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Optimization of α-Hydroxyketone and Pyrazine Syntheses Employing Preliminary Reactions of Glucose and Buffer Solutions*

by

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SUMMARY

Glucose and selected phosphate buffers have been reacted employing systematic variations in reaction temperature and time (150–160 °C for 60–90 min) to optimize the yield of acetol. This mixture was reacted further with NH_4OH, systematically varying reaction conditions and reagent ratios to optimize pyrazine yield. The highest yield of pyrazine was obtained when 1 g of glucose was reacted with 25 mL of buffer at 150–160 °C for 60 min, which in turn was reacted with 1 mL of concentrated aqueous NH_4OH at 120–130 °C for 17–18 h. Higher temperatures and higher concentrations of glucose caused a decrease in the yield of pyrazines. The addition of hydrolyzed tobacco-derived F1 protein as a secondary source of nitrogen increased the yield of pyrazines by 2–10% depending on F1 protein concentration. Furthermore, the addition of any α-hydroxyketone, similar in structure to acetol, as a pure reagent to the reaction mixture not only increased the yields of pyrazine by ranging from 25–100 % depending on the reagent concentration, but also significantly altered the qualitative and quantitative distribution of the pyrazines. With all of the reaction parameters examined (reaction time, temperature, reagent ratios, etc.) the most significant impacts on both pyrazine yield and distribution were noted when: 1) glucose was pre-reacted with buffer, 2) hydrolyzed F1 protein was added as a second nitrogen source, and 3) when pure α-hydroxyketones were employed as co-reagents. Use of these reaction parameters was found to dramatically shift the pyrazine distribution toward higher molecular weight resulting in a pyrazine array having more desirable physical and sensory attributes. [Beitr. Tabakforsch. Int. 28 (2019) 329–339]

ZUSAMMENFASSUNG

Es wurden Glukose und eine Auswahl an Phosphat-Puffern bei systematischer Variation der Reaktionszeit und -temperatur (60–90 min bei 150–160 °C) zur Reaktion gebracht, um die Ausbeute an Acetol zu optimieren. Dieses Gemisch reagierte anschließend mit NH_4OH weiter, wobei systematisch die Reaktionsbedingungen und das Reagenzverhältnis variiert wurden, um eine optimale Pyrazinausbeute zu erreichen. Die höchste Ausbeute an Pyrazinen wurde erzielt, wenn 1 g Glukose zunächst mit 25 mL Puffer für eine Dauer von 60 min bei 150–160 °C und anschließend mit 1 mL konzentrierter wässriger Ammoniaklösung NH_4OH über 17–18 h bei 120–130 °C reagierte. Höhere Temperaturen und höhere Glukosekonzentrationen bewirkten einen Rückgang der Pyrazinausbeute. Die Zugabe von hydrolisiertem Tabakprotein F1 als sekundärer Stickstoffquelle führte zu einer Zunahme der Pyrazinausbeute um 2–10% in Abhängigkeit von der Konzentration des Proteins F1. Zudem führte die Zugabe eines dem Acetol strukturell ähnlichen α-Hydroxyketons als reinem Reagenz zum Reaktionsgemisch nicht nur zu einer Erhöhung der Ausbeuten an Pyrazin um 25–100% in Abhängigkeit von der Reagenzkonzentration, sondern veränderte auch signifikant die quantita-

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tive und qualitative Verteilung der Pyrazine. Bei der Untersuchung aller Reaktionsparameter (Reaktionszeit, Temperatur, Reagenzverhältnis) ergaben sich die signifikantesten Auswirkungen auf die Pyrazinausbeute und -verteilung, wenn 1) die Glukose mit einem Puffer vorreagiert wurde, 2) hydrolisiertes Protein F1 als weitere Stickstoffquelle hinzugegeben wurde und 3) reine α-Hydroxyketone als Koreagenzien eingesetzt wurden. Es konnte bei der Anwendung dieser Reaktionsparameter eine deutliche Veränderung der Pyrazinverteilung hin zu einem höheren Molekulargewicht und damit zu einer Pyrazinreihe mit wünschenswerteren physikalischen und sensorischen Eigenschaften festgestellt werden. [Beitr. Tabakforsch. Int. 28 (2019) 329–339]

RESUME

Le glucose et une solution-tampon composée d'une sélection de phosphates furent soumises à des réactions avec des variations systématiques du temps et de la température de réaction (150–160 °C durant 60–90 min) afin d'optimiser le rendement d'acétol. Par l'adjonction de NH_4OH, ces mélanges furent ensuite soumis à une autre réaction durant laquelle nous avons systématiquement fait varier les conditions de réaction et les ratios de réactifs afin d'optimiser le rendement des pyrazines. Le rendement des pyrazines le plus élevé fut obtenu lorsqu'un gramme de glucose fut mis en réaction avec 25 mL de solution-tampon à une température de 150–160 °C durant 60 min, pour ensuite être mis en réaction avec 1 mL d'ammoniaque (solution aqueuse concentrée) à une température de 120–130 °C durant 17–18 h. Les températures et les concentrations plus élevées du glucose provoquèrent une baisse du rendement des pyrazines. L'adjonction de la protéine F1 hydrolysée dérivée du tabac en guise de source secondaire d'azote augmenta le rendement des pyrazines, dans une plage de 2–10%, selon la concentration de la protéine F1. Par ailleurs, l'adjonction d'une quelconque α-hydroxycétone, d'une structure similaire à celle de l'acétol, en guise de réactif pur, au mélange de réaction augmenta non seulement les rendements des pyrazines dans une plage de 25–100% selon la concentration du réactif mais altéra aussi de manière significative la distribution qualitative et quantitative des pyrazines. Après l'examen de tous les paramètres de réaction (temps de réaction, température, ratios de réactifs, etc.), il s'avéra que les effets les plus significatifs tant sur le rendement que sur la distribution des pyrazines purent être observés lorsque 1) le glucose fut soumis à une réaction préalable avec le tampon, 2) la protéine F1 hydrolysée fut ajoutée en guise de seconde source d'azote et 3) les α-hydroxycétones pures furent utilisées en guise de coréactifs. Selon nos observations, ces paramètres de réaction modifieraient de façon spectaculaire la distribution des pyrazines en faveur d'un poids moléculaire plus élevé et serait à l'origine d'une série de pyrazines présentant des attributs sensoriels et physiques plus attrayants. [Beitr. Tabakforsch. Int. 28 (2019) 329–339]

