4-Hydroxy-2,5-dimethyl-3(2*H*)-furanone Formation in Buffers and Model Solutions of Citrus Juice

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4-Hydroxy-2,5-dimethyl-3(2*H*)-furanone (DMHF, furaneol) is formed in stored citrus juice and has been reported to reduce juice quality. The source and pathways by which DMHF is formed in citrus products have not been delineated. In model orange juice solutions, DMHF was formed only when rhamnose (6-deoxyhexose) was present. Arginine was the major amino acid to react with rhamnose via the Maillard reaction. In buffer solutions, the formation of DMHF was affected by pH, temperature, and time of storage. Under acidic conditions, DMHF is formed only from arginine and rhamnose, whereas at pH 6−8, DMHF was also formed (as verified by GC−MS) from glucose and fructose, albeit in smaller amounts. The formation of DMHF from hexoses required a reducing step, which was apparently contributed by Maillard-producing reductones. It is concluded that the accumulation of DMHF to above taste-threshold levels, which occurs under the acidic conditions of citrus juices, is related, at least in part, to the reportedly small amounts of rhamnose interacting with arginine via the Maillard reaction.

Keywords: *Furaneol; 4-hydroxy-2,5-dimethyl-3(2H)-furanone (DMHF); rhamnose; Maillard; citrus; model solutions*

INTRODUCTION

Furanones, produced mostly via Maillard reactions, generally impart caramel-like, sweet, fruity flavors. 4-Hydroxy-2,5-dimethyl-3(2*H*)-furanone (DMHF, furaneol) is an important flavor compound having a caramel-like burnt-pineapple flavor, although at low concentrations its contribution is described as a strawberry-like note (Pickenhagen et al., 1981).

DMHF occurs naturally in various fruits, such as pineapple (Rodin et al., 1965), mango (Wilson et al., 1988), strawberry (Pickenhagen et al., 1981), and others (Honkanen et al., 1980; Kallio, 1976). Glucosidically bound DMHF has been reported in strawberry (Mayerl et al., 1989), pineapple (Wu et al., 1991), raspberry (Pabst et al., 1991), and tomato (Krammer et al., 1994). Due to its low odor threshold, it seems to be one of the character-impact compounds in many foods and it is used for flavoring.

DMHF is also found in processed foods and beverages such as pineapple and apple juices (Pisarnitskii et al., 1992), beef broth (Tonsbeek et al., 1968), and roasted almonds (Tei and Yamanishi, 1974). It is assumed that DMHF is formed in heat-processed foods containing hexoses (Schieberle, 1992). In processed fruit juices, its presence is proportional to the content of methylpentoses, suggesting that it is a product of a carbonyl amine interaction during the final stage of ripening (Pisarnitskii et al., 1992), or during thermal processing. DMHF has been reported to be produced during base-catalyzed fructose degradation (Shaw et al., 1968) and at pH 3.5 from rhamnose and alanine (Shaw and Berry, 1977). It has been identified by GC−MS and GC−MS/MS in Maillard-reaction systems employing pentoses (Blank and Fay, 1996) and is also formed from some 6-deoxy sugars and amino acids (Doornbos and Ouweland, 1982).

DMHF, along with PVG (4-vinylguaiacol) and α-terpineol, has been proposed to be responsible for the objectionable flavor in aged canned orange juice (Tatum et al., 1975). It was found to mask the aroma of orange juice with a detection threshold of 0.1 ppm (Tatum et al., 1975). Although UV−HPLC analyses have suggested that DMHF is accumulated during the storage of grapefruit (Lee and Nagy, 1987) and orange (Naim et al., 1993) juices, no direct evidence of its formation in citrus products by the Maillard mechanisms has been provided. The addition of low concentrations of L-cysteine reduced DMHF formation in commercial orange juice stored under accelerated conditions (Naim et al., 1993).

