Tofu whey permeate is an efficient source to enzymatically produce prebiotic fructooligosaccharides and novel fructosylated α-galactosides

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ABSTRACT

This work addresses a novel and efficient bioconversion method for the utilization of tofu whey permeate (TWP), an important by-product from the soybean industry, as a precursor of high value-added ingredients as prebiotic fructooligosaccharides and novel fructosylated α-galactosides. This process is based on the high capacity of the commercial enzyme preparation Pectinex Ultra SP-L to transfructosylate the main carbohydrates present in TWP as sucrose, raffinose and stachyose to produce up to a maximum of 82.1 g L^{-1} (equivalent to 57% with respect to initial sucrose, raffinose and stachyose content in TWP) of fructooligosaccharides and fructosylated α-galactosides. Raffinose- and stachyose-derived oligosaccharides were formed by the elongation from the non-reducing terminal fructose residue up to three fructosyl groups bound by β-(2→1) linkages. These results could provide new findings on the valorization and upgrading of the management of TWP and an alternative use of raw material for the production of FOS and derivatives.

KEYWORDS*:* tofu whey, fructooligosaccharides, prebiotics, α-galactosides, enzymatic synthesis, transfructosylation.

1. INTRODUCTION

In tofu-making, whey is a waste product derived from the coagulation of soy protein whereas the resulting curd is pressed to form tofu. Tofu whey (TW) is highly perishable due to its high water content and high content of nutritious substances for bacteria.[1] TW is normally used as animal feed, fertiliser,[1] coagulant for the next batch of tofu[2] or simply discarded causing an industrial, economic and environmental problem.[3,4] Considering that products made with soy, tofu being the main processed soybean product, are increasingly accepted worldwide, it is expected that the volume of by-products derived from processing of soy foodstuffs will increase and, therefore, an improvement in the management of these by-products should be required.

TW contains oligosaccharides, proteins and isoflavones that could be isolated and used as functional ingredients,[4] generating, thus, additional revenue and reducing disposal and/or post-treatment costs for this by-product. In this context, the protein fraction has been used to produce functional peptide enriched hydrolysates,[5] glycated proteins[1] or acylated peptides with improved surface functional properties.[6] Interestingly, soy or TW has been also used as a growth medium for the production of lactic acid starters, specifically for the genus *Lactobacillus*.[7-9] This behavior can be attributed to the capacity of the tested bacteria to metabolize, at least, part of the major fermentable carbohydrates present in TW. Several studies have previously reported the carbohydrate composition of soy/TW indicating that sucrose is the main carbohydrate, followed by α-galactosides such as stachyose or raffinose, in addition to monosaccharides as fructose or glucose.[3,8,9] As a consequence of its high sucrose content, TW could be a suitable, readily available and cost-effective substrate to produce high value sucrose-derived oligosaccharides, in a similar way than cheese whey is used as a precursor for the production of functional oligosaccharides derived from

lactose.[10,11] As far as we know, the only approach to produce bioactive oligosaccharides from TW has been recently reported and is based on the combined use of cheese whey permeate and TW to produce lactosucrose by a transfructosylation reaction catalyzed by levansucrase from *Bacillus subtilis* CECT 39.[12]

In this work, we explore the feasibility of using tofu whey permeate (TWP) as a single and efficient source of prebiotic fructooligosaccharides (FOS), as well as fructosylated derivatives of α-galactosides (FDG), by using the commercial enzyme preparation Pectinex Ultra SP-L. The conversion of TWP into value-added bioactive oligosaccharides could provide a new and more sustainable process to manage its reuse.

2. MATERIALS AND METHODS

2.1. Enzyme, chemicals and reagents. The commercial enzyme preparation Pectinex Ultra SP-L, a soluble preparation containing fructosyltransferase activity and produced by *Aspergillus aculeatus*, was a generous gift from Novozymes (Bagsvaerd, Denmark). Fructosyltransferase activity was 400 U mL^{-1}, where 1 U is defined as the amount of enzyme transferring 1 μmol of fructose per minute at a working temperature of 60 °C and a sucrose concentration of 300 g L^{-1} at pH 5.5.

Fructose, glucose, galactose, *myo*-inositol, pinitol, sucrose, melibiose, raffinose, and stachyose were purchased from Sigma–Aldrich (St Louis, MO, USA), whilst kestose and nystose were from Carbosynth (Compton, UK). Acetonitrile (HPLC grade) was purchased from Lab-Scan (Gliwice, Poland). Ultrapure water (18.2 MΩ cm, with levels of 1–5 ng mL^{-1} total organic carbon and <0.001 EU mL^{-1} pyrogen) was produced in-house with a laboratory water purification system (Milli-Q Synthesis A10, Millipore, Billerica, MA, USA). All other chemicals were of analytical grade.

2.2. Production of tofu whey permeate. Tofu whey (TW) was kindly provided by Natursoy (Barcelona, Spain). Immediately after reception, TW was freeze-dried for a properly storage until its use. For obtainment of tofu whey permeate (TWP), freeze-dried TW was reconstituted in ultrapure water at 24 mg mL^{-1} (simulating original dry matter concentration). To remove proteins, 10 mL portions were ultrafiltered through hydrophilic 3 kDa cutoff membranes (Amicon® Ultra-15, Millipore Corp., Bedford, MA) by centrifugation at 4,000*g* for 90 min. Finally, filtrate was recovered, freeze-dried and kept at -20 ºC until posterior analysis.

2.3. Enzymatic synthesis of oligosaccharides. Enzymatic synthesis of oligosaccharides was carried out by incubating TWP (600 g L^{-1}) with Pectinex Ultra SP-L at 60 ºC, pH 5.5 (using 1 M NaOH to adjust the pH value of the TWP aqueous solution) and continuous agitation at 1,350 rpm using an orbital shaker (Eppendorf Thermomixer Confort, Hauppauge, NY, USA). Optimum reaction conditions were according to literature.[13] Regarding enzyme concentration, optimization was carried out by testing three values, 1.7 U mL^{-1},[14] 9 U mL^{-1},[15] and 34 U mL^{-1}.[13] Evolution of FOS and FDG formation was determined taking aliquots from the reaction mixture at suitable time intervals, including 0, 0.5, 1, 3, 8 and 24 h. The enzyme was inactivated by heating at 100 °C for 5 min. Once the enzyme concentration was optimized to TWP, individual enzymatic synthesis from sucrose, raffinose or stachyose were developed under the same reaction conditions than TWP.

