Quantitation of Important Roast-Smelling Odorants in Popcorn by Stable Isotope Dilution Assays and Model Studies on Flavor Formation during Popping

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Stable isotope dilution assays were developed for the quantitation of the roast-smelling popcorn odorants, 2-acetyl-tetrahydropyridine (ACTPY) and 2-propionyl-1-pyrroline (PPY). Both and, in addition, the two further roast-aroma compounds, 2-acetyl-1-pyrroline (ACPY) and acetylpyrazine, were quantified in different popcorn samples. In fresh hot-air popped corn, ACTPY showed the highest concentration (437 μg/kg), followed by ACPY (24 μg/kg) which were established as the key contributors to the roasty popcorn odor. During storage of a popcorn sample for seven days in a sealed polyethylene bag, the concentrations of ACTPY, ACPY, and PPY decreased to about one third. Model studies using aqueous maize extracts and distinct precursor compounds revealed the pair proline/fructose as the most effective precursor system in ACTPY formation, while the pair 1-pyrroline/2-oxopropanal was most effective in the generation of ACPY. A reaction scheme suggesting that ACPY is formed by an "acylation" of the intermediate 1-pyrroline by 2-oxopropanal is discussed.

Keywords: *2-Acetyltetrahydropyridine; 2-acetyl-1-pyrroline; 2-propionyl-2-pyrroline; 2-benzoyl-1-pyrroline; stable isotope dilution assay; popcorn; flavor formation*

INTRODUCTION

By aroma extract dilution analysis, we could recently (Schieberle, 1991) identify the roast-smelling 2-acetyltetrahydropyridine (ACTPY; two tautomers), 2-acetyl-1-pyrroline (ACPY), and 2-propionyl-1-pyrroline (PPY) with high odor activities in the overall popcorn flavor. A fourth roasty odorant, acetylpyrazine, appeared with a lower flavor dilution (FD) factor among the 23 odorants identified. Very recently, ACPY was also established as the key crackerlike odorant in sweet corn products (Buttery et al., 1994) with 44 μg/kg of ACPY being found in a sample of canned cream corn.

The amounts of the four odorants present in fresh popcorn are as yet unknown. In a quantitative study of bread crust odorants, we (Schieberle and Grosch, 1987) previously developed a stable isotope dilution assay to quantitate the relatively unstable ACPY as well as acetylpyrazine. The purpose of the following investigation was to develop isotope dilution assays also for ACTPY and PPY and to quantitate the four roasty odorants in different popcorn samples. Furthermore, some investigations were performed to gain insights into the factors governing the formation of, especially, ACPY and ACTPY during popping of corn.

EXPERIMENTAL PROCEDURES

Popcorn. Corn grown in the United States was purchased in a local shop. Fresh popcorn (200 g) was prepared by using either a hot-air corn-popper (PP 105; IMEX Corp.) or by popping the kernels in a surface-coated pan containing a small amount (5 g) of commercial sunflower oil.

Chemicals. 2-Acetylpyridine, acetylpyrazine, 2-oxo-2-phenylacetaldehyde (phenylglyoxal hydrate), and 2-oxopropanal (40% in H_2O) were from Aldrich (Steinheim, Germany).

Synthesis. *[^{2}H]-2-Acetyltetrahydropyridine ([^{2}H]-ACTPY; d-I).* The synthesis was performed following the method described for the undeuteriated compound (Büchi and Wuest, 1971) with slight modifications: 2-Acetylpyridine (20 mmol) was dissolved in 20 mL of CH_3OD and deuteriated for 20 h at 1000 Pa in the presence of rhodium on alumina as the catalyst (0.8 g) by using a laboratory autoclave (Type I; Roth, Karlsruhe, Germany). The suspension was then filtered over Celite (type Celatom FW-50; Aldrich, Germany) and the d_1-methanol was distilled off. The residue was taken up in 200 mL of toluene which had been presaturated with nitrogen and, after addition of silver carbonate on Celite, heated and intensely stirred for 10 h. The suspension was then filtered over Celite and 50 mL of the dark-brown solution placed on the top of a water-cooled column (10–12 °C) filled with a slurry of 37 g neutral alumina (deactivated with 10% water, g/g) in pentane/diethyl ether (95 + 5, v/v). After elution of the toluene with 80 mL of pentane/diethyl ether (95 + 5, v/v), d-I was isolated with 150 mL of pentane/diethyl ether (1 + 1, v/v). To remove weakly bound deuterium atoms, the solution was shaken for 10 min with aqueous hydrochloric acid (50 mL; 2 mol/L), followed by aqueous sodium hydroxide (50 mL; 2.5 mol/L), which was added to the mixture with cooling. The organic layer was then washed with a saturated sodium chloride solution and dried over anhydrous Na_2SO_4. The concentration of d-I was determined by gas chromatography on capillary column CP-WAX/Amine (conditions cf. below) by using trimethylpyridine as the internal standard and by using an FID response factor (1.05) determined from a mixture of 2-acetylpyridine and trimethylpridine.

[^{2}H]-2-Propionyl-1-pyrroline ([^{2}H]-PPY; d-II). The labeled compound was prepared starting from 2-propionylpyrrole as previously reported for the unlabeled compound (Schieberle, 1991). The labeling was performed in CH_3OD by using deuterium instead of hydrogen in the preparation of the intermediate [^{2}H]-2-(1-hydroxypropyl)pyrrolidine. Purification by column chromatography and quantitation was performed as described above for d-I.

[^{2}H]-2-Acetyl-1-pyrroline ([^{2}H]-ACPY; d-III) and [^{2}H]-acetylpyrazine (d-IV) were synthesized as previously reported (Schieberle and Grosch, 1987). The following compounds were prepared according to the literature given in parentheses: 2-acetyltetrahydropyridine (Büchi and Wuest, 1971), 2-propionyl-1-pyrroline (Schieberle, 1991), and 2-acetyl-1-pyrroline (Buttery and Ling, 1982).

