Compositional Monosaccharide Analysis of Transgenic Corn Glycoproteins by HPLC with Fluorescence Detection and LC–MS with Sonic Spray Ionization

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Abstract

Transgenic corn offers an attractive, cost-effective means for the large-scale production of engineered glycoproteins suitable for pharmaceutical purposes. A glycoprotein expressed in transgenic corn theoretically should not contain glycans because glycosylation sites have been genetically altered. A sensitive and reliable analytical method is developed to investigate this particular protein for the presence of glycans by monitoring the monosaccharide composition. Identification and quantitation of low-level monosaccharides in the glycoprotein hydrolyzate are accomplished by derivatization prior to high-performance liquid chromatography (HPLC)–fluorescence and liquid chromatography (LC)–sonic spray ionization (SSI)-mass spectrometry (MS) analyses. LC–SSI-MS is used to confirm the results from HPLC–fluorescence analysis and to positively identify the compositional monosaccharides. Glucosamine, glucose, mannose, arabinose, xylose, and sialic acid are found in the transgenic corn derived glycoprotein at less than one moiety per protein, which indicates heterogeneity of the particular glycoprotein. In addition to the compositional analysis of low-level monosaccharides in glycoprotein by HPLC–fluorescence, the utility of SSI for the LC–MS analysis of derivatized monosaccharides is demonstrated in this paper.

Introduction

Glycans, or oligosaccharides, are found in a wide variety of proteins, including enzymes, immunoglobulins, carriers, hormones, toxins, lectins, and structural proteins. The most commonly occurring monosaccharides in glycans include glucose (Glc), mannose (Man), galactose (Gal), fucose (Fuc), *N*-acetylglucosamine, *N*-acetylgalactosamine, and sialic acids (1).

In recent years, glycoprotein research has drawn increasing interest in the fields of biotechnology, clinical chemistry, biochemistry, pharmaceutical, and food sciences (2–6). Glycoprotein plays important roles in biological processes, including antigenicity, transport, folding, recognition, defense or decoy functions, cellular adhesion, blood clotting, immunological protection, structural support, and others. Glycosylation of proteins is one of the most common and important post-translational modifications found in secretory proteins (7–14).

Glycoproteins are a class of proteins that exhibit an extraordinarly complex structure because of the presence of the surface carbohydrates. The structure diversity is derived not only from different linkages between proteins and carbohydrates, but also from the composition and the structure of the carbohydrate units.

Transgenic corn offers an attractive and cost-effective means for the large-scale production of therapeutic glycoproteins suitable for pharmaceutical purpose. The particular glycoprotein produced by transgenic corn should not contain glycans because the glycosylation sites have been genetically altered. A sensitive and reliable analytical method is needed to determine the glycans and monosaccharides in the glycoproteins for quality control purposes.

To determine whether a protein is glycosylated, the first step is to identify and quantitate the compositional monosaccharides in the glycoprotein. Several methods have been developed for analyzing monosaccharides derived from glycoproteins. Even though direct analysis of monosaccharides by gas chromatography (GC) has been reported (15,16), the derivatization of monosaccharides is preferred to enhance the volatility of monosaccharides for GC analysis (17–21). However, the instability of some of the derivatives renders this method less than ideal for an accurate quantitation of monosaccharide content.

High-performance liquid chromatography (HPLC) with various detectors, such as UV–vis, fluorescence, electrochemical, and mass spectrometry (MS), has become a more popular method for monosaccharide analysis. Reversed-phase (RP) HPLC analysis of derivatized monosaccharides with UV–vis or fluorescence detection is one category (22–25) and anion-exchange HPLC with different types of detection is another (25–28). For irrefutable identification of the monosaccharides released from glycoproteins, MS has also been used in conjunction with HPLC (29–32). Thermospray ionization and electro-

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spray ionization (ESI) were utilized as the source for liquid chromatography (LC)–mass spectrometry (MS), but reduced sensitivity was often encountered for direct sugar analysis (30, 33–35). Sonic spray ionization (SSI) can be an alternative ionization source for the LC–MS analysis of glycans and monosaccharides. SSI is a softer ionization technique compared with ESI; therefore, it is more efficient in the formation of molecular ions and provides a possibility of MS characterization of the monosaccharides (36,37). Because this ionization source operates with a much lower temperature and no needle voltage, it is well suited for the analysis of thermally labile and unstable compounds like carbohydrates. So far, it has been successfully applied only to a limited number of compounds in the environmental and bioanalytical field (36,37), and analysis of derivatized monosaccharides by LC–SSI-MS has not been reported.

