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Effect of sucrose concentration on the composition of enzymatically synthesized short-chain fructo-oligosaccharides as determined by FTIR and multivariate analysis

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ABSTRACT

Fructo-oligosaccharides (FOS) are mixtures of oligosaccharides composed of fructose and glucose units. As their composition is determined by the synthesis conditions, the goals of this work were: (a) to engineer FOS of different composition by adjusting the sucrose concentration used as initial substrate; (b) to define partial least square (PLS) based-models to quantify all the sugars present in the reaction medium directly from the FTIR spectra. The yield of each reaction was calculated as the percentage of initial sucrose converted to each oligosaccharide, as monitored by HPLC. In parallel, the reactions were followed by FTIR. Six different PLS models aiming to determine the concentration of each carbohydrate present in the reaction medium were calibrated and independently validated. The means of predicted values fitted well to those obtained by HPLC. Determining FOS composition directly from the FTIR spectra represents a useful tool to monitor enzymatic synthesis, with strong impact at both an academic and an industrial level.

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1. Introduction

Fructo-oligosaccharides (FOS) are small chain oligosaccharides composed of fructose units linked by (2→1)-β-glycosidic bonds and a single D-glucosyl unit at the non-reducing end. In most cases, FOS are mixtures of short chain oligosaccharides, namely 1-kestose [degree of polymerization (DP) equal to 3], nystose (DP4) and 1^F-fructofuranosylnystose (DP5) (Crittenden & Playne, 2009).

FOS are well recognized prebiotics, that is, non-digestible food components that beneficially affect the host health by selectively stimulating the growth and/or activity of one or a limited number of bacteria in the colon (Gibson & Roberfroid, 1995). They have a great economic importance as they are used in infant formula and other functional food products (Romano, Tymczyszyn, Mobili, & Gómez-Zavaglia, 2015). FOS are also low-calorie and non-cariogenic sweeteners, and effective protectants of biological structures during dehydration processes (Romano et al., 2014; Schwab, Vogel, & Gänzle, 2007). This latter property has been ascribed to their capacity to interact with lipid membranes, which in turn is dependent on their structure and on their DP (Hincha, Popova, & Cacela, 2006; Hincha et al., 2007).

Short chain FOS (*i.e.*: DP3, DP4, DP5) are generally produced from sucrose by transfructosylation reactions using fructosyltransferases (β-fructofuranosidase, EC 3.2.1.26 or β-D-fructosyltransferase, EC 2.4.1.9) as biocatalysts (Vega & Zuniga-Hansen, 2011, 2014; Vega-Paulino & Zuniga-Hansen, 2012). The composition of the FOS obtained can be modulated by adjusting the reaction parameters

Abbreviations: FOS, fructo-oligosaccharides; DP, degree of polymerization; DP3, degree of polymerization equal to three; DP4, degree of polymerization equal to four; DP5, degree of polymerization equal to five; HPLC, high performance liquid chromatography; FTIR, Fourier transform infrared spectroscopy; PLS, partial least square; FU, fructosyltransferase units; *X*, sucrose conversion; X_{max}, maximum sucrose conversion; *Y*, yield; MSC, mean centering correction; EMSC, extended multiplicative scatter correction; RMSEC, root mean square error of calibration; RMSEP, root mean square error of prediction; SEC, standard error of calibration; SEP, standard error of prediction; Glu, glucose; Fru, fructose; Tg, glass transition temperature.

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(*i.e.*, time, temperature, pH, enzyme source and substrate concentrations).

Commercial enzyme preparations have economic and technical advantages, including low price, versatility and stability under reaction conditions of industrial processes. Most of them have both transfructosylase and hydrolase activities, thus to synthesize short chain oligosaccharides, preparations with high transfructosylase activity are those to be selected. *Viscozyme L* from *Aspergillus aculeatus* (Novozyme, Denmark) is an adequate enzymatic preparation because it has both high transfructosylation activity and high transferase/hydrolase ratio (Lorenzoni, Aydos, Klein, Rodrigues, & Hertz, 2014; Lorenzoni et al., 2015; Vega-Paulino & Zuniga-Hansen, 2012).

The economic importance of FOS requires on quick and reliable methods to monitor their synthesis. Even though the usefulness of high performance liquid chromatography (HPLC) in determining the composition of sugar mixtures is unquestionable, Fourier transform infrared spectroscopy (FTIR) is nowadays a trustworthy technique to ascertain structural and physical properties of carbohydrates. As no exogenous chemical reagents are needed, samples require almost no preparation and analytical testing does not generate hazardous waste, FTIR is definitely a useful tool to determine the composition of complex oligo- and polysaccharides in a quick and environmentally friendly way (Anjos, Campos, Ruiz, & Antunes, 2015; Coimbra, Gonçalves, Barros, & Delgadillo, 2002; Santos, Gerbino, Tymczyszyn, & Gomez-Zavaglia, 2015). The use of FTIR in tandem with multivariate analysis has enabled an expeditious determination of the oligo and polysaccharide composition of different products, including commercial sugars (Kačurakóvá & Wilson, 2001), cellulose, pectins (Fellah, Anjukandi, Waterland, & Williams, 2009), starch, hemicelluloses, carrageenans, hyaluronates (Fellah et al., 2009; Kačurakóvá & Wilson, 2001), fruits (Bureau et al., 2009), cereals (Cozzolino, Roumeliotis, & Eglinton, 2014), honey (Anjos et al., 2015) and wine extracts (Coimbra et al., 2002). Moreover, different enzymatic reactions including sugars either as substrates or as products have been monitored using FTIR (Baum et al., 2013; Chiş, Fetea, Taoutaou, & Socaciu, 2010; Schindler, Le Thanh, Lendl, & Kellner, 1998).

