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JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Article

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Carbon Dioxide as a Traceless Caramelization Promotor: Preparation of Prebiotic Difructose Dianhydrides (DFAs)-Enriched Caramels from D-Fructose

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ABSTRACT: Activation of a concentrated solution of D-fructose with carbonic acid, generated from carbon dioxide, induces the formation of difructose dianhydrides (DFAs) and their glycosylated derivatives (glycosyl-DFAs), a family of prebiotic oligosaccharides. Under optimized conditions, up to 70% of the active DFA species were obtained from a highly concentrated solution of fructose, avoiding the filtration step and contamination risk associated with the current procedures that employ heterogeneous catalysis with acid ion-exchange resins. The optimized CO_2-promoted preparation of DFA-enriched caramel described here has been already successfully scaled up to 150 kg of D-fructose for nutritional studies, showing that implementation of this process is possible at a larger scale.

KEYWORDS: *D-fructose, carbon dioxide, caramelization, difructose dianhydrides, prebiotic*

INTRODUCTION

Prebiotics are nondigestible food ingredients that beneficially affect the host by selectively stimulating the growth and/or activity of one or a limited number of bacteria in the colon, improving host health.[1,2] The global prebiotics market was 500,000 t in 2013 and is expected to reach 1,100,000 t by 2020. Nondigestible oligo- and polysaccharides embody the most important group of prebiotics, among which fructose-based representatives such as inulin, fructooligosaccharides (FOS), and oligofructose are typical examples.[3,4] Difructose dianhydrides (DFAs) and their glycosylated derivatives (glycosyl-DFAs) constitute a relatively recent addition to this family (Figure 1).[5] Thirteen isomers of DFAs with five different tricyclic cores have been identified in caramel and used as tracers for caramel authenticity and for the detection of fraudulent addition of caramel to some foodstuffs (Figure 1).[6]

Their unique cyclic structure confers on DFAs much higher enzymatic and chemical stability as compared with classical fructosyl derivatives such as sucrose.[7,8] The growing interest in the prebiotic properties of DFAs has led to the search for new methodologies allowing the preparation of single isomers or DFA-enriched products. A method based on the enzymatic catalytic degradation of fructans was one of the first studied.[9−13] More recently, the enzymatic synthesis of a DFA diastereomer from sucrose, namely, α-D-fructofuranose β-D-fructofuranose 1,2′:2,3′-dianhydride, by coupling a sucrose fructosyltransferase and an inulin fructotransferase, has also been reported.[14] Major drawbacks of these syntheses are the low thermal stability, the substrate dependence of enzymes, and the high cost of these processes. Alternatively, controlled thermal and/or acidic activation of fructose were investigated. Under such conditions, a fructosyl oxocarbenium cation is formed, which can next glycosylate a second fructose molecule to form fructodisaccharides (fructobioses). A second intramolecular glycosylation process affords the DFAs, which can undergo further glycosylation reactions to give glycosyl-DFAs. This reaction pathway competes with unspecified intramolecular dehydration and condensation processes at the origin of the formation of furanic derivatives (e.g., 2-hydroxymethylfurfural, HMF) and oligomeric color compounds (melanoidins) characteristics of caramel.[15,16] Inhibition of these side reactions represents the main hurdle to achieving high DFA yields (Figure 2).[5] A product with up to 26% of DFAs has been previously produced by pyrolysis of inulin.[17] Thermal treatment of inulin or sucrose, alone or in admixture with an acid promoter, was also reported.[18−20] The highest conversions into DFAs and glycosyl-DFAs were obtained by activation of inulin, sucrose, fructose, or glycosylfructoses with anhydrous hydrogen fluoride (HF) and HF-based reagents.[21−25] Traces of HF or fluorine salts in the final product, however, represent a health risk that hampers the deployment of this process at an industrial level.

