroma formation from peptides and on peptide degradation is still unknown.

The objective of this study was to investigate the effect of peptide chain length on volatile generation at different water contents at 160 °C for 1 h. Glycine, diglycine, and triglycine with glucose were used as our model systems due to their simple structures. The remaining amounts of glycine, diglycine, cyclic diglycine, and triglycine were quantified to examine the degradation profile of oligopeptides in the Maillard reaction.

To overcome the difficulties in volatile extraction from a low water content medium, the stir bar sorptive extraction (SBSE) method was applied in our study for volatile analysis. SBSE is a recently developed solvent-free enrichment technique. Its principle is the same as solid-phase microextraction (SPME) but with higher sensitivity due to its thicker coating (*14*). The SBSE technique is reproducible and has been successfully applied to the volatile analysis of coffee brew by both liquid and headspace extraction (*15*).

MATERIALS AND METHODS

Model Reactions. Equimolar (0.0002 mol) amounts of glycine, diglycine, and triglycine (Sigma Chemical Co., St. Louis, MO) were heated, individually, with 0.0002 mol of glucose in an oil bath at 160 °C for 1 h. Glycerol (99.5%+, spectrophotometric grade, Sigma-Aldrich, Milwaukee, WI) was used as a reaction medium with water content ranges at 0, 10, 25, 50, 75, and 100% (v/v). The total volume of solution in the reaction vial (size 21 mm × 70 mm, Fisher Scientific, Pittsburgh, PA) was 5 mL. 4-Methylpyrimidine (10 ppm) (Sigma Chemical Co.) was used as a surrogate internal standard (IS) for semiquantitation. The quantities of identified compounds were relative amounts calculated by (peak area of identified compound/peak area of IS) × 10. The actual moisture content for the 0% water content reaction medium was 1.05% measured by Karl Fischer titration (Brinkmann Inc., Westbury, NY).

SBSE. An aliquot of the reaction product (1 mL) was transferred to the extraction vial. It was then diluted with 9 mL of phosphate buffer (pH 8 with 27% NaCl). In the dilution, the amount of glycerol, water,

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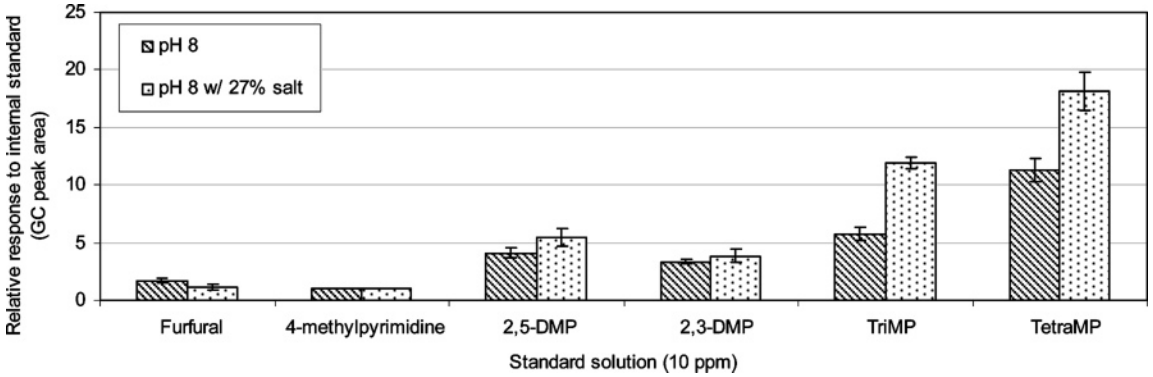

Figure 1. Effect of salt addition on the extraction efficiency of standard compounds. Standard aroma compounds used are furfural, 4-methylpyrimidine (IS), and 2,5-dimethyl-, 2,3-dimethyl-, trimethyl-, and tetramethylpyrazine.