Moreover, control experiments of enzyme in the absence of donor and acceptor carbohydrates were carried out with the purpose of checking any possible formation of products derived from the enzyme incubation.

All the synthesis reactions were done in duplicate and the corresponding analytical measurements were carried out twice for each enzymatic synthesis reaction.

2.4. Chromatographic determination of carbohydrates. *LC-RID.* Chromatographic separation and quantitation of carbohydrates present in the original sample of TWP and samples resulting from enzymatic synthesis was carried out by LC-RID.

Before chromatographic analysis, inactivated samples resulting from enzymatic synthesis were diluted with acetonitrile:water (50:50, v:v) at total carbohydrate concentration of ~10 mg mL^{-1} (~60-folds), filtered (0.45 μm PVDF filters, Symta, Madrid, Spain), and kept at 4 ºC until their analysis by LC with refractive index detection (RID) as described below.

LC analyses were carried out using an Agilent Technologies 1260 Series HPLC system (Böblingen, Germany). The separation of carbohydrates was carried out on a Kromasil column (100-NH_2; 250 mm × 4.6 mm, 5 μm particle size) (Akzo Nobel, Brewster, NY, USA) using acetonitrile/water (70:30 v/v) as mobile phase and elution in isocratic mode at a flow rate of 1 mL min^{-1} for 90 min. The injection volume was 50 μL (~ 550-850 μg of total carbohydrates). Data acquisition and processing were performed using Agilent ChemStation software. Carbohydrates in the reaction mixtures were initially identified by comparing their retention times (t_R) with those of pure standard sugars, including fructose, glucose, sucrose, melibiose, kestose, raffinose, nystose and stachyose. Quantitative analysis was performed by the external standard method, using calibration curves of each pure standard in the range 0.05–2.5 mg mL^{-1}.

All reactions and analyses were performed in duplicate (n = 4), obtaining relative standard deviation (RSD) values below 10% in all cases.

GC-FID and GC-MS. The trimethylsilyl oxime (TMSO) derivatives of mono-, di- and oligosaccharides for both GC-FID and GC-MS analyses were prepared as previously described by Corzo-Martínez et al.[16] A weight of 10 mg of sample was

added to 0.4 mL of internal standard (IS) solution, containing 0.5 mg mL^{-1} of phenyl-β-D-glucoside. The mixture was dried at 38-40 °C in a rotatory evaporator (Büchi Labortechnik AG, Falwil, Switzerland). Oximes were obtained by addition of 250 µL of a solution of 2.5% hydroxylamine chloride in pyridine to the carbohydrate mixture after 30 min at 70 °C incubation. Subsequently, the oximes were silylated with hexamethyldisilazane (250 µL) and trifluoroacetic acid (25 µL) at 50 °C for 30 min. Then, reaction mixtures were centrifuged at 10,000 *g* for 2 min. This derivatization procedure gives rise to a single chromatographic peak for non-reducing sugars, corresponding to their trimethylsilyl ethers, whereas two peaks are detected for reducing sugars, corresponding to their *syn*- (E) and *anti*- (Z) oxime isomers.

GC-FID analysis was performed following the method of Cardelle-Cobas et al.[17] on an Agilent Technologies 7890A gas chromatograph (Agilent Technologies, Wilmington, DE, EEUU) equipped with a flame ionization detector (FID). Separations were carried out using a fused silica capillary column HP-5MS (5% phenyl methylsilicone, 25 m x 0.32 mm x 0.25 µm thickness; J & W Scientific, Folsom CA, USA). Nitrogen was used as carrier gas at a flow rate of 1 mL min^{-1}. Injector and detector temperatures were 280 and 315 °C, respectively. The oven temperature was programmed from 180 to 315 °C at a heating rate of 3 °C min^{-1} and held 60 minutes. Injections were made in the split mode (1:20). Data acquisition and integration were done using the Agilent ChemStations Reb. 4B. 03.01 software (Wilmington, DE, USA). All analyses were done in duplicate. Response factors were calculated after the triplicate analysis of 5 standard solutions (*myo*-inositol, galactose, glucose, fructose, sucrose, raffinose and stachyose) over the expected concentration range in samples (0.01–1 mg mL^{-1}).

GC-MS analysis was performed on an Agilent Technologies 7890A gas chromatograph coupled to a 5975CMSD quadrupole mass detector (Agilent Technologies,Wilmington, DE, USA) in order to confirm the identification of all carbohydrates. Sugar separation was performed using helium as a carrier gas at 0.8 mL min^{-1}. The rest of chromatographic conditions (type of column, ramp rate, injector temperature and split mode) were the same as those described above for GC-FID analysis. The mass spectrometer was operated in electrospray ionisation mode at 70 eV. Mass spectra were acquired using Agilent ChemStation MSD software (Wilmington, DE, USA). Identification of trimethylsilyl oxime derivatives of carbohydrates was carried out by comparison of their relative retention times and mass spectra with those of standard compounds previously derivatized. In consequence, standard solutions of fructose, glucose, galactose, *myo*-inositol, pinitol, sucrose, melibiose, raffinose, kestose, stachyose, nystose, and fructosyl-nystose were used. In addition, the analysis of Raftilose®, commercial fructooligosaccharides from Beneo (Barcelona, Spain), allowed the identification of inulobiose and inulotriose.

In the particular case of the identification of fructosylated α-galactosides and due to the lack of commercially available standards, the mass spectra of the oligosaccharides resulting from the individual enzymatic synthesis from raffinose or stachyose were compared to those obtained under the same reaction conditions but using TWP as initial substrate.