More recently, a caramel containing about 30% by mass of DFAs (up to 70% considering the sum of DFAs and glycosyl-DFAs) was obtained from D-fructose using the strongly acidic sulfonic acid resin Lewatit S2328 as caramelization promotor.[26] The high viscosity of the reaction medium can represent a technical problem.[27] The use of microwave irradiation, aimed at heating the mixture to the core, was found advantageous,[28] but

Received: April 6, 2017
Revised: May 29, 2017
Accepted: May 30, 2017
Published: May 30, 2017

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DOI: 10.1021/acs.jafc.7b01601
J. Agric. Food Chem. 2017, 65, 6093−6099

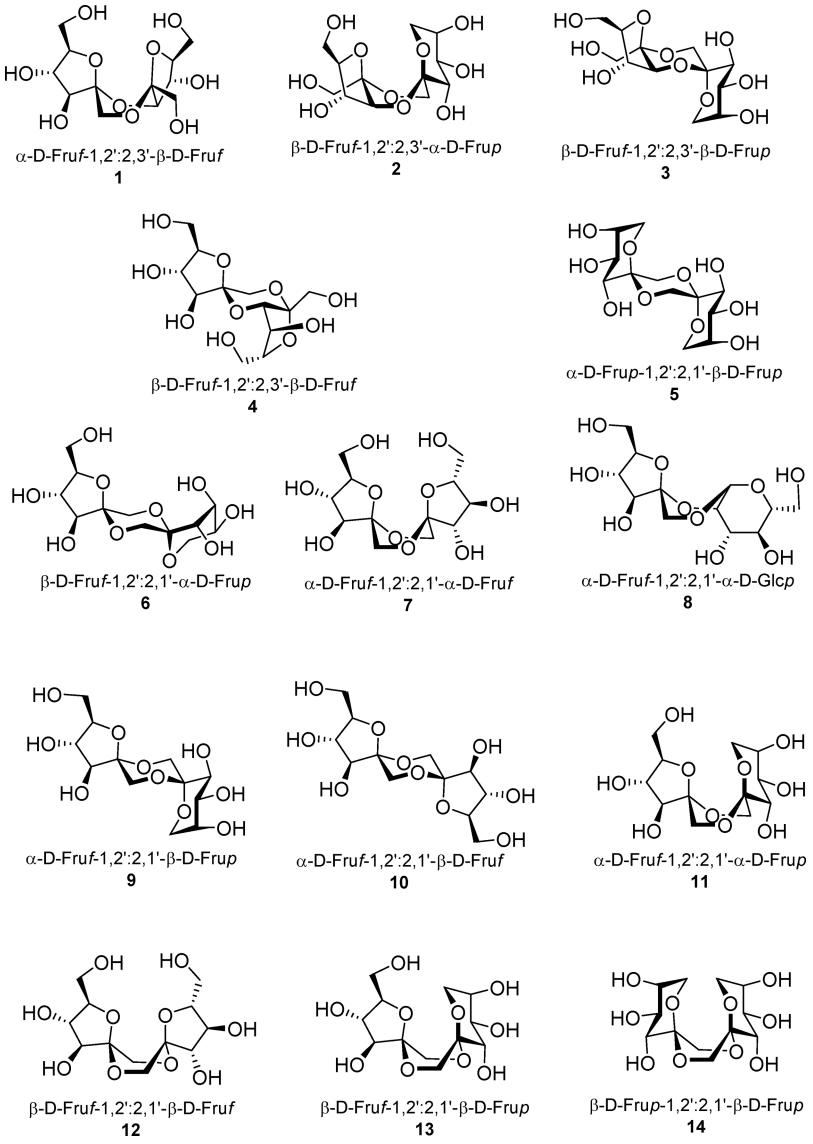

Figure 1. Structures of the 13 DFA diastereomers present in sucrose commercial caramel. For the sake of congruity they are numbered **1**−**7** and **9**−**14**, following the gas chromatography elution profile. Compound **8** corresponds to a mixed fructose−glucose dianhydride, which is not present in fructose caramel. The ring size (*f*, furanose; *p*, pyranose), anomeric configuration (*α* or *β*), and linking positions of the monosaccharide subunits are indicated.

the problem of separation of the catalyst from the final product remains difficult. Alternative technologies allowing transformation of D-fructose into prebiotic caramels using traceless promoters are thus greatly needed.