One may assume that the presence of DMHF in stored citrus products is due to sugar−amine reactions occurring during processing and storage, or to a release of DMHF from putative DMHF glycosides, as found in other fruits. The present study was designed to identify sugars and amino acids that may, due to the sugar−amine interaction, account for the increase in DMHF content in stored grapefruit and orange juices.

We verified the presence of rhamnose in orange and grapefruit juices as the likely sugar precursor for DMHF formation and investigated the latter's formation in model and buffer solutions under various conditions.

MATERIALS AND METHODS

Materials. DMHF, (hydroxymethyl)furfural (HMF), furfural, methylfurfural (MF), rhamnose, glucose, fructose, arginine, γ-aminobutyric acid (GABA), serine, aspartic acid, proline, hexamethyldisilazane, and trimethylchlorosilane were purchased from Sigma (St. Louis, MO). Commercial single-strength orange and grapefruit juices were purchased from "Rimon" (Kibbutz Givaat-Brenner, Israel).

Preparation of Orange and Grapefruit Juices. Rhamnose levels were determined in fresh, hand-squeezed orange

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and grapefruit juices and in commercial orange and grapefruit juices purchased at the local market. In addition, rhamnose level was determined in commercial orange juice stored for 3 months at 35 °C. DMHF was identified in commercial orange and grapefruit juices preserved with 500 ppm sodium benzoate and stored in 20-mL closed brown bottles for 48 h at 70 °C. Control samples were stored at 4 °C.

Preparation of Model and Buffer Solutions. Model orange juice solutions (MOJ) were prepared according to Peleg et al. (1992) with slight modifications. MOJ (100 g) fortified with 2.0 g of rhamnose contained (% w/w) 3.9 sucrose, 1.8 glucose, 1.8 fructose, 1 citric acid, 0.1 malic acid, 0.5 tripotassium citrate, 0.5 proline, 0.5 arginine, 0.2 GABA, and 0.1 aspartic acid in double distilled water. MOJ without rhamnose, but with the same total sugar concentration, was used as a control and contained (% w/w) 4.9 sucrose, 2.3 glucose, and 2.3 fructose.

In one experiment, the MOJ was modified by eliminating the amino acids and then fortified with 0.5% (w/v) of either arginine, GABA, proline, serine, or aspartic acid. Solutions were stored for 48 h at 70 °C.

The buffer solutions were either citrate phosphate (0.1 M, pH range 2.5−6) or phosphate (0.1 M, pH range 7−8). Buffer solutions (pH 3.5) were fortified with rhamnose and arginine (40 mM each) and stored for 1, 4, and 7 days at either 60, 70, 80, or 90 °C.

To investigate the effect of pH on DMHF formation, buffer solutions containing 55 mM rhamnose, fructose, or glucose were stored with or without arginine (55 mM) at 70 °C for 48 h, at different pHs (2.5−8). To investigate the effect of reductones on DMHF formation, ascorbic acid (0.1%, w/v) was added to buffer solutions (adjusted to pH 3.5, 6, 7, or 8) containing 50 mM glucose or rhamnose and 50 mM arginine. These solutions were then stored for 48 h at 70 °C.

Chemical Analyses. *Citrus Juice.* Fresh and stored citrus juice samples (10 mL) were filtered and centrifuged (15000*g*, 4 °C, 15 min). The resultant supernatant (1 mL) was added to a 5-mL vial and lyophilized to complete dryness. Rhamnose content was determined in the dried juice by GC of the trimethylsilyl (TMS) derivatives (Sweely et al., 1963). TMS reagent was prepared by mixing 1 volume of trimethylchlorosilane, 3 volumes of hexamethyldisilazane, and 9 volumes of pyridine. TMS reagent (1 mL) was added, and the mixture was heated in a closed vial for 30 min at 70 °C. Samples were then transferred to a 1-mL Eppendorf tube and centrifuged (5 min, 8740*g*). The supernatant (1 mL) was injected into a gas chromatograph (Hewlett-Packard Model 6890) equipped with a flame ionization detector and a 60-m fused silica nonpolar capillary column loaded with cross-linked 5% phenylmethyl silicone (film thickness 1.0 μm, 0.32 mm i.d., Rtx-5, Restek Corp, Bellefonte, PA). Nitrogen was used as the carrier gas. Conditions for the run were as follows: split ratio 1:10; flow rate 1.5 mL/min; injector temperature 280 °C; detector temperature 325 °C. The oven program was set for 150 °C (3 min) at 7 °C/min to 290 °C (20 min). Recovery of rhamnose was 40%. Authentic TMS-rhamnose samples prepared as described above were used for identification and the calibration curve.

