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Improved protocol for the formation of *N*-(*p*-nitrobenzyloxy)aminoalditol derivatives of oligosaccharides

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Abstract

An improved procedure has been developed for the rapid derivatization of oligosaccharides with UV-detectable *p*-nitrobenzylhydroxylamine (PNB). The improved conditions used result in quantitative derivatization of neutral oligosaccharides. Sialylated oligosaccharides can also be quantitatively PNB-derivatized without detectable desialylation. Of the oligosaccharides tested, only the derivatization of oligogalactosyluronic acids was incomplete (yield ~ 70%). PNB-derivatization of tamarind seed xyloglucan oligosacccharides results in products with improved chromatographic properties during HPAEC. These PNB derivatives were also subjected to hydrophilic interaction chromatography (HILIC) and analyzed by on-line LC–MS. On-line LC–MS is readily usable with HILIC, as this chromatographic technique does not require salt-containing solvents. Approximately 10 pmol of a PNB-derivatized oligosaccharide can be identified and quantitated utilizing this method.

Keywords: Alditol, *N*-(*p*-nitrobenzyloxy)amino- derivative; PNB derivative; Oligosaccharides xyloglucan; HILIC; LC–MS, on-line

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1. Introduction

Chromatographic separation of oligosaccharides is an important step in determining their structure, metabolism and function. Biologically important oligosaccharides are often available in only minute quantities and are typically difficult to separate using chromatographic techniques. Therefore, considerable effort has been expended to find derivatization methods that improve the chromatographic behavior of oligosaccharides while enabling their detection with high sensitivity. The value of a derivatization technique is increased if it is applicable to a wide variety of oligosaccharides while providing high yields of the products. The derivative should be amenable to chromatography, especially liquid chromatography combined with mass spectrometry (LC–MS), so that complex mixtures can be easily analyzed. One procedure that fulfills these requirements is the labeling of oligosaccharides by reductive amination with *p*-nitrobenzylhydroxylamine (PNB) to form *N*-(*p*-nitrobenzyloxy)aminoalditol derivatives (PNB derivatives) [1]. We now report an improved, high-yield procedure for PNB-derivatization using mild conditions. Xyloglucan oligosaccharide subunits, released from tamarind seeds by a fungal *endo*-β-(1 → 4)-glucanase [2], commercially available sialylated oligosaccharides, and oligogalatosyluronic acids released from polygalacturonic acids by a fungal *endo*-α-(1 → 4)-polygalacturonase [3] were derivatized and the products analyzed to evaluate the derivatization procedure.

2. Experimental

Preparation or purchase of oligosaccharides.—Xyloglucan oligosaccharides were generated as described [2] by treatment of tamarind seed xyloglucan with an *endo*-β-(1 → 4)-glucanase. Nonasaccharide **4** was purified using hydrophilic interaction chromatography (HILIC). 3′-Sialyllactose and di-sialyl-lacto-*N*-tetraose were purchased from Oxford GlycoSystems (Oxford, UK). α-D-Octagalactosyluronic acid was prepared as described [3].

Colorimetric assays.—The anthrone assay for hexoses was carried out as described [4]. The sample was dissolved in 250 μL of distilled water in a culture tube, and 500 μL of the anthrone reagent [0.2% anthrone (Sigma, St. Louis, MO, USA) in concd. H_2SO_4] was added. The tube was vortexed and heated (boiling water, 5 min). Subsequently absorbance at 620 nm was measured.

The meta-hydroxybiphenyl assay for uronic acids was carried out as described [5]. The sample was dissolved in 100 μL of distilled water in a culture tube and 600 μL of an ice-cold borate solution containing 4.77 g $Na_2B_4O_7 \cdot 10\ H_2O$ per liter of concd H_2SO_4 was added and thoroughly vortexed. The tube was heated (boiling water, 5 min), cooled to room temperature, 10 μL of 0.15% *m*-hydroxybiphenyl in 0.5% NaOH added, and the tube was vortexed. Absorbance at 540 nm was measured after 5 min.

