

1 **Tofu whey permeate is an efficient source to enzymatically produce prebiotic**  
2 **fructooligosaccharides and novel fructosylated  $\alpha$ -galactosides**

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25 **ABSTRACT**

26 This work addresses a novel **and efficient bioconversion** method for the utilization of  
27 tofu whey permeate (TWP), an important by-product from the soybean industry, as a  
28 precursor of high value-added ingredients as prebiotic fructooligosaccharides and novel  
29 fructosylated  $\alpha$ -galactosides. This process is based on the high capacity of the  
30 commercial enzyme preparation Pectinex Ultra SP-L to transfructosylate the main  
31 carbohydrates present in TWP as sucrose, raffinose and stachyose to produce up to a  
32 maximum of 82.1 g L<sup>-1</sup> (equivalent to 57% with respect to initial sucrose, raffinose and  
33 stachyose content in TWP) of fructooligosaccharides and fructosylated  $\alpha$ -galactosides.  
34 Raffinose- and stachyose-derived oligosaccharides were formed by the elongation from  
35 the non-reducing terminal fructose residue up to three fructosyl groups bound by  $\beta$ -  
36 (2→1) linkages. These results could provide new findings on the valorization and  
37 upgrading of the management of TWP and an alternative use of raw material for the  
38 production of FOS and derivatives.

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40 **KEYWORDS:** tofu whey, fructooligosaccharides, prebiotics,  $\alpha$ -galactosides, enzymatic  
41 synthesis, transfructosylation.

## 42 1. INTRODUCTION

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

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

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

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

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## 77 2. MATERIALS AND METHODS

78 **2.1. Enzyme, chemicals and reagents.** The commercial enzyme preparation  
79 Pectinex Ultra SP-L, a soluble preparation containing fructosyltransferase activity and  
80 produced by *Aspergillus aculeatus*, was a generous gift from Novozymes (Bagsvaerd,  
81 Denmark). Fructosyltransferase activity was 400 U mL<sup>-1</sup>, where 1 U is defined as the  
82 amount of enzyme transferring 1  $\mu$ mol of fructose per minute at a working temperature  
83 of 60 °C and a sucrose concentration of 300 g L<sup>-1</sup> at pH 5.5.

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

91           **2.2. Production of tofu whey permeate.** Tofu whey (TW) was kindly provided  
92 by Natursoy (Barcelona, Spain). Immediately after reception, TW was freeze-dried for a  
93 properly storage until its use. For obtainment of tofu whey permeate (TWP), freeze-  
94 dried TW was reconstituted in ultrapure water at 24 mg mL<sup>-1</sup> (simulating original dry  
95 matter concentration). To remove proteins, 10 mL portions were ultrafiltered through  
96 hydrophilic 3 kDa cutoff membranes (Amicon® Ultra-15, Millipore Corp., Bedford,  
97 MA) by centrifugation at 4,000g for 90 min. Finally, filtrate was recovered, freeze-dried  
98 and kept at -20 °C until posterior analysis.

99           **2.3. Enzymatic synthesis of oligosaccharides.** Enzymatic synthesis of  
100 oligosaccharides was carried out by incubating TWP (600 g L<sup>-1</sup>) with Pectinex Ultra SP-  
101 L at 60 °C, pH 5.5 (using 1 M NaOH to adjust the pH value of the TWP aqueous  
102 solution) and continuous agitation at 1,350 rpm using an orbital shaker (Eppendorf  
103 Thermomixer Confort, Hauppauge, NY, USA). Optimum reaction conditions were  
104 according to literature.<sup>13</sup> Regarding enzyme concentration, optimization was carried out  
105 by testing three values, 1.7 U mL<sup>-1</sup>,<sup>14</sup> 9 U mL<sup>-1</sup>,<sup>15</sup> and 34 U mL<sup>-1</sup>.<sup>13</sup> Evolution of FOS  
106 and FDG formation was determined taking aliquots from the reaction mixture at  
107 suitable time intervals, including 0, 0.5, 1, 3, 8 and 24 h. The enzyme was inactivated by  
108 heating at 100 °C for 5 min. Once the enzyme concentration was optimized to TWP,  
109 individual enzymatic synthesis from sucrose, raffinose or stachyose were developed  
110 under the same reaction conditions than TWP.

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

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

116           **2.4. Chromatographic determination of carbohydrates. LC-RID.**  
117   Chromatographic separation and quantitation of carbohydrates present in the original  
118   sample of TWP and samples resulting from enzymatic synthesis was carried out by LC-  
119   RID.

120           Before chromatographic analysis, inactivated samples resulting from enzymatic  
121   synthesis were diluted with acetonitrile:water (50:50, v:v) at total carbohydrate  
122   concentration of  $\sim 10 \text{ mg mL}^{-1}$  ( $\sim 60$ -folds), filtered (0.45  $\mu\text{m}$  PVDF filters, Symta,  
123   Madrid, Spain), and kept at 4 °C until their analysis by LC with refractive index  
124   detection (RID) as described below.

