Decomposition of Hydroxymethylfurfural in Solution and Protective Effect of Fructose

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Time-course changes were studied on both concentration and spectral curve shape of 5-(hydroxymethyl)furfural (HMF) in solution. A significant and progressive absorbance drop at 284 nm was observed as well as the concomitant appearance of a new band at 252 nm with standard HMF solutions and glucose, saccharose, maltose, and lactose solutions. These changes depended mainly upon HMF starting concentration and were affected by both the storage temperature and the time between the preparation of the solution and the spectrophotometric measurement. There were less significant changes in honey, high-fructose corn syrup, and fructose solutions. The results indicate that fructose has a protective effect on HMF decomposition in solution. The spectrophotometric measurement should be made within 6 h after sample preparation, and the solutions should be kept at 4−8 °C to avoid HMF decomposition. Honey solutions with high HMF starting concentration showed significantly lower concentrations when they were clarified by ultrafiltration, compared with the usual clarification with Carrez reagent. This last fact could be attributed to a deficient sample deproteinization, which is improved either by ultrafiltration or by using a larger amount of Carrez reagent.

Keywords: *Hydroxymethylfurfural; honey; syrup; fructose; glucose; sugars; food*

INTRODUCTION

5-(Hydroxymethyl)furfural [HMF; 5-(hydroxymethyl)-2-furaldehyde] is formed during dehydration of hexoses (Anam and Dart, 1995), nonenzymatic browning (the Maillard reaction), etc. Many foodstuffs containing hexoses may have variable amounts of such chemical, depending on the pH (Pérez et al., 1990), the temperature and storage conditions (Pérez et al., 1990; Sancho et al., 1992; Thrasyvoulou et al., 1994; Yilmaz and Küfrevioglu, 1994), and the presence of metal ions (Anam and Dart, 1995) or UV light (Faría, 1993). On the other hand, HMF is reported to be light- and air-sensitive (*Merck Index*, 1989).

HMF has been widely used both to predict honey freshness and to evaluate its quality upon treatment (Dustmann, 1993; Faría, 1993; Pérez et al., 1990; Sancho et al., 1992; Thrasyvoulou et al., 1994; White, 1992; Yilmaz and Küfrevioglu, 1994). HMF is also used to evaluate the stability of dextrose injection solutions (USP, 1995). Even though there are many methods available to measure HMF (Anam and Dart, 1995; Corradini and Corradini, 1994; Durán Meras et al., 1995; Espinoza Mansilla et al., 1993; Lo Coco et al., 1996; Rittgerodt, 1994), the UV spectrophotometric method has become the official AOAC method (AOAC, 1995) for the determination of HMF in honey since 1980 (White, 1979). Although it fails to measure accurately HMF in honey samples containing lower quantities of this compound (White et al., 1979), as is the case in honey with lower diastatic activity (Sancho et al., 1992), it could be considered a suitable method for regulatory purposes and quality control because it is easy to perform and no carcinogenic reagents are needed (White, 1992).

An accurate measurement of HMF is very important in connection with the determination of honey freshness and shelf life (Faría, 1993; Pérez et al., 1990; Sancho et al., 1992) as well as in the evaluation of the damage caused by overheating or storage abuse (Dustmann, 1993; Thrasyvoulou et al., 1994; White, 1992; Yilmaz and Küfrevioglu, 1994). The actual effectiveness of HMF to determine honey overheating and storage abuse must also be taken into account (Dustmann, 1993; White, 1992).

In the present work we investigate the behavior of HMF in solution as a contribution to the knowledge of chemical changes in honey and other foodstuffs containing sugars. We studied the spectrophotometric behavior of HMF in solutions containing HMF alone or mixed with glucose, glucose syrup, high-fructose syrup, fructose, saccharose, maltose, lactose, or honey (natural or supplemented with HMF).

MATERIALS AND METHODS

A Shimadzu (Kyoto, Japan) 1601 PC spectrophotometer, equipped with a PC interface and software, were used for spectral determination and quantitative analysis. Quartz glass cells from Hellma (Mülheim, Baden, Germany; light path 10.0 mm) were used in the spectrometer.

A Konik (Barcelona, Spain) KNK 500-A series HPLC chromatograph, equipped with refractive index detection, furnace, and Konik Datajet recorder−integrator, was used. The

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Table 1. Color and Sugar Composition of Honey Samples

sample	Córdoba dept	color classifn	glucose[a] (%)	fructose[a] (%)	saccharose[a] (%)
H1	San Justo (NE)	light amber	36.9 ± 0.0	37.4 ± 0.7	1.6 ± 0.4
H2	Cruz del Eje (NW)	amber	36.8 ± 1.7	40.2 ± 1.5	2.5 ± 0.1
H3	Gral. San Martín (S)	light amber	38.3 ± 1.2	39.7 ± 1.3	2.7 ± 0.1
H4	San Justo (NE)	amber	34.8 ± 1.5	38.2 ± 1.4	1.9 ± 0.3
H5	Unión (SE)	extra light amber	38.8 ± 2.1	40.4 ± 1.3	3.5 ± 0.2
H6	Unión (SE)	light amber	37.7 ± 1.6	41.0 ± 0.5	0.9 ± 0.1
H7	Sta. María (central)	light amber	34.8 ± 1.2	42.4 ± 1.0	2.6 ± 0.1
H8	Calamuchita (SW)	amber	33.4 ± 0.4	41.2 ± 0.2	3.1 ± 0.3

[a] Values were determined by HPLC and are averaged over at least two determinations. Error corresponds to one standard deviation ($\sigma n - 1$).

column used was an Aminex HPX87-P (Bio-Rad, Richmond, CA) with a Carbo-P (Bio-Rad) guard column (Assil et al., 1991).

5-(Hydroxymethyl)furfural (99%) was purchased from Aldrich Chemical Co. (Milwaukee, WI) and stored refrigerated at 0 °C in the dark under nitrogen. Fructose, glucose, maltose, lactose, saccharose, and sodium bisulfite were of analytical grade and are commercially available from Merck (Darmstadt, Germany). Water was ultrapure from a Millipore (Bedford, MA) Milli-Q water purification system. Glucose and high-fructose corn syrups were provided by ARCOR S.A. (Córdoba, Argentina) and were obtained by enzymatic hydrolysis of starch (glucose syrup) followed by enzymatic isomerization (high-fructose syrup). Honey was collected from local hives located in different regions in the Province of Córdoba, Argentina. Eight 1-kg samples were selected for the study on the basis of geographical origin, color, and sugar composition (Table 1) to take into account composition variation. Samples were kept protected from light at room temperature until analysis.

Solutions. *Solution A.* HMF stock solutions were prepared from ~13 mg of pure HMF diluted to 100 mL with water, covered with aluminum foil, and stored refrigerated at 4−8 °C (solution A). Fresh solutions were prepared weekly. Stock solutions were stable for at least 1 week, when stored refrigerated and light protected, as proved by spectral curve analysis before use.

Solution B. HMF standard solutions were prepared by diluting 1 mL of fresh solution A with either water (sample) or 0.2% sodium bisulfite (reference) to 100 mL (S1−3), 50 mL (S4−6), 25 mL (S7−9), or 10 mL (S10−12).

