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ARTICLE

Formation of Pyrazines in Maillard Model Systems of Lysine-Containing Dipeptides

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Whereas most studies concerning the Maillard reaction have focused on free amino acids, little information is available on the impact of peptides and proteins on this important reaction in food chemistry. Therefore, the formation of flavor compounds from the model reactions of glucose, methylglyoxal, or glyoxal with eight dipeptides with lysine at the N-terminus was studied in comparison with the corresponding free amino acids by means of stir bar sorptive extraction (SBSE) followed by GC-MS analysis. The reaction mixtures of the dipeptides containing glucose, methylglyoxal, and glyoxal produced 27, 18, and 2 different pyrazines, respectively. Generally, the pyrazines were produced more in the case of dipeptides as compared to free amino acids. For reactions with glucose and methylglyoxal, this difference was mainly caused by the large amounts of 2,5(6)-dimethylpyrazine and trimethylpyrazine produced from the reactions with dipeptides. For reactions with glyoxal, the difference in pyrazine production was rather small and mostly unsubstituted pyrazine was formed. A reaction mechanism for pyrazine formation from dipeptides was proposed and evaluated. This study clearly illustrates the capability of peptides to produce flavor compounds that can differ from those obtained from the corresponding reactions with free amino acids.

KEYWORDS: Peptides; Maillard; pyrazines; flavor; model reactions; SBSE

INTRODUCTION

The Maillard reaction comprises a set of complex chemical reactions, which are initiated when a free amino group of an amino acid, a peptide, a protein, or an amine reacts with the carbonyl group of a reducing carbohydrate. This nonenzymatic browning gives rise to modifications in color, aroma, taste, and nutritional value of thermally treated foods and is influenced by many factors, such as reactant concentration, temperature, time, pH, buffer, and water activity (*1*). The reaction between free amino acids and carbonyl compounds has been studied extensively (*1*), whereas only a minor part of the Maillard reaction studies focused on peptides and proteins. However, the amount of free amino acids in food is always very low as compared to the amounts of peptides and especially proteins (*2*). Therefore, it can be assumed that in food, the modifications caused by the Maillard reaction with peptides and proteins are much more important than the modifications caused by the Maillard reaction with free amino acids. As a first step to extend the current knowledge on the reactivity of free amino acids, this study was undertaken to investigate the formation of flavor compounds from dipeptides in the Maillard reaction.

A limited number of studies have already investigated the role of peptides in the Maillard reaction. The majority of these studies focused on the quantity, rate, and site specificity of glycation of peptides in model systems representing either physiological (*3*, *4*) or food-related conditions (*5*−*8*). Until now, the formation of flavor compounds due to the reaction between peptides and sugars has mainly been studied in model systems containing glutathione (*9*−*12*) or glycine-derived peptides such as diglycine, triglycine, and tetraglycine (*13*, *14*). Although these glycine-derived peptides can be used to represent di-, tri-, and tetrapeptides, it is known that peptides composed of other amino acids produce different flavor compounds. Possibly, these flavor compounds are more specific and more important than those produced by glycine-derived peptides. Oh et al. (*15*) already showed that proline-specific volatiles, such as pyrrolizines, were formed in model systems containing Pro-Gly or Gly-Pro and glucose. Strecker aldehydes from valine and leucine have been detected in reaction mixtures of glucose and Val-Gly or Gly-Val and Leu-Gly or Gly-Leu, respectively (*16*). In addition, the production of peptide-specific volatiles, such as 2(1*H*)-pyrazinones, has been reported (*17*, *18*).

To the best of our knowledge, the formation of flavor compounds from lysine-containing dipeptides has not been studied yet. However, because lysine contains a very reactive side chain, modifications of peptides and proteins are mostly situated at the lysine residues. In addition, lysine is known to produce high amounts of flavor compounds such as pyrazines and pyrroles (*19*−*21*). Therefore, in this study, eight dipeptides with lysine at the N-terminus (Lys-X) were reacted with glucose, methylglyoxal, and glyoxal, which are common and very reactive sugar degradation products. The C-terminal amino acid was

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varied to study the influence of the neighboring amino acid on the flavor production by the lysine residue, because, theoretically, only the two amino groups of lysine are able to react. The flavor compounds produced by these reactions were compared with those obtained from the mixture of the corresponding free amino acids and with those obtained from the reaction between lysine and the corresponding carbonyl compound. Attempts were undertaken to elucidate the mechanistic pathways that lead to differences in flavor patterns between free amino acid and peptide model reactions.

MATERIALS AND METHODS

Chemicals. Cysteine (97%), phenylalanine (98.5%), leucine (99%), and serine (99%) were purchased from Janssen Chimica (Geel, Belgium). Alanine (99%), lysine monohydrate (99%), and glutamic acid (99%) were purchased from Acros Organics (Geel, Belgium). Glycine (99%) and glucose (99.5%) were from Sigma-Aldrich (Bornem, Belgium). The peptides Lys-Ala hydrobromide (99%), Lys-Lys hydrochloride (99%), Lys-Leu acetate (99%), Lys-Gly hydrochloride (99%), and Lys-Ser hydrochloride (99%) were purchased from Bachem (Bubendorf, Switzerland). The peptides Lys-Cys (95.6%), Lys-Phe (99.4%), and Lys-Glu (95.5%) were from Genscript (Piscataway, NJ).

Model Reactions. For the model reactions, 1 mmol of peptide or of the corresponding free amino acids (1 mmol each) and 1 mmol of glucose or 0.1 mmol of methylglyoxal or glyoxal were dissolved in 4.7 mL of water. Reduced amounts were used for methylglyoxal and glyoxal to limit self-condensation of these reactive compounds. The pH was adjusted to 8 with aqueous NaOH (2 N) or HCl (2 N). Water was added to obtain a final volume of 5 mL. Final concentrations for peptides, amino acids, and glucose were 0.2 M, whereas final concentrations for methylglyoxal and glyoxal were 0.02 M. The reaction mixtures were transferred into Pyrex glass tubes (10 mL), tightly closed, heated for 120 min at 130 °C in a stirred oil bath, and immediately cooled in an ice bath afterward.

