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Formation of Odorants in Maillard Model Systems Based on L-Proline as Affected by pH

IMRE BLANK,* STÉPHANIE DEVAUD, WALTER MATTHEY-DORET, AND FABIEN ROBERT

Nestec Ltd., Nestlé Research Center, P.O. Box 44, 1000 Lausanne 26, Switzerland

Formation of the odorants acetic acid, 4-hydroxy-2,5-dimethyl-3-(2*H*)-furanone (HDMF), 6-acetyl-1,2,3,4-tetrahydropyridine (ATHP), and 2-acetyl-1-pyrroline (AP) was monitored by isotope dilution assays at pH 6, 7, and 8 in Maillard model reactions containing glucose and proline (Glc/Pro) or the corresponding Amadori compound fructosyl-proline (Fru-Pro). In general, higher yields were obtained at pH 7 and 8. Acetic acid was the major odorant with up to 40 mg/mmol precursor followed by HDMF (up to 0.25 mg/mmol), the formation of which was favored in the Fru-Pro reaction systems. On the contrary, ATHP (up to 50 μg/mmol) and AP (up to 5 μg/mmol) were more abundant in Glc/Pro. However, the sensory relevance of the two *N*-heterocycles was more pronounced on the basis of odor activity values, confirming their contribution to the overall roasty note of the reaction samples. It was also found that formation and decomposition of Fru-Pro were faster at pH 7 as compared to pH 6, explaining in part the preferred formation of the four odorants studied under neutral and slightly alkaline conditions. After 4 h of reaction at pH 7 in the presence of proline, about one-fourth of the glucose was consumed leading to acetic acid with a transformation yield of almost 40 mol %.

KEYWORDS: Maillard reaction; L-proline; Amadori compound; *N*-(1-deoxy-D-fructos-1-yl)-L-proline; isotope dilution assay; GC-MS; odorants; anion exchange chromatography

INTRODUCTION

Maillard reaction systems based on L-proline (Pro) are well-known to generate roasty notes (*1*−*4*) that contribute to the aroma of many thermally treated foods such as cereal products (*5*, *6*). 2-Acetyl-1-pyrroline (AP) and 6-acetyl-1,2,3,4-tetrahydropyridine (ATHP), also referred to as 2-acetyltetrahydropyridine in the literature, are two roasty smelling impact odorants (**Figure 1**) identified in proline-containing foods such as Basmati rice (*7*), bread crust (*8*), toast (*9*), popcorn (*10*), and sweet corn-based products (*11*). These *N*-containing volatile compounds, along with 2,3-butanedione (diacetyl) and 4-hydroxy-2,5-dimethyl-3-(2H)-furanone (HDMF), have been reported as important odorants generated in dry-heated model systems containing D-glucose (Glc) and proline (*12*). In addition, Hofmann and Schieberle (*13*) found further *N*-containing compounds such as 2-propionyl-1-pyrroline and 2-propionyltetrahydropyridine in Glc/Pro systems under boiling conditions. The formation mechanisms of these *N*-heterocyclic odorants have been studied in great detail; their common precursor is 1-pyrroline, the Strecker degradation product of proline and ornithine, which reacts with 2-oxopropanal (2-oxobutanal) and 1-hydroxy-2-propanone (1-hydroxy-2-butanone) to yield AP, ATHP, and their higher homologues (*5*, *13*−*16*). An excellent review on α-acetyl-*N*-heterocycles has recently been published by Kerler and co-workers (*17*).

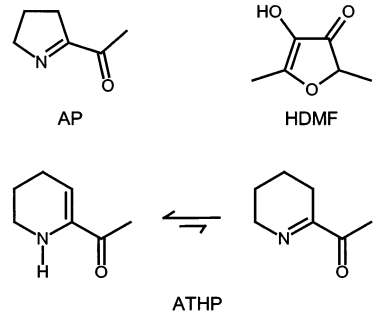

Figure 1. Chemical structures of AP, HDMF, and ATHP.

On the other hand, Amadori compounds have been claimed as nonvolatile flavor precursors in processed foods (*18*). These *N*-substituted 1-amino-1-deoxy-ketoses represent an important class of Maillard intermediates (*19*, *20*). They are formed in the initial phase of the Maillard reaction by Amadori rearrangement of the corresponding *N*-glycosylamines, the latter obtained by condensation of amino acids and aldoses, such as proline and glucose, for example (*21*). The Amadori compound *N*-(1-deoxy-D-fructos-1-yl)-L-proline (Fru-Pro) is a well-known constituent of dried apricots and peaches (*22*), cured tobacco leaves (*23*), white wine (*24*), malts, and beers (*25*). Fru-Pro has been the subject of several investigations dealing with its thermally

* To whom correspondence should be addressed. Tel: +21/785-8607. Fax: +21/785-8554. E-mail: imre.blank@rdls.nestle.com.

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induced degradation. Many volatiles have been identified after pyrolysis (*26*−*29*) or autoclave treatment (*30*) of Fru-Pro. However, there is not much known about their sensory relevance and the key odorants generated from Fru-Pro. Only recently, the roasty smelling odorants AP and ATHP were studied in Glc/Pro and Fru-Pro reaction samples (*31*).

In our previous work (*32*), we reported on the identification of odor active volatile compounds in Maillard systems based on Glc/Pro and Fru-Pro. Odorants of high intensity were screened by gas chromatography-olfactometry (GC-O) and identified by GC-mass spectrometry (GC-MS). The roasty smelling odorants AP and ATHP were perceived as more intense in Glc/Pro, whereas HDMF (caramel-like) and sotolone (seasoning-like) were more pronounced in Fru-Pro. No differences were observed for acetic acid (AcOH) and diacetyl. In this work, we focus on the quantification of four key odorants generated in Glc/Pro and Fru-Pro Maillard systems as affected by the pH of the reaction sample. In addition, an attempt is made to link the formation of key odorants to nonvolatile Maillard intermediates such as the Amadori compound Fru-Pro.

