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Characterization of carbohydrates using a combination of derivatization, high-performance liquid chromatography and mass spectrometry

Xiaodong Shen, Hélène Perreault*

Department of Chemistry, University of Manitoba, 144 Dysart Road, Winnipeg, Manitoba R3T 2N2, Canada

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Abstract

A combination of derivatization methods, chromatographic techniques and mass spectrometric ionization modes have been explored for the characterization of small sugars and medium-size oligosaccharides. Derivatization using 1-phenyl-3-methyl-5-pyrazolone (PMP) was preferred over pyridylamination (PA) owing to the simplicity of the reaction method, and also to enhanced ionization efficiency of the PMP derivatives relative to aminopyridyl sugars. The good quality and ease of separation of PMP derivatives by high-performance liquid chromatography were also advantages of using PMP derivatization rather than pyridylamination. PMP- and PA-monosaccharides produced abundant ions by either fast atom bombardment (FAB), electrospray ionization (ESI) or matrix-assisted laser desorption/ionization (MALDI). The PA and PMP derivatives of lactose, fucosyllactose and sialyllactose yielded FAB spectra with low S/N ratios, whereas ESI and MALDI produced better spectra with a hundredth of the material used for FAB. In general, PMP derivatives of these di- and trisaccharides gave rise to stronger signals than PA analogs. For oligosaccharides containing more than three sugar rings, only PMP was used for derivatization, FAB was dropped and only ESI and MALDI were utilized.

Keywords: Derivatization, LC; Interfaces, LC–MS; Carbohydrates; Phenylmethylpyrazolone; Aminopyridine

1. Introduction

The roles of carbohydrates in biological processes have been studied with increasing attention over the past recent years. For example, it has been of considerable interest to study in detail the structures and compositions of carbohydrates in glycoproteins, since they are known to assume very important biological functions. Structural elucidation of complex oligosaccharides remains challenging, as it involves characterization of sugar sequence, branching, linkage between the monosaccharide units and anomeric configuration.

A considerable amount of research has already been carried out in this area. A very reliable method so far has been nuclear magnetic resonance (NMR) spectroscopy, which is applicable when sufficient quantities of samples (mg levels) are available [1]. Mass spectrometry (MS) has also been remarkably useful for structural characterization of carbohydrates. Fast atom bombardment (FAB) (e.g., Refs. [2–5]), electrospray ionization (ESI) (e.g., Refs. [6,7]) and matrix-assisted laser desorption/ionization (MALDI) (e.g., Refs. [8–10]) have been extensively

*Corresponding author.

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used in the characterization of sub-nanomolar amounts of material. Intact native oligosaccharides are not ionized very efficiently by either FAB, ESI or MALDI since they are polar, thermally labile and relatively non-volatile. As a result, the amount of sample required to produce an informative spectrum of good quality often exceeds the quantity of native oligosaccharide available for analysis. To produce better ionization efficiency, the samples can be chemically derivatized. Permethylation (e.g., Refs. [11,12]) and peracetylation (e.g., Refs. [11–13]) among other methods have been used to enhance MS characterization of oligosaccharides at the sub-nanomole level. With the advent of on-line high-performance liquid chromatography–mass spectrometry (HPLC–MS) owing to continuous flow FAB and especially ESI, efforts have been focused on formation of derivatives which simultaneously enhance HPLC separation efficiency, ionization efficiency and detectability by conventional HPLC detectors. The combination of separation techniques with MS has become an invaluable tool in the characterization of biomolecules and MS is hardly ever used as a sole technique anymore.

HPLC has been widely used for the analysis of sugars. Although this technique does not allow for structure elucidation, it permits identification of unknowns relative to standards whose retention behavior vs. structure have been already established. In HPLC analysis as much as in MS, high sensitivity is essential when the oligosaccharides are available in very limited quantities. Carbohydrate analysis by HPLC or other separation techniques coupled to ultraviolet (UV) detectors is made difficult by the absence of effective chromophores or fluorophores [2,14]. Early studies relied on refractometry or absorption in the UV region at 190–210 nm [14]. However, refractive index (RI) detectors have several limitations, namely, sensitivity to changes in solvent composition, temperature and pressure. Following this, a major shortcoming of RI detectors is their incompatibility with gradient elution [15,16]. Pulsed amperometric detection (PAD), combined with high-pH anion-exchange chromatography, has become a popular method for the analysis of native carbohydrates because of its high sensitivity. However, the relatively high pH of eluents has been known to cause some epimerization and degradation of reducing carbohydrates [17], which would make further characterization difficult.

Where UV and/or MS detection is available, derivatization of carbohydrate samples plays a key role. Some chemical tagging methods convert carbohydrates into derivatives which can be detected at lower levels than their native analogs. Selectivity of detection is also enhanced. As an example, only reducing sugars can be derivatized upon modification of carbonyl groups [14]. Furthermore, derivatization can enhance HPLC separation characteristics of sugars. Because underivatized sugars are hydrophilic and have closely related structures, there are limitations in the ways they can differentially interact with the stationary and mobile phases. Tagging of sugars with aromatic groups renders oligosaccharides hydrophobic, thus permitting the use of reversed-phase HPLC, which enables separation of oligosaccharide mixtures [14,18]. Aromatic groups are also very effective chromophores.

For aqueous oligosaccharide samples, the derivatization reactions should be ideally rapid, mild, involve few transfer steps and proceed in aqueous media [19]. Several methods for derivatization of oligosaccharides into UV-absorbing compounds are available. Of these, a very useful and widely used method is reductive amination. For example, reducing oligosaccharides can be tagged with 2-aminopyridine to form pyridylamino (PA) derivatives [20,21]. This method is particularly valuable because of its highly sensitive fluorescence detection. However, it involves a two-step labelling process and has a few additional shortcomings [22], such as loss of sialic acid moieties.

Recently, 1-phenyl-3-methyl-5-pyrazolone (PMP) [23–25] and its methoxy analog, 1-(*p*-methoxy)-phenyl-3-methyl-5-pyrazolone (PMPMP) [22,26] have been used for pre-column derivatization of carbohydrates. The bis-PMP-sugars (Fig. 1), or PMPMP-sugars absorb strongly at 245 nm or 249 nm [22,23]. Both PMP and PMPMP derivatization methods can be used to label sialic acid-containing oligosaccharides without causing desialylation, which constitutes a great advantage over the PA-derivatization method [22].