Selection of the Extraction Technique. The comparison of different extraction techniques was performed with model systems containing lysine and glucose. For extraction with ethyl acetate, 5 mL of sample was extracted three times with 5 mL of ethyl acetate. Afterward, the extracts were combined and concentrated. For headspace solid phase microextraction (HS-SPME), 5 mL of sample was extracted for 30 min at 35 °C with both a divinylbenzene/Carboxen/poly(dimethylsiloxane) (DVB/Car/PDMS) and a PDMS fiber (Supelco, Bornem, Belgium). Both fibers were also used to perform direct SPME of 5 mL of sample for 30 min at 35 °C. The same extraction time and temperature were used to perform stir bar sorptive extraction (SBSE) of the 5 mL samples. The extracts obtained by these different techniques were analyzed by means of GC-MS and compared.

Analysis of Flavor Compounds. During all handlings, samples were kept in ice to ensure minimal losses of volatiles. First, 4.5 mL of cooled sample was transferred into a 15 mL headspace vial, and the pH was measured and adjusted to 8. This pH measurement was performed while the sample was kept in ice. However, a pH-meter with temperature sensor was used throughout all measurements, and calibration of the pH meter was performed at the corresponding temperature. After the vial had been closed, the sample was equilibrated for 30 min at 35 °C. Literature data reported little changes in the response obtained by SBSE when the extraction temperature was varied from 25 to 60 °C (*22*). To avoid continuation of the Maillard reactions during extraction at high temperature, but at the same time allowing the temperature to remain stable, 35 °C was chosen as the extraction temperature for SBSE measurements. Afterward, the reaction mixture was extracted with a conditioned Gerstel stir bar (10 mm length × 0.5 mm of PDMS film thickness, Twister, Gerstel GmbH, Mülheim a/d Ruhr, Germany) at 35 °C for 30 min at constant stirring. The repeatability of the extraction technique, SBSE, was checked by comparing the resulting peak area of the SBSE extracts of standard solutions of linear alkanes and pyrazines of triplicate measurements. Standard deviations of the resulting peak area were below 11%.

Thermal Desorption−GC-MS. After extraction, the PDMS stir bar was removed from the sample, rinsed with water, dried, and then placed in a glass thermal desorption tube. The analytes were thermally desorbed in the splitless mode using a Gerstel Thermo Desorption System (TDS2). The TDS2 oven was programmed from 20 to 250 °C at 60 °C min^{-1} (held for 10 min at 250 °C). The desorbed compounds were cryofocused in a CIS-4 PTV injector (Gerstel) at −100 °C. After complete desorption, the injector was programmed from −100 to 250 °C at 12 °C/s and held for 10 min.

GC-MS analyses of the SBSE extracts were performed with an Agilent 6890 GC Plus coupled to a quadrupole mass spectrometer 5973 MSD (Agilent Technologies, Diegem, Belgium) and equipped with a HP5-MS capillary column (30 m length × 0.25 mm i.d.; 0.25 μm film thickness). Working conditions were as follows: transfer line to MSD, 250 °C; carrier gas (He), 1.0 mL min^{-1}; ionization, EI 70 eV; acquisition parameters, scanned m/z 40−200 (2−10 min), 40−300 (> 10 min); oven temperature, start at 35 °C, hold for 5 min, programmed from 35 to 80 °C at 2 °C min^{-1} and from 80 to 250 °C at 20 °C min^{-1}, hold 2 min. Substances were identified by comparison of the mass spectrum with mass spectral libraries (NIST 98 and Wiley 6th) and by comparison of the calculated linear retention indices with literature values (*23*−*25*).

RESULTS AND DISCUSSION

Eight dipeptides with lysine at the N-terminus (Lys-X, with X = Gly, Ala, Leu, Lys, Ser, Glu, Phe, or Cys) or a mixture of the corresponding free amino acids were reacted with glucose, methylglyoxal, or glyoxal in unbuffered aqueous conditions at pH 8 (130 °C, 2 h). Afterward, the volatiles produced were sampled by means of SBSE-GC-MS. SBSE is a solventless extraction technique in which a stir bar coated with PDMS is placed in the liquid sample. Different extraction techniques, such as extraction with ethyl acetate, headspace SPME and direct SPME with different fibers and SBSE, were compared for model systems containing lysine and glucose. The highest recoveries of the flavor compounds were detected in the case of SBSE (data not shown).

It was decided to perform the experiments without buffer, because it has been shown that the anionic species of the buffer can exert a severe catalytic effect as has been extensively pointed out for the phosphate ion (*16*, *26*). Preliminary model reactions of free amino acids indeed showed a clear effect of several buffers tested (phosphate and citrate) on browning and flavor formation (data not shown). This was concluded from the clear difference in browning and flavor formation of model systems with different types or concentrations of buffers, but the same pH. In the unbuffered systems, the pH dropped from 8 to 4−5 during the reaction of the amino acids or peptides with glucose. The pH of the reaction mixtures containing methylglyoxal or glyoxal remained quite stable. The lower pH in the case of glucose reaction mixtures could be expected, because acids are formed during the reaction (*27*). To ensure identical extraction conditions of the volatiles, the pH was adjusted to pH 8 (measured in ice) before sampling of the volatiles.

Pyrazines were the most important volatiles detected in the case of reactions with the dipeptides, quantitatively as well as from a flavor point of view (*23*). The reaction mixtures of the dipeptides containing glucose, methylglyoxal, and glyoxal produced 27, 18, and 2 different pyrazines, respectively, whereas the reaction mixtures of the free amino acids produced 32, 22, and 8 different pyrazines, respectively (**Tables 1**−**3**).

