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Optimal Synthesis of Substituted and Branched Pyrazines via Reaction of Alpha Hydroxy Ketones with Selected Nitrogen Sources *

by

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SUMMARY

Employment of 1-hydroxy-acetone as a carbon source and NH_4OH as a source of base and nitrogen, has enabled arrays of pyrazines to be synthesized. Reaction conditions such as temperature, time, carbon/nitrogen mole ratios and pH were optimized to maximize the quantity of pyrazines, thereby providing the synthesis of at least 19–20 structurally different pyrazines. Addition of amino acids, selected aldehydes, and hydrolyzed tobacco-derived F1 protein has positively impacted the array of pyrazines from both qualitative and quantitative aspects. Results further showed that by changing the carbon source from 1-hydroxy-acetone to 1-hydroxy-2-butanone and/or 2-hydroxy-3-butanone, control of the type of pyrazines being synthesized could be realized in that the qualitative and quantitative distributions of the pyrazine array were shifted to higher molecular weight derivatives. A relatively large scale reaction (1.5 L) employing optimized parameters yielded > 2 g of a diverse array of pyrazines dominated by multiple dimethylpyrazine derivatives. While systematically varying reaction conditions and reagent mole ratios can predictably alter the distribution and yield of pyrazines, the two most overwhelmingly significant factors governing these two pyrazine product characteristics included the structure of the carbon source and the presence or absence of aldehydes and free amino acids. [Beitr. Tabakforsch. Int. 28 (2019) 267–277]

ZUSAMMENFASSUNG

Der Einsatz von 1-Hydroxyaceton als Kohlenstoffquelle und NH_4OH als Basen- und Stickstoffquelle hat die synthetische Herstellung von Pyrazinreihen ermöglicht. Die Reaktionsbedingungen wie Temperatur, Zeit, Molverhältnisse von Kohlenstoff/Stickstoff und pH-Wert wurden mit dem Ziel optimiert, die Menge der Pyrazine zu maximieren, so dass mindestens 19–20 strukturell unterschiedliche Pyrazine synthetisiert wurden. Das Hinzufügen von Aminosäuren, ausgewählten Aldehyden und hydrolysiertem aus Tabak gewonnenem F1-Protein führte sowohl in qualitativer als auch quantitativer Hinsicht zu positiven Auswirkungen auf das Pyrazinspektrum. Aus den Ergebnissen ging weiterhin hervor, dass durch Veränderung der Kohlenstoffquelle von 1-Hydroxyaceton zu 1-Hydroxy-2-butanon und/oder 2-Hydroxy-3-butanon die Art der synthetisierten Pyrazine gesteuert werden konnte, und zwar insofern, dass sich die qualitativen und quantitativen Verteilungen des Pyrazinspektrums zu Derivaten mit höherem Molekulargewicht verschieben ließen. Eine Reaktion in relativ großem Maßstab (1,5 L) mit optimierten Parametern ergab eine Ausbeute von > 2 g eines vielfältigen Pyrazinspektrums, das von mehreren Dimethylpyrazinderivaten dominiert war. Während die systematische Variation der Reaktionsbedingungen und Molverhältnisse der Reagenzien die Verteilung und Ausbeute von Pyrazinen vorhersagbar verändern kann, waren die beiden bei weitem signifikantesten Faktoren zur Beeinfluss-

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ung dieser beiden Eigenschaften der Pyrazinprodukte die Struktur der Kohlenstoffquelle und das Vorhandensein bzw. Fehlen von Aldehyden und freien Aminosäuren. [Beitr. Tabakforsch. Int. 28 (2019) 267–277]

RESUME

L'utilisation d'hydroxyacétone en guise de source de carbone et du NH_4OH comme source d'azote et de base permit la synthèse de séries de pyrazines. Les conditions de réaction telles que la température, le temps, les rapports moléculaires carbone/azote et le pH furent optimisées afin de maximiser la quantité de pyrazines et ainsi de permettre la synthèse d'au-moins 19–20 pyrazines de structures différentes. L'ajout d'acides aminés, d'une sélection d'aldéhydes et d'une protéine F1 de tabac hydrolysée eut une incidence positive sur la série de pyrazines tant du point de vue quantitatif que qualitatif. Les résultats démontrèrent également qu'en modifiant la source de carbone en remplaçant l'hydroxyacétone par du 1-hydroxy- butanone et/ou du 2-hydroxy-3-butanone, le contrôle du type de pyrazines synthétisées pouvait être assuré grâce au glissement des distributions qualitatives et quantitatives de la série de pyrazines vers des dérivés d'un poids moléculaire plus élevé. Une réaction à relativement grande échelle (1,5 L) recourant aux paramètres optimisés produisit > 2 g d'une série variée de pyrazines où prédominaient des dérivés multiples de diméthylpyrazine. Sachant que la variation systématique des conditions de réaction et des rapports moléculaires des réactifs put, de façon prévisible, altérer la distribution et le rendement de pyrazines, les deux facteurs les plus significatifs quant aux caractéristiques de ces deux productions de pyrazines furent la structure de la source de carbone et la présence ou l'absence d'aldéhydes et d'acides aminés libres. [Beitr. Tabakforsch. Int. 28 (2019) 267–277]

INTRODUCTION

To date, the vast majority of model reactions and fortified natural products which result in pyrazine-rich formulations upon heating have used sugars such as fructose, glucose, fructose/glucose mixtures, and rhamnose as the carbon source components of the formulations. These sugars have been shown to serve as carbon sources for the formation of the pyrazine aromatic ring structure (1–11). Most often these reactions have employed ammonium hydroxide and/or free amino acids as nitrogen sources that furnish the nitrogen contained within the pyrazine structure.

