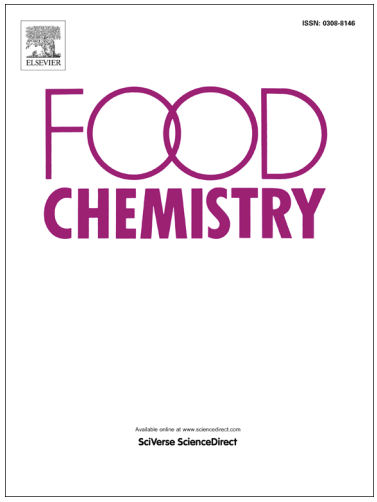


Frucooligosaccharides purification: complexing simple sugars with phenylboronic acid

R. Porras-Domínguez Jaime, Rodríguez-Alegría María Elena, Miranda Alfonso, Álvarez-Berber Laura Patricia, Castillo Edmundo, López Munguía Agustín

PII: S0308-8146(19)30205-5
DOI: https://doi.org/10.1016/j.foodchem.2019.01.130
Reference: FOCH 24225

To appear in: *Food Chemistry*

Received Date: 9 October 2018
Accepted Date: 23 January 2019

Please cite this article as: Jaime, R.P., Elena, R.M., Alfonso, M., Patricia, A.L., Edmundo, C., Agustín, L.M., Frucooligosaccharides purification: complexing simple sugars with phenylboronic acid, *Food Chemistry* (2019), doi: https://doi.org/10.1016/j.foodchem.2019.01.130

Frucooligosaccharides purification: complexing simple sugars with phenylboronic acid.

Porras-Domínguez Jaime R.[1], Rodríguez-Alegría María Elena[1], Miranda Alfonso[1], Álvarez-Berber Laura Patricia[2], Castillo Edmundo[1] & López Munguía Agustín[1] (*).

[1]Departamento de Ingeniería Celular y Biocatálisis, Instituto de Biotecnología, Universidad Nacional Autónoma de México. Av. Universidad 2001, Col. Chamilpa, C. P. 62210, Cuernavaca, Morelos, México.

[2]Centro de Investigaciones Químicas, Universidad Autónoma del Estado de Morelos. Av. Universidad 2001, Col. Chamilpa, C.P. 62210, Cuernavaca, Morelos, México

* Corresponding author (agustin@ibt.unam.mx).

Key words*: phenyboronic acid, fructooligosacarides, fructans, levans, prebiotics, sugar purification.*

Abstract

Prebiotic fructooligosaccharides (FOS) are currently obtained by enzymatic reaction with fructosyltransferases (FTFs) using sucrose as both donor and acceptor. In these reactions glucose results as the most abundant by-product, arising from each fructosyl transfer event and, together with fructose, because of the inherent hydrolytic activity of the FTFs. As FOS are mainly used as prebiotic in nutraceutical foods, the reduction or total elimination of monosaccharides is required. In this work the selective elimination of monosaccharides from a synthetic mixture FOS was achieved through the selective complexation of glucose and fructose with *phenyl boronic acid* (PBA) followed by ethyl-acetate extraction. The process was applied to a complex mixture of FOS obtained in an enzymatic synthesis reaction containing 40 % glucose, 15.8 % fructose and 35% of FOS,

elimination of the sugars was achieved through 3: 1 molar reactions, resulting in a levan-type FOS product with 97% purity.

1. Introduction

The use of fructooligosaccharides (FOS) as nutraceuticals has become an important trend in food science due to their prebiotic properties. Indeed, their application in the direct regulation of the human gut microbiota, the treatment and prevention of several diseases and the general well-being associated with their regular consumption have been extensively demonstrated (Ritsema & Smeekens, 2003). Evidence of these properties have been established through different *in vitro* and *in vivo* experimental approaches (M. Roberfroid, 2007), as well as the modern -omic techniques: transcriptomics, proteomics and genomics applied under a wide variety of experimental conditions (Candela, Maccaferri, Turroni, Carnevali, & Brigidi, 2010). Consequently, different strategies have been developed for the extraction and hydrolysis of plant inulins as well as for the direct synthesis of FOS of varied structure and molecular weight, through biocatalytic strategies (ref). Actually, the most popular industrial procedure adopted for FOS production is through enzymatic hydrolysis of inulins extracted from vegetal sources, mainly chicory and dahlia roots, using endo-inulinases (Sangeetha, Ramesh, & Prapulla, 2005).

The most abundant products of these reactions are FOS, also known as oligofructosides or oligofructose. Due to the nature of the hydrolytic reaction and considering the intrinsic inulin structure, FOS are characterized by a reducing fructosyl end and the absence of a terminal glucose. Actually, inulins are mainly

linear polydisperse polymers of fructose consisting of β(2–1) fructosyl–fructose linkages with usually a glucose moiety linked to the end of the chain by an α1–β2 bond as in sucrose (M. Roberfroid, 2007, Nollet, L. M., & Toldrá, F, 2012).

As these types of processes are carried out with inulin as substrate, depending on the nature and specificity of the inulinase preparation, either endo- or, to a minor extent, exo-inulinases, small quantities of fructose are obtained as by-product. This is also the case of chemical hydrolysis of inulin using organic or mineral acids, strong acid cation resins, zeolites or activated carbon as catalysts. Nevertheless, all these processes inevitably result in a certain amount of fructose contaminating the prebiotic FOS mixture (Sangeetha et al., 2005).

In contrast, synthesis of FOS may also be based on fungal FTFs synthesis, through transfer reactions using sucrose as substrate (M. B. Roberfroid, 2000; Sangeetha et al., 2005). The products of this kind of synthesis are mainly non-reducing-FOS with a terminal glucose molecule in one end (GFn) and a (n-1) fructose engaged in a C_2-OH glycosidic linkage with the last fructose in the other (C_1-OH or C_6-OH linkage). This is the case of 1- and 6-kestose, 1 and 6-nystose, 1 and 6 f-nystose), etc., corresponding to inulin or levan-type FOS respectively, among others. Nevertheless, and important feature in this type of synthesis is the presence of monosaccharides derived from sucrose hydrolysis, but also the important amount of glucose released as an intrinsic consequence of the FTFs mechanism, each time a sucrose molecule acts as a fructosyl donor. Inevitably, the final FOS mixture produced from FTFs contains glucose and fructose as by-products, which, due to their unselective effect on the hostage microbiota, require elimination from FOS when applied as prebiotics.

Methods to separate oligosaccharides from mixtures containing monosaccharides based on ultrafiltration and nanofiltration membranes have been reported. However, in this type of processes, are expensive and efficient separations are achieved only when low concentrations of monosaccharides are present (e.g. less than 5% w/v) otherwise, low productivity and/or large membrane surfaces are required (Kamada, Nakajima, Nabetani, Saglam, & Iwamoto, 2002).