1-Pyrroline. The compound was synthesized from L-proline following the method of Bragg and Hough (1958) with some modifications: L-proline, (40 mmol) dissolved in water (100

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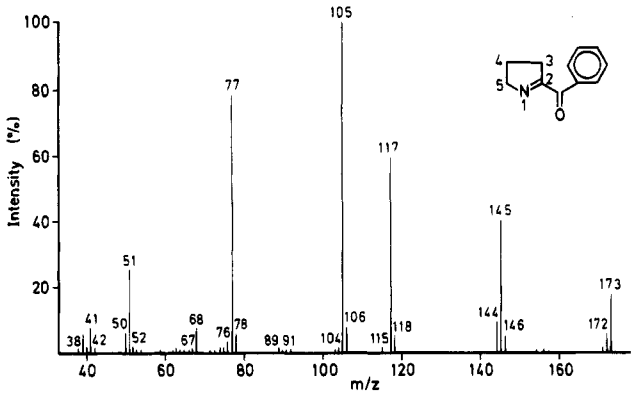


Figure 1. Mass spectrum of 2-benzoyl-1-pyrroline.

mL) was oxidized to 1-pyrroline by dropwise addition of an aqueous solution (100 mL) of sodium metaperiodate (0.42 mol/L). After stirring for 150 min in the dark, the mixture was adjusted to pH 10.5 (sodium hydroxide; 2 mol/L) with cooling and extracted with diethyl ether (total volume 200 mL). After drying over Na_2SO_4, the organic layer was concentrated to 20 mL on a Vigreux column (60 cm × 1 cm i.d.) and an aliquot (2 mL) was immediately applied onto a water-cooled column (20 cm × 1 cm; 12 °C) filled with neutral alumina (10% water content) in *n*-pentane. After flushing with *n*-pentane (50 mL), 1-pyrroline was eluted with *n*-pentane/diethyl ether (8 + 2 v/v; 100 mL) and its concentration determined by HRGC on capillary CP-WAX/A using pyrimidine as the internal standard. 1-Pyrroline was characterized by its mass spectrum (MS/EI) *m/z* 41 (100%), *m/z* 69 (68%), *m/z* 42 (40%), *m/z* 54 (8%), which was in good agreement with data published by Yoshikawa et al. (1965). For ^{1}H-NMR measurements, 1-pyrroline was purified by distillation (bp 91 °C), using a microdistillation apparatus (Mikrospaltrohrkolonne; Fischer, Meckenheim, Germany). The ^{1}H-NMR data were in very good agreement with those of Bock and Dammel (1987). It should be noted that, due to the high reactivity of the 1-pyrroline, very low yields were obtained by the distillation procedure. Therefore, in the model experiments, the eluates obtained by column chromatography were used immediately after blowing off the solvent.

2-Oxobutanal. Methyl dimethoxyacetate (40 mmol) was reacted with propionic acid methyl ester (40 mmol) in the presence of sodium methylate as described by Royals and Robinson (1956). The ethyl α-methyl-γ,γ′-dimethoxyacetoacetate formed was purified by microdistillation (Mikrospaltrohrkolonne; Fischer; bp 102 °C, 15 mbar) and then treated with aqueous potassium hydroxide (2 mol/L) to obtain the dimethylacetal of 2-oxobutanal. The 2-oxoaldehyde was freed from its acetal by treatment with H_2SO_4 (20 mL; 5% by weight) and, after adjusting of the pH to 7.0, this solution was immediately used in the model reactions.

2-Benzoyl-1-pyrroline. 1-Pyrroline (1 mmol) and phenylglyoxal (1 mmol) were reacted with reflux for 2 h in phosphate buffer (400 mL; 0.1 mol/L; pH 7.0). After cooling, the solution was extracted with diethyl ether (total volume 200 mL) and the organic layer, after drying over Na_2SO_4, concentrated to 1 mL by using a Vigreux column (60 cm × 1 cm). The solution was placed onto the top of a water-cooled (12 °C) column (20 cm × 1 cm) and filled with a slurry of 8 g of silica gel in *n*-pentane/diethyl ether (95 + 5 v/v). After flushing with this solvent mixture (25 mL), the target compound was isolated by elution with *n*-pentane/diethyl ether (85 + 15 v/v) and characterized by its mass spectrum (Figure 1). The molecular peak (M^+ *m/z* 173), which was established by MS/CI, as well as the fragments *m/z* 77 (phenyl) and *m/z* 105 (benzoyl) were well in line with the structure displayed in Figure 1. The fragment *m/z* 145, resulting from either elimination of C=O or C_2H_4, respectively, corresponds well to the fragments *m/z* 83 or *m/z* 97 in the respective spectra of 2-acetyl- (Schieberle and Grosch, 1987) or 2-propionyl-1-pyrroline (Schieberle, 1991). High resolution mass spectrometry revealed a sum formula of C_8H_7N for the fragment *m/z* 117, suggesting the indoyl cation.