It must be noted that complete chromatographic separation of 2,5-dimethylpyrazine and 2,6-dimethylpyrazine is not possible under the chromatographic conditions described. Because little difference can be found in their mass spectra, it was decided to report both compounds for one eluting peak and consider them together in the following discussion as 2,5(6)-dimethylpyrazine.

Pyrazine production is reported in terms of the obtained GC-MS peak area. Using SBSE as the extraction technique, reporting peak areas can be justified, because for SBSE all solutes have their own partitioning equilibrium into the PDMS phase and displacement does not occur (*28*). This is due to the fact that analytes are not retained on an active surface as is the case with adsorbents,

Table 1. Pyrazines (GC-MS Peak Area × 10^8) Detected in the Model Reactions of Glucose with Lysine-Containing Peptides or the Mixture of the Corresponding Free Amino Acids (2 h, 130 °C)

LRI exptl[a]	LRI lit.[b]	compound	Lys (0.2 M)	Lys (0.4 M)	LysLys	Lys + Gly	LysGly	Lys + Ala	LysAla	Lys + Leu	LysLeu	Lys + Ser	LysSer	Lys + Glu	LysGlu	Lys + Cys	LysCys	Lys + Phe	LysPhe	theoretical recovery (%)
750	760[c]	pyrazine	3.04	5.30	0.47	1.63	0.25	2.14	0.03	1.20	0.09	0.57	0.19	2.45	0.24	0.17	0.24	2.17	0.09	0.3
821	819[d]	methylpyrazine	1.82	3.06	1.26	2.02	2.77	0.85	0.67	1.40	1.24	0.29	0.48	3.03	7.77	0.42	1.78	0.90	1.50	0.8
902	908/909[d]	2,5(6)-dimethylpyrazine	0.85	2.84	11.81	2.27	21.05	0.74	6.23	1.73	13.78	0.36	4.16	5.85	71.46	–[g]	5.90	1.16	11.92	2.0/1.6
907	916[e]	2,3-dimethylpyrazine	0.03	0.31	0.29	0.57	4.96	0.38	1.11	0.50	0.78	0.05	0.22	0.62	6.64	0.28	0.98	0.29	2.04	1.6
991	997[c]	2-ethyl-6-methylpyrazine	0.02	–	0.79	0.05	0.15	0.06	0.02	0.06	0.09	0.01	0.03	0.05	–	0.28	2.30	0.09	0.67	
997	1000[c]	2-ethyl-5-methylpyrazine	–	0.33	–	0.21	1.33	0.17	0.45	0.45	1.42	0.06	0.61	1.13	8.15	–	0.35	0.07	–	4.1
998	1000[d]	trimethylpyrazine	0.09	0.25	1.80	0.80	5.22	0.12	0.82	0.15	1.82	0.02	0.58	0.69	7.80	0.16	1.32	0.19	2.05	
1005		2-ethenyl-6-methylpyrazine[f]	–	0.03	0.44	0.05	0.23	–	0.14	–	0.14	0.01	0.08	0.09	1.03	–	–	0.03	0.59	
1010		2-ethenyl-5-methylpyrazine[f]	–	0.04	–	0.12	0.84	0.04	0.41	0.20	0.53	0.02	0.33	0.24	2.42	–	–	–	–	
1011	1017[d]	2-acetylpyrazine	–	0.05	–	–	–	–	–	–	–	0.03	–	–	–	–	–	–	–	0.8
1074	1078[e]	3-ethyl-2,5-dimethylpyrazine	tr[h]	0.35	0.28	0.34	0.37	2.09	0.12	0.14	0.48	0.06	0.21	0.81	1.41	–	0.28	0.13	0.29	
1081	1083[d]	tetramethylpyrazine	–	–	–	0.19	–	–	–	0.07	–	–	–	–	–	–	–	–	–	8.4
1081	1083[e]	2-ethyl-3,5-dimethylpyrazine	tr	0.02	–	0.06	0.11	–	–	–	0.06	–	0.03	0.09	0.24	–	–	–	–	
1082	1084[e]	5-ethyl-2,3-dimethylpyrazine	–	–	–	–	0.22	–	0.04	–	0.09	0.01	0.04	0.17	0.39	0.04	0.02	–	–	
1086	1090[e]	2,5-diethylpyrazine	–	–	–	–	0.13	–	–	–	0.06	–	0.01	0.03	0.09	–	–	–	–	
1093	1095[e]	3-ethenyl-2,5-dimethylpyrazine	0.01	0.04	0.33	0.03	1.13	0.02	1.00	0.07	0.57	0.01	0.97	0.07	2.67	–	–	tr	1.31	
1128	1129[c]	2-acetyl-5-methylpyrazine	–	0.09	–	–	0.18	–	0.03	–	–	0.01	0.03	–	0.31	–	–	–	–	
1156	1155[e]	2,3-diethyl-5-methylpyrazine	–	–	–	–	–	0.09	–	–	0.01	–	0.01	0.05	0.07	–	–	–	–	30.0
1159	1157[e]	3,5-diethyl-2-methylpyrazine	–	–	–	0.02	0.02	0.16	–	–	0.03	–	0.01	0.08	0.09	–	–	–	–	
1161	1159[c]	2,3,5-trimethyl-6-ethylpyrazine	–	0.04	–	0.10	tr	0.36	–	–	–	0.01	0.01	0.07	–	–	–	–	–	
1163	1159[e]	2,5-dimethyl-3-propylpyrazine	–	–	–	–	–	–	–	–	0.03	–	0.01	0.11	–	–	–	–	–	
1166		2-(3-methylbutyl)pyrazine[f]	–	–	–	–	–	–	–	5.22	–	–	–	–	–	–	–	–	–	
1243		methyl-(3-methylbutyl)-pyrazine[f]	–	–	–	–	–	–	–	1.22	0.18	–	–	–	–	–	–	–	–	
1246		methyl-(3-methylbutyl)-pyrazine[f]	–	–	–	–	–	–	–	1.72	0.11	–	–	–	–	–	–	–	–	
1255		2-(2′-furyl)-pyrazine[f]	tr	0.10	–	0.12	0.05	0.09	–	–	–	0.04	0.01	0.21	–	–	–	–	–	
1259		methyl-(3-methylbutyl)-pyrazine[f]	–	–	–	–	–	–	–	1.34	0.07	–	–	–	–	–	–	–	–	
1295		2,5-dimethyl-3-(3-methylbutyl) pyrazine[f]	–	–	–	–	–	–	–	24.01	1.12	–	–	–	–	–	–	–	–	
1351		2-(2′-furyl)-5(6)-methylpyrazine[f]	–	–	–	–	0.20	–	0.03	–	0.13	–	0.02	0.20	0.80	–	–	–	–	
1382		1,4-dimethylpyrrole-(1,2A)-pyrazine[f]	–	0.58	–	0.29	0.09	0.38	0.04	–	0.06	0.06	0.04	2.17	–	–	–	–	–	
1382		2,3,5-trimethyl-6-(2-methylbutyl)-pyrazine[f]	–	–	–	–	–	–	–	4.03	–	–	–	–	–	–	–	–	–	
1390		2,3,5-trimethyl-6-(3-methylbutyl)-pyrazine[f]	–	–	–	–	–	–	–	6.67	–	–	–	–	–	–	–	–	–	
1585		(2-phenylethyl)pyrazine[f]	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	3.31	tr	
		total pyrazines	5.86	13.46	17.48	8.87	39.31	7.70	11.15	50.20	22.90	1.63	8.08	18.18	111.59	1.35	13.18	8.33	20.45	
		pyrazines (% of total GC-MS peak area)	29.8	27.4	87.3	31.9	70.6	14.8	63.7	19.0	39.7	22.2	66.4	37.0	63.6	3.4	33.3	9.8	64.4	

