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Generation of 4-Hydroxy-2,5-Dimethyl-3(2*H*)-Furanone from Rhamnose as Affected by Reaction Parameters: Experimental Design Approach

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The formation of 4-hydroxy-2,5-dimethyl-3(2*H*)-furanone (HDMF) was studied in aqueous model systems containing L-rhamnose and L-lysine. The approach consisted in systematically varying four reaction parameters (rhamnose concentration, rhamnose to lysine ratio, pH, and phosphate concentration) at 3 levels. A fractional factorial design was used to reduce the number of trials. The degradation of rhamnose was followed by high performance anion exchange chromatography and the formation of HDMF by solid phase extraction in combination with GC/MS. The study permitted the identification of critical reaction parameters that affect the formation of HDMF from rhamnose in aqueous systems. Although all studied parameters have some impact on the HDMF formation and rhamnose degradation kinetics, the effect of phosphate is by far the most important, followed by concentration of precursors and pH. The experimental design approach permitted us, with a limited number of experiments, to accurately model the effects of the four investigated reaction parameters on the kinetics of rhamnose degradation and HDMF formation ($R^2 > 0.93$). Overall, the results indicate that rhamnose can be an excellent precursor of HDMF (yield >40 mol%), if the reaction conditions are well mastered.

KEYWORDS: 4-Hydroxy-2,5-dimethyl-3(2*H*)-furanone; rhamnose; lysine; model systems; kinetics; experimental design; Maillard reaction; SPE; GC/MS

INTRODUCTION

4-Hydroxy-2,5-dimethyl-3(2*H*)-furanone (HDMF) is an important odorant of many fruits (e.g., strawberry and pineapple), fermented foods (e.g., soy sauce), and thermally treated foods (e.g., bread crust, beef broth, roasted beef, and roasted coffee) (*1*, *2* and references cited therein).

This caramel-like, sweet and fruity smelling compound has first been isolated by Hodge (*3*) from the reaction mixture of rhamnose with piperidine acetate. Since then, other studies have confirmed 6-deoxyhexoses (methylpentoses) as important precursors of HDMF (*4*−*7*). Its formation has been explained through the dehydration of a cyclic form of 1-deoxy-2,3-diulose of rhamnose (1-deoxyrhamnosone), which is formed via 2,3-enolization of rhamnose or the corresponding Amadori compound (*8*).

The majority of studies on the generation of HDMF from rhamnose were performed under aqueous conditions varying one or two reaction parameters and using one or two temperature/time combinations (*6*, *7*, *9*−*11*). HDMF formation from rhamnose was shown to be favored in the presence of phosphate ions (*6*). For example, the yield of HDMF (pH 7, 150 °C, 45 min) was increased by a factor of more than 40 when the reaction was performed in the phosphate buffer as compared to malonate (*6*). Under neutral conditions (pH 5−7), amino acids such as arginine or cysteine also enhanced the formation of HDMF (*7*). HDMF generation is highly dependent on the pH of the reaction media. It increases with increasing pH values. For example, the yield of HDMF increased by a factor of about 70 when the pH of the rhamnose/cysteine system (145 °C, 20 min) was increased from 3 to 7 (*11*). Even higher increase (by a factor of about 800) was observed in the rhamnose/arginine system (70 °C, 48 h) when pH was increased from 4 to 8 (*12*). Temperature is another parameter affecting HDMF generation from rhamnose. About 30 times more HDMF was formed during rhamnose/arginine incubation at pH 3.5 at 90 °C as compared to 70 °C (*12*). Similarly, about 14-fold increase of HDMF was

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Table 1. Reaction Mixtures Prepared According to the Experimental Design[a]