DMHF was extracted from orange and grapefruit juices according to Walsh et al. (1997). Juice samples were centrifuged (15000*g*, 4 °C, 15 min), and 4 mL of the supernatant was applied to C18 Sep-Pak cartridges (Waters Assoc., Milford, MA) which had been preconditioned with methanol (2 mL) and water (5 mL). Cartridges were then washed with water (1.5 mL) and eluted with methanol (1.5 mL). DMHF was identified by injecting 1 μL of the extract into a GC−MS (Finnigan MAT 4600) equipped with the aforementioned Rtx-5 column. Helium was used as the carrier gas. The oven program was set for 70 °C (1 min) at 6 °C/min to 200 °C and 15 °C/min to 250 °C; the injector temperature was 200 °C, split ratio 1:50 opened for 1 min. The ion source worked in electron impact mode at 70 eV. The MID was set at 50−500 or, for higher sensitivity in the juice samples, searching only for the relevant masses: 128, 85, and 57.

MOJ and Buffer Solutions. DMHF, HMF, and MF were extracted according to Lee and Nagy (1987) with slight modifications. Each solution (2 mL) was passed through a prewashed (2 mL of methanol and 5 mL of water) C18 Sep-Pak cartridge. Each cartridge was then washed with 0.5 mL of hexane and eluted three times with 3 mL of ethyl acetate. Residual water was removed by adding anhydrous sodium sulfate and the eluant was then concentrated by nitrogen flow to 1 mL. Each sample was filtered through a 0.45-μm filter (acrodisc CR PTFE, Gelman Sciences, Ann Arbor, MI), and 20 μL of the filtrate was injected to HPLC or 1 μL to GC−MS.

DMHF, HMF, and MF were analyzed by HPLC equipped with a Lichrospher 100 RP-18 column (5 μm, 250 mm, 4 mm, Merck) with an RP-18 precolumn (25 × 4 mm) (Naim et al., 1993). The mobile phase consisted of (A) 1.5% (v/v) acetic acid in water and (B) 50% (v/v) methanol, 50% (v/v) acetonitrile. The chromatographic conditions were as follows: 0−16 min gradient 20−40% A, 16−20 min 40−20% A; flow rate 0.5 mL/min. Twenty microliters of sample was injected. Peaks were identified and quantitated with known markers using a chromoscope UV−visible rapid-scanning detector set at 290 nm (Barspec, Rehovot, Israel). DMHF (extracted from a pH 8.0 buffer solution) was also identified by GC−MS, as described. Browning was determined in buffer solutions by measuring absorbance at 420 nm.

Data Analyses. Results of the chemical analyses were tested by one- or two-way analyses of variance of log DMHF data using a SAS statistical program. Tukey's studentized range test or paired *t*-test was used for comparisons among the means.

Table 1. Rhamnose Content in Different Juice Samples

juice type	rhamnose (mg/L)[a]
fresh orange juice (hand-squeezed)	0.77 ± 0.3
fresh grapefruit juice (hand-squeezed)	0.93 ± 0.3
commercial orange juice	2.28 ± 0.4
commercial grapefruit juice	3.30 ± 0.8
commercial orange juice stored for 3 months at 4 °C	2.05 ± 0.5
commercial orange juice stored for 3 months at 35 °C	2.49 ± 0.1

[a] Values adjusted to 11 °Brix are the mean ± SEM of two or three samples, each analyzed three times by GC.