Solution C. Honey solutions were prepared for HMF determination according to the AOAC method (AOAC, 1995). In some determinations we avoided the use of the Carrez reagent. In these cases the clarification and deproteinization were performed by filtering the honey solution first through a filter paper (Whatman No. 1) and then through a 0.45 μm nitrocellulose membrane (Millipore) and finally ultrafiltration in an Amicon (Lexington, MA) device equipped with a YM-10 membrane. The obtained solution was then treated and measured according to the AOAC method (AOAC, 1995). Solutions prepared for HMF measurement were also used for HPLC analysis.

Solution D. Syrup stock solutions were prepared by diluting the syrups with both water and a HMF solution (~5 mg/100 mL). The resulting stock solutions contained 17.6 g/100 mL glucose, 19.5 g/100 mL fructose, and ~0.5 mg/100 mL HMF. Syrup work solutions were prepared by diluting 5 mL of stock solution to 50 mL with either water (sample) or 0.2% sodium bisulfite (reference). Fresh solutions were prepared daily.

Solution E. Fructose, glucose, saccharose, maltose, and lactose work solutions were prepared by dissolving the appropriate amount of each sugar with 40 mL of water, followed by the addition of an HMF solution (~5 mg/100 mL) and further dilution to 100 mL with either water (sample) or 0.2% sodium bisulfite (reference). Fresh solutions were prepared daily.

Determinations. Spectral curves (230−340 nm) were obtained using the spectral mode of the UV spectrophotometer, using the solutions containing sodium bisulfite as both baseline and reference as described in the AOAC method (AOAC, 1995). Absorbances at 284 and 336 nm were taken from the spectral curve by point peak software resource. A band with λ_{max} at 252 nm was determined by peak-peaking software resource. All solutions (solutions B−E) were measured immediately after preparation and afterward at 6, 24, and 48 h, keeping them in cap-sealed test tubes at room temperature (22−25 °C) and in daylight or under refrigeration (4−8 °C) in the dark until the next analysis. Some experiments with standard solutions were carried out by keeping solutions protected from light at room temperature. All of the determinations were carried out either in duplicate or in triplicate (measuring and storing two or three different solutions prepared from the same sample). HMF concentration was calculated as follows:

$$\text{HMF (mg/100 mL)} = 0.7487 \times \Delta A \quad (1)$$

$$\text{factor} = \frac{126}{16830} \times \frac{1000}{10} = 0.7487 \quad (2)$$

ΔA = (absorbance at 284 nm − absorbance at 336 nm) = (A_{284} − A_{336}), 126 = molecular weight of HMF, 16830 = molar absorptivity of HMF at 284 nm (White et al., 1979), 1000 = mg/g, and 10 = cL/L.

HPLC. All runs were done using helium-degassed ultrapure water as mobile phase, flow rate of 0.6 mL/min, and furnace temperature of 80 °C. The injection volume was 20 μL, and quantitative analyses were done using mixed sugar standards as described by Assil et al. (1991).

Color determinations on honey samples were performed by the Ministerio de Agricultura, Ganadería y Recursos Renovables (Agriculture, Cattle and Renewable Resources Department) of the Province of Córdoba, Argentina, according to the AOAC method (AOAC, 1995).

RESULTS AND DISCUSSION

In our study, first, the spectrophotometric behavior of HMF in solutions was carried out with standard solutions. We used four different starting concentrations to verify the biggest error described at lower concentrations during spectrophotometric method validation (White et al., 1979). We should note that 0.1 mg of HMF/100 mL of solution corresponds to ~20 mg/kg HMF in honey (White et al., 1979; AOAC, 1995). So, from Table 2, solutions S1−S3 have concentrations similar to those expected for a solution from a commercial non-overheated honey sample prepared according to the AOAC method (AOAC, 1995), whereas S4−S6 have concentrations similar to a honey solution with HMF near the regulatory limit. Finally, S7−S12 correspond to the concentration of a solution from overheated or overstored honey (White et al., 1979; White, 1982). In the same experiment we also evaluated the incidence of the storage under three different conditions (dark/refrigerated; daylight/room temperature; dark/room temperature).

The results obtained in this experiment (Table 2) show that the HMF concentration of solutions, in particular those stored at room temperature, progres-

Table 2. HMF Concentration Variation in Standard Solutions Stored under Different Conditions

		av HMF (mg/100 mL) at time[a]			
solution	storage condition	0 h	6 h	24 h	48 h
S1	dark/refrig	0.126 ± 0.001	0.099 ± 0.002	0.071 ± 0.003	0.053 ± 0.004
S2	light/rt	0.127 ± 0.001	0.065 ± 0.002	0.000 ± 0.000	0.000 ± 0.000
S3	dark/rt	0.120 ± 0.001	0.074 ± 0.003	0.000 ± 0.000	0.000 ± 0.000
S4	dark/refrig	0.265 ± 0.002	0.215 ± 0.002	0.151 ± 0.003	0.110 ± 0.001
S5	light/rt	0.263 ± 0.001	0.213 ± 0.002	0.094 ± 0.004	0.000 ± 0.000
S6	dark/rt	0.259 ± 0.003	0.212 ± 0.003	0.095 ± 0.003	0.000 ± 0.000
S7	dark/refrig	0.573 ± 0.001	0.573 ± 0.004	0.582 ± 0.002	0.581 ± 0.002
S8	light/rt	0.603 ± 0.001	0.579 ± 0.002	0.476 ± 0.028	0.343 ± 0.037
S9	dark/rt	0.590 ± 0.002	0.572 ± 0.002	0.490 ± 0.015	0.320 ± 0.052
S10	dark/refrig	1.324 ± 0.015	1.325 ± 0.015	1.256 ± 0.016	1.198 ± 0.015
S11	light/rt	1.324 ± 0.003	1.294 ± 0.024	1.068 ± 0.053	0.497 ± 0.011
S12	dark/rt	1.322 ± 0.009	1.313 ± 0.006	0.999 ± 0.066	0.484 ± 0.060

[a] Storage time from starting measurement. HMF values are averaged over one determination on three different solutions with almost the same starting concentration ($n = 3$). Error corresponds to one standard deviation ($\sigma n - 1$).