sample description					amount (g)			
sample #	Rha conc.	ratio Rha/Lys	PO_4 conc.	pH	Rha · H_2O	Lys · HCl	$NaH_2PO_4 \cdot 2H_2O$	water
C01	0.2	1:2	0	6	3.64	7.29	0	89.07
C02	0.2	1:2	0.05	7	3.64	7.29	0.78	88.29
C03	0.2	1:2	0.20	8	3.64	7.29	3.12	85.95
C04	0.2	1:1	0	7	3.64	3.64	0	92.72
C05	0.2	1:1	0.05	8	3.64	3.64	0.78	91.94
C06	0.2	1:1	0.20	6	3.64	3.64	3.12	89.60
C07	0.2	2:1	0	8	3.64	1.82	0	94.54
C08	0.2	2:1	0.05	6	3.64	1.82	0.78	93.76
C09	0.2	2:1	0.20	7	3.64	1.82	3.12	91.42
C10	0.4	1:2	0	7	7.28	14.58	0	78.14
C11	0.4	1:2	0.05	8	7.28	14.58	0.78	77.36
C12	0.4	1:2	0.20	6	7.28	14.58	3.12	75.02
C13	0.4	1:1	0	8	7.28	7.29	0	85.43
C14	0.4	1:1	0.05	6	7.28	7.29	0.78	84.65
C15	0.4	1:1	0.20	7	7.28	7.29	3.12	82.31
C16	0.4	2:1	0	6	7.28	3.64	0	89.08
C17	0.4	2:1	0.05	7	7.28	3.64	0.78	88.30
C18	0.4	2:1	0.20	8	7.28	3.64	3.12	85.96
C19	1.0	1:1	0	8	18.2	18.22	0	63.58
C20	1.0	1:1	0.05	6	18.2	18.22	0.78	62.80
C21	1.0	1:1	0.20	7	18.2	18.22	3.12	60.46
C22	1.0	1:1	0	6	18.2	18.22	0	63.58
C23	1.0	1:1	0.05	7	18.2	18.22	0.78	62.80
C24	1.0	1:1	0.20	8	18.2	18.22	3.12	60.46
C25	1.0	2:1	0	7	18.2	9.11	0	72.69
C26	1.0	2:1	0.05	8	18.2	9.11	0.78	71.91
C27	1.0	2:1	0.20	6	18.2	9.11	3.12	69.57

[a] Initial rhamnose concentration (Rha conc.; mol/kg), initial rhamnose/lysine ratio (ratio Rha/Lys; mol/mol), phosphate buffer concentration (PO_4 conc.; mol/kg), L-rhamnose monohydrate (Rha · H_2O), L-lysine hydrochloride (Lys · HCl).

observed when the temperature of the rhamnose solution in phosphate (pH 7) was increased from 100 °C (60 min) to 150 °C (45 min) (*6*).

Usually, the effect of different reaction parameters on HDMF formation has been evaluated individually, i.e., one parameter at a time, which does not permit one to identify interactions between parameters. The study of Shaw et al. (*13*) is an exception, as the authors simultaneously evaluated the effect of several parameters including temperature, pH, concentration, and ratio of reactants on the generation of HDMF and some other volatile compounds from rhamnose in the presence of proline. The reaction time was fixed at 30 min. The highest yield (about 7 mol%) was observed for the reaction mixture containing equimolar concentration of rhamnose and proline (0.1 mol/L) heated at 152.5 °C at pH 6.3. The yield was mainly affected by temperature and pH. A strong drop of yield was observed when the temperature was increased to 180 or 190 °C or when the pH was changed to 2.9 or 9.7. Unfortunately, the effect of reaction time on the yield of HDMF was not evaluated. As a consequence, a better yield of HDMF using different reaction times cannot be excluded.

Only very limited attention has been given to studying HDMF formation from rhamnose under dry heating conditions. For example, Hofmann and Schieberle (*9*, *10*) compared the generation of HDMF from the rhamnose/cysteine system under aqueous conditions (phosphate buffer pH 5, 20 min, 145 °C) and under dry heating conditions (phosphate buffer pH 5, 6 min, 180 °C) and observed drastic decrease in flavor impact of HDMF in dry heated mixtures as compared to aqueous mixtures.

Apart from rhamnose, other precursors have been reported as precursors of HDMF including pentoses (*1, 14, 15*), hexoses, hexose phosphates (*6, 16*), and sugar fragments such as methylglyoxal and hydroxyacetone (*11, 17, 18*). Even though these classes of compounds are usually less efficient in generating HDMF as compared to rhamnose, their contribution to HDMF formation to thermally treated foods is often more important. This is due to the fact that the rhamnose concentration in food is generally very low. For example, glucose and fructose were identified as major precursors of HDMF in popcorn and sugar phosphates (fructose-1,6-biphosphate, glucose-6-phosphate, and fructose-6-phosphate) in bread crust (*6*).

The aim of this work was to identify the critical parameters favoring the conversion of rhamnose into HDMF under conditions simulating pressure cooking. This study did not aim to explain the reaction mechanism responsible for the observed results.

EXPERIMENTAL PROCEDURES

Materials. The following chemicals were commercially available: L-rhamnose monohydrate (RHA, >99%, Kaden Biochemicals, Hamburg, Germany); L-lysine·HCl (LYS, >99%, Aminolabs, Hasselt, Belgium); monosodium phosphate dihydrate (>98%, Riedel-de Haën, Seelze, Germany), 4-hydroxy-2,5-dimethyl-3(2*H*)-furanone (HDMF, 99.6%, Givaudan, Dübendorf, Switzerland); ethylmaltol (>99%, Sigma-Aldrich, Steinheim, Germany); methanol (>99.9%, Merck, Darmstadt, Germany); and disodium hydrogen phosphate dihydrate (>98%), methylacetate (99.5%), and sodium sulfate anhydrous (>99%, Fluka, Buchs, Switzerland).