3. RESULTS AND DISCUSSION

3.1. Carbohydrate composition of tofu whey permeate. Tofu whey (TW) was subjected to a previous ultrafiltration step to mainly remove residual proteins that may approximately correspond to 9% of the original soybean proteins.[8] The resulting tofu

whey permeate (TWP) could be an efficient substrate for the enzymatic synthesis of oligosaccharides given that the ultrafiltration process produces a substrate rich in carbohydrates. Thus, the total content in carbohydrates of TWP was close to 80% with respect to dry matter and the carbohydrate composition determined by LC-RID was mainly dominated by sucrose (34.4% of total carbohydrates), followed by stachyose (α-D-galactopyranosyl-(1→6)-α-D-galactopyranosyl-(1→6)-α-D-glucopyranosyl-(1→2)-β-D-fructofuranoside) (21.3%), fructose (12.3%), glucose (7.0%) and raffinose (α-D-galactopyranosyl-(1→6)-α-D-glucopyranosyl-(1→2)-β-D-fructofuranoside) (5.6%) as it is shown in Table 1. The major presence of these carbohydrates is in good agreement with previous reports also describing the carbohydrate composition of TW and/or TWP.[3,8,9,12] Interestingly, a broad and unresolved peak eluting in the monosaccharide area (t_R = ~ 7 min, Figure 1) and representing 16.9% of total content of carbohydrates was labelled as other monosaccharides and polyalcohols (Table 1).

Further GC-FID analyses were carried out to gain a deeper knowledge on the carbohydrate composition of TWP (Figure 2 (red profile)). Focusing on the separation of the monosaccharide fraction, substantial amounts of pinitol (peak 2), as well as *myo*-inositol in much lesser amounts (peak 6) were detected, in addition to citric acid (peak 1), fructose (peak 3), galactose (peak 4) and glucose (peak 5). Although pinitol was previously identified as a major soluble carbohydrate in soybean plant,[18] to the best of our knowledge, the presence of polyalcohols such as pinitol, inositol and *myo*-inositol in TW had not been reported up to date. Additionally, in good agreement with LC-RID data, GC-FID analysis confirmed the major presence of sucrose (peak 7), stachyose (peak 16) and raffinose (peak 10), as well as the minor presence of a series of unknown peaks eluting in the disaccharide zone (Figure 2).

3.2. Enzymatic synthesis of fructooligosaccharides and novel fructosylated α-galactosides. *Tofu whey permeate as starting substrate.* TWP could be an appropriate substrate for FOS according to the high content of sucrose (163 g L^{-1}) (Table 1). Additionally, TWP also contains substantial levels of α-galactosides such as raffinose (26.6 g L^{-1}) and stachyose (101 g L^{-1}) which was previously shown to be hydrolyzed and transfructosylated to product oligosaccharides of higher degree of polymerization (DP).[19]

The enzymatic source employed in this work was the commercial enzyme preparation Pectinex Ultra SP-L, produced by *Aspergillus aculeatus*, because is largely used in the food industry, has a low cost, good transfructosylation activity and high thermal stability.[15,20] Given that the optimum pH (5.0-5.5) and temperature (60-65 ºC) have been well-established in previous works that addressed the synthesis of FOS[14, 15, 21] or fructosylated derivatives of stachyose[19] catalyzed by Pectinex Ultra SP-L, the production of fructosylated oligosaccharides was studied as a function of the time and concentration of the enzyme preparation. Thus, three different enzyme concentrations, that is 1.7, 9 and 34 U mL^{-1}, were assayed whilst the concentration of TWP was set at 60% (w/v), which was the maximum concentration at which TWP was completely soluble in the medium reaction to favor the transglycosylation reaction. In general terms, the maximum production of fructosylated oligosaccharides was achieved after 24, 8 and 3 hours of reaction when 1.7, 9 and 34 U mL^{-1} of enzyme were, respectively, added. Nevertheless, the maximum production of FOS and FDG estimated by LC-RID was achieved when 9 U mL^{-1} of enzyme was used, i.e., 164 g L^{-1} (Table 1), being equivalent to a yield of 57% (in weight with respect to the determined initial amount of sucrose, raffinose and stachyose in TWP); whereas 150 g L^{-1} (51% of yield) and 128 g

238 L^{-1} (44% of yield) of fructosylated oligosaccharides were obtained with 1.7 and 34 U
239 mL^{-1} of enzyme, respectively.

240 *Sucrose, raffinose or stachyose as single starting substrates.* Once the
241 concentration of Pectinex Ultra SP-L was optimized, and considering that TWP
242 contains, in addition to sucrose, α-galactosides susceptible to act as donor and/or
243 acceptor of fructosyl units in transfructosylation reactions catalyzed by microbial
244 transglycosidases,[12,19] enzymatic syntheses under the optimum conditions using sucrose,
245 raffinose or stachyose as single substrates instead of TWP were carried out to allow the
246 identification of different compounds present in the complex mixture obtained with
247 TWP.

248 As it could be expected, sucrose was an efficient precursor of FOS with DPs
249 from 3 to 6. These oligosaccharides were formed by the transfer of β-2,1-linked fructose
250 units released from sucrose hydrolysis to the fructose moiety of sucrose to give rise to
251 inulin-type FOS (Figure 1). The maximum production of total FOS, achieved after 3
252 hours of reaction, was 432 g L^{-1} (Figure 3A), being equivalent to a yield of 80% (in
253 weight with respect to the determined initial amount of sucrose). 1-Kestose was the
254 predominant FOS present at 3 hours of reaction to, then, decrease until the end of
255 reaction probably due to its capacity to act, in turn, as acceptor. In good agreement with
256 this, Vergauwen et al.[22] indicated that 1-kestose is a suitable acceptor for 1-
257 fructosyltransferases which are the enzymes responsible for chain elongation of inulin-
258 type fructans. In consequence, nystose remained as the major FOS after 8 and,
259 particularly, 24 hours of reaction (Figure 3A).

260 Regarding raffinose-derived oligosaccharides, the detection by LC-RID of
261 fructose (peak 1, Figure 1) and melibiose (α-D-galactopyranosyl-(1→6)-D-glucose,
262 peak 4, Figure 1) demonstrated that raffinose (peak 6, Figure 1) was efficiently broken

down by Pectinex Ultra SP-L at the β(2→1) linkage between fructose and glucose. In addition, fructose was detected only at low levels which is indicative of its transfer to other raffinose molecules to give rise to a series of fructosylated-raffinosyl oligosaccharides with DP ranging from 4 to 6 (peaks 9, 12 and 16, Figure 1). Additionally, Figure 3B shows that raffinose was efficiently hydrolyzed by Pectinex Ultra SP-L since only 12% of raffinose remained unaltered after 24 hours of reaction. The highest production of fructosylated raffinose-derived oligosaccharides, also achieved at 3 hours of reaction, was 319.4 g L^{-1} (Figure 3B) and equivalent to a yield of 53% (in weight with respect to the determined initial amount of raffinose). Remarkably, the detection of galactose (detected by GC-FID) and sucrose (peak 3, Figure 1) from the third hour of reaction indicated that Pectinex Ultra SP-L had also the ability to cleave, although at a much lesser extent, raffinose at the α(1→6) bond between galactose and glucose. α-galactosidases from *Aspergillus* sp., as *A. terreus*, have been previously purified and characterized.[23]