With regard to FOS, FTIR and multivariate analysis were used to qualitatively characterize FOS in strawberries (Blanch, Goñi, Sanchez-Ballesta, Escribano, & Merodio, 2012), barley (Cozzolino et al., 2014) and agave (Mellado-Mojica & López, 2015). Trollope, Nieuwoudt, Görgens, and Volschenk (2014) used FTIR to quantify the consumption of sucrose as an indicator of the activity of β-fructofuranosidases from different origins. More recently, they developed partial least square (PLS) calibration models based on FTIR spectra for the screening of β-fructofuranosidases libraries generated by random mutagenesis (Trollope, Volschenk, Görgens, Bro, & Nieuwoudt, 2015).

Considering that the composition of FOS determines both their prebiotic properties and their capacity to interact with lipid membranes, and that this composition is in turn determined by the conditions of synthesis, the goal of this work was twofold: (a) to obtain FOS of different composition by adjusting the initial sucrose concentration; and (b) to define models based on multivariate analysis to determine the composition of FOS throughout the synthesis, directly from the FTIR spectra.

2. Materials and methods

2.1. Materials

Viscozyme L was donated by Blumos SA-Chile. 1-kestose (DP3), nystose (DP4) and 1^F-fructofuranosylnystose (DP5) standards were purchased from Wako Chemicals (Richmond, VA, USA). Sucrose, glucose, fructose and other reagents were obtained from Sigma Chemical (St. Louis, MO, USA).

2.2. Methods

2.2.1. Synthesis of FOS

Sucrose solutions within 10% and 60% w/v prepared in distilled water were used as substrate for the enzymatic synthesis. The pH was adjusted to 5.5 with 2 M NaOH and 4% v/v Viscozyme L (56 FU/mL; FU: fructosyltransferase units) was used as biocatalyst, according to Vega and Zuñiga-Hansen (2014). The volume of the reaction mixture was 15 mL. Transfructosylation reaction was performed for 6 h at 50 ± 1 °C in 25 mL Erlenmeyer flasks with stirring (100 rpm). One FU was defined as the amount of enzyme required to transfer 1 μmol of fructose per minute at 55 °C, pH 5.5 and stirred at 100 rpm.

The progress of each enzymatic reaction was followed by taking samples at regular intervals (every 30 min the first 3 h and every 1 h afterwards, up to a total of 6 h). The reactions were stopped after 6 h by heating at 100 °C for 2 min. A scheme representing the enzymatic production of short chain FOS is presented as Supplementary material (Scheme S1).

The composition of oligosaccharides was analyzed by HPLC and FTIR (see Sections 2.2.2 and 2.2.3 below). The following parameters were considered:

- *Sucrose conversion (X):* Represents the percentage of the initial sucrose consumed in the reaction (Eq. (1)):

$$X = [(S_0 - S)/S_0] \times 100 \quad (1)$$

  where S_0 and S represent the initial and final concentration of sucrose, respectively.
- *Yield [$Y_{DP(n)}$]:* Represents the percentage of initial sucrose converted to FOS (DP3, DP4 or DP5) at a given time during the reactions (Eq. (2)). It was evaluated every 30 min the first 3 h of reaction and every 1 h afterwards.

$$Y_{\mathrm{DP}(n)} = \mathrm{DP}(n)/s_0 \times 100 \quad (2)$$

  where DP(n) represents the mass of short chain FOS produced, (n) indicates their DP (3, 4 or 5), and s_0, the initial mass of sucrose.

The yield was also determined for glucose (Y_{glu}) and fructose (Y_{fruct}), which are byproducts of transfructosylation.

2.2.2. HPLC analysis

The composition of carbohydrates obtained throughout the syntheses (Section 2.2.1) was determined by HPLC in a Perkin-Elmer Series 200 equipment (Massachusetts, USA) with refractive index detector and autosampler. The chromatographic column used was a BP-100 Ag+ (300 × 7.8 mm) for carbohydrate analysis (Benson Polymeric, Reno, NV, USA). The column is composed of a stable high cross-linked styrene–divinylbenzene copolymer resin in the silver form that can resolve saccharides as large as DP-7.

Column and detector temperatures were maintained at 50 °C and 40 °C, respectively. The progress of each enzymatic reaction was followed by taking samples at regular intervals according to Section 2.2.1. Once collected, reactions were stopped by heating at 100 °C for 2 min, and samples diluted, filtered through 0.22 μm Millipore Durapore membranes (Billerica, MA, USA) and eluted with milli-Q water (mobile phase) at a flow-rate of 0.4 mL/min. Chromatograms were integrated using PeakFit software (version v4.12).

The composition of samples was determined by assuming that the area of each peak was proportional to the weight percentage of the respective sugar of the total sugar mass (Boon, Janssen, &

van der Padt, 1999) and the accuracy of such an assumption was checked by making a material balance. External standards of fructose, glucose, sucrose, 1-kestose (DP3), nystose (DP4) and 1^F-fructofuranosylnystose (DP5) were used to determine their retention times and check the linear range of the measurements.

2.2.3. FTIR

In parallel to HPLC determinations (Section 2.2.2), the synthesis of FOS was also monitored by FTIR. To this aim, samples collected throughout the syntheses were transferred into 5 mL vials, frozen for 48 h at −80 °C and freeze-dried for 48 h at −50 °C using a Heto FD4 freeze drier (Heto Lab Equipment, Denmark). Freeze-dried samples were kept in desiccators containing silica gel until analysis. A similar freeze-drying protocol was carried out on 20% w/v solutions of standards (DP3, DP4, DP5, sucrose, glucose and fructose).