Table 1. Volatile Compounds Identified in Glycine, Diglycine, and Triglycine Reactions with Glucose at Different Water Contents

		0%			10%			25%			50%			75%			100%			
compound	KI[c]	G[d]	Di[e]	Tri[f]	G	Di	Tri	G	Di	Tri	G	Di	Tri	G	Di	Tri	G	Di	Tri	I.D.
pyrazine	757	4.6	0.4	1.7	11.1	1.3	6.4	8.3	0.9	7.0	4.4	0.7	1.8	3.7	0.3	1.1	1.2	ND	0.4	*a*
1-methyl-1H-pyrrole	760	7.6	0.4	2.0	1.9	ND	4.5	trace	ND	0.2	ND	ND	ND	ND	ND	ND	ND	ND	ND	*a*
4,5-dimethyloxazole	769	2.0	0.4	0.7	2.8	0.8	3.0	2.0	0.3	0.9	0.4	trace	0.3	0.2	ND	ND	0.1	ND	ND	*b*
methylpyrazine	807	33.7	3.0	4.7	40.3	6.8	11.0	12.7	1.4	12.7	1.6	0.1	0.7	0.9	0.0	0.2	0.2	ND	trace	*a*
furfural	814	3.8	3.1	1.7	3.1	13.1	3.6	4.1	10.7	3.8	2.1	9.4	2.4	3.7	7.6	2.9	6.2	6.8	4.7	*a*
4-methyl-pyrimidine	853	10.0	10.0	10.0	10.0	10.0	10.0	10.0	10.0	10.0	10.0	10.0	10.0	10.0	10.0	10.0	10.0	10.0	10.0	*a*
2,5-dimethyl-pyrazine	926	38.7	77.2	58.7	76.2	39.1	71.8	77.7	27.7	62.2	36.5	8.6	21.9	20.7	2.9	9.2	5.3	ND	1.1	*a*
2,3-dimethyl-pyrazine	930	28.3	14.6	33.9	32.5	15.1	51.7	31.1	5.7	30.1	12.7	0.3	8.6	5.7.	0.1	2.5	1.9	trace	0.6	*a*
5-Methylfurfural	960	3.0	8.0	4.6	1.2	13.3	4.0	1.1	9.7	3.1	0.8	6.0	1.1	0.8	2.0	0.5	0.6	0.7	0.4	*b*
2-ethyl-5-methyl-pyrazine	985	18.5	12.3	21.1	30.8	28.8	49.2	25.6	17.3	21.9	10.3	1.2	2.0	2.4	0.5	1.5	1.3	ND	ND	*b*
trimethylpyrazine	989	46.3	63.4	80.6	68.0	41.8	110.4	121.9	23.3	91.6	107.5	5.5	58.5	55.2	1.4	25.7	12.0	ND	1.8	*a*
2-ethyl-3-methyl-pyrazine	990	14.6	8.1	17.8	19.4	5.3	32.4	16.3	1.9	14.4	3.7	1.5	3.3	3.3	0.6	1.8	1.4	ND	0.1	*b*
1-methyl-2-formyl-pyrrole	991	7.0	3.3	5.1	11.3	1.6	7.3	9.3	trace	5.2	2.8	trace	0.4	1.0	trace	0.3	0.3	ND	ND	*a*
2-ethenyl-6-methyl-pyrazine	1000	3.5	0.5	2.2	5.0	1.5	8.6	5.2	0.8	5.1	2.3	0.3	1.7	1.0	0.1	0.5	0.4	ND	0.1	*b*
2-acetylpyrrole	1038	ND	ND	ND	2.3	0.4	1.5	2.1	0.4	2.5	1.5	0.2	1.3	1.2	0.1	0.6	0.4	trace	0.2	*a*
2-acetyl-1-methyl-pyrrole	1050	10.2	0.8	2.4	13.2	0.7	6.2	9.7	0.2	3.5	1.9	0.0	0.7	0.4	ND	0.1	ND	ND	ND	*a*
3-ethyl-2,5-dimethyl-pyrazine	1055	4.3	1.7	6.7	5.7	2.7	10.7	6.7	0.8	3.5	1.6	ND	0.6	0.3	ND	0.6	0.0	ND	0.8	*b*
2-ethyl-3,5-dimethyl-pyrazine	1059	8.4	5.2	15.9	11.4	12.0	30.1	28.9	6.3	24.9	18.1	0.4	16.3	4.5	ND	1.8	0.7	ND	0.7	*b*
tetramethylpyrazine	1061	10.0	7.4	21.6	18.3	11.3	38.0	40.3	6.3	33.7	37.1	0.5	22.9	12.7	0.1	4.0	1.9	ND	0.3	*a*
5-(hydroxymethyl)-2-furfural	1200	ND	0.02	0.10	ND	0.58	ND	0.04	1.35	0.08	ND	1.52	ND	0.02	0.52	ND	ND	ND	ND	*a*

[a] Identification confirmed by GC retention index and mass spectra of authentic compounds; all authentic compounds were purchased from Sigma-Aldrich. [b] Tentatively identified by matching mass spectra library. [c] Linear retention indices were calculated according to Majlat et al. (*32*). [d] G, glycine with glucose. [e] Di, diglycine with glucose. [f] Tri, triglycine with glucose. All of the reactions were conducted at 160 °C for 1 h. The actual moisture content for the the 0% water content sample was 1.05%.

and buffer solution was kept constant at all extractions to eliminate a possible matrix effect on the extraction. The diluted samples were then extracted with a conditioned Gerstel stir bar [10 mm length × 0.5 mm of poly(dimethylsiloxane) (PDMS) film thickness, Twister, Gerstel GmbH, Mülheim and der Ruhr, Germany] at room temperature at 1100 rpm for 1 h.