Preparation of PNB derivatives.—The entire derivatization procedure was carried out in a borosilicate culture tube. The oligosaccharides (5–5000 pmol) were dissolved in water (200 μL), and a solution (200 μL) containing 2 mg of *p*-nitrobenzylhydroxylamine hydrochloride (Aldrich Chemical Co.) dissolved in pyridine was added to the tube. The solvent was immediately evaporated under a stream of nitrogen. The residue

was dissolved in 0.5 mL of toluene, and the solvent was evaporated to remove traces of pyridine. The toluene treatment was repeated twice. A solution (100 μL) containing 2 mg of cyanoborohydride (Aldrich) in a 9:1 mixture of methanol–glacial acetic acid was added, and the suspension was sonicated for 5 min. The solvent was evaporated under a stream of nitrogen, the residue was dissolved in 0.5 mL of 9:1 mixture of methanol–glacial acetic acid, and the solvent was evaporated under nitrogen. The methanol–acetic acid treatment was repeated two more times. Another 2 mg aliquot of cyanoborohydride dissolved in a 9:1 mixture of methanol–glacial acetic acid was added, and the mixture was sonicated, dried under nitrogen, and treated three times with methanol–glacial acetic acid as described above. Methanol (0.5 mL) was added and then evaporated under nitrogen to remove excess acetic acid. The methanol treatment was repeated two more times. Excess PNB reagent and cyanoborohydrate were removed from the residue by adding 500 μL of tetrahydrofuran (THF; J.T. Baker Chemical Co., Phillipsburg, NJ, USA), vortexing the resulting suspension, and centrifuging it at 2000 *g* for 15 min. The supernatant was decanted and discarded. The pellet was extracted three more times with THF. The residue containing the PNB oligosaccharides was dried under a stream of nitrogen and dissolved in 100 μL water.

High-pH anion-exchange chromatography (HPAEC) of underivatized and PNB-derivatized xyloglucan oligosaccharides.—Underivatized and PNB-derivatized xyloglucan oligosaccharides were analyzed by HPAEC on a CarboPac PA1 column (250 × 4 mm, Dionex, Sunnyvale, CA, USA) using standard Dionex hardware and a linear gradient increasing from 50 mM sodium acetate in 100 mM sodium hydroxide to 100 mM sodium acetate in 100 mM sodium hydroxide over 25 min. The underivatized and PNB-derivatized xyloglucan oligosaccharides were detected by sequential pulsed amperometric detection (PAD; Dionex) and UV absorption at 275 nm.

Hydrophilic interaction chromatography.—HILIC was performed as described [6] with an analytical PolyGlycoplex column (200 × 4.6 mm; PolyLc, Columbia, MD, USA) fitted with a gradient module (BioRad, Melville, NY, USA) controlled by a BioRad model 700 chromatography workstation. Elution was isocratic with aq acetonitrile (75%). UV absorbance of the eluant at 275 nm was monitored with a Beckman UV variable wavelength detector (Model 163).

On-line LC–MS of PNB derivatives.—The LC–MS system used is illustrated in Fig. 6. The apparatus consisted of an Applied Biosystems 140B solvent delivery system with dual 10 mL syringe pumps, a 75 μL dynamic mixer, and a Rheodyne model 8125 injector fitted with a 5 μL replaceable sample loop. The solvent was isocratic 70% aq acetonitrile at a flow rate of 50 μL/min. Separations were performed on a PolyGlycoplex microbore column (150 × 1.0 mm, PolyLc). Formation of the positively charged PNB derivative in the eluant was ensured by post-column addition of 0.1% acetic acid at 8 μL/min. This was accomplished with an Upchurch low-dead-volume tee using a Harvard apparatus model 22 syringe pump. After addition and mixing of the acetic acid, the effluent stream was split using a second Upchurch low-dead-volume tee with one outlet connected via a 50 μm diameter fused silica capillary to the electrospray mass spectrometer (ES–MS) and the other outlet connected via a 100 μm diameter fused silica capillary to the UV detector. The split ratio was adjusted by varying the lengths of the fused silica capillaries so that 90% of the flow went to the UV detector (Applied

Biosystems model 785A programmable absorbance detector fitted with a 2.4 μL flow cell) and 10% to the ES-MS. ES–MS was performed with a Sciex API III biomolecular mass analyzer (orifice voltage 35 kV) controlled with a Macintosh IIfx computer (Apple) using Tune version 2.12 software. The individual mass spectra as well as the total ion chromatogram were analyzed by MacSpec version 3.10 software.