The FTIR spectra were registered in the 4000–500 cm^{-1} range on KBr pellets, prepared with a ratio of 1 mg sample/200 mg KBr. For each sample, at least six FTIR spectra were registered. Spectra were recorded in a transmission mode by co-adding 64 scans with 4 cm^{-1} spectral resolution, using OMNIC software (version 8.3, Thermo Scientific, MA, USA) on a Thermo Nicolet iS10 spectrometer (Thermo Scientific, MA, USA).

2.2.4. Multivariate analysis

Multivariate analysis and data pre-processing as mean centering correction (MSC) and extended multiplicative scatter correction (EMSC) were performed on the FTIR spectra, using The Unscrambler® software (version 10.2, CAMO, Norway). Six different PLS models were calibrated to determine the percentage composition of DP3, DP4, DP5, glucose, fructose and sucrose in the mixtures (Esbensen, 2005; Martens & Næs, 1989). A group of 117 FTIR spectra was used to calibrate each of the six models. These spectra corresponded to different syntheses (carried out using different initial concentrations of sucrose) and were obtained from independent experiments, covering the whole range of sugar concentrations. The composition of FOS obtained by HPLC (Section 2.2.2) was used as reference method to define the PLS models. The reliability and robustness of the calibrated models were determined as a function of their correlation, *R*-square, BIAS and their calibration and prediction errors (RMSEC and RMSEP). A set of 119 spectra collected independently from those used for calibration were used to validate the models. All the information regarding the set up of PLS models is shown in Table S1.

2.2.5. Reproducibility of results

All experiments were performed on duplicate samples using three independent syntheses. The relative differences were reproducible irrespective of the synthesis used. Analysis of variance (ANOVA) was carried out using the statistical program Infostat v2009 software (Córdoba, Argentina). Differences were tested by paired sample *t* tests, and if $P < 0.05$ the difference was considered statistically significant.

3. Results

Sucrose concentrations of 10%, 20%, 40% and 60% (w/v) were tested to modulate the composition of oligosaccharides in the FOS syrup. The higher the sucrose concentration, the lower its percentage of conversion (X) at a given time (Fig. 1A). The exponential fitting of the experimental data enabled the description of X over time as shown in Eq. (3):

$$X = X_{max}(1 - e^{-t/k}) \quad (3)$$

where X is the sucrose conversion, X_{max} is the maximum sucrose conversion, t is the reaction time and k is the sucrose decay constant. X_{max} is determined by the kinetics of formation and/or hydrolysis of the reaction products (see later in Section 4). As the values of X_{max} did not differ significantly ($P > 0.05$) for the syntheses performed with different initial sucrose concentrations (Fig. 1A), the average value of X_{max} (89.21 ± 0.56) was used to calculate k for each reaction (Table S2). These values adjusted to a linear model for the concentration of sucrose as described in Eq. (4) and Fig. S1.

$$k = 0.026S_0 + 0.123 \quad (4)$$

where k is the sucrose decay constant and S_0 is the initial concentration of sucrose in w/v.

The values of k enable the determination of the sucrose conversion (X) for any initial concentration of this sugar within 10% and 60% w/v, at any time of the reaction course using Eq. (3).

The yield of total FOS synthesized (Y_{FOS}) as a function of time showed two different patterns (Fig. 1B). At low sucrose concentrations (10% and 20% w/v sucrose) the maximum Y_{FOS} was reached after 1.5 h of reaction time and thereafter, it slightly decreased.

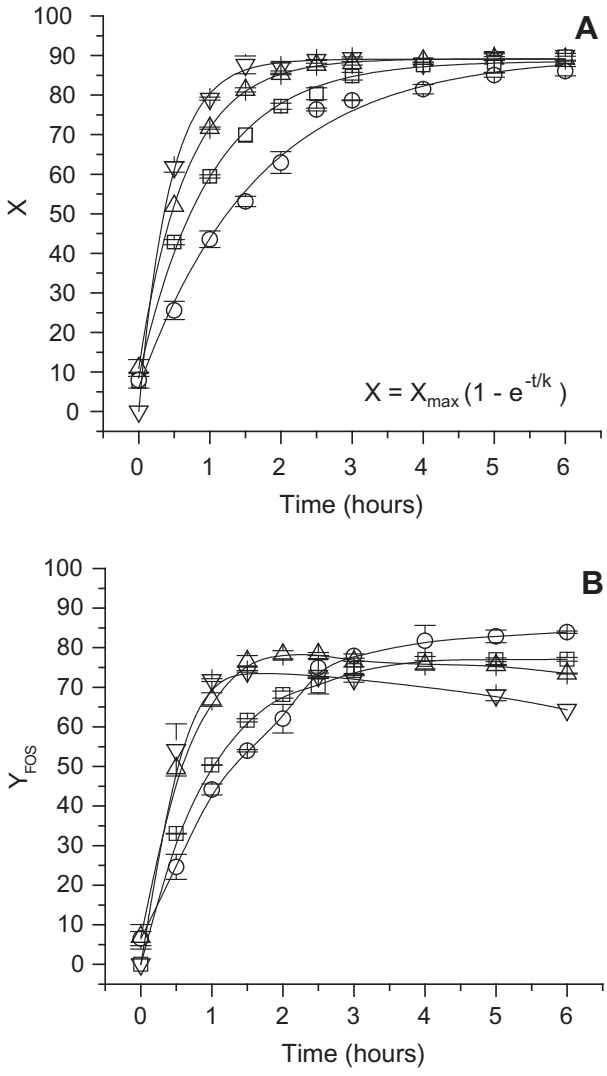

Fig. 1. (A) Percentage of sucrose conversion (X) as a function of time, using different initial concentrations of sucrose. Circles indicate 60% w/v initial sucrose; squares, 40% w/v; up triangles, 20% w/v; down triangles, 10% w/v. (B) Effect of the initial sucrose concentration on the synthesis of FOS at 50 °C and pH 5.5, using Viscozyme L as biocatalyst. Yield (Y) of total FOS *vs.* time of synthesis. Yield is expressed in grams of FOS/100 g sucrose. Total FOS include DP3, DP4 and DP5. Circles indicate 60% w/v initial sucrose; squares, 40% w/v; up triangles, 20% w/v; down triangles, 10% w/v.