An alternative available in the food industry is ion-exchange chromatography, commonly described in the production of high fructose corn syrups, to reduce the glucose content of glucose-fructose mixtures, among other processes. The physical-chemical basis of this separation is not exclusively ion-exchange, but rather the formation of a complex between metallic cations and hydroxyl groups of the carbohydrate molecule in a specific orientation, the stability of the complexes depending on the hydroxyl group orientation and the nature of the cation. The resins used to separate monosaccharides are charged with s-metal cations such as Na^{+} and Ca^{2+}. The complexes formed require 3 hydroxyl groups oriented in *cis* position. However, the stability constants are found in the 0.1-6.0 range, which are relatively low for an efficient separation (Alekseev, Garnovskii, & Zhdanov, 2007). Consequently, separation of glucose and fructose in a Ca^{2+} ions chromatography require very large columns and several steps of elution.

Boron and boronic compounds can also form complexes with carbohydrates. In fact, these compounds behave as Lewis acids due to their electron deficiency nature at the central boron atom possessing a vacant π-orbital, where the hydroxyl groups of sugars may act as electron pair donors (Sivaev & Bregadze, 2014).

The specificity of this kind of compounds is derived from the fact that the distance between the two hydroxyl groups in boron acids is equivalent to the distance between two vicinal hydroxyl groups of the carbohydrate in *cis*- position. A description of how sugars containing hydroxyl groups in *cis*- orientation may react with boron acids to obtain stables complexes has been described in detail (Hall, 2006).The equilibrium constants between boron acids with glucose and fructose are found in the range of 10^{-1} – 10^{2} M^{-1} in water at pH 5 to 8.5, which are higher than those reported for sugars and s-metal cations, suggesting a covalent character of the interaction between these compounds (Alekseev et al., 2007). In fact, several procedures to detect glucose involving colorimetric techniques and based on reactions with boron acids have been developed (Wu et al., 2013).

Despite the numerous reports dealing with boron complex formation with carbohydrates, the technical feasibility for FOS or saccharides purification from complex sugars mixtures using the boron complex approach has not been studied. In this paper, we propose a new method to purify FOS starting with a levan-type FOS mixture produced through a combination of levansucrase and endolevanase reactions from sucrose, as recently reported (Porras J et al, 2017), where the FOS product (35% w/v), contains a high concentration of glucose (40% w/v) and fructose (15% w/v). The process consists in the separation of fructose and glucose through the selective formation of non-soluble phenyl boronic acid complexes, easily extracted from the reaction bulk to render a FOS solution with high purity and low monosaccharides content suitable for consumption as prebiotic.

2.- MATERIALS AND METHODS

2.1 Expression and purification of fusion enzyme LevB$_1$SacB. The *Escherichia coli* Rosetta 2 strain was transformed with the plasmid pet22b+levb1sacb containing a fusion gene coding for the fusion enzyme LevB$_1$SacB, this containing both levansucrase and endolevanase activities designed for the synthesis of levan type FOS (Porras et al 2017). 50 ml of Luria Bertani medium containing triptone 10g/L (Bacto ™) , yeast extract (5 g/L Bacto ™,), NaCl 10g/L (J.T. BAKER® A.C.S) and ampicillin 200 µg/mL (Sigma Aldrich,), were inoculated with a single transformed colony and incubated for 18 h at 37°C and 200 rpm. The 50 ml served as inoculum of 2 L Luria Bertani medium with 200 µg/mL ampicillin distributed in two 2.5 L Fern Bach flasks. The cultures were incubated at 37°C and 200 rpm until an OD of 0.5 was reached (8h approximately) then the culture was induced with 0.4 mM IPTG (Gold Biotechnology, USA) and incubated for additional 18 h at 18°C and 200 rpm. Cells were then harvested by centrifugation at 8000 rpm for 20 min and washed twice with 50 mM, pH 6.0 potassium phosphate buffer (J.T. BAKER® A.C.S. Reagent), sonicated in an Ultrasonic Processor (Cole-Parmer,) with 3 pulses of 15 s with an amplitude of 70% and finally centrifuged at 9000 rpm for 30 min. The enzyme was recovered in the supernatant and purified by affinity chromatography in His-Trap columns (His-Trap 5 mL,) charged with nickel sulfate (J.T. BAKER® A.C.S.,). The enzyme was eluted in an ÄKTA Protein Purification Systems, GE Healthcare Life Science, using an imidazole (J.T. BAKER® A.C.S.) pH 6.5 buffer.

The pure enzyme fraction was dialyzed in dialysis bags with a 10 kDa molecular weight cut off against a 50 mM, pH 6.0 acetate sodium buffer, prepared with acetic acid (J.T. BAKER® A.C.S.,) and sodium acetate (J.T. BAKER® A.C.S.,) with a 1 mM of $CaCl_2$ (J.T. BAKER® A.C.S.). Finally, the levansucrase activity of the fusion enzyme was measured following the release of reducing sugars from sucrose through the DNS method as previously reported (Porras-Domínguez, Rodríguez-Alegría, Ávila-Fernández, Montiel-Salgado, & López-Munguía, 2017).

2.2 Synthesis of levan-type FOS. The standard conditions for the FOS synthesis were carried out in 50 ml volume reactors containing 7 U/ml $LevB_1SacB$ activity and 60% w/v sucrose as substrate (Sigma Aldrich,) in pH 6.0 acetate sodium buffer containing 1 mM $CaCl_2$ (J.T. BAKER® A.C.S). The reaction medium was incubated at 37°C and 300 rpm and monitoring for glucose, fructose and sucrose every hour by HPLC (6h or 90% conversion). The reaction was stopped by thermal inactivation, boiling the samples in a water bath for 10min.

2.3 Measurement of sugar concentration. The concentration of glucose, fructose and sucrose was measured by HPLC (Waters ™) in an aminated column (Prevail, Grace®, USA), with acetonitrile (J.T. Baker® HPLC-9012) / water 75:25 v/v, as mobile phase at a flow of 1.0 ml/min at 30°C, equipped with a refraction index detector. Glucose, fructose and sucrose (Sigma Aldrich,) were used as standards. Levan type FOS were measured as fructose equivalents after complete hydrolysis. For this purpose, 6 g/ml of total FOS samples in 50 mM pH 6.0 acetate buffer at 50°C were treated with 60 U/ml of Fructozyme (Novozyme). The reactions were stopped by

incubation in a boiling water bath for 10 minutes and fructose measured as described. FOS concentration is reported as fructose equivalents, considering that the molar mass of fructose incorporated in oligosaccharides correspond to 162 g/mol.

2.4 Levan type FOS purification.

In order to reduce the simple sugars content of the FOS solution, residual glucose and fructose were complexed with phenylboronic acid *(PBAc)*. Reaction conditions were explored in order to obtain an efficient separation.

