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**N-glycome profiling of patatins from different potato species of
Solanum genus**

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ABSTRACT

It is hypothesized that oligosaccharides are another potential source of immunological cross-reaction between different plant allergens. Patatin is the most abundant glycoprotein in potato and has been described to have oligosaccharide of composition $Man_3(Xyl)GlcNAc_2(Fuc)$. In this work N-glycosylation profiles of patatin proteins isolated from tubers of different potato species were investigated and compared. Oligosaccharides were released by enzymatic digestion with PNAGase A and analyzed primarily by matrix-assisted laser desorption ionization mass spectrometry. For glycan labeling a modified version of on-target derivatization with phenylhydrazine was applied. This study found the presence of glycan structures not described previously in patatins of potato tubers and their glycan profiles significantly differed. This knowledge about the glycosylation of potato patatins may be helpful for correct choice of potato species to decrease the presence of specific glycan epitopes causing food allergy as well as for utilization of potatoes for the manufacture of therapeutic proteins.

Keywords: glycans, mass spectrometry, patatin, plants, potato, Solanum

INTRODUCTION

N-glycosylation is one of the most ubiquitous types of post-translational modification (PTM). The structural diversity in glycoconjugates allows them to encode information for specific molecular recognition and serves as determinant of protein stability, folding and pharmacokinetics¹. Plants have emerged as alternative for the production of therapeutic proteins². However, modifications of plant oligosaccharides usually significantly differ from glycans found in vertebrates and sometimes result in immunogenic oligosaccharide structures³. This knowledge, together with the fact that cells are coated with saccharide molecules, makes the glycosylation of non-mammalian systems an interesting target in biochemical research.

N-linked oligosaccharides are synthesized in the endoplasmic reticulum through the oligosaccharide precursor, $Glc_3Man_9GlcNAc_2$, attached to the nitrogen of asparagine at specific sites of the protein⁴. Then asparagine linked oligosaccharides undergo several maturation steps and are transferred via Golgi apparatus to the cell⁵. High mannose glycans consist of five to nine mannose units ($Man_{5-9}GlcNAc_2$) and have a similar structure in mammals and plants. However, complex glycans in plants are structurally different from those found in mammalian glycoproteins. Most of the N-glycans in plants carry β 1,2-xylose (Xyl) residue bonded to the β -linked mannose moiety on the common conserved $Man_3GlcNAc_2$ core and α 1,3-fucose (Fuc) residue on GlcNAc reducing terminy⁶. These, β 1,2-Xyl and α 1,3-Fuc epitopes are not present in mammalian glycoproteins. They are documented to be highly immunogenic⁷; there are strong indications that these oligosaccharide structures are targets of immunoglobulin (IgE) in people suffering with allergy to plant foods and pollens⁸. Therefore, in order to use plant

sources for manufacturing therapeutic proteins, modification of their N-glycosylation pattern is required ⁹.

Potato (*Solanum tuberosum*) is an easy-to-grow plant and more than a billion people worldwide consume them daily ¹⁰. Because of this demand, a multitude of varieties have been developed to resist pre-or post-harvest diseases or target specific markets ¹¹. The major protein of potato is known as patatin, which amounts up to 40% of the total protein located in the vacuoles of potato tubers ¹². Patatins serve as storage proteins and have a lot of enzymatic activities as e.g. nonspecific lipid acyl hydrolase ^{13,14} phospholipase ¹⁵, β -1,3-glucanase ¹⁶ or act as defense against plant parasites ¹⁷. These characteristics make patatins an interesting protein source for use not only as a food, but for biotechnological applications as well ¹⁸. However, the real physiological role of this protein has not been still completely established. It has been shown that patatins of Kuras cultivar have five different glycosylated positions and all of them carry a complex N-glycan of composition *Man*₃(*Xyl*)-*GlcNAc*-*GlcNAc*(*Fuc*), typical plant oligosaccharide structure and the most abundant glycan in potato tubers ¹⁹. All sites of glycan attachment were located in loops or turns on the molecular surfaces.

Biochemical characteristics of patatins from tubers of different potato cultivars were recently reported ²⁰. The genotype differences of these proteins were evaluated with regards to utility groups: table potato cultivars and processing potato cultivars. Both groups showed similar values of protein content and three mass levels – 40.6, 41.8 and 42.9 kDa of purified patatins were found by MALDI-MS. The differences among these masses (~1.2 kDa) indicated a mass close to the molecular mass of patatin oligosaccharide as it was mentioned above. However, the present study aiming at detailed investigation of glycosylation profiles of patatins isolated from selected potato

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species showed the presence of more oligosaccharide structures. The data obtained by mass spectrometric analysis provided even evidence for differences in profiles of patatin N-glycans and these results will be discussed.

MATERIALS AND METHODS

Plant materials and growth conditions.

