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Di-D-fructose Dianhydride-Enriched Products by Acid Ion-Exchange Resin-Promoted Caramelization of D-Fructose: Chemical Analyses

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Caramelization commonly occurs when sugars, or products containing a high proportion of sugars, are heated either dry or in concentrated aqueous solutions, alone or in the presence of certain additives. Upon thermal treatment of sugars, dehydration and self-condensation reactions occur, giving rise to volatiles (principally 2-hydroxymethylfurfural, HMF), pigments (melanoidines) and oligosaccharidic material, among which di-D-fructose dianhydrides (DFAs) and glycosylated DFA derivatives of different degree of polymerization (DP) have been identified. This study reports a methodology to produce caramel-like products with a high content of DFAs and oligosaccharides thereof from commercial D-fructose based on the use of acid ion-exchange resins as caramelization promotors. The rate of formation of these compounds as a function of D-fructose concentration, catalyst proportion, temperature, catalyst nature and particle size has been investigated. The use of sulfonic acid resins allows conducting caramelization at remarkable low temperatures (70−90 °C) to reach conversions into DFA derivatives up to 70−80% in 1−2 h, with relative proportions of HMF < 2%.The relative abundance of individual DFA structures can be modulated by acting on the catalyst nature and reaction conditions, which offers a unique opportunity for nutritional studies of DFA-enriched products with well-defined compositions.

KEYWORDS: Caramelization; D-fructose; di-D-fructose dianhydrides (DFAs); ion-exchange resins; gas chromatography; mass spectrometry

INTRODUCTION

Di-D-fructose dianhydrides (DFAs) are a family of isomeric cyclic fructodisaccharides resulting, formally, from the dimerization reaction of D-fructose with the loss of two water molecules and generation of two reciprocal glycosidic linkages (*1*). The formation of DFAs by thermal and mineral acid activation of D-fructose and inulin was already known at the beginning of the 20th century (*2*), but they remained laboratory curiosities until recent times (*3*, *4*). Their isolation from several microorganisms and higher plants and, especially, the identification of DFAs as major nonvolatile components in caramel (up to 18% in an industrial sucrose caramel) (*5*) have strongly stimulated interest in this family of compounds. The presence of DFAs in roasted chicory (*6*), heat dried fruits (*7*), natural and sugar-roasted torrefacto coffee (*8*, *9*) and traditional tequila (*10*) has been also evidenced. Nowadays, it is well-established that the chemical transformations leading to DFAs take place universally during caramelization processes, even when glucose or glucose syrups are used as the starting sugar; D-glucose isomerizes to a certain extent to D-fructose through Lobry de Bruyn−Alberda van Eckenstein aldose → ketose rearrangement in the early stages of caramelization (*11*−*13*). Actually, DFAs have been proposed as chemical markers for caramel authenticity (*14*) and detection of adulteration by fraudulent addition of partially caramelized products to foodstuffs, e.g. honey (*15*).

The above findings imply that DFAs have been part of the human diet since prehistoric times; caramelization, together with the Maillard reaction, belongs to the group of nonenzymatic browning reactions taking place upon cooking of sugar-rich foods, and caramel itself has been used for millennia to impart color and flavor to food and beverages (*16*). Interestingly, some DFA representatives have been shown to exhibit favorable nutritional properties, promoting bifidobacteria growth and mineral/flavonoid absorption in the small and large intestine of rats (*17*−*21*) and humans (*22*). These results have fueled research in efficient methodologies for the preparation of DFAs. Enzymatic strategies using either inulin or levan fructotransferases and the corresponding fructan substrate have been developed for some particular DFA diastereomers (*23*−*26*). Among chemical methods, protonic activation of fructose (*27*), inulin, sucrose (*28*)

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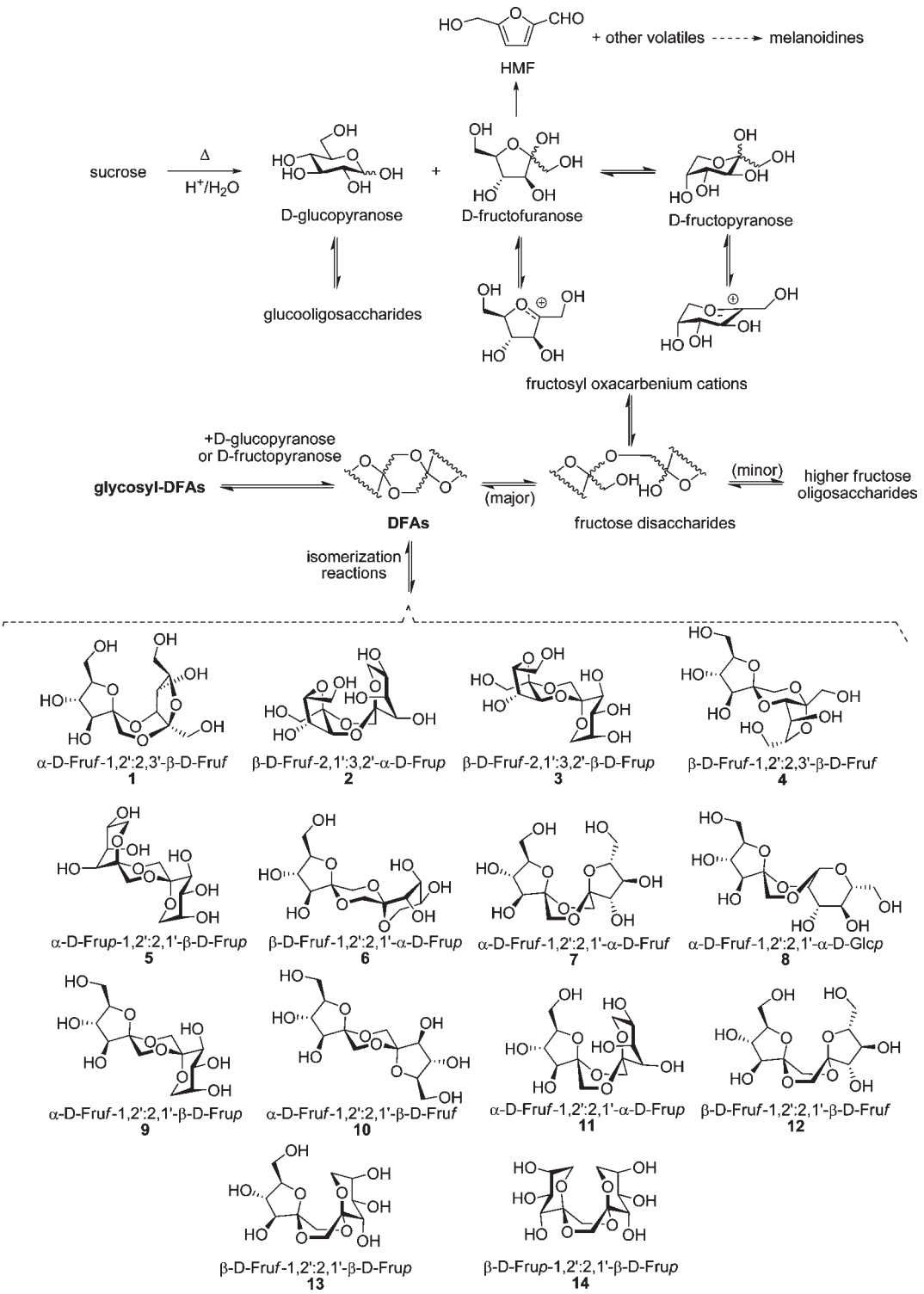
Scheme 1. General Mechanism for Thermal and Acid Promoted Caramelization of Sucrose[a]



[a] The structures of the DFA diastereomers identified in sucrose caramel are depicted.

or glycosylfructoses (*29*, *30*) with hydrogen fluoride (HF) or HF-based reagents was found extremely efficient in promoting conversion to DFAs, mimicking the reaction pathway that leads to nonvolatile saccharide components in caramel. Comparison of HF- and heat-induced (classical) caramelization allowed proposal of the general mechanism depicted in **Scheme 1** for the particular case of sucrose as the starting sugar.