A MS sensitivity study on PMP and PMPMP oligosaccharides, relative to other types of derivatives (including PA compounds), has been conducted

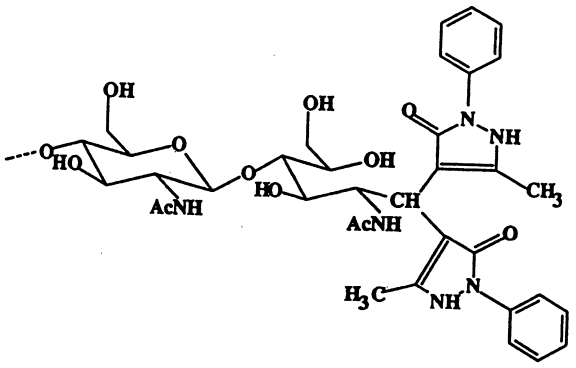

Fig. 1. General structure of the PMP derivatives.

previously [2]. The investigation involved continuous flow-FAB and ESI as HPLC–MS interfaces. Also, the oligosaccharides from thyroglobulin were analyzed by LC–ESI-MS as PMP derivatives [2].

This paper first reports on the HPLC and MS data obtained for the PMP and PA derivatives of a series of mono-, di- and trisaccharides in order to compare UV and MS sensitivities, and HPLC separation efficiencies. Secondly, based on the above findings, larger oligosaccharides obtained commercially were investigated. The results from MS experiments, especially involving MALDI, are emphasized. To our knowledge only one other MALDI study has been reported on the characterization of PMP derivatives of carbohydrates [27].

2. Experimental

2.1. Materials

The PMP reagent was purchased from ICN Biomedicals (Aurora, OH, USA) and used as obtained. 2-Aminopyridine and borane-dimethylamine complex were obtained from Aldrich (Wilwaukee, WI, USA). The small saccharides D-(+)-galactose, D-(+)-glucose, D-(+)-mannose, D-(+)-glucosamine, D-(+)-galactosamine, D-(+)-*N*-acetylglucosamine, L-(−)-fucose, β-lactose, 2′-fucosyllactose and sialyllactose (a mixture of 3′- and 6′-*N*-acetylneuramin-lactose) were purchased from Sigma (St. Louis, MO, USA). Larger oligosaccharide standards M3N2 (conserved trimannosyl core), NGA3 (asialo-, agalacto-, triantennary) and NGA4 (asialo-, agalacto-, tetraantennary) were obtained from Oxford Glycosystems (Rosedale, NY, USA) and used as received. All solvents were glass distilled, HPLC-grade and obtained from Mallinckrodt (Paris, KY, USA). Thin-layer chromatography (TLC) Baker-flex silica gel IB-F plates were obtained from J.T. Baker (Phillipsburg, NJ, USA).

2.2. Preparation of PA derivatives of small sugars [21,28,29]

The solutions of individual small sugars (0.3–5.5 μmol) or a mixture of six small sugars (1–5.5 μmol each) were brought to dryness in a 10×1.3 cm glass tube. The residue was mixed with 10–100 μl of coupling reagent (prepared by dissolving 552 mg of 2-aminopyridine in 200 μl of glacial acidic acid). The mixtures were heated at 90°C for 60 min. The reducing reagent (70–350 μl, freshly prepared by dissolving 200 mg of borane–dimethylamine complex in a mixture of 50 μl of water and 80 μl of acetic acid) was added to the reacted solutions. The solutions were mixed, and heated at 80°C for 60 min. Ammonium hydroxide (2.8 *M*) was then added in order to bring the pH to 10. Excess reagents were extracted three times with two volumes of chloroform. The pH of the aqueous phase was adjusted to 6.0 with acetic acid and the water phase was evaporated to dryness.

2.3. Preparation of PMP derivatives of small sugars [23]

The solutions of individual sugars (30 nmol–15 μmol) or a mixture of ten small sugars containing 1–5.5 μmol of each were brought to dryness and reacted by adding 10–500 μl of 0.3 *M* NaOH and 10–500 μl of 0.5 *M* PMP solution, in methanol. The mixtures were heated at 70°C for 2 h, then neutralized with 1 *M* hydrochloric acid. Water (0.5 ml) and chloroform (1 ml) were added. The organic phase was discarded after shaking; this extraction process was repeated three times. The aqueous layer was brought to dryness, leaving a residue of PMP-sugars.

2.4. PMP derivatization of larger oligosaccharide standards

Each of M3N2, NGA3 and NGA4 (10 μg) was subjected to PMP derivatization using the method described above, on a smaller scale.

2.5. Reversed-phase HPLC of PA derivatives of small sugars [30]

A mixture of D-(+)-mannose, D-(+)-galactose, D-(+)-glucose, L-(−)-fucose, β-lactose (5.5 μmol each) and 2′-fucosyllactose (1 μmol) was reacted with 2-aminopyridine as described above and the products were dissolved in 0.1 *M* ammonium acetate buffer. Aliquots corresponding to 1/400 of the total sample were used for injection into the HPLC system. A Waters Model 600 HPLC system (Millipore, Malborough, MA, USA) equipped with a Waters 486 Tunable Absorbance UV detector and a Waters 746 Data Module was used. A Vydac 218TP54 Protein and Peptide C_{18} column (25×0.46 cm, 5 μm particle size) was used (Separations Group, Hesperia, CA, USA). Elution was performed at 1 ml/min with solvents A and B. Solvent A was 0.1 *M* ammonium acetate buffer without pH adjustment (pH~6.0), and solvent B was 0.5% *n*-butanol in solvent A (pH~6.0). Initially, the mixture was A–B (95:5). The proportion of solvent B was then increased linearly to 15% over 10 min, and then to 85% over 40 min. Detection was performed at 318 nm.

2.6. Reversed-phase HPLC of PMP derivatives from small sugars [25]

A mixture of D-(+)-mannose, D-(+)-galactose, D-(+)-glucose, L-(−)-fucose, β-lactose, D-(+)-glucosamine, D-(+)-galactosamine, (D+)-*N*-acetylglucosamine (5.5 μmol each), 2′-fucosyllactose (1 μmol) and sialyllactose (0.76 μmol) was reacted with PMP as described above. Aliquots corresponding to 1/200 of the total sample were injected into the HPLC system. A Spectra-Physics HPLC system (Thermo Instruments, San Jose, CA, USA), equipped with a Model 8450 UV–Vis Spectra-Physics detector and a HP-3396A integrator (Hewlett-Packard Canada, Orangeville, Canada), was used. An Inertsil ODS-3 column (15×0.46 cm, 5 μm particle size) was used (Phase Separations, Franklin, MA, USA). The flow-rate was set at 1 ml/min and the wavelength at 245 nm. Solvents A and B were 0.1 *M* ammonium acetate (pH 5.5) with 10 and 25% acetonitrile, respectively. The gradient was 45 to 100% of B over 55 min.