125           LC analyses were carried out using an Agilent Technologies 1260 Series HPLC  
126   system (Böblingen, Germany). The separation of carbohydrates was carried out on a  
127   Kromasil column (100-NH<sub>2</sub>; 250 mm  $\times$  4.6 mm, 5  $\mu\text{m}$  particle size) (Akzo Nobel,  
128   Brewster, NY, USA) using acetonitrile/water (70:30 v/v) as mobile phase and elution in  
129   isocratic mode at a flow rate of 1 mL min<sup>-1</sup> for 90 min. The injection volume was 50  $\mu\text{L}$   
130   ( $\sim 550$ -850  $\mu\text{g}$  of total carbohydrates). Data acquisition and processing were performed  
131   using Agilent ChemStation software. Carbohydrates in the reaction mixtures were  
132   initially identified by comparing their retention times ( $t_R$ ) with those of pure standard  
133   sugars, including fructose, glucose, sucrose, melibiose, kestose, raffinose, nystose and  
134   stachyose. Quantitative analysis was performed by the external standard method, using  
135   calibration curves of each pure standard in the range 0.05–2.5 mg mL<sup>-1</sup>.

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

138           *GC-FID and GC-MS.* The trimethylsilyl oxime (TMSO) derivatives of mono-, di-  
139   and oligosaccharides for both GC-FID and GC-MS analyses were prepared as  
140   previously described by Corzo-Martínez et al.<sup>16</sup> A weight of 10 mg of sample was

141 added to 0.4 mL of internal standard (IS) solution, containing 0.5 mg mL<sup>-1</sup> of phenyl-β-  
142 D-glucoside. The mixture was dried at 38-40 °C in a rotatory evaporator (Büchi  
143 Labortechnik AG, Falwil, Switzerland). Oximes were obtained by addition of 250 µL of  
144 a solution of 2.5% hydroxylamine chloride in pyridine to the carbohydrate mixture after  
145 30 min at 70 °C incubation. Subsequently, the oximes were silylated with  
146 hexamethyldisilazane (250 µL) and trifluoroacetic acid (25 µL) at 50 °C for 30 min.  
147 Then, reaction mixtures were centrifuged at 10,000 g for 2 min. This derivatization  
148 procedure gives rise to a single chromatographic peak for non-reducing sugars,  
149 corresponding to their trimethylsilyl ethers, whereas two peaks are detected for reducing  
150 sugars, corresponding to their *syn*- (E) and *anti*- (Z) oxime isomers.

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

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

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

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### 186 3. RESULTS AND DISCUSSION

187 **3.1. Carbohydrate composition of tofu whey permeate.** Tofu whey (TW) was  
188 subjected to a previous ultrafiltration step to mainly remove residual proteins that may  
189 approximately correspond to 9% of the original soybean proteins.<sup>8</sup> The resulting tofu



190 whey permeate (TWP) could be an efficient substrate for the enzymatic synthesis of  
191 oligosaccharides given that the ultrafiltration process produces a substrate rich in  
192 carbohydrates. Thus, the total content in carbohydrates of TWP was close to 80% with  
193 respect to dry matter and the carbohydrate composition determined by LC-RID was  
194 mainly dominated by sucrose (34.4% of total carbohydrates), followed by stachyose ( $\alpha$ -  
195 D-galactopyranosyl-(1 $\rightarrow$ 6)- $\alpha$ -D-galactopyranosyl-(1 $\rightarrow$ 6)- $\alpha$ -D-glucopyranosyl-(1 $\rightarrow$ 2)-  
196  $\beta$ -D-fructofuranoside) (21.3%), fructose (12.3%), glucose (7.0%) and raffinose ( $\alpha$ -D-  
197 galactopyranosyl-(1 $\rightarrow$ 6)- $\alpha$ -D-glucopyranosyl-(1 $\rightarrow$ 2)- $\beta$ -D-fructofuranoside) (5.6%) as it  
198 is shown in Table 1. The major presence of these carbohydrates is in good agreement  
199 with previous reports also describing the carbohydrate composition of TW and/or  
200 TWP.<sup>3,8,9,12</sup> Interestingly, a broad and unresolved peak eluting in the monosaccharide  
201 area ( $t_R = \sim 7$  min, Figure 1) and representing 16.9% of total content of carbohydrates  
202 was labelled as other monosaccharides and polyalcohols (Table 1).

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

214           **3.2. Enzymatic synthesis of fructooligosaccharides and novel fructosylated**  
215  **$\alpha$ -galactosides.** *Tofu whey permeate as starting substrate.* TWP could be an appropriate  
216 substrate for FOS according to the high content of sucrose ( $163 \text{ g L}^{-1}$ ) (Table 1).  
217 Additionally, TWP also contains substantial levels of  $\alpha$ -galactosides such as raffinose  
218 ( $26.6 \text{ g L}^{-1}$ ) and stachyose ( $101 \text{ g L}^{-1}$ ) which was previously shown to be hydrolyzed  
219 and transfructosylated to product oligosaccharides of higher degree of polymerization  
220 (DP).<sup>19</sup>