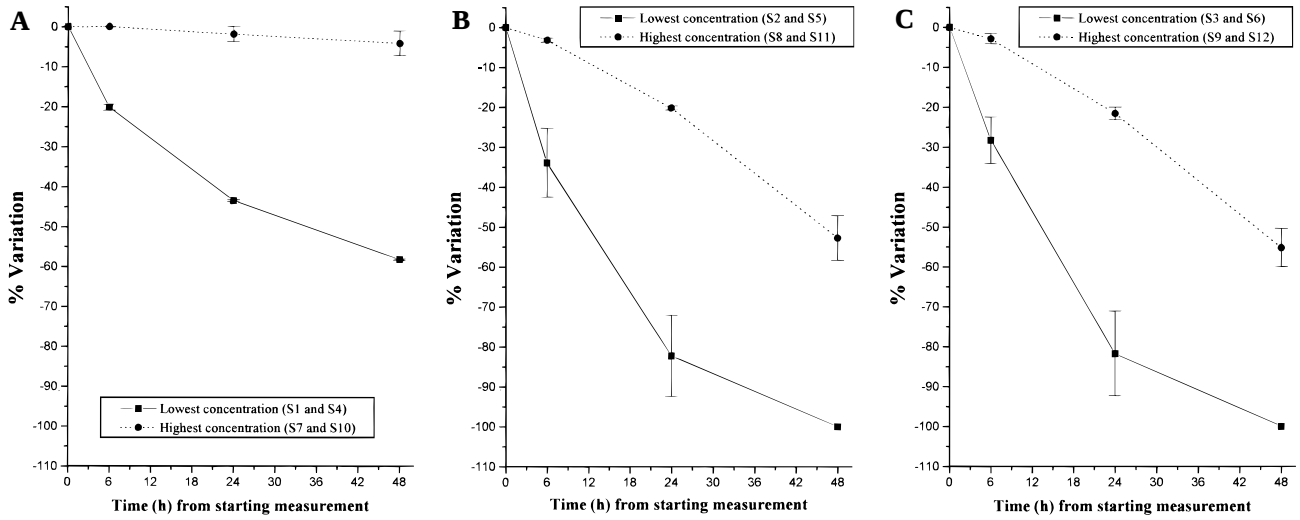

Figure 1. HMF variation as a function of concentration in standard solutions stored at different conditions: (A) stored refrigerated and in the dark; (B) stored at room temperature and in daylight; (C) stored at room temperature and in the dark. Percentages are calculated from Table 2 by statistical mean difference (paired observations between 0−6, 0−24, and 0−48 h from starting measurement, $n = 6$, hypothesized diff = 0). Error bars correspond to the percentage calculated from one standard error of mean difference.

sively drops in the course of time at any concentration (Table 2; Figure 1). Figure 1 also shows that the lower the starting concentration, the higher the drop magnitude. This fact resembles the results obtained in the collaborative study with honey containing low HMF values (White et al., 1979). We can also note that the refrigerated solutions (Figure 1A) have lower HMF concentration diminution compared with the solutions kept at room temperature (Figure 1B,C). However, there are no considerable differences between daylight (Figure 1B) and dark storage (Figure 1C) at room temperature. Therefore, we could conclude that the spectrophotometric behavior of HMF is more influenced by temperature than by light. It should be also noted that the magnitude of the change seems to be affected extensively by the starting concentration, more than by the storage temperature.

We should also consider the changes in the spectral curve shape (Figure 2). It should be noted that the reduction in the 284 nm band is simultaneous with the appearance of a new band at 252 nm due to the HMF decomposition product.

It has been widely reported that HMF levels increase in glucose solutions (USP, 1995), honey samples, and other foodstuffs with both temperature and time (Anam and Dart, 1995; Lo Coco et al., 1996; Sancho et al., 1992; Thrasyvoulou et al., 1994; White, 1992; Yilmaz and Küfrevioglu, 1994). However, we should consider some evidence which establishes that samples exposed to UV light have presented lower HMF values than those kept protected from light (Faría, 1993) and the advice to keep pure HMF protected from light and air (*Merck Index*, 1989). If HMF decomposes with temperature and light, we should not observe higher levels in sugar solutions. Therefore, as the next step, we decided to study the spectrophotometric behavior of honey solutions containing variable amounts of HMF. Even though a different behavior of HMF in honey compared with the corresponding to HMF in solution is expected, a honey solution has the honey components and could give a better idea of the behavior of HMF in honey than those derived from standard solutions.

Considering the differences in honey composition, we selected 8 honey samples from >100 collected from different locations in the Province of Córdoba, Argentina. Both honey color and sugar composition, measured by HPLC, were evaluated to determine differences between samples (Table 1).

First we have studied honey solutions clarified with the Carrez reagent according to the AOAC method (AOAC, 1995) (Table 3, Hn.1 and Hn.2). We have evaluated eight different solutions with an HMF start-

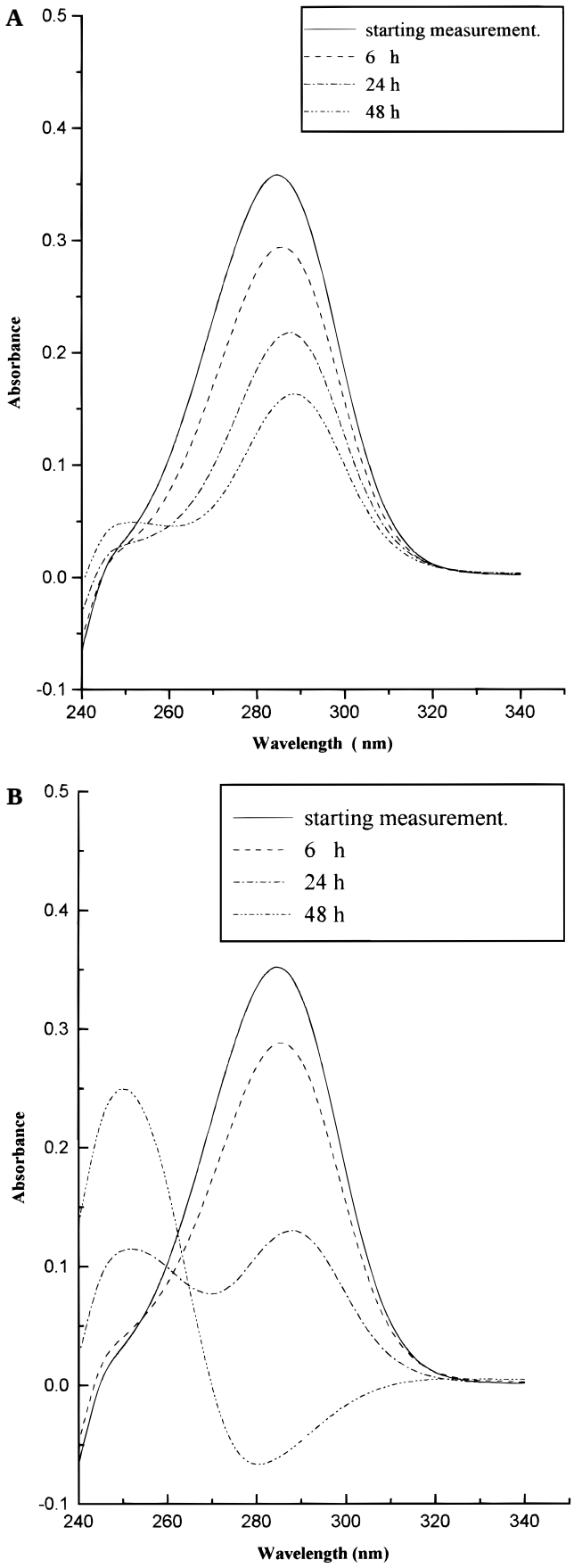

Figure 2. Spectral curve of standard solutionss: (A) stored refrigerated and in the dark; (B) stored at room temperature and in daylight.

ing concentration similar to those used for standard solutions (Table 2). Each solution was evaluated under two different storage conditions, either dark−refrigerated (Table 3, Hn.2) or daylight−room temperature (Table 3, Hn.1). We have found that honey solutions show a different behavior, compared with those observed for standards (Table 2, Figure 1). Therefore, most of the honey solutions stored in the dark under refrigeration show lower HMF concentration variation than the standard solutions with similar starting concentrations (Figures 1A and 3A). Also, honey solutions stored at room temperature show a different behavior. From Figure 3B we can note that HMF concentration is slightly increased during the first 24 h in honey solutions stored at room temperature and in daylight, instead of the decay observed with the standards of similar concentration (Figure 1B). Even when a concentration decay is observed after 48 h (Figure 3B), its magnitude is lower than those observed for standards with similar starting concentrations (Figure 1B); also, honey solutions with the lowest HMF starting concentration show the lowest decay (Figure 3B).

