Formation of Hydroxymethylfurfural in Domestic High-Fructose Corn Syrup and Its Toxicity to the Honey Bee (*Apis mellifera*)

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In the United States, high-fructose corn syrup (HFCS) has become a sucrose replacement for honey bees and has widespread use as a sweetener in many processed foods and beverages for human consumption. It is utilized by commercial beekeepers as a food for honey bees for several reasons: to promote brood production, after bees have been moved for commercial pollination, and when field-gathered nectar sources are scarce. Hydroxymethylfurfural (HMF) is a heat-formed contaminant and is the most noted toxin to honey bees. Currently, there are no rapid field tests that would alert beekeepers of dangerous levels of HMF in HFCS or honey. In this study, the initial levels and the rates of formation of HMF at four temperatures were evaluated in U.S.-available HFCS samples. Different HFCS brands were analyzed and compared for acidity and metal ions by inductively coupled plasma mass spectroscopy. Levels of HMF in eight HFCS products were evaluated over 35 days, and the data were fit to polynomial and exponential equations, with excellent correlations. The data can be used by beekeepers to predict HMF formation on storage. Caged bee studies were conducted to evaluate the HMF dose−response effect on bee mortality. Finally, commercial bases such as lime, potash, and caustic soda were added to neutralize hydronium ion in HMF samples, and the rates of HMF formation were compared at 45 °C.

KEYWORDS: High-fructose corn syrup; hydroxymethylfurfural; honey bees; *Apis mellifera*; HMF

INTRODUCTION

Since high-fructose corn syrup (HFCS) was first produced and introduced into the United States in 1968, its usage has become widespread in the processed food, beverage, and sweetener industries (*1*). This is because HFCS is less expensive to manufacture than sucrose. In the United States two classes of HFCS are manufactured, HFCS-42 and HFCS-90, which contain 42 and 90% fructose, respectively. HFCS-55 is produced by combining HFCS-42 and HFCS-90 (*1*). More modern HFCS plants employ immobilized enzyme technology to hydrolyze corn starch by α-amylase into eight glucose unit maltooligosaccharides. The maltooligosaccharides are treated with a second enzyme, amyloglucosidase, to yield high concentrations of liquefied glucose. The glucose is then transformed into fructose by a third enzyme, glucose isomerase (*2*). The reaction temperatures, metal ion concentrations, and pH of the reaction mixtures are some of the critical variables involved in HFCS manufacture (*2*). In addition, the processing steps involved require pressure filtration, cooling, ion exchange, evaporation, and mixing, so the manufacturing process is complex (*2*). HFCS-90 is manufactured from HFCS-42 by channeling the HFCS-42 through a fractionation unit with additional water. Most of the HFCS-90 is combined with HFCS-42 to produce HFCS-55. HFCS-55 has the advantage in cooler climates of being less likely to crystallize than HFCS-42, because it contains lower levels of glucose (*1*). In addition, HFCS-55 is usually produced at about 77% dissolved solids (Brix), whereas HFCS-42 is produced at about 71%, so the former has greater caloric value (*2*). HFCS-55 is used in most, but not all, HFCS-sweetened beverages, whereas HFCS-42 is more frequently used in the confectionary and baking industries. Since 1978, HFCS-55 has continually commanded an increase in the market share over HFCS-42 (*1*). In 1970, U.S. per capita consumption of HFCS was estimated at 318 g, according to a U.S. Department of Agriculture report (*1*), which increased to over 18 kg per capita (*3*), with total sucrose sweeteners at slightly less than 21 kg (*4*).

HFCS offers a variety of advantages to commercial users. Because HFCS is a liquid, as opposed to being crystalline, such as sucrose, the sweetener offers advantages in storage, transportation, and distribution logistics for industrial users (*1, 2, 5*), although some sucrose is delivered to companies as a liquid.

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HFCS is ideal for commercial beekeepers to stimulate brood rearing in the spring season when brood production is paramount, as many commercial beekeepers split strong colonies to double their colonies (*6*). HFCS supplementation is also incorporated after shipping bees for pollination, and when natural sources of nectar and pollen have diminished. In addition, HFCS is acidic and, therefore, resists fermentation, so it can be bought in advance and stored (*5*). This enables users to purchase in advance and store this product. Consequently, for these reasons, commercial beekeepers are large purchasers of HFCS. Early evidence of bees' attraction to HFCS came from spillages around HFCS plants where HFCS was being loaded onto railway tanks and spillage occurred (*6*). HFCS and honey are more similar in terms of carbohydrate constituents and levels than sucrose syrup. Early caged bee investigations, however, showed no significant increase in bee longevity over using HFCS compared to honey (*6*). In early investigations, when sucrose syrup was hydrolyzed to fructose and glucose (invert sugars) and fed to bees, the invert mixture was found to be toxic to bees when mineral acids or organic acids were used to hydrolyze the sucrose. In comparison, invertase-hydrolyzed sucrose syrup was found to be nontoxic to bees (*7*).

5-Hydroxymethylfuraldehyde or hydroxymethylfurfural (HMF) and its daughter hydrolysis products levulinic and formic acids were all approximately equally toxic to bees. These products are formed from the dehydration of fructose, which is catalyzed by mineral and organic acids; their toxicity to bees showed dysentery-like symptoms, so intestinal tract ulceration was suspected (*7*). The conversion of HMF into levulinic and formic acids proceeds by first-order kinetics (*7*), and Kuster found that fructose is about 40 times more reactive than glucose as a precursor to HMF. This is because glucose must proceed through a 1,2-enediol type intermediate, according to his proposed chemical mechanism (*8*).

HMF is also found in honey and, in addition to diastase, is a marker for aging in honey (*9*). The Codex Alimentarius Commission prohibits the sale of honey with HMF levels higher than 40 ppm for human consumption (*9*). Because honey is a valuable commodity, it is important to understand the effects of heat of pasteurization on aging and HMF levels. Ideally, with heat, microorganisms can be eliminated, vitamins preserved, and HMF minimized. Constant-temperature kinetic studies on honey to determine the rates of HMF were found to follow first-order kinetic models (*10*).

