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Note

Identification of monosaccharides by high-performance liquid chromatography using methanolysis and a light-scattering detector

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Structural studies of glycoconjugates rely heavily on chromatographic separations, which are of particular importance because of the great diversity of carbohydrates and large number of isomers. Before the separation of individual monosaccharides it is necessary to cleave glycosidic linkages of glycoconjugates by acid hydrolysis or methanolysis.

Acid hydrolysis of glycoconjugates followed by ion-exchange chromatography[1], high-performance liquid chromatography (HPLC)[2] with derivatization[3,4] or gas chromatography (GC)[5] has been extensively used. However, the conditions chosen for acid hydrolysis are usually a compromise between obtaining complete release of each sugar and preventing excessive destruction; liberated sugars differ in their stability to acids. Morever, separation usually requires derivatized samples.

Methanolysis offers the advantage of cleaving O-glycosidic linkages in a one-step procedure[6] and does not cause significant destruction of neutral and amino sugars[7]. Numerous analytical techniques using gas chromatography have been described[8,9], but all these methods require a derivatization of methyl glycosides and are often time consuming.

Various methods have been employed for the direct separation of methyl glycosides by HPLC[10,11], but analyses are limited by the detection of methyl glycosides. Some reports have appeared on the HPLC of methyl glycosides using mass spectrometric detection[12] or refractive index detection[10,13].

This paper describes a method for a rapid determination of neutral and amino sugars present in polysaccharides and glycoconjugates, involving methanolysis and HPLC analysis of the resulting methyl glycosides with a light-scattering detector. This method was applied successfully to the determination of monosaccharides of the well characterized glycoproteins α_1-acid glycoprotein and ovalbumin. It was also used for the determination of the sugar composition of polysaccharides such as gum arabic and gum karaya.

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EXPERIMENTAL

HPLC was carried out using a Perkin-Elmer L400 pump and a DDL 11 light-scattering detector (Cunow/Translab); data were collected on a Spectra-Physics SP4400 integrator. Nebulization of the eluent was provided by a stream of nitrogen at 29 p.s.i. The nebulized solvent was evaporated at 30 ± 1°C. The column was a Spherisorb ODS-2 (250 × 4.6 mm I.D.) (Societé Française de Colonnes Chromatographiques, SFCC). The samples were finally run at flow-rate of 0.5 ml/min with water–methanol (97:3) as the mobile phase. All solvents were of HPLC grade and were filtered through a 0.2-μm Millipore membrane; water was distilled.

Standard of monosaccharides, some of their methyl glycoside derivatives and ovalbumin were obtained from Sigma. Mesoinositol, used as an internal standard, was purchased from BDH Biochemicals. The α_1-acid glycoprotein was provided by Professor G. Durand (Laboratory of Biochemistry, Faculty of Pharmacy, Chatenay-Malabry, France) and gums were obtained from Iranex.

Methanolysis of the standard sugars, glycoproteins or polysaccharides was performed using the method of Zanetta *et al.*[9] with a few modifications. Methanolic hydrochloric acid (1.2 *M*) was diluted with anhydrous methanol to yield a concentration of 0.6 *M*. Samples containing glycoprotein or standard carbohydrate together with mesoinositol were dried over P_2O_5 under vacuum prior to methanolysis. Methanolysis was carried out by dissolving 100 μg of dried sugar or 500 μg of glycoprotein in 1 ml of the above solution in a sealed pyrex glass tube.

Samples were heated at 70°C for 20 h. Acid was removed under a stream of nitrogen at room temperature and the sample was dissolved in distilled water and injected into the chromatograph.

RESULTS AND DISCUSSION

Nine methyl glycosides, including neutral and amino sugars that commonly occur in glycoproteins and mesoinositol, often used as an internal standard in glycoprotein analysis[7], were separated on a Spherisorb ODS-2 column with water–methanol (97:3) (Fig. 1). Hexoses were eluted first, followed by pentoses, hexosamines, deoxyhexoses and uronic acids (Table I). Using a flow-rate of 0.5 ml/min the nine sugars were eluted within 16 min and detected using a light-scattering detector with an evaporation temperature of 30°C and a gas pressure of 29 p.s.i.