Dimerization of D-fructose to give DFAs and further DFA interconversion, via a transient fructodisaccharide, are reversible processes that compete with irreversible intramolecular dehydration leading to volatiles, especially to 5-hydroxymethylfurfural (HMF) (*31*, *32*). Although difructofuranose DFAs (e.g., compounds **1**, **7** or **10**) are kinetically favored, they isomerize to the thermodynamically more stable D-fructopyranose-containing derivatives (e.g., **5**, **9** or **14**). The relative abundance of the individual DFA diastereomers in the final product depends on the starting cyclic form of D-fructose (e.g., furanose in sucrose or inulin and pyranose in crystalline D-fructose) and on caramelization conditions. Up to 14 different dianhydride structures have been identified in sucrose caramel, 13 of which

(compounds **1**−**7** and **9**−**14**) are DFAs and one (compound **8**) is a mixed D-fructose D-glucose dianhydride. In a second step, DFAs can be glycosylated by monosaccharide (or oligosaccharide) units to give glycosyl-DFAs. Condensation and nonspecific dehydration processes involving volatile and nonvolatile components are in the origin of the high molecular weight melanoidines responsible for color development.

Several attempts to develop caramelization procedures that privilege the route leading to DFA formation have been reported. Products containing DFAs and glycosyl-DFAs in proportions up to 40% have been previously prepared by pyrolysis of sucrose (*33*), inulin (*29*, *34*) or isomaltulose (6-*O*-α-D-glucopyranosyl-D-fructofuranose, palatinose) (*35*) in the presence of citric acid. Interestingly, such products were found to exhibit beneficial nutritional properties in animals (*36*). More recently, it was found that dry heated (195 °C) inulin, containing DFAs (*37*), showed a much higher stimulation of bifidobacteria and other beneficial bacteria and a significant decrease of possibly pathogenic bacteria compared to unheated samples in *in vitro* fermentation experiments with pooled human fecal bacteria (*38*). However, the high temperatures used in the pyrolysis methodology make necessary a strict control of the procedure and its reproducibility. A caramelization method that would allow high conversions into DFAs with total control on the composition of the final product and technically appropriate for nutritional studies and food applications is, therefore, highly desirable.

In this study we have investigated the suitability of D-fructose caramelization under heterogeneous conditions, using acid ion-exchange resins as promoters, to obtain caramels (*39*) with a high content of DFAs and oligosaccharides thereof at temperatures well below the melting point of the monosaccharide. Ion-exchange resins are broadly used as catalysts in industrial processes, including the food industry; they can be easily separated from the final product by filtration, are reusable and well-adapted to continuous processes and allow a precise control of the reaction parameters (*40*). The rate of formation of DFAs and their diastereomeric distribution as a function of the initial D-fructose concentration, catalyst proportion and nature, particle size, temperature and reaction time will be evaluated in this paper. The use of water-soluble poly(*p*-toluenesulfonic acid) polymers has been included in the study to compare heterogeneous versus homogeneous caramelization conditions. Interestingly, the DFA-enriched products obtained by this methodology have shown a beneficial effect in a mouse model for the inflammatory bowel disease, (*41*) a protective and druglike (nutraceutical) activity that is shared by other prebiotics (*42*, *43*). The corresponding biological data will be the object of a separate paper.

EXPERIMENTAL PROCEDURES

Materials. Anhydrous D-fructose and 5-hydroxymethylfurfural (HMF) of 99% purity were purchased from Sigma-Aldrich (Steinheim, Germany) and were of HPLC grade. Phenyl β-D-glucopyranoside (internal standard, I.S.), hydroxylamine hydrochloride, hexamethyldisilazane and trimethylchlorosilane were purchased from Sigma-Aldrich (Steinheim, Germany) and were stored at room temperature. α-D-Fructofuranose β-D-fructofuranose 1,2′:2,1′-dianhydride (*44*), di-α-D-fructofuranose 1,2′:2,1′-dianhydride (*45*), di-β-D-fructofuranose 1,2′:2,1′-dianhydride (*46*), α-D-fructopyranose β-D-fructopyranose 1,2′:2,1′-dianhydride (*37*), di-β-D-fructopyranose 1,2′:2,1′-dianhydride (*47*), α-D-fructofuranose β-D-fructopyranose 1,2′:2,1′-dianhydride (*37*) and β-D-fructofuranose β-D-fructopyranose 1,2′:2,1′-dianhydride (*48*) 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 after the reference indicated in each case. Their identity and purity were confirmed by ^{1}H and ^{13}C NMR, gas chromatography (GC-FID) and melting point determination. Di-α-D-fructofuranose 1,2′:2,3′-dianhydride was a gift of Dr. C. Buttersack (Institute für Technologie der Kohlenhydrate e.V., Braunschweig, Germany) and was >99% pure as seen by NMR and GC.

The ion-exchange resins Lewatit S2328, K1131, K1469 and K2641 were obtain as gifts from LanXess (Barcelona, Spain). The resins Amberlite IRC50 (Rohm & Haas) and Dowex 50WX2 (Dow Chemical Co.) were purchased from Sigma-Aldrich (Steinheim, Germany). All resins were in the H^+ form and were conditioned by treatment with aqueous HCl prior to use in caramelization reaction as described hereinafter. Poly(*p*-toluenesulfonate) polymers (7×10^4 and 10^6 Da), in their Na^+ form, were purchased from Sigma-Aldrich (Steinheim, Germany). They were transformed into the H^+ form by treatment with excess of Amberlite IR120 (H^+) ion-exchange resin (Sigma-Aldrich; Steinheim, Germany) in deionized water (see below).

Conditioning and Titration of Acid Resins and Polymers. The corresponding ion-exchange resin (500 g) was stacked in a glass column (60 × 3 cm) and subsequently eluted (3 mL min^{-1}) with deionized water (2 L), 1 N aq HCl (2 L) and MeOH (1 L). Drying was effected first under air current at room temperature for 4 h and then in an oven at 90 °C for 16 h. The resin thus conditioned was stored in a desiccator under P_2O_5.

In the case of the poly(*p*-toluenesulfonate) polymers, the commercial product in the Na^+ form (2 g) was dissolved in deionized water (40 mL) and the resulting solution was magnetically stirred with Amberlite IR120 (H^+) ion-exchange resin (2 g) for 30 min. The resin was then separated by filtration. This process was repeated three times, but in the last one the stirring was allowed to continue for 24 h to warrant full $Na^+ \rightarrow H^+$ exchange. The filtered solution was then freezed-dried and stocked in a desiccator over P_2O_5.

To determine the exchange capacity of the conditioned resins, a back-titration procedure was carried out. The resin in the active H^+ form (1 g), in a glass column (30 × 1 cm), was eluted (2 mL min^{-1}) with a 0.1 N aqueous solution of Na_2SO_4. A 50 mL aliquot of the eluent was added onto 150 mL of 0.1 N NaOH (previously titrated with acid potassium phthalate). The resulting solution was then used to titrate 10 mL of 0.1 N H_2SO_4 previously titrated with the above NaOH standard solution. Phenolphthalein was used as pH indicator in all titration experiments. In the case of the water-soluble polymers, a direct titration with the standard solution of 0.1 N NaOH provided the exchange capacity.