There appears to be a dearth of knowledge on the thermal effects of the kinetics of HMF formation in HFCS. Published research in the area appears to be limited to a single paper reported by Korean investigators (*11*). Given the increasing use of HFCS with commercial beekeepers as a feed and the concerns of HMF production, more information is needed on the kinetics of HMF formation in HFCS. This prompted us to investigate the rates of HMF formation in samples obtained from U.S. HFCS manufacturers. The rates of HMF were observed spectrophotometrically at four constant temperatures. Because there are only two well-cited papers regarding the toxicity of HMF to bees, with the most recent one dating back to 1975 (*7*, *12*), caged bee studies were also conducted to determine the toxicity of HMF to bees. The initial pH and levels of HMF, percent C, H, N, S and transition and heavy metals were determined. Previous studies have shown that metals can catalyze the formation of HMF (*13*). Therefore, the levels of metals that could occur in HFCS as a result of manufacture or storage were determined by inductively coupled plasma mass spectrometry (ICP-MS). Finally, as a possible practical solution to commercial beekeepers who want to minimize the formation of HMF, the effects of the addition of industrial bases such as lime, potash, and caustic soda to neutralize the formation of HMF were also investigated.

MATERIALS AND METHODS

Materials. The HFCS samples were gifts from Roquette, Archer Daniels Midland, Mann Lake, Inc. (Cargill HFCS), and Tate & Lyle. All of the HFCS samples were received in quart containers, with the exception of Mann Lake, Inc. (Cargill HFCS), which were provided as 5 gal (18.5 L) samples. The chemicals, unless mentioned specifically, were of analytical grade from Aldrich, Fisher, and Fluka chemical companies. The microplates were Costar 96-well flat-bottom (Costar 9018). For ICP-MS analysis, the nitric acid was purchased from Fisher (Optima grade, Seastar Chemical Co.) and the hydrogen peroxide (31−33%, semiconductor grade) from Aldrich. ICP-MS 1000 ppm standards were purchased from High Purity Standards (Charleston, SC). The microplate reader used was a Synergy HT (Winooski, VT), and the inductively coupled mass spectrometer was a Perkin-Elmer (Shelton, CT) Elan DRC II ICP-MS. Elemental analysis (% C, H, N, and S) was conducted by Atlantic Microlab, Inc. (Norcross, GA).

Acidity of the High-Fructose Corn Syrup. The procedure employed was the AOAC approved method 962.19 (*14*). Standard solutions (0.0498 N KOH and HCl) were prepared using potassium hydrogen phthalate as a primary standard. The syrup sample (10.0 g) was dissolved in 75 mL of water (18 MΩ deionized, boiled) and the pH recorded; a few drops of ethanolic phenolphthalein indicator were added. Slow, dropwise, addition of 0.0498 N KOH was made with stirring until the pH was 8.50 (phenolphthalein end-point). The volume of NaOH was recorded and the %HCl calculated by determining the moles of NaOH from the titration data and the mass of the syrup. Average results are reported with standard deviations from three replicate measurements.

Spectrophotometric Analysis of Hydroxymethylfurfural. The procedure was based on the method of Winkler (*15*) that was scaled to the level of a microplate reader. To a tared 10 mL volumetric flask was added 1.000 g of the syrup with a Pasteur pipet. The volume was brought to the mark with DI water (18 M Ω, boiled). The suspension was then vortex mixed until homogeneous and then filtered through a 0.45 μm filter membrane (Whatman). For the sample, 500 μL of this solution was mixed with 500 μL of a *p*-toluidine solution [10 g of *p*-toluidine mixed with 10 mL of glacial acetic acid and brought to volume (100 mL) with isopropanol] in a 1.5 mL centrifuge tube. To the mixture was added 100 μL of a barbituric acid solution (500 mg of barbituric acid dissolved in 100 mL of deoxygenated water). A pink-red color is formed spontaneously. The spectrophotometric blank for the sample used the exact amount of sample and *p*-toluidine except 100 μL of DI water was added instead of barbituric acid. Next, the solution (200 μL) was transferred to a 96-well microplate in triplicate and the absorbance recorded at 550 nm after a 30 s shake cycle. The absorbance of the blank was subtracted from the sample. The concentrations of HMF were determined from standard curve created by plotting the concentration of HMF versus absorbance at 550 nm. The limit of detection of HMF for the developed method was < 0.006 mg/mL, and the percent recovery from a spiked sample was 95%. Sample to sample was 3%. A representative standard curve, fitted to a polynomial (below), is shown in **Figure 1**.
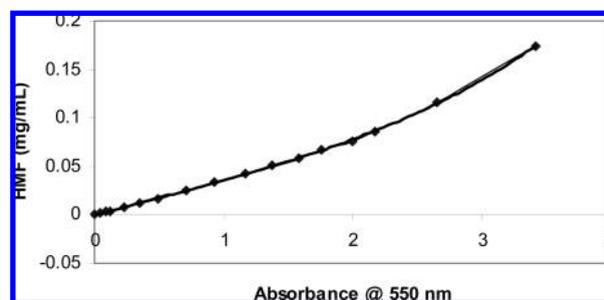

Figure 1. Standard curve for spectrophotometric analysis of HMF in HFCS samples. $[HMF]_{mg/mL} = 0.0001 \times (Abs)^4 + 0.0013 \times (Abs)^3 - 0.0019 \times (Abs)^2 + 0.0362 \times (Abs) - 0.0003$, $R^2 = 0.9997$.

For the HMF constant kinetic experiment and the effects of adding industrial base experiments, 10 centrifuge tubes (2 mL) were filled with the HFCS brand samples and placed in laboratory ovens maintained at 31.5, 40.0, 49.0, and 68.8 °C. Samples were removed periodically over 35 days and flash frozen. The samples were run together to ensure consistency. Oven and incubator temperatures were recorded daily with mechanical thermometers, and the reported values are time-weighted measurements using the following equation to account for temperature drift: $1/t_{total} \times \sum (t_n - t_{n-1}) \times T_n$, where t_{total} is the total amount of time, $t_n - t_{n-1}$ is the difference in time the samples were subjected to in the incubator or oven, T_n is the temperature recorded at the time (n) that the samples were removed and flash frozen and t_{n-1} is the time at the last recording. Ten temperature recordings were incorporated for the calculations to generate the time-weighted temperatures.