At high concentrations of sucrose (40% and 60% w/v), the maximum Y_{FOS} was reached after 3 h, which then stabilized for the remainder of the 6 h reaction time. Statistical comparisons revealed no significant differences for the maximum Y_{FOS} ($P > 0.05$) achieved in each condition of synthesis.

The analysis of Y_{DP3}, Y_{DP4} and Y_{DP5} in the course of the reactions shows that DP3 was the oligosaccharide produced with the highest efficiency regardless of the initial sucrose concentration (Fig. 2A, B and C). Although the maximum Y_{DP3} was not significantly different for the different conditions of synthesis ($P > 0.05$), the lower the initial sucrose concentration, the earlier it was reached (Fig. 2A). The similarity between Y_{DP3} and Y_{FOS} patterns (Figs. 1B and 2A, respectively) indicates that Y_{DP3} is the main determinant of Y_{FOS} behavior under the tested conditions of synthesis.

The production of DP4 started increasing later, attaining the maximum Y_{DP4} after 6 h of incubation regardless of the initial concentration of sucrose. In addition, DP4 was more efficiently produced at the lowest sucrose concentrations (10% and 20% w/v; up and down triangles, respectively in Fig. 2B). Finally, DP5 started being produced even later than DP4 (Fig. 2C). Y_{DP5} was rather low and in none of the conditions surpassed 3 g DP5/100 g sucrose. Moreover, it is worth mentioning that no DP5 was produced at the highest sucrose concentration during 6 h of incubation under the studied conditions of synthesis (60% w/v sucrose).

Besides FOS, transfructosylation reactions led to the formation of two byproducts: fructose and glucose. Y_{fruct} remained more or less constant and below 15 g Fru/100 g sucrose in all the conditions assayed (Fig. 3A). In turn, maximum Y_{glu} was about 30 g Glu/100 g sucrose at 40% and 60% w/v sucrose after 6 h of incubation, and noticeably higher (about 40–50 g Glu/100 g sucrose) at the lowest concentrations of sucrose (Fig. 3B).

The FTIR spectra of pure glucose, sucrose, fructose, DP3, DP4 and DP5 are typical for carbohydrates and look similar to each other in most of the regions (Fig. 4). The strong band at ~3500 cm^{-1} arises from the hydroxyl stretching vibrational modes (νOH), those in the 2980–2850 cm^{-1} region, from the –CH stretching vibrational modes (νCH), the band at 1643 cm^{-1} from water molecules embedded in the amorphous sugar matrix, and the group of bands at ~1400 cm^{-1}, from the –CH bending vibrational modes (δCH) (Fig. 4) (Santos, Araujo-Andrade, Tymczyszyn, & Gómez-Zavaglia, 2014). The main differences were observed in the so-called "fingerprint region" of sugars (1200–900 cm^{-1}) (Santos et al., 2014), a complex region rich in bands attributed essentially to the C–O–C glycosidic linkage, the δCOH and the νC–C vibrational modes. Even when it is difficult to assign the vibrational modes corresponding to each individual band, the bands in this region collectively provide a complex pattern that unequivocally characterizes the analyzed compound and can be used to identify it in a pure sample.

When "fingerprint regions" of FTIR spectra registered throughout the enzymatic reactions were compared, an evolution from spectra corresponding to pure sucrose at time 0 to largely different spectra resulting from sucrose consumption and products' emergence was noticed (Figs. 5 and S2). This leads to complex spectra of largely overlapped bands arising from different proportions of the sugars present in the reaction media (*i.e.*: DP3, DP4, DP5 glucose, sucrose and fructose). Figs. 5 and S2 show the results for the reaction using 20% w/v sucrose as a representative example. The main differences observed throughout the enzymatic reactions include the decrease of bands at 1136 and 996 cm^{-1}, distinctive for pure sucrose, and the increase of the band at 1034 and the shoulder at 1077 cm^{-1}, corresponding to mixtures of FOS (see arrows in Figs. 5 and S2).

To determine the amount of DP3, DP4, DP5, fructose, glucose and sucrose present in the samples, six different PLS models were