Caramelization Reactions. All caramelization reactions were carried out in a 250 mL round-bottom flask provided with a Teflon screwed-cap immersed in a silicon oil (polymethylphenylsiloxane) bath. Heating and magnetic stirring was effected with an IKA RTC Basic plate provided with a thermopar for temperature control. D-Fructose (50−100 g) was first dissolved in water to the desired concentration by mild heating (60 °C). The appropriate amount of catalyst was then added, the flask was placed in the bath and the mixture was magnetically stirred. For kinetics experiments, aliquots (100−150 mg) were withdrawn at intervals and stocked in eppendorf vials at −18 °C before their derivatization and GC analysis.

Derivatization. For GC analysis, caramel samples as well as the reference samples used for identification were transformed into their corresponding per-*O*-trimethylsilyl (TMS; nonreducing sugars) or per-*O*-trimethylsilylated oxime (TMS-oximes; reducing sugars) derivatives. Immediately after a caramelization experiment, the samples were diluted with deionized water (1 mL), the resin was separated by centrifugation (13000 rpm, 5 min) and the solution was decanted using a micropipet. This process was repeated twice, and the aqueous solutions were freeze-dried. To 15−20 mg of each sample, deionized water (1 mL) was added. To 100 μL of the resulting solution was then added 100 μL of internal standard (I.S.; 4 mg mL^{-1} 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^{-1}) 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 (13000 rpm, 5 min) before injection in the GC apparatus. It is worth noting that following oximation−trimethylsilylation derivatization, reducing compounds (e.g., residual D-fructose and HMF) provide two peaks in the GC

chromatograms, corresponding to the *syn*- and *anti*-TMS-oximes, while nonreducing derivatives (e.g., DFAs and the I.S.) provide a single peak.

GC-FID Analysis. GC-FID was carried out using an Agilent 6890 Series Plus chromatograph with an EPC injector fitted with a cross-linked 5% phenyl-dimethylsiloxane column (HP-5; 30 m × 320 μm × 0.25 μm). Operating conditions were: injection port temperature 310 °C; splitting ratio 25:1; injection volume 1 μL of derivatized samples; column oven temperature programmed from 180 to 310 °C at 5 °C min^{-1}, with a 25 min hold at 310 °C; carrier gas helium (constant flow at 1.2 mL min^{-1}); 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 (*14*). Response factors (RFs) for HMF (1.13), D-fructose (1.42) and eight individual DFAs (α-D-fructofuranose β-D-fructofuranose 1,2′:2,1′-dianhydride, 0.75; di-α-D-fructofuranose 1,2′:2,1′-dianhydride, 0.68; di-β-D-fructofuranose 1,2′:2,1′-dianhydride, 0.86; α-D-fructopyranose β-D-fructopyranose 1,2′:2,1′-dianhydride, 0.76; di-β-D-fructopyranose 1,2′:2,1′-dianhydride, 0.85; α-D-fructofuranose β-D-fructopyranose 1,2′:2,1′-dianhydride, 0.68; β-D-fructofuranose β-D-fructopyranose 1,2′:2,1′-dianhydride, 0.78; di-α-D-fructofuranose 1,2′:2,3′-dianhydride, 0.89), at concentrations similar to those encountered in the experiments, were determined relative to the internal standard phenyl β-D-glucopyranoside and use for quantification 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 DFA components in the mixtures.

UV−Vis Spectrometry Analysis. Caramel samples after centrifugation, filtering and freeze-drying were diluted to 50 mg mL^{-1} in deionized water to measured the absorbance at 420 nm by a diode array spectrophotometer (Hewlett Packard HP 8452). Deionized water was used as a reference.

Determination of pH. The pH of caramel samples were measured for solutions at 50 mg mL^{-1} in deionized water using a Crinson pH 2000 digital potentiometer calibrated with standard solutions of pH 7.0 and 4.0.

Acetylation and Column Chromatography. Samples were acetylated by treatment with Ac_2O−pyridine (1:1, 10 mL per 1 g of caramel) at room temperature for 16 h. The crude acetylated product was dropped into ice−water (100 mL per 1 g of starting caramel), extracted with CH_2Cl_2 (2 × 50 mL), the organic phase was washed successively with 1 N H_2SO_4 (2 × 50 mL), saturated aqueous $NaHCO_3$ (2 × 50 mL) and water (2 × 50 mL), dried by addition of anhydrous sodium sulfate (2 g), filtered and finally concentrated. The absence of organic material in the aqueous phase was checked during the separation and washing processes by TLC (aluminum plates, silica gel 60 F_{254}, E. Merck, 0.25 mm, eluent EtOAc−petroleum ether 2:1). The acetylation mixture (1 g) was subjected to column chromatography (glass column, 56.5 × 2.5 cm) with silica gel 60 (E. Merck, 230−400 mesh, 30 g) by eluting with a gradient of EtOAc−petroleum ether 1:1 (200 mL) → 2:1 (200 mL) → 4:1 (200 mL) → neat AcOEt (400 mL). Fractions (10 mL) were collected and checked by TLC (EtOAc−petroleum ether 2:1) in comparison with authentic standards obtained by acetylation of pure HMF, D-fructose and DFAs, as well as by ESI-MS. Fractions containing acetylated HMF, peracetylated D-fructose, DFAs/fructodisaccharides and higher oligosaccharides (mainly glycosyl-DFAs, though minor peaks for fructose oligosaccharides were also observed in the ESI-MS spectra) were combined separately and concentrated to give uncolored or yellow colored products. Most of the strongly colored melanoidines were retained in the column. In experiments with caramelization times lower than 2 h, the total recovered material accounted for >90% of the initial mixture, indicating that the mass product of the obtained caramels is essentially of glucidic nature. The relative proportions of the fractions corresponding to acetylated HMF, D-fructose and DFAs virtually matched the results obtained by GC quantification. The acetylation-column chromatography separation process was carried out typically for caramelization times of 0.5, 1, 1.5, 2, and 3 h in each experiment and allowed quantification of the relative proportion of glycosyl-DFAs and higher fructose oligosaccharides.

The above protocol did not allow separation of DFAs from fructodisaccharides. The latter are present in the reaction mixture at the early stages of the caramelization process. To confirm the correspondence of the peaks for the fructodisaccharides in the GC chromatograms and to quantify their abundance, the fraction containing DFAs and fructodisaccharides was subjected to deacetylation by treatment with 0.2 M NaOMe in methanol at room temperature for 4 h. The solution was neutralized with Amberlite IR120 (H^+) ion-exchange resin, filtered and concentrated. The residue was subjected to a second column chromatography (silica gel 60; 6:3 acetonitrile−water) to give separated DFAs and fructodisaccharides.

Electrospray Ionization Mass Spectrometry. Electrospray mass spectra (ESI-MS) in the positive ion mode were obtained on a Q-TOF Ultima Global hybrid quadrupole time-of-flight instrument (Waters), equipped with a pneumatically assisted electrospray (Z-spray) ionization source and an additional sprayer (Lock Spray) for the calibration solution (0.1% orthophosphoric acid in H_2O/CH_3CN 50/50, v/v). The caramel samples were dissolved in deionized water (0.01 mg mL^{-1}) and the solutions were directly introduced (5 μL min^{-1}) through an integrated syringe pump into the electrospray source. The source and desolvation temperatures were 80 and 150 °C, respectively. Nitrogen was used as the drying and nebulizing gas at flow rates of 350 and 50 L h^{-1}, respectively. Typically, the capillary voltage was 3.7 kV and the cone voltage 170 V. The mass range was 50−3000 Da, and spectra were recorded at 3 s/scan in the profile mode at a resolution of 10000 (fwhm). Data acquisition and processing were performed with MassLynx 4.0 software.