2.7. Reversed-phase HPLC separation of PMP derivatives of larger oligosaccharides (M3N2, NGA3 and NGA4)

A Varian 9010 HPLC system (Varian, Lexington, MA, USA), equipped with a Varian 9050 UV–Vis detector and a Varian Star data processor, was used. The reversed-phase column was Hypersil ODS 5 μm (25×0.46 cm) (Sigma–Aldrich). Solvent A was 0.01 *M* trifluoroacetic acid (TFA) in water, and solvent B was 0.01 *M* TFA in acetonitrile. Detection was at 245 nm. The gradient used consisted of increasing the proportion of B from 20 to 100% over 70 min at a flow-rate of 1 ml/min. Fractions were collected and concentrated for MALDI- and ESI-MS analysis.

2.8. Fast atom bombardment mass spectrometry (FAB-MS)

Positive-ion mode FAB experiments were carried out with a VG Analytical 7070E mass spectrometer (Micromass, Manchester, UK). Samples (ca. 10 nmol) were dissolved in glycerol or a mixture of glycerol–thioglycerol (1:1) and loaded onto a stainless steel target. The FAB gun (Xe°) was operated at 8 kV. Conventional scans were at the rate of 2 s/decade and the m/z range covered was from 45 to 1000.

2.9. Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF-MS)

MALDI-TOF-MS was performed in the linear mode on Manitoba II, an instrument built in the Time of Flight Laboratory, Department of Physics, University of Manitoba, Canada, and described elsewhere [31]. The samples (ca. 100 pmol) were loaded onto a stainless steel target with α-cyano-4-hydroxycinnamic acid [saturated in water–CH_3CN (50:50) with 0.1% TFA] as the matrix. The samples

were irradiated using a N_2 laser beam at 337 nm. Bovine insulin and substance P were used as external calibrants.

2.10. Electrospray ionization mass spectrometry (ESI-MS)

Sample-infusion ESI analyses were performed on a Micromass Q-Tof instrument (Micromass). Full spectra were acquired over a m/z range 100 to 2000. Collision induced dissociation (CID) MS–MS experiments were conducted with argon at a gas pressure of $3 \cdot 10^{-3}$ bar (300 Pa) and a collision energy of 28 eV. Product ion scans were recorded.

2.11. On-line HPLC–ESI-MS

A Micromass Quattro-II triple quadrupole mass spectrometer was used in these experiments. Full scan data were acquired in positive ion mode over the m/z range 100 to 2000 at 7 s/scan. The declustering voltage was set to alternate between 40 and 100 V every second scan. An ABI Aquapore RP 300 column (100×1 mm) was used; the amounts of sample injected were half of those used for HPLC–UV experiments. The flow-rate was 50 μl/min and gradients were as described above.

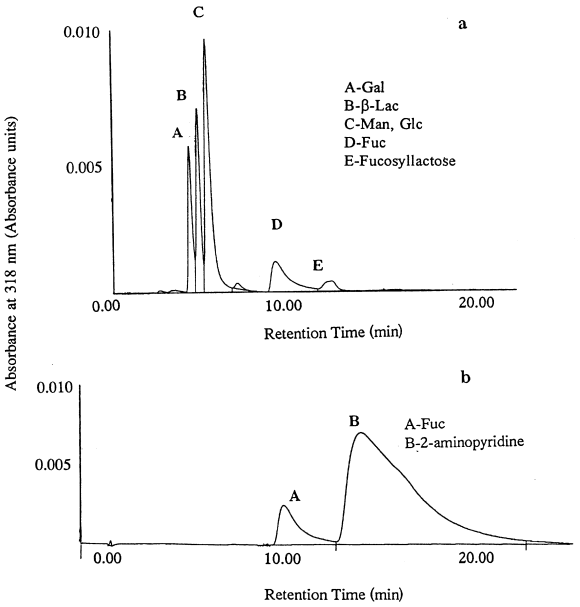

Fig. 2. (a) Reversed-phase HPLC–UV chromatogram of the PA derivatives of small sugars. (b) Chromatogram showing the elution of 2-aminopyridine and PA-L(−)-fucose. Column: Vydac Protein and Peptide C_{18}, (25×0.46 cm). Gradient: from 0.025% to 0.425% *n*-butanol in 0.1 *M* ammonium acetate over 50 min, 1 ml/min. Detection: 318 nm. Gal=Galactose, Lac=lactose, Man=mannose, Glc=glucose, Fuc=fucose. Sample injected: mixture of D-(+)-mannose, D-(+)-galactose, D-(+)-glucose, L-(−)-fucose, β-lactose (13 nmol each) and 2′-fucosyllactose (2 nmol).

3. Results and discussion

The PA and PMP derivatization methods were selected among a number of reported methods for labelling carbohydrates since both involve quantitative yields, rapid reactions and simple clean-up procedures [2]. Preliminary work was carried out on characterization of PA or PMP derivatives of small sugars prior to studying derivatives of larger oligosaccharides obtained commercially. These oligosaccharides were available in very limited quantities and therefore had to be characterized by methods which had been preassessed in our laboratory.

3.1. Reversed-phase HPLC of PA labelled small sugars

Fig. 2a shows the HPLC–UV chromatogram obtained after injecting a mixture of PA-mannose, galactose, glucose, fucose, β-lactose (14 nmol of each) and 2′-fucosyllactose (2.5 nmol). The retention times of individual compounds had been determined first. In previous studies [14,32], retention times of PA-labelled linear sugar chains were found to vary proportionally to the length of the chains. Fig. 2a shows that PA-β-lactose was eluted before some of the PA-monosaccharides, e.g., mannose and glucose. The elution order observed here is similar to that reported by Takemoto et al. [33]. A longer retention time is observed for 2′-fucosyllactose, which is predictable according to its three-ring composition. Fucose, or 6-deoxy-galactose, is less polar than galactose, resulting in a longer retention time relative to that of galactose. The peaks corresponding to PA-mannose and PA-glucose are superimposed, and all other components are resolved. The broad 2-aminopyridine peak obtained using the same conditions (Fig. 2b) was absent from the chromatogram

of the mixture, showing that extraction of the excess reagent with chloroform was quite efficient, as reported earlier [28]. Borane-dimethylamine complex was used instead of sodium cyanoborohydride ($NaBH_3CN$) for the reductive step of the derivatization reaction, since it is more volatile and thus more easily removed [14].