For this work, tubers from selected potato species (for details see Table 1) were donated by the Gene Bank of Potato Research Institute Havlíčkův Brod, Czech Republic. In both experimental years (2011, 2012), analyzed tubers were obtained by following approach of cultivation: Plants in vitro reproduction were made on Murashige and Skoog (MS) agar medium containing 30 g L⁻¹ of sucrose without growth hormones²¹. Rooted in vitro plants were transferred to in vivo conditions of greenhouse. Each potato plant (in total five plants from each species) was cultivated in a pot with 10 L of potting soil to ensure harmonic nutritive conditions for complete growing season. Potting soil in pots was kept in appropriate humidity by daily watering. Potato plants were cultivated during both seasons under chemical control against *Phytophthora infestans* and insect pests. All conditions of plant cultivation were the same in both experimental years. Obtained tubers were flushed in water and cut first longitudinally into identical halves and then into thin slices (~2 mm). The slices were frozen to -80°C and freeze-dried (-50°C, 0.040 mbar, 48 h, freeze-dryer Alpha 1-4 LSC, Martin Christ, Germany) and homogenized by using a laboratory mill. Freeze dried potato tuber meal (5 g) was extracted in 25 mM Tris HCl buffer (50 ml, pH 7.4) for 30 min. at 4°C. The mixture was centrifuged (4°C, 3600 g, 15 min), the supernatant filtered.

154 ***Purification of patatins***

155 Chromatographic purification of patatins was achieved with the following three steps
156 modified according to the procedure reported previously²⁰:

157 1. Ion-exchange chromatography was completed on DEAE 52-Cellulose Servacel (15 ml,
158 Serva, Germany) with 90 ml of starting buffer (25 mM Tris-HCl, pH 7.4). After applying
159 of protein extract (10 mL), the column was washed with 60 ml of starting buffer to
160 remove unbound proteins and isocratic elution of bound proteins was performed with 30
161 ml of elution buffer (25 mM Tris-HCl pH 7.4 + 0.5 M NaCl).

162 2. Affinity chromatography was performed on Concanavalin A Sepharose 4B (10 ml, GE
163 Healthcare, USA). The column was equilibrated with 60 ml of starting buffer (25 mM
164 Tris-HCl pH 7.4 + 0.5 M NaCl). Elution of bound patatins was performed with 20 ml
165 buffer consisting of 25 mM Tris-HCl pH 7.4 + 0.5 M NaCl + 100 mM α -methyl-D-
166 glucoside .

167 3. Desalting of patatin proteins was achieved by using MWCO filters (3 kDa) according
168 to the manufacturer's protocol (Millipore). The purity of patatins (~ 99 %) was verified
169 by a means of SDS-PAGE and Experion automated chip electrophoretic system
170 (Supplementary Figure 1).

171 ***Release of N-glycans from patatins***

172 Lyophilized powder of patatin protein (500 μ g) was dissolved in 25 mM ammonium
173 bicarbonate (100 μ L) and deglycosylated with N-glycosidase A (0.2 mU; Roche) at 37 °C
174 for 20 h. Alternatively, the samples were digested first with trypsin (20 μ g) at 37 °C for
175 4h. Then the digest was kept in a water bath at 85 °C for 5 minutes. After cooling N-
176 linked oligosaccharides were released from peptides again enzymatically by treatment
177 with PNGase A (37 °C, 20 h). The reaction samples were mixed frequently during

7

178 incubation.

179 ***Solid phase extractions on carbon clean cartridges***

180 Prior to an extraction, the carbon cartridge columns (Supelclean™-ENVI Carb SPE
181 cartridges, Sigma-Aldrich) were first washed with 100% ACN following deionized water
182 (5mL of each). Then, digest (100 µL) was applied to a column with wet carrier, left to
183 permeate and approximately after 10 min the carrier was rinsed with 5x1000 µL of
184 deionized water. After that glycans were eluted with 40 % of ACN (3 x 800 µL) and
185 collected into separate tubes without using any pressure. All glycan fractions were
186 evaporated and derivatized according to the procedure as described below.

187 ***Phenylhydrazine (PHN) labelling***

188 The derivatization with PHN (Sigma-Aldrich) was achieved according to the method
189 previously reported^{22 23}, with modified conditions as described next.

190 For MALDI-MS analysis, on-target labeling approach was applied: the fractions with
191 glycans were dissolved in 20 µL of deionized water and 1 µL of a sample solution was
192 spotted onto the pre-deposited matrix ATT/PHN.HCl (2-aza-2-
193 thiothymine/phenylhydrazine hydrochloride in molar ratio 2:1)²⁴. When the spot was
194 partially dried, 0.4 µL of derivatization reagent (10 µL of PHN in 50 µL of deionized
195 water and 5 µL of ACN) was added. During this time the target was kept at temperature
196 of 50 °C until the spot had dried. These conditions prevent smearing of the spot after
197 adding PHN reagent solution; speed up the reaction time and after 3-5 minutes the sample
198 is ready for measurement.

199 For ESI-MS analysis N-glycan pools were reconstituted in the mixture consisting from
200 deionized water (50 µL), PHN (1 µL) and ACN (3 µL). The tube with the reaction
201 mixture was kept at a temperature of 60°C for 30 min under vigorous mixing.