[a] LRI exptl, linear retention index determined experimentally on a HP5-MS stationary phase. [b] LRI lit., linear retention index value from the literature. [c] Adams and De Kimpe (*25*). [d] Adams (*24*). [e] Wagner et al. (*23*). [f] Tentatively identified. [g] –, not detected. [h] tr, trace.

Table 2. Pyrazines (GC-MS Peak Area × 10^8) Detected in the Model Reactions of Methylglyoxal with Lysine-Containing Peptides or the Mixture of the Corresponding Free Amino Acids (2 h, 130 °C)

LRI exptl[a]	LRI lit.[b]	compound	Lys (0.2 M)	Lys (0.4 M)	LysLys	Lys + Gly	LysGly	Lys + Ala	LysAla	Lys + Leu	LysLeu	Lys + Ser	LysSer	Lys + Glu	LysGlu	Lys + Cys	LysCys	Lys + Phe	LysPhe	theoretical recovery (%)
750	760[c]	pyrazine	–[g]	–	–	–	–	–	–	–	–	0.05	–	–	–	–	–	–	–	0.3
821	819[d]	methylpyrazine	0.12	0.13	0.12	0.18	0.24	0.18	0.06	0.10	0.14	0.36	0.03	–	0.08	–	–	0.04	0.09	0.8
902	908/909[d]	2,5(6)-dimethylpyrazine	3.61	8.98	44.22	5.13	25.70	7.50	17.91	6.18	33.44	1.71	6.22	3.96	24.17	2.18	45.62	6.56	68.22	2.0/1.6
907	916[e]	2,3-dimethylpyrazine	0.67	1.28	4.01	0.73	11.39	0.70	1.65	0.55	2.12	0.30	0.79	0.34	0.52	–	3.11	0.72	8.11	1.6
998	1000[d]	trimethylpyrazine	1.03	4.55	36.84	9.80	28.47	2.75	9.91	2.97	16.65	0.79	5.43	1.57	11.34	0.15	71.67	2.76	48.32	4.1
1074	1078[e]	3-ethyl-2,5-dimethylpyrazine	0.34	1.29	0.77	0.30	0.31	6.77	0.62	0.75	1.81	0.54	0.16	–	0.62	0.06	1.25	0.68	1.26	
1081	1083[e]	2-ethyl-3,5-dimethylpyrazine	–	0.12	0.08	0.20	0.41	0.11	0.05	0.03	0.10	–	0.05	–	–	–	0.50	0.03	0.34	
1081	1083[d]	tetramethylpyrazine	–	–	–	0.33	–	–	0.07	0.09	–	–	–	–	–	–	–	–	0.01	8.4
1082	1084[e]	5-ethyl-2,3-dimethylpyrazine	–	–	0.29	0.58	0.76	0.10	0.08	–	0.26	0.03	0.10	0.01	0.19	–	0.92	0.06	0.32	
1093	1095[e]	3-ethenyl-2,5-dimethylpyrazine	–	–	0.06	tr[h]	0.06	0.03	0.06	–	–	0.17	0.03	–	0.04	–	–	–	0.28	
1128	1129[c]	2-acetyl-5-methylpyrazine	–	–	–	–	–	–	–	–	–	0.04	0.06	0.06	0.23	–	0.31	–	–	
1130	1134[c]	2-acetyl-6-methylpyrazine	–	–	–	0.12	0.31	0.08	0.07	–	–	0.02	–	–	–	–	0.31	–	0.36	
1156	1155[e]	2,3-diethyl-5-methylpyrazine	–	–	–	–	–	0.06	–	–	–	0.01	–	–	–	–	–	–	–	30
1159	1157[e]	3,5-diethyl-2-methylpyrazine	–	–	–	–	–	0.12	–	–	–	0.02	–	–	–	–	–	–	–	
1161	1159[c]	2,3,5-trimethyl-6-ethylpyrazine	–	–	tr	0.14	–	0.19	tr	–	0.03	0.04	–	0.01	–	–	0.05	0.04	–	
1163	1159[e]	2,5-dimethyl-3-propylpyrazine	–	–	–	–	–	–	–	–	0.22	0.01	0.01	0.02	0.04	–	–	–	–	
1212		2-acetyl-3,5-dimethylpyrazine[f]	0.04	0.09	1.46	0.26	1.60	0.08	1.29	–	1.41	0.05	0.40	0.05	2.62	–	5.57	0.06	3.41	
1215	1200[e]	3,5-dimethyl-2-(*Z*-1-propenyl)-pyrazine	0.02	0.09	0.39	0.02	–	0.04	0.52	–	0.13	0.01	0.04	0.03	0.04	–	–	–	0.02	
1226	1217[e]	2,3-dimethyl-5-(*Z*-1-propenyl)-pyrazine	tr	0.09	1.18	0.02	0.83	0.04	0.76	0.09	1.10	0.01	0.20	0.03	0.69	–	1.97	0.03	1.18	
1245		3-isopropenyl-2,5-dimethyl-pyrazine[f]	tr	tr	0.14	0.01	0.08	0.05	0.07	0.04	0.14	0.01	0.03	0.03	0.04	–	1.38	tr	0.08	
1295		2,5-dimethyl-3-(3-methylbutyl)-pyrazine[f]	–	–	–	–	–	–	–	10.36	0.30	–	–	–	–	–	–	–	–	
1390		2,3,5-trimethyl-6-(3-methylbutyl)-pyrazine[f]	–	–	–	–	–	–	–	0.31	–	–	–	–	–	–	–	–	–	
		total pyrazines	5.83	16.62	89.56	17.82	70.17	18.81	33.13	21.48	57.85	4.15	13.54	6.11	40.61	2.39	132.67	10.98	131.99	
		pyrazines (% of total GC-MS peak area)	23.8	13.8	73.8	48.7	69.8	49.3	42.4	12.8	53.4	49.9	60.0	38.8	52.7	6.2	62.5	8.4	50.4	