To search for the reason for this different behavior, we added ∼3 mg of pure HMF to 100 g of a honey sample. We then repeated the experiment looking either for some difference between the natural and the added HMF or for some failure in the method that could report any other chemical with a similar spectral curve as HMF. We observed ∼100% recoveries of the added HMF as well as a behavior similar to those of the corresponding honey solutions without added HMF (Table 3, H4.1 and H4.A), so changes in the behavior due to the method itself should be rejected.

On the other hand, we also observed changes in the spectral curve shape of honey solutions (Figure 4). Even when spectral changes are not always so important as with standards (Figure 2), a signal at 252 nm is observed whenever the HMF signal at 284 nm drops (Figure 4B). Also, an absorbance increase is observed throughout the spectral curve from the starting measurement (Figure 4). Although this last fact could be attributed to mold and yeast activity in the sugar solutions, the absorbance difference between 284 and 336 nm should remain almost constant. However, we have observed that this difference is not constant, and it is one of the causes that produces the concentration increase within the first 24 h for many of the studied honey solutions (Figure 3). As we can also see from Figure 4B (spectral curve after 48 h), ΔA ($A_{284} - A_{336}$) could lead to the calculation of an incorrect HMF concentration, even when no band is observed at 284 nm. However, this is an error because the starting HMF was decomposed, probably into the same product observed with standard solutions. HMF decomposition in honey solutions stored at room temperature is statistically confirmed from concentration values at 48 h, even when these values are calculated from ΔA ($A_{284} - A_{336}$) and are affected by this mentioned error, which causes the observed value dispersion (Figure 3B).

From now on we should ask why HMF does not decompose in honey solutions either in the same way or with the same kinetics as in standards. We have thought of many probable reasons. First, we should consider that additional HMF could be formed within honey solutions, mainly from fructose. If so, we should observe the competition between formation and decomposition. If these processes are equal, no band disappearance should be observed at 284 nm when the new band at 252 nm appears; on the contrary, both bands

Table 3. HMF Concentration Variation in Honey Solutions

solution[a]	storage condition	clarification method	av HMF (mg/100 mL of soln) at time[b] 0 h	6 h	24 h	48 h
H1.1	light/rt	Carrez	0.145 ± 0.004	0.146 ± 0.001	0.160 ± 0.000	0.151 ± 0.002
H1.2	dark/refrig	Carrez	0.148 ± 0.005	0.149 ± 0.006	0.166 ± 0.004	0.160 ± 0.004
H1.3	light/rt	filtration	0.043 ± 0.004	0.058 ± 0.004	0.072 ± 0.003	0.026 ± 0.000
H1.4	dark/refrig	filtration	0.047 ± 0.001	0.059 ± 0.004	0.073 ± 0.007	0.093 ± 0.011
H2.1	light/rt	Carrez	0.161 ± 0.004	0.174 ± 0.001	0.170 ± 0.000	0.137 ± 0.008
H2.2	dark/refrig	Carrez	0.160 ± 0.004	0.169 ± 0.002	0.163 ± 0.004	0.161 ± 0.001
H2.3	light/rt	filtration	0.157 ± 0.004	0.160 ± 0.002	0.160 ± 0.001	0.098 ± 0.004
H2.4	dark/refrig	filtration	0.163 ± 0.001	0.163 ± 0.002	0.161 ± 0.003	0.160 ± 0.001
H3.1	light/rt	Carrez	0.206 ± 0.002	0.210 ± 0.004	0.213 ± 0.004	0.197 ± 0.024
H3.2	dark/refrig	Carrez	0.202 ± 0.001	0.205 ± 0.001	0.207 ± 0.001	0.216 ± 0.004
H3.3	light/rt	filtration	0.154 ± 0.003	0.159 ± 0.002	0.169 ± 0.004	0.170 ± 0.006
H3.4	dark/refrig	filtration	0.152 ± 0.000	0.157 ± 0.000	0.167 ± 0.001	0.173 ± 0.004
H4.1	light/rt	Carrez	0.218 ± 0.004	0.220 ± 0.006	0.226 ± 0.001	0.088 ± 0.023
H4.2	dark/refrig	Carrez	0.215 ± 0.003	0.217 ± 0.000	0.222 ± 0.001	0.224 ± 0.002
H4.3	light/rt	filtration	0.209 ± 0.004	0.220 ± 0.001	0.188 ± 0.018	0.066 ± 0.011
H4.4	dark/refrig	filtration	0.212 ± 0.002	0.222 ± 0.004	0.220 ± 0.004	0.220 ± 0.001
H4.A[c]	light/rt	Carrez	0.371 ± 0.003	0.351 ± 0.007	0.383 ± 0.005	0.381 ± 0.009
H5.1	light/rt	Carrez	0.230 ± 0.002	0.238 ± 0.004	0.244 ± 0.005	0.246 ± 0.005
H5.2	dark/refrig	Carrez	0.228 ± 0.001	0.229 ± 0.001	0.235 ± 0.001	0.235 ± 0.005
H5.3	light/rt	filtration	0.176 ± 0.003	0.187 ± 0.005	0.183 ± 0.006	0.042 ± 0.018
H5.4	dark/refrig	filtration	0.177 ± 0.000	0.189 ± 0.004	0.199 ± 0.004	0.195 ± 0.005
H6.1	light/rt	Carrez	0.224 ± 0.001	0.224 ± 0.002	0.233 ± 0.002	0.088 ± 0.001
H6.2	dark/refrig	Carrez	0.226 ± 0.001	0.218 ± 0.001	0.224 ± 0.000	0.230 ± 0.002
H6.3	light/rt	filtration	0.202 ± 0.004	0.217 ± 0.004	0.219 ± 0.006	0.068 ± 0.011
H6.4	dark/refrig	filtration	0.200 ± 0.000	0.205 ± 0.002	0.209 ± 0.002	0.211 ± 0.001
H7.1	light/rt	Carrez	0.327 ± 0.003	0.333 ± 0.004	0.343 ± 0.001	0.345 ± 0.016
H7.2	dark/refrig	Carrez	0.326 ± 0.004	0.328 ± 0.002	0.336 ± 0.003	0.346 ± 0.000
H7.3	light/rt	filtration	0.278 ± 0.003	0.294 ± 0.004	0.303 ± 0.004	0.191 ± 0.006
H7.4	dark/refrig	filtration	0.290 ± 0.002	0.291 ± 0.004	0.304 ± 0.001	0.293 ± 0.003
H8.1	light/rt	Carrez	0.414 ± 0.008	0.408 ± 0.004	0.414 ± 0.004	0.231 ± 0.027
H8.2	dark/refrig	Carrez	0.410 ± 0.004	0.404 ± 0.004	0.412 ± 0.001	0.399 ± 0.005
H8.3	light/rt	filtration	0.328 ± 0.001	0.342 ± 0.006	0.331 ± 0.007	0.181 ± 0.037
H8.4	dark/refrig	filtration	0.334 ± 0.006	0.336 ± 0.001	0.341 ± 0.009	0.355 ± 0.005
H1−2.1[d]	light/rt	Carrez	0.153 ± 0.010	0.160 ± 0.016	0.165 ± 0.006	0.144 ± 0.009
H1−2.3[d]	light/rt	filtration	0.100 ± 0.066	0.109 ± 0.059	0.116 ± 0.051	0.062 ± 0.041
H3−6.1[d]	light/rt	Carrez	0.219 ± 0.010	0.223 ± 0.014	0.229 ± 0.012	0.155 ± 0.075
H3−6.3[d]	light/rt	filtration	0.185 ± 0.023	0.195 ± 0.027	0.189 ± 0.021	0.086 ± 0.054
H7−8.1[d]	light/rt	Carrez	0.370 ± 0.056	0.370 ± 0.044	0.378 ± 0.041	0.288 ± 0.063
H7−8.3[d]	light/rt	filtration	0.303 ± 0.029	0.318 ± 0.028	0.317 ± 0.017	0.186 ± 0.022
H1−2.1,3[e]	light/rt		0.126 ± 0.052	0.134 ± 0.048	0.140 ± 0.042	0.103 ± 0.052
H3−6.1,3[e]	light/rt		0.202 ± 0.025	0.209 ± 0.024	0.209 ± 0.026	0.120 ± 0.072
H7−8.1,3[e]	light/rt		0.337 ± 0.052	0.344 ± 0.044	0.347 ± 0.044	0.237 ± 0.072
H1−2.2,4[e]	dark/refrig		0.129 ± 0.052	0.135 ± 0.048	0.141 ± 0.042	0.143 ± 0.032
H3−6.2,4[e]	dark/refrig		0.201 ± 0.025	0.205 ± 0.022	0.210 ± 0.020	0.213 ± 0.020
H7−8.2,4[e]	dark/refrig		0.340 ± 0.047	0.340 ± 0.044	0.348 ± 0.043	0.348 ± 0.040

