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Isotope Labeling Studies on the Formation of 5-(Hydroxymethyl)-2-furaldehyde (HMF) from Sucrose by Pyrolysis-GC/MS

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Although it is generally assumed that the reactivity of sucrose, a nonreducing sugar, in the Maillard reaction is due to its hydrolysis into free glucose and fructose, however, no direct evidence has been provided for this pathway, especially in dry and high temperature systems. Using specifically ^{13}C-labeled sucrose at C-1 of the fructose moiety, HMF formation was studied at different temperatures. Under dry pyrolytic conditions and at temperatures above 250 °C, 90% of HMF originated from fructose moiety and only 10% originated from glucose. Alternatively, when sucrose was refluxed in acidic methanol at 65 °C, 100% of HMF was generated from the glucose moiety. Moreover, the relative efficiency of the known HMF precursor 3-deoxyglucosone to generate HMF was compared to that of glucose, fructose and sucrose. Glucose exhibited a much lower conversion rate than 3-deoxyglucosone, however, both fructose and sucrose showed much higher conversion rates than 3-deoxyglucosone thus precluding it as a major precursor of HMF in fructose and sucrose solutions. Based on the data generated, a mechanism of HMF formation from sucrose is proposed. According to this proposal sucrose degrades into glucose and a very reactive fructofuranosyl cation. In dry systems this cation can be effectively converted directly into HMF.

KEYWORDS: Isotope labeling studies; HMF formation mechanism from glucose; fructose and sucrose; fructofuranosyl cation; levoglucosan formation; 3-deoxyglucosone

INTRODUCTION

Similar to the widespread occurrence of acrylamide in thermally processed food, 5-(hydroxymethyl)-2-furaldehyde (HMF) is also detected in variety of food products but in relatively higher concentrations (exceeding 1 g/kg). HMF is one of the major degradation products of carbohydrates that has been studied extensively as an indicator of heat damage (*1, 2*). HMF has been used successfully as a chemical index in ensuring adequate heat processing or for monitoring storage conditions for fruit juices, milk, honey, cereal products, cookies and jams (*3–5*). Formation of HMF from carbohydrates has been found to depend on many factors such as time, water activity, temperature, amount and type of catalyst and sugar used (*6*). Ketoses generate more HMF than aldoses and the yield increases with increase in the temperature and the concentration of the acid catalyst although, it can also be formed in slightly lower yields in the absence of a catalyst (*7*). Numerous studies have indicated that fructose is the most reactive sugar relative to sucrose and glucose, in the formation of HMF under acidic conditions. According to Lee and Nagy (*8*) at 50 °C and pH of 3.5, fructose was 31.2 times faster than glucose, whereas sucrose was 18.5 times faster than glucose in the rate of HMF formation. Furthermore, rates of HMF formation from glucose and sucrose showed slight enhancement in the presence of the amino acids, whereas virtually no enhancement occurred when fructose was the substrate. Without acid catalysis and at 250 °C the conversion rate of glucose into HMF was 24% and for fructose the rate was 36% (*7*). However, increasing the acid concentration significantly improved the rate of HMF formation from fructose relative to glucose. At 1 mM H_2SO_4, 42% of fructose was converted into HMF versus 31% for glucose. Interestingly, when sucrose was heated under identical conditions, the yield of HMF per mole of fructose increased from 36% to 47% for the uncatalyzed reaction and from 42% to 53% for the acid catalyzed reaction (*7*). The enhanced HMF formation from sucrose per mole of fructose moiety at high temperatures can be justified by the fact that the glycosidic bond of sucrose can be easily cleaved under mild acidic conditions to produce fructofuranosyl cation, the direct precursor of HMF (see **Figure 1**), however, it is much more difficult for the free fructose to generate the same cation (*9*) under identical conditions. Numerous studies have also indicated the formation of fructofuranosyl cation from fructose as the first step in the formation of HMF (*9–11*). On the other hand, glucose cannot be converted into HMF through dehydration from cyclic forms for obvious reasons and is therefore recognized to generate HMF through cyclization of 3-deoxyglucosone (3-DG) intermediate formed