MATERIALS AND METHODS

Chemicals. Glc, disodium hydrogenphosphate, sodium dihydrogenphosphate, sodium sulfate, sodium acetate (NaOAc), diethyl ether (Et_2O), methanol (MeOH), and *n*-hexane were from Merck (Darmstadt, Germany). Pro, HDMF, 2-acetylpyridine, rhodium (5% on active alumina, Rh/Al_2O_3), d_1-methanol (MeO^2H), heavy water (^2H_2O), deuterochloroform (C^2HCl_3), acetone, Celite 560, Florisil (100−200 mesh), silver carbonate (50% on Celite, Ag_2CO_3), sodium hydroxide (50−52%, NaOH), and AcOH were from Aldrich/Fluka (Buchs, Switzerland). [$^{13}C_1$]Acetic acid (c-AcOH) was from S. I. C. (Inneberg, Switzerland). Argon, hydrogen, and deuterium (grade 28) were obtained from Carbagaz (Lausanne, Switzerland). The chemicals were of analytical grade. The solvents were distilled prior to use on a Vigreux column (100 cm × 1 cm). AP and 4-hydroxy-2(or 5)[^{13}C]methyl-5(or 2)-methyl-3-(2*H*)-[2(or 5)-^{13}C]furanone (c-HDMF) were prepared as previously described (*33*, *34*).

Model Reactions. *Quantification of Odorants.* Equimolar amounts (2 mmol) of glucose and proline or Fru-Pro (2 mmol) were dissolved in a phosphate buffer (20 mL, 0.2 M), and the pH was adjusted to 6, 7, or 8. Then, the solutions were refluxed for 1, 2, or 4 h. Quantification was achieved using isotope dilution assay (IDA) (*14*). Defined amounts of labeled internal standards were added to the cooled reaction mixture: 0.4 mL of *c*-AcOH (1100 μg/mL Et_2O), 0.2 mL of *c*-HDMF (182.5 μg/mL Et_2O), 0.05 mL of *d*-AP (47.7 μg/mL PG), and 0.05 mL of *d*-ATHP (289.0 μg/mL PG). The samples were separated in acidic (pH 3) and basic (pH 10) fractions using HCl (2 N) and NaOH (2 N), and the odorants were extracted with Et_2O (2×, 5 mL). Finally, the combined organic extracts were dried over anhydrous Na_2SO_4 and concentrated to about 1 mL with a stream of nitrogen prior to analysis by GC-MS. Each sample was prepared at least in duplicate.

Quantification of Nonvolatiles. Samples for monitoring the formation of Fru-Pro were prepared by refluxing glucose (0.9 g, 5 mmol) and proline (0.575 g, 5 mmol) in a phosphate buffer (50 mL, 0.2 mol/L) for 7 h. Aliquots (0.6 mL) were taken at defined time intervals and passed through a filter (0.45 μm). Samples were diluted with water, to be in the linear range of the calibration curves, and then analyzed by high-performance anion exchange chromatography (HPAEC). Similarly, the decomposition of Fru-Pro was studied by refluxing Fru-Pro (1.39 g, 5 mmol) in a buffered solution as described above and analyzing Fru-Pro by HPAEC. All samples were prepared at least in duplicate.

GC. This was performed on a Carlo Erba Mega 2 gas chromatograph equipped with a cold on-column injector and a flame ionization detector (FID). A DB-1701 or DB-Wax fused silica capillary column was used, 30 m × 0.32 mm, with a film thickness of 0.25 μm (J&W Scientific, Folsom, CA). A splitter was attached to the end of the capillary column to split the effluent 1:1 into the FID and the sniffing port. The chromatographic conditions were as follows: temperature program, 35 °C (2 min), 40 °C/min to 50 °C, 8 °C/min to 180 °C, 10 °C/min to 240 °C (10 min).

Table 1. Waveforms Used for the Analysis of Glc, Pro, and the Amadori Compound Fru-Pro by HPAEC Coupled with Electrochemical Detection

	potential (V)			potential (V)	
time (s)	Fru-Pro	Glc, Pro	time (s)	Fru-Pro	Glc, Pro
0.00	+ 0.05	–0.10	0.61	–0.15	
0.20	+ 0.05[a]	–0.10[a]	0.70		–0.10[b]
0.30		+ 0.35	0.71		+ 0.90
0.40	+ 0.05[b]	+ 0.35	0.90		+ 0.90
0.41	+ 0.75		0.91		–0.90
0.50		–0.10	1.00	–0.15	–0.90
0.60	+ 0.75				

[a] Starting with integration. [b] Integration stopped.

GC-MS. This was carried out on a HP-6890A GC coupled to a HP-5973N mass selective detector (Hewlett-Packard). Samples were injected splitless (1 μL). The electron impact (EI) MS spectra were generated at 70 eV. Qualitative measurements were performed on a DB-Wax (60 m × 0.25 mm, 0.25 μm film thickness) or HP-PONA (60 m × 0.25 mm, 0.25 μm film thickness) using the temperature program 20 °C (1 min), 70 °C/min to 60 °C, 4 °C/min to 240 °C.

Quantification by IDA (*14*) was performed in the EI-MS mode using the chromatographic conditions described above. AcOH and HDMF were quantified on the DB-Wax capillary column by measuring the molecular ions of analyte and labeled internal standard at *m*/*z* 60 (61) and *m*/*z* 128 (130), respectively. The amounts of AP and ATHP were determined on the HP-PONA by monitoring the molecular ions at *m*/*z* 111 (113−115) and *m*/*z* 125 (127−130), respectively. Quantification of the *N*-heterocycles was based on the first eluting tautomer. Each sample was injected twice.