221           The enzymatic source employed in this work was the commercial enzyme  
222 preparation Pectinex Ultra SP-L, produced by *Aspergillus aculeatus*, because is largely  
223 used in the food industry, has a low cost, good transfructosylation activity and high  
224 thermal stability.<sup>15,20</sup> Given that the optimum pH (5.0-5.5) and temperature (60-65 °C)  
225 have been well-established in previous works that addressed the synthesis of FOS<sup>14, 15, 21</sup>  
226 or fructosylated derivatives of stachyose<sup>19</sup> catalyzed by Pectinex Ultra SP-L, the  
227 production of fructosylated oligosaccharides was studied as a function of the time and  
228 concentration of the enzyme preparation. Thus, three different enzyme concentrations,  
229 that is 1.7, 9 and  $34 \text{ U mL}^{-1}$ , were assayed whilst the concentration of TWP was set at  
230 60% (w/v), which was the maximum concentration at which TWP was completely  
231 soluble in the medium reaction to favor the transglycosylation reaction. In general  
232 terms, the maximum production of fructosylated oligosaccharides was achieved after  
233 24, 8 and 3 hours of reaction when 1.7, 9 and  $34 \text{ U mL}^{-1}$  of enzyme were, respectively,  
234 added. Nevertheless, the maximum production of FOS and FDG estimated by LC-RID  
235 was achieved when  $9 \text{ U mL}^{-1}$  of enzyme was used, i.e.,  $164 \text{ g L}^{-1}$  (Table 1), being  
236 equivalent to a yield of 57% (in weight with respect to the determined initial amount of  
237 sucrose, raffinose and stachyose in TWP); whereas  $150 \text{ g L}^{-1}$  (51% of yield) and 128 g

238 L<sup>-1</sup> (44% of yield) of fructosylated oligosaccharides were obtained with 1.7 and 34 U  
239 mL<sup>-1</sup> 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,  $\alpha$ -galactosides susceptible to act as donor and/or  
243 acceptor of fructosyl units in transfructosylation reactions catalyzed by microbial  
244 transglycosidases,<sup>12,19</sup> 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  $\beta$ -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<sup>-1</sup> (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.<sup>22</sup> 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 ( $\alpha$ -D-galactopyranosyl-(1 $\rightarrow$ 6)-D-glucose,  
262 peak 4, Figure 1) demonstrated that raffinose (peak 6, Figure 1) was efficiently broken

263 down by Pectinex Ultra SP-L at the  $\beta(2\rightarrow1)$  linkage between fructose and glucose. In  
264 addition, fructose was detected only at low levels which is indicative of its transfer to  
265 other raffinose molecules to give rise to a series of fructosylated-raffinose  
266 oligosaccharides with DP ranging from 4 to 6 (peaks 9, 12 and 16, Figure 1).  
267 Additionally, Figure 3B shows that raffinose was efficiently hydrolyzed by Pectinex  
268 Ultra SP-L since only 12% of raffinose remained unaltered after 24 hours of reaction.  
269 The highest production of fructosylated raffinose-derived oligosaccharides, also  
270 achieved at 3 hours of reaction, was  $319.4 \text{ g L}^{-1}$  (Figure 3B) and equivalent to a yield of  
271 53% (in weight with respect to the determined initial amount of raffinose). Remarkably,  
272 the detection of galactose (detected by GC-FID) and sucrose (peak 3, Figure 1) from the  
273 third hour of reaction indicated that Pectinex Ultra SP-L had also the ability to cleave,  
274 although at a much lesser extent, raffinose at the  $\alpha(1\rightarrow6)$  bond between galactose and  
275 glucose.  $\alpha$ -galactosidases from *Aspergillus* sp., as *A. terreus*, have been previously  
276 purified and characterized.<sup>23</sup>

277 Stachyose was hydrolyzed by Pectinex Ultra SP-L at similar extension than  
278 raffinose since a decrease of 87% in stachyose content was found after 24 hours of  
279 reaction (Figure 3C). Stachyose was mainly hydrolyzed at the sucrose moiety giving  
280 rise to 6'-galactosyl-melibiose ( $\alpha$ -D-galactopyranosyl-(1 $\rightarrow$ 6)- $\alpha$ -D-galactopyranosyl-  
281 (1 $\rightarrow$ 6)-D-glucose) (peak 8, Figure 1) and fructose (Peak 1, Figure 1), confirming  
282 previous findings.<sup>13,19</sup> The maximum production of FDG, tentatively identified as the  
283 pentasaccharide fructosyl-stachyose (peak 13, Figure 1) and the hexasaccharide  
284 difructosyl-stachyose (peak 14, Figure 1), was  $177.1 \text{ g L}^{-1}$  (equivalent to a yield of 30%,  
285 in weight with respect to the determined initial amount of stachyose) and achieved at 8  
286 hours of reaction (Figure 3C). Furthermore, low levels ( $6.1 \text{ g L}^{-1}$ ) of the heptasaccharide  
287 trifructosyl-stachyose could be determined only after 24 hours of reaction.