Exoglycosidase digestions

Selected glycan fractions were subjected to α -mannosidase or β -N-acetylglucosaminidase (Canavalia ensiformis, Jack beans; Sigma) treatment at 37 °C according to the protocol supplied by manufacturer. After each time of incubation (20 min – 4h), 1 μ L of digested mixture was spotted on the MALDI target, derivatized and analyzed under the same conditions as the glycan pool before exoglycosidase treatment.

Mass spectrometry (MS)

MALDI-MS analysis was performed on the MALDI-TOF/TOF instrument (Ultraflex III, Bruker) operated in the positive reflectron mode and for each sample spot, 1200 shots were summed. The spectra were acquired using the FlexControl software. Parent ions of interest were selected for LIFT (MS/MS) experiments. MS^n data were recorded on Orbitrap ELITE instrument (Thermo Fisher Scientific, Waltham, MA, USA) equipped with Nanospray Flex Ion Source using direct infusion in 50% acetonitrile at flow rate of 300 nl/min. The resolution of all MS^n spectra was 240,000 (at 400 m/z). AGC target value of 1×10^6 or 50,000 ions, maximum injection time of 200 ms or 5,000 ms were set for all MS or MS^n measurements, respectively. Higher-energy collisional dissociation (HCD) was used to generate MS^2 ions of glycans. In case of MS^3 fragmentation scheme, collision induced dissociation (CID) at relative energy of 40 % was used to generate glycan's fragments, from which one was further selected for HCD fragmentation at different energies. The isolation window for all MS^n experiments was set to 2 m/z. MS^n spectra were visualized and exported using Xcalibur Qual Browser (version 2.2, Thermo Fisher Scientific).

MS/MS spectra of N-glycans were interpreted manually. For the assignment of oligosaccharide fragment ions, the established nomenclature reported by Domon and

Costello was followed²⁵. The proposed oligosaccharide structures were derived from the single fragmentation patterns obtained for each precursor ions. For discrimination of single isomeric structures, previously reported rules were applied²⁶.

Statistical analysis

The MALDI measurements were repeated three times for each sample and all experiments were repeated in triplicates for samples with the exception of *Solanum Tuberosum* L (cv. Desirée). In the case of this cultivar, the experiments were repeated five times to confirm the presence of only one oligosaccharide structure. The recorded MALDI-MS spectra were base-line corrected and processed by using an instrument's software tool. The proportions of individual glycans that differentiated according to their m/z values were calculated from integrated MS peak intensities over the range of their isotopic envelopes and then normalized, by using Microsoft Excel software.

RESULTS and DISCUSSION

In this study we examined N-glycans of patatins isolated from tubers of selected potato species from *Solanum* genus as listed in Table 1. The selection of these potato groups was based on the knowledge reported recently that these types exhibit significant differences in the level of many tuber metabolites²⁷. The oligosaccharides were released from purified patatins enzymatically with PNGase A, cleaned on carbon cartridges and derivatized with PHN prior to mass spectrometric analysis. The principal method used for detection was high sensitive MALDI-MS. This instrumental technique allows for the discrimination of different oligosaccharide classes. Besides, the abundance ratios of oligosaccharides can be calculated from a comparison of relative ion intensities corresponding to individual glycans. To confirm the type of cross-ring cleavages

additional MS/MS data were recorded on Orbitrap ELITE instrument. The assignment of oligosaccharide types was based on monosaccharide composition, fragmentation patterns, exoglycosidase digestions, and knowledge of the glycan biosynthetic pathway in plants. Generally, only neutral oligosaccharides were detected and produced $[M+Na]^+$ ions when detected by MALDI-MS or by Orbitrap when using a direct infusion. Most of the identified peaks showed 3-linked fucose at the GlcNAc of the reducing end (the resistance to PNGase F cleavage). The most abundant oligosaccharide in all samples was observed at m/z 1301.5 (Figure 1a) and its fragmentation pattern clearly corresponded to glycan with a composition $Man_3(Xyl)GlcNAc_2(Fuc)$ (Figure 2a), the same structure as described previously in patatin isolated from potato tuber lipases¹⁹. This glycan was detected as only oligosaccharide in patatins isolated here from *Solanum tuberosum* cv. *Desirée* independently on the year of collection and from *Solanum goniocalyx* harvested in 2011 (Figure 1a). The glycoprotein obtained from *Solanum phureja* showed higher incidence of glycan at m/z 1139.4 (Figure 1b) and corresponded to the structure with composition $Man_2(Xyl)GlcNAc_2(Fuc)$ (Figure 2b). The peak with small intensity detected at m/z 1007.37 could be assigned to a glycan of similar structure as described above, however with no xylose linked on the core mannose (Figure 2c). The second highest peak in the spectra of most patatins was observed at m/z 1504.6 (e.g. Figure 1b-c) and MS/MS data were supportive for the structure of the composition $GlcNAcMan_3(Xyl)GlcNAc_2Fuc$ (Figure 3a). B, C-fragment ions resulted from the loss of chitobiose core - m/z values detected at 1047.3 (B_4) or 1065 (C_4); 844 (B_3) or 860 (C_3). Next the loss of 3-positioned hexose produced the fragment ions at m/z 682 ($B_3/Y_{3\beta}$) corresponding to the composition of $GlcNAcMan_2Xyl$ residue (Figure 3a). Although two isomers could be possible for this glycan, according to the rules published previously²³, only one isomer was detected: the