3.2. Reversed-phase HPLC of PMP labelled small sugars

For the PMP labelling reaction, the optimal concentrations of the reagent and the alkali (NaOH) to obtain quantitative yields were found to be 0.25 and 0.15 *M*, respectively [23]. These concentrations represent large excesses of PMP and NaOH relative to the substrates, which were ca. $1.5 \cdot 10^{-3}$ *M*. The excess PMP reagent thus had to be removed prior to HPLC analysis. Dibutyl ether as an extraction solvent was reported to give more complete recovery of the PMP-monosaccharides than chloroform [25]. However, we used chloroform in our experiments since it yielded much clearer two-phase separation than dibutyl ether.

Fig. 3 shows the elution pattern of PMP derivatives of small sugars. An aliquot containing the derivatives of D-(+)-mannose, D-(+)-galactose, D-(+)-glucose, L-(−)-fucose, β-lactose, D-(+)-glucosamine, D-(+)-galactosamine, D-(+)-*N*-acetylglucosamine (27.5 nmol each), 2′-fucosyllactose (5 nmol) and sialyllactose (3.8 nmol) was injected to obtain this chromatogram. The PMP-labelled sugar peaks were all sharp and baseline resolved. A similar pattern had been reported by Fu and O'Neill [25]. The deoxy-hexose (fucose) eluted later than its parent compound galactose, as observed with PA derivatives. The PMP derivative of fucose produced a much smaller peak (labelled K in Fig. 3) than the other monosaccharide derivatives, although equal amounts of all monosaccharides were used for derivatization. The PMP reagent appears as a minor peak at a retention time of ca. 5.6 min, which indicates that PMP can be almost completely removed by the extraction procedure. Our results show that a low initial acetonitrile concentration and a shallow gradient can provide good quality separation of the reagents and PMP-labelled products, as reported by Strydom [24].

3.3. Mass spectrometry of PA and PMP derivatives of small sugars

The derivatives have been characterized using three ionization techniques: FAB, ESI and MALDI. The purposes of this investigation were first to determine the molecular mass (M_r) of derivatization products, secondly to determine how much material was required to produce informative spectra, and

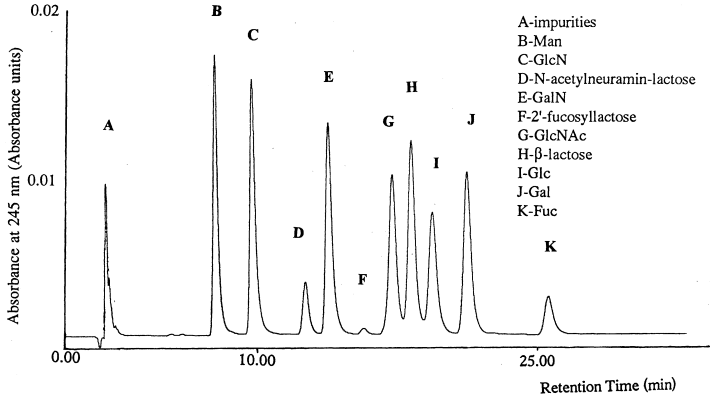

Fig. 3. Reversed-phase HPLC–UV chromatogram of the PMP derivatives of small sugars. Column: Inertsil ODS (25×0.46 cm). Gradient: from 16% to 25% acetonitrile in 0.1 *M* ammonium acetate over 55 min, 1 ml/min. Detection: 245 nm. GlcN=Glucosamine, GalN=galactosamine, GlcNAc=*N*-acetylglucosamine. Sample injected: mixture of D-(+)-mannose, D-(+)-galactose, D-(+)-glucose, L-(−)-fucose, β-lactose, D-(+)-glucosamine, D-(+)-galactosamine, D-(+)-*N*-acetylglucosamine (27 nmol each), 2′-fucosyllactose (5 nmol) and sialyllactose (3.8 nmol).

thirdly to assess the relevance of the information obtained with each technique, for both types of derivatives.

3.4. FAB-MS of PA derivatives

The PA-monosaccharides produced $[M+H]^+$ ions at m/z 259 for PA-mannose, glucose (Fig. 4a), galactose and at m/z 243 for PA-fucose. The resulting sensitivity enhancement obtained with these derivatives relative to underivatized sugars was around one-order of magnitude and thus consistent with data published previously [6]. Fig. 4b shows the FAB spectrum of PA-β-lactose, which contains characteristic $[M+H]^+$ ions at m/z 421. Ions at m/z 259 indicate the loss of the galactose residue (Y-type cleavage [3,34]). While molecular ions were relatively abundant, very few (and low-abundance) fragment ions were present, as reported previously for PA- and *p*-aminobenzoic ethyl ester (ABEE) derivatives [4]. PA-2′-Fucosyllactose and PA-sialyllactose produced low-abundance $[M+H]^+$ ions at m/z 567 and 712, respectively, as shown in Fig. 4c and Fig. 4d. Especially in Fig. 4d, the fragment ions are obscured by background signals, making the spectrum nearly uninterpretable. With such lack of sensitivity, it was decided that PA derivatives of sugars larger than trisaccharides would be characterized by ESI and/or MALDI.

3.5. ESI- and MALDI-MS of PA derivatives of small sugars

Full-scan ESI spectra of PA derivatives acquired at low declustering voltage (40 V, not shown) contained mainly $[M+H]^+$ ions. A higher declustering voltage (100 V) favored formation of $[M+Na]^+$ species and formation of Y-type fragments from $[M+H]^+$.

On-line HPLC–ESI-MS results were obtained for the same sample mixture as discussed in Fig. 2a. However, overlapping between the HPLC peaks made the interpretation of spectra rather difficult and these data will not be further discussed here. In MALDI, the PA-derivatives produced predominant $[M+H]^+$ ions, with less abundant $[M+Na]^+$ species. For PA-monosaccharides, these ions appeared in the matrix-region of the spectra, i.e., where most background ions are present, making their assignments more difficult. In the case of di- and trisaccharides, the $[M+H]^+$ and $[M+Na]^+$ ions were easily distinguished, although fragments were in turn obscured by the background in the matrix region. To summa-