2.4.1 Effect of phenylboronic acid (PBAc) **equivalents.** A volume of 5 ml of ethanol was added to 5 ml of the FOS samples with a given glucose and/or fructose content as specified in the next section. 1, 2 or 3 PBAc molar equivalents (Sigma Aldrich,) per sugar molar were added to the FOS mixture. PBAc added with continuous stirring until complete dissolution.

The pH was then adjusted to 7.0 with sodium hydroxide, and the solution evaporated in a rotatory evaporator first at 60°C under a 400 mmHg vacuum to evaporate ethanol, followed by an additional treatment at 70°C under 80 mmHg until complete dryness. The white powder obtained was washed with 10 volumes of ethyl acetate in a Büchner flask to eliminate the phenylboronic-monosaccharides complexes. Finally, the purified FOS obtained as powder were stored at room temperature.

2.4.2 Solvent effect. Alternative solvents were explored for the complex extraction: the procedure described in section 2.4.1 was repeated with acetonitrile or methanol

instead of ethanol, in extractions using 2.5 equivalents of PBAc per reducing sugar equivalent.

2.4.3 Monosaccharides extraction kinetics. The monosaccharides extraction kinetics was studied repeating the procedure described in section 2.4.1 using 2.5 equivalents of PBAc with incubation at room temperature, measuring the extraction reached after 0, 30, 60, 90 and 120 min of reaction after PBAc addition and pH adjustment. After each complexation time, the levan-type FOS purification procedure described above was applied.

2.4.4 Synthesis of glucose- and fructose- PBAc complexes. The procedure described in section 2.4.1 was applied to a 5ml standard solutions containing 60% w/v glucose or fructose in 50mM pH 6.0 acetate buffer to synthesize the phenyl boronic-glucose and phenyl boronic-fructose complexes 3 PBAc equivalents were added to monosaccharides solutions. The PBA-monosaccharides complexes were extracted with 10 volumes of ethyl-acetate- The extraction solution was evaporated in a rotatory evaporator under 400 mmHg vacuum at 50°C to concentrate the monosaccharide complexes and residual PBAc. The concentrated solution was kept in ice to crystalize the residual PBAc and removed later by filtration. The filtrate was further evaporated until complete dryness in the rotatory evaporator under 400 mmHg and 50°C. A sample of 100mg of each complex was used for RMN ^{1}H & ^{13}C spectroscopy analysis.

2.4.5 Thin Layer chromatography. Complexation reaction products were also analyzed by Thin Layer Chromatography in silica gel Plates 60 (MERCK) as the stationary phase and an ethanol:butanol:water (15:9:6) v/v mixture as the mobile

phase. Plates were eluted twice in the mobile phase and revealed with α-naftol solution and heat treatment.

2.4.6 Binding analysis of glucose and fructose with phenyl boronic acid via NMR Spectrometry.

NMR spectra were acquired on a Varian Unity NMR Spectrometers operating at 500 MHz for ^{1}H and 175 MHz for ^{13}C nuclei, respectively. Chemical shifts are listed in parts per million (ppm), referenced to $CHCl_3$ and were made based on ^{1}H–^{1}H COSY, HSQC, and HMBC spectral analyses as required. All coupling constants are given as numerical values.

Analytical:^{1}H-NMR (500 MHz, $CDCl_3$): δ (ppm) 8.31 – 7.20 (aromatic protons of complexed PBAC), 5.14 (dd, 1H, *J*=2.5 Hz, *J*=9.1 Hz, H-4), 4.88 (d, 1H, *J*=2.5 Hz, H-3), 4.72 (dd, 1H, *J*=1.75 Hz, *J*=8.25 Hz, H-5), 4.0 (d, 1H, *J*=13.5 Hz, H-1a), 3.88 (d, 1H, *J*=12.0 Hz, H-1b), 3.80 (dd, 1H, *J*=2.0 Hz, *J*=14.0 Hz, H-6a), 3.71(d, 1H, *J*=12.0 Hz, H-6b). ^{13}CNMR (175 MHz, $CDCl_3$): δ (ppm) 140-125 (aromatic carbons of complexed PBAC), C-2 104.55, C-4 86.47, C-5 75.04, C-3 70.86, C-1 64.15, C-6 61.91.

1D- and 2D-NMR spectroscopic measurements were performed to analyze the binding of monosaccharides D-glucose and D-fructose to PBAC. We used $CDCl_3$ as solvent for experiments under neutral non-aqueous conditions. Depending on the saccharide under investigation different binding complexes between the phenylboronic acid and monosaccharides were proposed. The structures were

deduced by comparing the chemical shifts and coupling constants of the boronic acid-glucose or fructose complexes with those described.

By mixing the ligand and monosaccharides in a 3:1 stoichiometric ratio, the ^{1}H NMR spectra of the solution showed signals from complexed sugars (δ 3.7 - δ 4.5 for D-glucose and δ 3.7- δ 5.5 ppm for D-fructose). Furthermore, different peaks could be detected which were shifted downfield. Signals corresponding to free fructose or glucose did not appear.

3.- Results

3.1 FOS synthesis and by-products

As in all FOS production processes where FTFs are involved using sucrose as substrate, glucose accumulates as the main by-product besides fructose and residual sucrose. Although there are no toxic consequences derived from these sugars, their presence in the product is not convenient considering that their final destination is the intestinal microbiota, where only soluble fiber and FOS favor the establishment of a beneficial microbiota and the overall gastric health. Considering that sucrose is used both as fructosyl donor and acceptor, one free glucose molecule will be obtained for each fructosyl unit incorporated to FOS. We have previously described the simultaneous levan synthesis/hydrolysis reaction involving the use of a levansucrase and a endolevanase for the production of a new levan-type FOS series from sucrose, where an inconvenient amount of monosaccharides are obtained as by products (Porras-Domínguez et al., 2017). In order to design a process for FOS purification, reaction conditions were stablished to ensure a systematic content of glucose, fructose and residual sucrose in the product reaction batches (M & M section 2.2). In this context, 1.7L reactors were employed for the synthesis of levan-type FOS, with a composition consisting of 35 % (w/w) of levan-type FOS with potential prebiotic properties, but also a simple sugars content including 40 %(w/w) glucose, 15 % (w/w) fructose and 10% (w/w) residual sucrose, as summarized in Table 1. This composition results from a compromise between the amount of residual sugars (glucose and fructose), sucrose conversion, and FOS diversity in the final product distribution. The larger the reaction time, the larger the

conversion of sucrose, but also the larger the reduction of FOS average molecular weight due to endolevanase hydrolysis.

This preparation was the basis to develop a specific FOS purification procedure eliminating glucose and fructose, through a phenyl boronic acid complexation extraction.