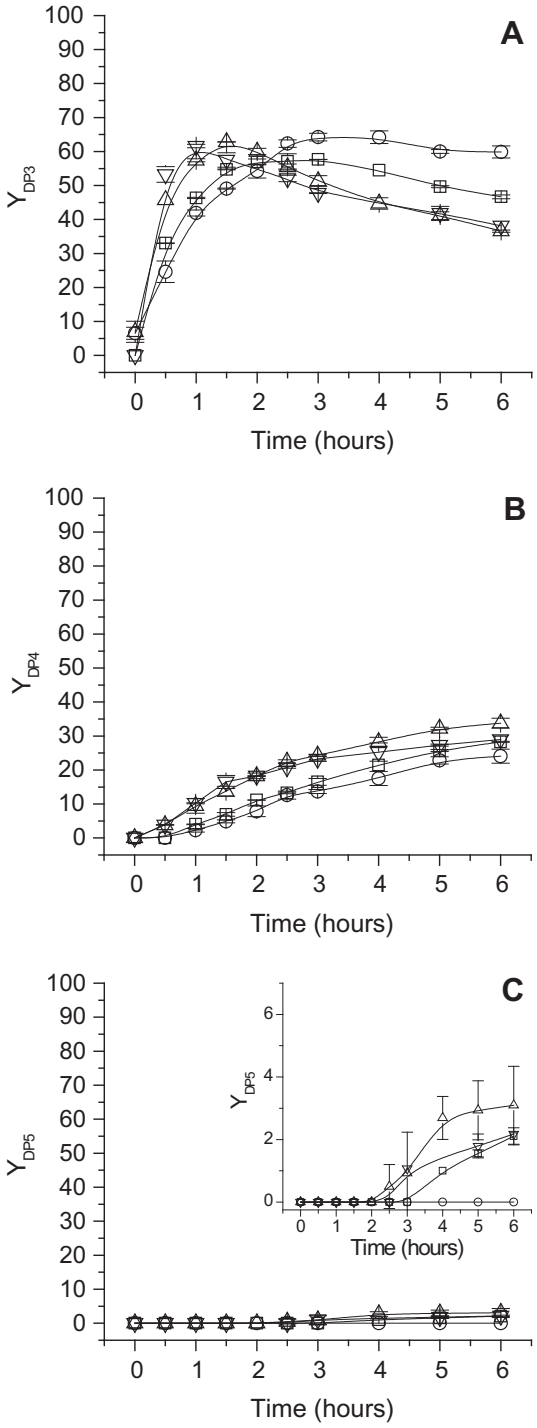

Fig. 2. Yield (Y) of short chain oligosaccharides *vs.* time of synthesis using different initial concentrations of sucrose. Yield is expressed in grams of $DP_{(n)}$/100 g sucrose, where (n) indicates the DP. Circles indicate 60% w/v initial sucrose; squares, 40% w/v; up triangles, 20% w/v; down triangles, 10% w/v. (A) Y_{DP3}; (B) Y_{DP4}; (C) Y_{DP5}.

defined. Results obtained by HPLC were used as reference. All PLS models were calibrated using 117 spectra and validated with an independent set of data composed of 119 spectra, collected under the same conditions (Fig. 6A–F). The means of predicted values agreed well with the results obtained by HPLC, thus supporting the use of the PLS models to investigate unknown samples. The complete data set of the performance calibration and validation processes are shown in Table S3.

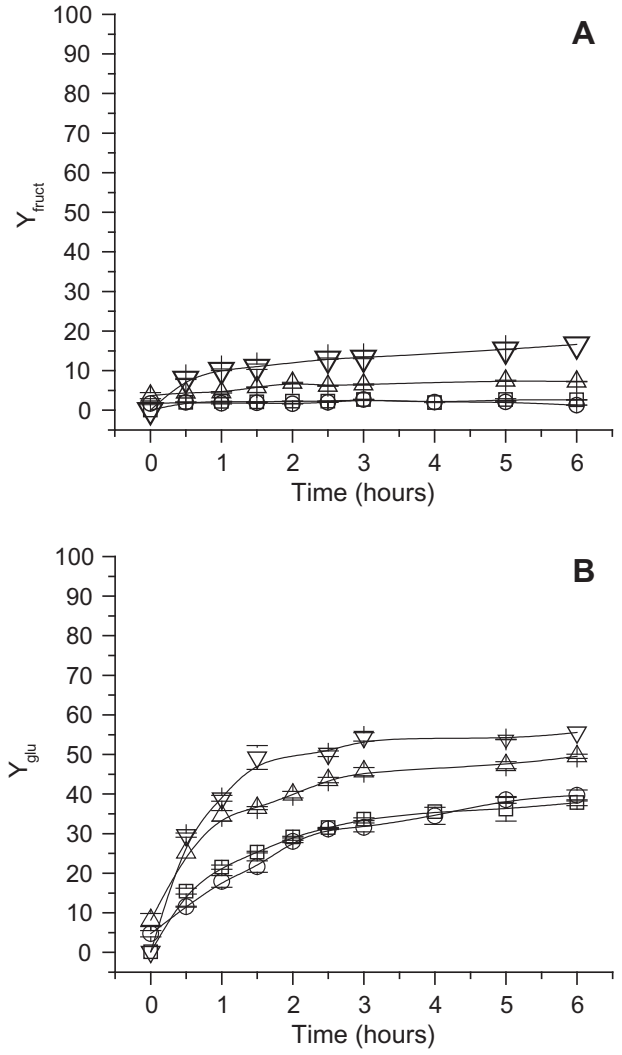

Fig. 3. Yield (Y) of monosaccharides *vs.* time of synthesis using different initial concentrations of sucrose. Yield is expressed in grams of fructose or glucose/100 g sucrose. Circles indicate 60% w/v initial sucrose; squares, 40% w/v; up triangles, 20% w/v; down triangles, 10% w/v. (A) $Y_{fructose}$ (Y_{fruct}); (B) $Y_{glucose}$ (Y_{glu}).

4. Discussion

The composition of FOS is directly related to their prebiotic and physico-chemical properties, and this composition can be modulated by adjusting the synthesis conditions. Bearing in mind this background, the results obtained in this work can be analyzed from two different points of view: (a) the effect of the initial sucrose concentration on the composition of FOS, and (b) the definition of quantification models based on FTIR and multivariate analysis to determine the composition of transfructosylation products in samples containing mixtures of FOS of different degrees of polymerization (DP), glucose, fructose and sucrose.

4.1. Effect of the initial sucrose concentration on the composition of FOS

The composition of the short chain FOS synthesized in this work was strongly determined by the initial concentration of sucrose, DP3 being the main product of reaction under all of the conditions assayed. DP3 is very important both in terms of prebiotic properties (Crittenden & Playne, 2009) and in the interaction with lipid membranes (Hincha et al., 2006, 2007). Therefore, determining the conditions at which its production is maximum is of great importance to engineer FOS with the desired properties.