Sample Settings. The reaction mixtures were prepared according to an experimental design (**Table 1**). This design systematically varied 4 reaction parameters at 3 levels each: (i) rhamnose concentration (0.2, 0.4, 1.0 mol/kg); (ii) ratio rhamnose/lysine (1:2, 1:1, 2:1 mol/mol); (iii) pH (6, 7, 8); and (iv) phosphate concentration (0, 0.05, 0.2 mol/kg). As all possible combinations would lead to 81 ($= 3^4$) mixtures, a fractional factorial design over 27 ($= 3^{4-1}$) mixtures was used to reduce the experimental effort without compromising on the quality of the results. Apart from reducing the number of experiments, this design allows easy estimation of all main effects (i.e., effect of individual reaction parameters) and all two-parameter interactions because none

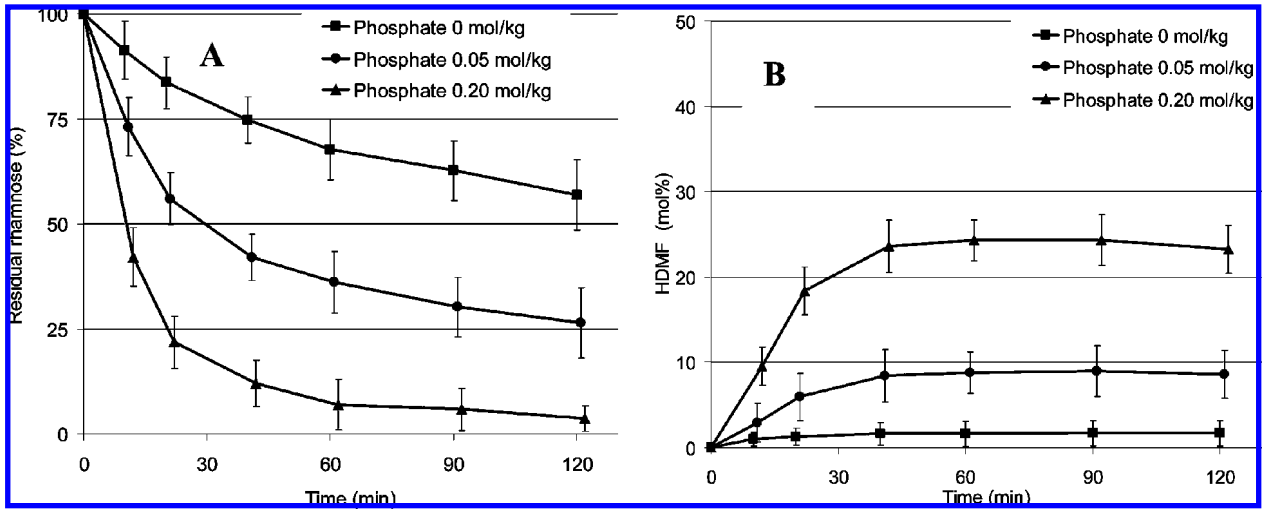

Figure 1. Mean rhamnose degradation kinetics (**A**) and HDMF formation kinetics (**B**) for each phosphate level.