3.2 Extraction stoichiometry.

The first step in the design of the extraction process was to define the required molar rate of PBAc for the removal of both sugars. To address this question a set of experiments was designed using molar ratios from 1:1 to 3:1 PBA: glucose & fructose in the reaction medium (Figure 1). The 3:1 molar ratio is equivalent to 6 M PBAc for the 2 M glucose & fructose present in the FOS solution (1333 mM and 526 mM of glucose & fructose respectively). The experiments were carried out following the procedure described in section 2.2 in Materials and Methods. In Figure 1 the distribution and relative amount of the main components of the FOS solution are shown before and after treatment with the different PBAc: sugar ratios. A control experiment is included, for an extraction in the absence of PBAc. In all the extractions no significant change in FOS concentration was observed, suggesting that PBAc does not interact with FOS. In all experiments, glucose content (1333 mM) was substantially reduced, particularly at high molar PBAc ratios. Although with a

different performance, it is important to point out that fructose concentration is also reduced. When looking at the results in detail, it is clear that equimolar amounts of PBAc and sugars (1 PBAc eq per reducing sugar equivalent) was not enough for an efficient removal of all monosaccharides. If a total removal is the objective in a reasonable time, then 3 PBAc equivalents are the best option. Moreover, it seems that it exists a particular selectivity for the different sugars or FOS present in the mixtures. Actually, from Figure 1, glucose was preferentially removed at all PBAc:sugars ratios evaluated, followed by fructose, which is removed when PBAc: sugars ratio is higher than 2:1;finally sucrose was removed only at the highest PBAc:sugars ratio. Therefore, all three sugars were almost completely removed at the 3:1 PBAc:sugars ratio.

This behavior may be explained by the set of different factors that influence the removal of sugars in solution: the PBAc-sugar equilibrium constant together with the individual concentration of each sugar and the equilibria of the different isomeric forms of the sugars in solution.

The multiple equilibrium PBAc-sugar forms present in solution have already been reported (Springsteen & Wang, 2002). These authors demonstrate the higher affinity of PBAc to form complexes with fructose than with glucose. In fact, the PBAc-fructose equilibrium formation constant is forty times higher (Keq = 160 M^{-1}) than the PBAc- glucose constant (Keq = 4.6 M^{-1}). Therefore, and in contrast to the results we have just described, complexes should be preferentially formed with fructose. However, glucose concentration in the FOS mixture is almost 4 times higher than

fructose, resulting in a preferential PBAc-glucose complex formation, even at low PBAc:sugars equivalents, particularly in ethanol/water 50:50 pH 7.0, where reduced differences between the equilibrium constants of PBAc with the two sugars have been reported. In fact, in Figure 1 it may be observed that fructose is removed once glucose concentration has been substantially reduced, at PBA/sugar equivalent ratios higher than 1.5.

In terms of the equilibria of the different structural isomeric forms of sugars in solution,

The formation of PBAc complexes with glucose, fructose and sucrose is mainly determined by the presence of *cis*-vicinal hydroxyl groups (Figure 2). Hence, there is an interaction of PBAc with the two vicinal hydroxyl groups of C1 and C2 positioned in *cis* orientation in the α-D glucopyranose configuration of glucose. Nevertheless, several alternative configurations of PBAc:glucose complexes have been proposed. The α-glucofuranose is a glucose conformer whose equilibrium proportion in water solution is less than 1%. Nevertheless, a recent report describes the formation of complexes, with 2 PBAc molecules in the hydroxyl groups in C5 - C6, and C1 - C2 (Wu et al., 2013), explaining the PBAc molar excess requirement for total glucose removal.

The PBAc selectivity for glucose and fructose in the FOS mixture is explained by the fact that in the structure of levan type FOS, oligofructosides composed mainly of β-fructofuranose conformers, the most abundant oligosaccharides, have no hydroxyl groups in *cis* configuration, with the C2 compromised in the glycosidic bond with the C6 hydroxyl group of the next fructose molecule. In the case of free fructose, an

equilibrium of 4 different conformers exists in solution, where β-fructopyranose, is the preferential isomer form (70% in water solutions) wherein pairs of hydroxyl groups (C4- C5, as well as C2- C3) may complex with PBA; an additional isomer, β-fructofuranose (25% in water solutions) may also form a complex with PBAc with its C2-C3 hydroxyl groups. In the case of the less abundant conformers, while α or β fructopyranose may react with their C4-C5 hydroxyls, α–fructofuranose does not form complexes, but its concentration may be reduced as a consequence of the overall isomers equilibrium during the complexation reaction. In spite of the 4 possible interactions between fructose and PBAc, only the β-fructopyranose linked through the C4- C5 hydroxyl groups has been reported in reactions between fructose and boronic acids (Kim et al., 2008).

In conclusion, the multiple equilibrium sugar isomers and sugar isomers-PBAc forms in the reaction between PBAc and sugars substantially explain why a large molar excess of PBAc is required to remove all monosaccharides. A Thin layer chromatography plate comparing the FOS distribution before and after the extraction with 3 PBAc equivalents and the recovered sugars demonstrates the efficiency of the process (Figure 3).

3.3 Extraction optimization.

The initial experiments were performed assuming an immediate interaction between PBAc and sugars to reach equilibrium. We therefore explored the evolution of the extraction profile with extraction time as described in M&M section 2.2.2. It was

verified that the complexation is instantaneous as no differences were found in the amount of glucose and fructose removed after mixing or after 120 min of interaction. We may conclude that the reaction equilibrium is reached almost immediately with a concomitant decay in pH as previously reported for reactions carried out in aqueous systems (Springsteen & Wang, 2002). We also explored alternative solvents for the reaction. Two additional solvents were selected: acetonitrile and methanol. For each alternative solvent the extent of monosaccharides removed was quantified. Although ethanol has a hydroxyl group, it is not able to react with PBAc due to the absence of 2 hydroxyl groups in *cis* position, separated by a C-C bond distance, a requirement already discussed (Hall, 2006). This explains the similar results found for the extractions carried out with both methanol and ethanol (supplementary material). Finally, no differences in monosaccharides efficiency removal were found neither in acetonitrile, a solvent with a similar polarity as ethanol, where PBAc is soluble (supplementary material). In conclusion, all three solvents are suitable for the extraction process.

3.4 RMN analysis and structures.

In order to clearly define the major complexes structure between glucose, fructose and PBAc among all possible structures described, fructose, glucose and PBAc complexes were synthetized and extracted as described in M&M, section 2.4.4, for RMN ^{1}H and ^{13}C analysis.