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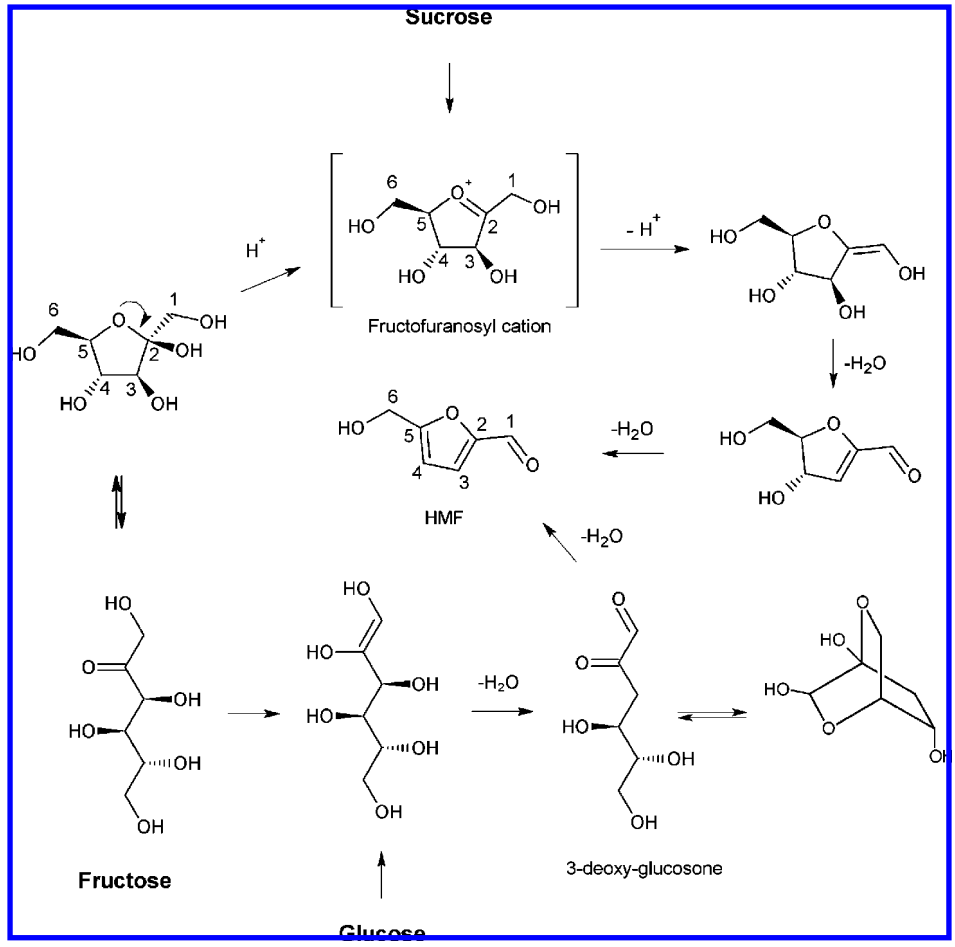

Figure 1. Proposed mechanism of HMF formation from glucose, fructose, 3-deoxyglucosone, and sucrose.

from the open-ring form of glucose (see **Figure 1**). Due to the low propensity of glucose to exists in open ring form and due to many other side reactions of 3-DG, the rate of its conversion into HMF is low compared to fructose especially at higher temperatures where fructose can directly dehydrate from its cyclic forms through the intermediacy of fructofuranosyl cation without the need to undergo thermodynamically controlled ring opening process.

Recent findings on the acute toxicity of HMF (*12–14*) and lack of evidence from isotope labeling studies confirming the above proposed mechanism of conversion of sucrose into HMF, prompted us to investigate the mechanism of HMF formation utilizing ^{13}C-labeled precursors.

MATERIALS AND METHODS

All reagents, chemicals were purchased from Aldrich Chemical company (Milwaukee, WI) and used without further purification. 3-Deoxyglucosone was purchased from Toronto Research Chemicals (Ontario, Canada). The [^{13}C]glucoses were purchased from Cambridge Isotope Laboratories (Andover, MA). [1-^{13}C]fructose and [1-^{13}Cfru]sucrose were purchased from Omicron Biochemicals Inc. (IN).

Pyrolysis-GC/MS Analysis of HMF. A Helwett-Packard (Palo Alto, California) GC with Mass selective detector (5890 GC/5971B MSD) interfaced to a CDS pyroprobe 2000 unit (CDS Analytical, Oxford, PA) was used for the Py-GC/MS analysis. One mg samples of reactants were mixed either with silica gel or introduced into the quartz tube (0.3 mm thickness) as is, plugged with quartz wool, and inserted inside the coil probe and pyrolyzed at indicated temperatures with a total heating time of 20s. The column was a fused silica DB-5 column (50 m length × 0.2 mm i.d. × 0.33 μm film thickness; J&W Scientific, Folsom, CA). The pyroprobe interface temperature was set at 250 °C. Capillary direct MS interface temperature was 280 °C; ion source temperature was 180 °C. The ionization voltage was 70 eV, and the electron multiplier was 2471 V. All injections were in splitless mode. The mass range analyzed was 33−650 amu. The initial temperature of the column was set at 37 °C for 2 min and was increased to 100 °C at a rate of 30 °C/min, immediately the temperature was further increased to 250 °C at a rate of 8 °C/min and kept at 250 °C for 5 min. The identity and purity of the chromatographic peaks were determined using NIST AMDIS version 2.1 (http://chemdata.nist.gov/mass-spc/amdis/). The reported percent label incorporation values (corrected for natural abundance and for % enrichment) are the average of duplicate analyses and are rounded off to the nearest multiple of 5%.