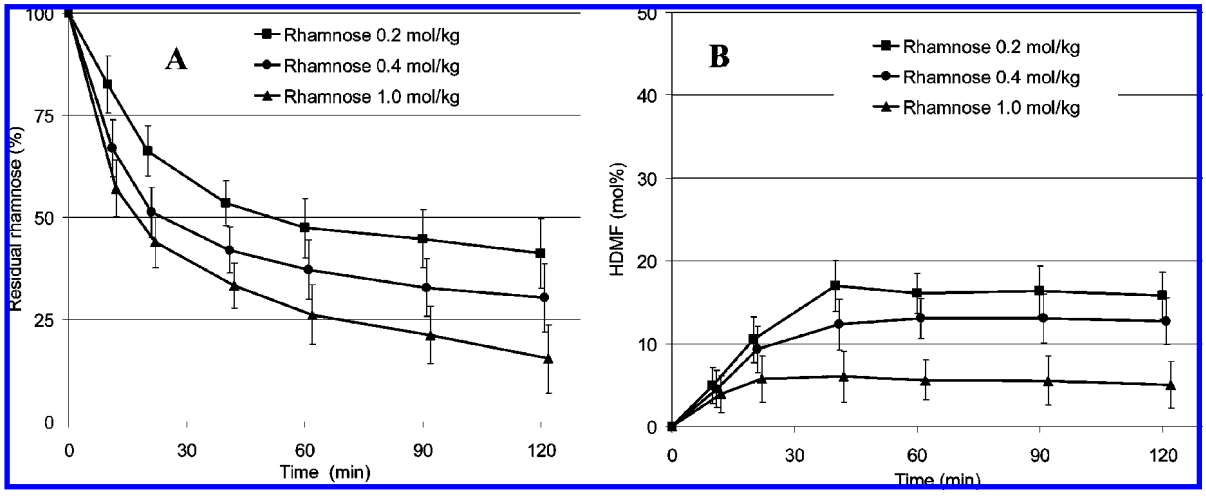

Figure 2. Mean rhamnose degradation kinetics (**A**) and HDMF formation kinetics (**B**) for rhamnose concentrations 0.2, 0.4, and 1.0 mol/kg.

of the effects to be estimated are confounded (*19*). The detection of interactions is not possible when a classical one parameter at a time approach is used.

Sample Preparation. The reaction mixtures were prepared by dissolving the compounds listed in **Table 1** in water. After adjustment of the pH to the target value with the solution of NaOH (0.1 mol/L), the reaction mixtures were adjusted to a total amount of 100 g with water. The solutions were dispatched in 5 mL portions into Pyrex tubes (200 × 20 mm) and thermally treated at 120 °C in a silicone bath for a defined period of time (10−120 min). To stop the reaction, the samples were taken out of the oil bath and put directly into ice water. After cooling down, the reaction mixture was analyzed by high performance anion exchange chromatography (HPAEC) for residual rhamnose and by gas chromatography−mass spectrometry (GC/MS) for HDMF.

Quantification of Rhamnose. An aliquot of the reaction mixture was diluted with water (100 to 2500 times, depending on the initial concentration of rhamnose and on reaction time), filtered through a PVDF filter (polyvinylidene fluoride, 0.22 μm/25 mm), and directly analyzed by HPAEC on a Dionex ion chromatography system (DX500, Dionex, Sunnyvale, CA) composed of an autosampler (model AS-50 with a 25 μL sample loop), a gradient pump (model GP-50) with online degas, an electrochemical detector (model ED-40), and a post column pump (HPLC Compact Pump, Bischoff, Germany). The separation was accomplished on a 250 mm × 4 mm i.d. CarboPac PA100 anion exchange column (Dionex) and a 50 mm × 4 mm i.d. CarboPac PA100 guard column (Dionex). Isocratic separation used water (A) and NaOH (100 mmol/L, B), 88% A and 12% B, as a mobile phase at a flow rate 1 mL/min for 9 min. Each analytical cycle was followed by cleaning and regeneration of the column with NaOH (1 mol/L) for 5 min and equilibration of the column with initial conditions for 10 min. L-Rhamnose was quantified with a pulse amperometric detector equipped with a gold working electrode. The electrode pulse potentials were as follows: $E_1 = 0.1$ V, 0−400 ms; $E_2 = -2.0$ V, 410−420 ms; $E_3 = 0.6$ V, 430 ms, and $E_4 = 0.1$ V, 440−500 ms. To increase the sensitivity, the column eluent was mixed with NaOH (300 mmol/L, 0.5 mL/min) prior to detection. Quantification was based on a calibration curve by comparing the peak area with that of standard solutions containing known amounts of pure compounds. Each sample was injected twice (variation coefficient <1%). The solutions and eluents were prepared using ultrapure deionized water (specific resistivity 18.2 MΩ·cm) from a Milli-Q-system (Millipore, Bedford, MA). NaOH solutions used as eluents were prepared by diluting a carbonate free 50−52% (w/w) NaOH solution in water previously degassed under vacuum.

Quantification of HDMF. The isolation of HDMF from the reaction mixtures was performed as described by Adahchour et al. (*20*) using some modifications. An aliquot of the reaction mixture (0.5 mL, 0.25, or 0.1 mL) was spiked with 1 mL of internal standard (4 mg of ethylmaltol in 1 mL of water) and diluted with water to a total volume of 20 mL. A SPE cartridge ENVI-Chrom P (Supelco, Bellefonte, PA, USA) was rinsed first with methanol (3 mL), then with water (2 mL), and finally loaded with sample (1 mL) containing the internal standard. The cartridge was rinsed with water (2 mL) and dried for at least 30