INTRODUCTION

Research into expansion of the potential of tobacco-derived materials in the preparation of ever widening types of organic substances, including flavors and fragrances, has yielded surprising findings. For example, the chemical conversion of residual tobacco biomass to pulp and lignin along with the isolation of tobacco F1 protein has represented the creation of a new first-generation set of tobacco-based components. The subsequent biotechnical conversion of the tobacco-derived pulp to glucose represents a second-generation tobacco-derived component. Chemical and/or biotechnical conversion of the glucose to arrays of new components have represented third-generation products. In a similar fashion, the isolation followed by the biotechnical conversion of tobacco F1 protein into free amino acids represents a new possibly fourth-generation product. A fifth-generation material could be represented by the synthesis and isolation of desirable flavor materials from the tobacco-derived glucose and free amino acids. To maximize the use of these new tobacco-derived reagents, fundamental research was necessary to further understand the chemical mechanisms and reaction conditions that would ultimately lead to the greatest yields of flavor substances such as pyrazines, known for their positive contribution to tobacco-based sensory responses. As the research itself progressed, coupled with detailed examination of the literature, indications were clear that glucose-derived α-hydroxyketones (AHK) were and are key reagents directly linked to the production of specific pyrazines. That is, the structure of the AHK was directly related to the structure of the resulting pyrazine. Furthermore, the structure of the pyrazine plays an overwhelming role in the pyrazine sensory attributes, thresholds and notes. Hence model reactions employing commercially available AHK's were studied extensively and efforts to maximize their yield from tobacco-derived glucose were examined in great detail. Thus, the question was, how might the reaction conditions be optimized by employing the tobacco-derived glucose and possibly free amino acids to make the maximum amount of the desired arrays of the now-tobacco-derived pyrazines? In summary, these research initiatives were directed in part so as to illustrate and define to some extent the potential for the creation of multiple generations of unique tobacco-derived substances.

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 (1–11). These sugars have been confirmed as carbon sources for the formation of the pyrazine aromatic ring structure. Most often these reactions have featured ammonium hydroxide and/or free amino acids as nitrogen sources that furnish the nitrogen contained within the pyrazine ring. In the vast majority of these cases, pyrazine and methylpyrazine dominate the quantitative and qualitative array of pyrazines produced in these model reactions. These two pyrazines, from a physical property and sensory perspective, are generally viewed as less desirable (8, 9). Reaction protocols that significantly reduce or eliminate their presence in a pyrazine array and result in a corresponding increase of other substituted pyrazines of higher molecular weight, thereby having desirable sensory properties, would be viewed very positively for flavor applications.

Detailed investigations of the pyrazine literature strongly suggest that the structure of the carbon precursor in pyrazine

ring formation reactions has a notable impact on structures of the pyrazine array (12–14). In addition, when amino acids capable of producing Strecker aldehydes are used in model pyrazine reactions, the alkyl portion of the Strecker aldehyde structures appear as carbon substituents on the aromatic ring of the resultant pyrazines (12). 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 literature also indicates that 3-hydroxy-2-butanone, an α-hydroxyketone, could be a sugar degradation product that could subsequently be reacted with nitrogen sources to produce pyrazines (12).

The current literature also indicates that α-hydroxyketones and similar molecules such as 1-hydroxy-2-propanone (acetol, an α-hydroxyketone) can be produced from a C_6 sugar such as glucose which is obtainable as derivatives of glucose. These references (15–19) report on the conversion of the sugars to 1-hydroxy-2-propanone using phosphate buffers at elevated temperatures; a strong base, NaOH at pH 11.5, under reflux; nickel catalysts under hydrogen pressure; and copper chromite catalysts in gas-phase heterogeneous reactions. In some cases, the yield of 1-hydroxy-2-propanone was 25% with small contributions from 1-hydroxy-2-butanone and acetone.

Cellulose, when viewed as a polymer of glucose, has been converted to acetol with a 30% yield using a tin-based catalyst system (20). NOVOTNY *et al.* synthesized α-hydroxycarbonyl and α-dicarbonyl compounds via degradation of monosaccharides (21). The maximum yield of α-hydroxycarbonyl (glycolaldehye, acetol, lactaldehyde, glyceraldehyde, 1,3-dihydroxyacetone and acetoin) when glucose or fructose was reacted with sodium hydroxide was approximately 4%. Acetol and 1,3-dihydroxyacetone had the highest yield (2.52% and 1.02%, respectively) when sodium hydroxide was used.

Previously, NODZU reported (22) the synthesis of acetol from the reaction of glucose and a phosphate buffer at a pH of 6.5–7.1. The yield of acetol varied from 3.3–4.4%. Preliminary experiments have indicated similar yields using a buffer solution with a pH 6.5–6.8, and when the product of this reaction was reacted with NH_4OH, neither pyrazine nor 2-methypyrazine were detected in meaningful quantities and the pyrazine reaction product was dominated by higher-molecular weight pyrazines.

The first objective of this research was to optimize conditions (time, pH, temperature, glucose concentration, buffer concentration) for maximum yield of α-hydroxyketones generated by hydrolysis of glucose in the presence of phosphate buffer. A secondary objective was to use these optimum conditions for synthesis of α-hydroxyketones followed by reaction *in situ* with and without free amino acids in the presence of diammonium phosphate and/or NH_4OH and H_3PO_4 to determine the optimum conditions for the synthesis of novel arrays of pyrazines, having generally higher molecular weight characteristics and containing branched alkyl side chains. A subset of these objectives was to focus on those pyrazine arrays whose quantitative distribution contained little or no detectable amounts of pyrazine and methylpyrazine. It was also essential to define the influence of the addition of enzymatically hydrolyzed tobacco-derived F1 protein (free amino acids) (23) as secondary nitrogen sources in the reaction and how this reagent(s) addition could change both the yields and types of pyrazines synthesized.

EXPERIMENTAL

Materials

Glucose, 1-hydroxy-2-butanone (acetoin), acetol, deuterated (d6) 2-methylpyrazine, sodium hydroxide, potassium phosphate dibasic, potassium phosphate monobasic, sodium chloride, sodium sulfate anhydrous, phosphoric acid, methanol, and dichloromethane (DCM) were obtained from Sigma-Aldrich (St. Louis, MO, USA) and were used as received. F1 protein and cellulosic-derived sugar (CDS) as an aqueous solution containing 78% glucose, were obtained from R.J. Reynolds Tobacco Co. F1 protein was enzymatically hydrolyzed using optimum conditions which were developed previously (23). Amino acid content in all of the hydrolyzed solutions were in the range of 50–55%. All pyrazine synthesis reactions were performed in a high-pressure 40-mL Parr vessel with heating control jacket.