(1-^{13}Cfru)Sucrose Reaction in Methanol. The (1-^{13}Cfru)sucrose (4 mg) was refluxed in methanol (200 μL) for 10 min in the presence of *p*-toluenesulfonic acid monohydrate (3 mg). A 20 μL portion was injected into the GC/MS and analyzed using the same method as described above.

Detection of Levoglucosan by Ion Chromatography. A Metrohm MIC-8 modular IC system (Herisau, Switzerland) consisting of a pulsed amperometric detector (E_1 = 0.15 v, t_1 = 400 ms, E_2 = 0.75v, t_2 = 200 ms; E_3 = −0.15v, t_3 = 400 ms), pump and a sample injection unit connected to Metrosep Carb1−150 anion exchange column thermostatted at 31 °C was used for the analysis of carbohydrate residue after pyrolysis. The mobile phase was 0.1N NaOH and flow rate of 1 mL/min. The retention time of the commercial levoglucosan was 2.62 min. The residue after pyrolysis of sucrose was dissolved in distilled water and diluted before injection. One of the major peaks had a retention time of 2.62 min identical to the levoglucosan standard.

RESULTS AND DISCUSSION

Sucrose Degradation and Origin of HMF. Although there are some reports in the literature (*6*) to indicate that fructose is the main moiety in sucrose that contributes to the generation

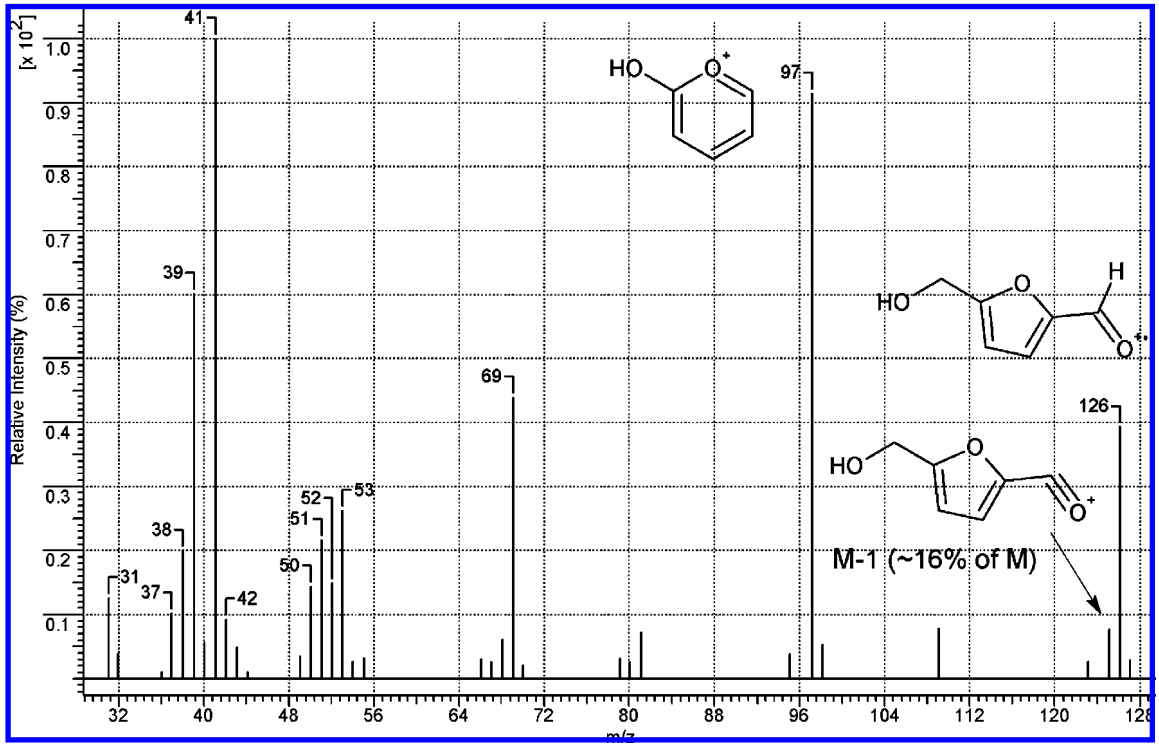

Figure 2. Mass spectrum of HMF and the structure of the major ions.