Thermal Desorption−Gas Chromatography (GC)−Mass Spectrophotometry (MS) Analysis. After extraction, the PDMS stir bar was removed from the sample solution using a nonmagnetic forcep, rinsed with Milli-Q purified water, dried with a kimwipe, and then placed in a glass thermal desorption tube. The analytes were thermally desorbed in the splitless mode using a Gerstel TDU system (Twister Desorption Unit) by programming from 40 to 280 °C at 40 °C/min and holding the final temperature for 7 min. The desorbed compounds were cryofocused in the CIS-3s PTV injector (cooled injection system, programmed temperature vaporization, Gerstel, Inc., Baltimore, MD) at −100 °C and programmed from −100 to 280 °C and held for 5 min at 12 °C/s to vaporize the trapped compounds in the injector. A Varian model 3800 gas chromatography with a 30 m × 0.25 mm × 0.25 μm ZB-5ms column (Phenomenex, Folsom, CA) was used to separate the compounds in the reaction products. Helium of chromatographic grade (99.9999%) (BOC Gases, Murray Hill, NJ) was used as the carrier gas and was maintained at constant flow of 1 mL/min. Chromatographic separation was achieved using a temperature program with the following conditions: the initial temperature of 50 °C was held for 1 min and then ramped at 3 °C/min to a temperature of 209 °C. A final temperature of 280 °C was reached by ramping at 20 °C/min. A Saturn 2000 Ion Trap Detector (Varian Instruments, Walnut Creek, CA) was interfaced to the GC for detection of eluting compounds. Detector conditions applied were as follows: ion trap, manifold, and transfer lines were held at 120, 40, and 175 °C, respectively. The Detector scan range (in EI mode) was set from 40 to 450 Da five times per second.

Table 2. Nonvolatile Compounds Quantified by LC-MS in the Diglycine and Triglycine Reactions with Glucose at Different Water Contents

Diglycine-Glucose

		amount (mol/mol) (%)					
compd	RT (min)	0	10	25	50	75	100
DKP	1.02	16.920	19.345	19.786	20.420	17.895	18.107
glycine	5.56	2.940	6.036	9.698	12.756	10.667	9.387
diglycine	6.90	0.693	1.488	3.787	12.139	23.841	28.505
triglycine	7.60	0.009	0.010	0.011	0.014	0.016	0.023

Triglycine-Glucose

		amount (mol/mol) (%)					
compd	RT (min)	0	10	25	50	75	100
DKP	1.02	47.019	59.563	67.766	74.496	79.469	80.656
glycine	5.56	2.755	4.149	15.432	41.243	55.122	59.867
diglycine	6.90	0.505	0.607	0.929	1.475	1.729	1.742
triglycine	7.60	0.070	0.035	0.035	0.056	0.065	0.061

Liquid Chromatography−Tandem Mass Spectrometry (LC-MS/MS). An aliquot (1 mL) of reaction product was dissolved into 200 mL of methanol/water (1:1, v/v) solution containing 0.5% formic acid for HPLC-ESI-MSMS (high-performance liquid chromatography−electrospray ionization−tandem mass spectrometry) analysis. Briefly, a TSQ Quantum tandem mass spectrometer (Thermo-Finnigan, San Jose, CA) was equipped with an ESI interface and Agilent 1100 HPLC system (Agilent Technologies, Palo Alto, CA). HPLC separation was performed using a ThermoHypersil-Keystone Silica column (Agilent Technologies), 21 mm × 2.1 mm i.d. with a particle size of 5 μm, at room temperature. The reaction components were eluted using a mobile phase of 25% acetonitrile−water containing 0.5% formic acid. The flow rate was 0.75 mL/min. The TSQ Quantum was operated in the positive ion mode under the following conditions: (i) nitrogen (>99.7%) was used for the sheath gas and auxiliary gas at a pressure of 30 psi and 5 units, respectively; (ii) the temperature of the heated capillary was maintained at 350 °C; (iii) the spray voltage of ESI was set at 4.5 kV; (iv) a collision-induced dissociation was achieved using argon as the collision gas at a pressure adjusted to 0.8 mTorr above the normal; and (v) the applied collision offset energy was set to −45 eV. Identification of reaction component was accomplished by comparing the HPLC retention time and parent−daughter and selected reactant monitoring analysis of the sample peaks with that of the authorized pure commercial compounds. The *m*/*z* from 76.1 (molecular ion) to 30.4 (responding major fragment ion) was set for glycine, 115.1 (molecular ion) to 30.4 (major fragment ion) was set for c (Gly-Gly), and *m*/*z* 133.1 → 76.1 and 190.1 → 76.1 were set for diglycine and triglycine, respectively. Data were acquired with Xcalibur software system (Thermo-Finnigan). The quantification for each compound was conducted using the total ion counts with an external standard.