[a] LRI exptl, linear retention index determined experimentally on a HP5-MS stationary phase. [b] LRI lit., linear retention index value from literature. [c] Adams and De Kimpe (*25*). [d] Adams (*24*). [e] Wagner et al. (*23*). [f] Tentatively identified. [g] –, not detected. [h] tr, trace.

Table 3. Pyrazines (GC-MS Peak Area × 10^8) Detected in the Model Reactions of Glyoxal with Lysine-Containing Peptides or the Mixture of the Corresponding Free Amino Acids (2 h, 130 °C)

LRI expt[a]	LRI lit.[b]	compound	Lys (0.2 M)	Lys (0.4 M)	LysLys	Lys + Gly	LysGly	Lys + Ala	LysAla	Lys + Leu	LysLeu	Lys + Ser	LysSer	Lys + Glu	LysGlu	Lys + Cys	LysCys	Lys + Phe	LysPhe	theoretical recovery (%)
750	760[c]	pyrazine	6.48	5.03	1.39	5.18	2.71	5.87	1.31	1.31	1.19	1.82	2.17	3.02	2.25	0.01	0.11	2.16	2.16	0.3
821	819[d]	methylpyrazine	0.15	0.15	0.04	0.20	0.10	0.15	0.03	0.05	0.06	0.05	0.05	0.06	0.09	tr[g]	0.04	0.03	0.13	0.8
913	912[d]	ethylpyrazine	–[f]	–	–	–	–	0.55	–	–	–	–	–	–	–	–	–	–	–	2.3
1055		(2-methylpropyl)-pyrazine[e]	–	–	–	–	–	–	–	0.03	–	–	–	–	–	–	–	–	–	
1166		2-(3-methylbutyl)-pyrazine[e]	–	–	–	–	–	–	–	2.10	–	–	–	–	–	–	–	–	–	
1243		methyl-(3-methylbutyl)-pyrazine[e]	–	–	–	–	–	–	–	0.07	–	–	–	–	–	–	–	–	–	
1585		(2-phenylethyl)-pyrazine[e]	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	4.43	–	
1733		5*H*-pyrazino(2,3-b)-indole[e]	0.28	0.11	–	0.16	–	0.16	–	0.07	–	–	–	–	–	–	–	0.01	–	
		total pyrazines	6.92	5.30	1.43	5.54	2.81	6.74	1.34	3.62	1.26	1.87	2.22	3.07	2.34	0.01	0.14	6.64	2.29	
		pyrazines (% of total GC-MS peak area)	44.1	46.6	91.0	58.5	57.0	52.2	92.2	9.2	24.9	84.3	89.1	81.5	89.6	5.9	38.8	20.2	49.2	

[a] LRI exptl, linear retention index determined experimentally on a HP5-MS stationary phase. [b] LRI lit., linear retention index value from literature. [c] Adams and De Kimpe (*25*). [d] Adams (*24*). [e] Tentatively identified. [f] –, not detected. [g] tr, trace.

but instead they are partitioned (or sorbed) into the PDMS phase. Thus, peak areas can be used to compare the amounts of a specific compound present in different solutions. However, different compounds have different extraction efficiencies, which makes the comparison of peak areas of different compounds more difficult. Partitioning coefficients between PDMS and water are proportional to octanol−water partitioning coefficients ($K_{o/w}$), and can be calculated. For some compounds, theoretical recovery values could be calculated with the Gerstel Twister Recovery Calculator software. These theoretical recovery values are also reported in the tables. Lipophilic compounds are retained more than hydrophilic compounds. Keeping this in mind, it is possible to compare the amounts of different compounds in a semiquantitative way.