Indications from the literature strongly suggest that control of the carbon source structure can dictate the structure of pyrazines when the carbon sources, other than sugars, are reacted with ammonium hydroxide (12, 13) to form pyrazines. Preliminary trials have indicated that a reaction between acetoin (1-hydroxyethyl-methylketone) and ammonium hydroxide produces almost exclusively tetramethylpyrazine (TMP) with small amounts of pyrazine and methylpyrazine (14). The absence of these lower molecular weight pyrazines has a positive impact on the sensory characteristics of the TMP and their absence in other pyrazine formulations could be similarly beneficial (10, 11). The literature suggests that acetoin is a sugar degradation intermediate that subsequently reacts with nitrogen sources to produce tetramethylpyrazine, since some tetramethylpyrazine can be detected at low levels in reactions between sugars such as fructose, glucose, and rhamnose with ammonium hydroxide (1–11). Earlier research (12, 13) supports this observation. When the carbon sources that were reacted with bases were 1-hydroxyacetone or other selected aldehydes, such as isovaleraldehyde, an array of alkyl substituted pyrazines was produced. However, the total qualitative and quantitative distribution of the pyrazine structures were not specifically delineated at the time. A re-examination of the qualitative distribution of pyrazines produced by the described reaction of 1-hydroxyacetone and ammonium hydroxide has recently been completed. Four reagent combinations were studied at a reaction temperature of 120 °C and reaction times of 2, 8, and 12 h: a) 1-hydroxyacetone/isovaleraldehyde/ammonium hydroxide (2 h); b) 1-hydroxyacetone/ammonium hydroxide (2 h); c) acetoin/ammonium hydroxide (2 h and 12 h); and d) 1-hydroxyacetone/acetoin/isovaleraldehyde/ammonium hydro-xide (8 h). The preliminary results revealed that pyrazines having at least two alkyl substituents were synthesized and, in addition, tri- and tetra-substituted pyrazines with up to seven total carbons were prepared. Pyrazines having the isobutyl moiety, attributable to the isovaleraldehyde, attached to the ring were identified within the array. No detectable amounts of pyrazine and or methylpyrazine were found.

For this current study, a number of general hypotheses was tested. The first hypothesis is based on the theory that acetoin (2-hydroxy-3-butanone) and acetol (1-hydroxyacetone) serve as carbon sources (substitutes for sugars) and can be readily reacted with and without free amino acids in the presence of diammonium phosphate and/or NH_4OH and H_3PO_4, to produce unique arrays of pyrazines, having multiple alkyl side chains and whose quantitative distribution contains little or no detectable amounts of pyrazine and methylpyrazine. The reactions were optimized using different mole ratios of carbon source to NH_4OH (C:N) ranging from 1:0.5 up to 1:2.5. Also, the effect of different reaction temperatures (100–140 °C), reaction times (4–24 h), and pH levels (8.0–11.0) were investigated. After optimization of the reaction, other sources of nitrogen such as amino acids or amino acids from hydrolyzed tobacco F1 protein (14) were investigated by using the optimized reaction conditions to study their effects on pyrazine distributions and yield. Optimized reaction conditions were also used for reactions of 1-hydroxy-2-butanone as a different source of carbon. Furthermore, the addition of isovaleraldehyde to one of the optimized reactions and its effects on pyrazine synthesis was also investigated.

EXPERIMENTAL

Materials

Acetoin (2-hydroxy-3-butanone), acetol (1-hydroxyacetone), 1-hydroxy-2-butanone, ammonium hydroxide (28–30%), dichloromethane, methyl-*t*-butylether (MTBE), dichloromethane (DCM), phosphoric acid (H_3PO_4), leucine, isoleucine, threonine, anhydrous sodium sulfate, solid phase extraction (SPE) C18 silica, and isovaleraldehyde were obtained from Sigma-Aldrich (St. Louis, MO, USA). Tobacco F1 protein

was obtained from R.J. Reynolds Tobacco Co. (Winston-Salem, NC, USA) and hydrolyzed using optimum conditions which were developed previously (14). The enzymatic catalytic conversion of F1 protein to free amino acids was in the range of 50–55%.

Reaction protocols

All pyrazine synthesis reactions were performed in a high pressure 40 mL Parr vessel, a sealed receptacle which could handle high pressure and temperature reactions. For all acetoin reactions, 0.8 g of acetoin was reacted with ammonium hydroxide (NH_4OH, 27–30%) (1.8 mL) and orthophosphoric acid (H_3PO_4) when 20 mL of water was used. Different reaction temperatures (90 and 120 °C), pH values (8.0–12.0), time (4–18 h) and addition of amino acids (0.4 g) were examined. For all acetol and 1-hydroxy-2-butanone reactions, 1 g of each compound was mixed with 0.25, 0.5, 1.0, and 1.25 mL of NH_4OH and 10 mL of H_2O. Each reaction was mixed and heated at different temperatures (100–140 °C) for a period of 4–24 h. The pH level for most of the reactions was approximately 11.0 (no adjustment was made). However, in reactions in which the pH level was adjusted to 8.0, concentrated H_3PO_4 was used to lower the pH. Two different amino acids were tested as an additional source of nitrogen. In each reaction, 0.2 g of amino acid was added separately to each reaction so that it would be possible to study how it would affect the qualitative and quantitative pyrazine distributions. In another reaction, isovaleraldehyde was added and optimized reaction conditions were used to study the effect on the qualitative and quantitative pyrazine distributions. When the hydrolyzed F1 protein was used as a source of additional nitrogen, 10 or 20 mL of hydrolyzed F1 protein (which contained approximately 0.2 or 0.4 g of different amino acids) were used. In this reaction, H_2O was not added since the hydrolyzed F1 protein was contained in 10 or 20 mL of H_2O. See Table 1 for a list of all reactions which were studied. After completion of each reaction, the mixture was extracted with 20–25 mL of dichloromethane (DCM). In each extraction, 250 µg of deuterated 2-methylpyrazine (only the methyl group was deuterated) was used as an internal standard for all quantifications. For all reactions, the mixtures were stirred using a magnetic stirrer during the reaction process.

Instrumentation

All GC/MS analyses were performed using a 6890 GC equipped with a 5973 mass selective detector (MSD) from Agilent (Wilmington, DE, USA). Separations were obtained using an Agilent J&W DB-WAXETR capillary column (30 m long × 250 µm I.D. with a film thickness of 0.25 µm) (Wilmington, DE, USA). The following operating parameters were used for each analysis:

- Injection port temperature 260 °C
- Purge valve 3 mL/min
- Purge time 1 min
- Total flow 24 mL/min
- Constant flow 1 mL/min
- Injection volume 2 µL, split 1:20
- Column oven initial temperature 50 °C
- Column oven initial time 3 min
- Column oven ramp rate 15 °C/min
- Column oven final temperature 250 °C
- Column oven final time 1 min
- MSD Transfer line temperature 260 °C

MS Wiley library was used to identify each pyrazine. For analysis, pyrazines were quantified using single ion monitoring mode. Each pyrazine was quantified against the response for a known mass of internal standard (250 µg) added to the extraction solvent.