In this paper, the HPLC–fluorescence and LC–SSI-MS based techniques for compositional monosaccharide analysis of glycoproteins in transgenic corn is described. The monosaccharides are released from glycoproteins by acid hydrolysis and derivatized with a fluorophore, and RP-HPLC with fluorescence detection is used to identify and quantitate these monosaccharides. Each monosaccharide is further confirmed by LC–SSI-MS. This approach shows an advantage over others in that it is possible to perform HPLC with fluorescence and MS detection simultaneously.

Experimental

Materials

The glycoprotein was a monoclonal antibody (IgG) expressed in corn and provided by Monsanto Protein Technologies (St. Louis, MO). This transgenic corn protein (product designation PR 390) was determined to be homogeneous by sodium dodecyl sulfate polyacrylamide gel electrophoresis. It was engineered in such a way that asparagine, normally *N*-glycosylated in mammalian cells, was substituted with alanine, therefore, the protein should not be glycosylated. Nevertheless, the protein was suspected to be *O*-glycosylated and non-enzymatically glycosylated as well. As a glycosylated reference standard, bovine fetuin of highest purity was obtained from Sigma Chemical Co. (St. Louis, MO). Arabinose (Ara), Glc, Gal, Man, xylose (Xyl), Fuc, glucosamine (GlcN), galactosamine (GalN), *N*-acetylneuraminic acid (Neu5Ac), *o*-phenylenediamine (OPD), and dichloride and anthranilic acid [2-aminobenzoic acid (AA)] were purchased from Sigma Chemical Co. Sodium cyanoborohydride was from Fluka Chemie GmbH (Steinbeim, Switzerland). Ultrafree-MC centrifugal filter devices with a Biomax-30 membrane was purchased from Millipore (Bedford, MA). Ultra-pure water was prepared with a Synergy 185 system (Millipore). HPLC-grade acetonitrile and tetrahydrofuran were purchased from Fisher Scientific (Pittsburgh, PA). Other reagents and solvents were of reagent grade.

Purification of glycoprotein samples

One milliliter of transgenic corn glycoprotein (9.33 mg protein/mL) or 5.1 mg of bovine fetuin (dissolved in 1.0 mL H_2O) was filtered through the Biomax-30 membrane using an Ultrafree-MC Centrifugal Filter Device to remove constituents with molecular weight less than 30,000. The protein residues collected on the filter were washed with 2.0 mL of ultra-pure water. The purified glycoprotein was recovered from the filter with 1–5 mL of ultra-pure water.

Acid hydrolysis of glycoprotein for neutral and basic monosaccharides analysis

A 100-µL aliquot of purified protein sample was mixed with 500 µL of 20% trifluoroacetic acid in a 2.0-mL screw-cap glass vial. The mixture was heated at 100°C for 7 h. The sample was freeze-dried after the completion of hydrolysis.

Derivatization of neutral and basic monosaccharides with anthranilic acid

The neutral monosaccharides were derivatized by reductive amination with the anthranilic acid following a procedure described previously (23). First, a solution of 4% sodium acetate·$3H_2O$ and 2% boric acid in methanol was prepared. Thirty milligrams of anthranilic acid and 20 mg of sodium cyanoborohydride were then dissolved in 1.0 mL of this methanol–acetate–borate solution.

Lyophilized glycoprotein hydrolyzate or 10 µL monosaccharide standard (0.25~100 µg/mL) was dissolved in 40 µL of 1% freshly prepared sodium acetate·$3H_2O$. The solution was mixed with 50 µL of the derivatizing reagent in a screw-cap glass vial and heated at 80°C for 1 h. After cooling to ambient temperature, the samples were centrifuged, and 10 µL of the supernatant was injected onto the HPLC column.