It is well-known that CO_2 forms carbonic acid with water, which can be used as an acid catalyst as already mentioned in the literature.[29−31] Herein, we demonstrate that DFA and related oligomer products can be synthesized from a highly concentrated solution of fructose using cheap, safe CO_2 as a reservoir of acid. At the end of the process CO_2 can be easily removed and pure prebiotic caramel obtained without any purification step, which is a significant advantage over existing methods.

MATERIALS AND METHODS

Materials. Anhydrous D-fructose (for analytical determinations) and HMF of 99% purity, phenyl *β*-D-glucopyranoside (internal standard, IS), hydroxylamine hydrochloride, hexamethyldisilazane, and trimethylchlorosilane were purchased from commercial sources (Sigma-Aldrich, Tres Cantos, Spain) and were stored at room temperature. Authentic samples of *α*-D-fructofuranose *β*-D-fructofuranose 1,2′:2,1′-dianhydride, **10**,[32] di-*α*-D-fructofuranose 1,2′:2,1′-dianhydride, **7**,[33] di-*β*-D-fructofuranose 1,2′:2,1′-dianhydride, **12**,[34] *α*-D-fructopyranose *β*-D-fructopyranose 1,2′:2,1′-dianhydride, **5**,[33] di-*β*-D-fructopyranose 1,2′:2,1′-dianhydride, **14**,[35,36] *α*-D-fructofuranose *β*-D-fructopyranose 1,2′:2,1′-dianhydride, **9**,[33] and *β*-D-fructofuranose *β*-D-fructopyranose 1,2′:2,1′-dianhydride, **13**, used for calibration of the analytical protocol, were prepared by boron trifluoride or trifluoromethanesulfonic acid (triflic acid)-promoted spirocyclization of suitably protected D-fructose precursors, column chromatography of the protected derivatives, and final deprotection of the individual DFAs following the reference indicated in each case. *α*-D-Fructofuranose *β*-D-fructofuranose 1,2′:2,3′-dianhydride, **1**, was obtained by a biotechnological process involving treatment of inulin with inulase II from *Arthrobacter* sp.[37] and was >99% pure as seen by NMR and GC.

General Procedure for the Caramelization Reaction. The caramelization reaction was carried out in a stainless steel batch reactor (Parr Instruments, France). The reagent (fructose) and water were introduced in the reactor in an appropriate amount. Carbon dioxide

DOI: 10.1021/acs.jafc.7b01601
J. Agric. Food Chem. 2017, 65, 6093−6099

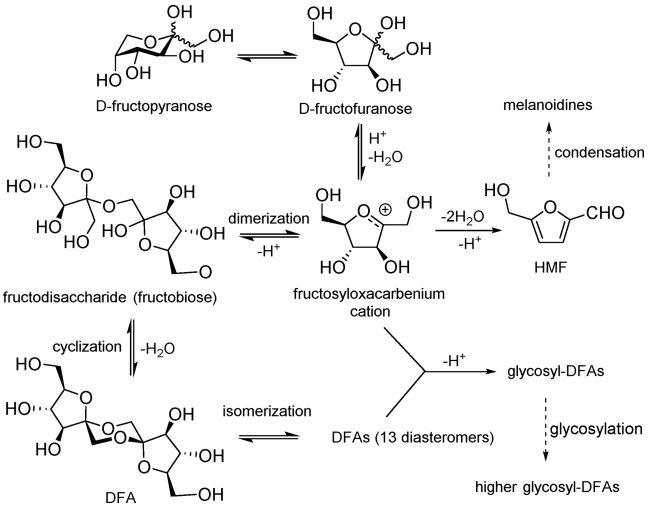

Figure 2. Main reaction pathways operating during the acid-catalyzed caramelization of D-fructose. The kinetically favored fructosyloxocarbenium cation and the major kinetic DFA (**10**) are depicted. Note that a variety of fructobioses (positional, ring size, and stereochemical isomers) can be initially formed, which reversibly interconvert and progress toward the thermodynamically more stable DFAs.