RESULTS AND DISCUSSION

DMHF and Rhamnose in Citrus Juice. DMHF is considered to be one of the major off-flavors in stored citrus products, having a very low detection threshold (0.1 ppm) (Tatum et al., 1975; Handwerk and Coleman, 1988). Its content in orange and grapefruit juices has been recorded (Lee and Nagy, 1987; Naim et al., 1993) and is known to be high especially at storage temperatures above 25 °C. Temperature appears to be a more critical factor for DMHF formation than storage time (Walsh et al., 1997). The presence of DMHF in stored orange and grapefruit juices was verified in the present experiments by GC−MS analyses which indicated the expected (Blank and Fay, 1996; Rodin et al., 1965) fragments: 128 (M^+), 85 ($M - CH_3OH$), and 57($M - CH_3OH - CO$) at a 0.94:0.33:1 ratio, respectively. Rhamnose, which is a sugar precursor for DMHF production under acidic conditions (Shaw and Berry, 1977), is present in small amounts in citrus juice (Stepak and Lifshitz, 1971; Lanza et al., 1991), but nevertheless was found to be a building block for glycosides and polysaccharides in orange juice (Kauschus and Thier, 1985). Quantification of rhamnose in orange and grapefruit juices is being reported (Table 1). Presumably the galacturonic acid and rhamnose found in citrus juice result from the enzymic degradation of pectin which occurs during processing and storage. Apparently, the enzymic release (e.g., via naringinase) of rhamnose from the flavonoids naringin (grapefruit) or hesperidin (orange) may contribute ad-

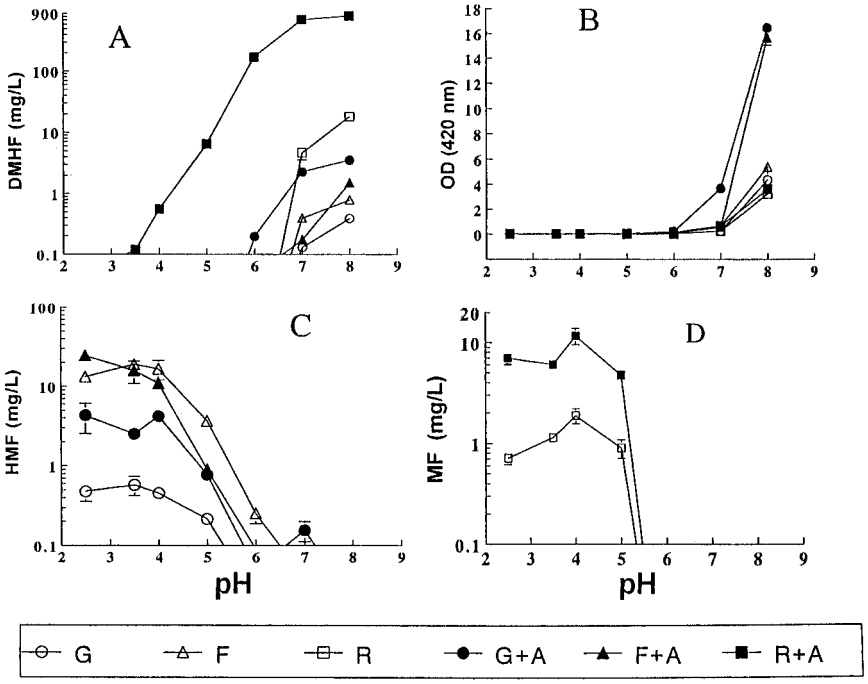

Figure 1. Effects of pH on DMHF formation (A), browning (B), HMF formation from glucose and fructose (C), and MF formation from rhamnose (D) in buffer solutions. Values for DMHF, HMF, and MF are the means ± SEM of two samples, each analyzed twice by HPLC. Values for optical density (OD) are the means and SEM of two samples. When SEM are not given, they were too small to be presented. Legends: rhamnose (R), glucose (G), fructose (F), and arginine (A).