Analysis of Metals by Inductively Coupled Plasma Mass Spectroscopy. The samples were ashed and analyzed as follows: The syrup sample (5.000 g) was added to a tarred 20 mL borosilicate scintillation vial. One hundred microliters of 2000 ppb Rh-103 was added as a recovery standard. Three blank scintillation vials were spiked with Rh-103 to subtract for any metallic impurities added during the ashing cycles. Nitric acid (Optima grade) (4 mL) was added and the sample allowed to stand at room temperature under a fume hood overnight. The following day, 2 mL of nitric acid was added and the sample allowed to stand for 48 h at ambient temperature. Next, 1 mL of hydrogen peroxide (31−33%) was added and the sample allowed to stand for 24 h. These procedures were repeated until bubbling had ceased. The samples were then warmed on a hot plate, and the steps were repeated until an off-white solid remained. The residue was dissolved in 10 mL of 4% nitric acid solution and allowed to soak for 24 h. Next 5 mL of the solution was mixed with 5 mL of DI water (18 MΩ), and 10 mL of the solution was tested for all metals except the aliquot for Hg analysis. For Hg analysis 5 mL was transferred to a scintillation vial with 4.9 mL of DI water (18 MΩ) and 100 μL of a 20000 ppb Au standard to form an amalgam with any Hg. The Rh-103 recovery standard ranged between 70 and 100%. A NIST (Gaithersburg, MD) standard reference material, 1566b, was also used as an additional quality control measure for this analysis. Analytes and percent recoveries of the NIST standard under the conditions described were as follows: [23]Na, 64.2%; [24]Mg, 71.1%; [56]Fe, 94.4%; [59]Co, 80.3%; [60]Ni, 77.4%; [64]Zn, 91.6%; [121]Sb, 109%; [38]Ba1, 56.1%; [206]Pb, 57%. Results were tabulated in parts per million (ppm) and parts per billion (ppb).

ICP experimental parameters: RF power, 1300 W; dwell time, 100 ms; sweeps per replicate, 40; replicates, 3; acquisition mode, peak hopping; argon flow rates, nebulizer (0.87 L/min), coolant (15 L/min), auxiliary (1.2 L/min); sweep uptake, 0.400 mL/min; nebulizer type, PFA in self-aspirating mode; spray chamber, cyclonic quartz, sample/skimmer cones; Pt detection limits [m/z − detection limit (3σ) ppb], [23]Na, 0.18; [24]Mg, 3.12; [27]Al, 6.05; [47]Ti, 51.89; [52]Cr, 1.92; [55]Mn, 0.06; [56]Fe, 7.14; [59]Co, 0.04; [60]Ni, 1.90; [63]Cu, 0.78; [64]Zn, 3.36; [120]Sn, 0.14; [202]Hg, 4.00; [208]Pb, 0.08.

Caged Honey Bee Experiments. A previously reported, a caged bee method was used (*17*). Approximately 100 freshly emerged Italian honey bees were placed into the cage for each caged bee trial (conducted in triplicate). Current research laws use committee approval for honey bee research. The caged trials were recorded in multiples of four, so that average and standard deviation counts can be reported. For all trials, the bees were fed water, ad libitum, and a plug of pollen−sugar. For the HFCS syrup formulation, we used A-55, which was determined to have 57 ppm

HMF. For the higher HMF concentration solutions (100, 150, 200, and 250 ppm), pure HMF was added to the 57 ppm HFCS to obtain the desired concentrations.

Effect of Adding Base To Increase the Initial pH of HFCS. Commercially used bases caustic soda (NaOH), lime (CaO), and sodium carbonate (Na$_2$CO$_3$) were added to the HFCS samples as 1 M aqueous solutions. The amount of each base added was determined on the basis of the %HCl, using the titrimetric method described in this section. For each syrup sample, five plastic centrifuge tubes (2 mL) were filled with the treated syrup samples and then heated to 45 °C in a laboratory incubator. The samples were removed periodically over 18 days, flash cooled, and analyzed together to minimize inconsistencies due to the precision of the spectrophotometric method for the determination of HMF described in this section.

Statistics. Statistical results for bee consumption and mortality were determined by using the ANOVA technique with XLStat 2009 (Addinsoft). Dunnett (two-sided) analysis of the differences between categories and the control sucrose with a confidence interval of 95% was chosen for all comparisons. All data are reported as mean ± standard deviation.

RESULTS AND DISCUSSION

The method employed for determining the acidity of the HFCS samples was specifically designed for honey and syrups to determine their hydronium ion concentrations and %HCl. One of the HFCS-42 samples (B-42) had a markedly lower pH than the other HFCS syrups analyzed (**Table 1**). HFCS samples A-42 and C-42 had similar pH values at 4.15 ± 0.04 and 4.18 ± 0.04, respectively. Sample B-55 had the lowest pH of the 55% HFCS samples at 4.16 ± 0.04 (**Table 1**). The 42% HFCS samples had an average pH of 4.06 ± 0.18, whereas the 55% HFCS samples had an average pH of 4.60 ± 0.41. The D-blend sample was a HFCS-55−sucrose syrup blend that is specifically formulated for bee-keepers. The higher pH of this D-blend syrup is due to its high sucrose concentration. Sucrose is less stable at pH values < 8.3 and particularly at even lower pH values (*18*). Multiple acid titrations were recorded for the syrups, and the average values and standard deviations are reported in **Table 1**. It was noticed that there was a delay between the time the titrant (NaOH$_{aq}$) was added and the pH-meter recorded the change in pH, and it was, therefore, important to maintain a uniform titrant drip rate when this method was employed. In this study the pH correlated (R^2 = 0.941) well with the theoretical %HCl in the samples (**Table 1**).

The Winkler method (*15*) is the most reported spectrophotometric method for the determination of HMF, and we found this method was more practical to scale down to the microlevel compared to White's (*16*) method (AOAC accepted method). With the White method, the Carrez I and Carrez II solutions (*16*), 0.355 M potassium ferrocyanide, and 1.37 M zinc acetate, respectively, need to be added to volumetric flasks (10 mL) that contain the HFCS diluted solution. A cloudy colloidal suspension forms that is difficult to filter through 0.45 μm membranes, due to back pressure. Afterward, the supernatant is treated with a 0.20% sodium bisulfite solution, and the absorbance is recorded at 284