HPAEC. The analyses were performed using a Dionex ion chromatography system (DX500, Dionex, Sunnyvale, CA) composed of an autosampler (model AS-50 with a 25 μL sample loop), a gradient pump (model GP-50) with on-line degas, and an electrochemical detector (model ED-40). The separation was accomplished on a 250 mm × 4 mm i.d. CarboPac PA10 anion exchange column (Dionex) and a 50 mm × 4 mm i.d. CarboPac PA10 guard column (Dionex). The solutions and eluents were prepared using ultrapure deionized water (specific resistivity ≥ 18.2 MΩ cm) from a Milli-Q-system (Millipore, Bedford, MA). NaOH solutions used as eluents were prepared by diluting a carbonate free 50−52% (w/w) NaOH solution in water previously degassed with helium gas. The following gradients were used, composed of water, NaOH (500 mM), and NaOAc (1 M) in %: (i) for Fru-Pro: 0.0 (87.5/10/2.5), 12.0 (52/40/8), 14.0 (30/50/20), 23.0 (30/50/20), 25.0 min (87.5/10/2.5); (ii) for glucose and proline: 0.0 (82/10/8), 7.0 (82/10/8), 9.0 (30/50/20), 18.0 (30/50/20), 20.0 min (82/10/8). The flow rate was 1 mL/min separating Glc (R_T = 2.5 min), Pro (R_T = 3.4 min), and Fru-Pro (R_T = 9.0 min), which were quantified with an electrochemical detector equipped with a gold working electrode. Each sample was injected twice. Quantification was based on calibration curves by comparing the peak areas with those of standard solutions containing known amounts of pure compounds. The waveforms used for the detection of Fru-Pro, glucose, and proline are shown in **Table 1**.

NMR Spectroscopy. The samples for NMR spectroscopy were prepared in WILMAD 528-PP 5 mm Pyrex NMR tubes, using deuterated water or chloroform (0.7 mL) as solvent. The NMR spectra were acquired on a Bruker AM-360 spectrometer, equipped with a quadrinuclear 5 mm probe head, at 360.13 MHz for ^{1}H and at 75.56 MHz for ^{13}C under standard conditions (*35*). Chemical shifts were measured relative to the solvent signal.

Syntheses. *Fru-Pro.* The Amadori compound Fru-Pro was synthesized as described in the literature (*20*, *26*, *29*) using some modifications. Glucose (39.3 g, 0.22 mol) and proline (28.2 g, 0.25 mol) were placed in a round flask. Anhydrous MeOH (300 mL) was added under a stream of argon and then refluxed for 7 h in an oil bath (72 °C). The reaction

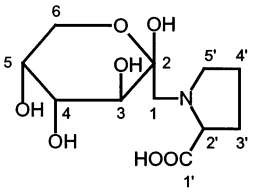

Figure 2. Chemical structure of the Amadori compound Fru-Pro.

mixture was cooled in a refrigerator, and the solvent was evaporated under reduced pressure to obtain about 200 mL of solution. The target compound was isolated by precipitation with dry acetone (350 mL) in a desiccator under argon. This procedure was repeated twice to purify Fru-Pro, finally obtaining a white powder after drying under reduced pressure (14 g, 20% yield) with a purity of 95% (HPAEC) containing residual glucose (4%) and proline (1%). The product is hygroscopic and must be stored under argon. ^{13}C NMR (90 MHz, ^2H_2O, δ/ppm): 23.8 (4′-CH_2), 28.7 (3′-CH_2), 57.8 (5′-CH_2), 61.1 (1-CH_2), 64.2 (6-CH_2), 69.3−70.4 (3-CH, 4-CH, 5-CH), 71.9 (2′-CH), 96.1 (2-CH_2), 174.3 (1′-COOH). The assignment of the signals was verified by a HETCOR experiment, in particular 2′-CH, 5′-CH_2, and 1-CH_2 (data not shown). In general, these data are in good agreement with those reported in the literature (*29*, *36*) and indicate that Fru-Pro occurred mainly in the β-pyranoid form (**Figure 2**), which usually represents the major conformation of Amadori compounds (*37*).

[$^2H_{2-5}$]ATHP. *d*-ATHP was prepared as a tautomeric mixture of isotopomers according to the Büchi and Wüst method (*38*) using some modifications. 2-Acetylpyridine (3 g, 24.8 mmol) was deuterated in MeO^2H (70 mL) in the presence of Rh/Al_2O_3 (3.54 g) for 12 h under pressure (5 bar) and gentle stirring using an autoclave with a glass vessel (100 mL). The reaction product was filtered through Celite (30 g) and concentrated at room temperature in vacuo (20 mbar, Rotavap, Büchi) yielding [$^2H_{5-11}$]-2-(1-hydroxyethyl)piperidine (3.7 g) as a crude orange oil with a purity of 95%. (GC): RI(DB-1701) = 1231, RI(HP-PONA) = 1078. MS-EI (*m*/*z*, %): ion clusters 134−140 (1−2, M^+), 115−123 (2−3, [M − H_2O]$^+$), 86−94 (10−100), 90 (100), 56−62 (5−30), 40−50 (2−10). ^{1}H NMR (360 MHz, C^2HCl_3, δ/ppm): 0.88 (s, 3H, CH_3), 0.90−1.00 (m, 1H, CH), 1.16−1.31 (m, 1H, CH), 1.40−1.52 (m, 1H, CH), 2.80−2.97 (m, 1H, N−CH). ^{13}C NMR (90 MHz, C^2HCl_3, δ/ppm): 18.8 (CH_3), 24.0, 25.6, 28.6, 46.8 (6-C), 61.9 (2-C), 70.0 (CH−O).

The intermediate (3.4 g) was dissolved in *n*-hexane (200 mL) and oxidized under a stream of argon with Ag_2CO_3 (13.5 g) by refluxing the solution at 72 °C overnight. The reaction product was filtered through Florisil (90 g) and eluted with hexane/Et_2O (1:1, by volume, 200 mL). The target compound was purified by distilling off the solvent under high vacuum (10^{-5} mbar) using the SAFE apparatus (*39*), leading to a solution of *d*-ATHP (215.5 μg/mL) with an overall yield of 1.4% and a purity of 85% (GC) represented by two tautomers, each of them composed of several isotopomers: [$^2H_{2-5}$]-6-acetyl-2,3,4,5-tetrahydropyridine (60%, RI(HP-PONA) = 1019) and [$^2H_{2-5}$]ATHP (40%, RI(HP-PONA) = 1115). The mass spectra of both tautomers (**Figure 3**) show characteristic ion clusters of deuterated compounds. On the DB-Wax column, *d*-ATHP was obtained as a broad peak (RI ≈ 1610), which is not recommended for quantification purposes due to poor chromatographic properties. The concentration of the *d*-ATHP solution was determined using *N*-acetylpiperidine as internal standard.