3. Results and discussion

Optimization of the PNB-derivatization procedure.—The conditions for the formation of PNB derivatives (Fig. 1) were optimized by varying reaction time, temperature, solvents, and reagents. Optimized reaction conditions (described in the Experimental section) were used to prepare PNB derivatives of nonasaccharide **4** isolated from tamarind seed xyloglucan (Fig. 2).

The improved protocol for PNB-derivitization was evaluated by HPAEC, using oligosaccharide **4** as a model compound. HPAEC of oligosaccharide **4** (Fig. 3A) and of its PNB derivative (Fig. 3B) both resulted in single peaks when the oligosaccharide content of the eluant was monitored by PAD. The PNB derivative (t_R = 23.0 min) is retained by the CarboPac PA1 column for a longer time than the underivatized oligosaccharide (t_R = 21.2 min). The PNB derivative absorbs UV at 275 nm, while the underivatized oligosaccharide is UV transparent, making it possible to distinguish the underivatized and derivatized oligosaccharides. Evaluation of the chromatograms depicted in Fig. 3 shows that virtually no starting material or secondary product is present in the derivatized sample.

Yield of PNB derivatization.—HPAEC of PNB-derivatized oligosaccharide **4** (**8**) provides evidence that the yield of the reaction is quantitative (Fig. 3). A total of 20 μg of underivatized oligosaccharide **4** was subjected to HPAEC (Fig. 3A), and the resulting peak was collected. The same amount of material (20 μg) was PNB derivatized according to the procedure described in the Experimental section, subjected to HPAEC (Fig. 3B), and the resulting peak collected. Both samples were reduced with 10 mg/mL of aq $NaBH_4$ for 4 h before assaying the amount of carbohydrate present in the samples with the anthrone assay (see the Experimental section for details). The reduction step is necessary because unreduced hexoses present at the reducing end of underivatized oligosaccharides and amino alditols present at the alditol end of the PNB derivatives respond differently in the anthrone assay. The yield of PNB-derivatized oligosaccharides was determined by comparing the absorbances of the anthrone assay of both peaks collected (Fig. 3A, Fig. 3B). The average yield of PNB-derivatized oligosaccharide in two different experiments was 97%. Thus, the reaction is essentially quantitative.

The formation of PNB derivatives of structurally diverse oligosaccharides.—The PNB derivatives of a structurally diverse group of oligosaccharides (including tamarind xyloglucan oligosaccharides, sialylated oligosaccharides isolated from milk, and oligogalactosyluronic acids from plant cell wall pectins) were prepared and analyzed by high-performance liquid chromatography (HPLC).

A mixture containing four tamarind xyloglucan oligosaccharides **1–4** was derivatized with PNB (to generate the corresponding PNB-derivatized compounds **5–8**), which was