275 preferential loss of 3-positioned hexose on the core mannose (m/z 682.21) was
276 supportive for the prevalence of an isomer with GlcNAc moiety at the 6-position in
277 mannose linked to the core mannose. Exoglycosidase treatment with β -N-
278 acetylglucosaminidase confirmed the presence of GlcNAc residue on the terminal end of
279 oligosaccharides and treatment with α -mannosidase produced glycans at m/z 1342.49,
280 1139.41 and 977.35 (e.g. Supplementary Figure 2a). The parent ions of these products
281 under MS/MS conditions produced the fragment ions supporting the attachment of Xyl
282 moiety on the core mannose (Supplementary Figure 2ab). Biantennary complex glycan
283 detected at m/z 1707.6 was observed with lower abundance in some samples (e.g. Figure
284 1b-c) and its precursor ions produced the fragment ions indicating a structure with two
285 GlcNAc residues at the non-reducing end (Figure 3b). The fragment ions recorded at m/z
286 682.2 (B_3/Y_{3a}) were consistent with a loss of GlcNAcMan residue from B_3 or C_3 ions and
287 provided evidence for GlcNAc moieties on both mannoses linked at the core mannose.

288 In the MS/MS spectra of all detected glycans the most dominant peak was
289 associated with a loss of 282 mass units from each parent precursor ions (e.g. fragment
290 ions detected at m/z 1019 in Figure 2a; fragment ions at m/z 1222 in Figure 3a or ions at
291 m/z 1425 in Figure 3b). The presence of these peaks in the MS/MS spectra of all detected
292 glycans was not influenced by the type of monosaccharide residue/s at the non-reducing
293 end. Moreover, these fragment ions under MS^2 (Orbitrap) conditions produced the
294 fragment ions as seen in the MS/MS spectra shown e.g. the Figures 2-3 and provided
295 again the support for cross-ring cleavages at the side from the reducing termini. Based on
296 the above mentioned facts, these dominant fragment ions could be explained by the loss
297 of Fuc altogether with the cleavage $^{0,1}A$. The loss of later mentioned residue from
298 reducing termini ($^{0,1}A$ -type cleavage) is commonly observed in the CID spectra of

299 mammalian N-glycans when fucose on GlcNAc of reducing end is linked at the 6-
300 position, but never produced as major fragment ions^{23,28}.

301 In the spectra of glycoproteins isolated from species *Solanum andigenum* and
302 *Solanum stenotomum* additional peaks appeared at m/z 1671.6 and 1833.7 (Figure 1c).
303 Tandem mass spectra showed that these N-glycans had both antennae occupied with
304 hexose residues only and the fragmentation pattern was similar to that assigned to high-
305 mannose structures as previously described^{28,29}. Besides, sequential exoglycosidase
306 treatment of the glycan pool with α -mannosidase caused the loss of hexose residue/s and
307 supported the structure with mannose residues on both antennae as depicted e.g. in Figure
308 1c (m/z 1671, 1833).

309 A lot of information regarding patatins, as a glycoprotein with interesting properties
310 was reported, however most of this information is limited to only one type. The first
311 report mentioning glycosylation pattern in patatin isolated from potato tubers was
312 published more than two decades ago; two glycosylation sites at *Asn60* and *Asn90* were
313 occupied with glycan of composition $Man_3(Xyl)GlcNAc_2(Fuc)$ ³⁰. Although a few papers
314 have studied the glycosylation of potato patatins, no evidence for the presence of other
315 glycan structures has been reported up to now. Our present study provides evidence for
316 the presence of other glycans in potato patatins and the profile can differ with species
317 level. The chart shown in Figure 4 illustrates identified N-glycans in the analyzed patatin
318 samples. The major glycan structure found in all samples corresponded to the typical
319 fucosylated plant glycan with xylose on the core mannose (m/z 1301). This glycan solely
320 as one saccharide structure was found in *S. tuberosum* cv. Désirée (from both seasons)
321 and *S. goniocalyx* (2011). It was reported previously that genotype (equally species and
322 cultivar) is the predominant factor playing a role in the final expression of patatin

proteins. However, the other factors such as year, climate and soil conditions, fertilization, production system, stress factors represent also running factors with an ability to modify patatin proteins³¹. The patatin of *S. tuberosum* cv. Désirée did not show any significant change from one year to another. However, N-glycome profiles of other species altered with period. The most obvious change on this level was observed for *S. goniocalyx* (Supplementary Figure 3a). This protein together with patatin of *S. phureja*, both collected in 2012, showed higher abundance of shorter glycans – $Man_2GlcNAc_2Fuc$ and $Man_2(Xyl)GlcNAc_2Fuc$. Whereas, in the samples from *S. stenotomum* and *S. andigenum* glycans with GlcNAc residue/s on arm/s were detected with higher intensity – $GlcNAc_{1-2}Man_3(Xyl)GlcNAc_2Fuc$. These two species also showed the additional presence of high-mannose glycans with composition $Man_{7-9}GlcNAc_2$ glycans. These oligosaccharides have also never been described in patatins of potato tubers, on the other side they are commonly found in mammals and not mentioned to be immunogenic.