ATHP. ATHP was synthesized as a tautomeric mixture as described for *d*-ATHP by hydrogenation of 2-acetylpyridine in MeOH yielding 2-(1-hydroxyethyl)piperidine with 95% purity: RI(DB-1701) = 1231, RI(HP-PONA) = 1078, followed by oxidation of the intermediate leading to the target compound with a conversion yield of 98% (GC) and a purity of 97% (GC). The mass spectra of the two tautomers were in good agreement with literature data (*13*).

[$^2H_{2-4}$]AP. *d*-AP was synthesized as reported by Buttery et al. (*7*), which is based on the Büchi and Wüst method (*38*), following the procedure described above for *d*-ATHP using some modifications: Deuteration of 2-acetylpyrrole (2 g, 18.3 mmol) was performed with Rh/Al_2O_3 (2.35 g) in MeO^2H (50 mL) resulting in [$^2H_{2-9}$]-2-(1-hydroxyethyl)pyrrolidine (1.7 g) as an orange oil with a purity of 80% (GC): RI(DB-Wax) = 1591, RI(HP-PONA) = 993. MS-EI (*m*/*z*, %):

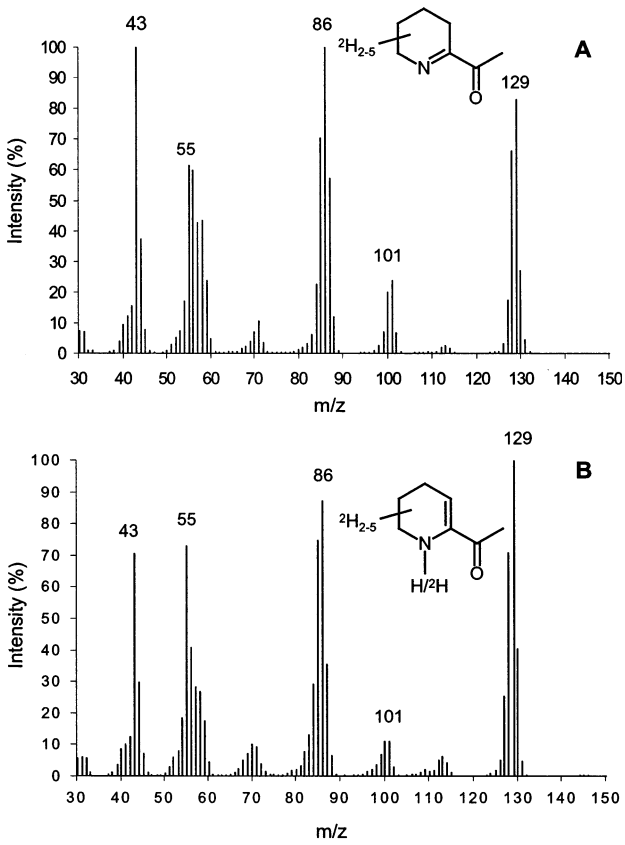

Figure 3. Mass spectra of the deuterated acetyltetrahydropyridine tautomers: (**A**) [$^2H_{2-5}$]-6-acetyl-2,3,4,5-tetrahydropyridine (60%); (**B**) [$^2H_{2-5}$]ATHP (40%).

ion clusters 118−123 (1, M^+), 101−106 (2−3, [M − H_2O]$^+$), 71−76 (10−100), 75 (100), 40−48 (2−10). ^{1}H NMR (360 MHz, C^2HCl_3) δ/ppm: 1.10 (s, 3H, CH_3), 1.56 (m, 1H, CH), 1.72 (m, 2H).

Oxidation of the intermediate was carried out in *n*-hexane (75 mL) with Ag_2CO_3 (13.5 g) by refluxing the solution at 72 °C overnight. Purification over Florisil and elution with hexane/Et_2O (1:1, by volume, 150 mL) gave a solution of *d*-AP (47.7 μg/mL) with an overall yield of 0.4% and a purity of 95% (GC) represented by two tautomeric forms: [$^2H_{3-5}$]AP (93%, RI(HP-PONA) = 891) and [$^2H_{3-5}$]-2-acetyl-2-pyrroline (7%, RI(HP-PONA) = 921). The mass spectra of both tautomers (**Figure 4**) show characteristic ion clusters of deuterated compounds. Only one peak was observed on the DB-Wax column at RI = 1355. The concentration of the *d*-AP solution was determined using *N*-methyl-2-acetylpyrrol as internal standard.

RESULTS

The effect of pH on the formation of odorants from Glc/Pro and Fru-Pro was studied in buffered aqueous solutions under boiling conditions using the same initial concentration of the precursors (0.1 mol/L). The experiments were carried out at pH 6, 7, and 8. As GC-O data only provide a rough estimation of the concentration, four odorants with high sensory intensities, AcOH, HDMF, AP, and ATHP (*32*), were quantified by IDA using the labeled analogues as internal standards (*14*) to substantiate differences between the Maillard systems Glc/Pro and Fru-Pro as affected by the pH. An HPEAC method coupled with electrochemical detection was developed to quantify the Amadori compound Fru-Pro.