RESULTS AND DISCUSSION

Reaction using acetoin (2-hydroxy-3-butanone)

In this reaction, acetoin (0.8 g) was reacted with NH_4OH (1.8 mL) and H_3PO_4 (0.6 mL) at pH 8.0 at 90 °C for 12–15 h. More than 90% of the acetoin was converted to tetramethylpyrazine, with no pyrazine or methylpyrazine, or any other pyrazines detected in the chromatographic total ion current profile. Next, the reaction was repeated using identical conditions, but instead of heating the reaction at 90 °C for 12–15 h, it was heated for 4 h at 120 °C. Results were similar to those obtained at 90 °C and 12–15 h.

In order to determine if branched pyrazines, that is pyrazines having Strecker aldehyde-like alkyl chains, could be synthesized, an amino acid (leucine) was added to the reaction mixture composed of 0.8 g acetoin, 1.8 mL of NH_4OH, 0.6 mL of H_3PO_4, and 0.25 g of leucine. These reagents were mixed with 20 mL of H_2O and pH was adjusted to 8.0. The Strecker degradation is a chemical reaction which converts an alpha amino acid into an aldehyde that contains the amino acid alkyl side chain. For example, should the alpha amino acid valine take part in a Strecker degradation then 2-methylpropanal would be the predicted corresponding aldehyde. The reaction mixture was heated for 18 h at 120 °C and then the reaction mixture was extracted with 30 mL of DCM and analyzed via GC/MS. In this reaction the only pyrazine detected was tetramethylpyrazine (TMP) and unreacted acetoin. Somewhat surprisingly, pyrazines with branched alkyl substituents were not detected, indicating that the reaction between acetoin and NH_4OH must be the dominant reaction pathway here and Strecker aldehyde formation does not play a significant role under these conditions.

A similar reaction was performed but instead of leucine and H_2O, 20 mL of hydrolyzed F1 protein was used for the reaction. The reaction was performed in a Parr vessel, similar to above reaction, at 120 °C for 18 h. After cooling the reaction mixture 30 mL of DCM was used to extract the pyrazines. Again, no pyrazines with branched alkyl substituents were observed, only TMP.

In order to make sure that ammonia was not consuming all the acetoin and thus preventing the amino acid(s) from reacting with acetoin, another base (NaOH) was used instead of NH_4OH to maintain a basic pH greater than 8.0 under the same reaction conditions. Two reactions were performed. In the first reaction, 0.8 g acetoin was mixed with 0.25 g of leucine and 20 mL of 0.1 N NaOH (pH = 12.0), while in the second reaction the pH was adjusted to 8.2 using H_3PO_4. Both reactions were heated at 120 °C for 8 h using the Parr

Table 1. Reaction conditions and reagents employed in this study.

Reaction	Volume hydroxy ketone (mL)	NH_4OH (mL) + any additional reagents	Time (h)	Temperature (°C)	H_2O (mL)	pH
1	0.8 mL Acetoin	1.8	12–15	90	20	8.0
2	0.8 mL Acetoin	1.8	4	120	20	8.0
3	0.8 mL Acetoin	1.8 + 0.25 g Leucine	4	120	20	8.0
4	0.8 mL Acetoin	1.8 + 20 mL of Hydrolyzed F1	4	120	0	8.0
5	0.8 mL Acetoin	0.1 N NaOH + 0.25 g Leucine	8	120	20	8.0
6	0.8 mL Acetoin	0.1 N NaOH + 0.25 g Leucine	8	120	20	12.0
7	1.0 mL Acetol	0.5	12	120	10	11.1
8	1.0 mL Acetol	0.5	12	120	10	8.0
9	1.0 mL Acetol	0.5	4	100	10	11.1
10	1.0 mL Acetol	0.5	4	110	10	11.1
11	1.0 mL Acetol	0.5	4	120	10	11.1
12	1.0 mL Acetol	0.5	8	100	10	11.1
13	1.0 mL Acetol	0.5	8	110	10	11.1
14	1.0 mL Acetol	0.5	8	120	10	11.1
15	1.0 mL Acetol	0.5	12	100	10	11.1
16	1.0 mL Acetol	0.5	12	110	10	11.1
17	1.0 mL Acetol	0.5	12	120	10	11.1
18	1.0 mL Acetol	1.0	4	100	10	11.1
19	1.0 mL Acetol	1.0	4	110	10	11.1
20	1.0 mL Acetol	1.0	4	120	10	11.1
21	1.0 mL Acetol	1.0	8	100	10	11.1
22	1.0 mL Acetol	1.0	8	110	10	11.1
23	1.0 mL Acetol	1.0	8	120	10	11.1
24	1.0 mL Acetol	1.0	12	100	10	11.1
25	1.0 mL Acetol	1.0	12	110	10	11.1
26	1.0 mL Acetol	1.0	12	120	10	11.1
27	1.0 mL Acetol	0.25	4	100	10	11.1
28	1.0 mL Acetol	0.25	4	110	10	11.1
29	1.0 mL Acetol	0.25	4	120	10	11.1
30	1.0 mL Acetol	0.25	8	100	10	11.1
31	1.0 mL Acetol	0.25	8	110	10	11.1
32	1.0 mL Acetol	0.25	8	120	10	11.1
33	1.0 mL Acetol	0.25	12	100	10	11.1
34	1.0 mL Acetol	0.25	12	110	10	11.1
35	1.0 mL Acetol	0.25	12	120	10	11.1
36	1.0 mL Acetol	1.0	12	140	10	11.1
37	1.0 mL Acetol	1.0	12	130	10	11.1
38	1.0 mL Acetol	1.25	12	120	10	11.1
39	1.0 mL Acetol	1.0	24	120	10	11.1
40	1.0 mL Acetol	1.0	16	130	10	11.1
41	1.0 mL Acetol	1.0	16	120	10	11.1
42	1.0 mL Acetol	1.0 + 0.2 g Isoleucine	16	120	10	11.1
43	1.0 mL Acetol	1.0 + 0.2 g Threonine	16	120	10	11.1
44	1.0 mL Acetol	1.0 + 100 µL Isovaleraldehyde	16	120	10	11.1
45	1.0 mL Acetol	1.0 + 10 mL of hydrolyzed F1	16	120	0	11.1

vessel. After cooling the reaction, the reactants were extracted with DCM and analyzed via GC/MS. No pyrazines (not even TMP) were detected.