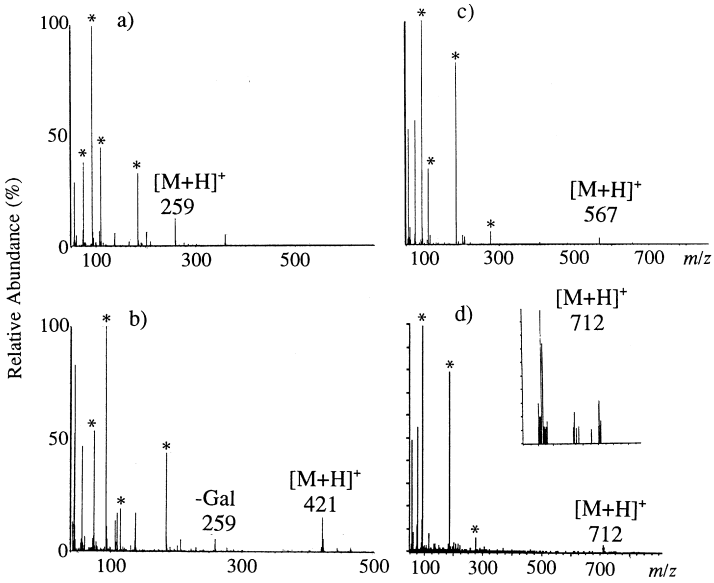

Fig. 4. Positive mode FAB mass spectra of the PA derivatives of small sugars. (a) PA-glucose, (b) PA-β-lactose, (c) PA-2′-fucosyllactose, (d) PA-sialyllactose. Details on conditions are given in Section 2.8.

rize, ESI produced good quality spectra for each of the mono-, di- and trisaccharides analyzed. Okamoto et al. [6] had made similar observations with ESI of PA-maltopentaose and had measured a 100-fold sensitivity improvement relative to ESI of the underivatized pentasaccharide. In the case of our mono-, di- and trisaccharides, the sensitivity improvement was more of the order of 50. On the other hand, MALDI signals for mono- and disaccharides were easily lost in the matrix background, making this technique more favorable to characterization of higher-M_r PA-oligosaccharides.

3.6. FAB-, ESI- and MALDI-MS of PMP derivatives of small sugars

FAB spectra of PMP derivatives are shown in Fig. 5 (monosaccharides) and Fig. 6 (di- and trisaccharides). The sensitivity enhancement obtained with these derivatives relative to underivatized sugars was of the order of 100. The PMP derivatives produced abundant $[M+H]^+$ and $[M+Na]^+$ ions, and also fragment ions, although low in abundance. The $[M+Na]^+$ species predominated, and also $(M-H+2Na)^+$ and $(M-2H+3Na)^+$ were present, due to excess sodium hydroxide in the derivatization reaction. The loss of one PMP residue from $[M+Na]^+$ ions produced abundant ions. The same loss from $[M+H]^+$ and $[M-H+2Na]^+$ ions produced ions varying in abundance, depending on the overall sodium content of the sample. Fig. 5c illustrates these trends. In Fig. 6b, loss of the fucose residue from the non-reducing end of PMP-fucosyllactose produced abundant ions at m/z 674 and 696. Ions due to further loss of a galactose residue appear at m/z 511 and 533, respectively.

Fig. 6c shows the FAB spectrum of PMP-sialyllactose. The peaks at m/z 966, 988, and 1010 correspond to the $[M+H]^+$, $[M+Na]^+$ and $(M-H+2Na)^+$ ions, respectively. The abundant ions at m/z 696 and 533 resulted from sequential losses of sialic acid and galactose residues from the non-reducing end of the $[M+Na]^+$ ions. The spectra of all PMP-sugars examined contained two high peaks at m/z 359 and 381 (Figs. 5 and 6). These signals were attributed to cleavage of bond 1 of the reducing-end sugar (between C1 and C2), producing fragments bearing two PMP residues (without and with Na attached). Also, for sugars with a hexose residue at their reducing end, ions at m/z 359 and 381 could be due to fragment ions corresponding to mono-PMP-hexose, with one and two Na attached, respectively.

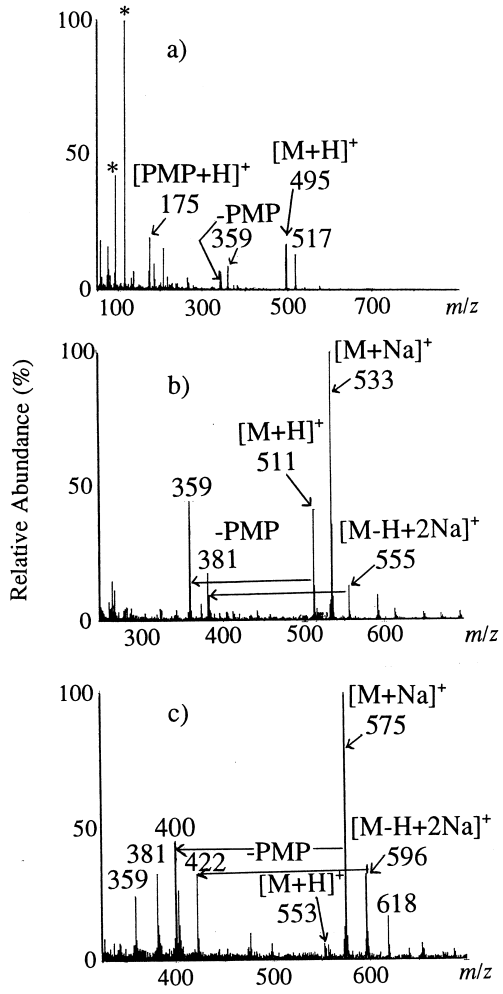

Fig. 5. Positive mode FAB mass spectra of the PMP derivatives of monosaccharides. (a) PMP-fucose, (b) PMP-galactose and (c) PMP-*N*-acetylglucosamine. Details on conditions are given in Section 2.8.

These results show that PMP labelling enhances sensitivity by almost an order of magnitude over PA derivatization for the analysis of small sugars. According to Suzuki et al. [2], PMP-labelled maltopentaose yielded 2- to 4-fold enhanced FAB sensitivity over its PA derivative, with a variety of matrices. PMP-labelling produces sugars with a more hydro-