Plants, among other properties, have great potentials as “biofactories” for the production of therapeutic proteins. Therefore, the number of plant recombinant proteins produced is increasing rapidly³². The immunogenicity of plant oligosaccharides in animals or humans is associated with the presence of an $\alpha 1,3$ -fucose and a $\beta 1,2$ -xylose attached to the glycan core³³. It was reported that disappearance of sugar allergenic epitopes from plant oligosaccharides is offering a new possibility to produce safe proteins for therapeutic applications⁹. Although further studies are needed to follow carefully more conditions which may also influence the glycosylation profile and to provide better insight into biology of patatins, our data showed that potato patatin’s glycan profile among other factors alters with genotype/cultivar variability. The observed

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changes in profiles suggest that correct choice of species with careful monitoring of all conditions can enable to decrease the presence of glycans with immunogenic sugar epitopes and potatoes as one of the most available and the low-cost crops can be used even more beneficially.

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Supporting Information

Additional supporting information is available in the Supplementary Data. Supplementary Figure 1 demonstrates the control of purity patatin samples by using Experion Automated Electrophoretic System. Supplementary Figure 2 shows MALDI-TOF/TOF-MS and MS/MS spectra of patatin N-glycans obtained from *Solanum stenotomum* after exoglycosidase treatment. Supplementary Figure 3 represents comparative MS spectra of N-glycans recorded from patatin of *Solanum goniocalyx*. This information is available free of charge via the Internet at <http://pubs.acs.org>.

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FIGURE LEGENDS

Figure 1. Representative MALDI-TOF/TOF-MS spectra of N-glycan profiles of patatin tubers from the following potato species: **a)** *Solanum tuberosum*, cv. Désirée; **b)** *Solanum phureja* (2012) and **c)** *Solanum stenotomum* (2012). All peaks are $[M+Na]^+$. N-glycans were released with PNGase A from native patatins and labeled with PHN (+90.05 Da).

Key symbols: ▲ Fuc; ■ GlcNAc; ● Man; ★ Xyl;

Figure 2. MALDI-TOF/TOF MS/MS spectra recorded for the glycans with precursor ions at: **(a)** m/z 1301.4; **(b)** m/z 1139.4 and **(c)** m/z 1007.4. Fragment ions are sodiated.

Figure 3. ESI-HCD spectra recorded for the glycans with precursor ions **(a)** at m/z 1504.5; and **(b)** m/z 1707.6. Fragment ions are sodiated.

Figure 4. The schematic chart illustrating the occurrence of N-glycans detected by MALDI-MS in patatins isolated from five potato species (collected in years 2011 and 2012). The relative amounts of glycans calculated based on their relative MS intensities as described in the experimental section are presented as the mean $\pm$ SD from at least three measurements.

476 **TABLES**

477

478 **Table 1.** The list of potato species used in this study for isolation of patatins from tubers.

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Species	Code of Gene Bank in Potato Research Institute Havlickuv Brod	Country of origin / geographic distribution
<i>Solanum tuberosum</i> L. cv. Desirée	EVIGEZ 07S0100243	Netherlands
<i>Solanum andigenum</i> Juz. & Bukasov	EVIGEZ 07S0300066	South America (Venezuela, Argentina)
<i>Solanum goniocalyx</i> Juz. & Bukasov	EVIGEZ 07S0300109	South America (Peru, Bolivia)
<i>Solanum phureja</i> Juz. & Bukasov.	EVIGEZ 07S0300136	South America (Bolivia)
<i>Solanum stenotomum</i> Juz. & Bukasov	EVIGEZ 07S0300001	South America (Columbia, Argentina)