was introduced in the reactor under gaseous form. The temperature was then increased to the desired temperature. The stirring was continued during the reaction time. After the completion of the reaction, the reactor was cooled to room temperature. The reactor was depressurized to atmospheric pressure, and the caramel was recovered and analyzed.

Sample Derivatization. For GC analysis, caramel samples as well as the reference samples used for calibration purposes were transformed into their corresponding per-*O*-trimethylsilyl (TMS; nonreducing sugars) or per-*O*-trimethylsilylated oxime (TMS-oximes; reducing sugars) derivatives. The crude samples were diluted with deionized water (1 mL), and the aqueous solutions were freeze-dried. To 15−20 mg of each sample was added deionized water (1 mL). To 100 μL of the resulting solution was then added 100 μL of IS consisting of 4 mg/mL phenyl β-D-glucopyranoside in acetone/water (1:9, v/v), and the final solution was evaporated to dryness at 60 °C (drying oven). The residue was treated with 1 mL of a solution of hydroxylamine in pyridine (20 mg/mL) at 60 °C over 50 min, with mixing at intervals. Neat hexamethyldisilazane (200 μL) and trimethylchlorosilane (100 μL) were then added, and the reaction mixtures were kept at 60 °C over a further 40 min period. Formation of a white precipitate was observed during this operation, which was separated by centrifugation (13,000 rpm, 5 min) before injection in the GC apparatus. It should be noted that following oximation-trimethylsilylation derivatization, reducing compounds (e.g., residual D-fructose) show two peaks in the GC chromatograms, corresponding to the *syn*- and *anti*-TMS-oximes, whereas nonreducing derivatives (e.g., DFAs and the IS) show a single peak.

GC-FID Analysis. GC-FID was carried out using a 7820A GC system (Agilent, Las Rozas, Spain) with a split/splitless EPC injector equipped with an autosampler fitted with a cross-linked 5% phenyl-dimethylsiloxane column. The column used was a 30 m × 320 μm, i.d. = 0.25 μm, HP-5. Operating conditions were as follows: injection port temperature, 310 °C; split ratio, 25:1; injection volume, 1 μL of derivatized samples; column oven temperature programmed from 180 to 310 °C at 5 °C/min, with a 25 min hold at 310 °C; carrier gas, helium (constant flow at 1.2 mL/min); detector port temperature, 310 °C. Total acquisition time was 56 min. The identity of DFAs in the samples was confirmed by comparison of the GC chromatograms with that of a sucrose industrial caramel and DFA authentic samples as reported.[15,16] Response factors (RFs) for D-fructose (1.42) and eight individual DFAs (**1**, 0.89; **5**, 0.76; **7**, 0.68; **9**, 0.68; **10**, 0.75; **12**, 0.86; **13**, 0.78; **14**, 0.85), at concentrations similar to those encountered in the experiments, were determined relative to the internal standard phenyl β-D-glucopyranoside and used for quantitation of their relative proportion in caramels. The average of the RF values for the eight DFAs available in pure form (0.78) was applied to the rest of the DFA components in the mixtures.