of HMF, however, there is no quantitative data. According to Antal et al. (*7*) at 250 °C and under 34.5 MPa, 30% of the uncatalyzed sucrose solution (0.05M) can be converted into HMF in 32 s. This conversion rate increases to 50% under acid catalysis. Using the data provided by the Antal et al. (*7*) regarding the amount of free glucose and fructose remaining after sucrose hydrolysis and the rates of HMF formation from glucose and fructose under identical conditions in addition to the amount of other products formed, we were able to estimate the amount of HMF formed from fructose moiety of sucrose to be ~84% during both uncatalyzed and acid catalyzed reactions. In order to verify this number, the ability of sucrose labeled only at C-1 of fructose moiety to generate HMF was analyzed by Py-GC/MS at various temperatures. If HMF can be produced only from glucose, no incorporation of ^{13}C-label will be observed in the parent ion of HMF at *m*/*z* 126, whereas if HMF was generated only from fructose moiety 100% ^{13}C-label incorporation should be observed in HMF and finally if both sugar moieties are responsible for HMF formation less than 100% incorporation will be observed. Moreover, percent label incorporation will indicate percent contribution of fructose to total HMF production from sucrose.

In order to extract mechanistic information from such labeling studies, knowledge of the elemental composition and the structures of the important mass spectral fragments are essential. Consequently, to gain insight into mass spectral fragmentation patterns of HMF singly labeled glucoses were pyrolyzed to generate HMF and label incorporation was analyzed (see **Figures 2** and **3** and **Tables 1** and **2**). These figures show the structures of the relevant fragment ions identified based on the label incorporation pattern listed in **Tables 1** and **2**. It is important to note that similar to any aldehyde, HMF exhibits an M-1 peak due to the loss of aldehydic hydrogen. During labeling studies the percent of M-1 peak will be used to indicate unlabeled HMF arising from the glucose moiety, therefore it is important to confirm this number accurately. **Table 1** indicates that based on six labeled glucoses and one labeled fructose this value is 15−16% of the intensity of the parent ion at *m*/*z* 126. When (1-^{13}Cfru)sucrose was pyrolyzed to generate labeled HMF the corresponding M-1 peak for the labeled HMF was 26% indicating that 10% was due to the unlabeled HMF arising from glucose and 16% due to loss of aldehydic proton. Therefore, 90% of HMF generated from sucrose arises from fructose moiety during pyrolysis at high temperatures (see **Table 1**). These results are consistent with the above estimation of 84% based on literature data generated at 250 °C.

H-Rearrangement and Scrambling of Labels in the Molecular Ion of HMF Generated under Electron Impact (EI) Conditions. During studies on the fragmentation patterns of HMF, inspection of ion at *m*/*z* 97 (see **Figures 2** and **3**) has indicated an unexpected label incorporation pattern as shown in **Table 2**. The ion at *m*/*z* 97 arises from ion at M − 1 by the loss of aldehydic CO as shown in **Figure 3**. According to **Figure 3**, when the precursor of HMF is either glucose-1-^{13}C or fructose-1-^{13}C, the ion at *m*/*z* 97 is expected to completely lose the label as ^{13}CO, however, both labeled sugars retained 20% of the label as shown in **Table 2**. In addition, the remaining 80% was incorporated into the C-6 as indicated from the data on pyrolysis of glucose-6-^{13}C (see **Table 2**). These observations can be explained by the formation of two molecular ions as shown in **Figure 3**, one by the loss of electron from carbonyl oxygen (60%) and the other by the loss of electron from hydroxyl oxygen (40%). The latter can initiate a series of two hydrogen rearrangement reactions to generate two isotopomers of *m*/*z* 126 in equimolar amounts (20% each) due to the symmetrical nature of the intermediate formed after the first rearrangement. Consequently, 80% of the HMF in the mass detector will incorporate C-1 as the aldehydic carbon and 20% will incorporate C-6 as the aldehydic carbon.