The binding of fructose to boronic acids has been reported both in neutral non-aqueous and alkaline aqueous solutions. Extensive NMR experiments have shown that furanose binds with boronic acids by the β-D-fructofuranose and β-D-

fructopyranose forms. In alkaline aqueous solution seven different complexes were observed; while, under non-aqueous conditions, signals emerged from 13 different compounds. The number of isomers obtained and the anomers involved depend on the ratio between fructose and the boronic acid concentration (Norrild & Eggert, 1996). In our case, only one complex fructose-phenylboronic acid was observed according with the following evidence: ^{1}H NMR spectrum of PBAC-fructose showed seven signals in the saccharide region, three of them shifted downfield. The signals in the aromatic region of proton spectrum indicated also the presence of complexed PBAc. (Figure 4a). The *J*-coupling were particularly useful to distinguish the conformation of the D-fructose moiety. By comparing the proposed binding complexes with the different anomers present during the mutarotation of fructose, signals corresponding to only one complex appeared in the ^{1}H NMR and ^{13}C NMR spectra. The data obtained provides evidence that the phenylboronic acid was complexed to the β-D-pyranose form of fructose, which exists in aqueous solution at around 75% (Norrild & Eggert, 1996). We deduce an 1,2:4,5-α-D-fructopyranose complex with PBAC on the basis of a contemporary NMR study and reports data (Figure 4b) (Nicholls & Paul,2004.; Wood, 1974). ^{13}C chemical shifts for the boronic acid part of the complexes are given in the Experimental Section. The chemical shift assignments were in agreement with information from 2D NMR spectra obtained for the complex studied. β-D-fructopyranose binds favorably to boronic acids due to the preponderant presence of syn-periplanar hydroxyl groups in this anomer.

As far as glucose interactions with PBAc, the situation is more complex. The ^{1}H NMR and ^{13}C NMR spectra of PBAC and D-glucose showed chemical shifts for the sugar

part of the complex. In general, the results show that protons within the saccharide are more deshelled after binding to PBAc. Nevertheless, for D-glucose a readily interpretation of the spectra was not possible since the measurement of coupling constants, which are necessary for a further analysis, cannot be performed due to peak broadening.

However, the ^{1}H NMR spectra showed signals corresponding to the presence of five α anomer protons (δ=6.26, 6.28, 6.29, 5.77 and 6.55 ppm), corresponding to five complexes (ratio 25:25:42:1.5:6.5). β anomers were not detected (Figure 5a)

The results from NMR spectrum analysis, results suggest that, under our conditions, the PBAc probably bound one and two pairs of hydroxyl groups. The positions at C-1 and C-2 were critical for binding, while the remaining hydroxyl groups played a secondary role at C-3,5, C-5,6 and C-4,5,6 respectively. As the boronate template was synthesized in organic solvent under azeotropic distillation, neutral trivalent boronate esters instead of negatively charged tetravalent structures are expected as reported in the literature (Norrild & Eggert, 1995; Shiomi & Saisho, 1993).

In conclusion NMR data indicated that four complexes in the furanose form and one in the pyranose form were formed by glucose and PBAc. As we mentioned before glucose could interact with two boronic acid units in its furanose form, and we suggest the formation of α-D-glucofuranose-1,2:3,5-bis(borate), α-D-glucofuranose-1,2:5,6-bis(borate), and α-D-glucofuranose-1,2:3,5,6-bis(borate), and with one

boronic acid unit to yield α-D-glucofuranose-1,2 mono(borate) and α-D-glucopyranose-1,2 mono (borate) complexes (Figure 5b).

There are reports demonstrating the formation of boronic acid-D-glucose complexes by using ^{1}H and ^{13}C NMR spectroscopy under both neutral non-aqueous and alkaline aqueous conditions. From these studies, it was concluded that the preferred binding form is the α–D-glucofuranose bound to two boronic acid groups. In both neutral non-aqueous and alkaline aqueous conditions, the first binding site was position (1,2) of the furanose form of D-glucose. The binding site for the second boronic acid group is (3,5) and (3,5,6). Other papers mention that there is an intramolecular arrangement of glucose between furanose and pyranose forms during complexes formation, and that is a prerequisite for strong boronate complexation (Norrild & Eggert, 1995; Shiomi & Saisho, 1993).

4.- Conclusions

The purification of FOS obtained by different enzymatic synthesis strategies, may be efficiently performed through complexation of glucose, fructose and residual sucrose in an extraction process with PBAc. The process has a high selectivity towards glucose, fructose, and eventually sucrose, which are eliminated from the solution without interaction with the FOS product. When a 3:1 molar PBAc : sugars ratio is used in the extraction, a purity of around of 97% is obtained. The efficiency in terms of individual sugars separation is affected by the PBAc:sugars ratio and is

strongly affected by the large excess of glucose present in the FOS solution. We demonstrate through RMN analysis, that an excess of PBAc is required for an almost complete elimination of sugars as multiple sugar isomer forms coexist in equilibrium, particularly in the case of glucose. This simple and strait forward methodology can be used obtain high purity FOS obtained from FTFs reactions with sucrose to avoid hindrance of the FOS prebiotic effect by the simple sugars.

5.-Aknowledgments.

Jaime Porras acknowledges the National Council of Science and Technology (CONACyT) support during his PhD thesis. This work was supported by CONACyT-BMBF-2015-267620 and the National Autonomous University of Mexico grants UNAM-PAPIIT IT200316 and IN209016. We particularly thank Fernando Gonzalez for analytical support, as well as Martín Patiño and Mario Trejo for their advice in technology transfer procedures.

4.-References

Alekseev, Y. E., Garnovskii, A. D., & Zhdanov, Y. a. (2007). Complexes of natural carbohydrates with metal cations. *Russian Chemical Reviews*, *67*(8), 649–669. https://doi.org/10.1070/RC1998v067n08ABEH000343

Candela, M., Maccaferri, S., Turroni, S., Carnevali, P., & Brigidi, P. (2010). Functional intestinal microbiome, new frontiers in prebiotic design. *International Journal of Food Microbiology*, *140*(2–3), 93–101. https://doi.org/10.1016/j.ijfoodmicro.2010.04.017

Hall, D. G. (2006). *Structure, Properties, and Preparation of Boronic Acid Derivatives. Overview of Their Reactions and Applications*. *Boronic Acids: Preparation and Applications in Organic Synthesis and Medicine*. https://doi.org/10.1002/3527606548.ch1

Kamada, T., Nakajima, M., Nabetani, H., Saglam, N., & Iwamoto, S. (2002). Availability of membrane technology for purifying and concentrating oligosaccharides. *European Food Research and Technology*, *214*(5), 435–440. https://doi.org/10.1007/s00217-001-0486-6

Kim, N. H., Kim, H. J., Kang, D. Il, Jeong, K. W., Lee, J. K., Kim, Y., & Oh, D. K. (2008). Conversion shift of D-fructose to D-psicose for enzyme-catalyzed epimerization by addition of borate. *Applied and Environmental Microbiology*, *74*(10), 3008–3013. https://doi.org/10.1128/AEM.00249-08

Nicholls, M. P., & Paul, P. K. C. (n.d.). Structures of carbohydrate-boronic acid complexes determined by NMR and molecular modelling in aqueous alkaline

media. https://doi.org/10.1039/b312760e

Nollet, L. M., & Toldrá, F. (Eds.). (2012). Handbook of analysis of active compounds in functional foods. CRC Press, Boca Raton, FL. USA.