The most accepted mechanism for pyrazine formation involves the condensation reaction of two α-aminocarbonyl compounds with the formation of a dihydropyrazine. This dihydropyrazine oxidizes spontaneously to the corresponding pyrazine. The initial α-aminocarbonyl compounds result mainly from the Strecker degradation between an amino acid and an α-dicarbonyl compound, being a product of carbohydrate degradation. When the intermediate dihydropyrazine reacts with a carbonyl compound in an aldol-type reaction, an alkylpyrazine with an additional substituent is formed and the oxidation step is not necessary due to dehydration followed by double-bond shifts to the aromatic species (*29*). When this reacting carbonyl compound is a Strecker aldehyde, amino acid specific pyrazines are formed, such as 3-ethyl-2,5-dimethylpyrazine in a methylglyoxal/alanine model system. Other pyrazines, however, such as 2,3-diethyl-5-methylpyrazine and 2,3,5-triethyl-5-methylpyrazine, require longer chain dicarbonyl compounds as starting materials, which can be formed through amino acid assisted chain elongation reactions of smaller dicarbonyl compounds (*30*, *31*).

The pyrazines produced during the reaction of the Lys-X dipeptides or the corresponding free amino acids with glucose are depicted in **Table 1**. It can be seen that pyrazines were produced more in the case of reactions with dipeptides as compared to reactions with free amino acids. Except for Lys-Leu, the total GC-MS peak area of pyrazines from reactions with dipeptides was 1.3−9.8 times higher than the total peak area of pyrazines from reactions with the corresponding free amino acids. For Lys-Leu, this difference can be explained by the high recoveries of the amino acid specific pyrazines in the model systems containing free amino acids, which have relatively high $K_{o/w}$ values and, therefore, create the impression that pyrazines were produced more in reactions with free amino acids. With regard to the relative share of pyrazines in the total peak area, it can be seen that pyrazines comprised a much bigger portion of the total volatiles produced in the case of the dipeptides as compared to the corresponding free amino acids. Especially 2,5(6)-dimethylpyrazine and trimethylpyrazine were produced more in reactions with dipeptides. In contrast to 2,5(6)-dimethylpyrazine and trimethylpyrazine, unsubstituted pyrazine was produced more in reactions with free amino acids. Conflicting results were found for methylpyrazine: in some model systems methylpyrazine was produced more in the case of free amino acids, whereas in other model systems the reactions with dipeptides resulted in the highest methylpyrazine amounts. Amino acid specific pyrazines, for instance, 3-ethyl-2,5-dimethylpyrazine from alanine, 2-(3-methylbutyl)pyrazine and 2,5-dimethyl-3-(3-methylbutyl)pyrazine from leucine, and 2-phenylethylpyrazine from phenylalanine, were produced more in the case of reactions with free amino acids (**Table 1**). In the reaction mixtures of the corresponding dipeptides, these pyrazines were also detected but only in small amounts.

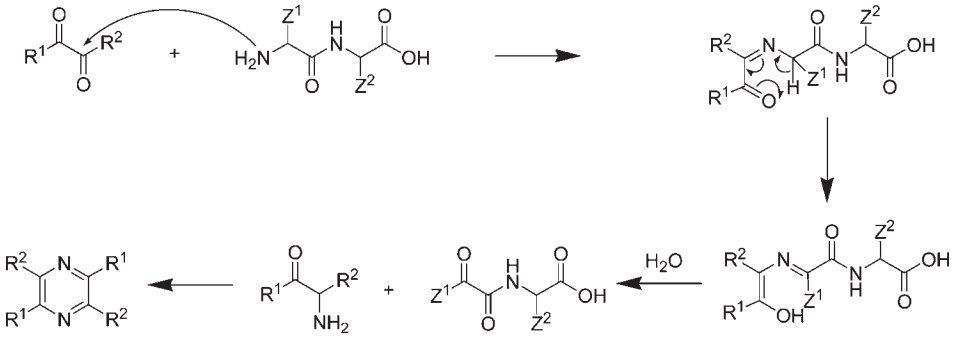
Besides glucose, model reactions were also performed with methylglyoxal and glyoxal, as the most common mechanism of pyrazine formation involves the condensation of two α-aminoketones resulting from the Strecker degradation of the amino acid initiated by an α-dicarbonyl compound and as methylglyoxal and glyoxal are the most common α-dicarbonyl compounds resulting from glucose degradation (*1*). Therefore, reaction of the dipeptides and amino acids with these compounds was expected to give a simpler pyrazine spectrum and, thus, a clearer view on the mechanism involved. In these cases, a 10-fold lower concentration of the dicarbonyl compound was used to avoid too much self-condensation reactions. Similar results were obtained for the model systems containing methylglyoxal or glyoxal.

Table 2 depicts the pyrazine formation of the reaction of the Lys-X dipeptides or the corresponding free amino acids with methylglyoxal. Also in this case, pyrazines were produced more in the case of the dipeptides compared to the free amino acids: the total GC-MS peak areas of pyrazines in the reactions with the dipeptides were 1.8−55.5 times the total GC-MS peak areas of pyrazines in the reactions with the corresponding free amino acids. In addition, pyrazines also comprised a bigger portion of the total volatiles in the case of reactions with dipeptides. It can be seen that, again, this difference is mainly caused by the high amounts of 2,5(6)-dimethylpyrazine and trimethylpyrazine produced in the model systems containing the peptides. As expected from the mechanism of pyrazine formation, unsubstituted pyrazine and methylpyrazine are almost not detected in methylglyoxal model systems because they require two or one two-carbon fragments, respectively. In case these compounds were detected, they are supposed to originate from the carbon skeleton of the amino acid, such as serine. The formation of pyrazines from serine and threonine without a carbohydrate source has been shown repeatedly (*25*,*32*,*33*). Possibly, other amino acids, such as lysine, can form reactive fragments that are incorporated in the pyrazine carbon skeleton. On the other hand, methylglyoxal might undergo fragmentation, resulting in less-substituted pyrazines. However, fragmentation of methylglyoxal has not been described in the literature yet. With regard to the amino acid specific pyrazines, only 3-ethyl-2,5-dimethylpyrazine from serine and 2,5-dimethyl-3-(3-methylbutyl)pyrazine from leucine were produced in substantial amounts. It can be seen that also for the model reactions with methylglyoxal, these amino acid specific pyrazines are produced much more in the case of the reactions with free amino acids.