Proposed Mechanism of Thermal Generation of HMF from Sucrose. In order to confirm the literature data (*7*) generated at 250 °C and under a pressure of 34.5 MPa regarding the relative conversion efficiency of sucrose into HMF; fructose, glucose and sucrose were pyrolyzed at 250, 300, and 350 °C

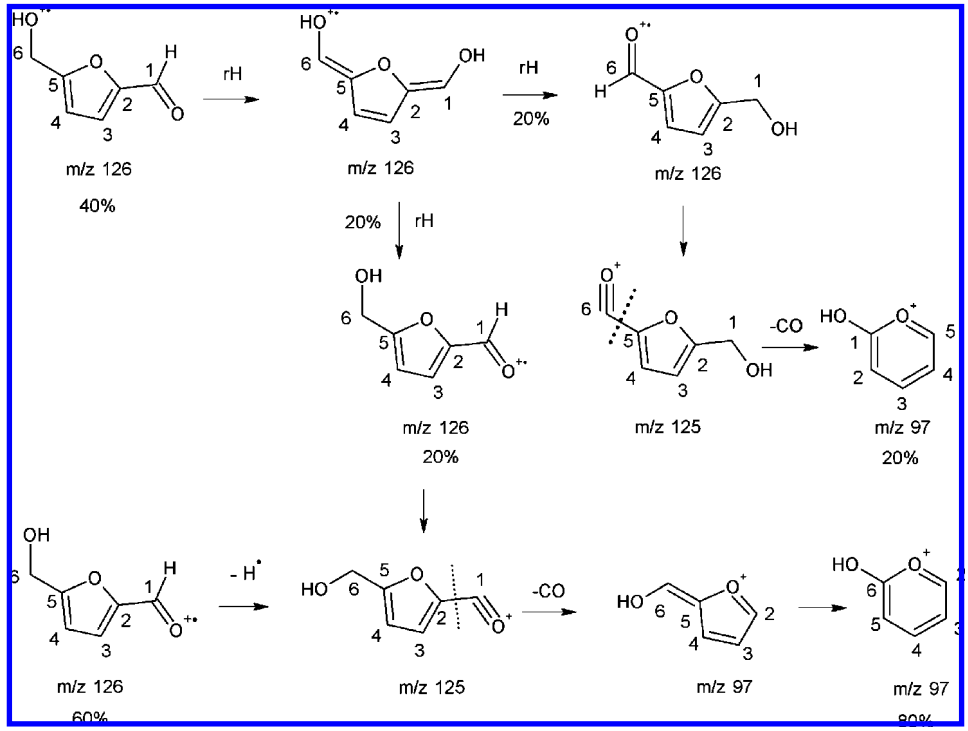

Figure 3. Mass spectral fragmentation pattern of HMF and formation of two molecular ions at *m*/*z* 126. rH = hydrogen rearrangement.

Table 1. Percent Label[a] Incorporation in M + 1, M, and M − 1 Ions[b] of HMF Generated from Various Precursors[c]

compound	M + 1, *m*/*z* 127	M, *m*/*z* 126	M − 1, *m*/*z* 125
3-deoxyglucosone	0	100	16 (% of M)
D-glucose	0	100	15 (% of M)
D-glucose-6-^{13}C	100	16 (% of M + 1)	
D-glucose-5-^{13}C	100	16 (% of M + 1)	
D-glucose-4-^{13}C	100	15 (% of M + 1)	
D-glucose-3-^{13}C	100	16 (% of M + 1)	
D-glucose-2-^{13}C	100	15 (% of M + 1)	
D-glucose-1-^{13}C	100	16 (% of M + 1)	
D-fructose-1-^{13}C	100	16 (% of M + 1)	
(1-^{13}Cfru)sucrose[d]	90	26 (% of M + 1)	
(1-^{13}Cfru)sucrose[e]	90	26 (% of M + 1)	
(1-^{13}Cfru)sucrose[f]	0	16 (% of M + 1)	

[a] All singly labeled and corrected for ^{13}C natural abundance. [b] See **Figures 3** and **4**. [c] Values represent average of two replicates with standard deviation of not more than 5%. [d] Pyrolyzed at 250 °C and corrected for loss of aldehydic hydrogen. [e] Pyrolyzed at 350 °C and corrected for loss of aldehydic hydrogen. [f] (1-^{13}Cfru)sucrose (4 mg) was refluxed in methanol for 10 min in the presence of *p*-toluenesulfonic acid monohydrate (3 mg).