Norrild, J. C., & Eggert, H. (1995). Evidence for Mono- and Bisdentate Boronate Complexes of Glucose in the Furanose Form. Application of1JC-CCoupling Constants as a Structural Probe. *Journal of the American Chemical Society*, *117*(5), 1479–1484. https://doi.org/10.1021/ja00110a003

Norrild, J. C., & Eggert, H. (1996). Boronic acids as fructose sensors. Structure determination of the complexes involved using 1 J CC coupling constants. *Journal of the Chemical Society, Perkin Transactions 2*, *0*(12), 2583. https://doi.org/10.1039/p29960002583

Porras-Domínguez, J. R., Rodríguez-Alegría, M. E., Ávila-Fernández, Á., Montiel-Salgado, S., & López-Munguía, A. (2017). Levan-type fructooligosaccharides synthesis by a levansucrase-endolevanase fusion enzyme (LevB1SacB). *Carbohydrate Polymers*, *177*(May), 40–48. https://doi.org/10.1016/j.carbpol.2017.08.040

Ritsema, T., & Smeekens, S. (2003). Fructans: Beneficial for plants and humans. *Current Opinion in Plant Biology*, *6*(3), 223–230. https://doi.org/10.1016/S1369-5266(03)00034-7

Roberfroid, M. (2007). Prebiotics: the concept revisited. *The Journal of Nutrition*, *137*(3 Suppl 2), 830S–7S. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/17311983

Roberfroid, M. B. (2000). Prebiotics and probiotics: are they functional foods? *The American Journal of Clinical Nutrition*, *71*(6 Suppl), 1682S–7S; discussion 1688S–90S. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/12495454

Sangeetha, P. T., Ramesh, M. N., & Prapulla, S. G. (2005). Recent trends in the microbial production, analysis and application of Fructooligosaccharides. *Trends in Food Science & Technology*, *16*(10), 442–457. https://doi.org/10.1016/j.tifs.2005.05.003

Shiomi, Y., & Saisho, M. (1993). Specific complexation of glucose with a diphenylmethane-3, 3′-diboronic acid derivative: correlation between the absolute configuration of mono-and di-saccharides. *J. Chem. Soc., Perkin …*. Retrieved from http://pubs.rsc.org/en/content/articlehtml/1993/p1/p19930002111

Sivaev, I. B., & Bregadze, V. I. (2014). Lewis acidity of boron compounds. *Coordination Chemistry Reviews*, *270–271*(1), 75–88. https://doi.org/10.1016/j.ccr.2013.10.017

Springsteen, G., & Wang, B. (2002). A detailed examination of boronic acid–diol complexation. *Tetrahedron*, *58*(26), 5291–5300. https://doi.org/10.1016/S0040-4020(02)00489-1

Wood, P. J., & S~diqui, I. R. (1974). *AND MASS SPE&RA OF SOME BORONIC ESTERS OF CARBOHYDRATES**. *Carbohydrate Research* (Vol. 36). Retrieved from https://ac.els-cdn.com/S0008621500830451/1-s2.0-S0008621500830451-main.pdf?_tid=ab8b4252-91ee-4c5d-8576-

3a0bb9e5e648&acdnat=1536867419_c968f3b5074e31956ba8b3ecfdb52aa1

Wu, X., Li, Z., Chen, X.-X., Fossey, J. S., James, T. D., & Jiang, Y.-B. (2013). Selective sensing of saccharides using simple boronic acids and their aggregates. *Chemical Society Reviews*, *42*(20), 8032. https://doi.org/10.1039/c3cs60148j

Figure Legends.

Table 1. Composition of the final enzymatic reaction after levan-type FOS synthesis with $LevB_1SacB$ (*)

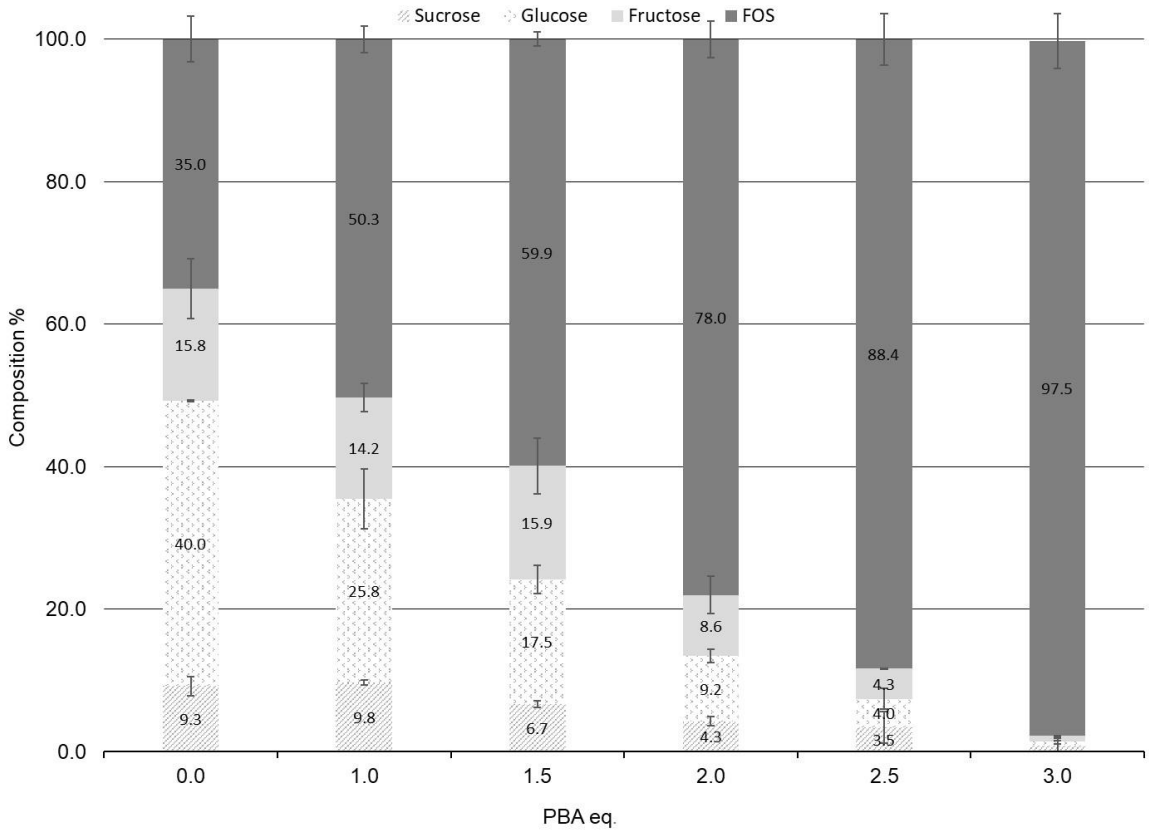
Figure 1. Effect of PBAc equivalents per sugars equivalent in the purification process to remove glucose and fructose (1M) in a solution containing FOS after an enzymatic synthesis from sucrose. Sucrose (diagonal lines), glucose (dots), fructose (light grey), and FOS (dark grey).