Stachyose was hydrolyzed by Pectinex Ultra SP-L at similar extension than raffinose since a decrease of 87% in stachyose content was found after 24 hours of reaction (Figure 3C). Stachyose was mainly hydrolyzed at the sucrose moiety giving rise to 6'-galactosyl-melibiose (α-D-galactopyranosyl-(1→6)-α-D-galactopyranosyl-(1→6)-D-glucose) (peak 8, Figure 1) and fructose (Peak 1, Figure 1), confirming previous findings.[13,19] The maximum production of FDG, tentatively identified as the pentasaccharide fructosyl-stachyose (peak 13, Figure 1) and the hexasaccharide difructosyl-stachyose (peak 14, Figure 1), was 177.1 g L^{-1} (equivalent to a yield of 30%, in weight with respect to the determined initial amount of stachyose) and achieved at 8 hours of reaction (Figure 3C). Furthermore, low levels (6.1 g L^{-1}) of the heptasaccharide trifructosyl-stachyose could be determined only after 24 hours of reaction.

Although sucrose was the best donor and acceptor leading to a high production of FOS, raffinose and, in a lesser level, stachyose were also efficient donors and acceptors for the transfructosylation reaction catalyzed by Pectinex Ultra SP-L which allowed the high yield-synthesis of FDG. According to the well-described mechanism of transfructosylation of Pectinex Ultra SP-L acting on sucrose,[24] as well as to the scarce studies dealing with the transfructosylation of stachyose by this commercial enzyme,[13,18] it can be inferred that raffinose- and stachyose-derived oligosaccharides were formed by the elongation of their respective chains from the non-reducing terminal fructose residue through a linear chain of up to three fructosyl residues bound by β-(2→1) linkages. Thus, the general chemical structure of fructosylated oligosaccharides derived from raffinose and stachyose was α-D-Gal-(1→6)-α-D-Glc-[(1→2)-β-D-Fru]^{n} and α-D-Gal-(1→6)-α-D-Gal-(1→6)-α-D-Glc-[(1→2)-β-D-Fru]^{n}, respectively, with n = 1-3.

The individual syntheses of FOS from sucrose and FDG from raffinose or stachyose were also useful to tentatively identify and chromatographically distinguish between FOS and FDG when TWP is used as precursor of bioactive oligosaccharides. In general terms, FOS and FDG were well resolved by LC-RID with the exception of 6'-galactosyl-melibiose (peak 8, Figure 1) and fructosyl-raffinose (peak 9, Figure 1), as well as fructosyl-nystose (peak 10, Figure 1) and stachyose (peak 11, Figure 1). Both pair of chromatographic peaks co-eluted during the LC chromatographic separation that hampered their accurate quantification.

Separation and identification of fructooligosaccharides and novel fructosylated α-galactosides by GC-FID and GC-MS. To prevent the coelution of peaks observed by LC-RID and to strengthen the carbohydrate identification previously carried out, the enzymatic reaction mixture resulting from the TWP transfructosylation

after 8 hours of reaction using 9 U mL^{-1} of Pectinex Ultra SP-L, which led to the maximum production of FOS and FDG, was analyzed by GC-FID (Figure 2 (blue line)). Under the developed chromatographic conditions, carbohydrates up to DP 5 were detected and 6'-galactosyl-melibiose (peak 13, Figure 2) and fructosyl-raffinose (peak 15, Figure 2), as well as stachyose (peak 16, Figure 2) and fructosyl-nystose (peak 17, Figure 2) could be well resolved, thus, allowing their accurate quantification (Table 1). Finally, the detection of both inulobiose (β-D-Fru-(2→1)-β-D-Fru) (peak 8, Figure 2) and inulotriose (β-D-Fru-(2→1)-β-D-Fru-(2→1)-β-D-Fru) (peak 12, Figure 2) could be attributed to the ability of fructose to act as a minor acceptor in the transfructosylation reaction.

Given the lack of commercially available standards for FDG, the structural confirmation was achieved by comparison of the mass spectra corresponding to synthetized FDG from TWP with the respective mass spectra of the FDG resulting from the enzymatic synthesis using raffinose or stachyose as single precursors. Figure 4, which shows the mass spectra of fructosyl-stachyose (main synthetized fructosylated α-galactoside) and fructosyl-raffinose, respectively, demonstrated the structural similarity of the synthetized FGD regardless the use of TWP or single raffinose or stachyose as initial substrates. The most abundant ions were those found at *m/z* 361 (which is characteristic of a glycosylated sugar ring) and at *m/z* 217 (typical of furanose rings) which contains two units of TMSOCH from C6 and C5 completed by the atom of C4.[12] Both fragment ions were previously identified in raffinose and stachyose by GC-MS.[25] Fragmentation behavior of FDG also resulted in characteristic ions at *m/z* 129, 169, 271, 437 and 451. Moreover, fragment ions detected at *m/z* 230 and 362 have been previously related to carbohydrates having fructose units bonded by β-(2→1) glycosidic linkages, such as kestose, inulobiose and inulotriose (Figure 4).[26] Furthermore, the mass

spectra of fructosylated derivatives of stachyose were essentially the same than those previously obtained (data not shown) and whose structural characterization was carried out by NMR approaches.[19]

Overall, the enzymatic production of FOS and FDG from TWP under the optimum conditions was quite balanced since the proportion of both type of oligosaccharides was 53.6% (44 g L^{-1}) and 46.4 % (38.1 g L^{-1}), respectively. This could be explained by the fact that the concentrations of sucrose, precursor of FOS, against raffinose and stachyose, precursors of FDG, maintained a similar ratio, i.e. 56% vs 44%, in the initial composition of TWP (Table 1). Likewise, this behavior supports the efficient capacity of either raffinose or stachyose to act as donors and acceptors in the transfructosylation reaction catalyzed by Pectinex Ultra SP-L, previously demonstrated when these carbohydrates were used as single substrates (Figure 3B and 3C).