Buffer solution

Buffer solutions with three different concentrations (2, 1.67 and 1 M) and three different pHs (6.1, 7.1, and 8) were prepared to study the effect of buffer concentrations and pH on the synthesis of acetol. The first buffer solution was prepared by mixing 18 g of potassium phosphate monobasic and 22 g of potassium phosphate dibasic in 60 mL of deionized water (40% buffer). This buffer solution had a pH of 7.1. The concentrations of potassium phosphate monobasic and potassium phosphate dibasic were 2.2 and 2.1 M, respectively. This buffer was diluted with H_2O to reduce the concentration of each phosphate to half while keeping the pH in the same range. A third buffer was prepared by mixing 23.8 g of potassium phosphate monobasic and 29 g of potassium phosphate dibasic in 100 mL of deionized water. This buffer solution had a pH of 7.1. The concentration of potassium phosphate monobasic and potassium phosphate dibasic were 1.75 and 1.67 M, respectively. To study the effect of pH on the synthesis of acetol, a pH of the same buffer solution (containing 18 g of potassium phosphate monobasic and 22 g of potassium phosphate dibasic in 60 mL of deionized water) was lowered to 6.1 using phosphoric acid. For the buffer with the higher pH close to 8, a fourth buffer solution was prepared by mixing 1.9 g of potassium phosphate monobasic and 15.0 g of potassium phosphate dibasic in 50 mL H_2O.

Acetol synthesis conditions

The synthesis of acetol was performed in a high-pressure 40-mL Parr vessel with heating control system. To synthesize acetol, different parameters (buffer concentrations, pH values, temperatures, mass of glucose and reaction times) were optimized to maximize the quantity of acetol. The initial reaction conditions were obtained from the literature for the synthesis of acetol using 0.1 N NaOH and glucose. In

this reaction, 0.5 g of glucose were mixed with 25 mL of buffer solution at 140 °C for 60 min. Next, the same conditions were used, but buffer solutions with different concentrations and different pHs were tested. The optimum buffer concentration and optimum pH were used to study the effect of different temperatures (120, 140, 180, and 200 °C) on the synthesis of acetol. All reactions were first equilibrated for 60 min and then heated for an additional 60 min. Next, different masses of glucose (0.125, 0.25, 0.5, 0.75 and 1 g) were used with the optimum temperature, pH, and buffer concentration for 60 min to determine the optimum mass of glucose required to maximize the mass of acetol. Finally, the reaction time (30, 60, 90 and 120 min) was optimized to obtain the maximum mass of acetol. To quantify acetol, each reaction was extracted twice with fixed volume (25 mL) of DCM and analyzed via GC/MS using same conditions specified in instrumentation section. An internal standard, 1-hydroxy-2-butanone, was used for quantification.

Pyrazine synthesis conditions using acetol

The synthesis of pyrazines was performed in a high-pressure 40-mL Parr vessel with heating control system. Reactions were performed by reacting the acetol synthesized from the reaction of glucose and buffer solution with NH_4OH at different conditions without isolation. In this reaction every 25 mL of solution was reacted either with 0.5, 0.1 or 2 mL of NH_4OH. Reaction temperatures were set at 100, 120, 140 and 160 °C for periods of 12, 17 and 24 h. After the completion of each reaction, the mixture was cooled and spiked with 0.25 mg of deuterated (d6) 2-methylpyrazine as an internal standard. Next, the solution was extracted with 30–35 mL of DCM as a solvent. The DCM solution was dried over sodium sulfate and analyzed via GC/MS for identification and quantification. Different extracted ions were used to quantify each pyrazine.

Instrumentation

All gas chromatography/mass spectrometry (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 a DB-WAXTER capillary column (30 m long × 250 µm I.D. with a film thickness of 0.25 µm) from J&W (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 peaks. For quantitative analysis, the calibration curve was prepared using a concentration of acetol and a response factor ratio of acetol/acetoin. It is important to note here that acetoin was not detected in any reactions performed at 120 and 140 °C. However, acetoin residue was found in the reaction where the temperature was set to 160 °C. Figure 1 shows the GC/MS of DCM extract of the reaction of 1 g of glucose with buffer solution with a pH of 7.1 at 160 °C for 60 min followed by addition of 1 mL of NH_4OH to the reaction and heating it at 120 °C for 17–18 h. After this time, the reaction mixture was spiked with internal standard and extracted with DCM. The characteristics of this chromatogram are representative of the chromatograms resulting from DCM extractions of all reactions discussed herein.

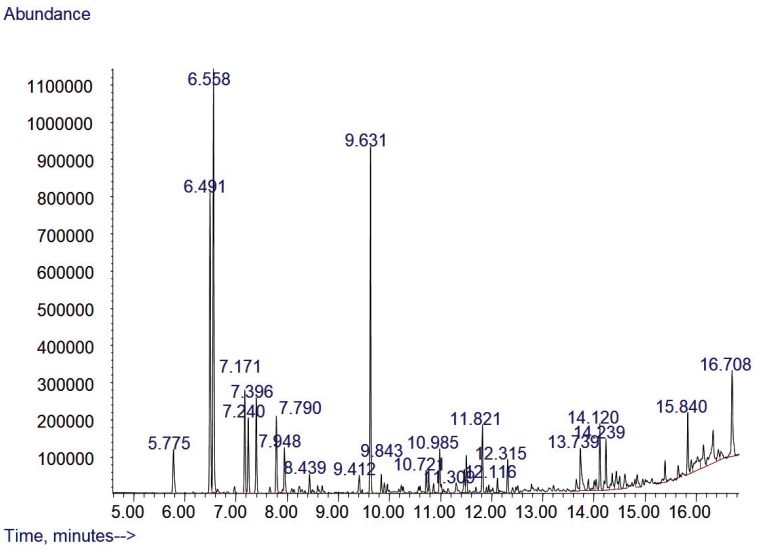

Figure 1. GC/MS of DCM extract of the reaction of 1 g of glucose with buffer solution with a pH of 7.1 at 160 °C for 60 min followed by addition of 1 mL of NH_4OH to the reaction and heating it at 120 °C for 17–18 h. See Table 1 for pyrazine structures correlated with retention times.