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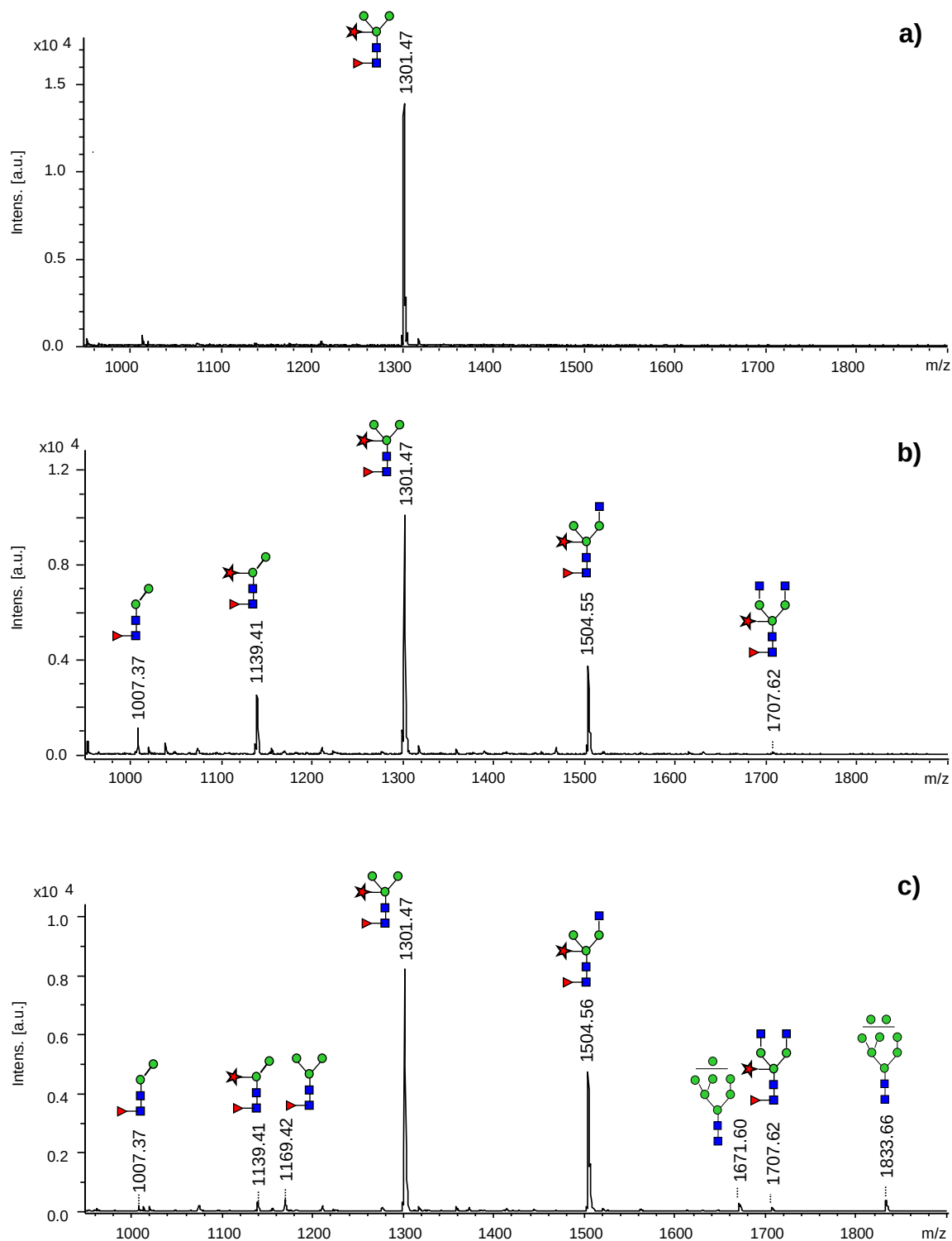