[a] Solutions H*n.m* were prepared as described under Materials and Methods from ∼5 g of H*n* honey samples (Table 1). [b] Storage time from starting measurement. HMF values are averaged over duplicate determinations and expressed as mg/100 mL of honey solution. 0.1 mg/100 mL corresponds to ca. 2 mg of HMF/100 g of honey. Error corresponds to one standard deviation ($\sigma n - 1$). [c] Solutions were prepared from H4 honey sample with 3.1 mg of pure HMF added and further homogenization. [d] Solutions stored at room temperature were grouped according to both starting HMF concentration (H1−2, H3−6, and H7−8) and clarification method (H*n*−*n*′.1 and H*n*−*n*′.3). Values are averaged over all the determinations of the group ($n \geq 4$). Error corresponds to one standard deviation ($\sigma n - 1$). [e] Solutions were grouped according to both starting HMF concentration (H1−2, H3−6, and H7−8) and storage condition (H*n*−*n*′.1,3 and H*n*−*n*′.2,4). Values are averaged over all the determinations of the group ($n \geq 8$). Error corresponds to one standard deviation ($\sigma n - 1$).

should be noticed, because additional HMF should be forming simultaneously with the decomposition product at 252 nm. Even though a significative amount of fresh HMF could be forming during the solution storage period, the decomposition rate is higher than the production rate; otherwise, the band at 284 nm should either remain almost constant or increase while the band at 252 nm increases. We have not observed this last fact in our experiments. So far, we reject the possibility that additional HMF, formed during the solution storage period, could produce the different behavior observed in honey solutions compared with standards.

We also thought of the differences in the solution preparation between honey and standards. The only difference was the use of the Carrez reagent to deproteinize as well as clarify honey solutions. Therefore, we carried out paired experiments with honey samples deproteinized−clarified with and without Carrez reagent as described under Materials and Methods. The concentration changes observed in solutions clarified without Carrez reagent have the same tendency as those observed for honey solutions treated according to the AOAC method (AOAC, 1995) (Table 3, Hn.1 versus Hn.3 and Hn.2 versus Hn.4). Furthermore, if we compare HMF concentration changes in honey solutions with similar starting concentrations (Table 3, H1-2, H3−6, and H7−8) clarified with or without Carrez and after 48 h of storage at room temperature, we can find a statistically significant difference ($P < 0.05$) only with the first group (Table 3, H1-2.1 versus H1−2.3). Therefore, we should discard the use of Carrez reagent as the main cause that led to avoidance or retardation of HMF decomposition.

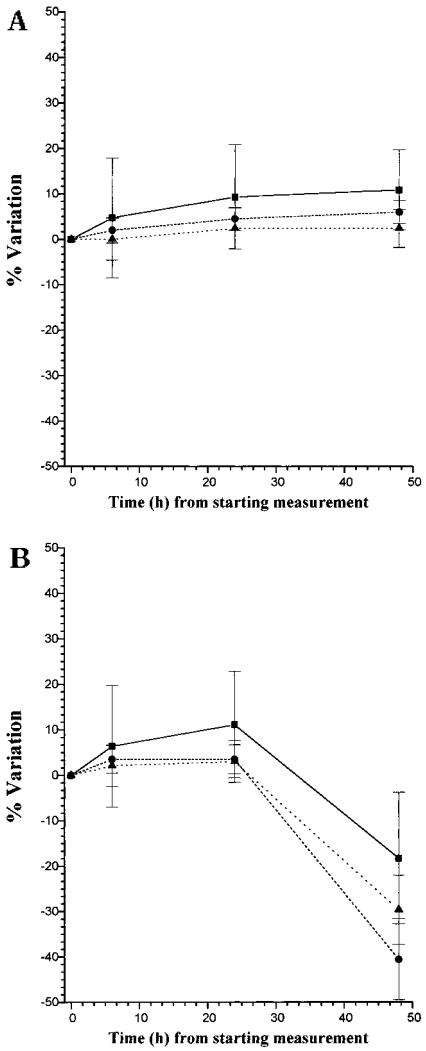

Figure 3. HMF variation as a function of concentration in honey solutions stored at different conditions: (A) stored refrigerated and in the dark; (B) stored at room temperature and in daylight; (■) lowest concentration (H1 and H2); (●) intermediate concentration (H3−H6); (▲) highest concentration (H7 and H8). Percentages are calculated from Table 3 by statistical mean difference (paired observations between 0−6, 0−24, and 0−48 h from starting measurement, $n \geq 4$, hypothesized diff = 0. Error bars correspond to the percentage calculated from one standard deviation of mean difference.