HPLC–fluorescence analysis of AA-monosaccharide derivatives

A Hewlett-Packard 1090 HPLC instrument (Palo Alto, CA) with a Shimadzu RF-551 fluorescence detector (Columbia, MD) was used for the analysis of anthranilic acid derivatives of monosaccharides. The separation conditions were similar to the reported method with some modifications (23). A Waters C18 column (300 × 3.9-mm i.d., 5 µm, Milford, MA) was used at ambient temperature with a flow rate of 1.0 mL/min. Solvent A consisted of 0.4% *n*-butylamine, 0.5% phosphoric acid, and 1.0% tetrahydrofuran in water. Solvent B consisted of 50% solvent A and 50% acetonitrile. The HPLC separation was performed at 5% B for 15 min followed by a linear gradient to 15% B at 50 min. After each run, the column was washed with mobile phase B for 15 min and equilibrated with the initial mobile phase for 10 min. Fluorescence detection was carried out at an excitation wavelength of 230 nm and an emission wavelength of 425 nm.

LC–MS analysis of AA-monosaccharide derivatives

The Hitachi M-8000 3DQ LC–MS^n system (San Jose, CA) was used for the MS confirmation of monosaccharides released from hydrolysis of the glycoprotein samples. The HPLC condition used for HPLC-fluorescence experiment was utilized with minor changes in the mobile phase composition and gradient program. Solvent A consisted of 0.12% *n*-butylamine, 0.12% formic acid, and 1.0% tetrahydrofuran in water. Solvent B consisted of 50% solvent A and 50% acetonitrile. The HPLC separation was per-

formed at 100% A for 30 min, followed by a linear increase to 20% B at 60 min. The 1 mL/min effluent from the column was split through a tee connector, and only 0.2 mL/min was directed to the SSI-MS. A divert valve was placed right before the ionization source to remove the HPLC fractions containing salts and excess AA derivatizing reagent and to prevent the contamination of the ionization source and MS. The SSI parameters were set to the following optimized values: nitrogen sheath gas, 3 kgf/cm^2; capillary voltage, 0 kV; drift plate, 45 V; focus plate, 30 V; cover plate temperature, 200°C; and aperture 1 temperature, 150°C. Ion trap MS was operated at the following conditions: accumulating time, 500 ms; accumulation voltage, 0.072V; low mass cutoff, 48.2 amu; and scan range, 250–350 amu. The remaining (0.8 mL/min) flow of the HPLC effluent was directed to a UV detector set at 250 nm.

Mild-acid hydrolysis of glycoprotein for sialic acid analysis

A 50-µL aliquot of purified glycoprotein sample was mixed with 50 µL 0.5M $NaHSO_4$ in a screw-cap glass vial. The mixture was heated at 80°C for 20 min and allowed to cool to ambient temperature.

Derivatization of sialic acids with OPD

Sialic acids were labeled with OPD to produce fluorescent quinoxaline derivatives via an optimized procedure previously reported (23). An aliquot (0.1 mL) of standard sialic acid solutions (0.20–4.0 µg/mL) or mild-acid hydrolyzates of glycoprotein samples were mixed with 0.1 mL of the 20 mg/mL OPD in 0.25M $NaHSO_4$. The mixtures in glass screw-cap vials were heated at 80°C for 40 min. After cooling to ambient temperature, the samples were centrifuged, and 10 µL of the supernatants were injected onto the HPLC column.

HPLC–fluorescence analysis of sialic acid

A Hitachi L-7000 series HPLC system was used for the analysis of the OPD derivatives of sialic acid. The separation conditions were similar to the reported method with some modifications (23). A Waters C18 column (300 × 3.9-mm i.d., 5 µm) was used at ambient temperature with a flow rate of 1.0 mL/min. Solvent A consisted of 0.15% *n*-butylamine, 0.5% phosphoric acid, and 1.0% tetrahydrofuran in water. Solvent B consisted of 50% solvent A and 50% acetonitrile. The HPLC separation of OPD derivatives of sialic acids was isocratic, with a solvent mixture of 89% A + 11% B. After each run, the column was washed with mobile phase B for 15 min and equilibrated with the initial mobile phase for 10 min. The excitation and emission wavelength for the fluorescence detector was 230 and 425 nm, respectively.