The modeling of the sucrose conversion (X) with time [Eqs. (3) and (4)] enabled the determination of the minimum time required to reach X_{max} starting from any concentration of sucrose between 10% and 60% w/v. To facilitate the conjoint analysis of X and Y_{DP3} during the course of the reaction, Figs. 1A and 2A were merged to construct Fig. S3. This figure shows that for all the initial sucrose concentrations assayed, Y_{DP3} started decreasing once X_{max} was reached. Thus, determining the parameter t from Eqs. (3) and (4) is very relevant because it provides information to optimize the production of DP3. The present study showed that the best conditions to obtain high DP3/DP4 ratios were 10% w/v initial sucrose and 1 h of reaction time. If a higher contribution of DP4 is needed, the best approach is to start from 60% w/v sucrose and carry out the reaction for up to 4 h (Figs. 2A, B and S3).

To analyze the evolution of both reagents and products in the course of the enzymatic reaction, it must be considered that transfructosylases normally have both fructosyltransferase and hydrolytic activities. The enzymatic production of short chain FOS is a complex process in which different reactions of synthesis and hydrolysis occur simultaneously both in parallel and in series (Vega & Zuñiga-Hansen, 2014) (see also Scheme S1). Jung, Yun, Kang, Lim, and Lee (1989) carried out independent enzymatic syntheses of FOS, starting from pure sucrose, DP3, DP4 and DP5. They reported that transfructosylations occur through consecutive sets of disproportionation reactions in which the FOS synthesized in the first steps act as fructosyl donors and acceptors leading simultaneously to the production of FOS with DP immediately higher (DPn + 1) and lower (DPn − 1) than those of the FOS acting as reagents.

The decrease of Y_{DP3} after having reached its maximum yield can be explained in the light of these mechanisms. When the availability of sucrose (DP2) was high, as occurred at the beginning of the four syntheses carried out in this work, the reaction was displaced to the production of DP3 (DP2 + 1), with liberation of glucose (DP2 − 1). Once X_{max} for sucrose was attained (Fig. S3), DP3 acted as both fructosyl donor and acceptor to produce DP4 (Vega & Zuñiga-Hansen, 2014). As a result, and considering that sucrose had been mostly consumed, DP3 was no longer produced. In addition, different authors reported that DP4 cannot act as a fructosyl moiety donor substrate when sucrose or DP3 are present in the reaction medium (Nishizawa, Nakajima, & Nabetani, 2001; Vega & Zuñiga-Hansen, 2014). This explains why Y_{DP4} started increasing only when Y_{DP3} started decreasing (Fig. 2A and B). In turn, DP4 also acted as fructosyl donor and acceptor to produce DP5. In this work, DP5 was produced with higher efficiency at lower initial concentrations of sucrose (10% and 20% w/v) and prolonged reaction times (greater than 2.5 h) (Fig. 2B and C). On the contrary, at higher initial concentrations of sucrose (40% and 60% w/v), DP4 were synthesized less efficiently (in terms of yield) and, consequently, the production of DP5 was also less efficient (Fig. 2B and C). This indicates that the production of DP5 was determined by the kinetics of synthesis of DP4.

It must not be forgotten that transfructosylation of sucrose also includes hydrolytic reactions generating glucose as the main byproduct (Fig. 3). The presence of glucose in the reaction medium inhibits transfructosylation reactions (Vega & Zuñiga-Hansen, 2014). In addition, as the initial sucrose concentration determines the trans fructosylation/hydrolysis ratio (Khandekar, Palai, Agarwal, & Bhattacharya, 2014), it becomes an important parameter in modulating the formation of FOS. At low initial concentrations of sucrose (10% and 20% w/v), the maximum Y_{FOS} occurred quite early (t = 1.5 h) (Fig. 1B). The decrease in Y_{FOS} after the maximum value was reached, was probably due to the low availability of sucrose. Moreover, a decrease in the transfructosylation/hydrolisis ratio