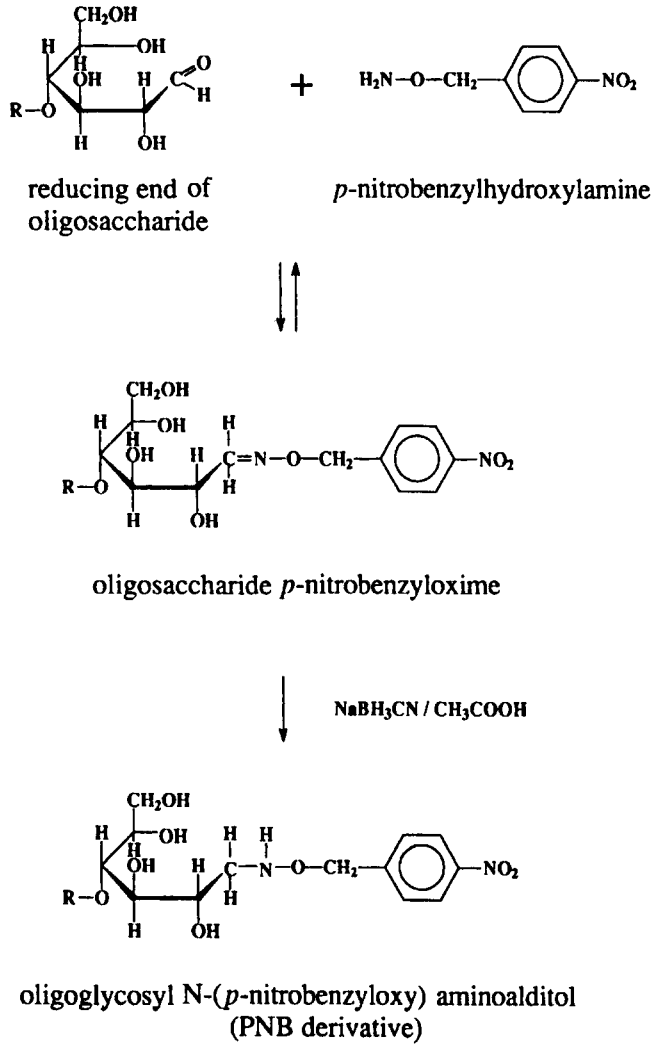
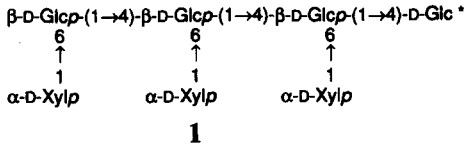


Fig. 1. Reductive alkoxyamination (PNB derivatization) of oligosaccharides. "R" represents the remainder of the oligosaccharide.

separated by HPAEC and detected by PAD (Fig. 4B) and UV absorption at 275 nm. Each peak was assigned to a specific xyloglucan oligosaccharide by comparison to the published retention times of the underivatized oligosaccharides [2,7], and the assignments were confirmed by ES–MS analysis. Table 1 shows a comparison of the peak area ratios of the derivatized oligosaccharides with those of the underivatized oligosaccharides. The ratio of PNB-derivatized xyloglucan oligosaccharides, determined by either PAD or UV absorption, matches the ratio of the underivatized oligosaccharides determined by PAD. This establishes that the efficiency and specificity of the derivatization reaction is not significantly affected by differences in the structures of the



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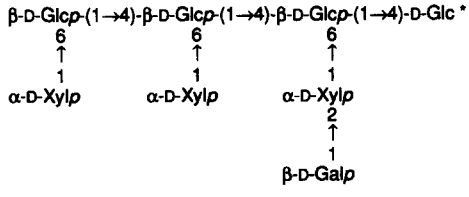

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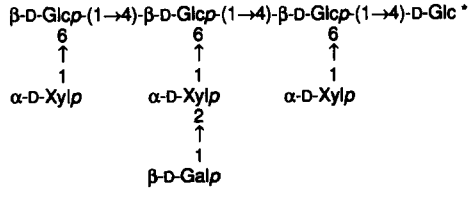

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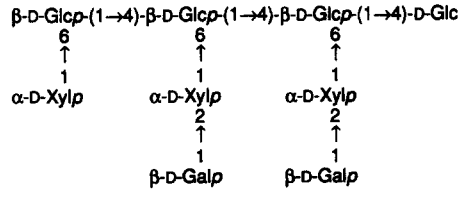

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Fig. 2. Structures of the xyloglucan oligosaccharides used in this study (compounds **1–4**). Compounds **5–8** in the text are the corresponding PNB derivatives of **1–4**, respectively. The * indicates the reducing end of the oligosaccharides.

xyloglucan oligosaccharides and, therefore, that the peak area of a PNB-derivatized xyloglucan oligosaccharide is proportional to the molar amount of the oligosaccharide present in the initial mixture.