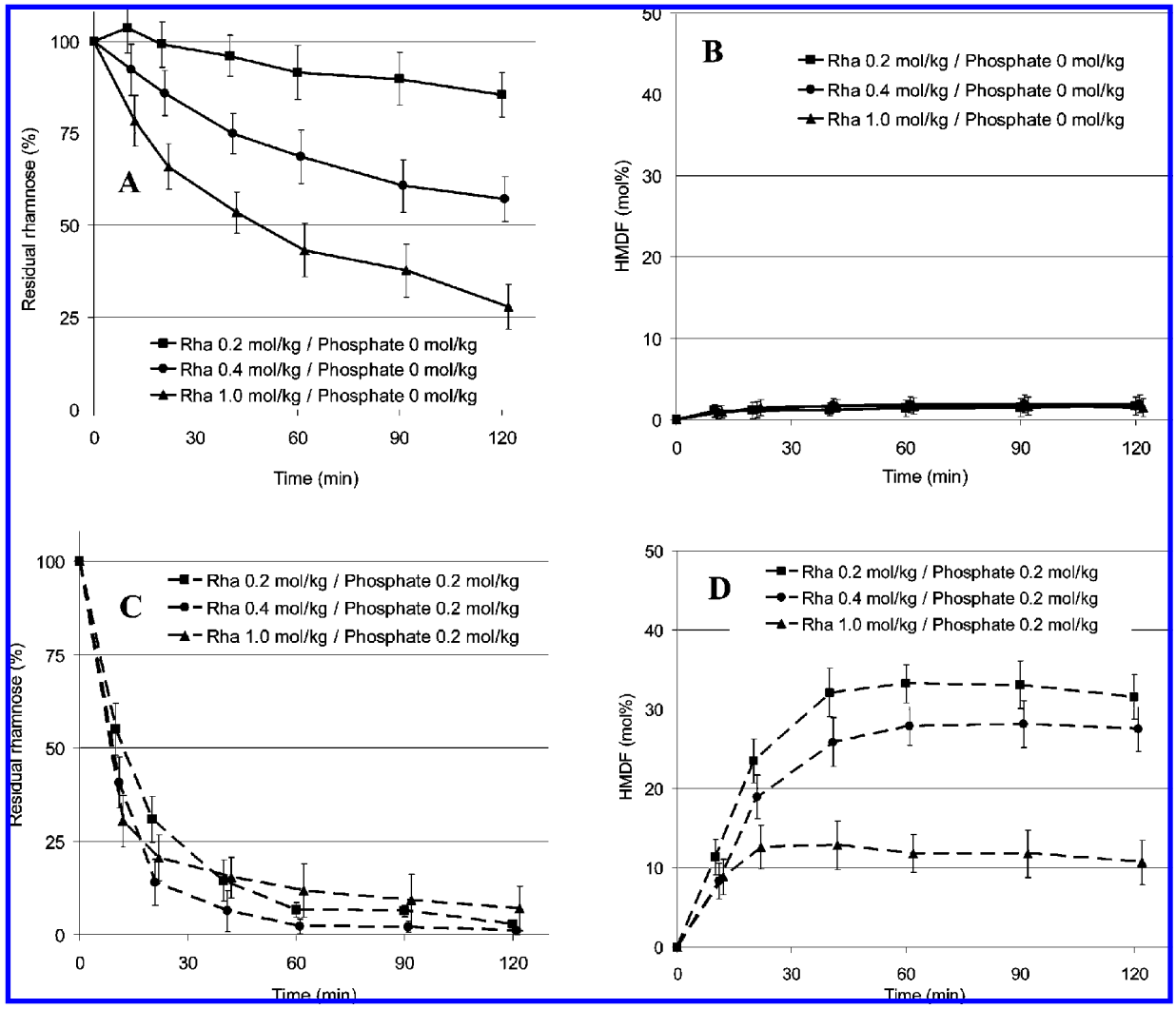

Figure 3. Mean rhamnose degradation kinetics (**A**,**C**) and HDMF formation kinetics (**B**,**D**) for each rhamnose concentration as affected by phosphate concentration 0 and 0.2 mol/kg.