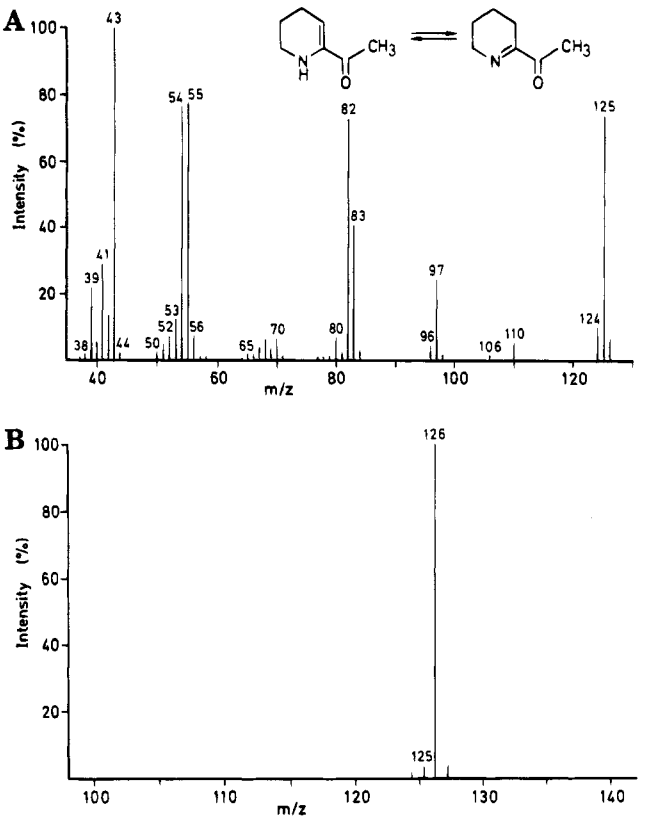


Figure 2. Mass spectra (MS/EI: A; MS/CI: B) of 2-acetyltetrahydropyridine (ACTPY).

Table 1. Selected Ions Used in the Quantitation of the Four Roast Aroma Compounds by Mass Chromatography (MS/CI)

compound	m/z unlabeled	m/z labeled	fraction[a]
2-acetyltetrahydropyridine[b]	126	128–132	C
2-propionyl-1-pyrroline	126	129–130	C
2-acetyl-1-pyrroline	112	114–118	D
acetylpyrazine[c]	123	126	D

[a] Fraction in which the odorant and the internal standard were isolated by column chromatography. [b] The intensities of both tautomers separated by HRGC (cf. Figure 5) were summed up. [c] Analysis was performed on column OV-1701.

The ^{1}H-NMR spectrum (360 MHz, CD_2Cl_2) showed three signals of the pyrroline ring at δ 1.89 (m, 2H, H at C-4 (Figure 1)), δ 2.83 (m, 2H at C-3), and δ 4.10 (m, 2H at C-5) and three signals of the phenyl ring at δ 7.38 (m, 2H), δ 7.50 (m, 1H) and δ 8.07 (m, 2H). The chemical shifts of the protons in the pyrroline ring were in good agreement with data obtained for the homologues, 2-acetyl-1-pyrroline (Buttery and Ling, 1982) and 2-propionyl-1-pyrroline (Schieberle, 1991).

Isolation of the Popcorn Volatiles. *Vacuum Sublimation (VS).* Two samples of fresh popcorn, either prepared by hot-air popping or by pan-frying, were frozen in liquid nitrogen and then powdered in a commercial blender. The powder (200 g each) was soaked overnight in diethyl ether (2 L), containing the labeled internal standards d-I, d-II, d-III, and d-IV (20 μg each), and then extracted in a Soxhlet apparatus for 8 h. The combined extracts were dried over Na_2SO_4, concentrated to about 150 mL on a Vigreux column (60 cm × 1 cm i.d.) and the odorants, the internal standards, and the solvent were isolated by sublimation *in vacuo* (Sen et al., 1991). The sublimate was concentrated to 1 mL for separation by column chromatography.

Simultaneous Steam Distillation/Extraction (SDE). A mixture of d-I (30 μg) and d-II to d-IV (10 μg each) in 5 mL of ethanol was added to a suspension of ground popcorn (200 g) in 2.3 L of water. The suspension was distilled and continuously extracted for 2 h with 150 mL of diethyl ether in the

apparatus according to Nickerson and Likens (1966). After drying over Na_2SO_4, the extract was concentrated to 1 mL on a Vigreux column (60 cm × 1 cm i.d.).

The concentrates obtained by VS and SDE were fractionated into four fractions (A–D) on silica gel by using the solvent mixtures described previously (Schieberle, 1991).

Isolation of the Water-Soluble, Low Molecular Weight Compounds from Corn. Maize kernels (1 kg) were frozen in liquid nitrogen, ground in a commercial blender, defatted with *n*-hexane, and air-dried. The material obtained was extracted with phosphate buffer (1.5 L; 0.05 mol/L; pH 7.0) by using an Ultra-turrax (Jahnke and Kunkel, Hohenstaufen, Germany). The suspension was centrifuged for 30 min (5000 g; 4 °C) and, after decanting, the extraction was repeated three times by using a total volume of 1.5 L of water. The combined aqueous extracts were freeze-dried. Aliquots were dissolved in water and filtered with a membrane (MW cutoff: 1000; YM2; Amicon, Witten, Germany) to obtain a fraction of the water-soluble, low molecular weight compounds (LMW-fraction). This solution was either freeze-dried or used immediately in the model experiments.

High Resolution Gas Chromatography (HRGC)/Mass Spectrometry (MS). HRGC/MS was performed by means of a gas chromatograph (Type 5160; Fisons, Mainz, Germany) coupled to an MS 8230 (Finnigan, Bremen, Germany). The samples were injected "on-column" at 40 °C on capillary columns, either OV-1701 (30 m × 0.32 mm fused silica capillary DB-7 J&W; distributed by Fisons, Mainz, Germany) or CP-Wax for amines (CP-WAX/Amine; 30 m × 0.32 mm fused silica capillary; Chrompack, Frankfurt, Germany). The temperature of the oven was raised at 40 °C/min to 60 °C, held 2 min isothermally and then raised at 4 °C/min to 220 °C. Mass spectra were recorded in the electron-impact mode (MS/EI) at 70 eV and in the chemical-ionization mode (MS/CI) at 115 eV with isobutane as reagent gas.