288           Although sucrose was the best donor and acceptor leading to a high production  
289 of FOS, raffinose and, in a lesser level, stachyose were also efficient donors and  
290 acceptors for the transfructosylation reaction catalyzed by Pectinex Ultra SP-L which  
291 allowed the high yield-synthesis of FDG. According to the well-described mechanism  
292 of transfructosylation of Pectinex Ultra SP-L acting on sucrose,<sup>24</sup> as well as to the  
293 scarce studies dealing with the transfructosylation of stachyose by this commercial  
294 enzyme,<sup>13,18</sup> it can be inferred that raffinose- and stachyose-derived oligosaccharides  
295 were formed by the elongation of their respective chains from the non-reducing terminal  
296 fructose residue through a linear chain of up to three fructosyl residues bound by  $\beta$ -  
297 (2 $\rightarrow$ 1) linkages. Thus, the general chemical structure of fructosylated oligosaccharides  
298 derived from raffinose and stachyose was  $\alpha$ -D-Gal-(1 $\rightarrow$ 6)- $\alpha$ -D-Glc-[(1 $\rightarrow$ 2)- $\beta$ -D-Fru]<sup>n</sup>  
299 and  $\alpha$ -D-Gal-(1 $\rightarrow$ 6)- $\alpha$ -D-Gal-(1 $\rightarrow$ 6)- $\alpha$ -D-Glc-[(1 $\rightarrow$ 2)- $\beta$ -D-Fru]<sup>n</sup>, respectively, with n =  
300 1-3.

301           The individual syntheses of FOS from sucrose and FDG from raffinose or  
302 stachyose were also useful to tentatively identify and chromatographically distinguish  
303 between FOS and FDG when TWP is used as precursor of bioactive oligosaccharides.  
304 In general terms, FOS and FDG were well resolved by LC-RID with the exception of  
305 6'-galactosyl-melibiose (peak 8, Figure 1) and fructosyl-raffinose (peak 9, Figure 1), as  
306 well as fructosyl-nystose (peak 10, Figure 1) and stachyose (peak 11, Figure 1). Both  
307 pair of chromatographic peaks co-eluted during the LC chromatographic separation that  
308 hampered their accurate quantification.

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

313 after 8 hours of reaction using  $9 \text{ U mL}^{-1}$  of Pectinex Ultra SP-L, which led to the  
314 maximum production of FOS and FDG, was analyzed by GC-FID (Figure 2 (blue line)).  
315 Under the developed chromatographic conditions, carbohydrates up to DP 5 were  
316 detected and 6'-galactosyl-melibiose (peak 13, Figure 2) and fructosyl-raffinose (peak  
317 15, Figure 2), as well as stachyose (peak 16, Figure 2) and fructosyl-nystose (peak 17,  
318 Figure 2) could be well resolved, thus, allowing their accurate quantification (Table 1).  
319 Finally, the detection of both inulobiose ( $\beta$ -D-Fru-(2 $\rightarrow$ 1)- $\beta$ -D-Fru) (peak 8, Figure 2)  
320 and inulotriose ( $\beta$ -D-Fru-(2 $\rightarrow$ 1)- $\beta$ -D-Fru-(2 $\rightarrow$ 1)- $\beta$ -D-Fru) (peak 12, Figure 2) could be  
321 attributed to the ability of fructose to act as a minor acceptor in the transfructosylation  
322 reaction.

323 Given the lack of commercially available standards for FDG, the structural  
324 confirmation was achieved by comparison of the mass spectra corresponding to  
325 synthesized FDG from TWP with the respective mass spectra of the FDG resulting from  
326 the enzymatic synthesis using raffinose or stachyose as single precursors. Figure 4,  
327 which shows the mass spectra of fructosyl-stachyose (main synthesized fructosylated  $\alpha$ -  
328 galactoside) and fructosyl-raffinose, respectively, demonstrated the structural similarity  
329 of the synthesized FGD regardless the use of TWP or single raffinose or stachyose as  
330 initial substrates. The most abundant ions were those found at  $m/z$  361 (which is  
331 characteristic of a glycosylated sugar ring) and at  $m/z$  217 (typical of furanose rings)  
332 which contains two units of TMSOCH from C6 and C5 completed by the atom of C4.<sup>12</sup>  
333 Both fragment ions were previously identified in raffinose and stachyose by GC-MS.<sup>25</sup>  
334 Fragmentation behavior of FDG also resulted in characteristic ions at  $m/z$  129, 169, 271,  
335 437 and 451. Moreover, fragment ions detected at  $m/z$  230 and 362 have been  
336 previously related to carbohydrates having fructose units bonded by  $\beta$ -(2 $\rightarrow$ 1) glycosidic  
337 linkages, such as kestose, inulobiose and inulotriose (Figure 4).<sup>26</sup> Furthermore, the mass

338 spectra of fructosylated derivatives of stachyose were essentially the same than those  
339 previously obtained (data not shown) and whose structural characterization was carried  
340 out by NMR approaches.<sup>19</sup>

341 Overall, the enzymatic production of FOS and FDG from TWP under the optimum  
342 conditions was quite balanced since the proportion of both type of oligosaccharides was  
343 53.6% (44 g L<sup>-1</sup>) and 46.4 % (38.1 g L<sup>-1</sup>), respectively. This could be explained by the  
344 fact that the concentrations of sucrose, precursor of FOS, against raffinose and  
345 stachyose, precursors of FDG, maintained a similar ratio, i.e. 56% vs 44%, in the initial  
346 composition of TWP (Table 1). Likewise, this behavior supports the efficient capacity  
347 of either raffinose or stachyose to act as donors and acceptors in the transfructosylation  
348 reaction catalyzed by Pectinex Ultra SP-L, previously demonstrated when these  
349 carbohydrates were used as single substrates (Figure 3B and 3C).