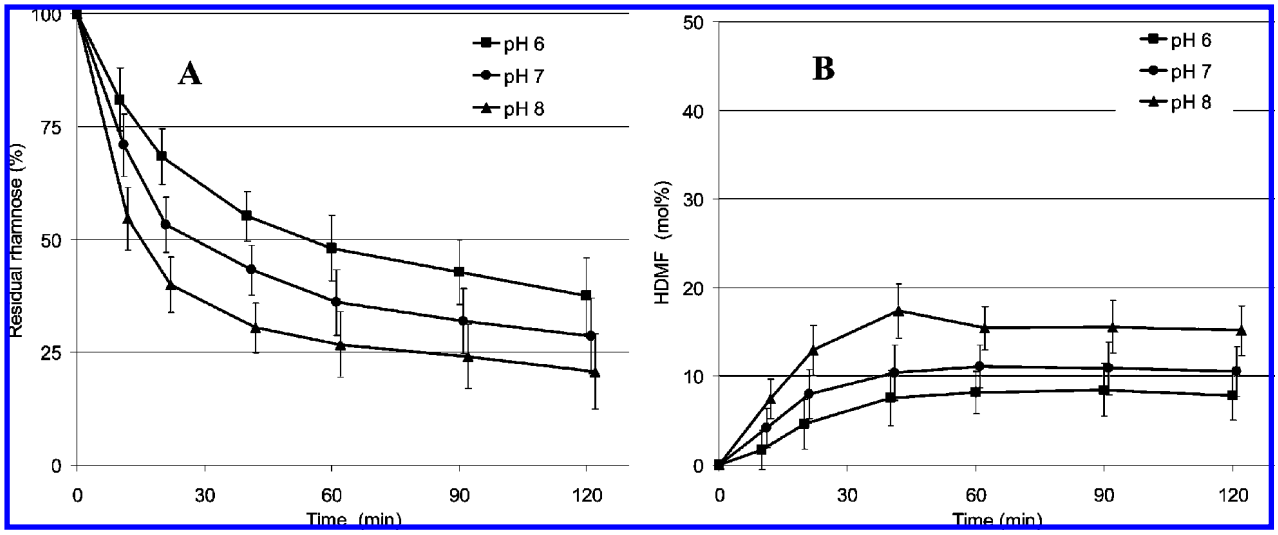

Figure 4. Mean rhamnose degradation kinetics (**A**) and HDMF formation kinetics (**B**) for each pH value.

min under vacuum (ca. 35 mbar). After drying, the HDMF and internal standard were eluted with methyl acetate (2 mL). The eluent was dried over anhydrous sodium sulfate, diluted 5 times with methyl acetate, and immediately analyzed by GC/MS on a Finnigan Trace gas chromatograph coupled to a Finnigan Trace mass spectrometer (both from ThermoQuest, Italy) equipped with an MPS2 autosampler (Gerstel, Switzerland). The separation was achieved on a ZebronWAX capillary column (30 m × 0.25 mm, film thickness 0.25 μm; Phenomenex, USA)

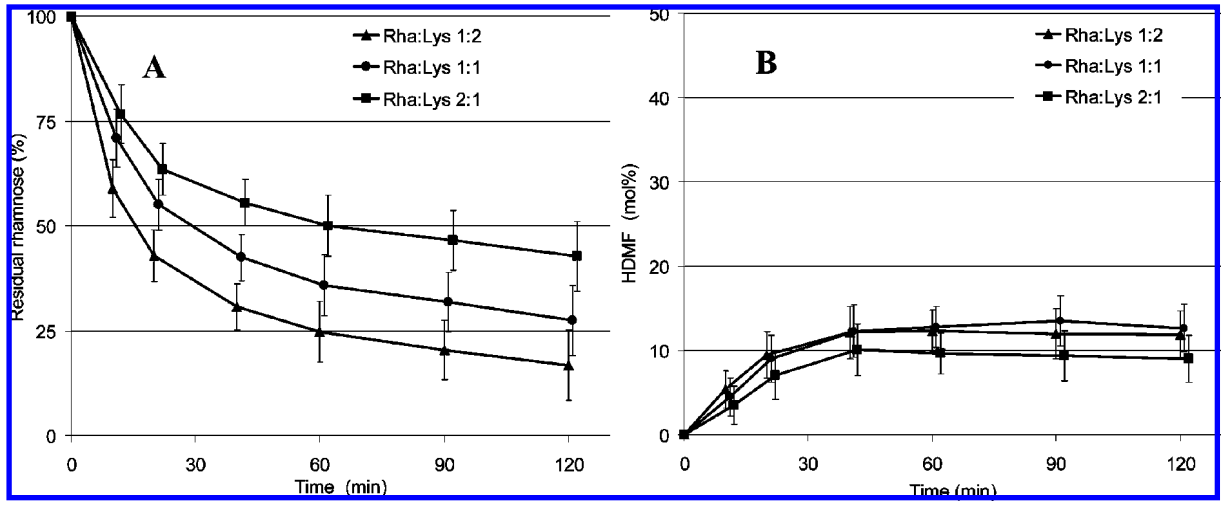

Figure 5. Mean rhamnose degradation kinetics (**A**) and HDMF formation kinetics (**B**) for each reactant ratio.