Quantitation by MS was performed on the CP-WAX/Amine column as detailed in Table 1 by means of the ion-trap detector 800 (Finnigan, Bremen, Germany), running in the chemical ionization mode with methanol as the reagent gas. Comparison of the integrated abundances of the ions of the labeled and unlabeled compounds provided the data for the quantification of the four odorants. Calibration factors were calculated as described recently (Sen et al., 1991).

RESULTS AND DISCUSSION

Stable Isotope Dilution Assays. *Comparison of MS Data.* As previously reported (Schieberle, 1990) 2-acetyltetrahydropyridine (ACTPY) is separated by HRGC on a specially deactivated stationary phase into two tautomers (RI: 1433 and 1635; CP-WAX/A) which showed identical MS/EI and MS/CI spectra (Figure 2, parts A and B). [^{2}H]-ACTPY was also separated into two tautomers with identical mass spectra (Figure 3, parts A and B), showing a cluster of molecular ions. A comparison with the spectra of the unlabeled ACTPY (Figure 2) indicated the presence of three main isotope-isomers (m/z 127–129; Figure 3A) containing two to three deuterium atoms. The MS/CI resulted in intense $M^+ + 1$ ions for the labeled (m/z 128–130; Figure 3B) and the unlabeled ACTPY (m/z 126; Figure 2B), confirming the incorporation of two to three deuterium atoms in the labeled ACTPY.

The mass spectra (MS/EI) of the labeled ([^{2}H]-PPY)- and the unlabeled 2-propionyl-1-pyrroline (PPY) are compared in Figure 4. The molecular peaks m/z 128 and 129 in the labeled PPY (Figure 4A) indicated the presence of two main isotope-isomers containing three or four deuterium atoms. Intense protonated molecular ions at m/z 129 and 130 ([^{2}H]-PPY) obtained by MS/CI (data not shown) confirmed these results and suggested this ionization technique for the quantitation of the PPY.

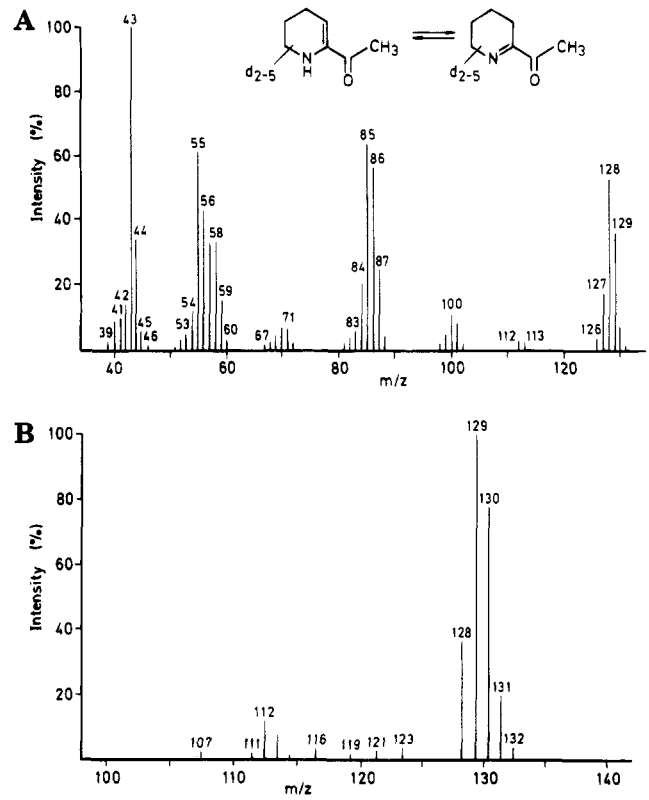


Figure 3. Mass spectra (MS/EI: A; MS/CI: B) of [^{2}H]-2-acetyltetrahydropyridine ([^{2}H]-ACTPY).

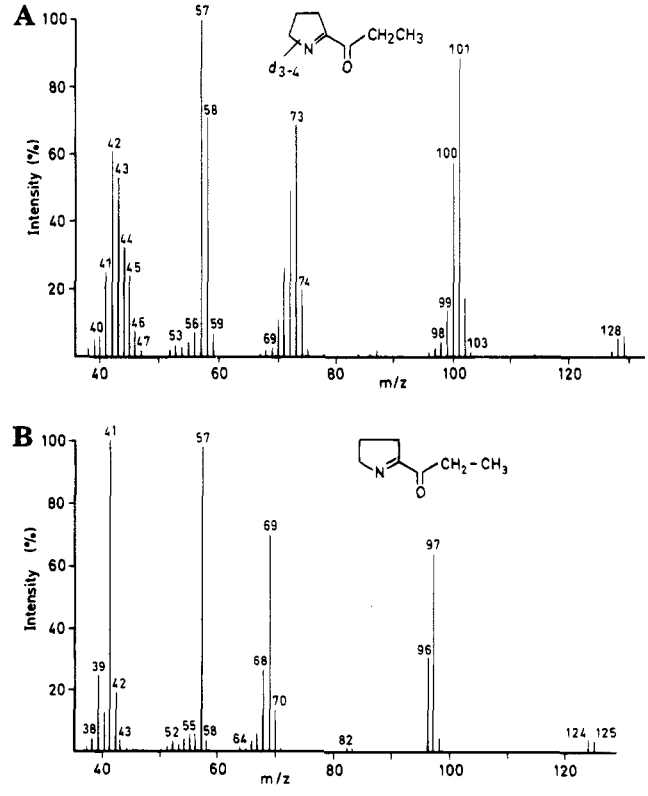


Figure 4. Mass spectra of [^{2}H]-2-propionyl-1-pyrroline ([^{2}H]-PPY; A) and 2-propionyl-1-pyrroline (PPY; B).