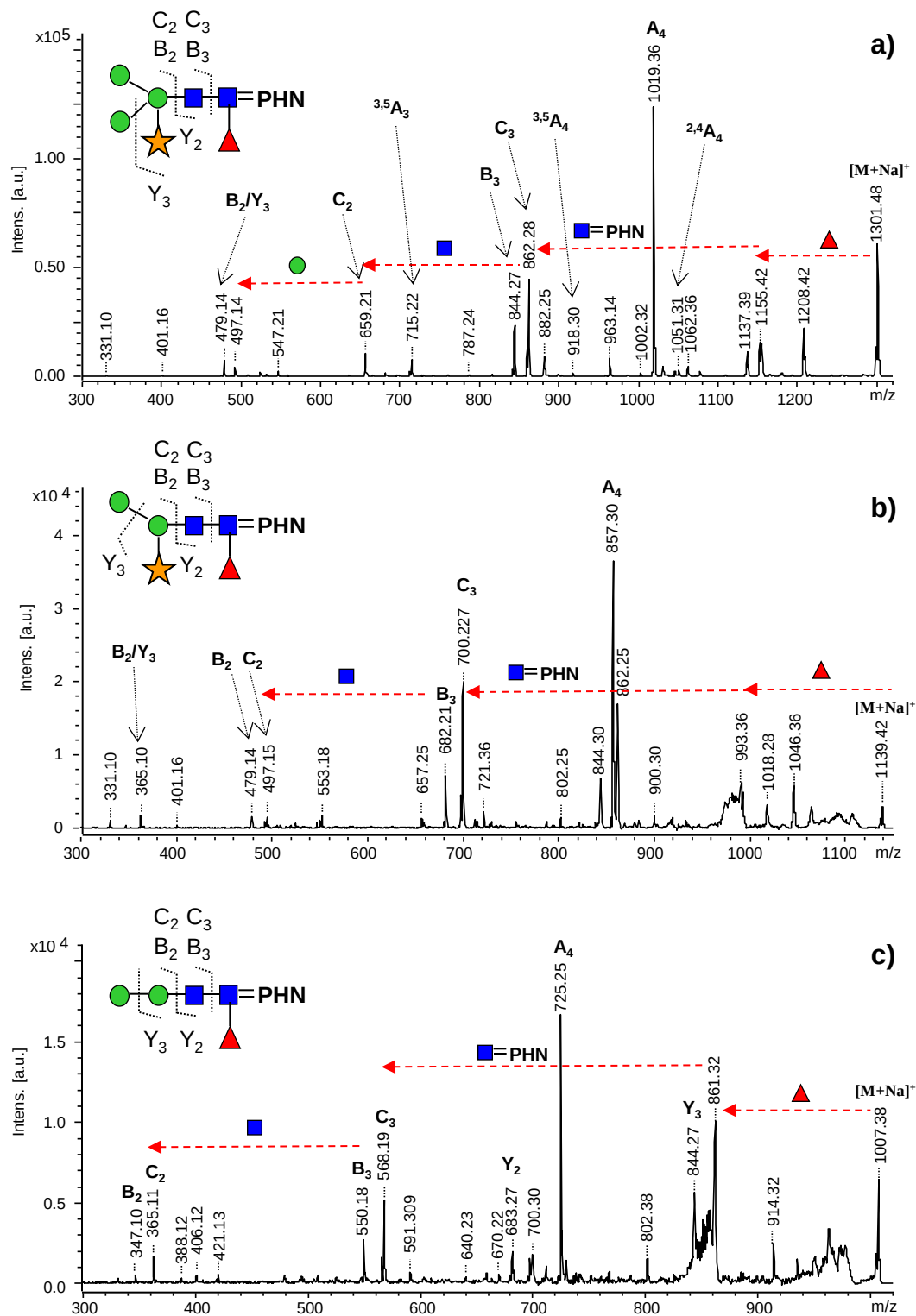
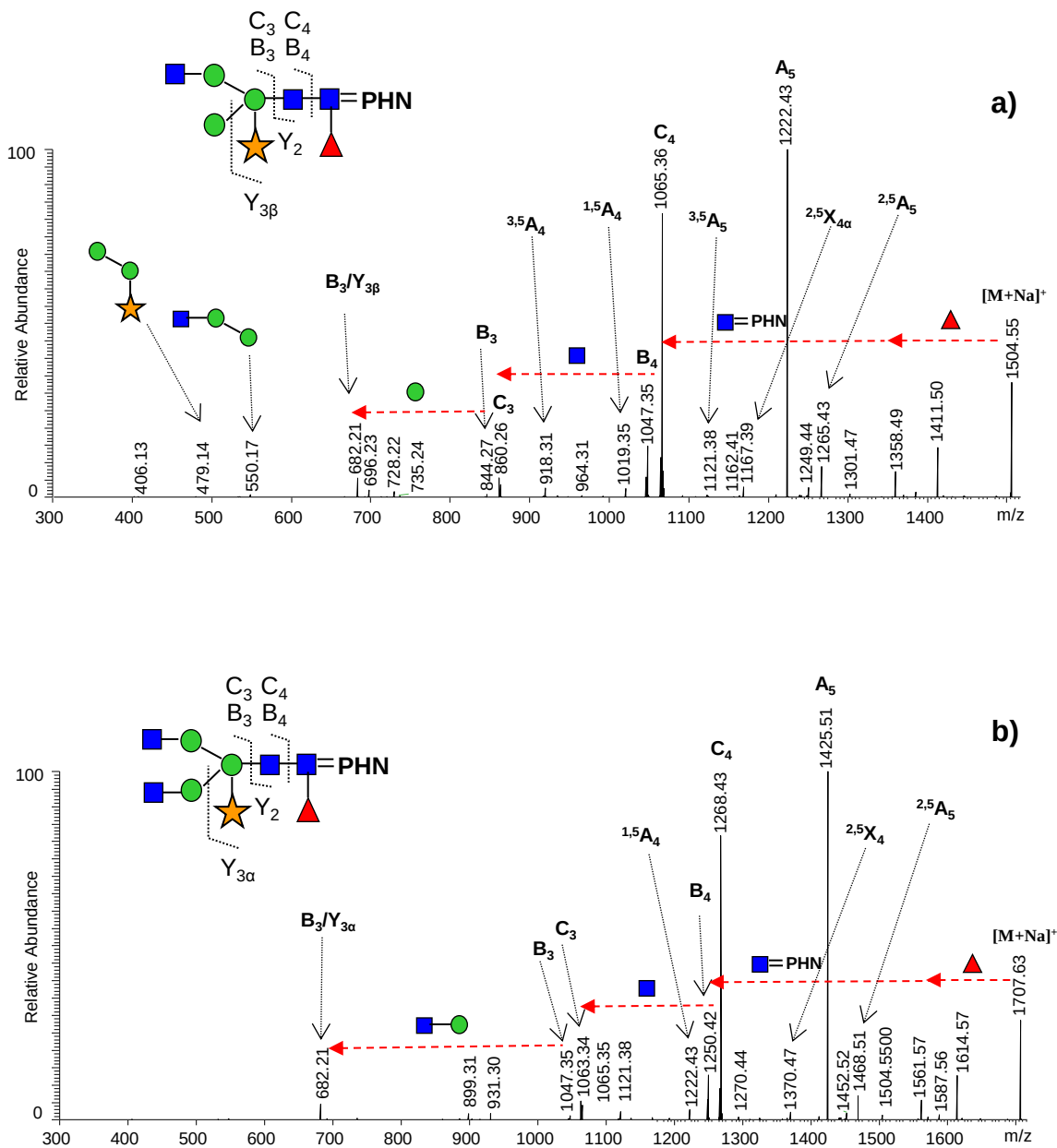