Table 2. Percent label[a] incorporation in fragment at *m*/*z* 97[b] of HMF Generated from Different Precursors[c]

compound	*m*/*z* 97	*m*/*z* 98
3-deoxyglucosone	100	0
D-glucose	100	0
D-glucose-6-^{13}C	20	80
D-glucose-5-^{13}C	0	100
D-glucose-4-^{13}C	0	100
D-glucose-3-^{13}C	0	100
D-glucose-2-^{13}C	0	100
D-glucose-1-^{13}C	80	20
D-fructose-1-^{13}C	80	20
(1-^{13}Cfru)sucrose[d]	81	19
(1-^{13}Cfru)sucrose[e]	81	19

[a] All singly labeled and corrected for ^{13}C natural abundance. [b] See **Figures 3** and **4**. [c] Values represent average of two replicates with standard deviation not more than 5%. [d] Pyrolyzed at 250 °C. [e] Pyrolyzed at 350 °C.

and the areas of HMF peaks produced are reported as area per *m*mol of the starting sugar (see **Table 3**). The data indicated that at all temperatures studied sucrose indeed generated more HMF per mol relative to both fructose and glucose. For mechanistic considerations the efficiency of HMF formation from these sugars at 300 °C relative to 3-deoxyglucosone (3-DG) was also studied (see **Table 4**). According to this table and relative to 3-DG, sucrose generated 4.5 fold more HMF and fructose generated 2.4 fold more HMF, on the other hand, glucose generated only 0.16 fold relative to 3-DG. These results clearly show that 3-DG is not the main precursor of HMF in the case of fructose and sucrose otherwise it would have generated more HMF as is the case relative to glucose. These conclusions are consistent with the above assertion (shown in **Figure 1**) that sucrose and fructose generate HMF through fructopyranosyl cation pathway and glucose generates HMF through 3-DG pathway. Furthermore, to confirm the ability of glycosidically linked terminal fructose (as in sucrose) to generate more HMF relative to free fructose, other oligosaccharides such as raffinose and stacchiose having similar fructose linkages were also analyzed and the results are shown in **Table 5**. According to this table, both raffinose and stacchiose exhibited higher efficiency of HMF formation compared to lactose a disaccharide lacking a terminal fructose moiety as in sucrose.

Based on the above observations it can be proposed that the major pathway of sucrose decomposition is the direct formation of fructofuranosyl cation in addition to glucose and 1,6-anhydroglucose (levoglucosan), a known degradation product of glucose and cellulose (see **Figure 4**). To confirm the formation of levoglucosan from different sugars, the sugars were pyrolyzed at 250, 300, and 350 °C and the data are reported in **Table 6**. According to this table, glucose is the most efficient precursor of levoglucosan followed by sucrose. Fructose however, did not generate any levoglucosan. Pyrolysis of levoglucosan itself indicated that it is volatile enough to be detected at high temperatures (see **Table 6**) and that it did not produce any HMF (see **Table 3**). Furthermore, the formation of levoglucosan from sucrose was also confirmed by ion chromatography, when the