RESULTS AND DISCUSSION

SBSE Extraction Efficiency. Before the SBSE technique was applied to model reaction samples, its extraction efficiency on heterocyclic aroma compounds and our IS (4-methylpyrimidine) was examined by using 10 ppm of standard solution mixtures. **Figure 1** shows the extraction efficiency of the standard aroma compounds and IS in phosphate buffer solution at pH 8 and pH 8 with 27% salt. The bar graph represents the relative GC peak area to the IS. The error bars in the graph represent the standard error of three replications. Results showed that each aroma compound at 10 ppm has a different GC response due to the extraction efficiency by SBSE. Tetramethylpyrazine showed a higher response than other compounds. The addition of salt increased the extraction efficiency of the aroma compounds. The salt addition was also reported to increase the extraction efficiency of Maillard reaction flavors using SPME with a Carboxen/PDMS coating (*16*).

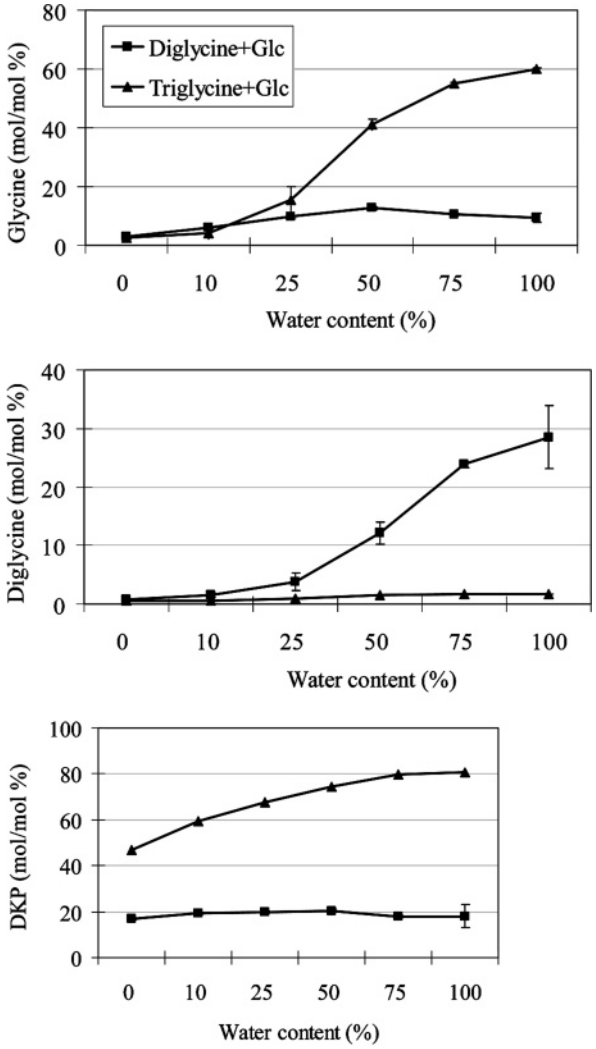

Figure 2. Triglycine with glucose reaction mixture (▲) and diglycine with glucose reaction mixture (■). The actual moisture content for the 0% water sample was 1.05%.