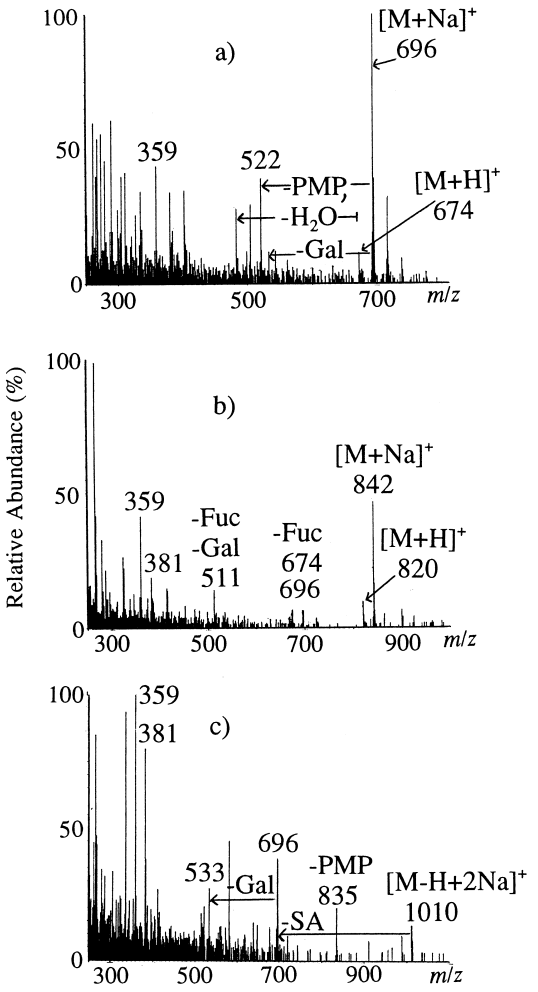

Fig. 6. Positive mode FAB mass spectra of the PMP derivatives of di- and trisaccharides. (a) PMP-β-lactose, (b) PMP-2′-fucosyllactose, (c) PMP-sialyllactose. Details on conditions are given in Section 2.8.

phobic tail than the PA method, and thus enhances surface activity of the analytes during FAB experiments. Poulter et al. [35] reported the same phenomenon with the labelling of sugars using *n*-alkyl *p*-aminobenzoates for liquid secondary ion mass spectrometry (LSI-MS). Our results also show that all types of small saccharides (hexoses, fucose, aminohexoses, acetoaminohexoses, fucosylated and sialyllated lactose) can be labelled with PMP to form relatively stable bis-PMP derivatives. The extensive loss of one PMP residue observed in the mass spectra may indicate the higher relative stability of mono-PMP derivatives, as indicated previously for PMPMP compounds [22]. Overall, the PMP derivatives were more readily fragmented than PA derivatives under FAB conditions. In the case of PMP-di- and trisaccharides, more information on the sugar sequence was obtained than with PA derivatives, which provided M_r information almost exclusively.

Because of the limited mass range and sensitivity available with our FAB-equipped mass spectrometer, no significant spectra could be obtained for sugar derivatives with M_r above 800. The use of ESI- and MALDI-MS was thus investigated for characterization of PMP-sugars. Suzuki et al. [2] have previously reported that PMP derivatives of maltopentaose and oligosaccharides from glycoproteins gave the highest ESI sensitivities among other types of derivatives investigated, such as PA-, ABEE, 2-aminoethanethiol (AET) and 2-aminobenzenethiol (ABT).

The ESI spectra of PMP-β-lactose and 2′-fucosyllactose are shown in Fig. 7a and Fig. 7b. The full-scan ESI spectrum of β-lactose (not shown) contained many unknown ions due to impurities and thus an ESI-MS–MS spectrum of the $[M+H]^+$ ions at m/z 674 was recorded instead (Fig. 7a). The peaks at m/z 511 and 337 correspond to successive losses of a galactosyl residue and of one PMP group. The peak at m/z 373 has been interpreted as corresponding to a X_0 ion originating from cleavage of the C2–C3 bond, followed by loss of H_2O. Fig. 7b shows the full-scan ESI spectrum of PMP-fucosyllactose. The ionic pattern somehow differs from that of Fig. 6b (FAB). For example, the peak observed at m/z 696 in the FAB spectrum and corresponding to loss of a fucosyl residue from $[M+Na]^+$ ions is small in the ESI spectrum and dominated by an ion peak at m/z 667, which indicates the preferential loss of a PMP residue from $[M+Na]^+$. The ESI spectrum of Fig. 7b, obtained at relatively high declustering voltage (100 V), required 100× less material than the FAB spectrum, and the absence of matrix ions in ESI yielded a much cleaner spectrum. Fig. 7a and Fig. 7b, show that Y-type cleavages from $[M+H]^+$ are more readily produced by ESI, in the specific declustering conditions used here. At a low declustering voltage (40 V), $[M+H]^+$ ions dominated the spectra of all sugars investigated in this study, whereas higher values (100 V) favored the $[M+Na]^+$ ions. The additional ions created by

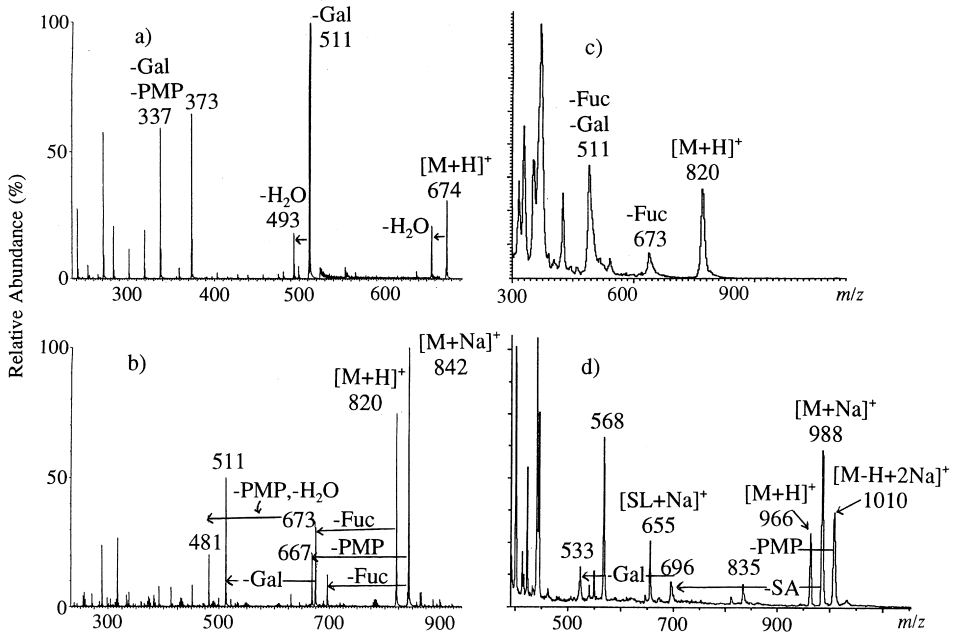

Fig. 7. (a) Positive ESI-MS–MS spectrum of the $[M+H]^+$ ions of PMP-β-lactose. (b) Full scan ESI-MS of 2′-fucosyllactose. (c) Linear mode MALDI-TOF mass spectrum of PMP-2′-fucosyllactose. (d) Linear mode MALDI-TOF mass spectrum of PMP-sialyllactose. Details on conditions are given in Sections 2.9 and 2.10. SL=Sialyllactose.

increasing the declustering voltage are mostly due to fragmentation of $[M+H]^+$, as suggested by MS–MS experiments on the latter.