Table 1. Hydronium Ion Concentration, Hydroxymethylfurfural Concentration, and Elemental Analysis of Domestically Produced High-Fructose Corn Syrup

| HFCS	pH	EP pH	% HCl	HMF$_o$ (μg/g)	% fructose[a]	% C, H, N, S
A-42	4.15 ± 0.04	8.49 ± 0.03	0.0104 ± 0.0013	20.75 ± 0.004	42	29.30 ± 0.09, 5.55 ± 0.08, 0.0, 0.0
B-42	3.86 ± 0.02	8.50 ± 0.06	0.0108 ± 0.0007	3.07 ± 0.002	42	29.50 ± 0.06, 7.65 ± 0.04, 0.0, 0.0
C-42	4.18 ± 0.04	8.46 ± 0.01	0.0092 ± 0.0005	8.13 ± 0.000	42	29.53 ± 0.11, 7.55 ± 0.04, 0.0, 0.0
A-55	4.86 ± 0.17	8.48 ± 0.03	0.0776 ± 0.0004	28.65 ± 0.005	55	31.72 ± 0.14, 7.39 ± 0.04, 0.0, 0.0
B-55	4.16 ± 0.04	8.50 ± 0.06	0.0092 ± 0.0030	20.77 ± 0.006	56	31.56 ± 0.08, 7.42 ± 0.0, 0.0, 0.0
C-55	5.02 ± 0.02	8.50 ± 0.06	0.0074 ± 0.0004	7.89 ± 0.004	56	31.69 ± 0.13, 7.43 ± 0.07, 0.0, 0.0
D-55	4.34 ± 0.06	8.47 ± 0.01	0.0085 ± 0.0005	27.47 ± 0.003	55	31.70 ± 0.09, 7.41 ± 0.01, 0.0, 0.0
D-blend	6.09 ± 0.06	8.52 ± 0.02	0.0062 ± 0.0008	4.05 ± 0.001	50	32.79 ± 0.05, 7.19 ± 0.03, 0.0, 0.0

[a] Denoted from certificate of analysis.
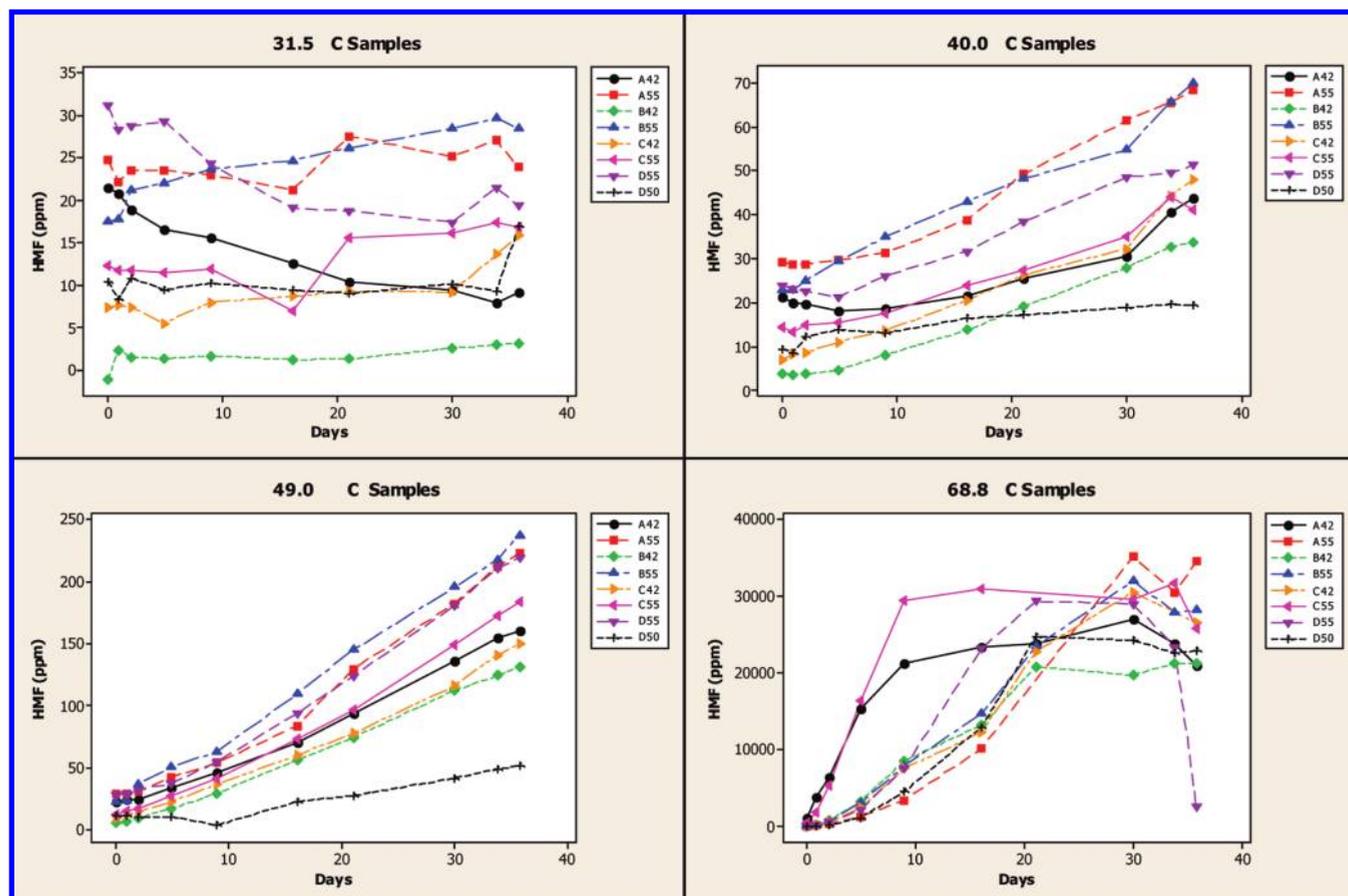
Figure 2. Rates of increase of HMF (ppm) with respect to time over 35 days at 31.5, 40.0, 49.0, and 68.8 °C.

and 336 nm. In comparison, the Winkler method requires filtration after the syrup is diluted to remove fine particulate, and afterward *p*-toluidine (10% w/w) in 2-propanol and aqueous barbituric acid 0.5% (w/w) are added. The blank is treated with an equal volume of DI water instead of the latter. One of the main criticisms of the Winkler method is the use of *p*-toluidine, which is carcinogenic (*15*). We observed that with the Winkler method the reaction product began to fade after about 10 min, so the absorbance had to be recorded immediately. We investigated warming the Winkler method reaction product. On warming the reaction product became orange with an absorbance maximum of 434 nm. Substituting thiobarbituric acid instead of barbituric acid was also investigated, but the reaction mixture had an unpleasant aroma. For the HFCS samples (**Table 1**), all of the samples fell within specifications for the amount of HMF as listed on Certificates of Analysis, which were provided by the manufacturer and accompanied the samples.