As we also observed lower starting HMF values in samples clarified by ultrafiltration (Table 3, Hn−n'.1 versus Hn−n'.3), we decided to check for statistical significance of the observed differences. We carried out a statistical mean comparison between samples grouped according to starting concentration, treated with or without the Carrez reagent (Table 3, Hn−n'.1 versus Hn−n'.3). We observed significant differences only in solutions of higher starting concentration. The group with the lowest HMF level (Table 3, H1 and H2) has an average starting concentration of 0.153 mg/100 mL (SD = 0.010) when Carrez reagent was used and 0.100 mg/100 mL (SD = 0.066) when clarified by ultrafiltration (n = 4; T = 1.58; P = 0.082). The group with intermediate HMF level (Table 3, H3−H6) has an average starting concentration of 0.219 mg/100 mL (SD = 0.009) when Carrez reagent was used and 0.185 mg/100 mL (SD = 0.023) when clarified by ultrafiltration (n = 8; T = 3.80; P = 0.001). Finally, the group with the highest HMF level (Table 3, H7 and H8) has an average starting concentration of 0.370 mg/100 mL (SD = 0.050) when Carrez reagent was used and 0.303 mg/100 mL (SD = 0.029) when clarified by ultrafiltration (n = 4; T = 2.32; P = 0.030). Therefore, the use of the

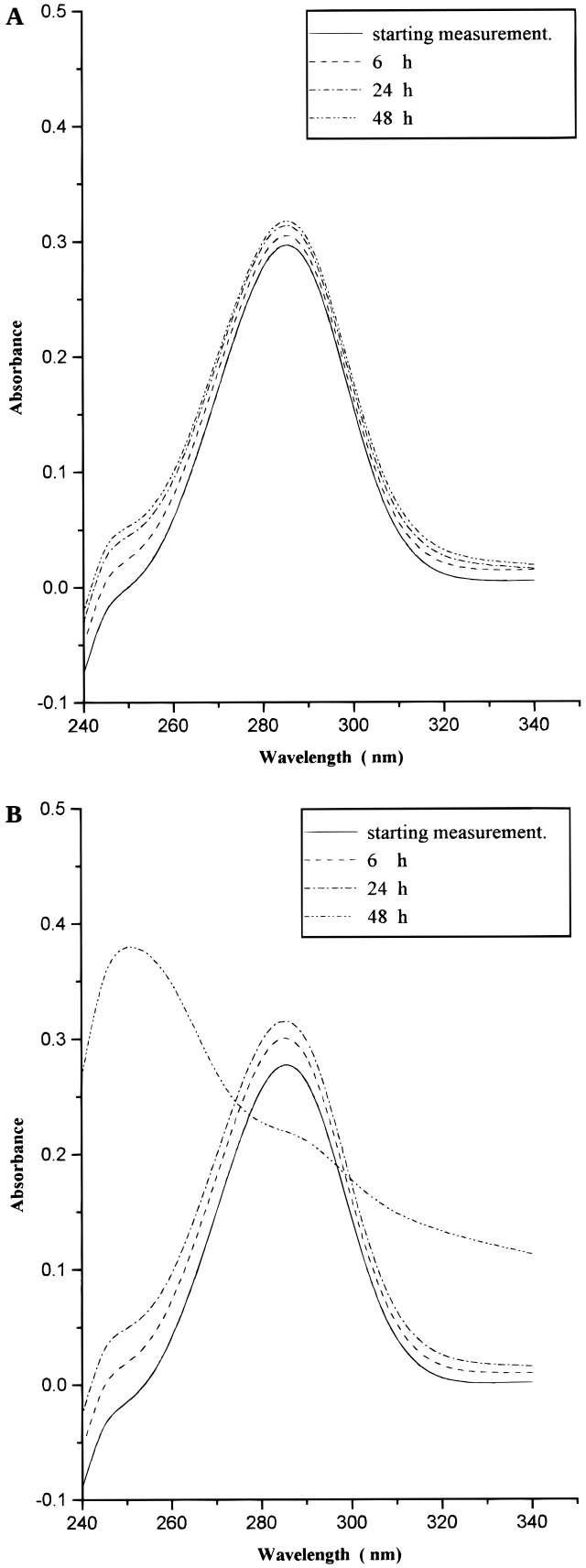

Figure 4. Spectral curve of honey solution: (A) stored refrigerated and in the dark; (B) stored at room temperature and in the daylight.

Table 4. HMF Concentration Variation in Syrup and Sugar Solutions Stored under Different Conditions

solution[a]	storage condition	av HMF (mg/100 mL) at time[b] 0 h	6 h	24 h	48 h
HFS.1	dark/refrig	0.048 ± 0.003	0.049 ± 0.002	0.054 ± 0.001	0.054 ± 0.001
HFS.2	light/rt	0.049 ± 0.001	0.052 ± 0.001	0.057 ± 0.001	0.054 ± 0.000
GS.1[c]	dark/refrig	0.078 ± 0.001	0.074 ± 0.000	0.072 ± 0.000	0.065 ± 0.003
GS.2[c]	light/rt	0.077 ± 0.000	0.070 ± 0.004	0.044 ± 0.001	0.000 ± 0.000
F.1	dark/refrig	0.016 ± 0.001	0.020 ± 0.001	0.020 ± 0.000	0.023 ± 0.001
F.2	light/rt	0.015 ± 0.001	0.016 ± 0.001	0.015 ± 0.001	0.012 ± 0.002
F15.2	light/rt	0.245 ± 0.003	0.240 ± 0.001	0.240 ± 0.000	0.237 ± 0.000
F30.1	dark/refrig	0.297 ± 0.001	0.298 ± 0.001	0.290 ± 0.003	0.279 ± 0.005
F30.2	light/rt	0.241 ± 0.004	0.241 ± 0.001	0.244 ± 0.000	0.211 ± 0.012
F60.2	light/rt	0.233 ± 0.002	0.236 ± 0.002	0.236 ± 0.002	0.226 ± 0.002
SAC30.1	dark/refrig	0.299 ± 0.003	0.299 ± 0.004	0.288 ± 0.004	0.270 ± 0.004
SAC30.2	light/rt	0.292 ± 0.004	0.286 ± 0.003	0.245 ± 0.004	0.017 ± 0.024
SAC31.2	light/rt	0.191 ± 0.004	0.174 ± 0.004	0.000 ± 0.000	0.000 ± 0.000
GLU30.1	dark/refrig	0.297 ± 0.001	0.297 ± 0.004	0.292 ± 0.004	0.281 ± 0.005
GLU30.2	light/rt	0.297 ± 0.000	0.293 ± 0.001	0.258 ± 0.001	0.062 ± 0.050
GLU31.2	light/rt	0.194 ± 0.004	0.175 ± 0.002	0.008 ± 0.002	0.000 ± 0.000
MAL30.1	dark/refrig	0.321 ± 0.002	0.319 ± 0.002	0.319 ± 0.001	0.319 ± 0.004
MAL30.2	light/rt	0.323 ± 0.004	0.314 ± 0.002	0.288 ± 0.006	0.000 ± 0.000
LAC30.1	dark/refrig	0.299 ± 0.002	0.292 ± 0.002	0.287 ± 0.002	0.276 ± 0.003
LAC30.2	light/rt	0.299 ± 0.002	0.293 ± 0.002	0.252 ± 0.002	0.000 ± 0.000

[a] HFS, high-fructose syrup; GS, glucose syrup; F, fructose solutions (F = 0.05%, F15 = 1.5%, F30 = 3%, F60 = 6%); GLU, glucose solutions (3%); SAC, saccharose solutions (3%); MAL, maltose solutions (3%); LAC, lactose solutions (3%). [b] Storage time from starting measurement. HMF values are averaged over one determination on two different solutions with almost the same starting concentration ($n = 2$). Error corresponds to one standard deviation ($\sigma n - 1$). [c] Difference with HFS starting concentration corresponds to HMF present in glucose syrup as determined from controls without added HMF.