Quantification of Selected Odorants. AcOH was the major odorant formed in both Maillard systems at all pH values studied (**Figure 5A**). The concentrations after 2 h of reaction varied from about 400 to 38 000 μg/mmol, which corresponds to up

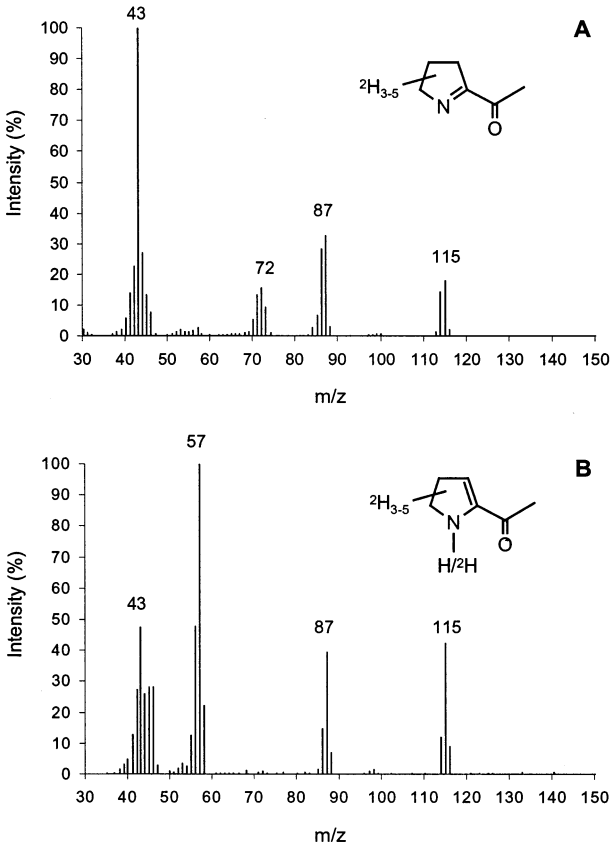

Figure 4. Mass spectra of the deuterated AP tautomers: (**A**) [$^2H_{3-5}$]AP (93%); (**B**) [$^2H_{3-5}$]-2-acetyl-2-pyrroline (7%).

to 60 mol % yield. However, in all cases, more AcOH was found in the samples containing the Amadori compound (Fru-Pro) as compared to those containing Glc/Pro. As a consequence, the pH in these samples dropped after 4 h of reaction by 1 unit from the original pH value (**Table 2**). In contrast, the pH remained unchanged in the Glc/Pro reaction systems over the whole reaction period, most likely due to the lower amounts of AcOH formed. The increase in AcOH concentration from Glc/Pro was particularly drastic at pH 8 (**Figure 5A**) where almost 20 mg/mmol was formed already after 1 h of reaction. Such high amounts were produced from Fru-Pro under milder conditions, namely, at lower pH, thus suggesting that the Amadori compound is readily decomposed leading to fragmentation products such as AcOH.

After 2 h of reaction, 0 to about 250 μg/mmol of the caramel-like smelling odorant HDMF was produced (**Figure 5B**), which is 100 times lower than AcOH, representing yields of up to 0.2 mol %. In general, the formation of HDMF was favored from the Amadori compound as compared to Glc/Pro. However, at pH 8, both Maillard systems resulted in comparable amounts of HDMF with similar kinetic curves. The Amadori system showed a drastic increase at pH 7 whereas Glc/Pro required pH 8 to significantly enhance the HDMF concentration. This behavior was similar to that observed for AcOH, indicating that their formation may follow similar pathways. However, HDMF can be generated by several mechanisms: with or without sugar fragmentation.

The formation of the roasty smelling odorant ATHP is shown in **Figure 5C**. Concentrations after 2 h of reaction varied from 2 to 45 μg/mmol, which was approximately 5 times less as compared to HDMF, representing yields of up to 0.04 mol %. In contrast to AcOH and HDMF, higher amounts were obtained from Glc/Pro as compared to the Amadori compound Fru-Pro, in particular at pH 6 and 7, whereas similar amounts were found in both Maillard systems at pH 8. These data also suggest that ATHP may be formed by other reaction pathways than AcOH and HDMF. Another difference to AcOH and HDMF was the observation that the formation of ATHP was favored at pH 7. It seems that at pH 8 more ATHP is decomposed than formed.

As shown in **Figure 5D**, AP was generated in very low amounts, i.e., about 0.5−4 μg/mmol, with overall yields of up to 0.004 mol % after a reaction period of 2 h, which is about 10 times less as compared to ATHP. Similar to ATHP, the formation of AP was favored in Glc/Pro as compared to Fru-Pro at all pH values studied. However, under slightly alkaline conditions (pH 8), the amounts of AP generated were similar in both Maillard systems. This was also observed for ATHP, but there, the concentrations were lower as compared to pH 7. The Amadori system gave the highest AP amounts at pH 8.

Quantification of Nonvolatile Flavor Precursors. The formation of the Amadori compound Fru-Pro in Maillard systems containing glucose and proline was monitored by HPAEC (**Figure 6A**). The concentrations were very low, up to 1.3 mg of Fru-Pro per mmol glucose, representing about 0.5 mol % yield. A continuous formation was observed at pH 6 with a rapid increase within 2 h that then flattened off to reach a plateau after about 4 h. Therefore, Fru-Pro can be accumulated at pH 6. On the contrary, at pH 7, the increase in Fru-Pro was faster, reaching a maximum after already 1 h. However, thereafter, the Amadori compound that formed was more rapidly decomposed: only half of the maximum amount could be detected after 7 h of reaction.

The amount of glucose was monitored during the Maillard reaction as it is consumed by the formation of Fru-Pro, which in turn can further react and give rise to many sugar degradation products. As shown in **Table 3**, more glucose was consumed at pH 7 and more rapidly than at pH 6. After a reaction period of 7 h, about 40% of glucose was lost at pH 7 as compared to 10% at pH 6.

Degradation of Fru-Pro was more pronounced at pH 7 (**Figure 6B**). Less than 10% of the Amadori compound was left at pH 7 after a reaction period of 1 h, whereas at pH 6 about 40% was still available. Therefore, it is not surprising that rapid formation of Fru-Pro at pH 7 cannot compensate for the losses.

DISCUSSION

Reaction of glucose and proline leads via pathway A to the corresponding *N*-glycosyl derivative **I**, which then enolizes to various intermediates such as the enaminol derivative **II**, the Amadori compound **III** (Fru-Pro), and the endiol derivative **IV** as shown in **Figure 7** (*19*, *40*). Pathway B is favored under acidic conditions and results by 1,2-enolization in 3-deoxy-2-hexosulose (**V**), with formic acid and 5-hydroxy-2-furaldehyde as characteristic degradation products. Pathway C preferably proceeds under alkaline conditions leading by 2,3-enolization to 1-deoxy-2,3-hexodiulose (**VI**) with AcOH and 2,3-dihydro-3,5-dihydroxy-6-methyl-4(*H*)-pyran-4-one as typical reaction products.