Various mobile phase compositions were studied in order to show the effect of adding an organic solvent to an aqueous eluent on chromatographic behaviour of methyl glycosides. Using water as eluent, the less polar methyl glycosides (deoxyhexoses) such as methyl rhamnoside and methyl fucoside were eluted after 21 min and the two methyl glycosides of fucose overlapped with the α-methyl glycoside of N-acetylglucosamine. Much better resolution was obtained by adding methanol to the eluent. We adopted 3% aqueous methanol because the derivatives of N-acetylglucosamine, fucose and rhamnose were partially separated with lower percentages of methanol, and with higher percentages of methanol the selectivity for the first eluted methyl glycosides was poor. Acetonitrile was also tested and gave the same elution profile except that a good separation required an acetonitrile content lower than 1%.

Methanolysis produces a mixture of methyl glycosides which do not reveal the

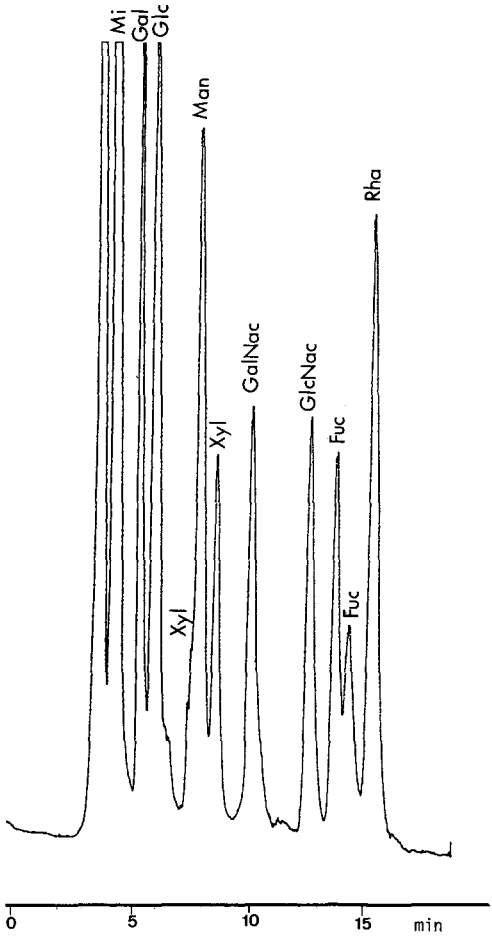

Fig. 1. Separation of a mixture of methanolysed monosaccharides on a 250 × 4.6 mm I.D. Spherisorb ODS-2 column at a flow-rate of 0.5 ml/min. Eluent, water–methanol (97:3); detector temperature, 30°C; pressure, 29 p.s.i.; photomultiplier gain, 9. Mi = Mesoinositol; Gal = galactose; Glc = glucose; Xyl = xylose; Man = mannose; GlcNac = N-acetylglucosamine; GalNac = N-acetylgalactosamine; Fuc = fucose; Rha = rhamnose.

anomeric configuration of the glycosidic linkage in polysaccharides or glycoproteins. The anomeric effect associated with a methoxy group causes an increase in the proportion of the anomer with an axial methoxy group on the more stable conformer of a methylpyranoside. Hence there is a higher proportion of α-anomer with galacto-, gluco-, manno- and xylo- configurations, as showed in previous studies[14]. Some of the isomers of monosaccharides were identified by comparison between the retention times obtained by methanolysis of a mixture of monosaccharides (Table I) (Fig. 1) and those obtained from standard methyl glycosides (Fig. 2). Methanolysis of individual sugars was used to determine whether or not some anomeric methylglycosides were formed and in what amounts; both galactose and mannose gave rise to a secondary anomer (less than 2%) which was eluted in the shoulder following the methyl glucoside

TABLE I

RETENTION TIMES AND CAPACITY FACTORS OF METHYL GLYCOSIDES

Column, Spherisorb ODS-2; eluent, water–methanol (97:3); flow-rate, 0.5 ml/min.