ditional free rhamnose (Romero et al., 1985). Although overall rhamnose content is low, it is higher in commercial juices than in fresh juice (Table 1), probably due to processing and pasteurization of the former. After storage, rhamnose content does not change significantly. Glycosidically bound DMHF, which might be formed with reducing sugars if present in significant concentrations, has not been reported in citrus fruit and is unlikely to be present in citrus juice. No release of free DMHF was identified in a preliminary experiment applying β-glucosidase to grapefruit juice extract following removal of free sugars and volatiles (Amberlite XAD-2). Since only minor amounts of a bound form, if any, would be expected to be present, further investigation is required to verify this question. If present in citrus juice, it may also contribute to the free DMHF in processed products.

DMHF Formation in MOJ Solutions. Although the main sugars in citrus juices are sucrose, fructose, and glucose, small amounts of other sugars, such as rhamnose, xylose, trehalose, mannose, ribose, and arabinose, have been reported (Stepak and Lifshitz, 1971; Lanza et al., 1991). DMHF was not formed in stored acidic MOJ containing the main sugars and amino acids but lacking rhamnose. The addition of rhamnose to MOJ resulted in 6.57 ± 0.42 ppm DMHF after storage for 24 h at 70 °C, 19.5 ± 2.2 after 48 h at 70 °C, and only 0.1 ± 0.01 after 2 weeks at 45 °C. Experiments employing modified MOJ solutions in which amino acids were added separately showed arginine and GABA to be the most reactive in terms of DMHF formation (Table 2). Stored MOJ solutions containing aspartic acid or serine did not contain DMHF, and only traces of DMHF were found when proline was present. The fact that the basic amino acids arginine and GABA, which are abundant in citrus products (Rouseff and Ting, 1986), were very reactive is thus relevant to DMHF formation in citrus juice during storage. The small rhamnose concentrations in citrus juices reported elsewhere (Stepak and Lifshitz, 1971; Lanza et al., 1991) and the range of 2−3 ppm found in commercial juice in this study may be sufficient for the formation of the above-taste-threshold level (0.1 ppm) of DMHF occurring in citrus juices stored for long periods at temperatures above 25 °C (Lee and Nagy, 1987; Naim et al., 1993).

Table 2. Effect of Amino Acids on DMHF Formation in MOJ Solutions Lacking Amino Acids and Fortified with Rhamnose (2%, w/v)

amino acid (0.5%, w/v)	DMHF (mg/L)[a]
arginine	0.270 ± 0.04[a]
proline	0.017 ± 0.005[c]
GABA	0.060 ± 0.008[b]
aspartic acid	nd
serine	nd

[a] Values are the mean ± SEM of three samples, each analyzed twice by HPLC. Values not sharing the same superscript letters are different by at least the $p < 0.05$ level; nd, not detected. Samples were stored for 48 h at 70 °C.

DMHF, HMF, and MF Formation and Browning in Buffer Solutions. The formation of DMHF was heavily dependent on pH (Figure 1A): under acidic conditions, DMHF was formed only in the presence of rhamnose and arginine, and its level increased with increasing pH. Neither rhamnose alone nor other sugars added to acidic solutions (pH 3.5) resulted in DMHF formation. At pHs above 6, all sugars, with or without amino acids, were active substrates for DMHF formation (verified by GC−MS). The order of the magnitude of DMHF formation from sugars in buffers not containing amino acids was rhamnose > fructose > glucose. When amino acids were present, the order of the magnitude of DMHF formation from sugars was rhamnose > glucose > fructose. In fact, the combination of rhamnose and arginine resulted in DMHF formation which was 40−50-fold higher than in any other sugar−amine combination.