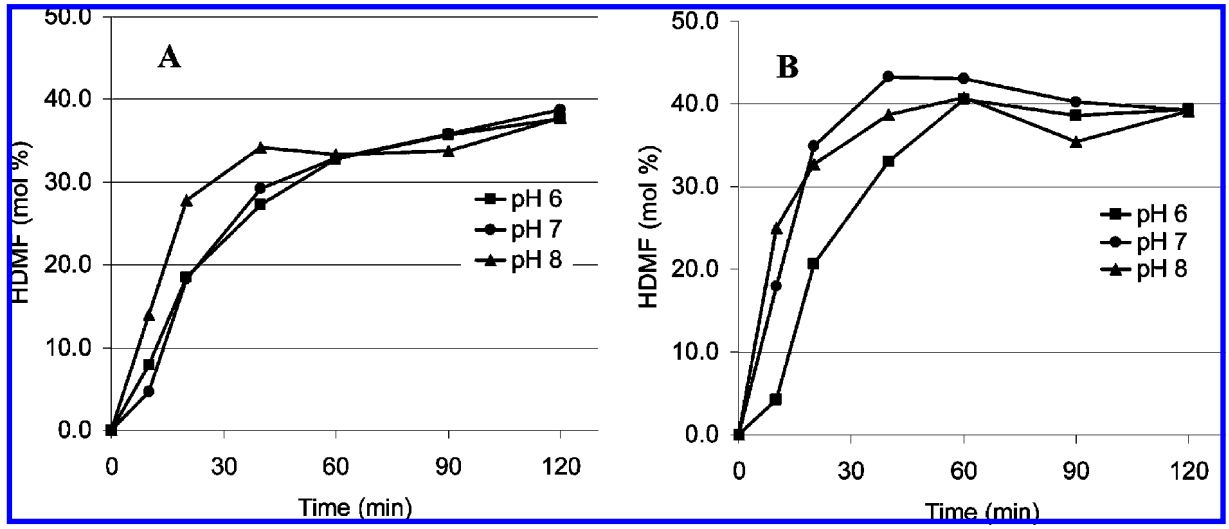

Figure 6. HDMF formation kinetics for rhamnose/lysine ratio 1:1 (**A**) and 1:2 (**B**) and different pH values.

using helium as a carrier gas with a constant flow of 2 mL/min. Samples were introduced via splitless injection at 240 °C (1 μL). The oven temperature program was as follows: 40 °C (2 min), 40 °C/min to 120 °C, 6 °C/min to 185 °C, and 10 °C/min to 240 °C (10 min). The temperature of the ion source was 200 °C. Mass spectra in the EI mode were generated at 70 eV over a mass range of 30 to 220 Da. Quantification of HDMF (in duplicate) was performed in the scan mode by measuring the molecular ions of HDMF (*m*/*z* 128) and that of internal standard (ethylmaltol; *m*/*z* 140).

Data Analysis. The two kinetics (i.e., rhamnose degradation and HDMF formation) were described as a function of the four investigated reaction parameters. According to the experimental design used, the mathematical description consists of all main effects and two-factor interactions, for which the significance was tested using 4-way analysis of variance. Mean kinetics, and their 95% confidence intervals, are visualized using bivariate charts (*21*). All statistical analyses were performed using NCSS (*22*).

RESULTS AND DISCUSSION

The formation of HDMF was studied in model systems simulating pressure cooking conditions (120 °C). The kinetics of rhamnose degradation and HDMF formation was measured for all 27 trials. Both the degradation of rhamnose and the formation of HDMF were highly dependent on the reaction parameters studied. For example, the yield of HDMF varied from 2 mol% to 41 mol% (mol% are relative to rhamnose), and the degradation of rhamnose varied from 12% to 100%.

To evaluate the impact of individual parameters on the level of HDMF and rhamnose, the main effects were calculated for all parameters studied and visualized as the mean kinetics (i.e., a mean of 9 individual kinetics obtained at the given parameter value, e.g., phosphate concentration = 0.2 mol/kg). The higher the observed differences between these mean kinetics, the larger the effect of the investigated parameter.

Effect of Phosphate Concentration. The effect of phosphate on the degradation of rhamnose and on the generation of HDMF is shown in **Figure 1**. Out of the studied parameters, the phosphate concentration had the strongest effect on both HDMF formation and rhamnose degradation. The rate of both reactions strongly increased with increasing phosphate levels. These results are well in line with the literature data as phosphate is known to enhance the Maillard reaction, and better yields of HDMF from rhamnose were reported in its presence as compared to malonate (*6*, *11*). The highest degradation of rhamnose as well as the highest HDMF formation was observed at the highest phosphate level of 0.2 mol/kg. Interestingly, although substantial degradation of rhamnose was observed

already in the absence of phosphate, practically no HDMF was formed under these conditions, indicating the key role of phosphate in HDMF generation.