Elemental analyses among HFCS samples with the same fructose levels were consistent (**Table 1**). This reveals similar intercompany manufacturing methods. Elemental analysis is also an acceptable AOAC method for the determination of protein in foods, with an absence of nitrogen revealing that protein and, therefore, enzymes were not detected (*19*). In addition to the latter, the absence of proteins in the HFCS samples was confirmed by spectrophotometric analysis using the Coomassie blue method, which is capable of detecting proteins as low as 1 μg/mL (*21*). Therefore, with the absence of detectable nitrogen, the formation of HMF in acidic HFCS must be formed by a carbocation-type dehydration mechanism. To form HMF, 3 mol of water is lost from each fructose molecule to form 1 molecule of HMF. In the presence of nitrogen-containing

molecules, the competing mechanism for the formation of HMF would be through the formation of Schiff base-type intermediates, such as with Amadori rearrangements, as is found in nitrogen-containing products (*21*). According to Kuster (*8*), fructose is about 40 times more reactive than glucose in forming HMF. In addition to the absence of detectable nitrogen by elemental analysis, there was an absence of any detectable sulfur (**Table 1**), which was reported as SO_2 with the certificates of analysis that were provided with the HFCS from the manufacturers (*1*).

For the study of the formation of HMF over ∼36 days, in eight different HFCS products at four constant, time-weighted temperatures (Materials and Methods), interesting results were observed (**Figures 2**). Temperatures near 30, 40, 50, and 70 °C seemed to be appropriate because the Korean investigators reported HMF formation in 55% HFCS at 20, 40, and 60 °C (*11*). At 31.5 °C, in samples A-42 and D-55, the amount of HMF decreased (**Figure 2**). The pH values of these samples were 4.15 ± 0.04 and 4.34 ± 0.06, respectively (**Table 1**), so it is possible that at this temperature and pH the rate of destruction of HMF was greater than the rate of formation. The studies were repeated three times to confirm the findings. In terms of the rate of destruction of HMF, Fallico et al. reported that at 35 °C, the rates of degradation of HMF in citrus, chestnut, and multifloral honeys were 1.95, 3.25, and 1.35 ppm of HMF per day (*23*). These samples had corresponding pH values of 3.6, 6.5, and 3.2 (*23*). Therefore, it seems that the higher pH chestnut honey had a higher rate of HMF degradation. For the HMF increase with time in the HFCS samples in this study, polynomial and logarithmic equations were fitted for the plots in **Figure 2** for the eight HFCS products so that bee keepers and other consumers of

Table 2. Equations Describing the Rate of Formation of Hydroxymethylfurfural in Eight Domestic High-Fructose Corn Syrup Samples with Respect to Time at Four Isotherms[a]

| brand	T = 31.5 °C	T = 40.0 °C	T = 49.0 °C	T = 68.8 °C
A-42	$-0.0002[x]^3 + 0.0219[x]^2 - 0.872[x] + 21.32$ $R^2 = 0.985$	$0.0007[x]^3 + 0.0013[x]^2 - 0.1596[x] + 20.257$ $R^2 = 0.9798$	$21.253E^{0.09598[x]}$ $R^2 = 0.9977$	$-21716[x]^3 + 365259[x]^2 - 544769[x] - 70784$ $R^2 = 0.9992$
A-55	$-0.0006[x]^3 + 0.0303[x]^2 - 0.3267[x] + 24.283$ $R^2 = 0.5442$	$-0.0012[x]^3 + 0.105[x]^2 - 0.6226[x] + 30.17$ $R^2 = 0.9985$	$24.748E^{0.123[x]}$ $R^2 = 0.9911$	$48.777[x]^3 - 259.53[x]^2 + 466.33[x] - 43.309$ $R^2 = 0.9999$
B-42	$-0.0001[x]^3 + 0.0114[x]^2 - 0.2154[x] + 2.1327$ $R^2 = 0.9451$	$-0.0005[x]^3 + 0.0404[x]^2 + 0.1372[x] + 3.4403$ $R^2 = 0.9988$	$7.5188E^{0.1157[x]}$ $R^2 = 0.9776$	$1648.2[x]^3 - 10019[x]^2 + 13434*[x] - 1471.6$ $R^2 = 0.9999$
B-55	$0.0004[x]^3 - 0.0284[x]^2 + 0.8394[x] + 18.269$ $R^2 = 0.9566$	$0.0027[x]^3 - 0.117[x]^2 + 2.6444[x] + 21.094$ $R^2 = 0.9909$	$24.107E^{0.1377[x]}$ $R^2 = 0.9931$	$1572.8[x]^3 - 9456.1[x]^2 + 12605[x] - 1335.3$ $R^2 = 0.9999$
C-42	$0.0009[x]^3 + 0.0374[x]^2 + 0.4584[x] + 7.0123$ $R^2 = 0.9392$	$0.0017[x]^3 - 0.0632[x]^2 + 1.4234[x] + 6.2649$ $R^2 = 0.9879$	$11.608E^{0.1074[x]}$ $R^2 = 0.9937$	$1045.6[x]^3 - 6735.7[x]^2 + 9309.8[x] - 1000.3$ $R^2 = 0.9998$
C-55	$0.0005[x]^3 - 0.0285[x]^2 + 0.6384[x] + 12.686$ $R^2 = 0.9932$	$-0.0002[x]^3 + 0.0252[x]^2 + 0.2375[x] + 13.826$ $R^2 = 0.9809$	$13.275E^{0.1224[x]}$ $R^2 = 0.9982$	$36526[x]^3 + 32784[x]^2 - 139298[x] + 22400$ $R^2 = 1$
D-55	$0.0006[x]^3 - 0.0108[x]^2 - 0.6442[x] + 31.305$ $R^2 = 0.9353$	$-0.0017[x]^3 + 0.1047[x]^2 - 0.6697[x] + 23.77$ $R^2 = 0.9964$	$24.238E^{0.123[x]}$ $R^2 = 0.9957$	$1222.4[x]^3 - 7746.5[x]^2 + 10598[x] - 1176.4$ $R^2 = 0.9999$
D-blend	$0.001[x]^3 - 0.0416[x]^2 + 0.3828[x] + 9.3911$ $R^2 = 0.8894$	$0.0003[x]^3 - 0.0224[x]^2 + 0.7207[x] + 9.4059$ $R^2 = 0.9492$	$9.8122E^{0.0496[x]}$ $R^2 = 0.9861$	$1522.5[x]^3 - 17878[x]^2 + 59535[x] - 47585$ $R^2 = 1$

[a] [x] = time in days.