The suitability of PNB derivatization for the analysis of sialylated oligosaccharides was also investigated. PNB derivatives of 3′-sialyllactose and di-sialyl-lacto-*N*-tetraose were formed, separated by HPAEC, and detected by PAD and UV detection. Individual peaks were collected and analyzed by ES–MS (data not shown). Only the expected

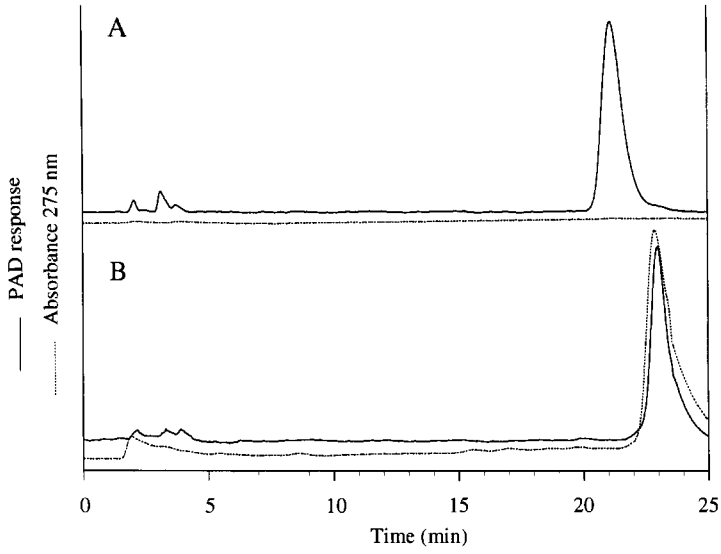

Fig. 3. Chromatographic evidence that the PNB-derivatization procedure goes to completion. Elution profile on HPAEC of underivatized **4** (A) and of corresponding PNB derivative **8** (B) detected by PAD (solid line) and UV absorption at 275 nm (dashed line). Chromatography was on a CarboPac PA1 column (250×4 mm) eluted at 1 mL min^{-1} with a 25 min linear gradient increasing from 50 mM sodium-acetate in 100 mM sodium hydroxide to 100 mM sodium acetate in 100 mM sodium hydroxide. (A) Injection of 20 μg of underivatized xyloglucan oligosaccharide **4**; (B) injection of PNB-derivatized xyloglucan oligosaccharide **8** (20 μg of **4** were used as starting material for the PNB reaction). (The baseline disturbances at 1–5 min are also seen in blank injections and are not due to degraded oligosaccharides.)

PNB-derivatized oligosaccharides were detected, establishing that the PNB reaction conditions are mild enough to prevent desialylation of the oligosaccharides. No trace of underivatized oligosaccharide was found, indicating that the reaction went to completion. Approximately 96% of the sialylated oligosaccharide was recovered as PNB derivatives as determined by the anthrone method described in the *Yield of PNB-derivatization* section.

The PNB derivative of α-(1 → 4)-D-octagalactosyluronic acid was also prepared and analyzed by HPAEC, using both PAD and UV detection (data not shown). Individual peaks were collected and analyzed by ES–MS. Only approximately 70% of the oligogalacturonide was recovered as the PNB derivative as determined by the method described in the *Yield of PNB derivatization* section utilizing the meta-hydroxybiphenyl assay for uronic acids (for details see the Experimental section) instead of the anthrone assay. The remaining 30% of the oligogalactosyluronic acid was underivatized. Therefore, PNB derivatization is incomplete for uronic acids under the derivatization conditions used.

Separation of PNB-derivatized tamarind xyloglucan oligosaccharides.—The abilities of HPAEC, reversed-phase chromatography, and HILIC [8] to separate a mixture of PNB-derivatized xyloglucan oligosaccharides were investigated. The PNB oligosaccharides are not fully separated by reversed-phase chromatography (data not shown). The