Effect of Precursor Concentration. The effect of precursor concentration was also important (**Figure 2**). Similarly to phosphate, rhamnose degradation increased with increasing levels of precursors. However, the opposite trend was observed for the formation of HDMF. The highest HDMF yield was observed for the lowest precursor concentration (0.2 mol rhamnose/kg). It was not significantly different from that obtained for a medium level of precursor, but significantly higher than the yield obtained for high precursor levels.

The behavior of rhamnose can be explained by the increased probability of the collisions between rhamnose and lysine at higher concentrations. To explain the behavior of HDMF, one hypothesis could be that the reactions leading to the HDMF formation are accelerated with increasing concentration of reactants; however, the reactions causing its degradation (e.g., interaction of HDMF with lysine) are even faster. As a result, the yield of HDMF decreases with increasing concentrations of rhamnose and lysine. However, the increasing concentration of precursors could also lead to a higher number of side reactions, which would result in a lower rate of HDMF formation.

Interaction of Phosphate and Precursor Concentrations. Analysis of variance revealed only one significant two-parameter interaction (i.e., phosphate and precursor concentrations). In the absence of phosphate, the degradation of rhamnose strongly depends on the initial precursor level, whereas the HDMF formation is negligible and independent of the initial precursor level. However, in the presence of high levels of phosphate, HDMF formation is highly dependent on the initial precursor level, whereas the degradation of rhamnose is very rapid (catalytic effect of phosphate) and almost independent of the initial precursor level (**Figure 3**).

Effect of pH. The effect of pH on both rhamnose degradation and HDMF formation was similar to that of phosphate but less pronounced. Rhamnose degradation and also the yield of HDMF increased with increasing pH (**Figure 4**). This observation is coherent with the formation pathway of HDMF (2,3-enolization) that is favored at higher pH (*8*).

Effect of Reactant Ratio. The degradation of rhamnose increased with the decreasing ratio of rhamnose/lysine (**Figure 5**). This can be explained by the increased probability of the collision of rhamnose with lysine as the concentration of lysine increases. However, HDMF formation was much less affected by the addition of lysine. The best yield of HDMF was obtained for the rhamnose/lysine ratio between 1:2 and 1:1. The yield obtained for ratio 2:1 was slightly lower, but this difference was not significant.

Model to Maximize HDMF Yield. The calculated main effects and the only significant two-parameter interaction (phosphate−precursor concentration interaction) were further used to model rhamnose degradation and HDMF generation kinetics as a function of the 4 investigated reaction parameters. The developed model that explains 93% of the observed variability (R^2 >0.93) was used to maximize HDMF yield. About 40 mol% should be achieved with relevant conditions (0.2 mol/kg phosphate, 0.2 mol/kg rhamnose, Rha/Lys = 1:1, pH 8, and 40−90 min).

The used fractional factorial design permitted one to screen a broad experimental region with a limited number of experiments, and as a consequence, the screening was not very detailed. Therefore, further experiments were performed around the predicted optimal conditions to find out the exact conditions favoring HDMF formation. A full factorial design keeping the concentration of phosphate and rhamnose constant (both at 0.2 mol/kg) and systematically varying the rhamnose/lysine ratio (1:1 and 1:2) and pH (pH 6, 7 and 8) was used.

The results shown in **Figure 6** indicate that in the presence of a high level of phosphate (0.2 mol/L) the pH value has relatively small effect on the yield of HDMF. Globally better yields were obtained with the rhamnose/lysine ratio of 1:2 at each pH value studied. The highest yield of HDMF (about 43 mol%) was obtained for a rhamnose/lysine ratio of 1:2 at pH 7, 0.2 mol/kg rhamnose and 0.2 mol/kg phosphate.

In conclusion, the study permitted us to identify critical reaction parameters that affect the formation of HDMF from rhamnose under conditions simulating pressure cooking. Although all studied parameters have some impact on HDMF formation and rhamnose degradation kinetics, the effect of phosphate is by far the most important, followed by the concentration of precursors and pH. The combination of the experimental design to screen a broad experimental region combined with well-targeted experiments around predicted optima is an efficient way to study the effect of processing parameters on flavor generation. HDMF is an important component of many complex flavors. The data obtained in this study will help to select the conditions that should be varied in order to optimize HDMF formation during food processing.

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