RESULTS AND DISCUSSION

The choice of D-fructose as the raw material for the preparation of DFA-enriched caramel-like products was motivated from technological, mechanistic and physicochemical considerations. Caramelization of D-fructose parallels the scheme presented in **Scheme 1** for sucrose where the routes involving the D-glucose moiety are canceled; fructose → glucose isomerization is very limited under caramelization conditions. Activation of D-fructose via fructosyl oxacarbonium cations, with formation of fructodisaccharides and their subsequent cyclization to DFAs, is much more readily achieved than activation of D-glucose due to the tertiary character of the ketose derived cation. Last, but not least, neat D-fructose exhibits outstanding water solubility, much higher than other D-fructose sources such as sucrose, inulin or levan. Since the first step of DFA formation is a bimolecular reaction, it was predictable that concentration would be a critical parameter for optimization of the reaction conditions. We hypothesized that heterogeneous caramelization of D-fructose would occur preferentially at the surface of the catalyst. High local concentrations would then translate into higher caramelization rates as compared with homogeneous reaction conditions, which should allow lowering caramelization temperature and, thereby, limiting the nonspecific dehydration processes leading to volatiles and melanoidines.

Caramelization of D-Fructose Promoted by Lewatit S2328 (H^+) Ion-Exchange Resin. The ion-exchange resin Lewatit S2328 was selected for our initial studies on ion-exchange resin promoted caramelization of D-fructose. It is a strongly acidic, macroporous type resin based in the styrene−divinylbenzene matrix, with low cross-linking degree and possessing sulfonic acid (−SO_3H) catalytic groups. The large internal surface allows access of relatively big molecules to the catalytic centers. This resin is currently used, for instance, to promote sucrose hydrolysis during the industrial preparation of invert sugar syrups. Before use, it was conditioned as described in Experimental Procedures, showing an exchange capacity of 5.39 mequiv of H^+ per 1 g of dry resin.

Influence of Temperature, Catalyst Proportion and D-Fructose Concentration in the Outcome of Caramelization Reactions. ESI-MS monitoring of caramelization experiments carried out with 70% (w/v) D-fructose solutions in deionized water, using a 25:1 w/w relative proportion of Lewatit S2328 (H^+) at 70 °C, indicated the formation of fructodisaccharides (*m*/*z* 365 for $[M + Na]^+$) and DFAs (*m*/*z* 347 for $[M + Na]^+$). GC-FID analysis of aliquots withdrawn at intervals showed the presence of low proportions of HMF, residual fructose, DFAs and a set of peaks

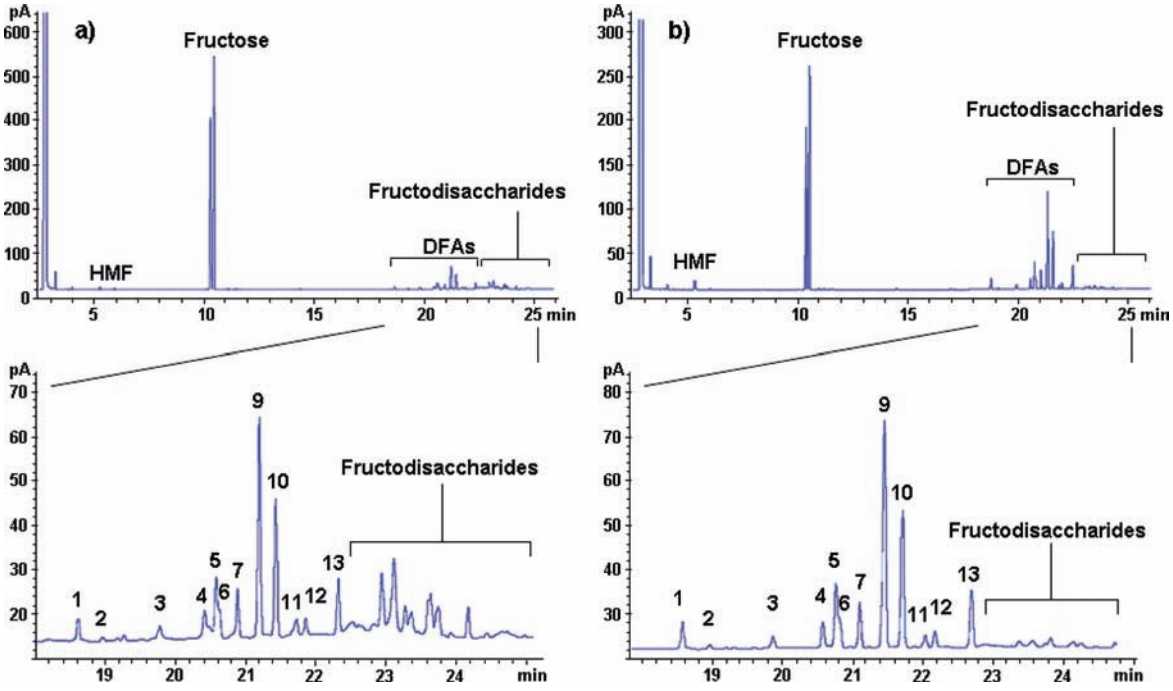


Figure 1. FID-GC profiles of caramel samples obtained by heating D-fructose (70% in deionized water) at 70 °C in the presence of Lewatit S2328 (H^+) resin (D-fructose:resin 25:1, w/w) during 1 h (**a**) and 5 h (**b**). The numbers over the peaks for DFAs correspond to the chemical structures in **Scheme 1**.

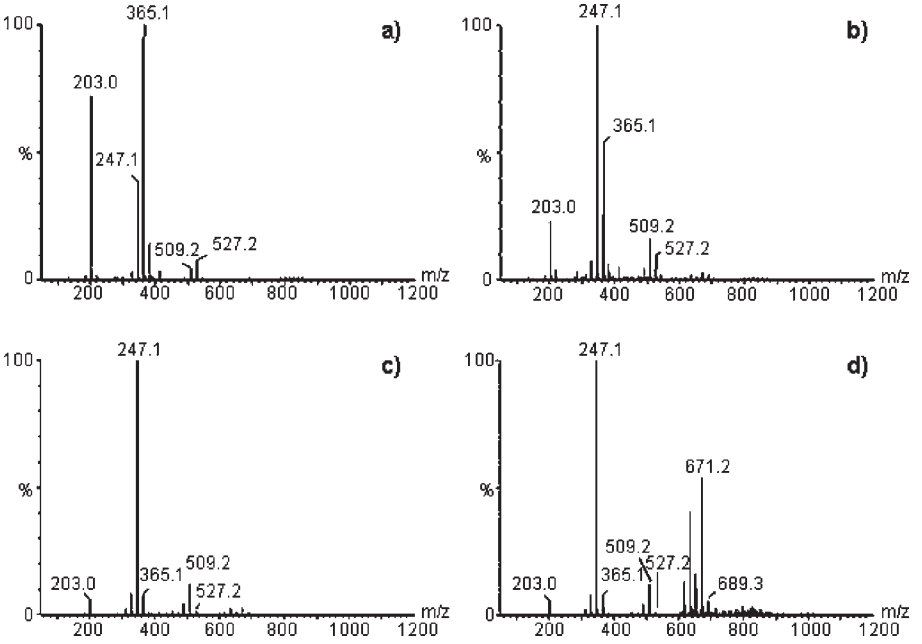
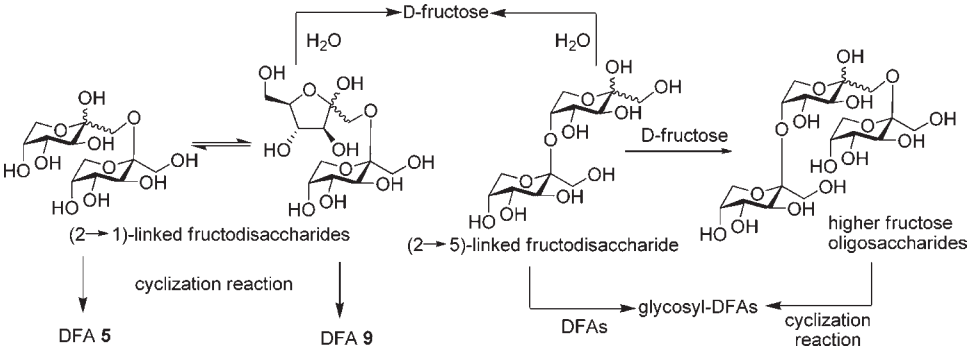