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

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

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

365

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

**A**

Time (hours)	Fructose	Glucose	Galactose	Other monosaccharides and polyalcohols	Sucrose	Melibiose	Other disaccharides	Raffinose	Stachyose
0	58.4 $\pm$ 0.9	33.2 $\pm$ 0.2	0.0 $\pm$ 0.0	80.2 $\pm$ 0.5	163.0 $\pm$ 0.8	0.0 $\pm$ 0.0	11.0 $\pm$ 0.4	26.6 $\pm$ 0.4	101.0 $\pm$ 0.5
0.5	69.8 $\pm$ 3.4	75.6 $\pm$ 1.8	9.6 $\pm$ 0.5	43.2 $\pm$ 4.7	89.0 $\pm$ 2.6	3.6 $\pm$ 0.0	11.4 $\pm$ 0.6	22.8 $\pm$ 0.5	101.0 $\pm$ 0.1
1	80.2 $\pm$ 1.3	97.6 $\pm$ 0.3	16.6 $\pm$ 0.7	91.8 $\pm$ 2.8	62.4 $\pm$ 1.6	4.6 $\pm$ 0.1	11.4 $\pm$ 0.2	22.4 $\pm$ 0.6	100.4 $\pm$ 0.3
3	83.6 $\pm$ 4.7	120.8 $\pm$ 3.5	17.6 $\pm$ 3.1	92.0 $\pm$ 6.4	36.4 $\pm$ 1.3	5.6 $\pm$ 0.1	10.0 $\pm$ 0.6	12.6 $\pm$ 0.1	90.2 $\pm$ 4.1
8	99.4 $\pm$ 1.0	150.6 $\pm$ 2.4	27.2 $\pm$ 2.8	120.2 $\pm$ 4.8	36.2 $\pm$ 1.9	8.6 $\pm$ 0.3	10.4 $\pm$ 0.4	12.0 $\pm$ 0.1	83.0 <sup>a</sup> $\pm$ 4.7
24	104.2 $\pm$ 1.0	151.0 $\pm$ 1.1	24.8 $\pm$ 0.6	119.2 $\pm$ 2.3	36.0 $\pm$ 0.2	9.0 $\pm$ 0.2	10.0 $\pm$ 0.1	9.2 $\pm$ 0.2	86.6 $\pm$ 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 $\pm$ 0.0	0.0 $\pm$ 0.0	0.0 $\pm$ 0.0	0.0 $\pm$ 0.0	0.0 $\pm$ 0.0	0.0 $\pm$ 0.0	0.0 $\pm$ 0.0	0.0 $\pm$ 0.0
0.5	4.8 $\pm$ 0.0	53.0 $\pm$ 1.0	4.4 $\pm$ 0.1	n.q	n.q	0.0 $\pm$ 0.0	10.0 $\pm$ 0.3	6.0 $\pm$ 0.1
1	8.4 $\pm$ 0.0	75.2 $\pm$ 1.6	13.2 $\pm$ 0.4	n.q	n.q	0.0 $\pm$ 0.0	17.2 $\pm$ 0.7	7.6 $\pm$ 0.2
3	12.4 $\pm$ 0.2	54.8 $\pm$ 1.1	32.0 $\pm$ 0.7	n.q	n.q	0.0 $\pm$ 0.0	30.2 $\pm$ 0.9	7.0 $\pm$ 0.0
8	6.0 <sup>a</sup> $\pm$ 0.2	37.0 $\pm$ 1.0	45.6 $\pm$ 1.1	5.4 $\pm$ 0.1 <sup>a</sup>	5.0 $\pm$ 0.2 <sup>a</sup>	5.0 $\pm$ 0.0	54.6 $\pm$ 1.3	5.6 $\pm$ 0.1
24	9.2 $\pm$ 0.3	31.0 $\pm$ 0.6	46.0 $\pm$ 0.5	n.q	n.q	4.8 $\pm$ 0.1	51.0 $\pm$ 1.0	6.0 $\pm$ 0.0

<sup>a</sup> Quantified by GC-FID.

n.q. Not quantified.

**FIGURE CAPTIONS**

**Figure 1.** LC-RID profiles of transfructosylation reactions catalyzed by Pectinex Ultra SP-L ( $9 \text{ U mL}^{-1}$ ) after 8 h at  $60 \text{ }^\circ\text{C}$  and pH 5.5 using  $600 \text{ g L}^{-1}$  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 ( $600 \text{ g L}^{-1}$ ) catalyzed by Pectinex Ultra SP-L ( $9 \text{ U mL}^{-1}$ ) after 8 h at  $60 \text{ }^\circ\text{C}$  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- $\beta$ -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 ( $9 \text{ U mL}^{-1}$ ) at  $60 \text{ }^\circ\text{C}$  and pH 5.5 using  $600 \text{ g L}^{-1}$  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 synthesized from raffinose (a) and tofu whey permeate (b) and fructosyl-stachyose from stachyose (c) and tofu whey permeate (d).