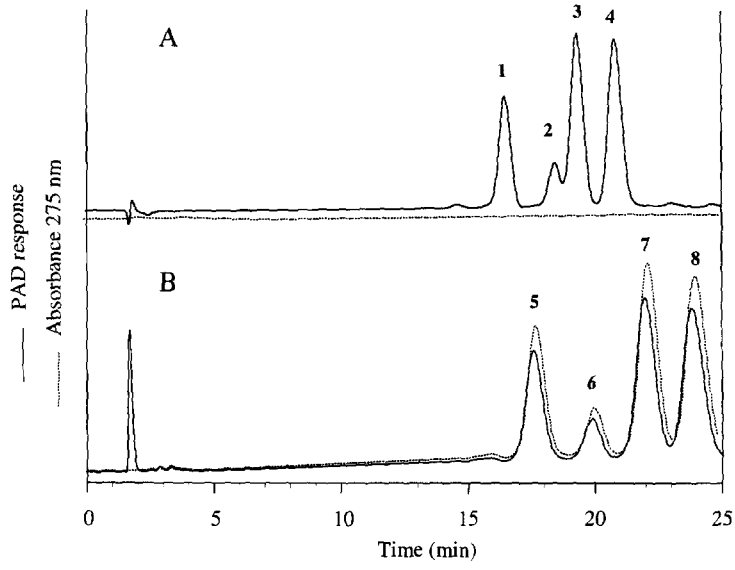

Fig. 4. HPAEC elution profile of underivatized xyloglucan oligosaccharides **1–4** (A) and PNB-derivatized xyloglucan oligosaccharides **5–8** (B) (for structures see Fig. 2) detected by PAD (solid line) and UV absorption (dashed line). Chromatography was on a CarboPac PA1 column (250×4 mm) eluted at 1 mL min^{-1} with a 25 min linear gradient increasing from 50 mM sodium acetate in 100 mM sodium hydroxide to 100 mM sodium acetate in 100 mM sodium hydroxide. The components in each peak were identified by comparing retention times to those of [2,7] and by their electrospray mass spectra. (The peak at about 2 min in the lower panel did not contain carbohydrates, as determined by the anthrone assay, and might be due to salt formed during the reaction.)

best separation of PNB-derivatized tamarind xyloglucan oligosaccharides is accomplished by HPAEC (Fig. 4B), where the two octasaccharide isomers **6** and **7** are completely separated. However, the high salt content in the HPAEC solvent (see the Experimental section for details) makes it necessary to desalt the sample prior to ES–MS analysis [9,10]. In contrast, HILIC is a procedure that does not require

Table 1
Integrated peak areas obtained by HPAEC (see Fig. 4) of xyloglucan oligosaccharides and their corresponding PNB derivatives

Oligosaccharide [a]	PAD detection peak area (%) [b]		UV detection peak area (%) [b]
	Underivatized oligosaccharides	PNB-derivatized oligosaccharides	PNB-derivatized oligosaccharides
1 (5)	20.8 ± 0.3	20.5 ± 0.3	20.7 ± 0.4
2 (6)	8.1 ± 0.2	7.6 ± 0.4	8.3 ± 0.2
3 (7)	34.9 ± 0.2	34.3 ± 0.8	34.6 ± 0.6
4 (8)	36.1 ± 0.3	37.5 ± 1.1	36.4 ± 0.5

[a] For structures, see Fig. 2; corresponding PNB derivatives are in parentheses.
[b] The standard error was calculated by averaging three HPAE chromatograms.

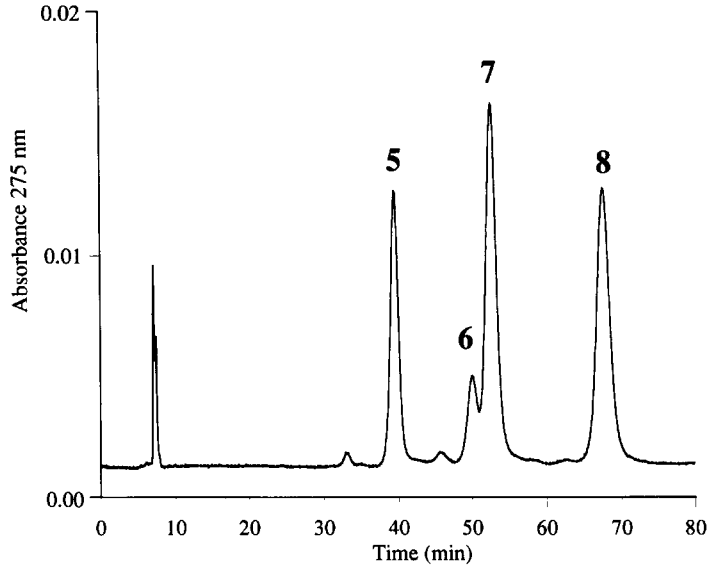

Fig. 5. Elution profile of ~15 μg of the mixture of PNB-derivatized xyloglucan oligosaccharides **5–8** separated by HILIC. Chromatography on a PolyGlycoplex column (200×4.6 mm) eluted isocratically with 75% aq acetonitrile at 1 mL min^{-1}. The components in each peak were determined, after collection and evaporation of the solvent, by comparing retention times to those of standards and by ES–MS.