Sugar	*Retention time (min)*	*Capacity factor*	*Anomer*[a]
Mesoinositol	5.04	0.22	–
D-Galactose	6.23	0.51	α-Pyranoside
D-Glucose	6.78	0.64	α-Pyranoside
D-Xylose	7.80	0.89	β-Pyranoside
	9.31	1.25	α-Pyranoside
L-Arabinose	7.93	0.92	–
D-Mannose	8.64	1.09	α-Pyranoside
N-Acetyl-D-galactosamine	10.84	1.62	–
N-Acetyl-D-glucosamine	13.24	2.20	α-Pyranoside
L-Fucose	14.65	2.55	α-Pyranoside
	15.03	2.64	–
L-Rhamnose	15.99	2.87	α-Pyranoside
D-Galacturonic acid	16.75	3.05	–

[a] The configuration of isomers was defined using standard methyl glycosides.

peak (retention times 6.81 and 6.97 min, respectively). However, this is of minor importance as the heights of the main peaks can be readily measured without interference from other sugars. Under the conditions of trimethylsilyl ether formation, used in GC, sugars give rise to a mixture of α- and β-anomers and when dealing with a mixture overlap is inevitable. Using our method, all the sugars except fucose and xylose give rise to only a main peak and it is therefore more suitable for quantitative analysis.

The influence of some parameters of the evaporative light-scattering detector on the response factor was investigated. Fig. 3 shows a plot of detector response *versus* the atomiser inlet nitrogen pressure between 17 and 34 p.s.i. The curves showed no trend with increasing temperature. This phenomenon was previously observed with pyrene by Righezza and Guiochon[15], who noticed that when the solvent flow-rate was below 0.7 ml/min the response did not vary significantly with the gas flow-rate.

The effect of the evaporator temperature setting on the detector response was investigated for several methyl glycosides. We observed a similar behaviour towards increasing temperature for similar sugars (Fig. 3); neutral methyl glycosides such as methyl glucopyranoside and methyl galactopyranoside showed nearly a 50% decrease in response at elevated temperature, whereas methyl glycosides of deoxyhexoses such as fucose and rhamnose showed a rapid decline with a very small temperature increase. In contrast, amino sugar derivatives and mesoinositol showed a slight decrease. In previous studies with other solutes[16,17] the various decreases in detector response with increasing temperature were attributed to a decrease in particule size. Methyl fucopyranoside and methyl rhamnopyranoside are relatively volatile, hence the decrease in response at elevated temperature is greater because it is also due to partial volatilization; above 38°C these derivatives are hardly detectable. The noise level was

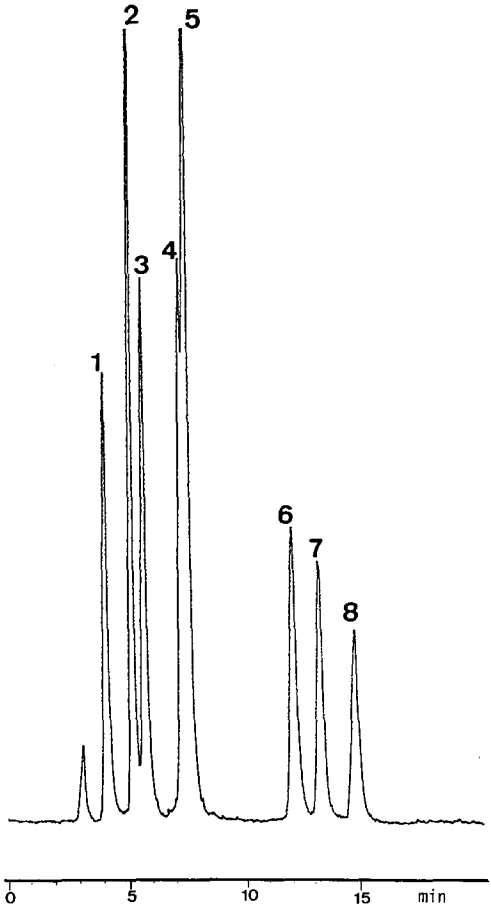

Fig. 2. Separation of a mixture containing standard methyl glycosides (conditions as in Fig. 1). Peaks: 1 = mesoinositol; 2 = methyl-α-D-galactopyranoside; 3 = methyl-α-D-glucopyranoside; 4 = methyl--β-D-xylopyranoside; 5 = methyl-α-D-mannopyranoside; 6 = methyl-α-D-N-acetylglucosamine; 7 = methyl-α-L-fucopyranoside; 8 = methyl-α-L-rhamnopyranoside.