RESULTS AND DISCUSSION

Acetol synthesis optimization

Effect of buffer concentration and pH on acetol synthesis

Three different buffer concentrations with the same pH and three buffer solutions with different pHs were evaluated for the synthesis of acetol using glucose (Figure 2). It can be observed that the maximum quantity of acetol was obtained when 0.5 g of glucose was reacted with 25 mL of phosphate buffer with concentrations of 2.2 and 2.1 M at pH of 7.1 for 60 min at 140 °C. It is important to note here that the reaction temperature and time were not optimized for this particular set of reactions. Lowering the buffer concentration lowered the yield of acetol. At the same time, lowering the pH of the buffer from 6.8–7.1 to 6.1, while using the same conditions as previously described, lowered the yield of acetol from ~10 to ~6.5 mg. Increasing the pH from 6.8–7.1 to 8.0 did not have a major effect on acetol yield. From the results obtained of this part of the study, the remaining experiments were performed using phosphate buffer solutions with concentrations of 2.2 and 2.1 M with a pH of 7.1.

Effect of temperature on acetol synthesis using glucose and phosphate buffer

Next, 0.5 g of glucose were reacted with 25 mL of a phosphate buffer solution with a concentration of 2.2 and 2.1 M of KH_2PO_4 and K_2HPO_4 for 60 min at different temperatures (140 °C, 160 °C and 200 °C). In all these reactions the temperature of the Parr vessel was first equilibrated for 60 min and then the sample was heated for an additional set time. The maximum mass of acetol, 75 mg, was obtained when the reaction temperature was set to 160 °C. Increasing the temperature to 200 °C, not only decreased the mass of acetol to 65 mg but also produced a detectable amount of acetoin. At a temperature of 220 °C, no acetol was detected and the yield at 140 °C was relatively low at 22 mg.

Effect of glucose mass on acetol synthesis using phosphate buffer

To determine the optimum mass of glucose that is required to be reacted with a phosphate buffer to obtain the maximum amount of acetol, a different mass of glucose (0.125, 0.25, 0.5, 0.75 and 1 g) was reacted with 25 mL of phosphate buffer at 160 °C for 60 min (Figure 3). The highest percent yield of acetol/glucose was obtained when 0.25 g of glucose were used in the reaction. In addition, it appears that increasing the mass of glucose in the reaction above 0.25 g did not lead to a higher percent yield but that the increased mass of glucose raised the acetol yield above the 70 mg-level. Thus, even though the percent yield was lower employing the higher amount of glucose, investigations were undertaken by varying reaction times to see if more acetol could be synthesized. The assumption here rested with the hypothesis that a higher acetol concentration would translate directly into higher pyrazine concentration.

Effect of reaction time on acetol synthesis using glucose and phosphate buffer

In this part of our study, 0.5 g of glucose were reacted with 25 mL of phosphate buffer at 160 °C for different times and then the acetol concentrations were measured in each reaction. The results showed that the maximum amount of acetol (75 mg) could be obtained when the reaction mixture was heated to 160 °C for 60 min. Increasing the reaction time for more than 90 min marginally reduced acetol yield to 68 mg. A further yield reduction, to 37 mg, occurred when the reaction time was extended to 120 min. A reaction time of 30 min produced only 9 mg of acetol.

Pyrazines synthesis optimization using acetol

Effect of glucose concentration on synthesis of pyrazine using different buffers

Different parameters such as glucose concentration, reaction temperature, NH_4OH concentration, and reaction time were optimized after the reaction of glucose and buffer to synthesize different pyrazines. Table 1 lists the names of the identified pyrazines, their retention times, and the masses of the extracted ions that were used to quantify each compound from a reaction mixture of glucose and buffer with NH_4OH.

Table 1. Pyrazines detected: Their retention time (RT) and quantifying extracted ion. Glucose and buffer reacted with NH_4OH at 120 °C for 17 h.

Pyrazine RT (min)	Ion	Peak	Pyrazine structure
5.779	100	1	Istd*, d_6-2-methylpyrazine
5.779	94	2	2-methylpyrazine
6.492	108	3	2,6-dimethylpyrazine
6.559	108	4	2,5-dimethylpyrazine
6.763	108	5	2,3,dimethylpyrazine
7.172	122	6	2-ethyl-5-methylpyrazine
7.241	122	7	2-ethyl-6-methylpyrazine
7.398	122	8	2,3,5-trimethylpyrazine
7.665	135	9	2,6-diethylpyrazine
7.792	135	10	2-ethyl-3,5-dimethylpyrazine
7.914	135	11	2,5-diethylpyrazine
7.949	135	12	2-ethyl-2,5-dimethylpyrazine
8.090	136	13	2,3,5,6-tetramethylpyrazine
8.520	122	14	2,3,5-trimethyl-6-ethylpyrazine
8.710	122	15	2,6-dimethyl-3-propylpyrazine
8.810	164	16	2,5-diethyl-3,6-dimethylpyrazine
9.406	134	17	5-methyl-6,7-dihydro-cyclopentylpyrazine
9.760	122	18	2-methylbutyl-3,5-dimethylpyrazine
9.840	148	19	dimethyl-*cis*-propenylpyrazine isomer
9.960	136	20	2-acetyl-3-methylpyrazine
9.970	148	21	2,5-dimethyl-3-propylpyrazine
10.080	148	22	2,5-dimethyl-*cis*-propenylpyrazine isomer
10.183	134	23	2-methyl-5H-6,7-dihydrocyclopentylpyrazine
10.238	148	24	2,5-dimethyl-3-*trans*-propenylpyrazine
14.120	108	25	2-isoamyl-6-methylpyrazine

* Istd: Internal standard

In one part of the subsequent discussion the quantitative distribution of the pyrazines detected in the reaction mixtures will be grouped so as to somewhat simplify the presentation. The grouped quantitative distribution of the pyrazines is described in the following manner:

The contribution from the dimethylpyrazines is labeled C2, representing pyrazines with a total of two carbons attached to the ring. The contribution from pyrazines having a total of three attached to the ring is labeled C3, representing pyrazines such as e.g., ethylmethylpyrazines. The contribution of pyrazines having a total of four carbons attached to the ring is labeled C4, with representative pyrazines being diethylpyrazines, tetramethylpyrazine and the like. The contribution from pyrazines having a total of five or more carbons attached to the ring is labeled C5, representing pyrazines such as diethylmethylpyrazines, trimethylethylpyrazines and those pyrazines having nonlinear alkyl groups attached, such as isopropylmethylpyrazines, acetyl pyrazines, and the like.