Figure 1
ACS Paragon Plus Environment





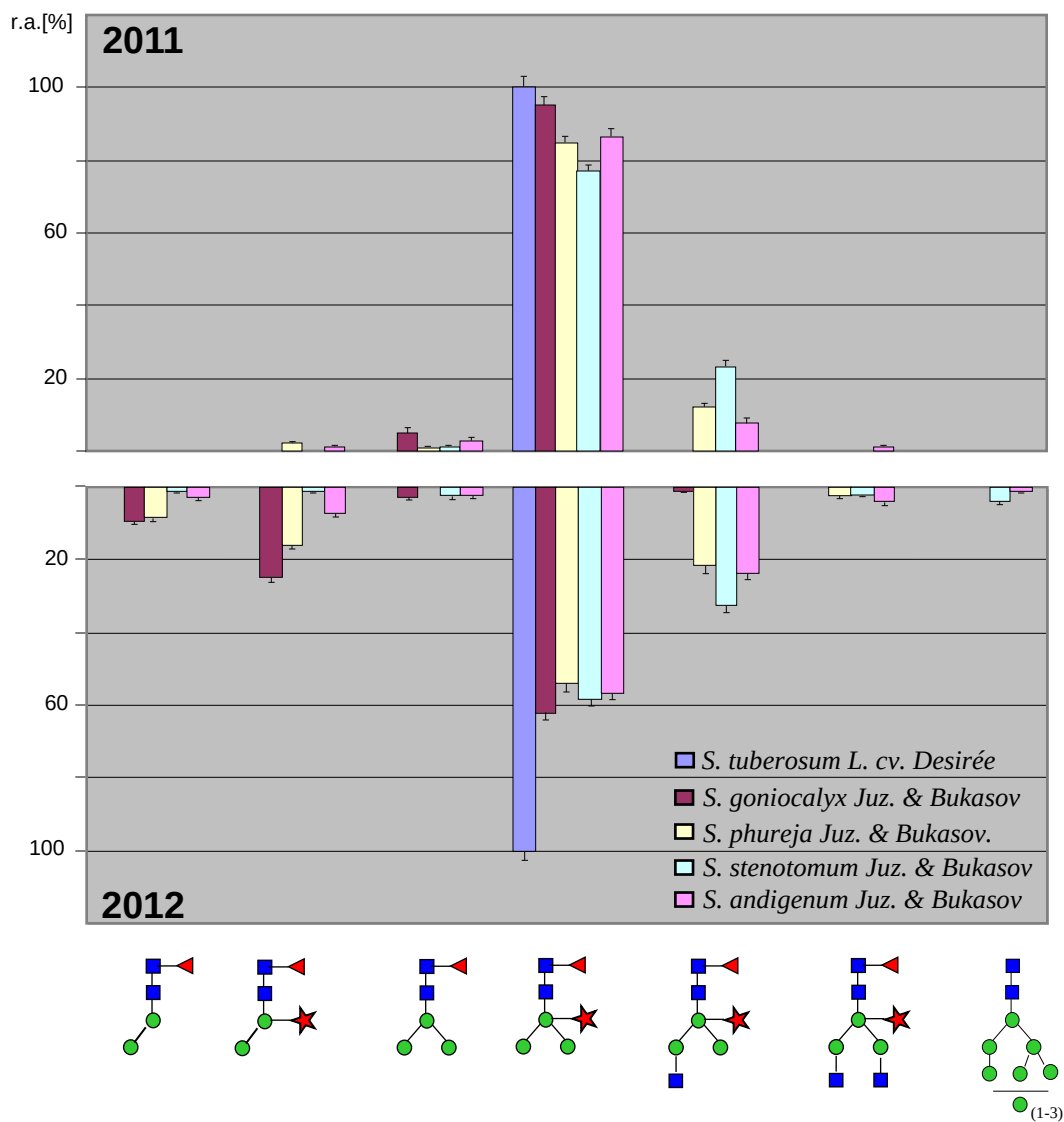


Figure 4

TOC Graphic