Reaction using acetol (1-hydroxyacetone)

Initially, 1-hydroxyacetone (acetol) was reacted with NH_4OH at different mole ratios, temperatures, pH levels, and reaction times in order to define the optimum conditions for maximum pyrazine yields. Table 1 shows a list of conditions that were used. Table 2 shows a list of all detected DCM extracted pyrazines from the reaction between 1-hydroxyacetone with NH_4OH. In Tables 2–4 appears in *italics* peak #23 which represents the amount of hydroxyketone left behind in the reaction mixture after the reaction had been completed. In almost all cases the amount of hydroxyketone (1-OH acetone) decreased significantly during the reaction. Figure 1 shows the total ion current chromatogram (GC/MS) for the DCM extract of this reaction and is representative of the qualitative profile of the pyrazines produced from all reactions. The 1-hydroxyacetone reactions were performed at two different pH levels (8.0 and 11.0) to determine which pH level would provide the highest yield and largest number of pyrazines. In the first reaction, 1 mL of 1-hydroxyacetone

Table 2. Synthesized pyrazines from a reaction of acetol and NH_4OH using different conditions and reagents.

Retention time (min)	Peak #	MW	Extractionion	Pyrazine
5.82	1	100	100	Internal standard
6.57	2	108	108	2,6-dimethylpyrazine
6.64	3	108	108	2,5-dimethylpyrazine
7.257	4	122	122	2-ethyl-5-methylpyrazine
7.323	5	122	122	2-ethyl-6-methylpyrazine
7.48	6	122	122	2,3,5-trimethylpyrazine
7.87	7	136	136	ethyldimethylpyrazine isomer
8.032	8	136	136	ethyldimethylpyrazine isomer
8.18	9	136	136	2,3,5,6-tetramethylpyrazine
8.52	10	150	122	2,3,5-trimethyl-6-ethylpyrazine
8.71	11	150	122	2,6-dimethyl-3-propylpyrazine
8.81	12	164	164	2,5-diethyl-3,6-dimethylpyrazine
9.57	13	178	122	2,6-dimethyl-3-(2-methylbutyl)pyrazine
9.76	14	178	122	2,5-dimethyl-3-(2-methylbutyl)pyrazine
9.96	15	178	122	2,5-dimethyl-3-(3-methylbutyl)pyrazine
9.94	16	148	148	2,5-dimethyl-3-propylpyrazine
10.07	17	148	148	dimethyl-3-*cis*-propenylpyrazine isomer
10.146	18	148	148	dimethyl-3-*cis*-propenylpyrazine isomer
10.338	19	148	148	2-isopropenyl-3,6-dimethylpyrazine
12.57	20	164	122	2-(2-methylpropyl)-3,5-dimethylpyrazine
12.74	21	164	122	2,6-dimethyl-3-isobutylpyrazine
12.94	22	178	136	2-(2-methylpropyl)-3,5,6-trimethylpyrazine
6.26	*23*	*74*	*43*	*1-OH-acetone remaining after reaction*

was reacted with 0.5 mL of NH_4OH and 10 mL of H_2O. The pH level for this reaction was measured to be around 11.0. In the second reaction, the same volume of reactant was mixed, but the pH level was adjusted to 8.0 using concentrated H_3PO_4. Both reactions were heated at 120 °C for 12 h (Figure 2). As can be observed, the yield of pyrazines was higher when the pH level was highest, approximately 11.0. At the lower pH of approximately 8.0, the quantitative contribution from lower molecular weight pyrazines increased significantly. The quantitative distribution of the pyrazines is described below. The contribution from the dimethylpyrazines is labeled C2, representing pyrazines with a total of 2 carbons attached to the ring. The contribution from pyrazines having a total of 3 to 4 carbons attached to the ring is labeled C3–C4, representing pyrazines such as ethylmethylpyrazines, diethylpyrazines, tetramethylpyrazine and the like. The contribution from pyrazines having a total of 5 or more carbons attached to the ring is labeled C5 representing pyrazines such as diethylmethylpyrazines, trimethylethylpyrazines and those pyrazines having nonlinear alkyl groups

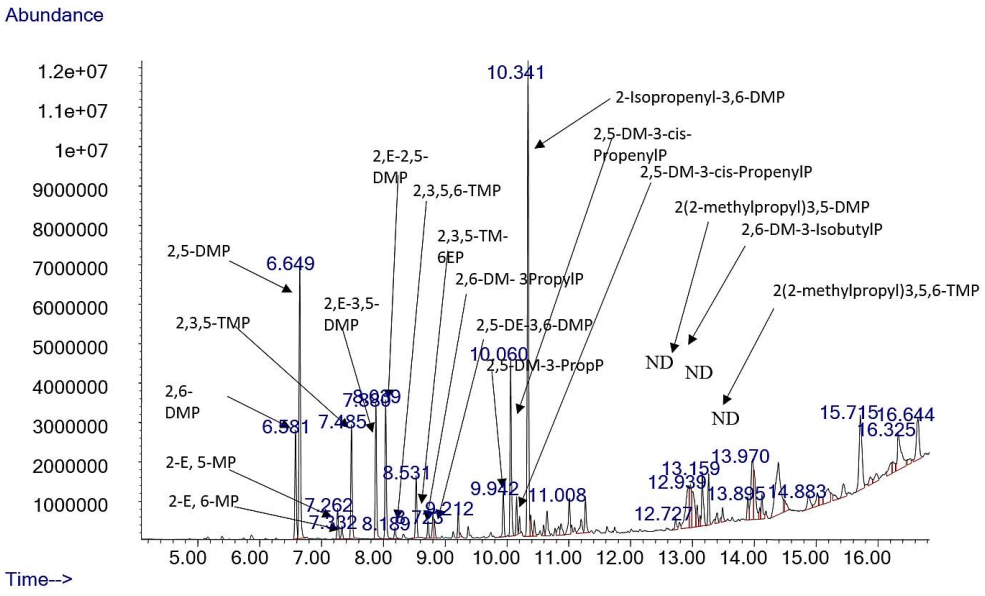

Figure 1. Total ion current (GC/MS) chromatogram of pyrazines produced as a result of the reaction between acetol and NH_4OH.

attached such as isopropylmethylpyrazines and the like. No pyrazine or methylpyrazine was detected in either of these reactions mimicking the results with acetoin, see previous page. Based on these results, the pH level of later experiments was held at pH 11.0 or higher.