The highest concentrations of AcOH were obtained at pH 8 as compared to pH 7 and 6, the latter being in general the least efficient reaction system in generating odor active compounds. The formation of AcOH was favored from Fru-Pro, in particular at pH 6 and 7, which means under milder conditions, suggesting that the Amadori compound is readily decomposed leading to AcOH by fragmentation. This reaction is one of the main

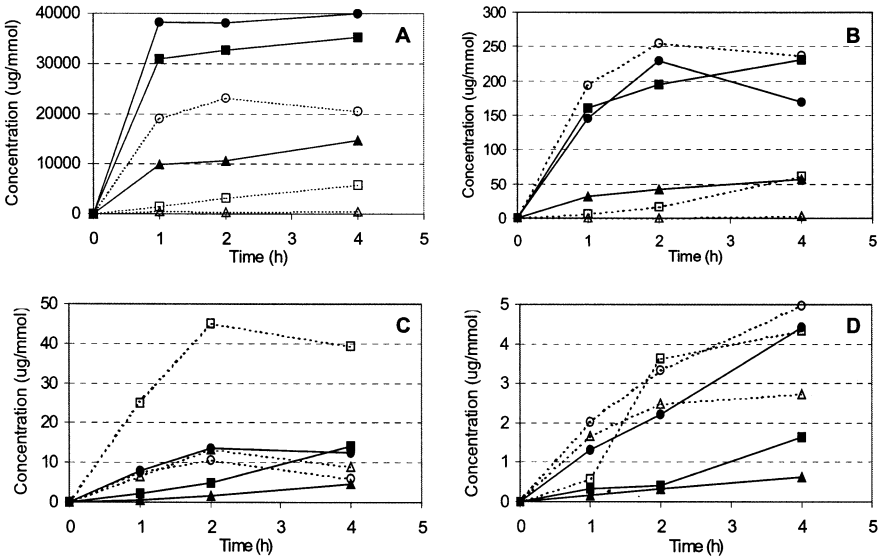

Figure 5. Generation of AcOH (**A**), HDMF (**B**), ATHP (**C**), and AP (**D**) from Glc/Pro (· · ·) and the Amadori compound Fru-Pro (—) under boiling conditions at pH 6 (△ ▲), pH 7 (□ ■), and pH 8 (○ ●). The coefficient of variation was lower than 20% for concentrations (μg/mmol) above 300 (**A**), 5.0 (**B**), 1.0 (**C**), and 0.3 (**D**).

Table 2. Evolution of the pH in the Maillard Reaction Samples Based on Glc/Pro and the Amadori Compound Fru-Pro[a]

reaction time (h)	pH 6		pH 7		pH 8	
	Glc/Pro	Fru-Pro	Glc/Pro	Fru-Pro	Glc/Pro	Fru-Pro
0	6.00	6.00	7.00	7.00	8.00	8.00
1	6.15	5.80	7.04	6.70	8.04	7.30
2	6.16	5.42	7.00	6.30	8.02	7.02
4	6.13	5.05	7.00	6.20	8.02	6.97

[a] The samples were buffered with phosphate (0.2 mol/L). The pH values were measured in an aliquot of the reaction sample after rapidly cooling to room temperature.

degradation pathways of 1-deoxy-2,3-diuloses resulting in up to 60 mol % of AcOH, which is mainly responsible for the pH drop observed particularly in the Amadori system (**Table 2**). These high yields are in line with recent literature data. Degradation of the casein-bound Amadori compound (120 °C, 40 min, pH 6.8) yielded 50 mol % of AcOH (*41*) and *N*-(1-Deoxy-D-fructos-1-yl)glycine gave rise to about 60 mol % of AcOH (90°C, 5 h, pH 7.0) (*40*).

The relatively high amounts of AcOH in the Glc/Pro system at pH 8 can be explained by the preferred formation and decomposition of the Amadori compound under alkaline conditions (**Figure 7**). In the Maillard system Glc/Pro at pH 7, AcOH was continuously generated reaching 3.3 and 5.8 mg/mmol sugar after 2 and 4 h (**Figure 5A**), respectively, which corresponds to about 5 and 10 mol % yield (**Table 4**). In the same period of time, about 10 and 25 mol % of glucose was consumed after 2 and 4 h of reaction (**Table 3**), respectively, thus indicating that almost half of the sugar consumed was transformed into AcOH at pH 7. Fru-Pro is rapidly decomposed under the same reaction conditions (**Figure 6B**), of which 50−60 mol % was transformed to AcOH (**Table 4**). The absolute yields at pH 6 in the Amadori system increased from 17 (1 h) to 25 mol % (4 h) with transformation yields of about 30 mol % (**Table 4**). These data suggest that at pH 6 less AcOH is formed in total and that the transformation rate from sugar to AcOH is half as much as compared to pH 7.

HDMF showed a behavior similar to that of AcOH. The formation of HDMF was also favored from Fru-Pro, in particular

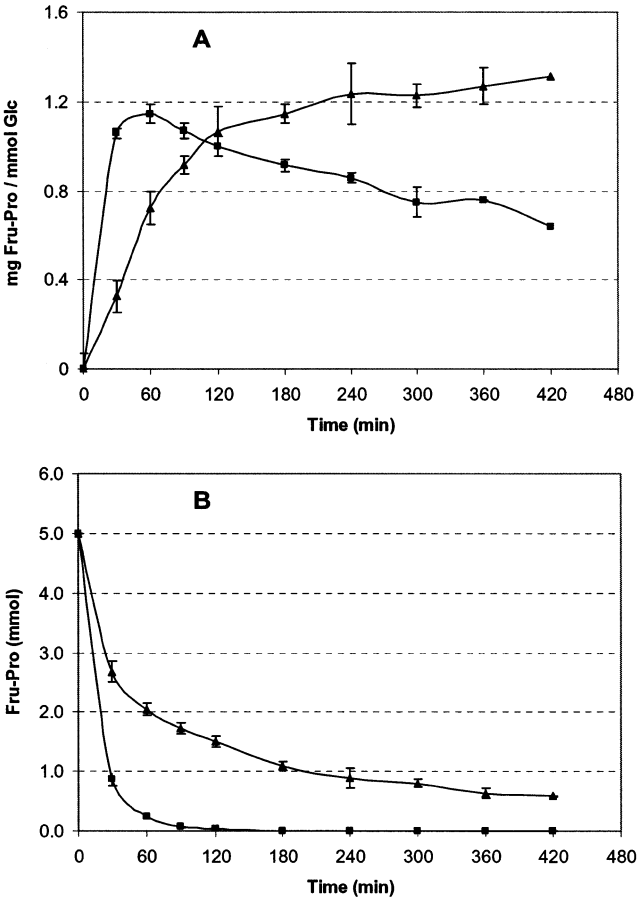

Figure 6. (**A**) Formation of the Amadori compound Fru-Pro from Glc and Pro and (**B**) degradation of Fru-Pro under boiling conditions at pH 6 (▲) and pH 7 (■).