HFCS can more accurately predict the rates of formation of HMF with time (**Table 2**). At 40.0 °C, samples B-42, A-55, B-55, and D-55 increased markedly in HMF content compared to the other HFCS samples (**Figure 2**). The C-55 sample had a relatively higher pH of 5.02 ± 0.02 (**Table 1**), so HMF formation was not as acid-catalyzed initially and, therefore, the rate of increase was slower. The D-blend sample (D-50), a blend of 50% HFCS-55 and sucrose syrup, gave the least increased formation of HMF of all the samples because it contained less fructose reactant and had the highest pH (6.09 ± 0.06), which is required to maintain the composition of the sucrose (*18*). At 49.0 °C, HFCS sample B-55, which is very acidic (**Table 1**) had the highest rate of HMF formation, with C-55 having the most modest rate of the 55% HFCS samples (**Figures 2**). The latter has the highest pH of any of the 55% HFCS samples tested, with the exception of D-blend. However, D-blend contains sucrose, which is nonreactive in terms of HMF (**Table 1**). Among the HFCS-42 samples, A-42 had the most accelerated rate of formation of HMF, whereas C-42 had the lowest rate. Our results were similar to the Korean findings in that his rate of increase of HMF was linear, with respect to time at 40 °C, but was exponential at 60 °C (*11*). In our paper, the 49.0 °C plot (**Figure 2**) became exponential with respect to time. In addition, the data for the 49.0 °C data had the highest R^2 coefficients when the data were fit to exponential equations (**Table 2**). At 68.8 °C, the syrups darkened after only 1 day and the rate of formation appeared to be erratic (**Figure 2**), especially after 20 days. This was due to the formation of humus (high molecular weight colorant) in the syrup, which interfered with the HMF spectrophotometric assay, even after dilution of the sample matrix to 100 mg of HFCS in 10 mL of water. Therefore, for the equations presented in **Table 2**, for the 31.5, 40.0, and 49.0 °C

data, all of the data were included in the regression analysis, whereas for the 68.8 °C data set, only the first 9 days (five data points) were used to generate the equations (**Table 2**).

For the metals analysis by ICP-MS, the eight HFCS samples were spiked with a Rh-103 recovery standard, wet ashed to remove carbon, and analyzed for metal ions. This was performed because it was reported that metal ions catalyze the formation of HMF in honey (*22*). For instance, it was reported that Mn, Zn, Mg, and Fe catalyze the formation of HMF in honey, with the Mn exhibiting the greatest catalytic effect (*22*). This may explain why in **Figure 2**, for the 40.0 and 49.0 °C plots, sample B-42 had a lower increase of HMF compared to A-42 and C-42. Sample B-42 was also more acidic than any of the 42% HFCS samples (**Table 1**), so the Mn, which is 1.73 and 4.15 times greater with A-42 and C-42, respectively (**Table 3**), could account for the difference in acidity, as B-42, the most acidic samples (**Table 1**) would be expected to form more HMF. The 55% HFCS samples formed HMF as expected, based of the levels of Mn (**Table 3**) and pH (**Table 1**), except that sample D-55 formed HMF at a more accelerated rate than expected (**Figures 2**). With the high levels of Mn in D-55, it is surprising that not more HMF formed. In addition to contributing to the pH, metal ions are a critical storage parameter for users of HFCS. For instance, the railway tank cars from HFCS manufacturers are reported to be epoxy lined (*5*), so this would limit the effect of the acidic HFCS dissolving metal ions from storage containers. In the samples that we tested, Co, Ni, and Pb were most likely from metallic vessels used to store and transport HFCS in manufacturing plants. The levels of Pb in manufacturer D samples were below EPA thresholds for the values in drinking water pertaining to the Safe Water Drinking Act (*24*). It should be mentioned that

Table 3. Metals Analysis of Domestic High-Fructose Corn Syrup Samples by Inductively Coupled Plasma Mass Spectroscopy

sample	Na (ppm)	Mg (ppm)	Ti (ppb)	Cr (ppb)	Mn (ppb)	Cp (ppb)	Ni (ppb)	Pb (ppb)
A-42	1.58 ± 0.011	008 ± 0.006	22.0 ± 0.998	nd	2.77 ± 1.106	nd	2.03 ± 0.321	nd
A-55	nd	0.049 ± 0.004	6.31 ± 1.50	nd	1.72 ± 0.301	0.434 ± 0.249	1.42 ± 0.212	nd
B-42	4.04 ± 0.18	0.619 ± 0.072	43.6 ± 1.76	nd	1.106 ± 0.104	nd	1.82 ± 0.615	nd
B-55	1.06 ± 0.181	0.040 ± 0.007	17.8 ± 2.79	nd	2.38 ± 0.244	nd	1.69 ± 0.684	nd
C-42	0.182 ± 0.005	0.067 ± 0.015	16.2 ± 3.74	nd	4.40 ± 0.016	0.189 ± 0.066	nd	nd
C-55	1.27 ± 0.846	0.462 ± 0.007	26.6 ± 1.57	nd	1.18 ± 0.013	nd	1.17 ± 0.070	nd
D-55	0.08 ± 0.000	0.432 ± 0.013	15.1 ± 1.82	3.33 ± 0.445	6.15 ± 0.169	nd	3.28 ± 0.129	1.68 ± 0.661
D-blend	4.38 ± 0.555	3.87 ± 0.059	5.19 ± 1.28	5.39 ± 0.062	3.81 ± 0.050	nd	2.06 ± 0.321	1.75 ± 1.20
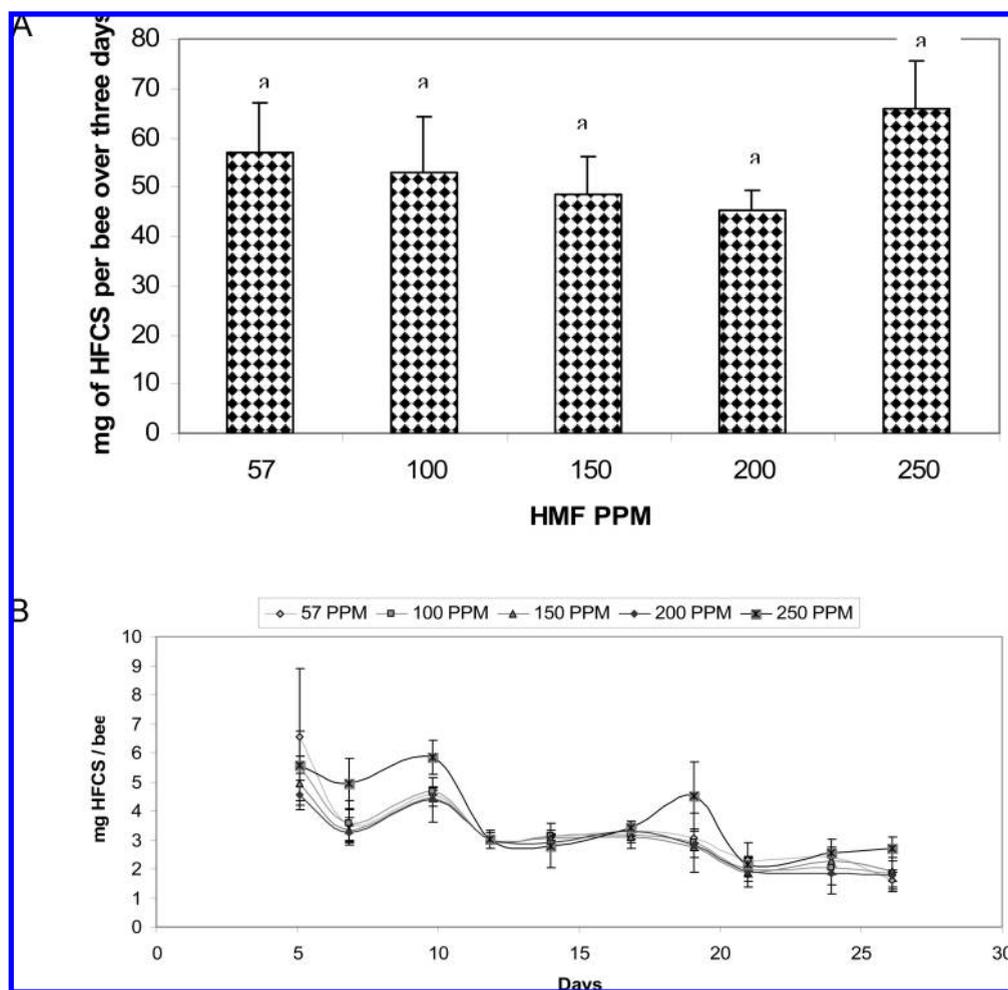
Figure 3. Consumption of HMFS in milligrams of HFCS per bee at 3 days (**A**) and over 27 days (**B**). Different letters in the bar graph indicate significant differences in mortality between different HMF dosages, ANOVA, Dunnet two-sided ($P < 0.005$).