Results and Discussion

HPLC–fluorescence analysis of neutral and basic monosaccharides

Initially, a mixture of eight monosaccharides commonly-occurring in glycoproteins were used in the optimization of the method (38). HPLC separation and fluorescence detection of AA-derivatized monosaccharide standards and monosaccharides hydrolyzed from transgenic corn glycoprotein are shown in Figure 1. A number of large peaks from excess derivatizing reagent and artifacts were present, but they were separated clearly from monosaccharide peaks and did not interfere in the quantitation. Glucosamine and neutral monosaccharides (including Man, Glc, Ara, and Xyl) were found in the glycoprotein sample. GalN and Gal levels were below the detection limits of 0.25 and 1.0 ng, respectively. All of the AA-monosaccharide peaks in the chromatogram were confirmed by the standard addition method. Because the glycoprotein sample contained no galactosamine, it was employed as an internal standard for an accurate quantitation of other monosaccharide constituents. A calibration curve of each AA-derivatized monosaccharide was generated using peak area ratio, and the regression coefficients (r^2) of all curves ranged between 0.9983 and 0.9999. The limit of detection (LOD) was calculated as the minimum amount of monosaccharide giving a signal-to-noise ratio of 3. The LODs on column for monosaccharides were as follows: 0.25 ng for GlcN and 1.0 ng for neutral monosaccharides.

Validation of the method was conducted by monitoring the recoveries of spiked monosaccharides. The results are shown in Table I. The monosaccharide recoveries were relatively lower for the highest level in the study, most likely because of the insufficient fluorescent labeling of monosaccharides. However, the specified derivatization condition should be adequate for the transgenic corn glycoprotein, whose monosaccharide concentrations are expected to be very low. The validity of the method was also evaluated with fetuin, whose monosaccharide composition of glycoproteins was already well known. The fetuin monosaccharide content determined with the method is shown in Table II, and the result is similar to the published data (39–43).

The neural and basic monosaccharide contents in the glycoprotein sample from transgenic corn are shown in Table III. The

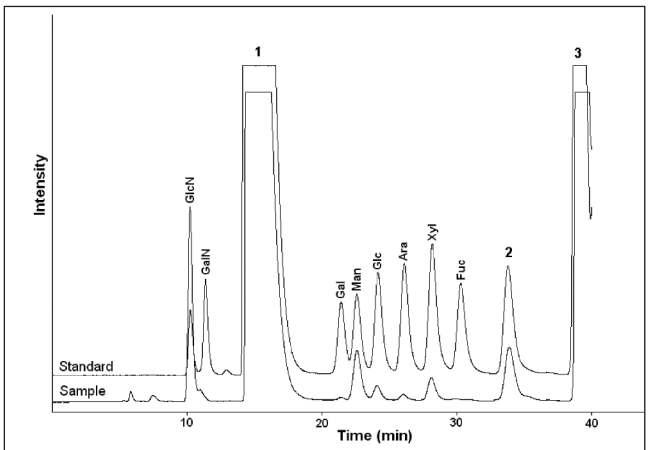

Figure 1. HPLC–fluorescence profiles of AA-derivatized neutral and basic monosaccharides from a standard mixture and a transgenic corn glycoprotein sample. Peaks 1, 2, and 3 are artifacts from derivatization. The experimental conditions on a C-18 column were: solvent A consisted of 0.4% *n*-butylamine, 0.5% phosphoric acid, and 1.0% tetrahydrofuran in water; and solvent B consisted of 50% solvent A and 50% acetonitrile. The HPLC separation was performed at 5% B for 15 min, followed by a linear increase to 15% B by 50 min. Excitation wavelength was 230 nm and emission wavelength was 425 nm.

contents of individual monosaccharides were measured in a µg/mg protein sample. The number of monosaccharide molecules per glycoprotein molecule was calculated based on the molecular weight (150,426 Da) of transgenic corn glycoprotein (number of GlcN molecule per glycoprotein = content × 150,426/179 and the number of neutral monosaccharide molecules = content × 150,426/180). This molecular weight was determined previously from the deconvolution of a positive ion ESI-MS spectrum obtained for the same glycoprotein (44).