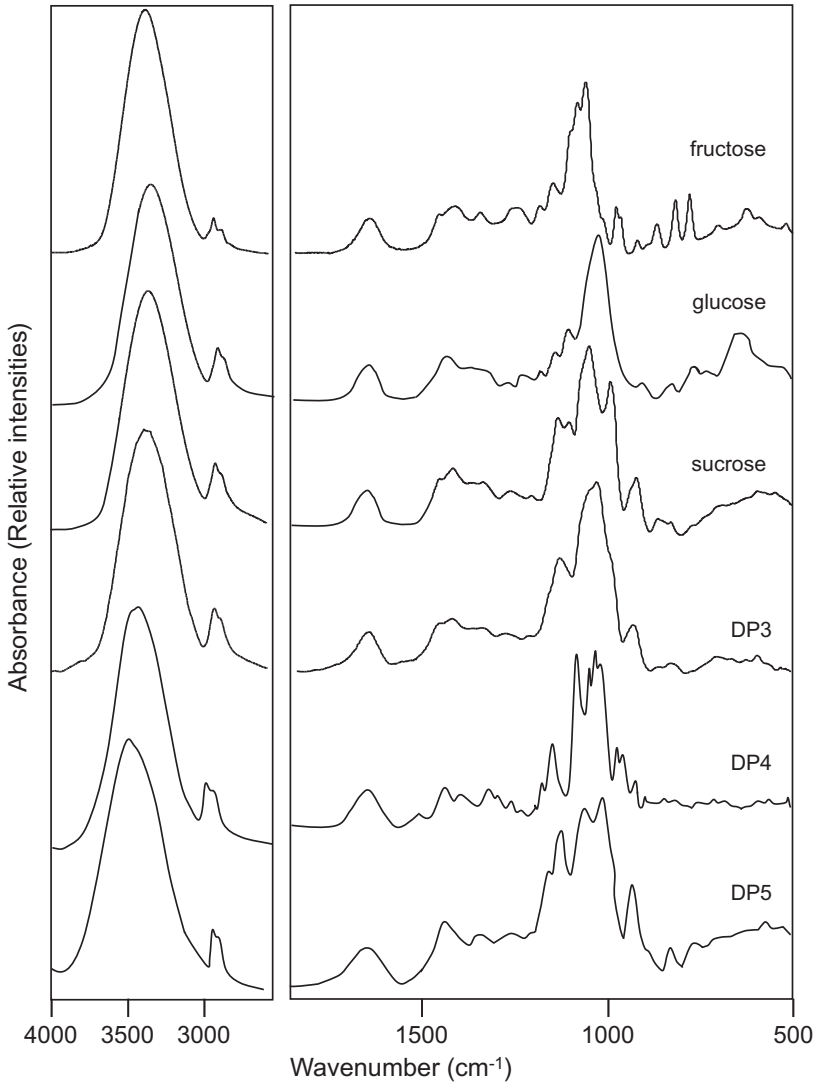

Fig. 4. FTIR spectra of fructose, glucose, sucrose, DP3, DP4 and DP5.

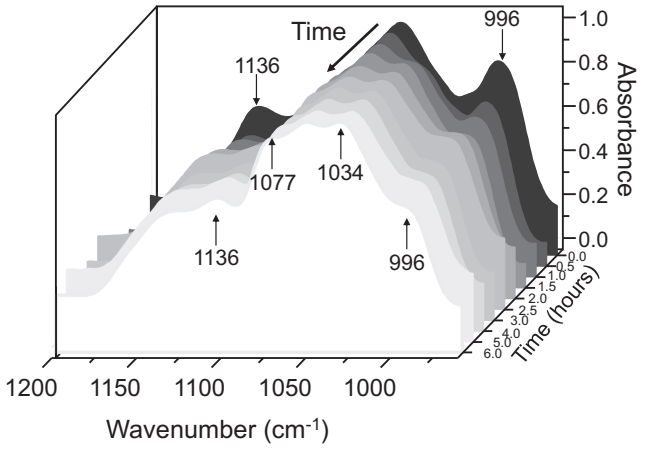

Fig. 5. Evolution of the FTIR spectra throughout the synthesis of FOS in the 1200–900 cm^{-1} region. Arrows indicate the bands increasing and decreasing throughout the synthesis (bands at 996 and 1136 cm^{-1} for sucrose, and band at 1034 and shoulder at 1077 cm^{-1}, for FOS). The spectra corresponding to the synthesis carried out with 20% w/v initial sucrose is shown as representative example.

may lead to the hydrolysis of FOS, which increases the concentration of fructose and especially glucose in the reaction medium (Figs. 1B and 3). To increase the purity of the FOS synthesized, and thus their prebiotic properties, glucose can be removed using a mixed enzymatic system with glucose oxidase (Vega & Zuñiga-Hansen, 2014). However, when FOS are synthesized to protect lipid membranes exposed to dehydration processes, the presence of glucose as a byproduct of transfructosylation could be potentially useful. In this sense, there are two accepted hypotheses to explain the protective capacity of sugars: (a) the interaction of sugars with lipid membranes by replacing water molecules and (b) vitrification (Romano et al., 2015). The water replacement hypothesis (a) considers that sugars directly interact with the polar heads of lipids in the dried state decreasing the membrane phase transition temperature. Smaller molecular weight sugars, like glucose or sucrose, are particularly efficient for this purpose. The vitrification hypothesis (b) explains protection by considering the capacity of sugars to form glassy states and maintain cells in a vitreous state at storage temperatures. In general, larger molecular weight sugars have higher Tg (Crowe, Carpenter, & Crowe, 1998). These two hypotheses are not exclusive. In fact, some articles suggest that the conjoint use of high Tg sugars alongside small molecular weight sugars with lower Tg values that still interact with membranes (*i.e.*: glucose or sucrose) may be a good protection strategy (Crowe et al., 1998;