AOAC methods utilize graphite furnace techniques specifically for analyzing food-grade syrups for Pb, and the ashing technique that was utilized in this study was similar to the method reported (*25*).

Caged Bee Studies. During caged bee studies, bees consume unusually higher than average amounts of syrup feed (*17*). Therefore, in this study, the consumption was reported for the cages for the first 3 days (**Figure 3A**) and then for 5−26 days. It was important to measure the syrup consumption early in the study as mortality increases and the estimates of the consumption per bee are likely not as accurate. HFCS A-55, which contained 57 ppm HMF, was spiked with a HMF standard to produce 100, 150, 200, and 250 ppm HMF HFCS. The bees were not repelled by the 250 ppm HMF dosage versus 57 ppm, as the latter was consumed significantly less during the first 3 days (**Figure 3A**). As time progressed, the bees consumed less syrup at all dosages

(**Figure 3B**). In some of the cages where drinking water was supplied ad libitum and the water supply expired without being replenished, more syrup was consumed and the mortality increased dramatically. For instance, in a 250 ppm HMF cage replicate where the water became expired, all of the bees were dead within 2 weeks. It should be mentioned that in the caged bee studies in this study, a commercially available pollen food was also fed to the bees as a pollen "food plug". As the bees become accustomed to a new environment, they may preferentially eat the pollen because it contains additional nutrients and, therefore, could provide satiety. In the commercial setting, a pollen food source is typically fed to bees in the spring to promote brood rearing, so the caged bee consumption and toxicity are not synonymous with a commercial beehive setting. This is because the bees in the cage have additional pollen nutrients and the open colony bees in the commercial setting would be required to

Article

J. Agric. Food Chem., Vol. 57, No. 16, 2009 **7375**

forage. Thus, in the commercial setting bees would likely consume more syrup and consequently HMF before they forage for pollen, because they are not fed with a pollen substitute or supplemented with water.

There are several reports on the toxicity of HMF and its hydrolysis products, levulinic and formic acid, in the literature (*7, 12*). Bailey (*7*) demonstrated increasing mortality to bees with fresh and 4 and 8 year stored honey, which had correspondingly high levels of HMF. Bailey (*7*) also found that there was higher mortality associated with concentrated solutions of 8 year stored honey versus dilutions. Bailey performed these studies because in the winter of 1962−1963 many commercial beekeepers lost many colonies in England due to acid-hydrolyzed sucrose, so his studies did not attempt to estimate the toxicity of HMF but, rather, HMF + levulinic acid + formic acid cumulatively, as toxins found in acid-hydrolyzed sucrose (*7*). It was observed that HMF caused gut ulceration, which resulted in dysentery in bees (*7*). Jachimowicz studied the toxicity of HMF, as a separate component, in caged bee studies and found that 30 ppm HMF levels had no adverse effects on bees (*12*). According to his report, the amount of HMF that killed 50% of the bees in his assay (LD-50) was near 100 ppm, and 150 ppm HMF in foraging formulation resulted in 50% mortality in 16 days (*12*). In the caged bee studies described in this paper, it was found that for the HFCS-55, 150 ppm HMF treatment, after 19 days 50% of the bees died (**Figure 4A**). This is very close to the 16 days that Jachimowicz reported for the 150 ppm HMF (*12*). The 250 ppm treatment resulted in higher mortality, over time, than any of the treatments. A comparison on 26 day mortality revealed that the 57, 100, 150, and 200 ppm treatments were not found to differ significantly (**Figure 4B**). However, the 250 ppm HMF, HFCS-55 enriched treatment indicated a significantly lower survival after 26 days (**Figure 4B**). In the caged bee studies we performed, sucrose was used as an external reference. However, it is invalid to compare estimated toxicity of HFCS compared to sucrose syrup as the chemical matrix and makeup are different. Refined, white sucrose is a crystalline solid before mixing with water to produce a syrup and is ≥99.9% pure (*18*), whereas HFCS contains fructose, glucose, oligosaccharides, SO_2 from the bleaching step, hydrolysis products, and pectins (*1, 5*). Therefore, it is not surprising that in our caged bee assays, bees lived longer on sucrose syrup of higher purity. Similarly, Barker observed greater longevity for bees fed sucrose compared to HFCS (*6*). It is interesting that Severson and Erickson (*26*) conducted open bee colony studies from 1982 to 1983 and compared HFCS-42 and HFCS-55 from one manufacturer versus sucrose and found no statistical difference in colony performance between the HFCS samples and sucrose. Moreover, the HFCS-55 produced the highest seasonal honey production. However, they noted significantly higher brood cluster size in the spring with the sucrose syrup (*26*).