In conclusion, this work provides novel findings on the renewable use of an important vegetal by-product such as TWP for the efficient production of high value-added ingredients as prebiotic FOS and novel FDG. This process is based on the high capacity of the food-grade, commercial and inexpensive enzyme preparation Pectinex Ultra SP-L to transfructosylate the main carbohydrates present in TWP as sucrose, raffinose and stachyose to produce up to a maximum of 82.7 g L^{-1} of FOS and FDG in a balanced proportion. While FOS is one of the most recognized prebiotics, the prebiotic potential of FDG is still to elucidate. Nevertheless, according to their chemical structure, FDG shares important structural features with FOS as they can be considered as galactosylated derivatives of FOS. In consequence, further studies evaluating the prebiotic potential of this novel oligosaccharide mixture are warranted.

Finally, the efficient bioconversion of TWP into value-added oligosaccharides could contribute to the revalorization and improvement of the management of this by-

product, as well as to open up the use of renewable and alternative raw material for the production of FOS and derivatives.

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Table 1. Carbohydrate composition (g L^{-1}) determined by LC-RID (A) and produced upon the transfructosylation reaction catalyzed by Pectinex Ultra SP-L (9 U mL^{-1}) at 60 ºC and pH 5.5 (B) using 600 g L^{-1} of tofu whey permeate. Values shown as mean ± SD (n =4).

A

Time (hours)	Fructose	Glucose	Galactose	Other monosaccharides and polyalcohols	Sucrose	Melibiose	Other disaccharides	Raffinose	Stachyose
0	58.4±0.9	33.2±0.2	0.0±0.0	80.2±0.5	163.0±0.8	0.0±0.0	11.0±0.4	26.6±0.4	101.0±0.5
0.5	69.8±3.4	75.6±1.8	9.6±0.5	43.2±4.7	89.0±2.6	3.6±0.0	11.4±0.6	22.8±0.5	101.0±0.1
1	80.2±1.3	97.6±0.3	16.6±0.7	91.8±2.8	62.4±1.6	4.6±0.1	11.4±0.2	22.4±0.6	100.4±0.3
3	83.6±4.7	120.8±3.5	17.6±3.1	92.0±6.4	36.4±1.3	5.6±0.1	10.0±0.6	12.6±0.1	90.2±4.1
8	99.4±1.0	150.6±2.4	27.2±2.8	120.2±4.8	36.2±1.9	8.6±0.3	10.4±0.4	12.0±0.1	83.0[a]±4.7
24	104.2±1.0	151.0±1.1	24.8±0.6	119.2±2.3	36.0±0.2	9.0±0.2	10.0±0.1	9.2±0.2	86.6±1.3

B

Time (hours)	6'Gal-melibiose	FOS			FDG			
		Kestose	Nystose	Fructosyl-nystose	Fructosyl-raffinose	Difructosyl-raffinose	Fructosyl-stachyose	Difructosyl-stachyose
0	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0
0.5	4.8±0.0	53.0±1.0	4.4±0.1	n.q	n.q	0.0±0.0	10.0±0.3	6.0±0.1
1	8.4±0.0	75.2±1.6	13.2±0.4	n.q	n.q	0.0±0.0	17.2±0.7	7.6±0.2
3	12.4±0.2	54.8±1.1	32.0±0.7	n.q	n.q	0.0±0.0	30.2±0.9	7.0±0.0
8	6.0[a]±0.2	37.0±1.0	45.6±1.1	5.4±0.1[a]	5.0±0.2[a]	5.0±0.0	54.6±1.3	5.6±0.1
24	9.2±0.3	31.0±0.6	46.0±0.5	n.q	n.q	4.8±0.1	51.0±1.0	6.0±0.0

[a] Quantified by GC-FID.
n.q. Not quantified.

FIGURE CAPTIONS

Figure 1. LC-RID profiles of transfructosylation reactions catalyzed by Pectinex Ultra SP-L (9 U mL^{-1}) after 8 h at 60 ºC and pH 5.5 using 600 g L^{-1} of a) stachyose, b) raffinose, c) sucrose and d) tofu whey permeate (TWP) as starting substrates; e) LC-RID profile of tofu whey permeate before transfructosylation reaction. Peaks identification: 1: fructose, 2: glucose, 3: sucrose, 4: melibiose, 5: kestose, 6: raffinose, 7: nystose, 8: 6'-galactosyl-melibiose, 9: fructosyl-raffinose, 10: fructosyl-nystose, 11: stachyose, 12: difructosyl-raffinose, 13: fructosyl-stachyose, 14: difructosyl-stachyose, 15: difructosyl-nystose, 16: trifructosyl-raffinose.

Figure 2. Gas chromatographic profile of the TMS oximes of carbohydrates present in tofu whey permeate (red) and formed during transfructosylation reaction of tofu whey permeate (600 g L^{-1}) catalyzed by Pectinex Ultra SP-L (9 U mL^{-1}) after 8 h at 60 ºC and pH 5.5 (blue). Peaks identification: 1: citric acid, 2: pinitol, 3: fructose, 4: galactose, 5: glucose; 6: *myo*-inositol, I.S.: Internal standard (phenyl-β-D-glucoside), 7: sucrose, 8: inulobiose, 9: melibiose, 10: raffinose, 11: kestose, 12: inulotriose, 13: 6'-galactosyl-melibiose, 14: nystose, 15: fructosyl-raffinose, 16: stachyose, 17: fructosyl-nystose, 18: difructosyl-raffinose, 19: fructosyl-stachyose. DP: degree of polymerization.

Figure 3. Concentrations of a) sucrose and fructooligosaccharides, b) raffinose and fructosyl-raffinose oligosaccharides and c) stachyose and fructosyl-stachyose oligosaccharides upon transfructosylation reaction catalyzed by Pectinex Ultra SP-L (9 U mL^{-1}) at 60 ºC and pH 5.5 using 600 g L^{-1} of starting substrate. Vertical bars represent standard deviations (SD) (n = 4).

Figure 4. Mass spectra obtained by gas chromatography coupled to mass spectrometry (GC-MS) analysis using the corresponding trimethylsilyl oximes (TMSO) of the

fructosyl-raffinose synthetized from raffinose (a) and tofu whey permeate (b) and fructosyl-stachyose from stachyose (c) and tofu whey permeate (d).