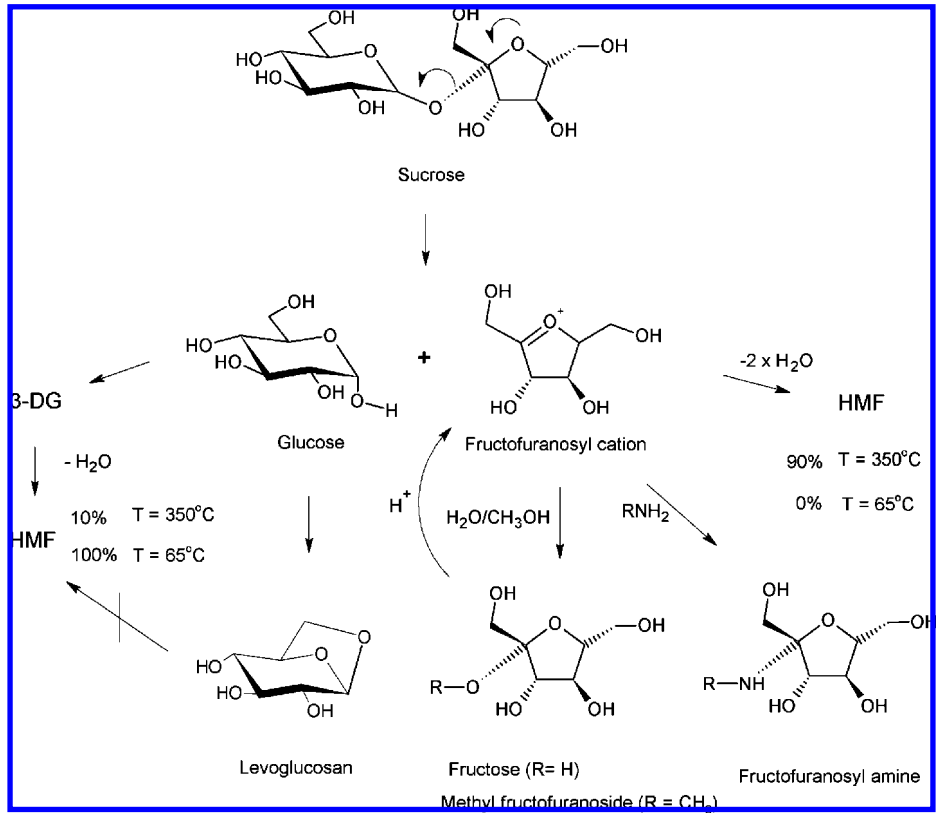

Figure 4. Proposed mechanism of thermal generation of glucose, levoglucosan, and fructofuranosyl cation, showing percent contribution of glucose and fructose moieties to HMF formation at 350 and 65 °C.

Table 3. Efficiency[a] ($\times 10^9$) of HMF Formation at Different Temperatures from Selected Sugars

	temperature		
sugar[b]	250 °C	300 °C	350 °C
sucrose	1.79	5.94	7.49
fructose	1.07	3.13	3.78
glucose	0.78	1.51	1.78
levoglucosan	0.0	0.0	0.0

[a] Expressed as chromatographic peak area of HMF/mmol of the sugar. Values represent average of two replicates with percent standard deviation <5%. [b] Sugars were homogenized with silica gel (60%) to maximize reproducibility.

Table 4. Comparison of Relative Efficiency[a] of 3-DG in HMF Formation Relative to Glucose and Fructose at 300 °C

sugar	relative efficiency
glucose	0.16
3-DG	1
sucrose	4.5
fructose	2.4

[a] Based on chromatographic peak area of HMF/mmol of the sugar in the absence of silica. Values represent average of two replicates with percent standard deviation <5%.

sucrose residue generated after pyrolysis was dissolved in water and analyzed. In addition to glucose, comparable amounts of levoglucosan were also detected using commercially available levoglucosan as a standard.

Finally, to confirm that HMF mainly arises from fructofuranosyl cation, (1-^{13}Cfru)sucrose was heated in refluxing methanol in the presence of *p*-toluenesulfonic acid as catalyst. According to Moody and Richards (*15*) when methanol is used as solvent under acidic conditions, the fructofuranosyl cation, if formed, will immediately react with the solvent to produce methyl

Table 5. Comparison of Relative Efficiency[a] of HMF Formation from Different Oligosaccharides Containing Terminal Fructose (Except Lactose) at 250 °C

sugar[b]	relative efficiency
sucrose[c]	1
raffinose[d]	1.3
stacchyose[e]	0.8
lactose[f]	0.2

[a] Based on chromatographic peak area of HMF/ mmol of the sugar. Values represent average of two replicates with percent standard deviation <5%. [b] Sugars were homogenized with silica gel (60%) to maximize reproducibility. [c] A disaccharide (Glu-Fru). [d] A trisaccharide (Gal-Glu-Fru). [e] A tetrasaccharide (Gal-Gal-Glu-Fru). Decomposes at 250 °C. [f] As control (Gal-Glu).

Table 6. Efficiency[a] ($\times 10^8$) of Levoglucosan Formation at Different Temperatures from Selected Sugars

	temperature		
sugar[b]	250 °C	300 °C	350 °C
sucrose	0.0	0.57	2.87
fructose	0.0	0.0	0.0
glucose	2.74	3.58	8.35
levoglucosan	0.0	9.66	19.7

[a] Expressed as chromatographic peak area of levoglucosan/mmol of sugar. Values represent average of two replicates with percent standard deviation <5%. [b] Sugars were homogenized with silica gel (60%) to maximize reproducibility.

fructofuranoside, thus preventing the formation of HMF from the fructose moiety through the 3-DG pathway and the low temperature of refluxing methanol (65 °C) will prevent formation of HMF through fructofuranosyl cation pathway. Alternatively, if sucrose was being hydrolyzed into glucose and fructose, without the formation of fructofuranosyl cation as an intermediate, then both fructose and glucose moieties can generate HMF