The rhamnose−amine interaction leading to DMHF formation probably involves Amadori-compound formation via the loss of an amine group, forming 2,3-

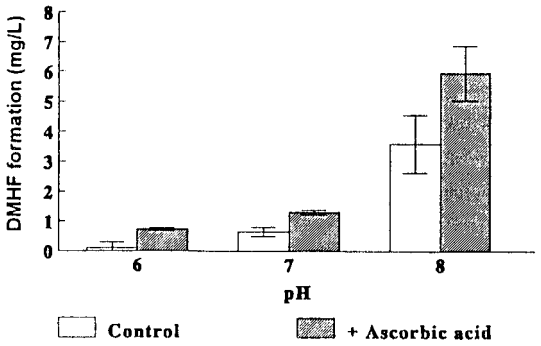

Figure 2. Effects of ascorbic acid on DMHF formation from glucose and arginine in buffer solutions adjusted to pH 6, 7, or 8. Values are the means ± SEM of three samples, each analyzed twice by HPLC.

enolization leading to a diketone, which eventually leads to DMHF after dehydration and cyclization (Pisarnitskii et al., 1992). The 2,3-enolization favored at higher pH values (Amadori compounds are unprotonated) is apparently an important stage in DMHF formation (Figure 1A). On the other hand, 1,2-enolization with further dehydration of sugars to HMF (glucose and fructose) and MF (rhamnose) favored at lower pH (Amadori compounds are protonated) (Doornbos and Ouweland, 1981) was indeed accelerated under acidic conditions whereas an increase in pH reduced HMF and MF contents (Figure 1C,D). Similar pathways may be considered for HMF and 2,3-dihydro-3,5-dihydroxy-6-methyl-4*H*-pyran-4-one produced from glucose and fructose under acidic and basic conditions, respectively (Lee and Nagy, 1990; Kim and Baltes, 1996).

The formation of small amounts of DMHF from fructose and glucose under basic but not acidic conditions has been shown previously (Shaw et al., 1968). In alkaline medium, fructose and glucose form mainly the homologue isomer 2,3-dihydro-3,5-dihydroxy-6-methyl-4*H*-pyran-4-one, which gives rise to the isomeric 4-hydroxy-5-(hydroxymethyl)-2-methyl-3(2*H*)-furanone and a much smaller amount of DMHF (Mills, 1978). This last transformation apparently involves dehydration and reduction (Kim and Baltes, 1996). Maillard-producing reductones are expected to be formed due to 2,3-enolization (Mills and Hodge, 1976), and they could be the source of the reducing agents for DMHF production from glucose− or fructose−amine interactions.

As shown in Figure 2, the addition of ascorbic acid, a natural reductone which, structurally, is closely related to Maillard reductones, significantly enhanced DMHF formation from glucose and arginine at pH 6, 7, and 8 (5-fold, 50%, and 30%, respectively) in the incubated buffer solutions. In contrast, the addition of the same amount of ascorbic acid to rhamnose−arginine solutions adjusted to pH 3.5 did not enhance DMHF formation above that observed in the absence of ascorbic acid (data not shown). The lack of detectable DMHF in stored glucose−arginine solutions under acidic conditions was not affected by the addition of ascorbic acid.

The present results further support those of Lee and Nagy (1990), who showed that the rate of HMF formation is 30-fold higher when fructose alone is present as compared to glucose (Figure 1C). Evidently, 5-HMF formation from fructose is more rapid due to the higher percentage of the acyclic form, which is enolized faster than glucose (Isabell et al., 1969). Under these conditions, the catalytic effect obtained by adding arginine to fructose was less marked whereas the addition of arginine to glucose significantly enhanced HMF formation (Figure 1C).