at pH 6 and 7. The Amadori compound *N*-(1-deoxy-D-fructos-1-yl)glycine has also been demonstrated to generate more HDMF at pH 6 and 7 as compared to its precursors, glucose and glycine (*42*). The reaction goes through 1-deoxy-2,3-hexodiuloses (**Figure 7**, pathway C) with acetylformoine as the key intermediate (not shown). This pathway requires a reduction step, which may take place in the presence of reductones, readily

Table 3. Consumption of Glc in the Presence of Pro[a]

reaction time (min)	concentration (mmol/50 mL)		loss of glucose (mol %)	
	pH 6	pH 7	pH 6	pH 7
0	5.00	5.00	0	0
30	4.95 ± 0.10	4.47 ± 0.10	1	11
60	4.93 ± 0.06	4.75 ± 0.33	1	5
90	4.59 ± 0.10	4.49 ± 0.10	8	10
120	4.74 ± 0.09	4.44 ± 0.24	5	11
180	4.66 ± 0.05	4.02 ± 0.15	7	20
240	4.65 ± 0.05	3.68 ± 0.40	7	26
300	4.33 ± 0.11	3.45 ± 0.05	13	31
360	4.44 ± 0.08	3.37 ± 0.06	11	33
420	4.44 ± 0.13	3.05 ± 0.07	11	39

[a] Glucose and proline were refluxed in a phosphate buffer (0.2 mol/L) for up to 7 h. Aliquots were analyzed by HPAEC as described in the Experimental Section.

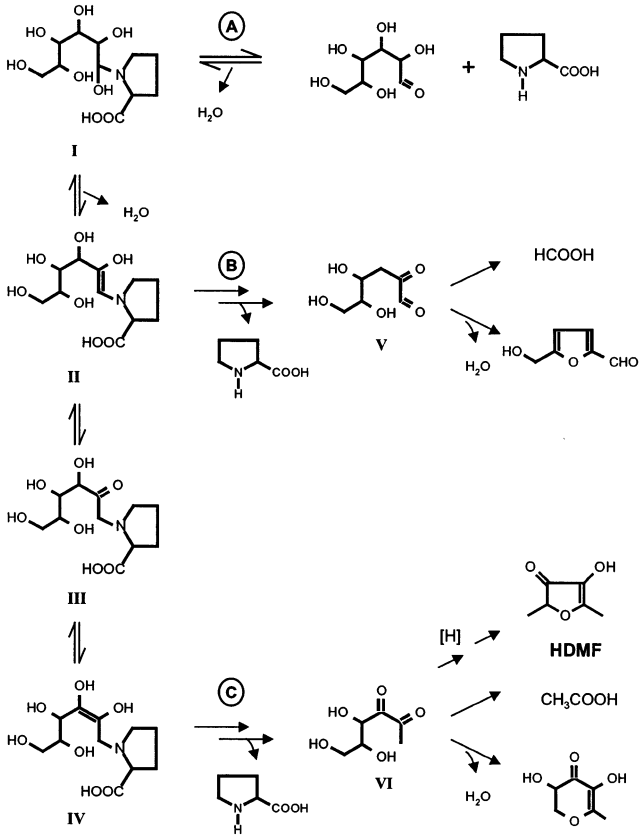

Figure 7. Simplified schematic presentation of parts of the Maillard reaction focusing on the formation of early Maillard intermediates (**A**) and their degradation via the 1,2- (**B**) and 2,3-enolization (**C**) pathways. The structures are shown in the open chain form, which are in equilibrium with cyclic species.

available in Maillard reactions (*42*, *43*). On the other side, HDMF can also be formed by recombination of sugar fragmentation products such as hydroxy-2-propanone (**VII**) and 2-oxopropanal (**VIII**) (**Figure 8**), which are readily available in Maillard reaction samples (*44*). Aldol reaction of **VII** and **VIII** leads to an intermediate with two methyl groups that can easily generate HDMF by enolization, dehydration, and cyclization. Indeed, HDMF could easily be identified by GC-MS after reacting **VII** and **VIII** at 90 °C in a phosphate-buffered solution at pH 7 for 1 h (data not shown). Thus, the favored sugar fragmentation under alkaline conditions leading to various reactive C2 and C3 units may explain the high amounts of

Table 4. Absolute and Relative Yields (mol %) of AcOH Generated from Glc/Pro and the Amadori Compound Fru-Pro in the Maillard Reaction[a]

time (t)	Glc/Pro (pH 6)		Glc/Pro (pH 7)		Fru-Pro (pH 6)		Fru-Pro (pH 7)	
	abs	rel	abs	rel	abs	rel	abs	rel
1	nd		2.5	50	16.6	28	51.6	54
2	nd		5.4	50	17.8	25	54.6	55
4	1.0	13	9.7	37	24.5	30	58.7	59

[a] The absolute (abs) yield was calculated on the basis of AcOH determined in the sample as related to the amount of Glc or Fru-Pro used in the reaction. The relative (rel) yield was calculated on the basis of AcOH determined in the sample as related to the amount of Glc or Fru-Pro consumed in the reaction. Not detected, nd.