Carrez reagent could lead to the prediction of higher HMF values than those really present in honey. To get more evidence on the differences between Carrez and ultrafiltration, we performed a new recovery assay. We added ~5 mg of pure HMF to 100 g of an overstored honey sample. We then determined HMF using both deproteinization methods on the natural honey as well as on the sample supplemented with HMF. We also decided to check the use of 1 mL of Carrez A and 1 mL of Carrez B, which is twice the amount described by the AOAC method (AOAC, 1995). Duplicate solutions coming from the natural honey sample showed 0.469 ± 0.002 mg of HMF/100 mL (9.38 mg of HMF/100 g of honey) when determined according to the AOAC method (AOAC, 1995). The same sample produced solutions with 0.434 ± 0.006 mg of HMF/100 mL (8.68 mg of HMF/100 g of honey) when the Carrez amount was duplicated; finally, it showed 0.435 ± 0.001 mg of HMF/100 mL (8.70 mg of HMF/100 g of honey) when prepared by ultrafiltration. Recoveries obtained from the supplemented sample, using both deproteinization methods, were always >95%. Considering this last result, the difference between Carrez and ultrafiltration, which produces higher HMF values, could be attributed to the presence of some amount of proteins with aromatic groups. Such proteins, when not removed, give a contribution to the band at 284 nm and lead to calculation of higher HMF values than those really present. As the absorbance due to such proteins remains constant in both natural honey solutions and supplemented honey solutions, almost quantitative recoveries are expected and observed with both methods. Therefore, we recommend the use of either ultrafiltration or 1 mL of Carrez A and 1 mL of Carrez B instead of the 0.5 mL described in the original technique (AOAC, 1995; White, 1979).

As we have discarded the protective effect of the Carrez reagent, we should now look for other differences between standards and honey solutions that produce the different spectrophotometric behavior. There are at least two important differences: sugars as well as enzymes and other minor components present in honey. We decided to use both glucose and high-fructose corn syrups containing sugars in similar proportions as honey (especially high-fructose syrup). Syrup solutions were supplemented with pure HMF, to simulate synthetic honey solutions, and studied in the same way as honey solutions (Table 4). Also, fructose (at different concentrations), glucose, saccharose, maltose, and lactose solutions, supplemented with pure HMF, were

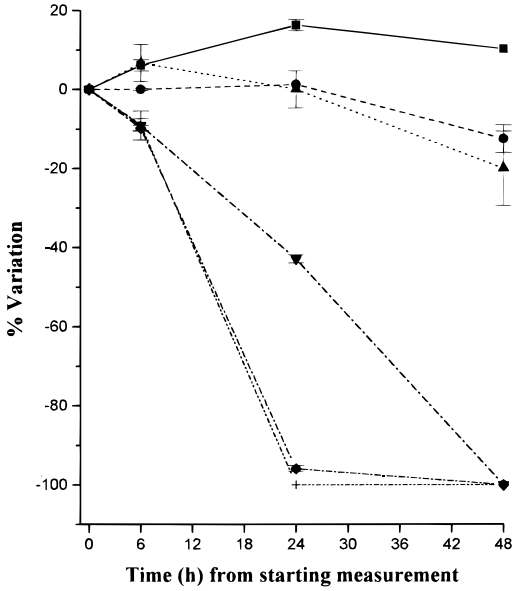

Figure 5. HMF variation in syrup and sugar solutions: (■) high-fructose syrup solution (HFS.2); (●) fructose solution (F30.2); (▲) fructose solution (F.2); (▼) glucose syrup solution (GS.2); (◆) glucose solution (GLU31.2); (+) saccharose solution (SAC31.2). Percentages are calculated from Table 4 by statistical mean difference (paired observations at 0−6, 0−24, and 0−48 h, $n = 2$, hypothesized diff = 0). Error bars correspond to the percentage calculated from one standard error of mean difference.

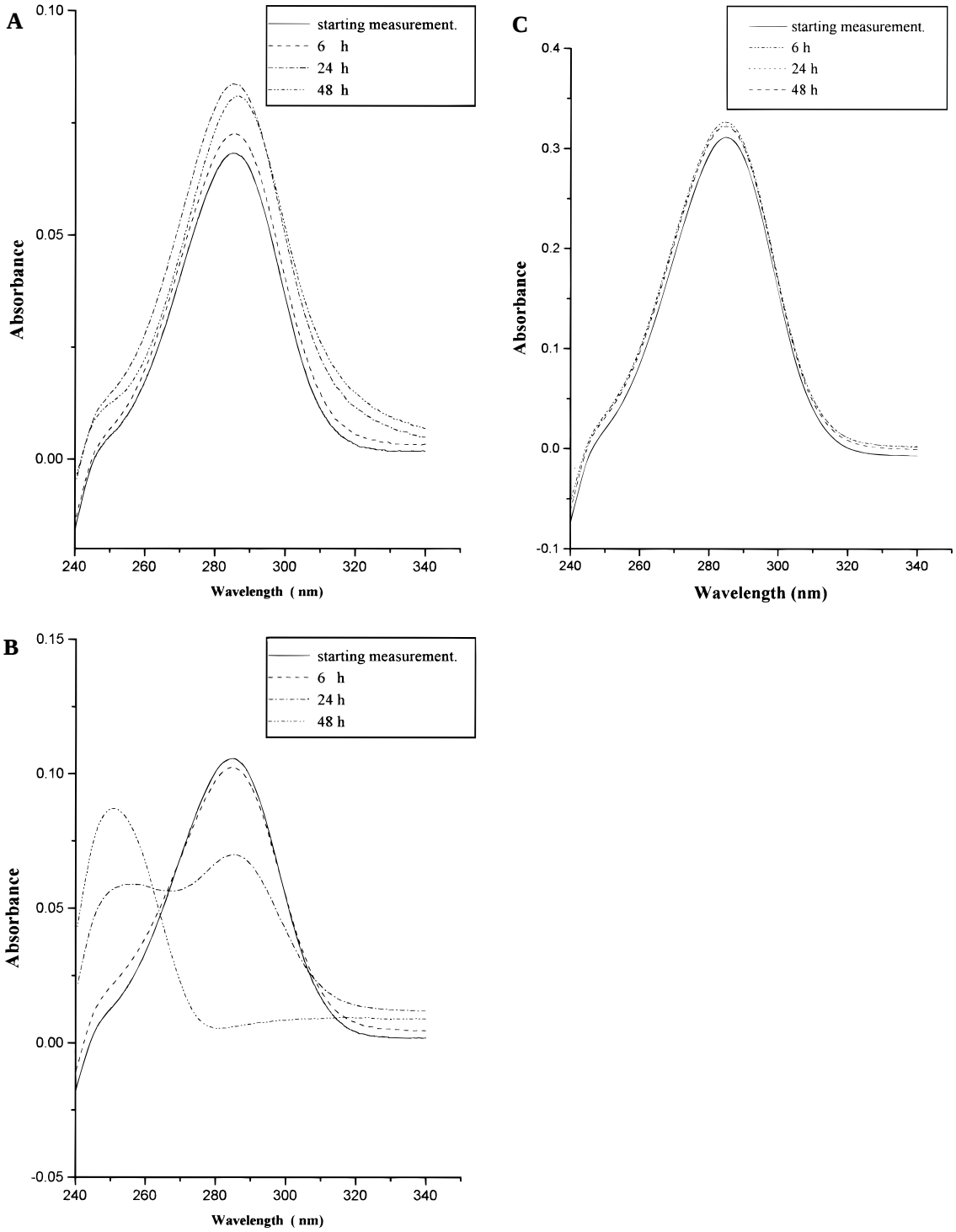

Figure 6. Spectral curve of syrups and fructose solutions: (A) high-fructose syrup; (B) glucose syrup; (C) fructose.