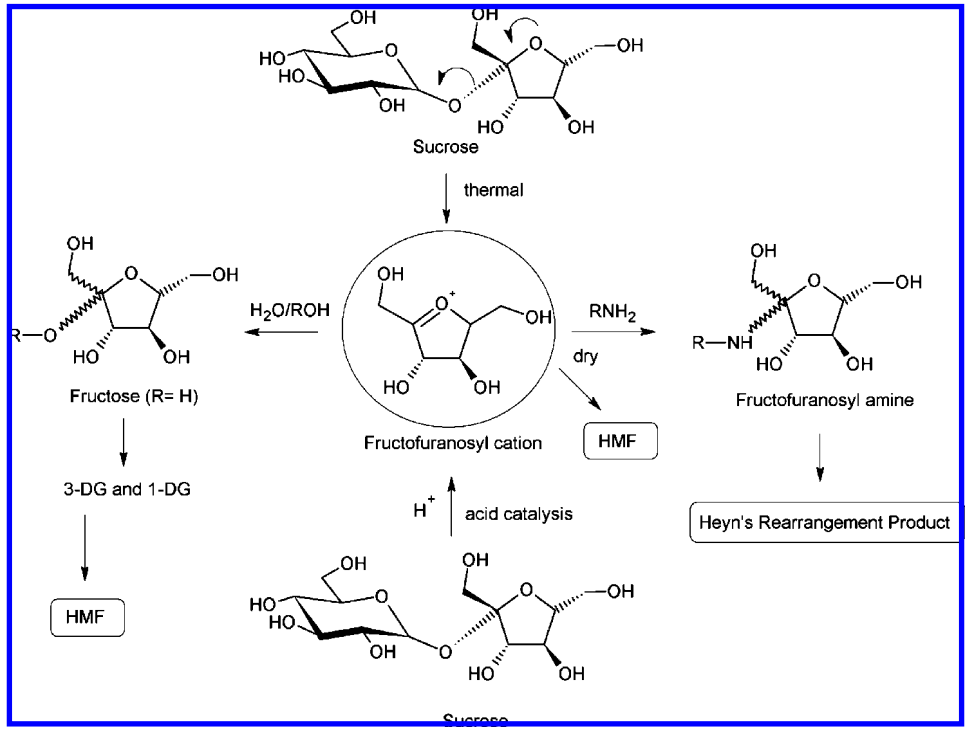

Figure 5. Fate of fructofuranosyl cation under various conditions.

through the less efficient 3-DG pathway as shown in **Figure 1**. In effect, generation of HMF exclusively from the glucose moiety in the refluxing methanol solution of (1-^{13}Cfru)sucrose can be considered as evidence for the fructofuranosyl cation formation and its generation from both glucose and fructose moieties can be considered as evidence against the fructofuranosyl cation formation.

Consequently, when (1-^{13}Cfru)sucrose was refluxed in methanol for 10 min in the presence of *p*-toluenesulfonic acid monohydrate and the solution was analyzed by GC/MS, the data indicated that the low concentration of HMF detected was exclusively produced from glucose alone. This was illustrated by the lack of any label incorporation in the HMF (see **Table 1**), thus confirming that hydrolysis of sucrose proceeds exclusively through the formation of fructofuranosyl cation. Based on the above observations, a mechanism of HMF formation from sucrose is proposed as shown in **Figure 4**. According to this figure, sucrose under thermal treatment and/or acid catalysis can easily cleave the glycosidic bond of the fructose moiety with the assistance of the lone pair electrons of the fructofuranosyl ring oxygen to release a free glucose and a fructofuranosyl cation as a reactive intermediate. At high temperatures and in dry systems this cation can quickly be converted into HMF, whereas in methanol and at low temperatures it can be trapped as methyl fructofuranoside and therefore only free glucose moiety can be converted into HMF through 3-deoxyglucosone pathway following ring opening and enolization steps (see **Figure 1**). This pathway is less efficient than direct dehydrations from cyclic forms (see **Table 4**).

The facile formation of fructofuranosyl cation from sucrose either under acid catalysis or just thermally generated, can also explain the unusual reactivity of sucrose observed in the Maillard reaction (*16*). The fate of this cation depends on the condition of its generation. When it is generated at high temperatures under dry conditions it can directly dehydrate into HMF or react with nucleophiles such as amino acids if present. The fructofuranosyl amine formed as a result of this interaction, can rearrange into the well-known Heyns product (see **Figure 5**). When sucrose was pyrolyzed in the presence of asparagine for example, more acrylamide was generated compared to glucose and fructose combined (*17*). However, if the fructofuranosyl cation is generated under catalysis by dilute acid and at lower temperatures it will mainly be converted into fructose due to its fast reaction with water. As the temperature increases, it is more likely for the fructofuranosyl cation to be converted into HMF or react with nucleophiles especially in low moisture systems. The amount of HMF formed from sucrose is expected therefore to be higher than that of fructose or glucose at higher temperatures due to the more efficient conversion pathway of fructofuranosyl cation into HMF relative to less efficient 3-DG pathway that glucose and fructose follow at lower temperatures under dilute aqueous conditions.

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