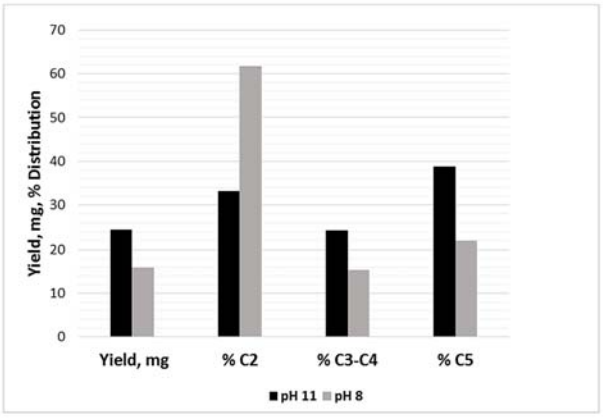

Figure 2. Effect of pH on synthesis of pyrazines using acetol and NH_4OH.

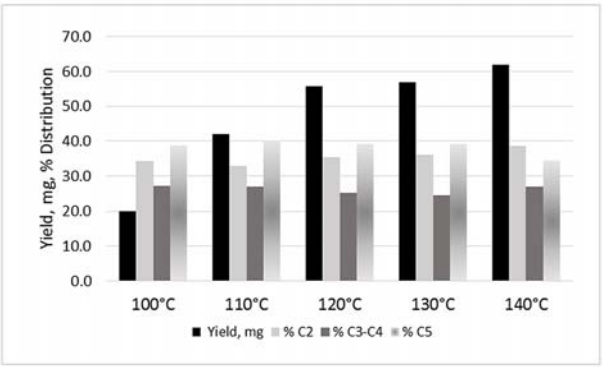

Figure 3. Effect of temperature (100, 110, 120, 130, and 140 °C) on synthesis of pyrazines using acetol and NH_4OH.

Figure 3 shows the effects of temperature (100, 110, 120, 130, and 140 °C) on the synthesis of pyrazines using 1-hydroxyacetone (1 g) and NH_4OH (1 g) with a 1:2 mole ratio of C:N in 10 mL of H_2O for 12 h. The pyrazine yield increased as the temperature increased, continuing to show an increase in pyrazine yields at the highest temperature tested, 140 °C. No notable consistent shift in pyrazine distribution was observed as a function of reaction temperature. Figure 4 shows the effect of varying reaction times (4, 8, 12, 16, and 24 h) on the synthesis of pyrazines using 1-hydroxyacetone (1 g) and NH_4OH (1 g) with a 1:2 mole ratio of C:N in 10 mL of H_2O. In this part of the study, maximum yields of pyrazines were obtained when the reaction time was 16 h with an accompanying shift in distribution to pyrazines having higher molecular weights as the reaction time increased. Figure 5 shows the effect on pyrazine yields of an acetol:NH_4OH reaction at mole ratios of 1:0.5, 1:1, 1:2, and 1:2.5 when the reaction conditions were: 10 mL of H_2O, 120 °C and 12 h. The optimum ratio for maximum pyrazine yield was 1:2, which was equal to 1 g

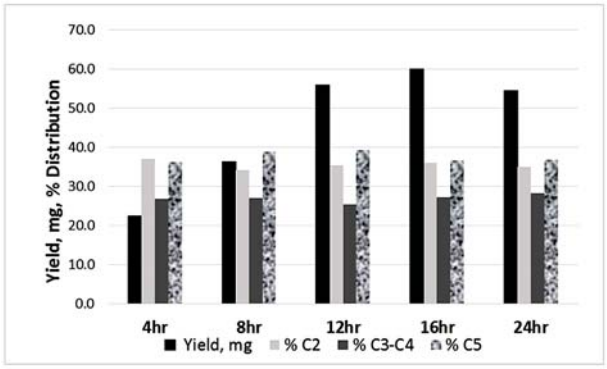

Figure 4. Effect of reaction time on synthesis of pyrazines using acetol and NH_4OH.

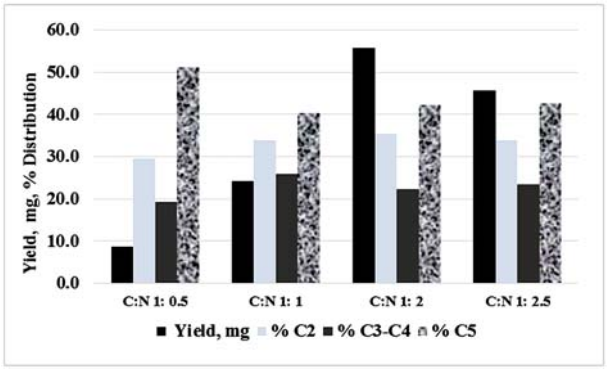

Figure 5. Effect of C:N mole ratio on synthesis of pyrazines using acetol and NH_4OH.

of 1-hydroxyacetone and 1 mL of NH_4OH. When a higher volume of NH_4OH was used in the reaction, the pyrazine yield of the reaction dropped by more than 10%. Close examination of the structures of the pyrazines produced when acetol served as the carbon source deserves attention and reveals the absence of any detectable amounts of pyrazine and/or methylpyrazine in all of these reactions. Of significant note with this particular set of reaction conditions and reagent ratios is that when the amount of NH_4OH was increased systematically, a meaningful increase in the contribution of one pyrazine, 2-isopropenyl-3,6-dimethylpyrazine, from 3.4 to 14.1% was observed.