Effect of Water Content on Volatile Formation. Table 1 lists the volatile compounds identified in the reaction mixtures of glycine, diglycine, and triglycine with glucose at 160 °C for 1 h. The experiments were done in triplicate. The amounts of volatiles formed at 0, 10, 25, 50, 75, and 100% (v/v) water content in glycerol medium were relative to the IS, which presented as 10. The relative amounts were calculated as (peak area of identified compound/peak area of IS) × 10. The substituted pyrazines dominated in all of the reactions and increased as the water content decreased. However, a slight inhibition of the volatile formation at 0% water content was observed and it may be partly due to the highly viscous properties of glycerol, which restrained the solubility and the mobility of the reactants (*17*). The optimal formation at the range of low water content was due to the fact that water is a product of several condensation steps in the Maillard reaction (*17*). Our results showed that the volatile profile changed with low water content medium, and some extra compounds were identified as compared to Oh et al.'s study (*8*).

Pyrroles. In our study, besides the 2-acetylpyrrole, which was reported by Oh et al. (*8*), 1-methyl-1H-pyrrole, 1-methyl-2-formylpyrrole, and 2-acetyl-1-methylpyrrole were found at low water content in all glycine peptide systems. The 1-methyl-2-formylpyrrole and 2-acetyl-methylpyrrole were reported in brewed coffee volatiles and were found to exhibit antioxidant

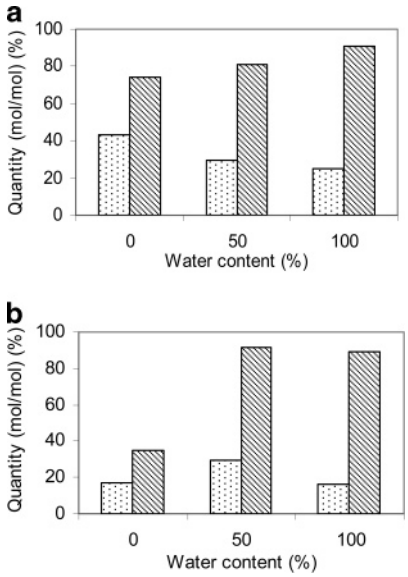

Figure 3. (**a**) Abundance of DKP from peptide only. (**b**) Abundance of glycine from peptide only. Peptides are heated without glucose under 160 °C for 1 h at different water contents. The actual moisture content for the 0% water sample was 0.05%. Experiments were done in duplicate; the bar graphs represent the average value.

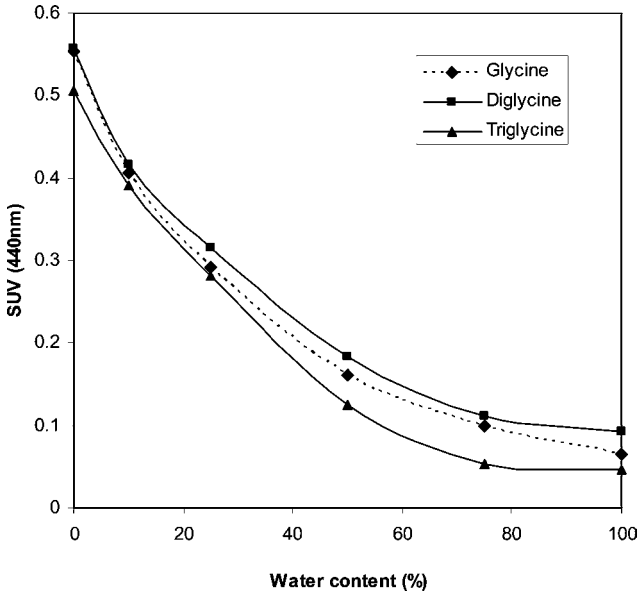

Figure 4. Color of the peptide reaction mixtures determined by UV at 440 nm. *The actual moisture content for the 0% water sample was 1.05%.

activity (*18*, *19*). In the glycine−glucose reaction, the optimal water content for the formation of pyrrole with a carbonyl function group was found to be 10% water content, whereas the maximum amount of pyrrole with only alkyl substitution was formed at 0% water content. This phenomenon was also reported in Ames' glycine−glucose model system in which 13% of water promoted the formation of pyrrole with a substituted carbonyl group (*13*). The optimum condition for pyrrole formation from diglycine and triglycine was different.