Effect of glucose concentration on synthesis of pyrazine

Initially it was demonstrated that the highest percent yield for acetol could be obtained when 0.25 g of glucose reacted with 25 mL of phosphate buffer. Increasing the glucose mass up to 0.5 g increased the total mass of acetol synthesized, but any further augmentation of the glucose within the reaction did not affect the total mass of synthesized acetol. In this part of the study, as the mass of glucose added to the initial reaction increased, the total mass of pyrazine synthesized rose likewise, coupled with a decrease in contribution of 2,6-dimethylpyrazine and a rising contribution from ethylmethyl pyrazines. However, at a certain point, increasing the amount of glucose resulted in a lower concentration of pyrazines and the formation of large dark particulates in the reaction vessel.

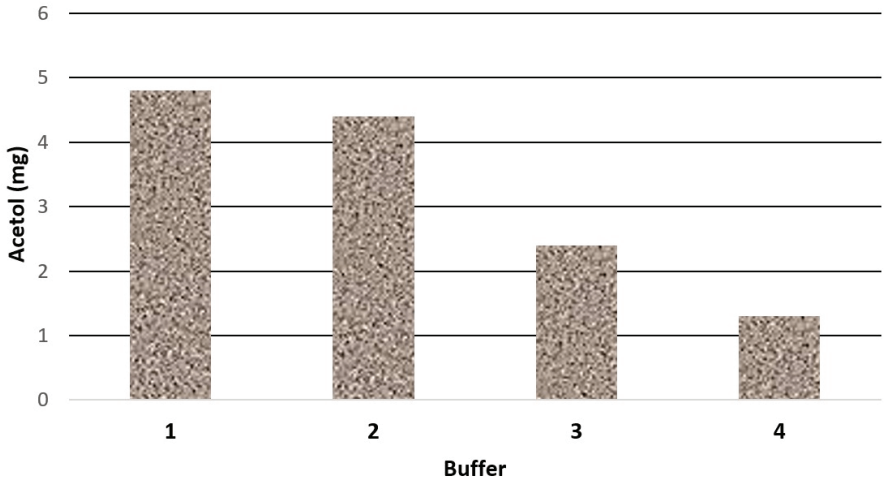

Figure 2. Yield of acetol as a function of buffer concentration using 0.5 g glucose as starting material, reaction time 60 min, reaction temperature 140 °C, reaction volume 25 mL, pH 7.1. Buffer 1 = 2.20 M KH_2PO_4 + 2.10 M K_2HPO_4, Buffer 2 = 1.75 M KH_2PO_4 + 1.67 M K_2HPO_4, Buffer 3 = 1.10 M KH_2PO_4 + 1.05 M K_2HPO_4, Buffer 4 = 0.55 M KH_2PO_4 + 0.5 M K_2HPO_4.

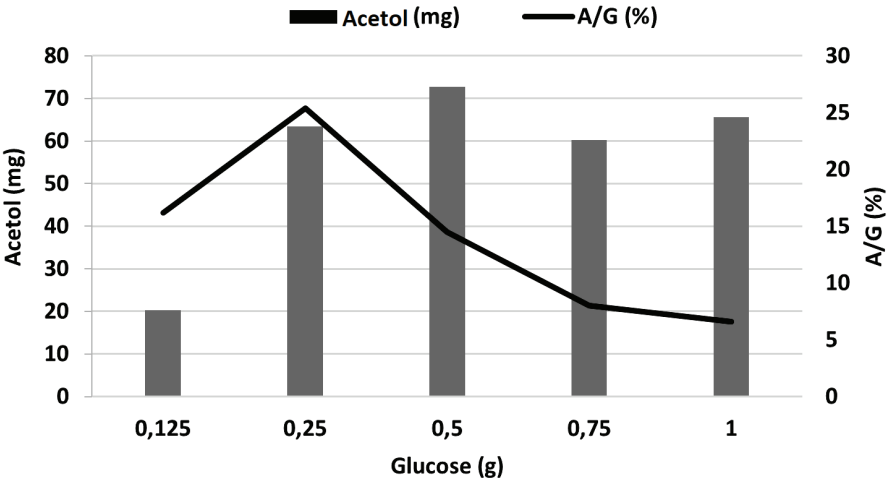

Figure 3. Effect of glucose mass on synthesis of acetol using phosphate buffer (2.2 M KH_2PO_4, 2.1 M K_2HPO_4), reaction volume 25 mL, reaction time 60 min, reaction temperature 160 °C, pH 7.1.

Table 2. Mass and percent distribution of synthesized pyrazines using different mass of glucose in initial buffered reaction. All other conditions were: reaction volume 15 mL, initial reaction time 60 min at 160 °C after equilibrium for reaction of buffer with the glucose, followed by cooling the reaction and adding 1 mL NH_4OH and heating the reaction mixture for 17 h at 140 °C.

Peak	Glucose (g)									
	0.25	(%)	0.6	(%)	1.2	(%)	2.0	(%)	1.0	(%)**
1	0.250		0.250		0.250		0.250		0.250	
2	0.017		0.084		0.088		0.055		1.663	
3	0.597	(22.3)*	0.621	(20.9)	0.994	(20.7)	0.749	(19.6)	0.865	(25.9)
4	1.154	(43.1)	1.384	(46.6)	2.163	(45.1)	1.595	(41.8)	1.796	(53.7)
5	0.002	(0.1)	0.008	(0.3)	0.009	(0.2)	0.005	(0.1)	0.065	(1.9)
6	0.147	(5.5)	0.209	(7.0)	0.359	(7.5)	0.313	(8.2)	0.127	(3.8)
7	0.083	(3.1)	0.112	(3.8)	0.192	(4.0)	0.170	(4.5)	0.071	(2.1)
8	0.233	(8.7)	0.210	(7.1)	0.396	(8.3)	0.321	(8.4)	0.171	(5.1)
9	0.020	(0.7)	0.043	(1.4)	0.048	(1.0)	0.052	(1.4)	0.009	(0.3)
10	0.184	(6.9)	0.137	(4.6)	0.236	(4.9)	0.256	(6.7)	0.050	(1.5)
11	0.000		0.000		0.000		0.000		0.000	
12	0.117	(4.4)	0.126	(4.2)	0.210	(4.4)	0.183	(4.8)	0.042	(1.3)
13	0.010	(0.4)	0.008	(0.3)	0.018	(0.4)	0.017	(0.5)	0.004	(0.1)
14	0.031	(1.2)	0.000		0.000		0.065	(1.7)	0.008	(0.2)
15	0.011	(0.4)	0.000		0.000		0.005	(0.1)	0.003	(0.1)
16	0.010	(0.4)	0.000		0.000		0.000		0.000	
17	0.005	(0.2)	0.007	(0.2)	0.024	(0.5)	0.023	(0.6)	0.033	(1.0)
18	0.000		0.000		0.000		0.000		0.000	
19	0.027	(1.0)	0.020	(0.7)	0.070	(1.5)	0.017	(0.4)	0.016	(0.5)
20	0.000		0.020	(0.7)	0.004	(0.1)	0.003	(0.1)	0.050	(1.5)
21	0.010	(0.4)	0.009	(0.3)	0.023	(0.5)	0.009	(0.2)	0.006	(0.2)
22	0.000		0.000		0.000		0.000		0.000	
23	0.007	(0.3)	0.011	(0.4)	0.020	(0.4)	0.026	(0.7)	0.020	(0.6)
24	0.032	(1.2)	0.012	(0.4)	0.021	(0.4)	0.002	(0.1)	0.006	(0.2)
25	0.000		0.036	(1.2)	0.006	(0.1)	0.004	(0.1)	0.004	(0.1)
Pyrazine total (mg)	2.680		2.973		4.791		3.816		3.345	