Figure 2. ESI-mass spectra of caramel samples obtained by heating D-fructose (70% in deionized water) at 70 °C in the presence of Lewatit S2328 (H^+) resin (D-fructose:resin 25:1, w/w) during 1 h (**a**), 3 h (**b**), 5 h (**c**) and 10 h (**d**). The peaks at *m*/*z* 203, 347, 365, 509, 527, 671, and 689 correspond to the $[M + Na]^+$ pseudomolecular ions for D-fructose, DFAs, fructodisaccharides, monoglycosyl-DFAs, fructotrisaccharides, diglycosyl-DFAs and fructotetrasaccharides, respectively. Their elemental composition was confirmed by accurate mass measurements as being $C_6H_{12}O_6Na$, $C_{12}H_{20}O_{10}Na$, $C_{12}H_{22}O_{11}Na$, $C_{18}H_{30}O_{15}Na$, $C_{18}H_{32}O_{16}Na$, $C_{24}H_{40}O_{20}Na$ and $C_{24}H_{42}O_{21}Na$, respectively.

for unknown compounds at higher retention times (RTs) (**Figure 1**). The intensity of those peaks increased at the beginning of the caramelization reaction and decreased with caramelization time, which correlated with a decrease of the peak corresponding to fructodisaccharides in the MS spectra (**Figure 2**).

The correspondence between the fructodisaccharides and the high RT peaks was unequivocally documented by MS after separation by a sequence involving (i) acetylation of samples obtained with reaction times <5 h followed by silica gel column chromatographic separation of the fraction containing peracetylated DFAs and fructodisaccharides and (ii) deacetylation and further separation of both families of unprotected compounds by a second column chromatography. Although individual components suitable for structure elucidation could not be isolated, glycosylation is expected to occur preferentially at the sterically less hindered primary positions, namely C-1 in the pyranose form and C-6 or C-1 in the furanose form. Nevertheless, the complexity of the region for fructodisaccharides in the GC chromatogram

Scheme 2. Schematic Representation of the Different Transformations that can Undergo the Fructodisaccharides Initially Formed during Caramelization[a]



[a] All combinations of cyclic forms of the fructose units and of glycosidic linkages between them are probably possible.

indicates that glycosylation at secondary positions also must occur to a significant extent. The (2→1)-linked disaccharides are direct precursors of DFAs following intramolecular cyclization. Fructodisaccharides with other type of linkages can undergo different transformations including (i) further fructosylation to give higher fructose oligosaccharides, (ii) glycosylation/cyclization to give a glycosyl-DFA, (iii) glycosylation of a DFA molecule in the reaction medium, which would also lead to a glycosyl-DFA, or (iv) reversion to D-fructose. All these processes are detailed in **Scheme 2**. Since the glycosidic bond in single-linked fructose oligosaccharides is much more labile than the glycosidic linkages in DFAs (*2*, *3*), thermodynamic equilibration favors higher proportions of DFAs and glycosyl-DFAs.

The proportion of HMF in the above experiment after 30 h remained rather low (1.2%), with 31% of DFAs and 64% of residual D-fructose (**Figure 3**). Small proportions of glycosyl-DFAs (major) and fructose oligosaccharides (minor) were also detected in the ESI-MS of the final product (m/z 324 + n·162 + 23 and m/z 180 + n·162 + 23 for the corresponding $[M + Na]^+$ pseudomolecular ions), which could be separated for quantification in the peracetylated form (see Experiental Procedures).

Temperatures below 60 °C or catalyst proportions below 3% by weight were ineffective to promote caramelization unless very long reaction times were used. Conversely, increasing the catalyst proportion and reaction temperature resulted in much faster conversions. Dimerization to give DFAs and oligomerization to give fructose oligosaccharides and glycosyl-DFAs (reversible) compete with dehydration to give HMF and polycondensation reactions leading to strongly colored compounds (irreversible). Di- and oligomerization are privileged at the first stages of caramelization, whereas long reaction times favored the last transformations. Actually, when caramelization of a 70% D-fructose solution using a 5:1 proportion of catalyst by weight at 90 °C was conducted for 3 days, a dark product was obtained consisting almost exclusively of HMF and melanoidines. Temperatures over 90 °C and catalyst proportions over 20% by weight did accelerate HMF and melanoidine formation to a greater extent that the reaction pathways leading to DFA derivatives and fructose oligosaccharides, making more difficult the control of the reaction.

It was anticipated that the initial concentration of D-fructose should have an important effect in the relative rate of the different processes that take place during caramelization. Thus, comparison of the composition of caramels obtained using initial D-fructose concentrations of 70, 80 and 90% (w/v) in deionized water at 90 °C using a 5:1 (w/w) D-fructose:Lewatit S2328 ratio evidenced an increase in the proportion of DFAs and glycosyl-DFAs/fructose oligosaccharides for the higher concentrated

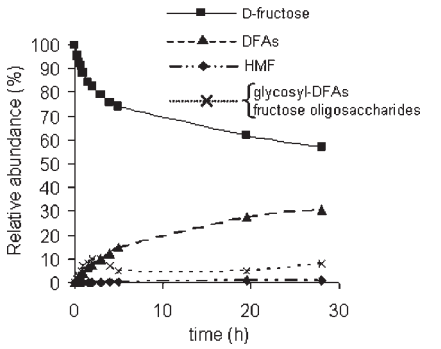


Figure 3. Variations of the relative proportions of residual D-fructose, HMF, DFAs and glycosyl-DFAs/fructooligosaccharides during caramelization of 70% D-fructose in deionized water at 70 °C in the presence of Lewatit S2328 (H^+) resin (D-fructose:resin 25:1, w/w). Note that the initial increase in the abundance of the glycosyl-DFAs/fructooligosaccharides components corresponds in fact to the fructodisaccharides, which become undetectable after 5 h.

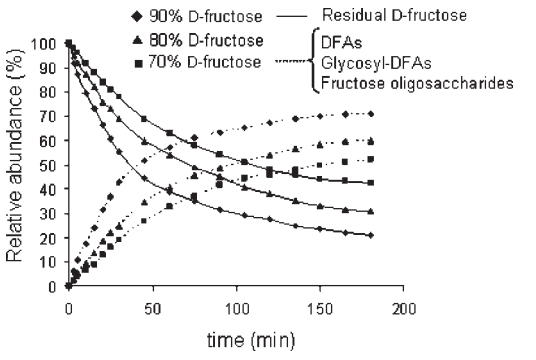


Figure 4. Variations of the relative proportions of residual D-fructose and of the ensemble of DFAs/glycosyl-DFAs/fructooligosaccharides during caramelization of D-fructose at different initial concentrations in deionized water at 90 °C in the presence of Lewatit S2328 (H^+) resin (D-fructose:resin 5:1, w/w).

syrups (**Figure 4**). Under these conditions, high conversions (>70% for the ensemble of DFAs/glycosyl-DFAs/fructose oligosaccharides) were achieved in short reaction times (2 h). At higher D-fructose concentrations (95% D-fructose in deionized water) a decrease in the conversion rate was observed, probably due to hampered diffusion in the highly viscous medium.