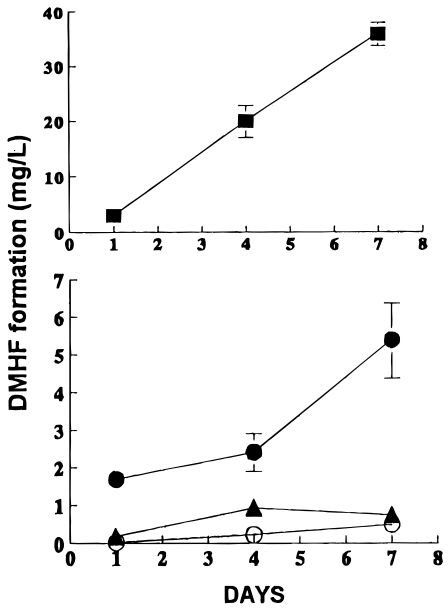

Figure 3. Effect of temperature on DMHF formation during incubation of rhamnose and arginine in a buffer solution at pH 3.5, 60 °C (○), 70 °C (▲), 80 °C (●), and 90 °C (■). Values are the means ± SEM of three samples, each analyzed twice by HPLC.

During the storage of buffer solutions, both caramelization and Maillard reactions contributed to browning with expected pH dependency, especially when amines were involved (Figure 1B). Browning due to glucose− and fructose−arginine interactions was most intense, whereas the rhamnose−arginine interaction resulted in browning which was identical to that produced in the presence of rhamnose alone. Since free radicals in the early stage may be important for browning and because rhamnose produces a smaller amount of free radicals than glucose (Namiki and Hayashi, 1982), free radical formation may in fact determine browning. The lack of browning under acidic conditions compared to previous studies (Shaw and Berry, 1977) was probably due to the relatively low sugar concentrations used in the present experiments.

As found with actual citrus juices, temperature was critical for DMHF accumulation in buffer solutions containing rhamnose and arginine (Figure 3). Under our experimental conditions, with buffers we were unable to detect DMHF at temperatures below 50 °C, while higher temperatures accelerated DMHF formation significantly (Figure 3). It should be noted that, under these circumstances, DMHF degradation and formation occur concomitantly. Storing buffer solutions (pH 3.5) containing 1 mg/L DMHF at 70 °C for 48 h resulted in a loss of 75% of the added DMHF (data not shown).

CONCLUSIONS

In DMHF formation, one needs to differentiate between hexoses and 6-deoxyhexoses. Mechanistically, steps of possible Amadori reaction (in the presence of amino acids), dehydration, and cyclization are suggested for both cases; hexoses, however, require also reduction. Under thermal conditions, both classes of sugars are expected to form DMHF through the 2,3-enediol pathway and, therefore, favor basic rather than acidic conditions (Figure 1A). When rhamnose (6-deoxyhexose) was present (with or without amino acid), DMHF

was formed under a wide range of pH conditions (2.5−8.0) tested here. On the other hand, no DMHF was formed in either the acidic model or acidic buffer solutions containing amino acids, glucose and/or fructose, with or without ascorbic acid fortification. When hexoses such as glucose and fructose were present, DMHF was formed in the pH range 6.0−8.0 (no higher pH conditions were applied). Evidently, the formation of DMHF from hexoses is more difficult than from 6-deoxyhexoses due to the necessity of reduction. This reduction can occur in the presence of ascorbic acid or due to the formation of other enoloxo compounds (reductones) via Maillard reactions (Rhee and Kim, 1975; Ninomiya et al., 1992). The small amounts of rhamnose present in citrus juices appear to be the substrates for DMHF formation during Maillard reactions in citrus products. Ascorbic acid and other reductones may further protect DMHF as it is generally unstable in air and in aqueous solutions (Shu et al., 1985; Hirvi et al., 1980).

ABBREVIATIONS USED

DMHF, 4-hydroxy-2,5-dimethyl-3(2*H*)-furanone; HMF, (hydroxymethyl)furfural; MF, methylfurfural; GABA, γ-aminobutyric acid; TMS, trimethylsilyl; MOJ, model orange juice solutions.

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