Analysis of a Model Mixture. Although labilely bound deuterium atoms had been removed by acid and alkaline treatment during synthesis of the internal standards, the analytical procedure might cause further protium/deuterium exchanges. To exclude this possibility, freshly ground nonpopped maize kernels were spiked with an ethanolic solution containing known amounts of the four

Table 2. Quantitation of the Four Roasty Odorants in a Model Mixture[a]

compound	amt added, μg	amt measd, μg[b]
2-acetyltetrahydropyridine	40	36
2-acetyl-1-pyrroline	40	38
2-propionyl-1-pyrroline	40	40
acetylpyrazine	40	42

[a] A mixture of the unlabeled and labeled odorants (40 μg each), dissolved in ethanol (5 mL), was added to freshly ground nonpopped corn and the volatiles were isolated by vacuum sublimation and enriched by column chromatography, as described for the popcorn sample. Preliminary experiments had revealed that the nonpopped sample did not contain the four odorants (detection limit: 0.1 μg/kg). [b] Mean values of triplicates.

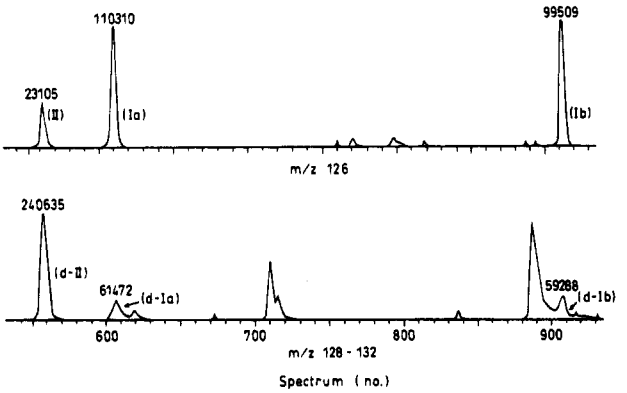


Figure 5. Ion abundances recorded by HRGC/MS simultaneously at m/z 126 (upper trace) and m/z 128–132 (lower trace) in fraction C of hot-air popped corn. Intensity units calculated by the MS software are displayed above each compound. II: PPY; Ia and Ib: tautomers of ACTPY; d-II: [^{2}H]-PPY; d-Ia and d-Ib: tautomers of [^{2}H]-ACTPY.

odorants (Table 2) and the corresponding internal standards (d-I–d-IV). The volatile fraction was then isolated by vacuum sublimation and, after enrichment by column chromatography, the odorants were quantified by mass chromatography.

The differences between the theoretical values and the values measured differed by not more than 10% (2-acetyltetrahydropyridine; Table 2), indicating that no significant protium/deuterium exchange occurred and, in addition, that the method is very effective, especially for the quantitation of the labile 2-acetyltetrahydropyridine at the microgram per kilogram level.

Analysis of Popcorn Samples. The method was then used to quantitate the four roast-flavor compounds in different samples of popcorn. As an example, the mass chromatograms recorded for fraction C of fresh hot-air popped corn, obtained by column chromatography of the flavor extract and containing 2-propionyl-1-pyrroline and 2-acetyltetrahydropyridine, are shown in Figure 5. From the ion intensities (m/z 126) of the unlabeled PPY (II in Figure 5) and the two unlabeled tautomers of ACTPY (Ia and Ib in Figure 5) in combination with the ion intensities (m/z 128–132) of the labeled PPY (d-II) and the two labeled tautomers of ACTPY (d-Ia and d-Ib), high amounts of ACTPY (437 μg/kg; Table 3) and comparatively lower amounts of PPY (17 μg/kg; Table 3) were determined in the fresh popcorn sample. Compared to ACTPY, 2-acetyl-1-pyrroline and acetylpyrazine were present in lower amounts of 24 and 8 μg/kg, respectively (Table 3).

A calculation of odor activity values (OAV; ratio of concentration to odor threshold), giving an idea of the contribution of the individual odorants to the overall odor, established 2-acetyltetrahydropyridine followed by 2-acetyl-1-pyrroline as the key contributors to the roasty odor note of fresh popcorn (Table 3), whereas acetylpyrazine did not contribute much to this odor note.

Table 3. Concentrations (μg/kg)[a] of the Four Roast Flavor Compounds in Fresh and Stored Hot-Air Popped Corn (HAP) and in Fresh Pan-Popped (PP) Corn (Odor Activity Value)[d]

		HAP stored for[b]		
odorant	HAP (fresh)	2 days	7 days	PP (fresh)
2-acetyltetrahydropyridine[c]	437 (7283)	237	132	138 (2300)
2-acetyl-1-pyrroline	24 (1200)	19	6	20 (1000)
2-propionyl-1-pyrroline	17 (850)	15	5	11 (550)
acetylpyrazine	8 (20)	7	8	25 (63)

[a] Mean values obtained by analysis of three different batches. Analysis was performed in extracts obtained by vacuum sublimation. [b] After cooling, the fresh hot-air popped corn was sealed in polyethylene bags (specified for food use) and stored in the dark at room temperature. [c] The sum of both isomers (cf. Figure 5) is given. [d] Odor activity values (ratio of concentration to odor threshold) were calculated by using the following odor thresholds in air: 2-acetyltetrahydropyridine: 0.06 ng/L; 2-acetyl- and 2-propionyl-1-pyrroline: 0.02 ng/L; acetylpyrazine: 0.4 ng/L (Schieberle, 1990 and 1991).