* Percent distribution
** No buffer pretreatment

Sugar may have been pyrolyzed, forming char and consequently being unavailable for reaction. Also, the particulates may have hindered pyrazine extraction from the reaction mixture. In the last right hand column of Table 2 the mass due to methylpyrazine is reported. For this reaction condition, 1.66 mg of methylpyrazine was produced and this level was significantly higher than in all the other trials wherein the glucose had been pre-reacted with the phosphate buffer. The level of methylpyrazine in reactions wherein the glucose had been pre-reacted with buffer was much lower, ranging between ~0.02 and ~0.08 mg. This low yield of methylpyrazine was consistent throughout all of the reactions wherein the sugar/carbon source had been pre-reacted with buffer. Thus, conversion of glucose using a pre-reaction between glucose and a phosphate buffer, offers the opportunity to dramatically shift the qualitative and quantitative distribution of pyrazines away from the lower-molecular weight, less desirable pyrazines like methylpyrazine and toward the higher molecular weight more desirable pyrazines.

Effect of temperature on synthesis of pyrazine

In the next part of the study, the reaction product of glucose and the phosphate buffer was reacted with 1 mL of NH_4OH for 17 h at different temperatures (100, 120, 140 and 160 °C) (Figure 4). The total mass of pyrazines did not change significantly despite increasing the temperature from 100 to 140 °C nor was there any identifiable trend in pyrazine distributions. However, increasing the temperature from 140 °C to 160 °C decreased the mass of synthesized pyrazines by 80%.

Effect of NH_4OH concentration on synthesis of pyrazine

Next, the reaction product of glucose and phosphate buffer (1 g of glucose and 25 mL of buffer reacted at 160 °C for 60 min) was reacted with different volumes of NH_4OH (0.5, 1 and 2 mL) at 120 °C for 17 h (Figure 5). The highest yield of pyrazines was obtained when the reaction product of glucose and buffer was reacted with 1 mL of NH_4OH. Increasing or decreasing the volume of NH_4OH decreased the yield of pyrazines by 25–35%. However, as the amount of NH_4OH increased, there was a shift in distribution away from the lower molecular weight pyrazines to the higher-molecular weight pyrazines.

Effect of reaction time on synthesis of pyrazine

Finally, the reaction products of glucose and a phosphate buffer (1 g of glucose and 25 mL of buffer reacted at 160 °C for 60 min) were reacted with 1 mL of NH_4OH at 120 °C for

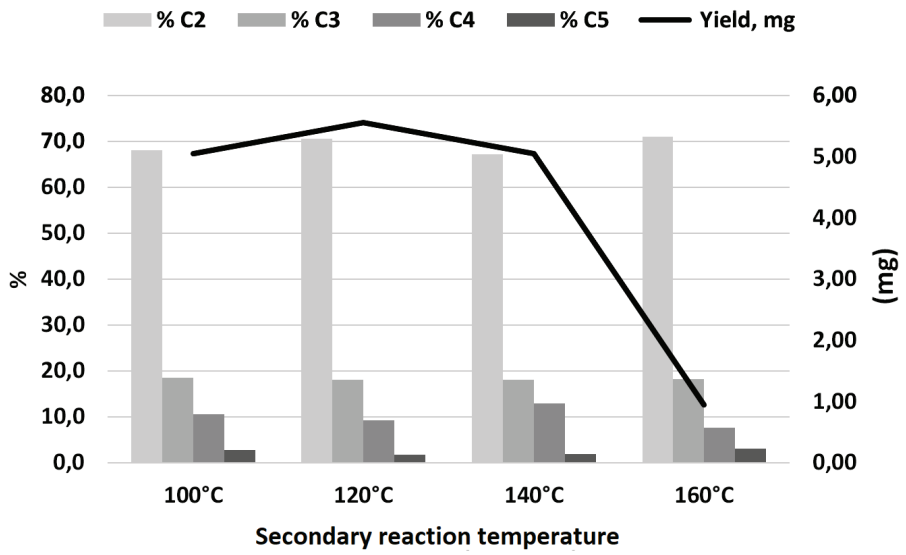

Figure 4. Mass and percent distribution of synthesized pyrazines using different second reaction temperatures. All other conditions were 60 min of heating at 160 °C with 1 g glucose and buffer, reaction volume 25 mL followed by cooling the reaction and adding 1 mL NH_4OH and heating the reaction mixture for 17 h at different temperatures.

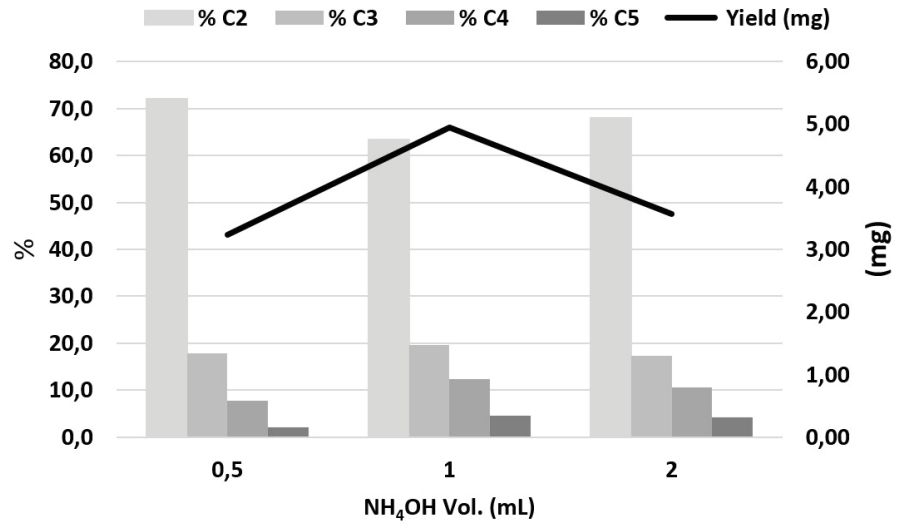

Figure 5. Mass and percent distribution of synthesized pyrazines using different volume of NH_4OH. See Figure 4 for reaction conditions.