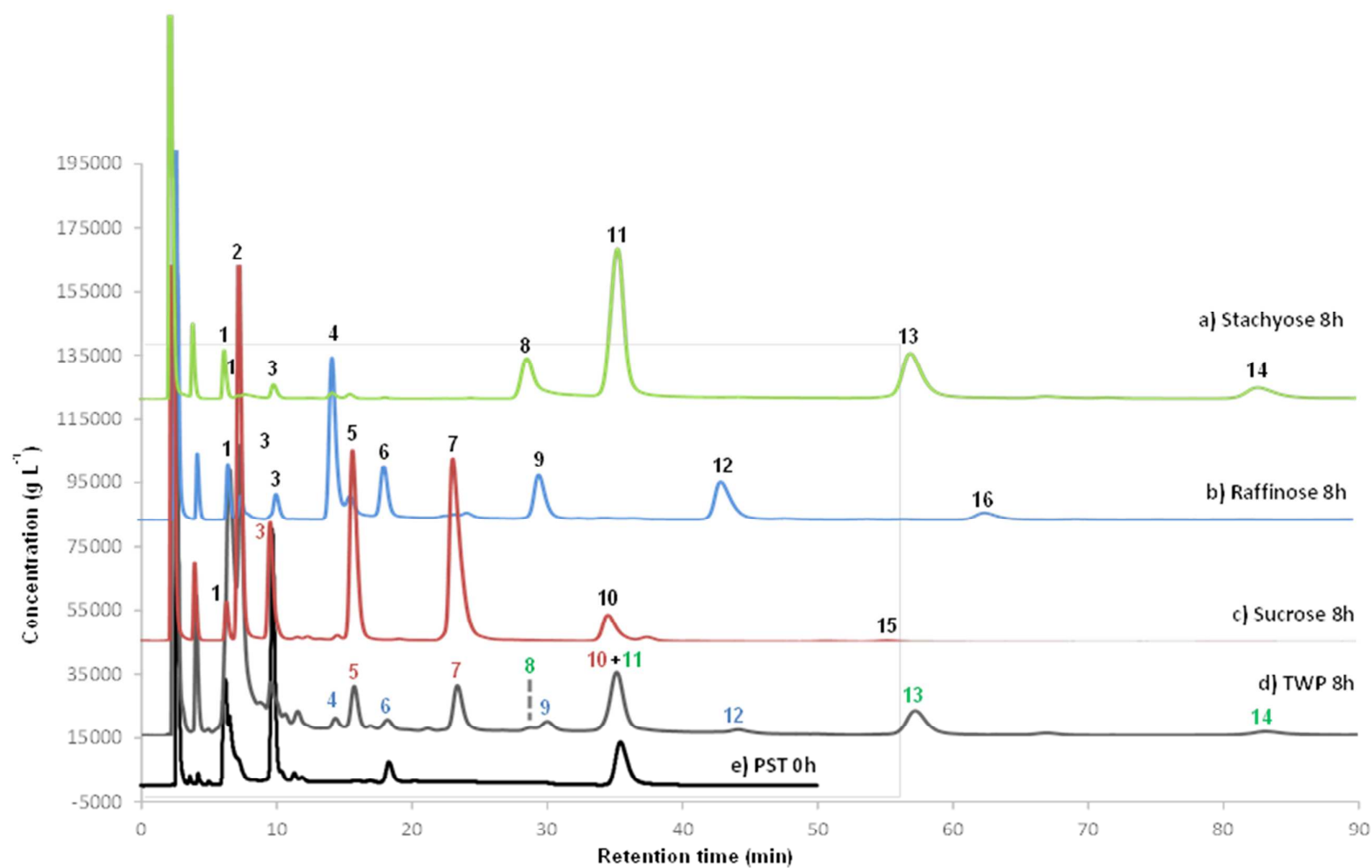


Figure 1



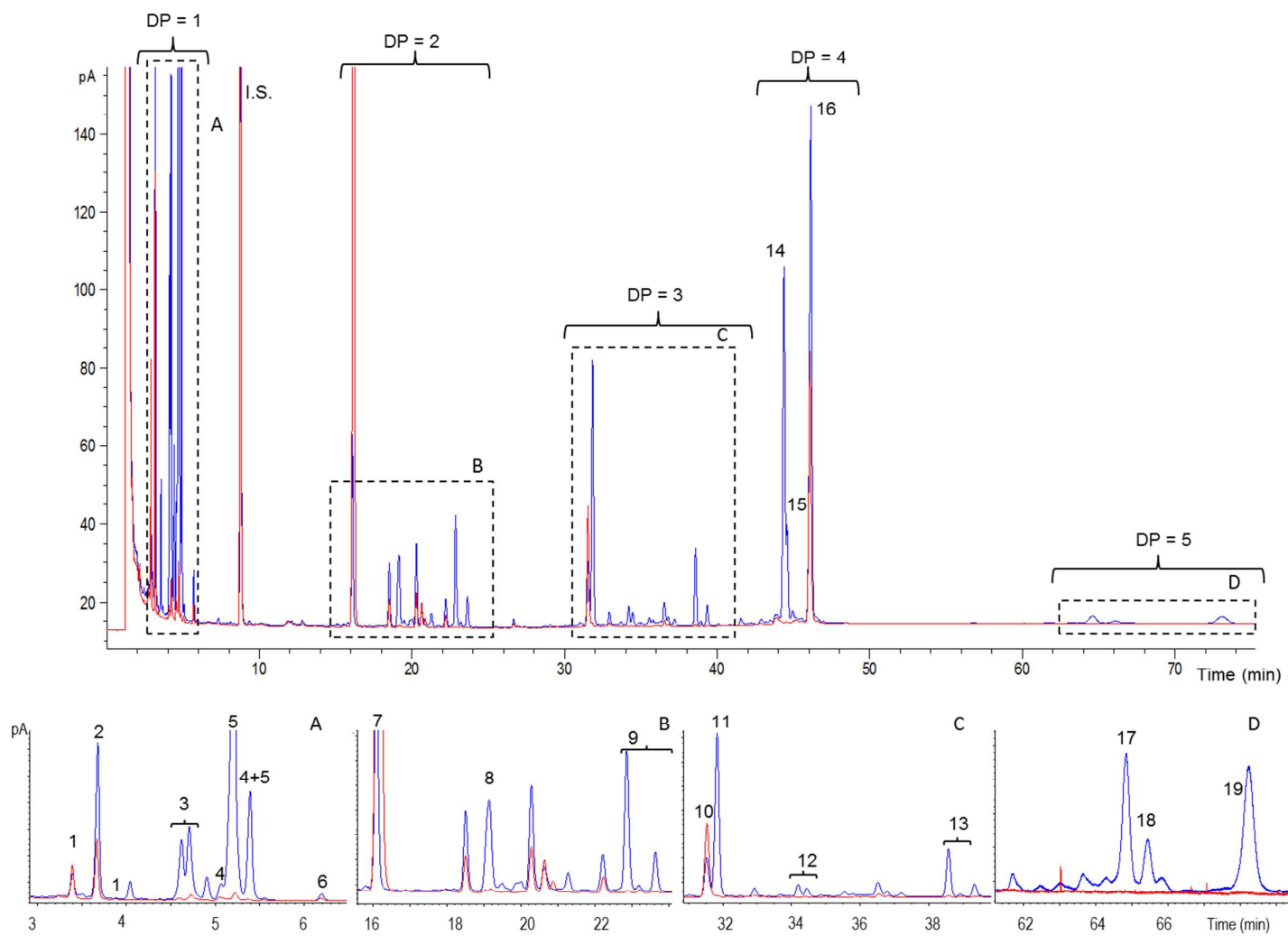


Figure 2

Figure 3.