Effect of amino acids and aldehyde addition

Table 3 shows the effects of amino acids and aldehyde additions on the reaction of 1-hydroxyacetone and NH_4OH with C:N mole ratio 1:2 mixed with 10 mL H_2O at 120 °C for 16 h. For this purpose, two different amino acids, as well as isovaleraldehyde were used. It was observed that when isoleucine and threonine were used as additional nitrogen sources, the contribution from C4 and C5 pyrazines increased slightly. Decreases in the contribution from the highest molecular weight pyrazines were found to have occurred. It was interesting to note that the total yields of pyrazines were similar when threonine or isoleucine was added to the reaction, both compounds having increased the total yield of pyrazine by more than 7%. The addition of isovaleraldehyde to a reaction of 1-hydroxyacetone and NH_4OH caused the

Table 3. Effect on pyrazines yields and distribution of added amino acids and aldehydes to reaction of acetol and NH_4OH.

Reaction time (h) Peak #	16 h Reaction 41 (mg)	16 h + 0.1 g Isoleucine Reaction 42 (mg)	16 h + 0.2 g Threonine Reaction 43 (mg)	16 h + 100 µL Isovaleraldehyde Reaction 44 (mg)
1	0.25	0.25	0.25	0.25
2	5.57 (9.3) [a]	7.79 (12.1) [a]	7.12 (10.9) [a]	6.11 (7.6) [a]
3	16.05 (26.8)	20.27 (31.4)	18.68 (28.5)	14.85 (18.5)
4	1.11 (1.9)	1.12 (1.7)	0.78 (1.2)	1.18 (1.5)
5	0.47 (0.8)	0.48 (0.8)	0.26 (0.4)	0.46 (0.6)
6	4.88 (8.1)	4.68 (7.3)	4.63 (7.1)	4.20 (5.2)
7	3.56 (5.9)	3.34 (5.2)	7.53 (11.5)	3.04 (3.8)
8	3.54 (5.9)	3.03 (4.7)	7.15 (10.9)	2.52 (3.1)
9	0.42 (0.7)	0.29 (0.5)	0.32 (0.5)	0.33 (0.4)
10	1.03 (1.7)	1.50 (2.3)	2.05 (3.1)	0.74 (0.9)
11	0.77 (1.3)	1.32 (2.1)	1.82 (2.8)	0.57 (0.7)
12	0.55 (0.9)	0.46 (0.7)	1.07 (1.6)	0.42 (0.5)
13	0.00 (0)	1.02 (1.6)	0.00 (0)	0.00 (0)
14	0.00 (0)	0.06 (0.1)	0.00 (0)	14.11 (17.6)
15	0.00 (0)	0.00 (0)	0.00 (0)	12.96 (16.1)
16	0.52 (0.9)	0.45 (0.7)	0.28 (0.4)	0.30 (0.4)
17	2.12 (3.5)	2.12 (3.3)	1.38 (2.1)	1.61 (2.0)
18	0.42 (0.7)	0.48 (0.8)	0.42 (0.6)	0.33 (0.4)
19	8.16 (13.6)	5.90 (9.1)	3.92 (6.0)	5.68 (7.1)
20	4.60 (7.7)	4.98 (7.7)	4.02 (6.1)	4.82 (6.0)
21	4.75 (7.9)	4.21 (6.5)	3.19 (4.9)	4.81 (6.0)
22	1.38 (2.3)	0.97 (1.5)	0.91 (1.4)	1.25 (1.6)
23 [b]	*0.77*	*0.81*	*7.12*	*7.37*
Total P [c] (mg)	59.91	64.49	65.53	80.28

[a] The number in the brackets represents the (%) distribution
[b] remaining 1-OH-acetone in mg after completed reaction
[c] P = Pyrazines

yield of 2,5-dimethyl-3-(2-methylbutyl) pyrazine and 2,5-dimethyl-3-(3-methylbutyl) pyrazine to increase from non-detected (0) to 14 and 13 mg, respective-ly. The total yield of pyrazines increased by more than 20%. These results were very similar to those obtained when high fructose corn syrup (HFCS) served as the carbon source, suggesting that from a mechanistic perspective, HFCS may convert to alpha-hydroxy ketone(s) when reacted under similar conditions with similar reagents. The appearance of pyrazines with alkyl side chains having the structure of the added aldehyde was consistent with previous findings (4).

Table 4 shows the results of a similar study, but instead of using a pure amino acid, a mixture of free amino acids prepared from the hydrolysis of F1 protein was used in the reaction. Since hydrolyzed F1 protein was already in an aqueous solution, no water was added to the mixture. For this purpose, 10 mL of hydrolyzed F1 protein which contained about 0.2 g of amino acid and 10 mL of H_2O was reacted with 1-hydroxyacetone and NH_4OH with a C:N mole ratio of 1:2 at 120 °C for 16 h. The yield of 2,5-dimethylpyrazine increased by more than 80% and the yield of 2,5-dimethyl-3-(2-methylbutyl) pyrazine and 2,5-dimethyl-3-(3-methylbutyl) pyrazine increased from 0 to more than 1 mg. Thus, most likely, the alkyl portion of specific amino acids was converted to a Strecker aldehyde which in turn reacted with the ammonium hydroxide to form an imine which subsequently was incorporated into a pyrazine structure. This observation is consistent with the literature (1–15).

Figure 6 shows additional results from a study concerning the effects of different temperatures and C:N mole ratios (1:1 and 1:2) on the synthesis of pyrazines using 1-hydroxyacetone and NH_4OH. In these studies, increasing the temperature from 100 to 120 °C and manipulating the C:N mole ratio, increased the yield of pyrazines. Increasing reaction temperatures systematically shifted the distribution of pyrazines away from C2 toward the higher molecular weight pyrazines represented by C3–C4 and C5 pyrazines.

Reaction using 1-hydroxy-2-butanone instead of acetol

Table 5 shows results for the reaction of 1-hydroxy-2-butanone as a different source of carbon with NH_4OH at optimum conditions obtained from an initial optimization using acetol. For this purpose, 1 g of 1-hydroxy-2-butanone was reacted with 1 mL of NH_4OH and 10 mL of H_2O at 120 °C for 16 h. No pyrazines or methyl pyrazines were formed. All pyrazines contained ethyl or higher branched alkanes. The yield of pyrazines was, however, not as high as when acetol was used. As mentioned earlier, with acetol as the carbon source, no pyrazine or methylpyrazine was produced. With 1-hydroxy-2-butanone, no pyrazines, methylpyrazines, or standalone dimethylpyrazines were produced, confirming that the carbon source is dictating the structure of the pyrazines. The structures of acetol and 1-hydroxy-2-butanone are shown in Figure 7. It is important to note that for acetol a methyl group is bound to one side of the carbonyl and for 1-hydroxy-2-butanone an ethyl group is bound to one side of the carbonyl. These structural features of the two α,β-hydroxyketones obviously dictate the structure of the

Table 4. Effect on pyrazines yields and distribution of hydrolyzed F1 protein (10 mL instead of H_2O = 0.2 g mixture of AA's) and amino acids as nitrogen sources on reaction of acetol and NH_4OH.