12, 17, and 24 h (Figure 6). Results showed that increasing the reaction time from 12 to 18 h increased the yield of pyrazines by almost 30%. Increasing the reaction time from 18 to 24 h decreased the yield of pyrazines by approximately 6%. No consistent shift in the pyrazine distribution as a function of reaction time was observed.

Effect of hydrolyzed F1 protein as a secondary source of nitrogen

In this part of the study, hydrolyzed F1 protein was added as a secondary source of nitrogen in addition to NH_4OH into the pre-reacted solution of glucose and buffer to synthesize pyrazines. Different masses of hydrolyzed F1 protein (0.15, 0.3, 0.45 and 0.6 g of free amino acids) equivalent to 1.5, 3.0, 4.5 and 6.0 mL of hydrolyzed F1 protein solution were added to the reaction of glucose and phosphate buffer (1 g of glucose and 25 mL of buffer reacted at 160 °C for 60 min) including 1 mL of NH_4OH. The mixture was heated at 120 °C for 17–18 h with mixing. Each reaction was cooled and the pyrazines were extracted and quantified via GC/MS. The addition of hydrolyzed F1 protein into the reaction mixture increased the mass of the synthesized pyrazines. The stepwise addition of 0.15, 0.30, 0.45, and 0.6 g of hydrolyzed F1 protein increased the total of mass of pyrazines in increments of 5% each time. The pyrazine distribution was found to slightly shift from the low-molecular C2 weight pyrazines, like dimethylpyrazines, to the higher molecular weight C5 pyrazines, as the amount of hydrolyzed F1 protein was increased.

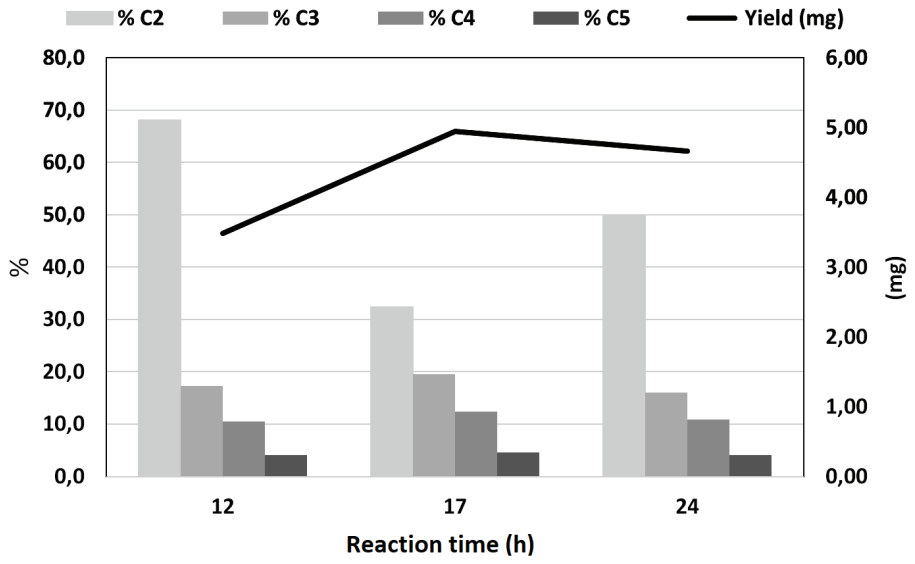

Figure 6. Mass and percent distribution of synthesized pyrazines using different second reaction times. See Figure 4 for reaction conditions.

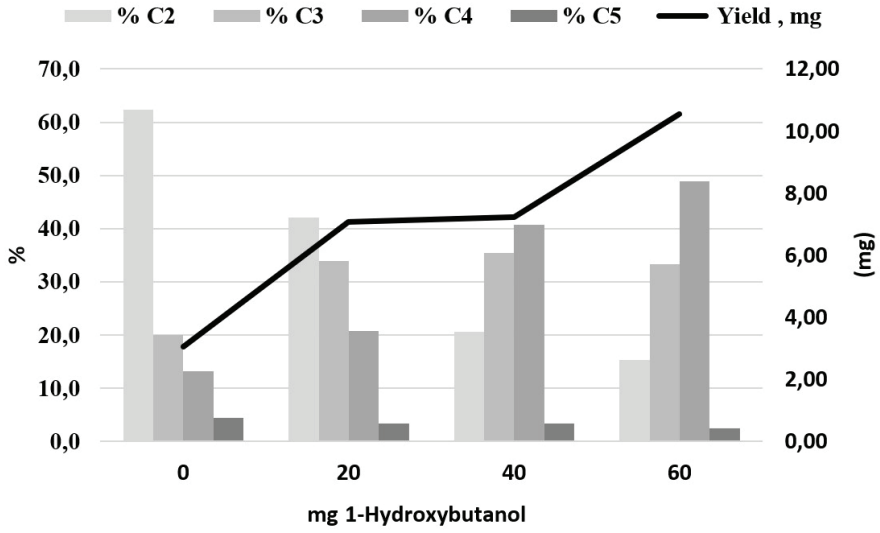

Figure 7. Mass and percent distribution of synthesized pyrazines with addition of 1-hydroxy-2-butanone to the reaction product of 1 g glucose and 25 mL buffer. See Figure 4 for reaction conditions.