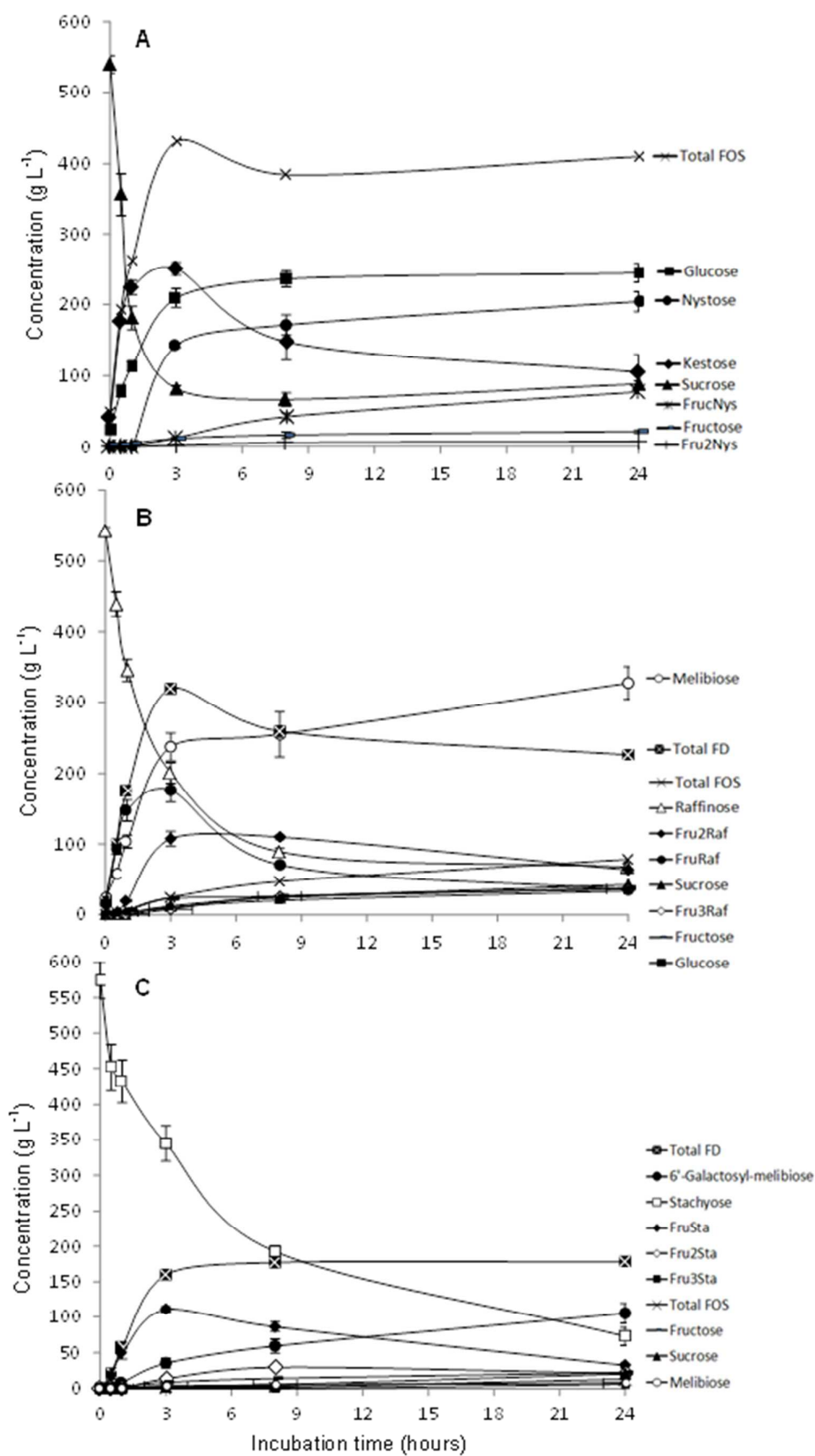