In all cases, fructodisaccharides were present in the reaction mixture at the beginning of caramelization, but they were progressively transformed into DFAs and higher fructooligosaccharides with caramelization advancement. The proportion of

DFAs increases rather fast at the beginning of the reaction and then start to decrease as a consequence of *in situ* fructosylation to give glycosyl-DFAs (**Figure 5**). Glycosyl-DFAs have been identified previously in sucrose caramel, and a fructosyl-DFA derivative has been isolated from the product of pyrolysis of inulin in the presence of citric acid (*33*).

Absorbance at 420 nm (A_{420}) has been used by researchers as a fast and reasonably accurate way to determine the brown color formed during caramelization (*13*). In our study, A_{420} was used as an indicator of the relative weight of the routes leading to glucidic materials and melanoidines, respectively. Using 50 mg mL^{-1} caramel solutions, A_{420} values higher than 4.0 indicated strong color development, with nonglucidic products accounting for more than 15% by weight of the total mass. Conversely, A_{420} values lower than 3.0 corresponded to amber/mahogany colored caramels with >90% of glucidic material.

The pH of the caramel became increasingly acidic with caramelization progress, going down to about 2.7−3.0. In principle, concomitant heterogeneous and homogeneous caramelization might then occur. However, in experiments carried out in parallel it was confirmed that caramelization did not proceed at this pH to any significant extent under homogeneous conditions (citric acid or *p*-toluenesulfonic acid) at these relatively low temperatures. It was concluded that acid resin-catalyzed caramelization is essentially a heterogeneous catalytic process, which is responsible for the outstanding increase in caramelization rate.

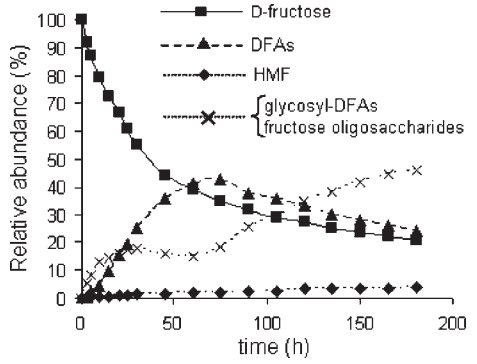


Figure 5. Variations of the relative proportions of residual D-fructose, HMF, DFAs and glycosyl-DFAs/fructooligosaccharides during caramelization of 90% D-fructose in deionized water at 90 °C in the presence of Lewatit S2328 (H^+) resin (D-fructose:resin 5:1, w/w).

Individual DFAs and Their Distribution as a Function of Caramelization Conditions. The relative distribution of individual DFA diastereomers upon caramelization of 90% D-fructose at 90 °C using 20% by weight of Lewatit S2328 resin at different reaction times is shown in **Figure 6**. 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**) are the most abundant DFAs at any stage of caramelization. Their relative proportion in the mixture follows divergent trends: the abundance of DFA **9** increases with time while that of DFA **10** decreases. The profile of DFAs obtained, with predominance of isomers containing at least a fructopyranoside moiety, is radically different from that encountered for citric acid-promoted caramelization of D-fructose or sucrose, where compound **10** was the major structure present in the DFA fraction (*14*, *29*), and from that obtained by pyrolysis of inulin in the presence of citric acid, where the difuranose derivatives **1**, **4** and **10** accounted for > 80% of the total DFAs (*34*). The obtained profile is instead very similar to that obtained by hydrogen fluoride activation of fructose or fructose-containing sugars and is indicative of a close-to-thermodynamic distribution (*27*−*31*).

The relative abundance of any individual DFA is a balance between its rate of formation and its rate of disappearance. Fructofuranose structures are kinetically favored due to the higher reactivity of the corresponding fructosyloxacarbenium cation. Yet, D-fructopyranose/D-fructofuranose interconversion is slower than glycosidation and subsequent DFA formation when very efficient promoters, such as HF, are used. In such circumstances, the reaction outcome depends, to a certain extent, on the initial cyclic form of D-fructose (*49*). Since crystalline commercial D-fructose used in our studies is in the pyranose form and the resin-supported sulfonic acid catalytic centers proved to be very efficient activators, the incorporation of fructopyranose moieties to the initial fructodisaccarides should be much favored (see **Scheme 2**). Tautomerization of the reducing fructose unit to the furanose form in the transient fructodisaccharides seems to be relatively fast, the driving force being probably the much higher thermodynamic stability of **9** over dipyranose structures such as **5** or **14**.

The rate of isomerization of DFAs during caramelization was relatively slow as compared to DFA formation and further glycosylation. Thus, when pure DFA **1** was subjected to caramelization under heterogeneous conditions with Lewatit S2328 the profile of DFAs obtained after 2 h (**Figure 7**) was still rather far from that obtained from D-fructose under identical conditions

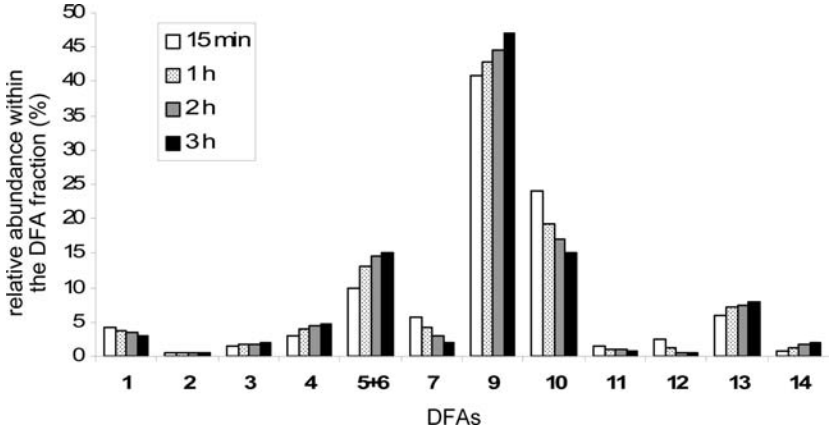


Figure 6. Variations of the relative proportions of the individual DFA isomers during caramelization of 90% D-fructose in deionized water at 90 °C in the presence of Lewatit S2328 (H^+) resin (D-fructose:resin 5:1, w/w). The proportion of DFAs **5** and **6** is given together since they are not fully resolved in the GC chromatograms.

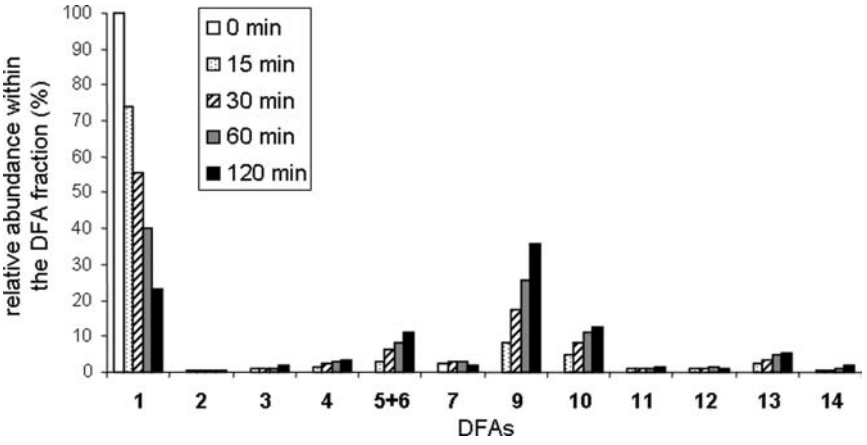


Figure 7. Isomerization of DFA **1** after treatment with Lewatit S2328 (H^+) resin under the conditions indicated in **Figure 4** caption. The proportion of DFAs **5** and **6** is given together since they are not fully resolved in the GC chromatograms.