Added amino acid Peak #	— Reaction 41, Yield (mg)	0.1 g Isoleucine Reaction 42, Yield (mg)	0.2 g Threonine Reaction 43, Yield (mg)	0.2 g hydrolyzed F1 Reaction 45, Yield (mg)
1	0.25	0.25	0.25	0.25
2	5.57 (9.3) [a]	7.79 (12.1) [a]	7.12 (10.9) [a]	8.37 (11.1) [a]
3	16.05 (26.8)	20.27 (31.4)	18.68 (28.5)	31.01 (41.3)
4	1.11 (1.9)	1.12 (1.7)	0.78 (1.2)	0.82 (1.1)
5	0.47 (0.8)	0.48 (0.8)	0.26 (0.4)	0.34 (0.4)
6	4.88 (8.1)	4.68 (7.3)	4.63 (7.1)	5.61 (7.5)
7	3.56 (5.9)	3.34 (5.2)	7.53 (11.5)	4.18 (5.6)
8	3.54 (5.9)	3.03 (4.7)	7.15 (10.9)	3.50 (4.7)
9	0.42 (0.7)	0.29 (0.5)	0.32 (0.5)	0.34 (0.5)
10	1.03 (1.7)	1.50 (2.3)	2.05 (3.1)	2.31 (3.1)
11	0.77 (1.3)	1.32 (2.1)	1.82 (2.8)	2.10 (2.8)
12	0.55 (0.9)	0.46 (0.7)	1.07 (1.6)	0.55 (0.7)
13	0.00 (0)	1.02 (1.6)	0.00 (0)	0.13 (0.2)
14	0.00 (0)	0.06 (0.1)	0.00 (0)	1.09 (1.5)
15	0.00 (0)	0.00 (0)	0.00 (0)	1.01 (1.3)
16	0.52 (0.9)	0.45 (0.7)	0.28 (0.4)	0.35 (0.5)
17	2.12 (3.5)	2.12 (3.3)	1.38 (2.1)	1.53 (2.0)
18	0.42 (0.7)	0.48 (0.8)	0.42 (0.6)	0.40 (0.5)
19	8.16 (13.6)	5.90 (9.1)	3.92 (6.0)	4.40 (5.9)
20	4.60 (7.7)	4.98 (7.7)	4.02 (6.1)	3.96 (5.3)
21	4.75 (7.9)	4.21 (6.5)	3.19 (4.9)	2.32 (3.1)
22	1.38 (2.3)	0.97 (1.5)	0.91 (1.4)	0.85 (1.1)
23 [b]	*0.77*	*0.81*	*7.12*	*0.67*
Total P [c] (mg)	59.91	64.49	65.53	75.15

[a] The number in the brackets represents the (%) distribution
[b] remaining 1-OH-acetone in mg after completed reaction
[c] P = Pyrazines

Table 5. Mass and distribution of synthesized pyrazines using 1-hydroxy-2-butanone and NH_4OH.

Retention time (min)	MW	Ion	Analyte	Reaction 46, Yield (mg)
8.01	136	136	2,5-diethylpyrazine	7.28 (24.7) [a]
8.35	150	150	2-ethyl-3,5,6-trimethylpyrazine	1.75 (5.9)
8.49	150	150	dimethyl-2-N-propylpyrazine isomer	0.25 (0.8)
8.56	150	150	2,5-diethyl-3-methylpyrazine	1.91 (6.5)
8.70	164	164	2,3-diethyl-5,6-dimethylpyrazine	0.38 (1.3)
10.76	190	190	*trans*-3-methyl-2-N-propyl-6(1-butenyl)pyrazine	0.29 (1.0)
14.07	136	136	2,6-dimethyl-3-ethylpyrazine isomer	2.85 (9.7)
			Total mass of pyrazines (mg)	29.4

[a] The number in the brackets represents the (%) distribution

resulting pyrazines, yielding only pyrazines which have multiple methyl and ethyl alkyl substituents. Studies with high fructose corn syrup instead of hydroxyketones as carbon sources have revealed a much larger array of pyrazines produced under similar reaction conditions including the less than desirable pyrazine and methylpyrazine which together have accounted for more than 50% of the pyrazine yield.

Large-scale reaction of 1-hydroxyacetone and NH_4OH

To test the capability for this reaction to be scaled higher, a Parr reactor with greater reaction volume was employed. Thus, 100 g of 1-hydroxyacetone was reacted with 100 mL of NH_4OH and 1000 mL of H_2O at 120 °C for 16 h in a 1.5-L Parr high-pressure vessel. After the reaction was complete, the mixture was cooled and transferred into a glass bottle. Of note here was that the bottom of the vessel contained a noticeable amount of a "tar"-like material which was soluble in MeOH. The addition of H_2O to the top of this solid material caused it to become hard. This "tar"-like material was well segregated from the aqueous solution. In addition, reagent concentration studies in a related effort have shown that overall reagent concentrations can play a significant role in the presence or absence of the "tar"-like material. For all optimization studies, vide supra, the amount

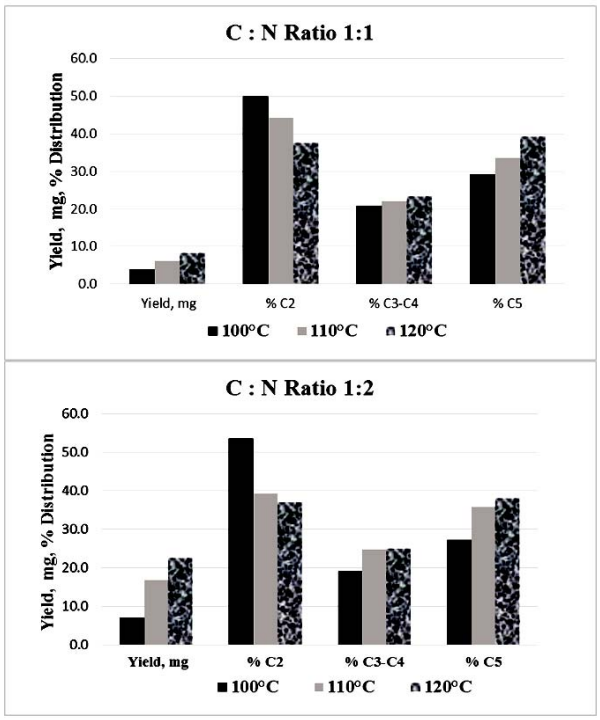

Figure 6. Effect of temperature and C:N mole ratio on synthesis of pyrazines using acetol and NH_4OH.

of "tar" at the bottom of 40 mL-reaction vessel was found to be very low. For this reason, a small volume of methanol (1 mL) was sufficient to dissolve everything and include it with the remaining reaction material.