The MALDI spectra of 2′-fucosyl- and sialyllactose are shown in Fig. 7c and Fig. 7d. These spectra show simpler patterns than FAB and ESI spectra. For instance, two high peaks are observed at m/z 673 and 511 (Fig. 7c) and correspond to the sequential loss of fucosyl and galactosyl residues from the non-reducing end of fucosyllactose. The ions due to loss of one PMP residue from either molecular or fragment ions are predominant in the ESI and MALDI spectra of the derivatives. The peak at m/z 568 (Fig. 7d) corresponds to protonated trimeric matrix ions. The signal-to-noise ratios observed with both ESI and MALDI were in general higher than those obtained by FAB, while using only 1% of the amount of sample used for FAB.

Overall, a sensitivity improvement of one-order of magnitude in MALDI and ESI was obtained by using the PMP-labelling method rather than preparing the PA derivatives.

On-line HPLC–ESI-MS experiments performed on a mixture of PMP-labelled small sugars produced better quality chromatograms and mass spectra than on-line work achieved with the PA mixture. Baseline chromatographic separation (similar to that obtained in Fig. 3) provided for unambiguous ESI spectra, with characteristics similar to those discussed above.

3.7. Application to larger oligosaccharides

So far, our results have indicated that: (i) PMP derivatives are more readily separable by HPLC than PA compounds; (ii) our FAB-MS system does not offer sufficient sensitivity for analysis of nanomolar quantities of sugars with M_r>800; (iii) MALDI or ESI are better suited than FAB to high-M_r analysis on small amounts of material; and (iv) the PMP derivatives yield an at least 10-fold sensitivity improvement over PA compounds under ESI and MALDI conditions. The present study has thus been extended to well-characterized *N*-linked oligosaccharides obtained commercially.

Three oligosaccharide standards (M3N2, NGA3 and NGA4) were thus labelled with PMP and

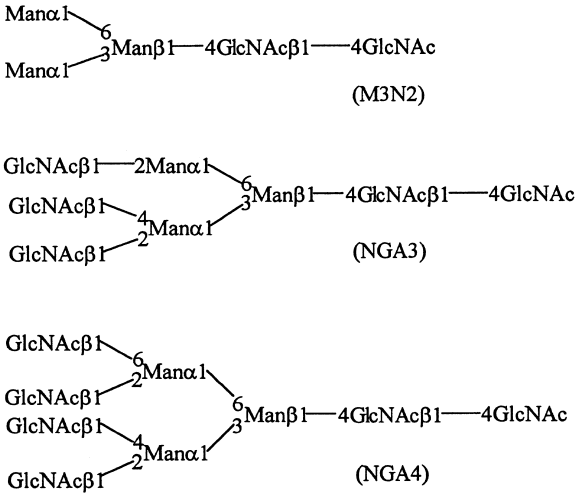

Fig. 8. Structures of three standard oligosaccharides obtained commercially.

characterized by linear mode MALDI-TOF-MS in order to ascertain observation of molecular ions at the low pmol level. Fig. 8 shows the structures of these three sugars. Fig. 9 shows the MALDI-TOF-MS spectra of PMP-M3N2 and NGA4, obtained with ca. 1 pmol of material. Mainly $[M+Na]^+$ and/or $[M+H]^+$ ions appeared and no fragment ions were observed. The measured and calculated m/z values for the $[M+Na]^+$ ions of the three sugars are given in Table 1.

The PMP standards (1 nmol of each, total) were then eluted on the C_{18} column. Separation of the PMP-sugars on a C_{18} reversed-phase column was effected using a water–acetonitrile system containing TFA at a concentration of 0.01 *M*, instead of ammonium acetate as suggested in the literature [25] and used here for smaller sugars. The presence of ammonium acetate in the collected fractions gave unsatisfactory results under MALDI conditions, since the presence of salts in samples caused formation of a wide distribution of adduct ions and thus disabled accurate M_r measurements. The HPLC procedure with the use of TFA on the other hand allowed for preparation of samples devoid of salts, e.g., NaCl, which was eluted within the dead volume.

In order to obtain more efficient isolation of fractions without overloading the column, the derivatized sugars were injected several times in a row,

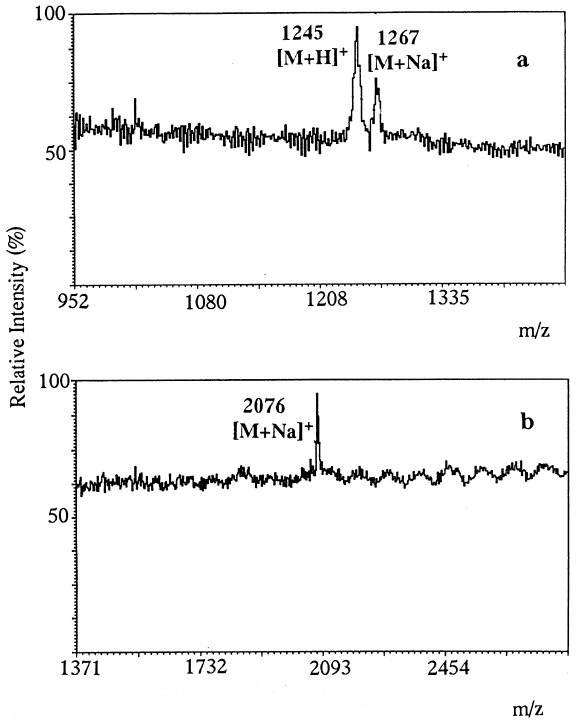

Fig. 9. Linear mode MALDI-TOF mass spectra of (a) PMP-M3N2, (b) PMP-NGA4. For conditions, see Section 2.9.

with only 50–100 μl aliquots each time. Fractions of several runs were combined and concentrated in order to obtain 10 μl of solution. Aliquots of these solutions (1 μl) were used as samples for ESI. The resulting ESI spectra displayed weak $[M+H]^+$ signals sufficient for the purposes of identification by M_r, but not sufficient for MS–MS experiments to be performed. The estimated quantity of each PMP derivative used for ESI is 5 pmol. As a general observation, the PMP-oligosaccharides yielded better sensitivity in MALDI relative to ESI, but more accurate M_r measurement was available with ESI. The m/z values of the $[M+H]^+$ measured with the latter were within 0.05% of the predicted monoisotopic values.