Electrospray Ionization Mass Spectrometry. Electrospray mass spectra (ESI-MS) in the negative ion mode were obtained on an Esquire 6000 instrument (Bruker, Madrid, Spain). The caramel samples were dissolved in Milli-Q water (1 mg/mL) and then diluted with methanol to a final concentration of 0.1 mg/mL. The solutions were directly introduced (0.2 mL/h) through an integrated syringe pump into the electrospray source. The source and desolvation temperatures were 300 °C, respectively. Nitrogen was used as the drying and nebulizing gas at flow rates of 5 mL/min. Typically, the capillary voltage was 4.5 kV and the skimmer voltage, −40 V. The mass range was 65−3000 Da, and spectra were recorded as averages of seven scans in the profile mode, with an ion change control (ICC) of 1000.

RESULTS AND DISCUSSION

Effect of CO_2 Pressure. In a first set of experiments, the reaction was carried out under an atmosphere of air or nitrogen with a fructose/water (w/w) ratio of 6.0 at 90 °C for 24 h (Table 1). The conversion of fructose was similar as well as the

Table 1. Effect of CO_2 Pressure on D-Fructose Caramelization[a]

entry	gas	pressure (MPa)	D-fructose conv (%)	DFAs (%)	oligos[b] (%)
1	air	0.1	22	21	0.7
2	N_2	0.1	21	19	2
3	CO_2	0.1	27	17	11
4	CO_2	0.2	28	15	12
5	CO_2	2.0	35	16	18

[a]Fructose/water (w/w) ratio = 0.6, T = 90 °C, t = 24 h. [b]Fructooligosaccharides and glycosyl-DFAs.

DFAs and oligosaccharides. These results correspond to thermal oligomerization of fructose. Under a CO_2 atmosphere, the conversion of fructose was slightly higher and the selectivity was changed. Thus, 11 and 17% of oligosaccharides and DFAs were, respectively, obtained against 2 and 19% under nitrogen atmosphere (Table 1). When the pressure of CO_2 was increased from 0.1 to 2.0 MPa, the fructose conversion was slightly increased from 27 to 35% (Table 1). The proportion of DFAs in the final product remained constant (15−17%). No significant differences were observed in the relative proportions of the different DFA diastereoisomers among the three experiments (data not shown). Instead, the fraction corresponding to other oligosaccharides increased from 11 to 12% when using a CO_2 pressure of 0.1 or 0.2 MPa and to 18% when the CO_2 pressure was 2.0 MPa. These data can be interpreted assuming that in the presence of CO_2 and with the increase of the pressure, higher concentrations of carbonic acid are generated in the medium, thus accelerating the formation of reactive fructosyloxocarbenium ions. Intermolecular glycosylation reactions to produce fructobioses (Figure 2) or glycosyl-DFAs then occur more quickly than intramolecular glycosylation processes of the fructobioses to give the DFAs under the reaction conditions of the experiments. These results demonstrate that CO_2 can promote the formation of DFA and related oligomers by forming carbonic acid from a concentrated solution of fructose.