After the completion of the reaction, portions of the aqueous solution were distilled (3 × 375 mL) at an oil bath

Figure 7. The structure of acetol or 1-hydroxyacetone (left) and 1-hydroxy-2-butanone (right).

temperature of 130–140 °C (16). In each distillation (375 mL), approximately 175 mL of aqueous solution containing different pyrazines was collected (light yellow color, total volume collected amounted to about 500 mL). The distilled materials (3 ×175 mL) were combined and passed through a C18 column (15 × 2.5 cm packed with SPE material) in order to remove the pyrazines from the water. After removal of the water from the C18 column using a gentle stream of dry nitrogen, the trapped pyrazines were eluted using ethanol. Ethanol was removed using a rotary evaporation and vacuum. Residual water in the final product was removed from the pyrazines by dissolving them in MTBE and drying over anhydrous sodium sulfate. MTBE was then removed using a rotary evaporator and vacuum. Three pyrazines were detected in a DCM extract of the reaction mixture after distillation. These pyrazines thus remained behind in the reaction mixture and were not distilled, possibly due to their non-volatility with steam and their higher molecular weight. The failure of these pyrazines to be distilled contributed to a lower percent

Table 6. Mass and distribution of isolated pyrazines after distillation and isolation from a relatively large scale reaction of acetol and NH_4OH.

Peak #	Pyrazine	Yield (mg)
1	ISTD [a]	0.25
2	2,6-dimethylpyrazine	290.1 (9.9) [b]
3	2,5-dimethylpyrazine	838.9 (28.6)
4	2-ethyl-5-methylpyrazine	45.3 (1.5)
5	2-ethyl-6-methylpyrazine	20.3 (0.7)
6	2,3,5-trimethylpyrazine	297.1 (10.1)
7	ethyldimethylpyrazine isomer	198.3 (6.8)
8	ethyldimethylpyrazine isomer	208.4 (7.1)
9	2,3,5,6-tetramethylpyrazine	25.9 (0.9)
10	2,3,5-trimethyl-6-ethylpyrazine	54.7 (1.9)
11	2,6-dimethyl-3-propylpyrazine	41.9 (1.4)
12	2,5-diethyl-3,6-dimethylpyrazine	26.9 (0.9)
16	2,5-dimethyl-3-propylpyrazine	45.6 (1.6)
17	dimethyl-3-*cis*-propenylpyrazine isomer	151.9 (5.2)
18	dimethyl-3-*cis*-propenylpyrazine isomer	27.1 (0.9)
19	2-isopropenyl-3,6-dimethylpyrazine	656.0 (22.4)
20	2-(2-methylpropyl)-3,5-dimethylpyrazine	4.7 (0.2)
	Total mass of pyrazines (mg)	2932.9

[a] ISTD: Internal standard
[b] The number in the brackets represents the (%) distribution

yield. These "non-steam-volatile" pyrazines were identified as 2-(2-methylpropyl)-3,5-dimethylpyrazine (12.57 min), 2,6-dimethyl-3-isobutylpyrazine (12.74 min), and 2-(2-methylpropyl)-3,5,6-trimethylpyrazine (12.95 min). The presence of those pyrazines in the reaction mixture confirms the participation of the free amino acids from the hydrolyzed F1 protein in the pyrazine synthesis. Table 6 shows the quantity of each individual pyrazine and its percent distribution after distillation, isolation, and extraction with MTBE. Of note is the observation that four pyrazines: 2,6-dimethylpyrazine; 2,5-dimethylpyrazine; 2,3,5-trimethylpyrazine; and 2-isopropenyl-3,6-dimethylpyrazine accounted for 71% of the pyrazine distribution, in the final product. A group of dimethylpyrazine derivatives, ethyldimethylpyrazine isomers and dimethylisopropenylpyrazine isomers accounted for an additional 20% of the total. The yield of total pyrazines from distillation of 1200 mL of reaction solution (100 g of 1-hydroxyacetone + 100 mL of NH_4OH) was about 60% of the value that would have been projected from the yield obtained from the 12 mL reaction (1 g of 1-hydroxyacetone + 1 mL of NH_4OH). This yield calculation does not include contribution from the pyrazines that remained in the post distillation reaction mixture. The lower % yield could be due to a variety of causes among them being loss of sample during the clean-up process and differences in isolation/extraction efficiencies. Also, the lower percentage could be due to formation of the "tar"-like material at the bottom of the large reaction vessel which was only soluble in methanol.

CONCLUSIONS

Optimized reactions of alpha-hydroxyketones with different nitrogen bases, aldehydes, and free amino acids as secondary nitrogen sources have been shown to produce a wide array of pyrazines. Changes in pH, reaction temperature and reaction time had nominal but consistent impacts on the pyrazine distribution, both qualitatively and quantitatively. The results showed that using optimized conditions (C:N mole ratio = 1:2, temperature = 120 °C, reaction time = 16 h, and pH = 11–12) at least 19–20 different pyrazines could be readily synthesized and isolated using a combination of atmospheric distillation and column chromatography. Addition of amino acids or a selected aldehyde altered the qualitative distribution of synthesized pyrazines, and also the pyrazine yield. Results further confirmed the hypothesis that steering the structure(s) of the alpha hydroxyketone carbon source(s) coupled with the presence or absence of free amino acids and aldehydes can offer unprecedented control of the types of pyrazines being synthesized.

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