HPLC–fluorescence analysis of sialic acid

Representative HPLC-fluorescence chromatograms of sialic acid from standard and glycoprotein samples are shown in Figure 2. The calibration curve regression coefficient (r^2) of sialic acid was 0.9998. The detection limit was 0.5 ng. The sialic acid content of the fetuin was determined to be 71.3 µg/mg protein, and this agreed well with the expected value. The sialic acid content of the glycoprotein sample from transgenic corn was in average 0.072 µg/mg protein. The number of sialic acid molecules per protein molecule was then calculated to be 0.035 based on the previously determined molecular weight of the particular transgenic corn glycoprotein.

Table I. Recovery of Different Monosaccharides

Spiked amount (ng) (amount injected)	% Recovery (n = 3)						
	GlcN	Gal	Man	Glc	Ara	Xyl	Fuc
2–5	83.2	89.8	87.6	105	78.5	83.9	87.0
RSD*	5.4	3.5	3.7	3.3	4.3	8.6	5.1
10–20	81.5	79.6	80.0	96.2	76.3	72.6	79.6
RSD	2.2	4.4	4.7	9.9	1.7	8.9	4.6
50–100	66.4	66.5	65.9	75.8	62.0	54.6	65.3
RSD	4.8	5.0	6.6	10.0	5.1	7.9	6.6

* RSD = relative standard deviation.

Table II. Monosaccharide Composition of Fetuin Determined with Current Method and Compared With Published Data

	Monosaccharide content (µg/mg protein)				
	GlcN	GalN	Gal	Man	Neu5Ac
Current method	45.3–48.5	7.2–7.9	30.5–33.7	21.7–22.9	69.1–73.4
Reported in the literature*	26.7–56.0	5.4–7.0	34.9–45.9	23.0–30.5	70.0–76.0

*See the literature (45,46).

Confirmation of the monosaccharides found in glycoprotein samples by LC–MS

For LC–MS analysis of AA-derivatized monosaccharides, the mobile phase solvents and gradient used for HPLC-fluorescence separation were slightly modified to accommodate the SSI-MS operation. The concentrations of buffers in the mobile phase were lowered and the length of initial isocratic condition was increased. Accordingly, the total time necessary for complete separation of all eight monosaccharide derivatives was greatly increased as can be seen in the UV-detected chromatogram in Figure 3. The 1:5 split of the same LC effluent was directed to SSI-MS, but diverted to waste during the time segments when two large peaks corresponding to excess derivatization reagent and byproduct eluted. Non-retained solvent front segment was also diverted to waste to prevent contamination of the ionization source and MS from the non-volatile buffer salt used in the derivatization of the sample. The resulting total ion chromatogram of monosaccharide standards from LC–SSI-MS is shown in Figure 4. The extracted ion chromatograms corresponding to different monosaccharides are also displayed in Figures 4A–4D. Based on the derivatization reaction process involving reductive amination of monosaccharides (45,46), the positive ion SSI-MS should yield a protonated molecular ion at *m/z* 301 for GalN and GlcN derivatives, which are optical isomers. Similarly, a protonated molecular ion at *m/z* 302 was expected for the AA-derivative of optical isomers (Gal, Man, and Glc). Another set of optical isomers, Ara and Xyl, should produce a protonated molecular ion at *m/z* 272. The protonated molecular ion of AA-derivatized fucose should have *m/z* 286.