DOI: 10.1021/acs.jafc.7b01601
J. Agric. Food Chem. 2017, 65, 6093−6099

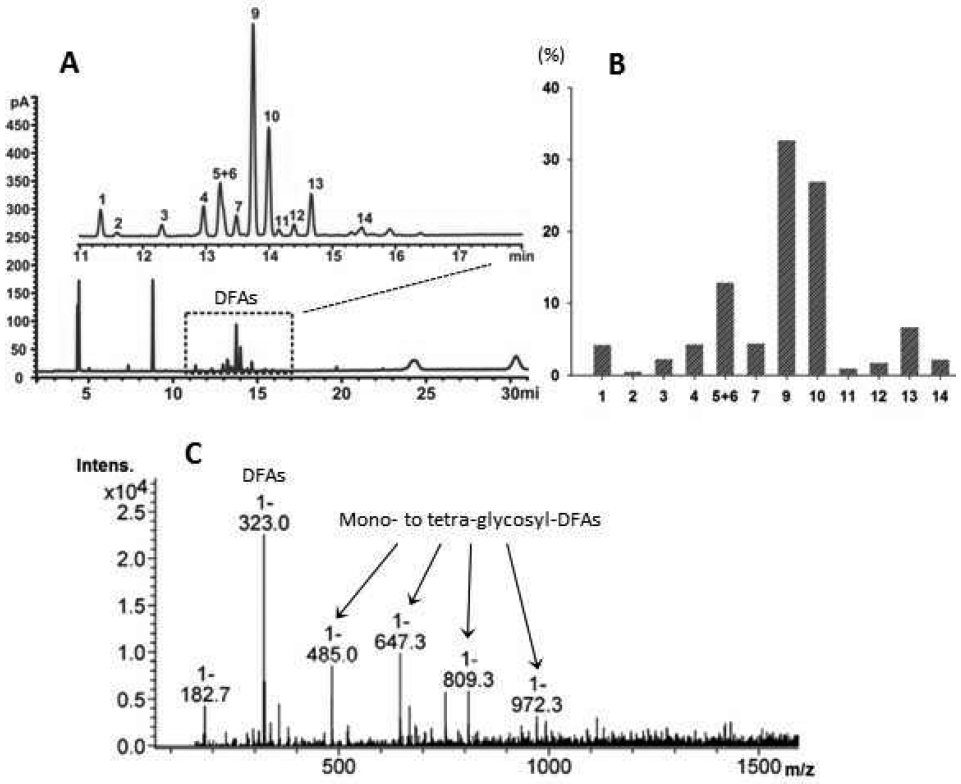

Figure 3. (A) GC chromatogram of the product obtained by CO_2-promoted (2 MPa) of D-fructose (6.0 fructose/water ratio) at 100 °C for 48 h. (B) Relative proportions of the individual DFA diastereomers in the corresponding DFA fraction. (C) Peaks for DFAs **5** and **6** are not resolved and were measured together and electrospray ionization−mass spectrum (negative mode; $[M - H]^-$ pseudomolecular peaks) of the crude caramel.

Effect of Reaction Temperature. Changing to higher temperatures obviously accelerated very significantly the CO_2-promoted caramelization of D-fructose. Indeed, when the temperature was increased from 90 to 100 °C, keeping the other reaction parameters unchanged (initial fructose/water ratio = 6.0; CO_2 pressure = 2 MPa), a 72% fructose conversion was achieved in 48 h, the final product containing 47% of DFAs and 23% of glycosyl-DFAs. The diastereomeric distributions of the individual DFAs in the final caramel products were analyzed by gas chromatography (GC), using a flame ionization detector (FID) after derivatization of the samples by oximation−trimethylsilylation. Using this protocol, reducing carbohydrates (e.g., D-fructose) afforded two peaks in the chromatogram corresponding to the *anti* and *syn* per-*O*-trimethylsilylated oximes. Nonreducing sugars such as DFAs produce instead a single peak for the per-*O*-trimethylsilylated derivative.[38] As a characteristic example, the GC profile (DFAs region) for the experiment conducted with an initial fructose/water ratio of 6 is depicted in Figure 1A, with peak assignments. Quantitation was performed by the IS (phenyl β-D-glucopyranoside) method. Authentic samples of D-fructose and of individual DFA diastereomers were used to obtain the corresponding response factors.