4. Conclusions

The labelling of small sugars with 2-aminopyridine and PMP provided sensitive detection by

Table 1
MALDI-TOF–MS molecular mass measurements on the PMP derivatives of some oligosaccharide standards

PMP derivatives of...[a]	Observed m/z, $[M+Na]^+$	Calculated m/z[b], $[M+Na]^+$
M3N2	1267	1264.2
NGA3	1875	1873.8
NGA4	2076	2077.0

[a] Structures of the oligosaccharides given in Fig. 9.
[b] Calculated using average values.

either UV absorption or MS (FAB, MALDI, ESI). Both derivatization methods involve reactions which are quantitative and simple to carry out. The PMP-derivatives allowed for better chromatographic resolution and efficiency than the PA-labelled sugars. Also, PMP compounds brought the sensitivity up by a factor of 100 relative to underivatized sugars, and by a factor of 10 relative to PA derivatives. We have chosen the PMP-labelling method to carry our study further and characterize larger oligosaccharides obtained commercially. It was possible to separate the PMP-sugars by HPLC using acetonitrile–water eluents with low percentages of TFA instead of less volatile buffer salts, which would hamper MALDI analysis. Since the eluents are compatible with peptide mapping, there is no need for reconfiguration of the HPLC system in a laboratory where several types of biological analyses are conducted. Linear MALDI-TOF-MS analyses of HPLC fractions can only confirm the sugar compositions of these oligosaccharides, based on $(Hex)_x(GlcNAc)_y(Fuc)_z$ formulae. We are currently developing a methodology involving PMP derivatization, on-line HPLC–ESI-MS and HPLC–ESI-MS–MS. These techniques are complementary to linear mode MALDI-TOF-MS and will provide more detailed information on the structures and sequences of glycans from glycoproteins, e.g., chicken egg ovalbumin. The results from the application of this methodology will be the object of a future publication.

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References

[1] J.F.G. Vliegenthart, L. Dorland, H.V. Halbeek, Adv. Carbohydr. Chem. Biochem. 41 (1983) 209.
[2] S. Suzuki, K. Kakehi, S. Honda, Anal. Chem. 68 (1996) 2073.
[3] W. Wang, N.C. LeDonne Jr., B. Ackerman, C.C. Sweeley, Anal. Biochem. 141 (1984) 366.
[4] S.A. Carr, V.N. Reinhold, B.N. Green, J.R. Hass, Biomed. Mass Spectrom. 12 (1985) 288.
[5] A. Dell, N.H. Carman, P.R. Tiller, J.E. Thomas-Oates, Biomed. Environ. Mass Spectrom. 16 (1988) 19.
[6] M. Okamoto, K. Takahashi, T. Doi, Rapid Commun. Mass Spectrom. 9 (1995) 641.
[7] K.L. Duffin, J.K. Welply, E. Huang, J.D. Henion, Anal. Chem. 64 (1992) 1440.
[8] F. Hillenkamp, M. Karas, R.C. Beavis, B.T. Chait, Anal. Chem. 63(24) (1995) 1193A.
[9] J. Lemoine, F. Chirat, B. Domon, J. Mass Spectrom. 31 (1996) 908.
[10] T. Takao, Y. Tambara, A. Nakamura, K. Yoshino, H. Fukuda, M. Fukuda, Y. Shimonishi, Rapid Commun. Mass Spectrom. 10 (1996) 637.
[11] A. Dell, Methods Enzymol. 193 (1990) 647.
[12] A. Dell, H. Egge, H. Von Nicolai, G. Strecker, Carbohydr. Res. 15 (1983) 41.
[13] V.N. Reinhold, B.B. Reinhold, C.E. Costello, Anal. Chem. 67 (1995) 1772.
[14] S. Hase, in: Z. El Rassi (Ed.), Carbohydrate Analysis (Journal of Chromatography Library, Vol. 58), Elsevier, Amsterdam, 1995, Ch. 15, pp. 555–71.
[15] K.B. Hicks, Adv. Carbohydr. Chem. Biochem. 46 (1988) 17–72.
[16] K.B. Hicks, Adv. Carbohydr. Chem. Biochem. 46 (1988) 327–329.
[17] Y.C. Lee, Anal. Biochem. 189 (1990) 151.

[18] Y.S. Kim, J. Liu, X.J. Han, A. Pervin, R.J. Linhardt, J. Chromatogr. Sci. 33 (1995) 162.
[19] K. Mopper, L. Johnson, J. Chromatogr. 256 (1983) 27.
[20] S. Hase, T. Ibuki, T. Ikenaka, J. Biochem. 95 (1984) 197.
[21] A. Kondo, J. Suzuki, N. Kuraka, S. Hase, I. Kato, J. Ikenaka, Agric. Biol. Chem. 54(8) (1990) 2169.
[22] K. Kakehi, S. Suzuki, S. Honda, Y.C. Lee, Anal. Biochem. 199 (1991) 256.
[23] S. Honda, E. Akao, S. Suzuki, M. Okuda, K. Kakehi, J. Nakamura, Anal. Biochem. 180 (1989) 351.
[24] D.J. Strydom, J. Chromatogr. A 678 (1994) 17.
[25] D. Fu, R.A. O'Neill, Anal. Biochem. 227 (1995) 377.
[26] K. Kakehi, M. Ueda, S. Suzuki, S. Honda, J. Chromatogr. 630 (1993) 141.
[27] D.S. Ashton, C.R. Beddell, D.J. Cooper, A.C. Lines, Anal. Chim. Acta 306 (1995) 43.
[28] S. Hase, Methods Enzymol. 230 (1994) 225.
[29] J. Toyota, K. Omichi, K. Fukase, S. Kusumoto, S. Hase, Biosci. Biotech. Biochem. 58 (1994) 567.
[30] S. Hase, S. Koyama, H. Daiyasu, H. Takemoto, S. Hara, Y. Kobayashi, Y. Kyogoku, T. Ikenaka, J. Biochem. 100 (1986) 1.
[31] W. Ens, V. Spicer, K.G. Standing, J. Zhou, Organic Mass Spectrom. 28 (1993) 1430.
[32] N. Tomiya, J. Awaya, M. Kurono, S. Endo, Y. Arata, N. Takahashi, Anal. Biochem. 171 (1988) 73.
[33] H. Takemoto, S. Hase, T. Ikenaka, Anal. Biochem. 145 (1985) 245.
[34] B. Domon, C.E. Costello, Glycoconjugate J. 5 (1988) 397.
[35] L. Poulter, R. Karrer, A.L. Burlingame, Anal. Biochem. 195 (1991) 1.