studied to evaluate the incidence of individual sugars, which could be present in honey solutions, on the spectrophotometric behavior. Sugar solutions were prepared at 3% (w/v), which is close to the concentration of glucose and fructose coming from 5 g of honey prepared to measure HMF according to the AOAC method (AOAC, 1995). Fructose solutions were also prepared at different concentrations (0.05, 1.5, and 6%) to evaluate the probable incidence of fructose amount on HMF stability. Sugar solutions were studied in the same way as honey solutions (Table 4 and Figure 5). Spectral curves for syrups and fructose solutions stored at room temperature are shown in Figure 6. From Figures 5 and 6B it can be observed that, when glucose is the only sugar present, HMF decomposes as observed for standard solutions (Figure 2B). From Table 4 and Figure 5 we can see that both syrup and sugar solutions without fructose show significant HMF degradation, particularly those stored at room temperature. However, only small changes in both concentration and spectral curve shape are observed in solutions containing fructose (Table 4; Figures 5 and 6A,C). Besides, fructose shows a protective effect used at different concentrations (Table 4, F15, F30 and F60), even at extremely low starting HMF concentrations (Table 4, F.1 and F.2). We also noted that the biggest changes were observed in solutions stored at room temperature compared with those kept at 4−8 °C. Furthermore,

sugar solutions only have HMF, sugar, and water, so a protective effect from amino acids, proteins, or any other component should be discarded. Despite the incidence of storage temperature, a protective effect of fructose was the only reason we were able to find to explain the different spectrophotometric behaviors of HMF in standards and honey solutions. We have to point out that the accumulation of HMF in sugar-containing foodstuffs can be affected by many other factors, so results obtained with HMF in solution cannot be directly applied to such foodstuffs. However, the presence of fructose should be considered as a factor that could modify the HMF accumulation in foodstuffs in addition to other parameters such as pH, temperature, and moisture.

CONCLUSIONS

HMF decomposes in water solutions, especially when they are stored at room temperature. The decomposition could lead to an important concentration drop when solutions are not measured immediately after preparation. Therefore, it is recommended that HMF measurements be made within 6 h after sample preparation with the solutions kept in the dark at 4−8 °C until analysis. To avoid incorrect HMF reports, it is also advised to verify the presence of a spectral band at 284 nm previous to the analysis.

The lower the starting HMF concentration, the higher the concentration decay. Also, the storage temperature plays an important role; thus, the greater changes are observed in solutions stored at room temperature compared with those kept at 4−8 °C. No important differences were observed in solutions stored at room temperature with or without daylight.

The biggest decomposition was observed in syrup and sugar solutions without fructose, whereas in solutions containing fructose the HMF concentration changes were significantly lower. Therefore, we suggest that fructose has a protective effect which prevents or delays HMF decomposition in solution.

The statistically significantly lower HMF starting concentrations found in honey solutions clarified without the use of Carrez reagent, compared with those clarified with such reagent, suggest the need to ensure protein removal previous to the measurement. We recommend the use of either ultrafiltration or 1 mL of Carrez A and 1 mL of Carrez B instead of the 0.5 mL described in the original technique.

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LITERATURE CITED

Anam, O. O.; Dart, R. K. Influence of Metal Ions on Hydroxymethylfurfural Formation in Honey. *Anal. Proc.* **1995**, *32*, 515−517.

AOAC. *Official Methods of Analysis*, 16th ed.; Association of Official Analytical Chemists: Arlington, VA, 1995; Sections 960.44, 980-23.

Assil, H. I.; Sterling, R.; Sporns, P. Crystal Control in Processed Liquid Honey. *J. Food Sci.* **1991**, *56*, 1034−1041.

Corradini, C.; Corradini, D. Determination of 5-Hydroxymethyl-2-furaldehyde in honey by micellar electrokinetic capillary chromatography. *J. Microcolumn Sep.* **1994**, *6*, 19−22.

Durán Meras, I.; Espinosa Mansilla, A.; Salinas Lopez, F. Simultaneous kinetic spectrophotometric determination of 2-furaldehyde and 5-hydroxymethyl-s-furaldehyde by application of a modified Winkler's method and partial least squares calibration. *Analyst (Cambridge U.K.)* **1995**, *120*, 2567−2571.

Dustmann, J. H. Honey, Quality and its Control. *Am. Bee J.* **1993**, *133*, 648−651.

Espinoza Mansilla, A.; Muñoz de la Peña, A.; Salinas, F. Semiautomatic determination of furanic aldehydes in food and pharmaceutical samples by a stopped-flow injection analysis method. *J. AOAC Int.* **1993**, *76*, 1255−61.

Faría, J. A. F. Shelf-life testing on honey. *Cienc. Tecnol. Aliment.* **1993**, *13*, 58−66.

Lo Coco, F.; Valentini, C.; Novelli, V.; Ceccon, L. High-perfomance liquid chromatographic determination of 2-furaldehyde and 5-hydroxymethyl-2-furaldehyde in honey. *J. Chromatogr. A* **1996**, *749*, 95−102.

Merck Index, 11th ed.; Merck & Co. Inc.: Rahway, NJ, 1989; p 4765.

Pérez, C.; Conchello, P.; Ariño, A.; Ucar, A.; Herrera, A. Evaluation of the freshness grade in honey samples from Zaragoza. *Anal. Bromatol.* **1990**, *XLII-1*, 99−105.

Rittgerodt, B. Furfural and hydroxymethylfurfural determination in foods. *GIT Spez. Chromatogr.* **1994**, *32*, 14, 79−80.

Sancho, M. T.; Muniategui, S.; Huidobro, J. F.; Simal Lozano, J. Aging of Honey. *J. Agric. Food Chem.* **1992**, *40*, 134−138.

Thrasyvoulou, A.; Manikis, J.; Tselios, D. Liquefying crystallized honey with ultrasonic waves. *Apidologie* **1994**, *25*, 297−302.

USP (The United States Pharmacopeia). *USP23 − NF 18*; United States Pharmacopeia Convention, Inc.: Rockville, MD, 1995; pp 483−484.

White, J. W., Jr. Quality evaluation of honey: Role of HMF and diastase assays. *Am. Bee J.* **1992**, *132*, 737−743 and 792−794.

White, J. W., Jr.; Kushnir, I.; Doner, L. W. Charcoal column/thin layer chromatographic method for high fructose corn syrup and spectrophotometric method for hydroxymethylfurfural in honey: Collaborative studies. *J. AOAC Int.* **1979**, *62*, 921−927.

Yilmaz, H.; Küfrevioglu, Ö. I. The effect of temperature on diastase activity and HMF content of honey. *J. Fac. Sci., Ege Univ., Ser. A* **1994**, *17*, 39−43.

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