The above reaction conditions thus allow a DFA-enriched caramel to be obtained that is comparable, in terms of D-fructose conversion and combined DFA and other oligosaccharide content, to the prebiotic DFA-enriched product obtained by heterogeneous catalysis using Lewatit S2328 acid ion-exchange resin, but with the important advantage that no filtration of the promoter or any additional manipulation (other than decompressing the reactor) was necessary. α-D-Fructofuranose β-D-fructopyranose 1,2′:2,1′-dianhydride, **9**, and α-D-fructofuranose β-D-fructofuranose 1,2′:2,1′-dianhydride, **10**, were the major DFAs formed in the product obtained by CO_2-promoted caramelization of D-fructose. The DFA derivative, **10**, is a kinetically favored diastereomer that rearranges into the furanose−pyranose derivative, **9**, under conditions of thermodynamic control.[5] The fructofuranose−fructopyranose DFA, **9**, was the major diastereomer in the DFA fraction, meaning that the profile of isomeric DFAs in the caramel is closer to a thermodynamic distribution (Figure 3A,B). However, the proportion of the major kinetic difructofuranose DFA, **10**, is still significantly higher as compared with literature data for the prebiotic DFA-enriched product obtained by Lewatit S2328-promoted caramelization (**9**:**10** ratio of 1.2 with CO_2 vs 2.2 with Lewatit S232). The extent of DFA glycosylation is also different, the DFAs fraction amounting to 48% in the CO_2 caramel (vs 32% with Lewatit S232) and to 25% for the oligosaccharide fraction (vs 38% in the acid ion-exchange resin caramel). The latter fraction was essentially constituted by glycosyl-DFAs, as seen in the corresponding mass spectrum of the crude caramel product (Figure 3C). Given that each of the 13 DFA diastereomers present in caramel possesses three (for C_2-symmetric derivatives) or up to six hydroxyl groups (nonsymmetrical DFAs) susceptible to acting as glycosyl acceptors in reversion (self-glycosylation) reactions, the oligosaccharide fraction of the caramel products is expected to be extremely complex. Nevertheless, considering that the major representatives **9** and **10** account for about 60% of the total DFA fraction and that the more accessible primary hydroxyls are preferentially engaged in reversion processes,[24,39] derivatives of **9** and **10**

DOI: 10.1021/acs.jafc.7b01601
J. Agric. Food Chem. 2017, 65, 6093−6099

fructosylated at the *O*-6 (or *O*-6′) position of the fructosyl moieties are expected to be the preferred substructures in the oligomeric material.

The simplified downstream process and the absence of contamination represents very significant technological advantages that can also facilitate approval for commercial development.

A further increase in the temperature to 115 °C accelerated fructose caramelization, but traces of furanic compounds were observed even if relatively short reaction times were used (Table 2). Given the concerns about the potential genotoxicity of HMF,[40−42] we have privileged conditions affording <1% of HMF in the final caramel product while ensuring fructose conversions into prebiotic DFAs and glycosyl-DAFs >60%. Thermal input reduction is indeed a well-established strategy to mitigate HMF formation.[43]

Table 2. Effect of the Initial D-Fructose Concentration on the CO_2-Promoted Caramelization[a]

entry	fructose/water (w/w)	time (h)	D-fructose conv (%)	HMF (%)	DFAs (%)	oligos[b] (%)
1	0.9	12	42	5	18	24
2	1.2	12	41	4	18	22
3	6	2	39	<1	16	23

[a] T = 115 °C, P = 2 MPa. [b] Fructooligosaccharides and glycosyl-DFAs.

Reaction Kinetics. The kinetics of the carbon dioxide-promoted caramelization of D-fructose was next investigated by running parallel experiments for 2, 10, 24, and 72 h in which the initial fructose/water (w/w) ratio was fixed at 6.0, the CO_2 pressure at 2 MPa, and the reaction temperature at 90 °C. GC analysis showed a sustained increase in the fructose conversion to 61% after 72 h of reaction, as well as a concomitant steady increase in the proportion of DFAs up to 45%.

The proportion of other oligosaccharidic products remained almost constant (17−19%) during the whole period presumably because the amount of in situ produced fructobioses subsequently converted to DFAs is compensated by the formation of glycosyl-DFAs (Figure 4A). The relative proportions of DFA diastereomers revealed the expected evolution toward a thermodynamic distribution, showing opposite up and down trends in the data for the major DFAs **9** and **10**, that is, a sustained decrease in the corresponding **9**:**10** ratio (Figure 4B). Higher reaction times led to unwanted high ratios of HMF and melanoidins and were judged unpractical.