The pyrazines produced during the reaction of the Lys-X dipeptides or the corresponding free amino acids with glyoxal are depicted in **Table 3**. Again, a larger portion of the total volatiles consisted of pyrazines in the case of reactions with dipeptides compared to the corresponding free amino acids: the share of pyrazines in the reactions with the dipeptides was 1.0−6.6 times the share of pyrazines in the reactions with the corresponding free amino acids. However, in terms of absolute peak area, in most cases, pyrazines were produced less in the case of reactions with dipeptides. As can be seen from **Table 3**, unsubstituted pyrazine was the main pyrazine detected. The lower amounts of unsubstituted pyrazine in reactions with dipeptides compared to reactions with the corresponding free amino acids are in accordance with the results obtained for the model systems containing glucose. In contrast to the reactions with glucose and methylglyoxal, for which low amounts were detected in the dipeptide model mixtures, the amino acid specific pyrazines ethylpyrazine, 2-isopentylpyrazine, and (2-phenylethyl)pyrazine were exclusively detected in the glyoxal model systems containing free amino acids and not in the model systems containing the corresponding dipeptides. These results indicate that the Strecker aldehydes from the C-terminal amino acid are not or to a much lesser extent produced in the model reactions containing glyoxal.

Comparison of these results with literature data suggests that this difference in pyrazine production could be caused by differences in reactivity of the α- and ε-amino group of free lysine and N-terminal peptide-bound lysine. Hwang et al. (*19*) studied the reaction of α-^{15}N-labeled free lysine with glucose, more specifically, the relative contribution of the α- and ε-amino groups of lysine in pyrazine production. These authors reported that in aqueous unbuffered model systems at pH 8.5, thermal treatment of reaction mixtures of lysine and glucose for 1 h at 180 °C leads mainly to the production of 2,6-dimethylpyrazine (2.4 μg/g), methylpyrazine (2.2 μg/g), unsubstituted pyrazine (1.5 μg/g), and trimethylpyrazine (0.4 μg/g). Besides, it was shown that unsubstituted pyrazine and methylpyrazine incorporated the highest relative amounts of nitrogen from the ε-amino group (about 40%), whereas this group was almost not incorporated (±2%) in 2,6-dimethylpyrazine and trimethylpyrazine. These two last compounds were almost exclusively produced from the α-amino group. The authors suggested that different mechanisms for transamination from α- and ε-amino groups of lysine lead to these results: α-amino groups produce α-aminoketones through Strecker degradation, whereas ε-amino groups form α-aminoketones by an intramolecular rearrangement followed by hydrolysis of the imine. It must be noted that, although the formation of 2,5-dimethylpyrazine is not mentioned by the authors, it most probably overlaps with 2,6-dimethylpyrazine under the chromatographic conditions described. Several authors reported the formation of 2,5-dimethylpyrazine from lysine before (*29*,*34*,*35*). Considering the findings of Hwang et al. (*19*) and the fact that, as compared to free lysine, Lys-X dipeptides produced less unsubstituted pyrazine, about equal amounts of methylpyrazine, and more of all other non-amino acid specific pyrazines, especially 2,5(6)-dimethylpyrazine and trimethylpyrazine (**Table 1**), it is hypothesized that, within the dipeptide, the α-amino group of lysine is more reactive, whereas the ε-amino group is less reactive as compared to free lysine. However, in the case of dipeptides, typical Strecker degradation involving decarboxylation followed by hydrolysis of the imine is not possible due to the absence of the free carboxyl group. Therefore, the formation of α-aminoketones must occur through a different mechanism. A hypothesized reaction mechanism for the formation of α-aminoketones from a dipeptide and an α-dicarbonyl compound is depicted in **Scheme 1**. In accordance to the reaction with free amino acids, the reaction of the α-dicarbonyl compound with the dipeptide starts with the formation of an imine. Afterward, deprotonation occurs at the α-position of the amide moiety. This proton is very acidic due to the presence of both the imine and the amide function. A 1,5-H-shift leads to enolization of the carbonyl of the α-aminoketone and formation of a 4-hydroxy-2-azadiene. Hydrolysis of the imino moiety of this 2-azadiene produces the α-aminoketone, without formation of the Strecker aldehyde of the N-terminal amino acid. Instead, the reaction results in the formation of a complex α-dicarbonyl compound. It can be expected that this α-dicarbonyl compound is less reactive than a Strecker aldehyde and therefore leads to fewer side reactions. It is assumed that this explains why pyrazines comprised a much bigger portion of the total volatiles in the case of dipeptides compared to free amino acids. An opposite behavior for unsubstituted pyrazine and 2,5-dimethylpyrazine and borderline behavior for methylpyrazine were also reported by Negroni et al. (*36*). These authors studied the effect of some important edible oils, for example, olive oil, canola oil, and sunflower oil, on the formation of volatiles from the Maillard reaction of lysine with xylose and glucose. A decreased production of unsubstituted pyrazine was

Scheme 1. Hypothetical Formation Mechanism of α-Aminoketones from the Reaction of Dipeptides with a Dicarbonyl Compound



always accompanied with an increased production of 2,5-dimethylpyrazine, suggesting that different mechanisms are responsible for their formation. The authors could not find a satisfying explanation for this observation.