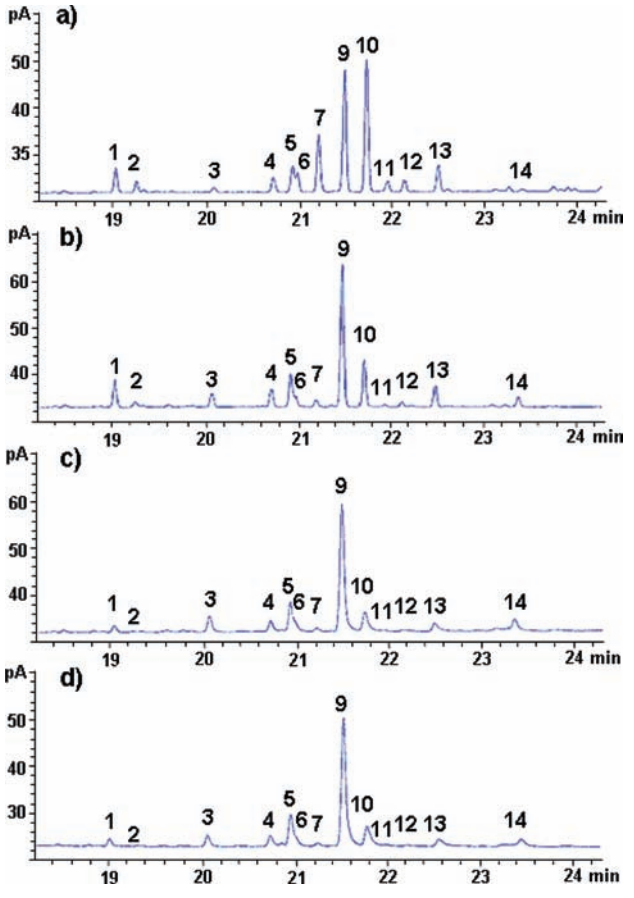


Figure 8. GC-FID profiles (DFA region) of caramels obtained by heating D-fructose at varying initial concentrations in deionized water at 90 °C in the presence of Lewatit S2328 (H^+) resin (D-fructose:resin 5:1, w/w). The numbers over the peaks for DFAs correspond to the chemical structures in **Scheme 1**.

(23% remaining **1** as compared with 3.2% in the DFA-enriched caramel from D-fructose; see **Figure 6**).

The isomerization rate was found to decrease with D-fructose concentration, which translated into higher proportions of the kinetic difuranose derivative **10** at the expenses of the thermodynamic furanose-pyranose isomer **9** (**Figure 8**). Glycosylation to give glycosyl-DFAs is another cause of disappearance of DFAs with time. Since **10** possesses two primary positions and **9** only one, a faster consumption of **10** by this route would be expected. Notwithstanding, the proportion of both **10** and glycosyl-DFAs increased with D-fructose concentration, which seems to indicate that the differences of reactivity between the primary and secondary positions in DFAs toward fructosylation over the resin are not so pronounced. Consequently, the relative distribution of individual DFAs is essentially dictated by the interplay of their rate of formation from the starting D-fructopyranose substrate, their rate of isomerization (a function of concentration) and their thermodynamic stability.

Effect of the Acid Ion-Exchange Resin Nature in Caramelization of D-Fructose. The efficacy of a series of commercial strongly acidic Lewatit ion-exchage resins with different mechanical properties was next investigated. In addition to the S2328 resin, the resins of references K1131, K1468, and K2641 were assayed. All of them bear strongly acidic sulfonic acid groups as the active centers and were conditioned before use. The characteristics as well as the combined proportions of DFAs and glycosyl-DFAs obtained for caramelization reactions of 90% D-fructose solutions in deionized water carried out in parallel using 10% catalyst by weight at 90 °C after 3 h are shown in **Table 1**. It can be seen that all resins behaved quite alike, indicating very similar accessibility of the reacting molecules to the active sites. The relative distribution of individual DFAs was also virtually identical, corresponding to a thermodynamic profile with the α-D-fructofuranose β-D-fructopyranose 1,2′:2,1′-dianhydride (**9**) as the most abundant diastereomer.

In order to assess the influence of the nature of the acid centers present in the resin in D-fructose conversion to DFAs, the ability of the weakly acidic Amberlite IRC50 ion-exchange resin to promote caramelization was additionally studied. This resin possesses a methacrylic matrix incorporating carboxylic instead of sulfonic acid groups, and showed an H^+ exchange capacity of 4.1 mequiv per gram of dry resin. Not unexpectedly, the higher pK_a of carboxylic acid groups as compared with sulfonic acid groups translated into a much lower efficiency in activating D-fructose. Under caramelization conditions identical to those used in the experiments with the sulfonic acid resins (vide supra), only the formation of a small proportion of fructodisaccharides was detected after 3 h. After 24 h the presence of DFAs became evident, and it took 3 days to attain 38% of DFAs, with 48% of unreacted D-fructose. Interestingly, the relative abundance of individual DFAs differed significantly from that obtained with sulfonic acid resins, with almost identical proportions of compounds **9** and **10**. This is consistent with a kinetic scenario in which D-fructopyranose/D-fructofuranose interconversion is faster than dimerization. The reaction proceeds then preferentially

Table 1. Conversions of Commercial D-Fructose[a] by Caramelization Reactions[b] Using Different Lewatit Ion-Exchange Resins[c]

Lewatit resin	resin type	particle size (mm)	exchange capacity (mequiv of H^+/g)	conversion[d] (%)
S2328	porous	0.4−1.25	5.39	50 ± 4
K1131	gel	0.5−1.6	5.35	50 ± 4
K1469	gel	0.61 (±0.05)	5.10	51 ± 2
K2641	porous	0.315−1.3	5.35	54 ± 4

[a] At 90% in deionized water. [b] At 90 °C during 1 h. [c] D-fructose:resin ratio 5:1 w/w. [d] Combined proportion of DFAs, glycosyl-DFAs and fructooligosaccharides in the final caramel.

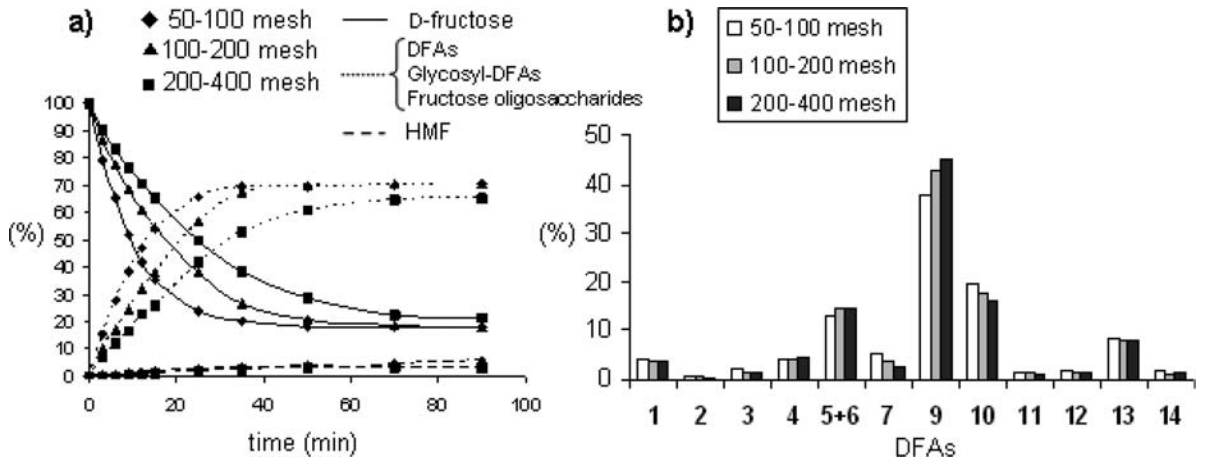


Figure 9. Variations of the relative proportions of residual D-fructose, ensemble of DFAs/glycosyl-DFAs/fructooligosaccharides and HMF (**a**) and relative abundance of the individual DFAs in the DFA fraction after 1 h (**b**) for caramelization of 90% D-fructose in deionized water at 90 °C in the presence of Dowex 50WX2 (H^+) resin (D-fructose:resin 5:1, w/w) with varying particle size.