Pyrazines. Trimethylpyrazine and 2,5-dimethylpyrazine were the major volatiles produced in all of the reaction systems. The 2,5-dimethyl group was proposed to be formed from pyruvaldehyde (*20*, *21*). It was very interesting to note that at 0% water content, diglycine generated more 2,5-dimethylpyrazine than triglycine followed by glycine. Although the mono-, di-, tri, and tetramethylpyrazine constituted a large portion of total volatiles formed, other ethyl-substituted pyrazines are relatively more important to the roast aroma due to their sufficiently low threshold (*22*). The 2-ethyl-5-methyl-, 2-ethyl-3-methyl-, 2-ethenyl-6-methyl-, 3-ethyl-2,5-dimethyl-, and 2-ethyl-3,5-dimethylpyrazines were identified in our study at both low and high water content reaction systems of glycine and triglycine. The 2-ethyl-5-methylpyrazine was reported in glycine−glucose model systems (*13*, *23*, *24*) and considered as a major contributor of sesame oil flavor (*25*). 2-Ethyl-3,5-dimethylpyrazine has been identified in glycine−glucose model systems (*24*) and heated foods (*26*, 27). The formation of the ethyl-substituted pyrazines from the condensation of amino acids fragments was proposed by Shibamoto and Bernhard (*28*).

Furans. Yeo and Shibamoto (*29*) suggested that the formation of furan derivatives involved the dehydration mechanism. The formation of 5-hydroxymethylfurfural in our study was insignificant, most likely due to the low extraction efficiency of the PDMS stir bar. Nevertheless, the formation of furan derivatives from diglycine was more than those from glycine and triglycine.

Quantitation of Glycine, Diglycine, Cyclic Gly-Gly, and Triglycine in the Peptide−Glucose Reaction Mixtures. The experiments in nonvolatiles study were done in duplicate. The remaining amounts (mol/mol %) of glycine, diglycine, cyclic Gly-Gly, and triglycine in the peptide−glucose reaction system were quantified (**Table 2** and **Figure 2**). The error bars in the graph represent the standard error of three replicates of analysis. The stability of two peptides can be determined by the remaining amount of peptides in the Maillard reaction. Triglycine remained only in a trace amount, and diglycine remained at 0.693−28.505% (mol/mol) in the triglycine−glucose and diglycine−glucose reaction mixtures, respectively. In the Maillard reaction, triglycine was mainly degraded into c(Gly-Gly) and glycine. In the diglycine−glucose reaction, c(Gly-Gly) was the dominant compound. It was found that diglycine generated less glycine than a triglycine system (**Table 2** and **Figure 3b**), and the reason may be due to the high electron density of the bond, which suppresses hydrolysis.

c(Gly-Gly) was the most abundant compound in both the diglycine−glucose and the triglycine−glucose reaction mixtures and was produced more in the triglycine than in the diglycine system (**Table 2** and **Figure 3a**). The formation pathway of cyclic dipeptide, also called diketopiperazine (DKP), from a tripeptide was proposed by Rizzi (*10*). He suggested that DKP was formed intramolecularly through cyclization of the first two N-terminal amino acids and then cleavage of the amino acid at the C-terminal. Rizzi (*10*) reported the yield of DKP varies with peptides; Val-Ala was almost quantitatively converted into c(Val-Ala), whereas Pro-Gly-Gly was converted only 15% into c(Pro-Gly). DKPs have been identified in many thermally processed foods and may contribute to sensory properties. Our results showed that in the Maillard reaction diglycine was converted into c(Gly-Gly) at about a 20% (mol/mol) yield and was not significantly affected by water content. Triglycine was converted into 50−80% (mol/mol) c(Gly-Gly) from low to high water content. The conversion of c(Gly-Gly) from peptides heated without glucose (**Figure 3a**) was slightly higher than that in Maillard reaction.

The higher reactivity of triglycine than diglycine in volatiles formation may be related to the availability of free glycine during the reaction. **Table 2** with figure shows that at low water content the amounts of nonvolatiles identified in peptide−glucose reaction mixtures were low except c(Gly-Gly). Melanoidins may be formed as main products at low water content. The browning was determined by a spectrophotometer (**Figure**

4), and data showed that the diglycine−glucose reaction mixtures had a higher degree of browning, followed by glycine−glucose and triglycine−glucose reaction mixtures.

Reactivity of Peptides. In our study, the reactivity of peptides was determined by the amounts of pyrazines formation. Our study showed that the overall reactivity of peptides for flavor formation was glycine ≈ triglycine > diglycine. It cannot be predicted by the pK_2 value of the glycine peptides, which are 9.8, 8.25, and 7.91 for glycine, diglycine, and triglycine, respectively (*30*). It was also reported by Mori et al. (*31*) that the pK_a value of the amino group does not seem to be the sole determinant in the reaction rate. Our study in diglycine and triglycine Maillard reactions suggests that a high hydrolysis rate of the peptide bond may be one of the main factors that enhance volatile formation.

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