salt-containing solvents and is suitable, therefore, for ES–MS analysis of the LC eluant (on-line LC–MS). The PNB-derivatized oligosaccharides are sufficiently separated by HILIC for further analysis (Fig. 5). HILIC separation of derivatized xyloglucan oligosaccharides of different degrees of polymerization (dp) is superior to separation by HPAEC, but the isomeric octasaccharides **6** and **7** are not completely separated. We estimate that as little as 1 μg (~ 1 nmol) of a PNB oligosaccharide can be detected by UV using HILIC.

On-line microbore LC–MS of the PNB-derivatized tamarind xyloglucan oligosaccharides.—On-line microbore LC–MS shortens analysis time and minimizes sample loss compared to collection and pooling of the HPLC eluent prior to MS analysis [11]. Therefore, the feasibility of detecting and structurally identifying picomol amounts of PNB-derivatized oligosaccharides by on-line microbore LC–MS was investigated.

The PNB-derivatized oligosaccharides generated from tamarind seed xyloglucan were separated by microbore HILIC and analyzed by ES–MS, as depicted in Fig. 6 (see the Experimental section for details). A total of approximately 150 pmol of the oligosaccharide mixture was injected. The UV absorption, total ion chromatogram, and the reconstructed ion chromatograms for the expected masses of each PNB oligosaccharide are presented in Fig. 7. These data allow the components of each peak to be assigned. However, the resolution of the microbore HILIC column under the conditions used is lower than that of the analytical size column (Fig. 5). Furthermore, peak areas in the total ion chromatogram do not accurately reflect the amounts of the individual compounds present in the mixture. Therefore, the mixture must also be analyzed by UV

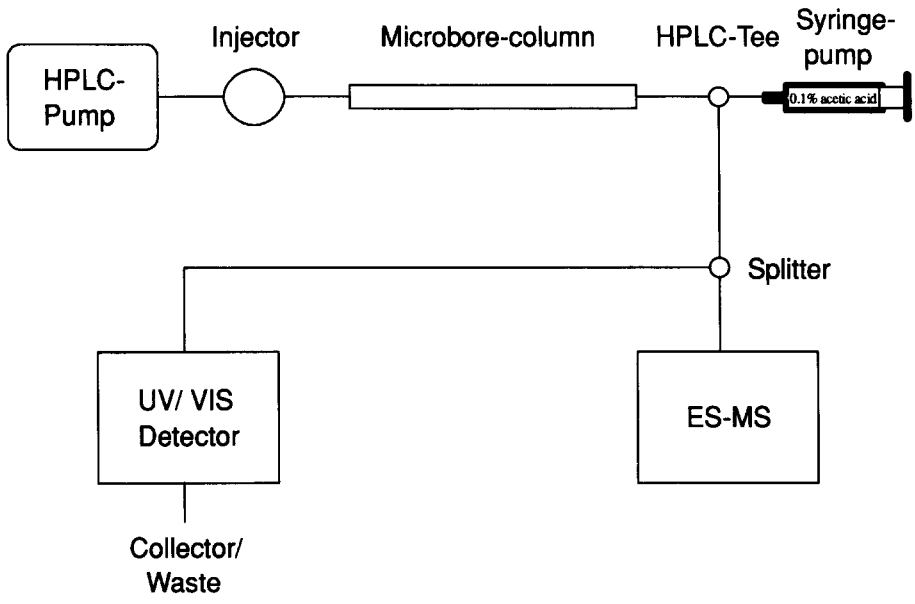

Fig. 6. Schematic diagram of LC–MS set-up. Separations were performed with a PolyGlycoplex microbore column (150 × 1.0 mm). Post-column addition of 0.1% acetic acid was accomplished with a syringe pump. After addition of acetic acid, the effluent stream was split with a tee with one outlet connected to the ES–MS and the other outlet connected to the UV detector. The split ratio was adjusted by varying the lengths of the fused silica capillaries so that 90% of the flow went to the UV detector.

absorption to determine the quantity of each component. We estimate that approximately 10 pmol of each PNB oligosaccharide can be identified and quantitated utilizing on-line microbore LC–MS combined with UV detection.