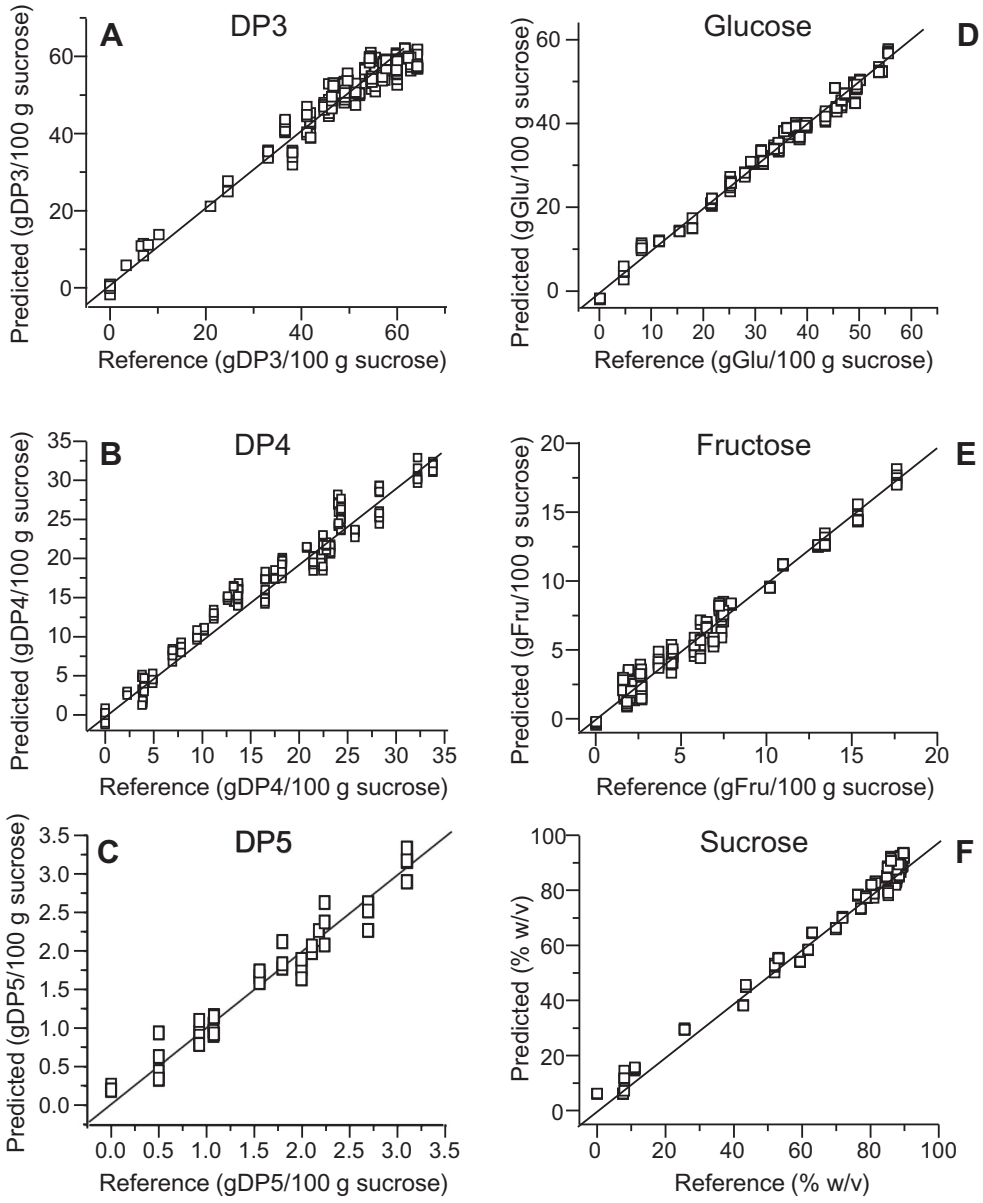

Fig. 6. Predicted *vs.* reference values of the enzymatic reactions' products. (A) DP3; (B) DP4; (C) DP5; (D) fructose; (E) glucose; (F) remaining sucrose.

Oldenhof, Wolkers, Fonseca, Passot, & Marin, 2005). In this context, the synthesis of FOS from low concentrations of sucrose (10% and 20% w/v sucrose) led to mixtures containing DP3, DP4, DP5 and glucose. Blanch et al. (2012) reported that Tg for pure kestopentaose (DP5) is high (121 °C). On the other hand, Hincha et al. (2007) reported that the protective effect of fructans is size and species dependent, and mixtures of fructans containing low and high DP can have a better protectant capacity. Therefore, the presence of glucose together with FOS could potentially improve the protective capacity of FOS obtained from lower concentrations of sucrose (Figs. 2 and 3). Nevertheless, this statement requires further research to be proved.

4.2. Definition of quantification models based on multivariate analysis to determine the composition of transfructosylation products

Carbohydrates strongly absorb in the so-called "fingerprint region" (1200–900 cm^{-1}) (Santos et al., 2014). This region is rich in bands arising from the C–O–C glycosidic linkage, the δCOH and the νC–C vibrational modes, that provide unique patterns that collectively characterize pure samples of sugars, as shown in Fig. 4. The FTIR spectra collected during enzymatic reactions (Figs. 5 and S2) correspond to mixtures of sugars, resulting from the absorption bands of each individual sugar. This increases their complexity and hence, their analysis requires the assistance of multivariate based methods.

For this reason, we calibrated and independently validated six robust PLS models that provided simple and cost-effective methods to simultaneously quantify substrates and products involved in the enzymatic synthesis of FOS. These models also provided additional information related to the evolution and yield of the synthesized FOS (Fig. 6). For example, quantifying the sucrose remaining in the reaction mixture (Fig. 6F) provides a direct idea about its conversion (X) (Figs. 1A and S3) and can prove useful when screening the activity of transfructosilases. Determining the concentration of glucose (Fig. 6D) gives information related to the inhibition of transfructosylation reactions. In turn, determining the concentration of each FOS (and thus, their ratio) is directly related to the properties of the obtained mixtures.

In summary, the PLS models defined in the present work are not restricted to the current conditions of synthesis, and can be used to determine the composition of FOS in other samples containing mixtures of sucrose, glucose, fructose, DP3, DP4 and DP5. This includes not only the monitoring of enzymatic syntheses but also the determination of the composition of unknown samples, with a clear application in the quality control of commercial FOS.

5. Conclusions

Even though FOS are mainly known for their prebiotic properties, they have other interesting properties, including their role as low-calorie and non-cariogenic sweeteners, and their capacity to protect lipid membranes during dehydration processes. Each of these properties is mainly ascribed to a particular type of FOS in the mixture of oligosaccharides.

In this context, this work integrates two complementary aspects related to the production of FOS: a rationalization of their enzymatic synthesis and the development of accurate models to quantify them. Understanding the effect of sucrose concentration on the final composition of FOS enables the possibility of engineering FOS according to the pursued goals.

One of the biggest challenges for the use of FTIR in foodomics is the development of adequate multivariate analysis based models to interpret the huge amount of information contained in the spectra. The PLS models developed in this work provide a quick, reliable and environmentally friendly tool to both monitor the enzymatic production of FOS and perform quality control analysis, in a much simpler way than traditional methods such as HPLC.

Considering the economic importance of the functional foods' market, the integrated approach developed in this work represents a useful and valuable tool both in academic research and in the food industry.

Competing interests

The authors declare that they have no competing interests.

Author's contributions

N.R. did the experimental work. M.S. did the multivariate analysis and R.V., the enzymatic analysis. A.G.-Z. and P.M. coordinated the work (analysis of results, discussion and writing of the manuscript). All authors have approved the final version of the manuscript.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.foodchem.2016.02.002.

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