Effect of 1-OH-2-butanone for yield and distribution of pyrazines

Next, it was of interest to determine how 1-hydroxy-2-butanone, an α-hydroxyketone similar in structure to acetol, when added to the reaction of glucose and buffer, could impact the pyrazine array. Reactions were performed first with 1 g of glucose and buffer at 160 °C for 60 min. The reactions were cooled and different masses of 1-hydroxy-2-butanone (20, 40 and 60 mg) were added along with 1 mL of NH_4OH to each reaction. Each reaction was then heated to 120 °C for 17–18 h (Figure 7). The increase in amount of 1-hydroxy-2-butanone systematically increased the total mass of pyrazines as well as it altered the quantitative distribution of the pyrazines. The pyrazine distribution dramatically shifted toward C3 and C4 pyrazines having ethyl alkyl substituents, as the amount of 1-hydroxy-2-butanone was stepwise increased. This observation is consistent with our earlier work with α-hydroxyketones (13).

Use of cellulose-derived sugar (CDS) instead of glucose for synthesis of pyrazines

In the last part of this study cellulose-derived sugar (CDS) was used instead of glucose for the synthesis of pyrazines. A previous study showed that CDS could be used for the synthesis of pyrazines (24). In order to optimize the reaction, different masses of CDS were mixed with 15 mL of phosphate buffer and reacted at 160 °C for 60 min. The reaction was cooled and then 1 mL of NH_4OH was added to the reaction and the solution was heated for 17–18 h at 120 °C with continuous mixing. After cooling, each reaction was spiked with internal standard and extracted with 30–40 mL of DCM. The total mass of synthesized pyrazines increased from 3.2 to 6.2 mg as the mass of CDS increased from 1 to 3 g (Table 3).

Table 3. Mass and percent distribution of synthesized pyrazines using CDS instead of glucose. All other conditions were: 60 min of heating at 160 °C after equilibration, followed by cooling the 15 mL reaction mixture and 1 mL NH_4OH and heating the reaction mixture for 17 h at 120 °C.

Peak	CDS (g)							
	1	(%)	2	(%)	3	(%)	5	(%)
1	0.250		0.250		0.250		0.250	
2	0.095		0.055		0.132		0.205	
3	0.662	(20.1)	0.909	(19.0)	0.985	(15.8)	1.198	(20.5)
4	1.425	(43.3)	1.788	(37.4)	2.026	(32.4)	2.464	(42.2)
5	0.001	(0.0)	0.007	(0.1)	0.013	(0.2)	0.0172	(0.3)
6	0.209	(6.4)	0.384	(8.0)	0.716	(11.5)	0.535	(9.2)
7	0.107	(3.3)	0.209	(4.4)	0.401	(6.4)	0.294	(5.0)
8	0.286	(8.7)	0.438	(9.1)	0.402	(6.4)	0.420	(7.2)
9	0.024	(0.7)	0.135	(2.8)	0.691	(11.1)	0.101	(1.7)
10	0.209	(6.4)	0.302	(6.3)	0.231	(3.7)	0.239	(4.1)
11	0.000		0.000		0.190	(3.0)	0.000	
12	0.156	(4.8)	0.294	(6.1)	0.308	(4.9)	0.270	(4.6)
13	0.012	(0.4)	0.025	(0.5)	0.020	(0.3)	0.018	(0.3)
14	0.045	(1.4)	0.080	(1.7)	0.057	(0.9)	0.056	(1.0)
15	0.017	(0.5)	0.008	(0.2)	0.027	(0.4)	0.024	(0.4)
16	0.016	(0.5)	0.026	(0.5)	0.020	(0.3)	0.013	(0.2)
17	0.011	(0.3)	0.025	(0.5)	0.028	(0.5)	0.029	(0.5)
18	0.000		0.011	(0.2)	0.000		0.000	
19	0.044	(1.3)	0.069	(1.4)	0.055	(0.9)	0.057	(1.0)
20	0.000		0.000		0.012	(0.2)	0.024	(0.4)
21	0.015	(0.5)	0.021	(0.4)	0.020	(0.3)	0.023	(0.4)
22	0.000		0.000		0.000		0.000	
23	0.013	(0.4)	0.026	(0.5)	0.025	(0.4)	0.028	(0.5)
24	0.027	(0.8)	0.013	(0.3)	0.007	(0.1)	0.005	(0.1)
25	0.010	(0.3)	0.018	(0.4)	0.019	(0.3)	0.027	(0.5)
Pyrazine total (mg)	3.288		4.786		6.252		5.841	

Increasing the mass of CDS to 5 g caused a decreased pyrazine yield. Similar results were obtained when the mass of glucose was increased under similar reaction conditions, *vide supra*. Again, the decreased yield/recovery of pyrazines could be due to either the formation of large dark particulates (similar to burnt sugar) in the reaction chamber at high concentrations of CDS, or, because of poor extraction of pyrazines during liquid-liquid extraction since the reaction mixture contained many solid particulates. A modest shift in pyrazine distribution was observed reflected by small increases in the contribution due to ethylmethylpyrazines as the CDS content increased.

CONCLUSIONS

By creating optimized reaction conditions, the highest yield of acetol could be obtained when 0.25 g of glucose was reacted with 25 mL of 30% phosphate buffer solution at 120 °C for 17–18 h. However, when the above reaction mixtures was further reacted with NH_4OH, the highest yield of pyrazines was obtained when 1 g of glucose had been reacted with 25 mL of 30% buffer solution using the same reaction parameters. This clearly demonstrates that the acetol is not the only compound acting as a carbon source in the reaction with NH_4OH to produce pyrazines. Reaction of the glucose with the buffer prior to the addition of NH_4OH, induced a notable shift in the distribution of pyrazines toward those pyrazines with a greater number of alkyl substituents and consequently to those pyrazines having more desirable physical and sensory properties. The addition of hydrolyzed F1 protein into the optimized reaction mixture of buffer and glucose increased the yield of pyrazines by 2–10%, depending on the mass of the added hydrolyzed F1 protein. Accompanying this increase in yield was a meaningful shift in the pyrazine array toward those pyrazines having a higher molecular weight. The addition of 1-hydroxy-2-butanone, an α-hydroxyketone, increased the mass of pyrazines by 25–100% and shifted the qualitative and quantitative pyrazine distribution, depending on the mass of the added 1-hydroxy-2-butanone.

Use of pure α-hydroxyketones as the sole carbon source was confirmed to have the capacity to dictate the structure of the resultant pyrazines. For example, the reaction of pure acetoin with NH_4OH produced only tetramethylpyrazine. With all of the reaction parameters examined (reaction time, temperature, reagent ratios, etc.) the most significant impacts on both pyrazine yield and distribution were: 1) glucose being pre-reacted with buffer, 2) hydrolyzed F1 protein added as a second nitrogen source and 3) inclusion of pure α-hydroxyketones as co-reagents.

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