Table 4. Concentrations of the Four Roast Aroma Compounds in Fresh Hot-Air Popped Corn Determined in an Extract Obtained by Simultaneous Distillation/Extraction (SDE)

odorant	concn (μg/kg)
2-acetyltetrahydropyridine	1207
2-acetyl-1-pyrroline	57
2-propionyl-1-pyrroline	21
acetylpyrazine	7

Table 5. Formation of 2-Acetyltetrahydropyridine and 2-Acetyl-1-pyrroline from Water-Soluble Precursors in Maize

	concn (μg/kg maize) generated in expt		
compound	I[a]	II[b]	III[c]
2-acetyltetrahydropyridine	40	166	nd[d]
2-acetyl-1-pyrroline	0.3	8.7	12.7

[a] The freezed-dried material (from 200 g of flour) was dissolved in water (200 mL) and continuously steam-distilled and extracted by SDE for 2 h. [b] See footnote a. 2-Oxopropanal (4 mg) was added prior to boiling. [c] The freeze-dried maize flour extract (from 200 g of flour) was mixed with silica gel (10 g) and heated for 10 min at 150 °C. [d] nd: not detectable.

The flavor of fresh popcorn is not stable and it might be assumed that a loss of the roast-aroma compounds is involved in the flavor changes observed during storage. The quantitation of the odorants in a sample of hot-air popped corn, which had been stored for 7 days (Table 3) in polyethylene bags, showed that the storage led to a significant decrease in the concentrations of ACTPY, ACPY, and PPY in the popcorn. Only 25–30% of their amounts present in the fresh popcorn survived storage of the sample for one week. In contrast, acetylpyrazine remained constant (Table 3).

In a sample prepared by popping the corn in a pan, much lower concentrations of ACTPY were obtained than in the fresh hot-air popped corn. On the other hand, the amounts of acetylpyrazine increased by a factor of 3. These results imply that the longer heating time during pan-popping leads to degradation, especially of the most labile roast odorant ACTPY. However, on the basis of its high odor activity value, ACTPY was the most odor-active compound also in this popcorn sample.

To study the influence of the workup procedure on the amounts of the odorants, in a further experiment,

Table 6. Formation of 2-Acetyltetrahydropyridine (ACTPY) and 2-Acetyl-1-pyrroline (ACPY) from Proline and Carbohydrates[a]

expt	carbohydrate	amount (μg) ACTPY	amount (μg) ACPY
1	glucose	150	<0.3
2	fructose	717	1.0
3	2-oxopropanal	170	41.0
4	fructose[b]	n.d.	15.0

[a] Proline (4 mmol) and the corresponding carbohydrate (2 mmol) or 2-oxopropanal (0.1 mmol) were boiled in phosphate buffer (400 mL; pH 7.0; 0.1 mol/L) and continously steam distilled and extracted (SDE). [b] The reaction mixture was intimately mixed with silica gel and then dry-heated (10 min, 150°C). n.d.: not detectable.

the four odorants were quantitated in an extract obtained by simultaneous distillation/extraction (SDE) of fresh hot-air popped corn. The results revealed (Table 4) that the concentrations of 2-acetyltetrahydropyridine and 2-acetyl-1-pyrroline were higher by factors of 2.7 or 2.3, respectively, than the concentrations determined in the extract obtained by extraction/vacuum sublimation (cf. Table 3), whereas the amounts of 2-propionyl-1-pyrroline and acetylpyrazine hardly differed.

These results suggest that, on the one hand, swelling of the popcorn matrix in boiling water might allow more odorant to escape. On the other hand, the results imply the presence of unreacted precursors which, upon boiling of the popcorn suspension, give rise to a "secondary" formation of ACTPY and ACPY.

Model Studies on the Formation of Roast Odorants. In a study on wheat bread crust flavor we could recently show (Schieberle, 1990) that significant amounts of ACPY were formed by boiling aqueous solutions of either the amino acids proline or ornithine in the presence of the reactive sugar-degradation product 2-oxopropanal (methylglyoxal). On the contrary, ACTPY was formed exclusively from proline. To localize the precursors in the maize kernel, a fraction of the water-soluble, low-molecular-weight compounds (LMW fraction) was isolated from freshly ground maize by extraction with a buffer solution, centrifugation, and ultrafiltration. In the freeze-dried material, relatively high amounts of free proline were found (155 mg/kg maize), but ornithine was not detectable (<5 mg/kg), excluding the latter amino acid as a precursor in maize. In addition, significant amounts of free glucose (1190 mg/kg) and fructose (880 mg/kg) were present. After boiling of the LMW-fraction in water and continuous extraction of the volatiles for 2 h by SDE, the amounts of ACTPY and ACPY liberated were determined by isotope dilution assays. The results (expt I; Table 5) revealed that simply by boiling of the maize extract distinct amounts of ACTPY were generated, whereas no significant formation of ACPY was observable. By contrast, addition of 2-oxopropanal prior to boiling of the maize fraction led to an increase in the amounts of ACPY by a factor of 29 (cf. expts I and II; Table 5). These results corroborated our previous findings (Schieberle, 1990), indicating the oxoaldehyde as a key intermediate in the formation of the ACPY. It may be assumed that during popping of the corn the oxoaldehyde is formed by a retro-Aldol cleavage of an appropriate C-6 α-dicarbonyl compound, such as 1-deoxyosone. This implies that a process simulating the popping procedure would generate the oxoaldehyde needed to liberate ACPY from the free proline present in the maize extract. This assumption is corroborated by results of a third experiment (expt III; Table 5), showing that dry-heating conditions initiate significant formation of the odorant from the LMW-fraction.

Table 7. Parameters Influencing the Yields of 2-Acetyl-1-pyrroline (ACPY) from 1-Pyrroline and 2-Oxopropanal[a]

expt	pH	reaction time (min)	ACPY (μg)
1	7.0	120	562
2	7.0	30	432
3	7.0	15	204
4	4.5	120	33
5	5.5	120	115
6	6.5	120	505
7	8.0	120	447
8[b]	7.0	120	187

[a] 1-Pyrroline (0.25 μmol) and 2-oxopropanal (0.25 μmol) were refluxed in phosphate buffer (100 mL) for 2 h. The ACPY was determined in an ethereal extract by stable isotope dilution assay. [b] The phosphate buffer was replaced by malonate buffer (pH 7.0; 0.1 mol/L).