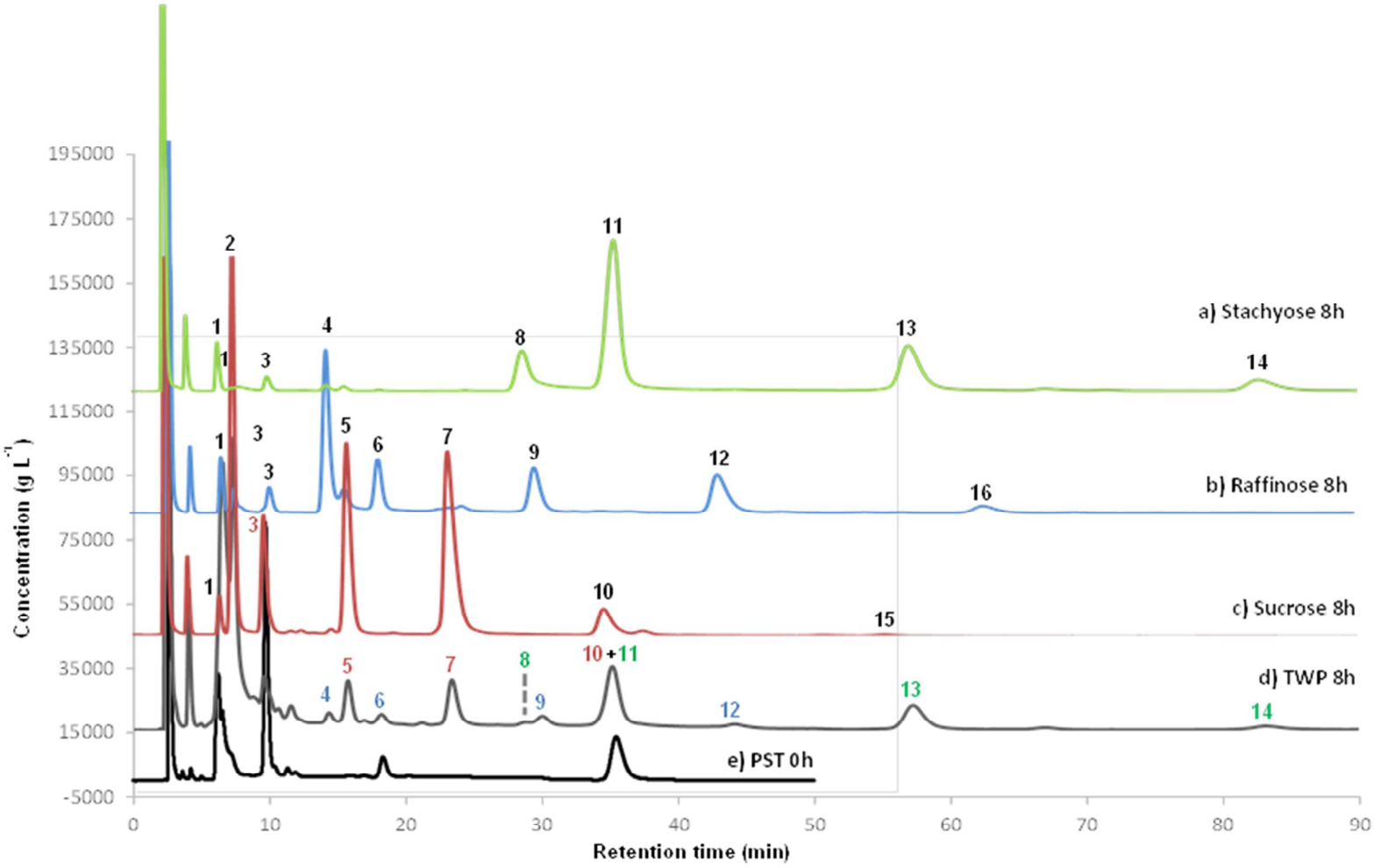
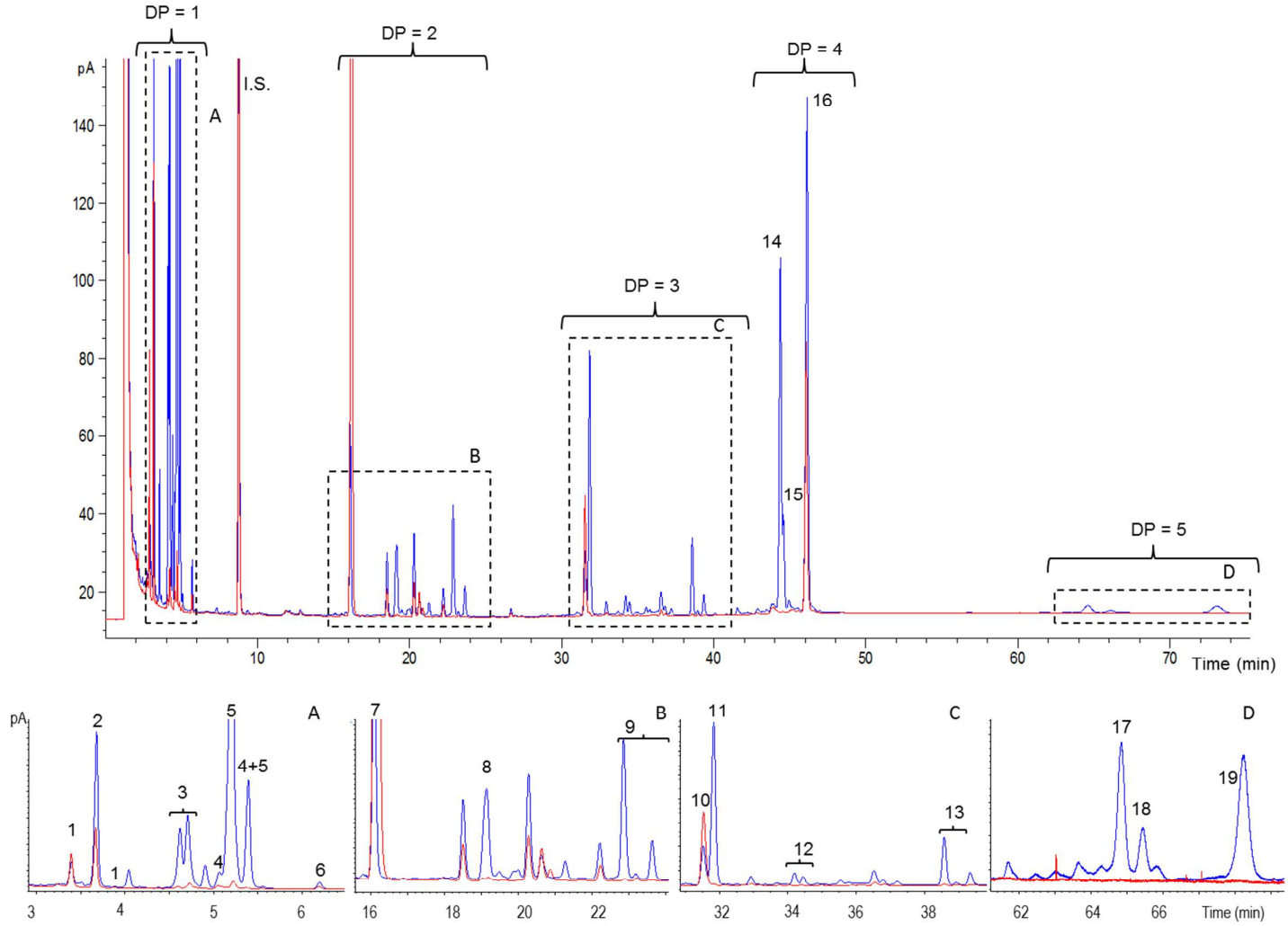