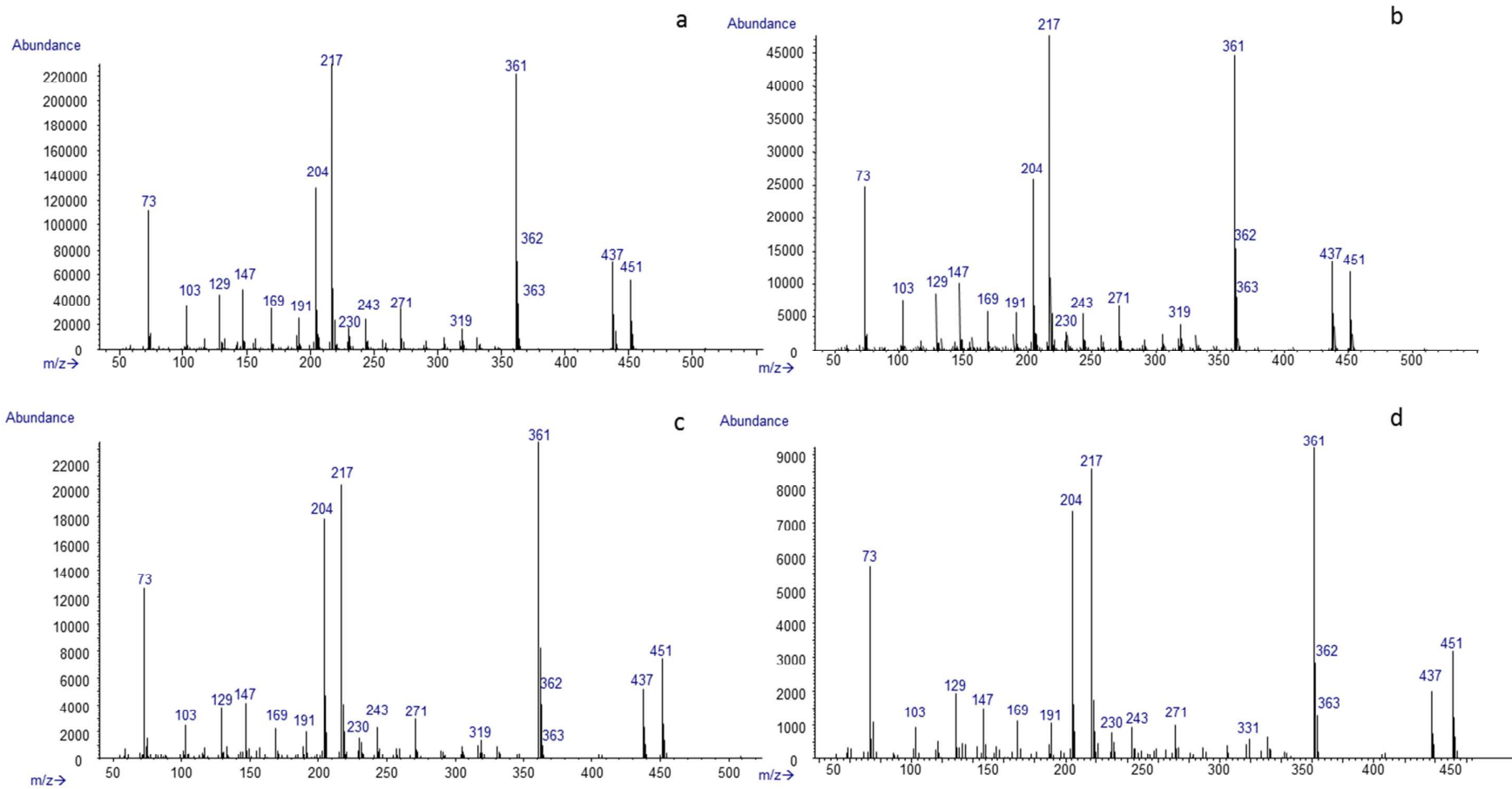


Figure 4



## TOC Graphic