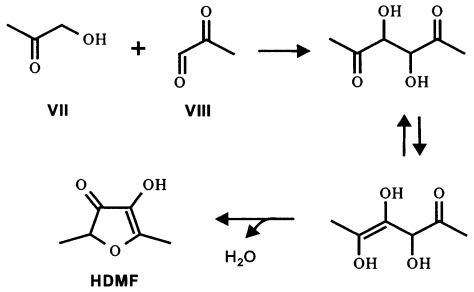

Figure 8. Formation of HDMF by recombination of the C_3 sugar fragmentation products **VII** and **VIII**.

HDMF found in the Glc/Pro system at pH 8. However, this may also be due to the preferred formation and decomposition of the Amadori compound under alkaline conditions (**Figure 7**).

The concentrations of the roasty smelling odorants ATHP and AP were significantly lower as compared to AcOH and HDMF. They were preferably generated from Glc/Pro, less from Fru-Pro, thus suggesting that there is no benefit in using the Amadori compound for the formation of ATHP and AP, which is in agreement with the recent observation of Weenen and van der Ven (*31*). All samples contained more ATHP than AP, confirming data reported by Schieberle (*14*) who obtained 38 μg of ATHP per mmol proline (pH 7, 2 h, boiling), which is close to the 45 μg/mmol found in this work.

In contrast to AcOH and HDMF, *N*-heterocyclic odorants were more abundant in the Glc/Pro samples, indicating that they are formed by other reaction pathways. As shown in **Figure 9**, the common precursor of ATHP and AP is 1-pyrroline (**IX**), the Strecker degradation product of proline, which reacts with the sugar degradation products **VII** (pathway D) and **VIII**, shown in the hydrated form (pathway E), to generate ATHP and AP, respectively (*16*). It seems that Glc/Pro represents a better system than the Amadori compound for generating these intermediates in high concentrations at a given reaction time. However, the reaction yields reported in the literature are moderate, about 1 mol % ATHP and 5 mol % AP when reacting equimolar amounts of **IX** with **VII** and **VIII** (pH 7, 30 min, boiling), respectively (*16*). In view of the high instability of **IX** and the probably low amounts generated in the Maillard reaction, **IX** seems to be the limiting factor in forming ATHP and AP. However, reliable quantitative data of **IX** under Maillard reaction conditions are missing in the literature.

Despite the low yields, both *N*-heterocyclic odorants play an important role for the overall aroma due to their low odor thresholds. The sensory relevance of odorants can be estimated by comparing their odor activity values (OAV), i.e., the ratio

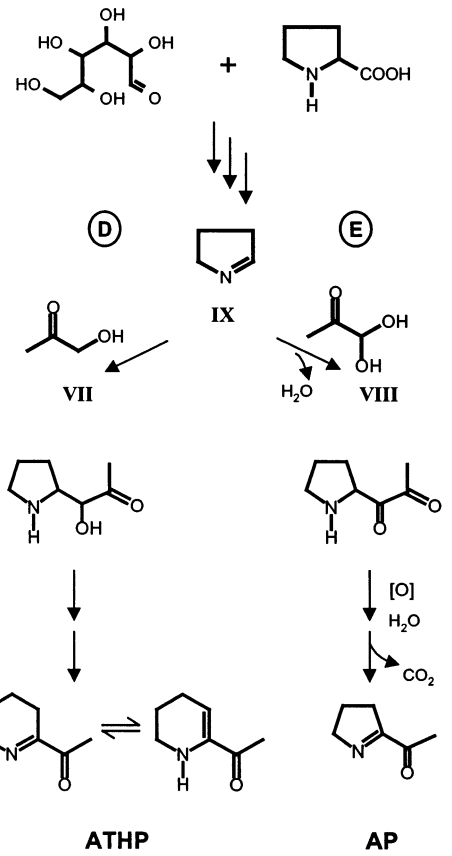

Figure 9. Simplified scheme showing the formation of AP and ATHP by reacting **IX** with **VII** and **VIII**, the latter shown in the hydrated form (adapted from (*4*)).

Table 5. OAV of AcOH, HDMF, ATHP, and AP in Maillard Reaction Samples Based on Glc/Pro and the Amadori Compound Fru-Pro[a]

	Maillard	AcOH		HDMF		ATHP		AP	
	systems	concn	OAV	concn	OAV	concn	OAV	concn	OAV
pH 6	Glc/Pro	41	<1	<0.05	<1	1.3	810	0.25	2500
	Fru-Pro	648	13	4	67	0.2	125	0.03	300
pH 7	Glc/Pro	325	6.5	1.6	27	4.5	2810	0.36	3600
	Fru-Pro	3274	65	20	330	0.5	310	0.04	400
pH 8	Glc/Pro	2310	46	26	430	1.1	690	0.33	3300
	Fru-Pro	3810	76	23	380	1.3	810	0.22	2200

[a] The concentrations were taken from samples reacted for 2 h. The OAVs were calculated by dividing the concentration (concn, in mg/L) of each odorant by its nasal odor threshold (mg/L) determined in water: AcOH, 50 (*45*); HDMF, 0.06 (*46*); ATHP, 0.0016 (*47*); and AP, 0.0001 (*7*). Note that the odor thresholds in water were determined without adjusting the pH to 6, 7, and 8.

of concentration to odor threshold determined in water. As shown in **Table 5**, both *N*-heterocyclic odorants showed high sensory relevance based on high OAVs and dominated especially in the Glc/Pro samples. These results also show that AP, occurring in very low concentrations, does significantly contribute to the roasty note. On the contrary, AcOH plays a minor role as aroma compound. Its major contribution is guiding Maillard reaction pathways by lowering the pH. Finally, the importance of HDMF is evidenced by its high OAV, particularly in the Fru-Pro samples.

In conclusion, AcOH was the most abundant odorant in both Glc/Pro and Fru-Pro Maillard systems formed by major reaction pathways in the Maillard reaction. On the contrary, HDMF and in particular the two *N*-heterocyclic odorants were minor reaction products formed by side reactions, which require various intermediates to be available at a given time. Consequently, aroma generation of such odorants may be improved on the basis of a more precise insight into dynamic changes of key intermediates as affected by pH, temperature, and other reaction parameters.

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