through the D-fructofuranosyl oxacarbenium cation, leading to higher proportions of difructofuranose DFA isomers, among which the α-D-fructofuranose β-D-fructofuranose 1,2′:2,1′-dianhydride isomer **10** is the most stable one. The already discussed impaired isomerization at high concentration explains that thermodynamic equilibration is not achieved in spite of the long reaction time.

The possibility of modulating the DFA distribution in DFA-enriched caramels by acting on the initial D-fructose concentration and the nature of the resin catalyst is interesting. It might have an impact on both the organoleptic and the nutritional properties of the final product. For instance, it is known that difuranose dianhydrides such as **1** and **10** are sweet (*50*), whereas DFAs incorporating pyranose units have no taste. Prebiotic properties have been reported for the first ones, while, to the best of our knowledge, the second have not been investigated yet (*4*, *23*, *25*).

Effect of the Resin Particle Size in Caramelization Rate. The accessibility of the reacting species to the catalytic centers is a critical parameter in heterogeneous catalysis. In order to evaluate the influence of diffusion processes in the acid resin catalyzed caramelization of D-fructose, experiments aimed at determining the influence of the particle size in conversion rate were carried out.

Caramelization of D-Fructose Using Milled (< 80 μm) Lewatit S2328 (H^+) Resin. Freshly conditioned and dried Lewatit S2328 (H^+) resin was milled in a grinder provided of a filter to a particle size < 80 μm. When this material was used as caramel promoter, a spectacular increase in the caramelization rate was observed. Thus, for caramelization of 90% D-fructose in deionized water at 90 °C, analogous conversions were obtained with 20% by weight of intact Lewatit S2328 resin in 1 h (63.1% DFAs/glycosyl-DFAs/fructose oligosaccharides, 2.2% HMF, 29.9% residual D-fructose) than with 10% by weight of milled (< 80 μm) resin in just 3 min (59.9% DFAs/glycosyl-DFAs/fructose oligosaccharides, 2.7% HMF, 35.4% residual D-fructose). Catalyst proportions over 10% by weight relative to the initial D-fructose favored HMF formation. A better control of the reaction was achieved by decreasing the proportion of milled catalyst (6.67%) and the caramelization temperature (70 °C). Under these conditions, a product containing 70.1% of DFAs/glycosyl-DFAs/fructose oligosaccharides, 1.67% HMF and 24.8% residual D-fructose was obtained.

Caramelization of D-Fructose Using Dowex 50WX2 (H^+) Resin. The above experiment evidenced the tremendous impact of particle size in the kinetics of acid resin-promoted caramelization of D-fructose. To have a more precise evaluation of this parameter, the resin Dowex 50WX2 (H^+), commercially available in three different particle sizes (50−100 mesh, equivalent to 297−149 μm; 100−200 mesh, equivalent 149−74 μm; 200−400 mesh, equivalent to 74−37 μm) was assayed. This resin possesses a styrenic matrix with sulfonic acid groups and is, consequently, strongly acidic. After conditioning, an exchange capacity of 4.8 mequiv of H^+ per gram of dry resin was measured. **Figure 9** shows the evolution trends of HMF, DFA/glycosyl-DFAs and residual D-fructose after caramelization of 90% (w/v) D-fructose in deionized water at 90 °C using a 6.67% proportion by weight (relative to D-fructose) of each catalyst. Although conversions about 70% into DFA derivatives were achieved in all cases, the effect of particle size in the reaction rate is quite evident. Results obtained with the 74−37 μm Dowex 50WX2 (H^+) catalyst virtually matched those obtained with the milled (<80 μm) Lewatit S2328 (H^+) resin and confirm the relevance of this parameter in view of high-scale preparations.

The effect of particle size in the relative abundance of the individual DFAs in the resulting caramel is much less pronounced than the effect in dimerization and glycosylation rate, as seen in **Figure 9b**. A slight increase in the proportion of the thermodynamic

DFA **9** at the expenses of the difuranose kinetic DFA **10** was observed upon decreasing the catalyst particle size, which is consistent with the hypothesis that in the case of very efficient activators DFA formation proceeds through the D-fructopyranosyl oxacarbenium cation, fructopyranose/fructofuranose interconversion being comparatively slow.

Caramelization of D-Fructose Using Water-Soluble Polymer-Supported Catalysts. The above experiments seem to indicate that diffusion of the reacting species through the resin matrix or the accessibility of the catalytic centers at the surface of the resin particles is the limiting factor of caramelization under heterogeneous conditions. In order to investigate whether or not this limitation could be overcome using homogeneous reaction conditions, the suitability of two water-soluble commercial polymers bearing *p*-toluenesulfonic acid groups (MW 7×10^4 and 10^6 Da) to promote caramelization of D-fructose was assayed. The exchanged capacity of these poly(*p*-toluenesulfonic acid) polymers was 6.6 and 6.8 mequiv of H^+ per gram of dry polymer, respectively. Both polymers were found to be very efficient catalysts to promote D-fructose dimerization into DFAs, achieving conversions much higher than those reported for mineral acids (*2*, *3*). Results were very similar to those obtained with milled (< 80 μm) Lewatit S2328 (H^+) resin or 74−37 μm Dowex 50WX2 (H^+) resin for formation of DFAs/glycosyl-DFAs/fructose oligosaccharides and HMF. It is probable that for particle sizes smaller than 80 μm diffusion is no more the limiting factor in caramelization rate.

In conclusion, the present results demonstrate that acid ion-exchange resins are very efficient catalysts to promote D-fructose caramelization under heterogeneous conditions. Temperatures much lower than those classically used in caramelization or roasting processes of edible sugars can be employed, which allows a remarkable control of the different processes taking place during caramelization. Dimerization to give DFAs, which can be further fructosylated to give glycosyl-DFAs, is a preferred pathway when highly concentrated D-fructose solutions are used. The reaction proceeds through transient fructodisaccharides that undergo subsequent intramolecular cyclization. Small proportions of fructose oligosaccharides are also detected by ESI-MS, the whole ensemble of DFAs, glycosyl-DFAs and fructose oligomers reaching proportions above 70% when strongly acidic sulfonic acid resins are used as promotors. The route leading to glucidic materials (reversible) competes with dehydration to give HMF and polycondensation reactions affording melanoidines (irreversible). Using short enough reaction times relatively low proportions of HMF (< 2%) and colored products are produced. Most interestingly, the relative abundance of individual DFA structures in the caramel can be modulated by acting on reaction conditions and the nature of the catalyst, offering a unique opportunity to investigate the food properties of well-characterized DFA-enriched products, a most appealing goal in view of the amount of evidence pointing to beneficial nutritional effects of some DFAs. Details that illustrate the potential of this technology to produce caramels with functional food properties will be published in due course.

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