Table 8. Yields of the Main Reaction Product Obtained by Reacting 1-Pyrroline with 2-Oxoaldehydes[a]

2-oxoaldehyde	main product	yield μg	yield mol %
2-oxobutanal	2-propionyl-1-pyrroline	312	1.0
phenylglyoxal	2-benzoyl-1-pyrroline	1590	3.7

[a] 1-Pyrroline (250 μmol) and the corresponding 2-oxoaldehyde (250 μmol) were refluxed for 2 h in phosphate buffer (100 mL; pH 7.0; 0.1 mol/L).

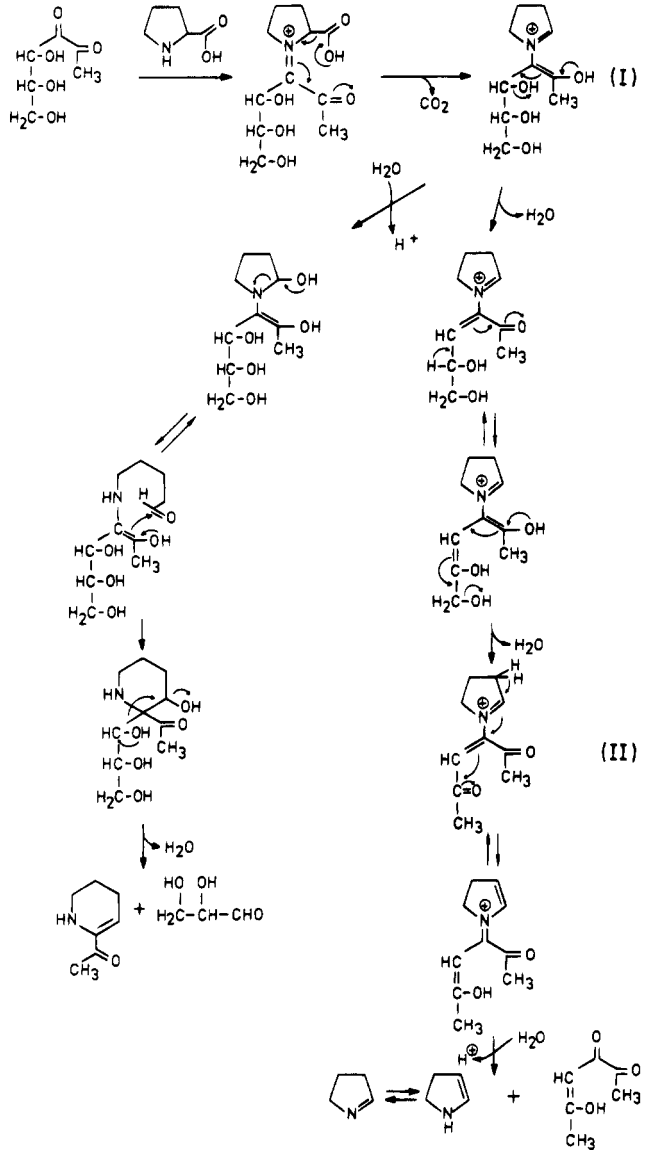
To investigate their potency in generating ACTPY and ACPY from proline, glucose and fructose, as well as 2-oxopropanal, were reacted with this amino acid in model solutions under the same conditions as used in the experiments with maize extract (Table 6).

Assuming proline as the most important precursor of both roast-odorants, the results summarized in Table 6 were well in line with the data obtained for the maize extract (cf. Table 5). Boiling of either the glucose/proline or the fructose/proline mixture resulted in the formation of significant amounts of the ACTPY, whereas under these conditions the generation of ACPY was negligible. However, during boiling of a 2-oxopropanal/proline solution (expt 3; Table 6), as well as during dry-heating of the fructose/proline mixture (expt 4; Table 6) significant amounts of ACPY were formed.

The reaction between 2-oxopropanal and proline had earlier been suggested (Hodge et al., 1972) as a key step in the formation of 2-acetyltetrahydropyridine. Very recent synthetic work by De Kimpe et al. (1994), however, has shown that the key intermediate *N*-acetonyl-4-aminobutanal, postulated by Hodge et al., did not generate ACTPY upon heating. Our results show that the ACTPY is preferentially formed in the presence of fructose (Table 6). With reference to Hodge's mechanism, we, therefore, suggest an alternative pathway (Scheme 1) for ACTPY formation. The reaction starts with the formation of an enamine from proline and the carbonyl group at carbon-3 of 1-deoxyosone, an intermediate, which may easily be generated by dehydration of fructose (Ledl and Schleicher, 1990). Decarboxylation and subsequent hydrolysis of the pyrroline ring in the key intermediate **I**, followed by a ring enlargement, would result in a spiro-compound, which, upon retro-Aldol cleavage, would generate ACTPY.

1-Pyrroline had been proposed by Hodge et al. (1972) as a further degradation product of proline. The cyclic imine may be formed in an alternative reaction also starting from intermediate **I** (Scheme 1; right-hand

Scheme 1. Hypothetical Reaction Pathway Leading from Proline and 1-Deoxyosone to 2-Acetyltetrahydropyridine and 1-Pyrroline



side). Elimination of two molecules of water from the carbohydrate skeleton of **I** by a retro-Michael reaction yields the oxidized intermediate **II**, from which simply by hydrolysis the 1-pyrroline can be generated.