The LC–SSI-MS analysis of the transgenic corn glycoprotein sample was performed with same conditions, and the result is shown in Figure 5. Comparing the retention time, standard addition, and mass spectra obtained for monosaccharide derivatives, GlcN, Man, glucose arabinose, and Xyl in glycoprotein samples of transgenic corn were identified. Based on the concentrations of monosaccharides and the number of monosaccharide molecules determined per glycoprotein, it was highly unlikely that the particular protein samples of transgenic corn contained any known *N*-linked or *O*-linked glycans. All of the *N*-linked oligosaccharides should have a common pentasaccharide core consisting of three Man and two *N*-acetylglucosamine residues (45,46). The absence of any *N*-linked glycans in the particular transgenic corn derived glycoprotein was also confirmed by a previous

Table III. Neutral and Basic Monosaccharide Composition of Transgenic Corn Glycoprotein Determined as Concentrations and Numbers of Monosaccharide per Protein (n = 3)

	GlcN	Man	Glc	Ara	Xyl
Monosaccharides content (µg/mg protein)	0.062 ± 0.002	0.728 ± 0.047	0.600 ± 0.158	0.350 ± 0.012	0.377 ± 0.006
No. of monosaccharide molecules per glycoprotein	0.052 ± 0.001	0.609 ± 0.039	0.502 ± 0.132	0.293 ± 0.009	0.316 ± 0.005

hydrolysis study with an enzyme specific for *N*-linked glycans (44). The protein also lacked the core structural component of *O*-linked glycans, *N*-acetylgalactosamine. Each sugar residue found in the transgenic corn glycoprotein was less than one unit per glycoprotein, which suggested the heterogeneous nature of this particular glycoprotein sample. It implied that not all of the glycosylation sites of protein molecules were genetically blocked, leading to the post glycosylation of some proteins. Another possibility could be the random linking of individual sugar to the amino acid residues by an unknown mechanism that require further investigation.

Conclusion

The compositional monosaccharides in glycoproteins of transgenic corn were quantitatively determined and identified following derivatization of protein hydrolyzate by RP-HPLC with fluorescence detection, and they were positively confirmed by LC–MS with a SSI source. The numbers of individual monosaccharide molecules bonded to the transgenic corn glycoprotein were found to be less than one for each glycoprotein, which indicated that these monosaccharides may come from the heterogeneous glycans in the glycoproteins or may be randomly linked to some amino acid residues in the proteins. Although this phenomenon deserves further study, it implied that not all of the glycosylation sites in the protein molecules were genetically blocked.

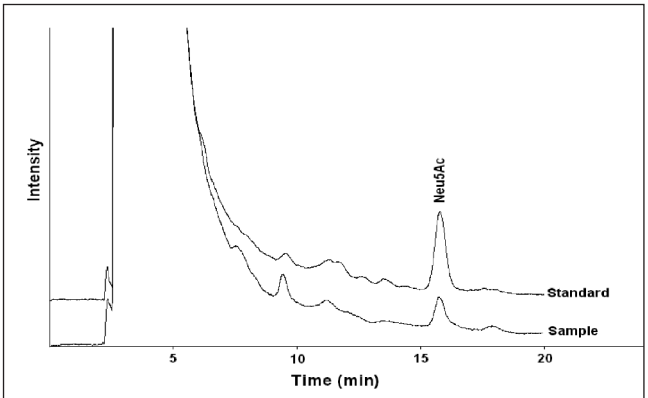

Figure 2. HPLC–fluorescence profiles of OPD-derivatized sialic acids from a Neu5Ac standard, a transgenic corn glycoprotein sample, and a blank. The experimental conditions on a C-18 column were: solvent A consisted of 0.15% n-butylamine, 0.5% phosphoric acid, and 1.0% tetrahydrofuran in water; and solvent B consisted of 50% solvent A and 50% acetonitrile. The separation was performed at an isocratic (89% A + 11% B) elution. The excitation and emission wavelength for the fluorescence detector was 230 and 425 nm, respectively.