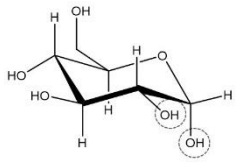
Figure 2. α-Glucose and β-fructose isomer forms in solution, highlighting the *cis*-vicinal hydroxyl groups required for interaction with phenylboronic acid. Complexes are not formed with fructooligosaccharides (levantetraose is included as an example).

Figure 3. Thin layer chromatography of A) Initial mixture of FOS reaction, B) FOS mixture purified using 3 equivalents of PBAc, and C) monosaccharides complexes removed from the mixture.

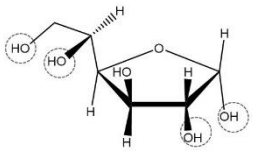
Figure 4. (a) ^{1}H NMR spectroscopic data (500 MHz) for the interaction between PBAC and D-fructose in $CDCl_3$. Displayed are here the aromatic region A. (b) Proposed structure of D-fructose complex formed with phenylboronic acid.

Figure 5. (a) ^{1}H NMR spectrum (500 MHz) for the interaction between PBAC and D-glucose in $CDCl_3$. (b) Proposed structures of D-glucose complexes formed with phenylboronic acid.

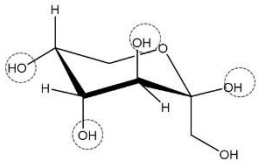