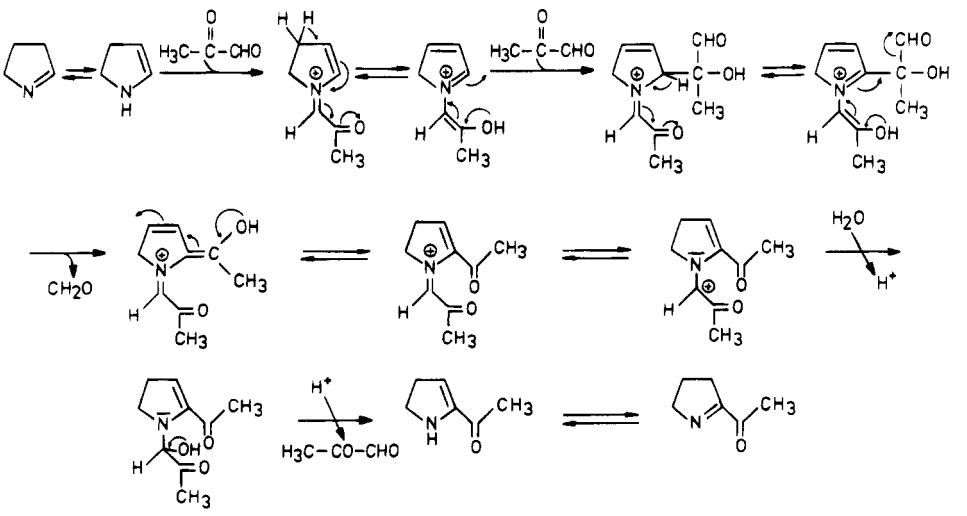
The reaction of 1-pyrroline with 2-oxopropanal had been previously shown (Schieberle, 1990) to be a key step in the generation of exclusively ACPY. Since both reactions, the formation of 2-oxopropanal from carbohydrates as well as the formation of 1-pyrroline from proline according to Scheme 1, require elimination of water, which is favorable especially under dry-heating conditions, the preferential formation of ACPY, e.g., from proline/fructose in the absence of water (expt 4; Table 6) is a logical consequence.

In the present study, the 1-pyrroline/2-oxopropanal system was studied in more detail to elucidate the influence of different reaction parameters on the yields of ACPY. The results summarized in Table 7 indicate that the formation of the odorant from the two precursors proceeds relatively fast, because 76% of the amounts present after 120 min were already formed within 30 min (cf. expts 1 and 2; Table 7). Furthermore, the formation of the odorant from the 1-pyrroline/2-oxopropanal-reaction system is extremely pH-dependent, since in the pH range 4.5–8.0 the yields went through a maximum at pH 7.0 (cf. expts 1, 4–7; Table 7). Under the latter conditions, about 2 mol % of ACPY were formed, compared with only 0.1 mol % at pH 4.5 (cf. expts 1 and 4). Furthermore, phosphate ions favored the formation of ACPY, because replacement of the phosphate by malonate buffer decreased the yields of the odorant to about one-third (cf. expts 1 and 8; Table 7).

To gain an insight into the reaction mechanism, the cyclic imine was then reacted with two further oxoaldehydes, 2-oxobutanal and phenylglyoxal. 2-Propionyl- and 2-benzoyl-1-pyrroline, which were characterized by their mass (cf. Figures 1 and 4B) as well as their ^{1}H-NMR spectra, were identified as the main reaction products. The yields of the pyrroline derivatives were in the same order of magnitude as those obtained for ACPY (cf. Table 8).

Results of previous investigations with labeled carbohydrates (Schieberle, 1989; Tressl et al., 1993) have indicated that in 2-acetyl-1-pyrrolines, generated from either proline and U-^{13}C-glucose (Schieberle, 1989) or proline and 1-^{13}C-glucose (Tressl et al., 1993), the labeling was present either in both carbon atoms of the acetyl group (U-^{13}C-glucose) or only in the methyl group (1-^{13}C-glucose), respectively. These findings support the view (Schieberle, 1989) that the 2-acyl-1-pyrrolines are

Scheme 2. Reaction Pathway Leading from 1-Pyrroline and 2-Oxopropanal to 2-Acetyl-1-pyrroline



formed by an "acylation" of 1-pyrroline by the respective 2-oxoaldehyde with elimination of formaldehyde.

In Scheme 2, a hypothetical formation pathway starting with the formation of a *Schiff* base between the tautomer 2-pyrroline and 2-oxopropanal is shown. Enolization and a subsequent nucleophilic attack of this intermediate at carbon-2 of a second molecule of 2-oxopropanal provides a 1,2-disubstituted pyrrole cation, which, upon elimination of formaldehyde, is hydrolyzed into 2-oxopropanal and 2-acetyl-2-pyrroline, which then tautomerizes into 2-acetyl-1-pyrroline.

The key step in this reaction is the formation of the *Schiff* base which may explain the significant influence of the pH (cf. Table 8). In this intermediate, the first molecule of the oxoaldehyde acts as a catalyst to facilitate the reaction with the second molecule. This "activation" of the pyrroline ring may be initiated also by further α-dicarbonyls generated in sugar degradation reactions, such as the 1-deoxyosone. However, because ACPY is formed preferentially in the presence of 2-oxopropanal (Table 6), 2-oxoaldehydes seem to be most effective as "acylating" agents.

As previously shown (Schieberle, 1990), ACPY is formed in significant yields also by reacting the amino acid ornithine with 2-oxopropanal. Because 1-pyrroline may easily be formed from 4-aminobutanal, the *Strecker* degradation product of ornithine, the present results are well in line with our previous data on ACPY formation.

Furthermore, on the basis of these results, it seems likely that in popcorn also the 2-propionyl-1-pyrroline may be formed from a reaction of 1-pyrroline with the homologous 2-oxobutanal.

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