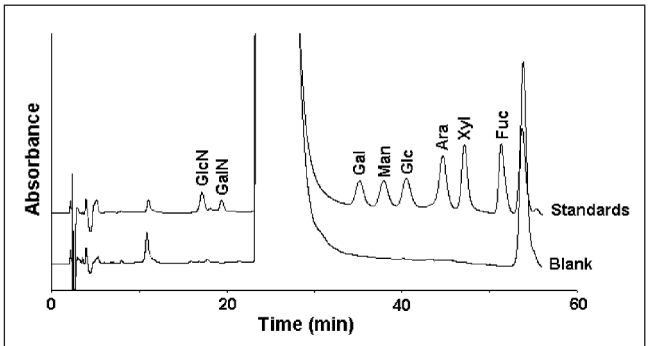

Figure 3. HPLC chromatogram of AA-monosaccharide derivatives with UV detector. The experimental conditions were: the analytical column was the same as that for HPLC–fluorescence experiments; solvent A consisted of 0.12% *n*-butylamine, 0.12% formic acid, and 1.0% tetrohydrofuran in water; and solvent B consisted of 50% solvent A and 50% acetonitrile. The HPLC separation was performed at 100% A for 30 min followed by a linear increase to 20% B by 60 min. The UV detector wavelength was set at 250 nm.

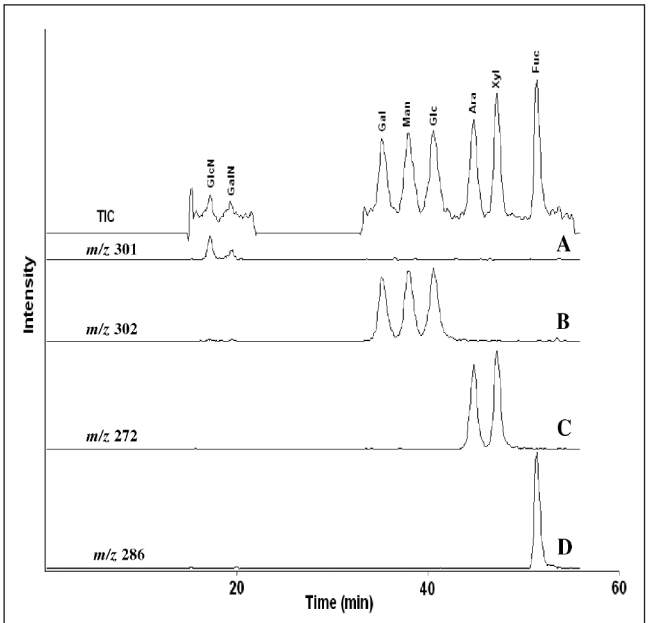

Figure 4. Reconstructed ion chromatograms of AA-derivatives of monosaccharide standards. The HPLC condition was same as given in Figure 3. The SSI-MS parameters were the optimized conditions stated in the Experimental section.

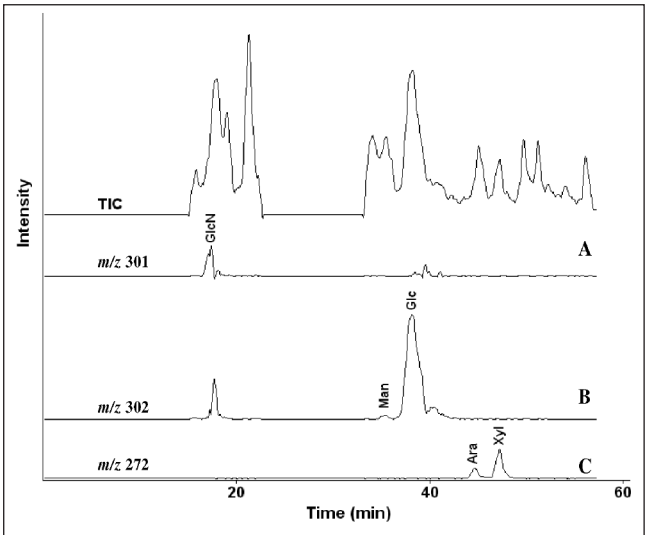

Figure 5. Reconstructed ion chromatograms and selected mass spectra corresponding to AA-derivatives of monosaccharides in a transgenic corn glycoprotein sample. Experimental conditions were same as described in Figure 4.

Acknowledgment

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