Concentration (g L-1)
Retention time (min)
a) Stachyose 8h
b) Raffinose 8h
c) Sucrose 8h
d) TWP 8h
e) PST 0h
195000
175000
155000
135000
115000
95000
75000
55000
35000
15000
-5000
0
10
20
30
40
50
60
70
80
90

Figure 1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DP = 2
DP = 4
DP = 3
DP = 5
pA
I.S.
A
B
C
D
14
15
16
Time (min)
1
2
3
4+5
5
6
7
8
9
10
11
12
13
17
18
19

Figure 2

Figure 3.

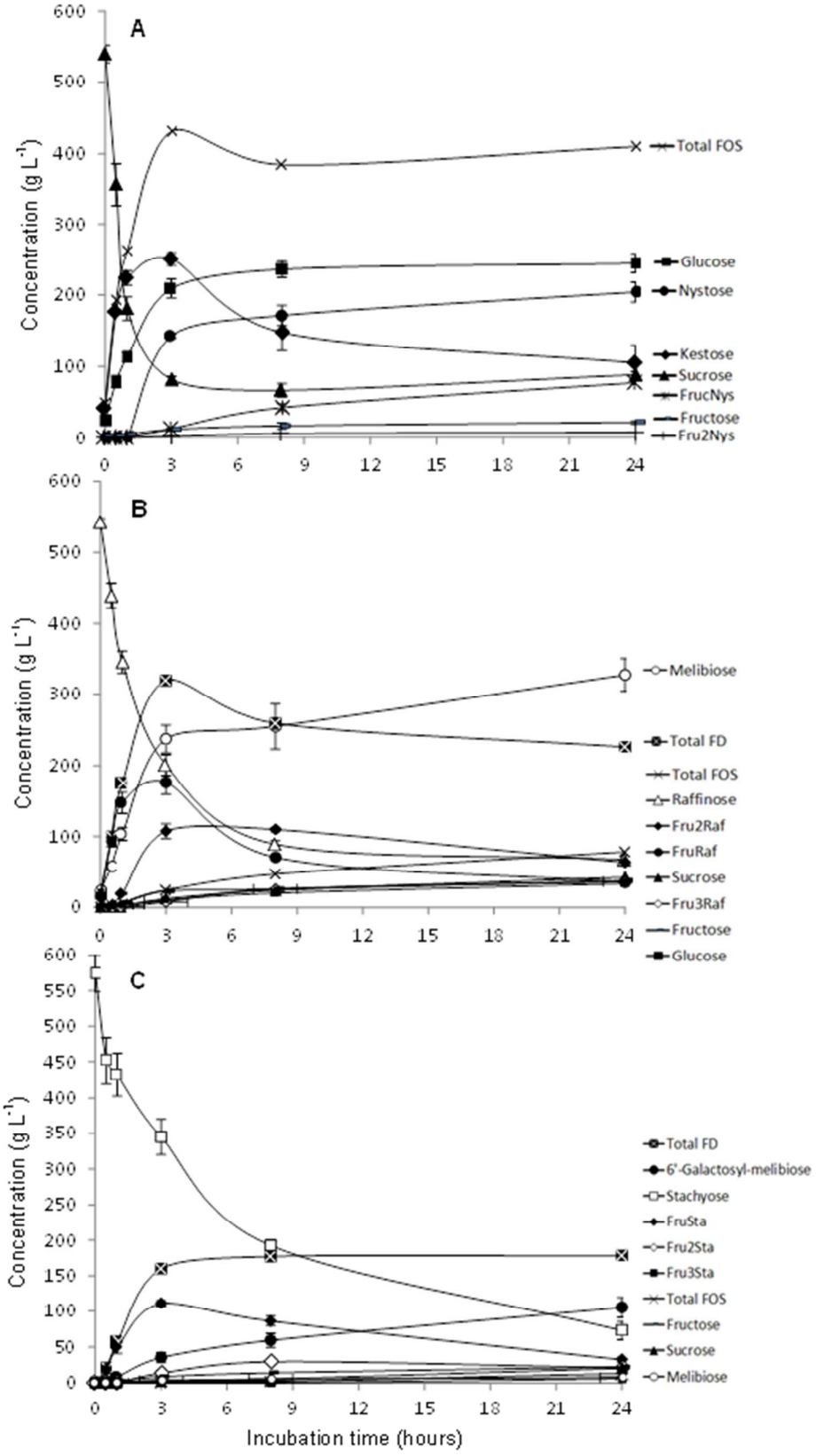
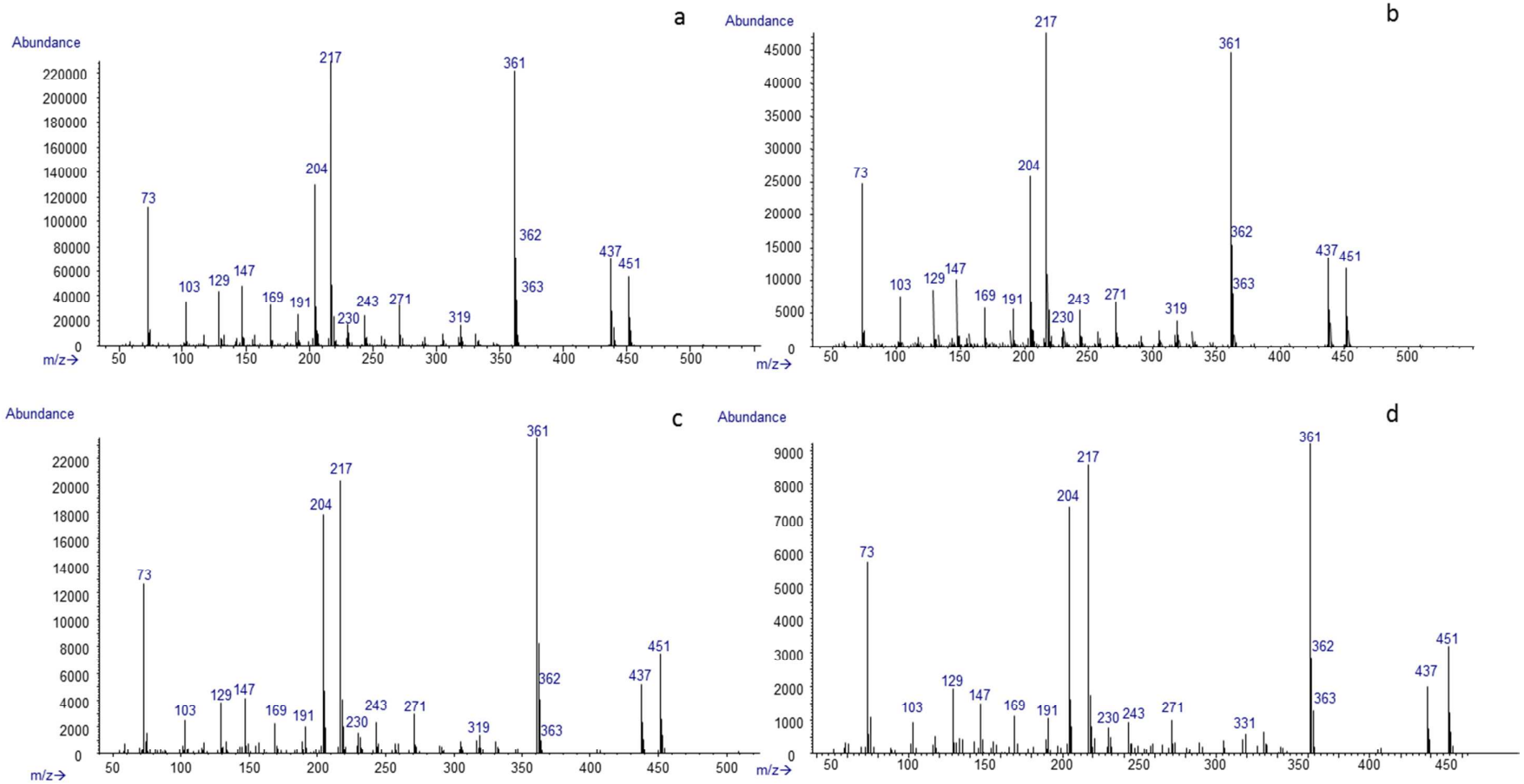
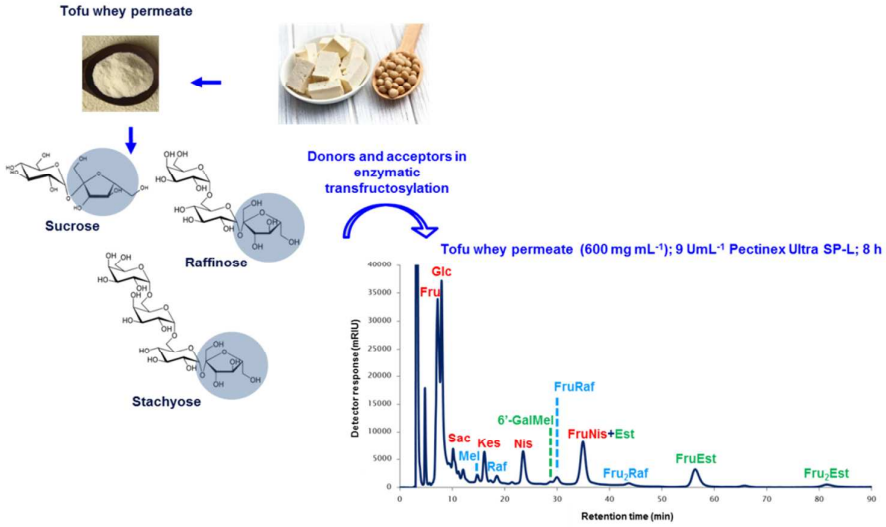

A
Concentration (g L-1)
Total FOS
Glucose
Nystose
Kestose
Sucrose
FrucNys
Fructose
Fru2Nys
B
Concentration (g L-1)
Melibiose
Total FD
Total FOS
Raffinose
Fru2Raf
FruRaf
Sucrose
Fru3Raf
Fructose
Glucose
C
Concentration (g L-1)
Total FD
6'-Galactosyl-melibiose
Stachyose
FruSta
Fru2Sta
Fru3Sta
Total FOS
Fructose
Sucrose
Melibiose
Incubation time (hours)

Figure 4


a
Abundance
m/z→
73
103
129
147
169
191
204
217
230
243
271
319
361
362
363
437
451
b
Abundance
m/z→
73
103
129
147
169
191
204
217
230
243
271
319
361
362
363
437
451
c
Abundance
m/z→
73
103
129
147
169
191
204
217
230
243
271
319
361
362
363
437
451
d
Abundance
m/z→
73
103
129
147
169
191
204
217
230
243
271
331
361
362
363
437
451

TOC Graphic


Tofu whey permeate
Sucrose
Raffinose
Stachyose
Donors and acceptors in enzymatic transfructosylation
Tofu whey permeate (600 mg mL-1); 9 UmL-1 Pectinex Ultra SP-L; 8 h
Glc
Fru
Sac
Kes
Nis
6'-GalMel
Mel
Raf
FruRaf
FruNis+Est
Fru2Raf
FruEst
Fru2Est
Detector response (mRIU)
Retention time (min)