Effect of Fructose to Water Ratio. The effect of the initial concentration of D-fructose on the CO_2-promoted caramelization reaction was investigated. Keeping in mind that DFA formation is a reversible dimerization process involving intermolecular dehydration, the fructose to water ratio is of prime importance both for the kinetics and for the thermodynamics of the reaction. Suárez-Pereira et al.[24] have shown that when the concentration of fructose was raised from 70 to 90% (w/w), the DFA proportion in the final caramel was increased by 20% when using Lewatit S2328 as the acid catalyst. In our case, increasing the sugar concentration implies a decrease in the water amount available to form carbonic acid, which was expected to be detrimental for fructose activation and the DFA formation rate. A compromise was thus necessary.

To explore the optimal sugar concentration, a series of tests starting from 30 g of commercial D-fructose were carried out. Three different fructose/water (w/w) ratios, 0.9, 1.2, and 6.0, were initially assayed keeping the pressure of CO_2 (2 MPa) and the reaction temperature (115 °C) unmodified. No significant differences were observed between the two first experiments (Table 2), which afforded virtually identical fructose conversions (42 and 41%, respectively) and DFA yields (18%) after 12 h of reaction. When the fructose/water ratio was increased to 6.0, we were pleased to see that a similar fructose conversion (39%) was attained after only 2 h of reaction, providing a similar yield (16%) of DFAs (Table 2). It is worth noting that formation of 5 and 4% of HMF by intramolecular dehydration occurred in the two first experiments but dropped to <1% in the third case. This can be explained by less formation of carbonic acid in the highly concentrated solution of fructose (less water), preventing the formation of undesired products even if they are favored by the temperature used (115 °C).

However, this process is of interest owing to the traceless production of DFAs using CO_2 compared to the use of heterogeneous catalysts.

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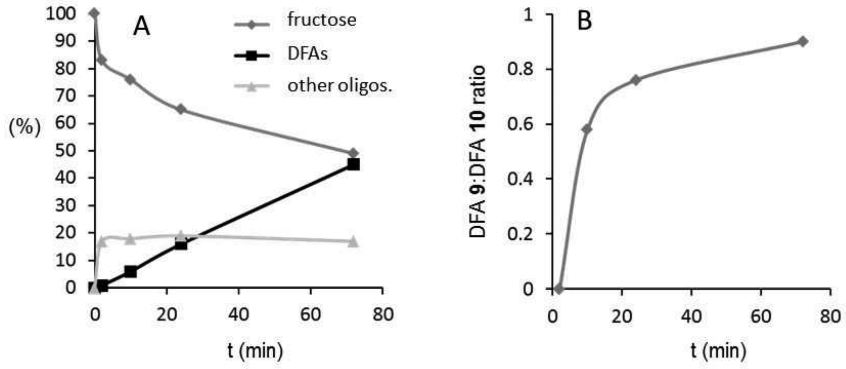

Figure 4. Plots of the relative proportions of D-fructose, DFAs, and other oligosaccharides (fructobioses and glycosyl-DFAs) for the CO_2-promoted (CO_2 pressure = 2 MPa) caramelization of D-fructose (initial fructose/water ratio 6.0 at 90 °C (A) and of the quotient between the major thermodynamic, **9**, and kinetic, **10**, DFAs as a function of reaction time (B)).

DOI: 10.1021/acs.jafc.7b01601
J. Agric. Food Chem. 2017, 65, 6093−6099

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Funding

M.A. received funding from the Poitou-Charentes region. C.O.M. and J.M.G.F. received funding from MINECO (Contracts SAF2016-76083-R and CTQ2015-64425-C2-1-R), the Junta de Andalucía (Contract FQM2012-1467), and the European Regional Development Funds (FEDER and FSE). L.A.-G. received funding from Fundación Danone.

Notes

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

The CITIUS (Universidad de Sevilla) and the analytical services of IIQ are thanked for technical support.

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DOI: 10.1021/acs.jafc.7b01601
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