Addition of Commercially Available Bases. Provided that commercial users of HFCS are cognizant of the pH of the HFCS syrup, they should be able to estimate the concentrations of HMF formed with time and temperature from the equations provided in **Table 2**. For instance, the HFCS 55 that we used in the toxicity studies in this paper was obtained as a 55 gal (203.5 L) drum and initially contained 18 ppm HMF; within 1 year the HMF level increased to 57 ppm at ambient uncontrolled temperature. Because HMF formation is catalyzed by acid, a logical and practical approach would be to neutralize the HFCS with commercially available and relatively inexpensive bases such as lime (CaO), potash (Na_2CO_3), and soda ash (NaOH) and then treat the syrup with antifermenting agents. The effects of adding molar equivalent (**Table 1**) amounts of bases to neutralize A-42 and A-55 were studied with CaO, Na_2CO_3, and NaOH (**Figure 5**).
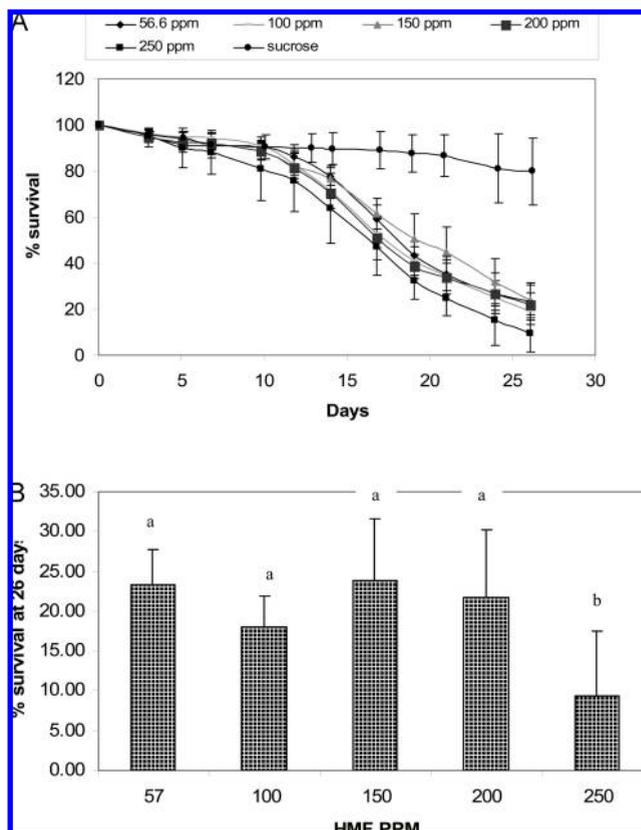

Figure 4. Mortality data from caged bee studies for bees dosed with 57, 100, 150, 200, and 250 ppm HMF. Different letters indicate significant differences in mortality between different HMF dosages, ANOVA, Dunnet two-sided (*P* < 0.005).
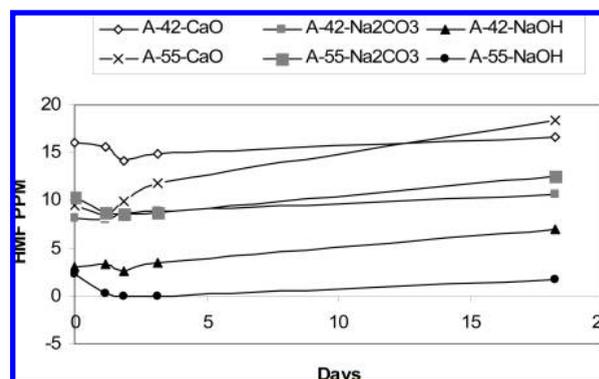

Figure 5. Rates of formation of HMF in HFCS A-55 after base equivalents were added.

NaOH had the effect of suppressing HMF formation at 20 days at 45 °C (**Figure 5**). The initial amount of HMF also decreased with NaOH, followed by Na_2CO_3 (**Figure 5**). In the case of NaOH, the initial destruction of HMF could be achieved chemically by OH^- nucleophilic attack on HMF, which could lead to furan ring-opening reactions. Although neutralizing HFCS with commercial bases effectively reduces HMF, microbiological growth will become much more problematic, although these bases may be toxic to microbes.

In this paper, four HFCS products were evaluated for rates of formation of HMF at four constant temperatures. The data generated are important for commercial beekeepers, for manufacturers of HFCS, and for purposes of food storage. Because HFCS is incorporated as a sweetener in many processed foods in

7376 *J. Agric. Food Chem.*, Vol. 57, No. 16, 2009

LeBlanc et al.

the United States, the data from this study are important for human health as well. For instance, recently it was reported for in vitro studies that HMF damaged DNA (*27*). Furthermore, daughter metabolites of HMF, such as 5-sulfoxymethylfurfural, are potentially more of a threat to humans and have been detected in urine shortly after being exposed to HMF in the diet (*28*).

In the southern United States, from the spring through fall, temperatures of 40 °C and higher occur. Therefore, provided beekeepers know the initial amount of HMF in their HFCS and its pH is similar to that of the brand evaluated in this study, the amount of HMF can be estimated from the equations presented in **Table 2**. For instance, for sample A-42 at 40 °C it would take approximately 69 days to achieve levels of HMF that are considered to be toxic (250 ppm) to bees by our methods. Future work will be directed at estimating the levels of HMF on the basis of Arhenius thermodynamic parameters, such that the levels of HMF can be predicted at selected temperatures. For instance, Tosi studied the rates of formation of HMF in Argentine honey from 100 to 160 °C, in 10 °C increments (*10*). From these data, Tosi was able to determine the energy of activation (226 kJ/mol) to form HMF and the enthalpy and entropy of the reaction (*10*). Fallico et al. studied the rates of formation of HMF in orange, sulla, eucalyptus, and chestnut honey samples at 50, 70, and 100 °C (*29*). They determined the energies of activation for the formation of HMF to be 136.5, 139.8, 141.1, and 182.5 kJ/mol, respectively (*29*). With this information the rates of formation of HMF can be predicted at selected temperatures. This type of information would be highly desirable and practical for industial manufacturers and users of HFCS because it is a more consistent product than honey, because honey varies with floral sources.

ABBREVIATIONS USED

HFCS, high-fructose corn syrup; HMF, hydroxymethylfurfural; ICP-MS, inductively coupled plasma mass spectroscopy.

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