4. Conclusions

Derivatization with PNB provides several favorable attributes for the analysis of oligosaccharides. The improved method is fast, easy to implement, and amenable to automation. The entire procedure takes place in a single reaction vessel, and no transfers are involved, thus minimizing the potential for sample loss. We prefer this approach to more complicated chromatographic sample workup protocols that may lead to selective loss of individual components of the analyte. The reaction conditions are sufficiently mild to avoid the formation of secondary products, even when sialylated or uronic acid-containing oligosaccharides are derivatized. Except for oligogalactosyluronic acids ($\sim$ 70% yield), the reaction is virtually complete for the oligosaccharides examined. Less than 10 pmol of each product can be quantitatively measured by UV absorbance at 275 nm. The PNB derivatives show improved chromatographic separation using HPAEC compared to the underivatized oligosaccharides. The PNB derivatives are also well separated by HILIC, allowing HILIC–ES–MS to be performed. The derivatization

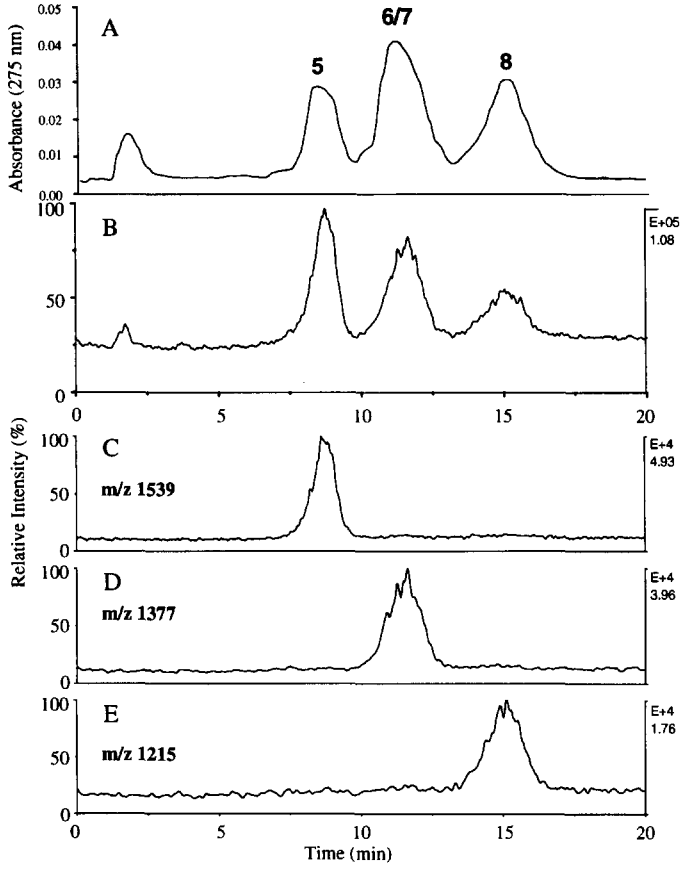

Fig. 7. UV absorption profile (A), total ion chromatogram (B), and selected mass chromatograms (C–E) of the eluant of microbore HILIC of a mixture of the PNB-derivatized xyloglucan oligosaccharides **5–8** (~ 150 pmol of mixture applied). The LC–MS system was configured as in Fig. 6. Chromatography on a PolyGlycoplex column (200 × 1.0 mm) eluted isocratically with 75% aq acetonitrile at a flow rate of 50 μL min^{-1}. The ion at m/z 1539 (C) represents the [M + H] ion expected for compound **8**, the ions at m/z 1377 (D) are the [M + H] ions expected for compounds **6** and **7**, and the ion at m/z 1215 is the [M + H] ion expected for compound **5**.

procedure, involving the formation and reduction of an oxime intermediate, is not limited to PNB-derivatization. Indeed, this chemistry is applicable to a wide range of other derivatives embodying favorable attributes, such as fluorescence or avid binding, provided that an appropiate *O*-substituted hydroxylamine reagent can be prepared.

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