The mechanism that leads to the production of the amino acid specific pyrazines, such as 3-ethyl-2,5-dimethylpyrazine from alanine, involves the reaction between the intermediate dihydropyrazine, which is formed by the condensation reaction of two α-aminocarbonyl compounds, and the Strecker aldehyde of the specific amino acid. The formation of 3-ethyl-2,5-dimethylpyrazine, 2,5-dimethyl-3-(3-methylbutyl)pyrazine, and 2-phenylethylpyrazine requires the presence of the Strecker aldehydes of alanine, leucine, and phenylalanine, respectively. Because of the peptide-bound NH_2 group, the Strecker aldehyde cannot be formed via the classical Strecker degradation from the peptides studied. Formation of the Strecker aldehyde of the amino acid at the C-terminus of the dipeptides seems unlikely. Therefore, detection of the Strecker aldehyde may indicate that peptide hydrolysis takes place to a limited extent during thermal treatment of 2 h at 130 °C. However, because the overall flavor profiles of the reaction mixtures with dipeptides and the reaction mixtures with free amino acids differ strongly, it can be assumed that this peptide hydrolysis is far from complete. This is in accordance with literature data, from which it is known that dipeptides are rather stable compounds which do not hydrolyze when they are heated in the absence of sugars at 100 °C (*26*), but which can hydrolyze when they are heated in the presence of sugars (*37*). It has been suggested that this peptide hydrolysis is caused by an intramolecular catalysis within the peptide−glucose Amadori rearrangement product (*38*). Although it would be interesting to know the exact percentage of hydrolysis under Maillard reaction conditions, it is impossible to measure because the resulting free amino acids immediately participate in the Maillard reaction and thus disappear.

As already mentioned before, the formation of amino acid specific pyrazines in the reaction mixtures with Lys-X dipeptides and glucose or methylglyoxal requires the presence of the Strecker aldehydes of the C-terminal amino acids and, therefore, probably indicates the hydrolysis of the peptide bond. Because it is not possible to determine the exact percentage of hydrolysis, the amount of Strecker aldehyde produced was used to estimate and compare the degree of hydrolysis. Under the analytical and chromatographic conditions applied, only the stable Strecker aldehydes of leucine and phenylalanine were detected. The peak areas of the Strecker aldehydes obtained from the reactions of glucose, methylglyoxal, or glyoxal with Lys-Leu, Lys-Phe, or the corresponding free amino acids are depicted in **Table 4**. It can be seen that in all cases, the peak areas of the Strecker aldehydes were much higher for the reactions between the carbonyl compound and the free amino acids as compared to the dipeptides. These results are in accordance with the above-described observation

Table 4. Strecker Aldehydes (GC-MS Peak Area × 10^8) Detected in the Model Reactions of Lys-Leu, Lys-Phe, or the Corresponding Free Amino Acids with Glucose, Methylglyoxal, or Glyoxal

		3-methylbutanal	phenylacetaldehyde
glucose	Lys-Leu	9.82	
	Lys + Leu	111.98	
	Lys-Phe		0.34
	Lys + Phe		4.30
methylglyoxal	Lys-Leu	0.62	
	Lys + Leu	81.98	
	Lys-Phe		0.05
	Lys + Phe		4.85
glyoxal	Lys-Leu	0.02	
	Lys + Leu	17.75	
	Lys-Phe		0.01
	Lys + Phe		0.75

that amino acid specific pyrazines are produced more in reactions with free amino acids and indicate that the peptide bond is hydrolyzed only to a limited extent. Peak area ratios of the Strecker aldehydes in model systems containing free amino acids as compared to model systems containing dipeptides were about 10, 100, and 100−1000 for glucose, methylglyoxal, and glyoxal, respectively. Although it is not possible to determine the exact percentage of hydrolysis, these peak area ratios indicate that the hydrolysis of the peptide bond was less pronounced in reactions with methylglyoxal and glyoxal as compared to reactions with glucose. Two possible causes may be hypothesized for this observation. On the one hand, 10 times lower amounts of methylglyoxal and glyoxal were used in the model reactions as compared to glucose. On the other hand, hydrolysis of the peptide bond can be higher in model systems containing glucose due to a mechanism of intramolecular catalysis within the peptide−glucose Amadori rearrangement product as described before (*38*). Because the Amadori product cannot be formed in reactions with methylglyoxal or glyoxal, this catalysis cannot occur in those cases. The range of the peak areas of the Strecker aldehydes produced in reactions with free amino acids suggests that the latter hypothesis is more likely. Because the peak areas of the Strecker aldehydes in the model reactions with free amino acids are comparable for glucose and methylglyoxal, the production of the Strecker aldehydes does not seem to be greatly influenced by the 10 times lower amounts of methylglyoxal used. Lower amounts of Strecker aldehydes were produced in the model reactions with glyoxal. These results suggest a lower reactivity of glyoxal as compared to methylglyoxal.

Although it is known that peptides are present in many food products and participate in the Maillard reaction (*2*), their contribution to flavor formation has been studied only rarely.

In this study, it was shown that the reaction of Lys-X dipeptides with glucose and two α-dicarbonyl compounds produced mainly pyrazines. Pyrazines are known to contribute significantly to the unique roasted aroma of many heated food products. The pyrazines described in this study generally have pleasant roasted, nutty flavor characteristics (*23*). Especially trimethylpyrazine, for example, which was produced much more in model systems containing dipeptides as compared to model systems containing free amino acids, has a very low odor threshold. These results indicate that for heat-treated food, also the production of flavor compounds from peptides should be taken into account. In this study, the C-terminal amino acid of lysine dipeptides was varied to study the influence of the neighboring amino acid on the flavor production of lysine. However, no clear influence of the neighboring amino acid could be distinguished. In general, all dipeptides revealed similar flavor profiles. Most likely, the differences that do occur are caused by reactive side chains of the C-terminal amino acid or by hydrolysis of the peptide bond and subsequent reaction of the liberated amino acid.

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