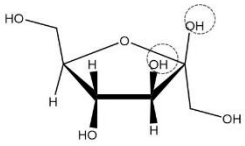
α-D-Glucopyranose



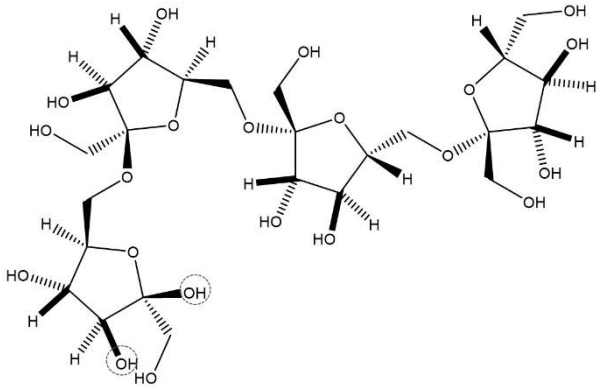
α-D-Glucofuranose



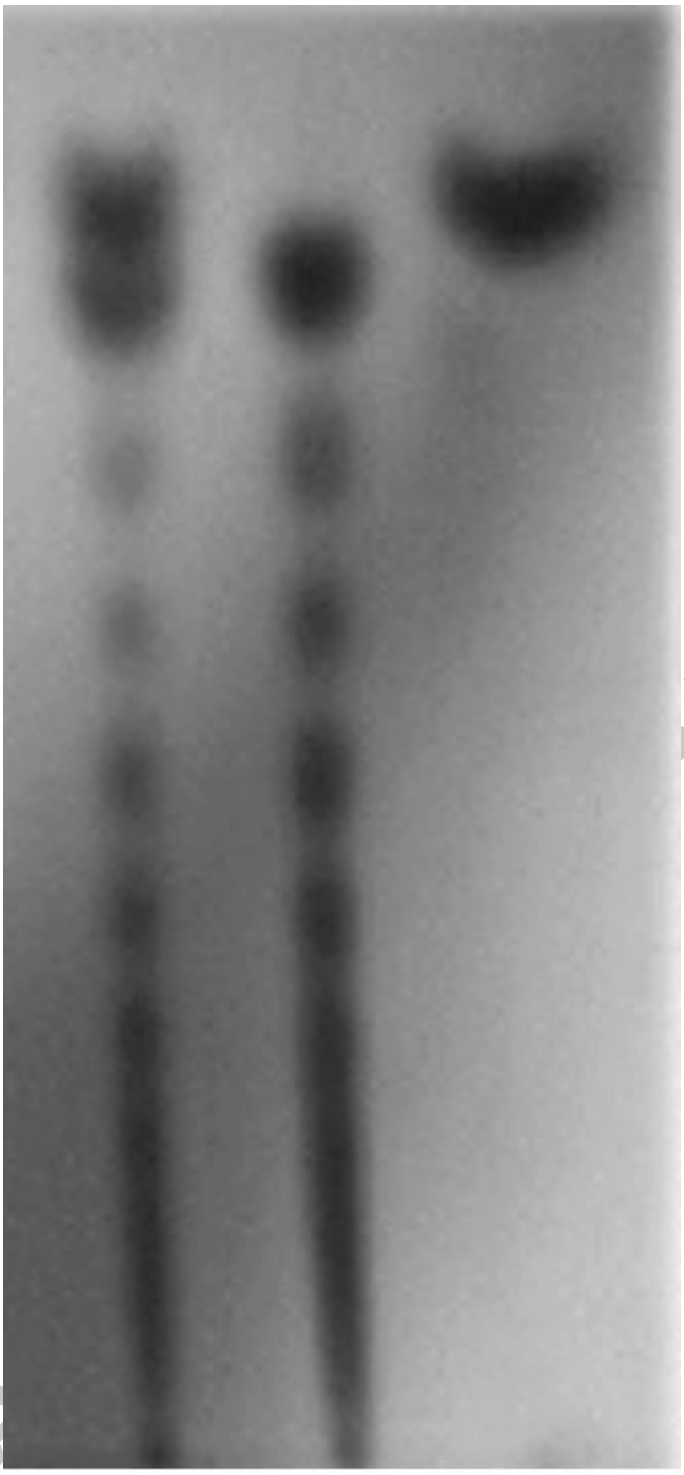
β-D-Fructopyranose



β-D-Fructofuranose

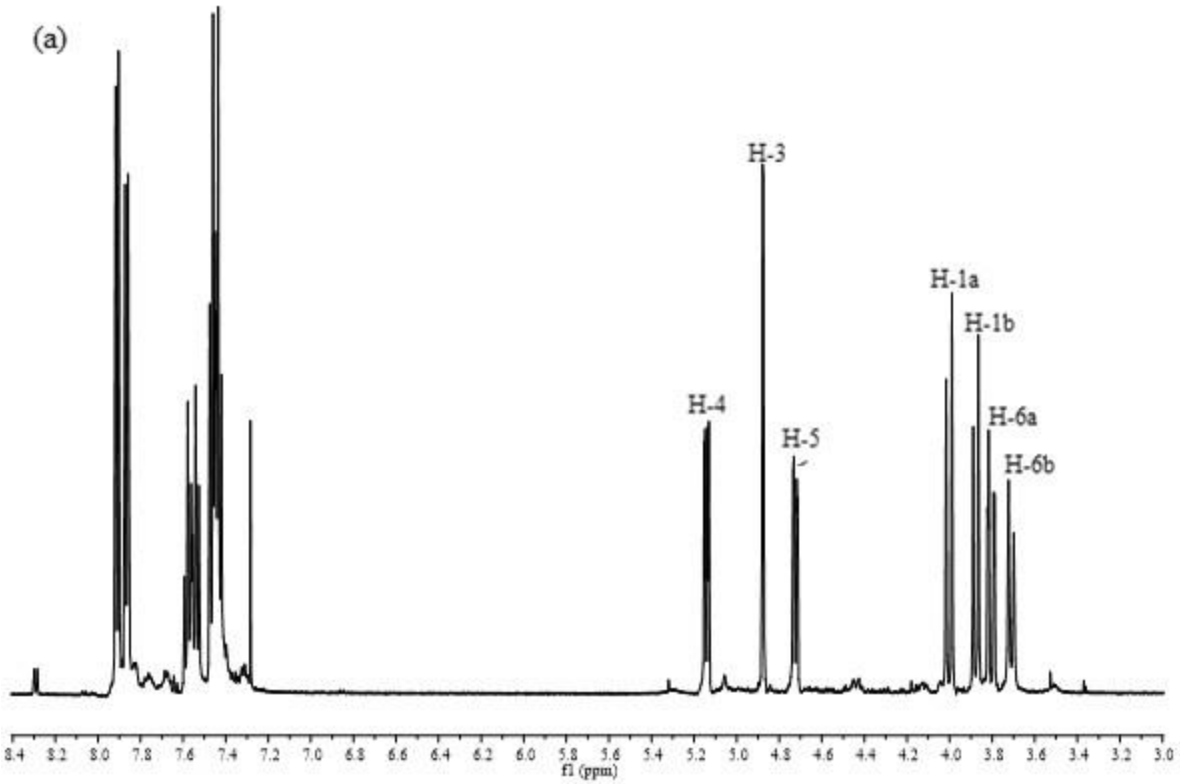


Levantetraose

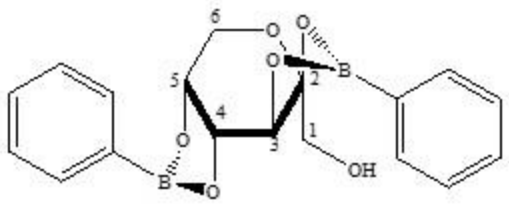


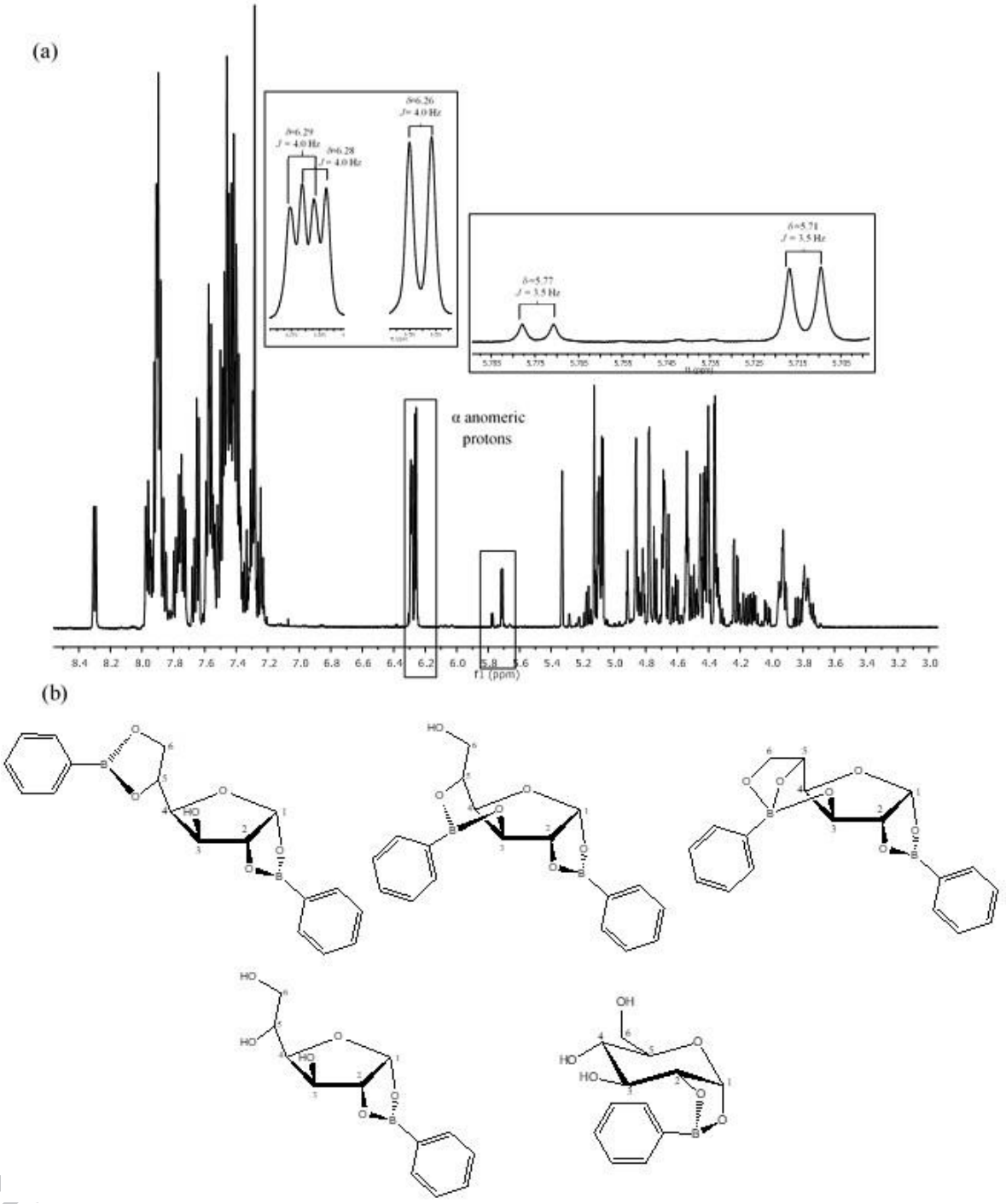
A) B) C)

(a)



(b)





Highlights

- FOS can be purified by a selective chemical method using phenyl boronic acid.

- PBAc react selectively with glucose and fructose to change their polarity.
- Only the free fructose pyranose conformer can react with PBAc.
- Free furanose and pyranose α–glucose conformers may react with PBAc
- NMR reveals five different glucose-PBAc extraction complexes

	Fructose	Glucose	Sucrose	FOS	Conversion %	Yield (%)
Concentration (mM)	526 ± 10	1333 ± 18	163 ± 8	1296.4 ± 7		
Percentage composition (w/w)	15.80%	40.00%	9.20%	34.00%	90.8 ± 2	34 ± 1.3

(*) Reaction conditions: 600 g/L of sucrose, pH 6.0, 37°C and 7.0 U/mL of enzyme activity