found to be dependent on the evaporation temperature, so it was necessary to work above 28°C to allow an acceptable noise level. The temperature had to be low enough to avoid solute vaporization and high enough to ensure complete vaporization of the solvents; the optimum temperature for analysing the mixture of methyl glycosides was 30°C. As the latent heat of vaporization of water is very important[18], we chose a low gas velocity in order to permit complete vaporization of the solvent.

Detection limits, expressed as twice the baseline noise, were less than 30 ng for most methyl glycosides (methyl glucoside, methyl galactoside and methyl mannoside) and for mesoinositol, whereas for the other compounds they ranged from 50 to 100 ng. This method is at least as sensitive as some other methods using HPLC of methyl glycosides[13] or derivatized methyl glycosides[19].

The technique was applied to the determination of monosaccharides of two glycoproteins, α-GPA, with a high content of sugar (40%, w/w), and ovalbumin, which

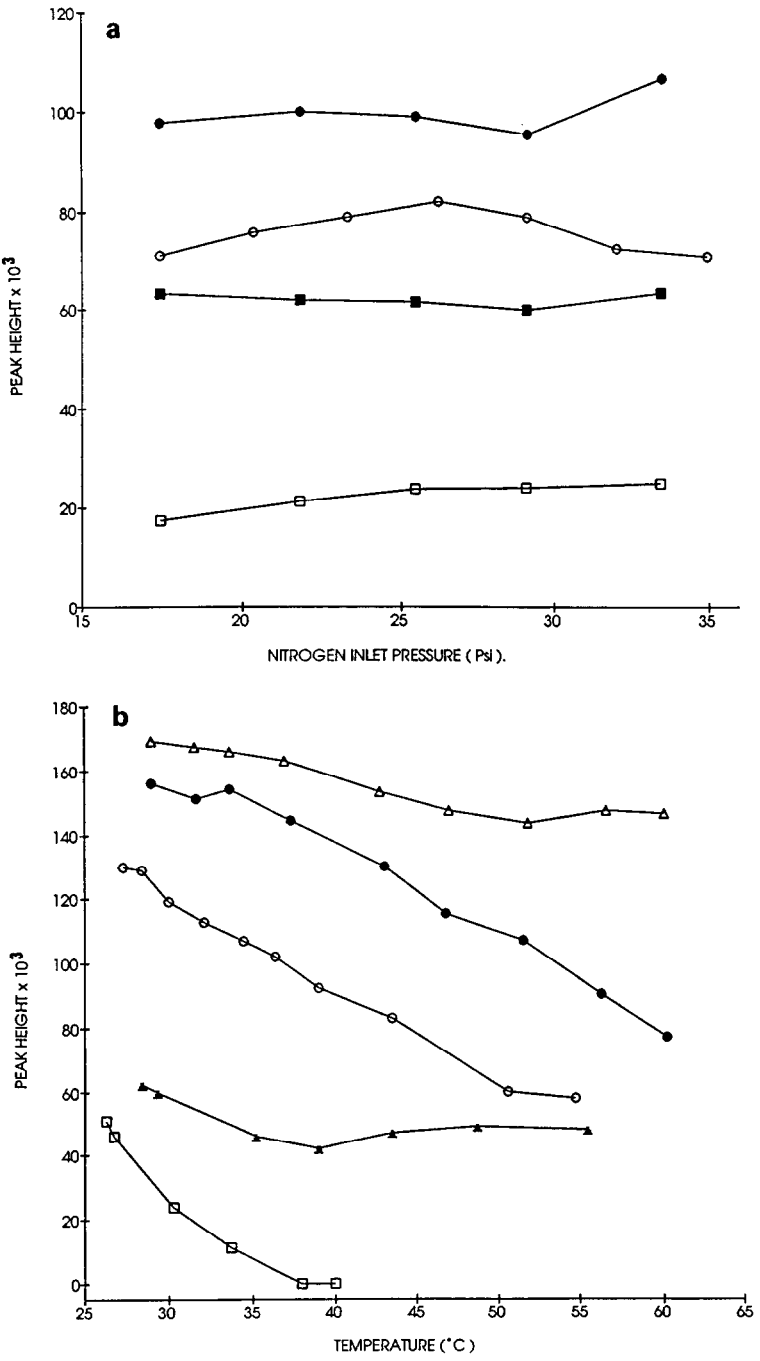

Fig. 3. Effect of (a) nitrogen inlet pressure and (b) temperature on the detector response for mesoinositol and several methyl glycosides. ● = Methyl-α-D-glucopyranoside; ○ = methyl-α-D-galactopyranoside; ■ = methyl-α-D-mannopyranosides;□ = methyl-α-L-fucopyranoside; ▲ = methyl-α-D-N-acetylglucosamine; △ = mesoinositol. Values are averages of three successive measurements for each temperature or each pressure in terms of peak height.

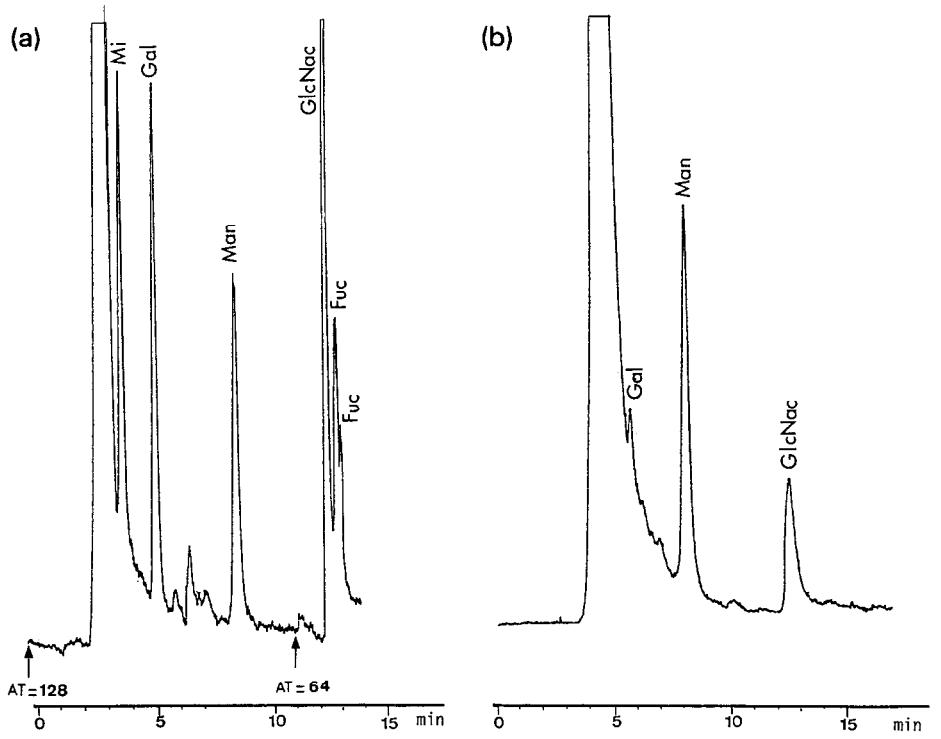

Fig. 4. Analyses of (a) α_1-acid glycoprotein and (b) ovalbumin. Conditions as in Fig. 1 with photomultipier at gain 10. AT = Attenuation.

contains less carbohydrate (5%, w/w). Two typical chromatograms are shown in Fig. 4. α-GPA contained mannose, galactose, N-acetylglucosamine and fucose, whereas methanolysis of ovalbumin gave rise mainly to mannose, some N-acetylglucosamine and traces of galactose. The method was also applied to the determination of the monosaccharide constituents of two polysaccharides. The chromatograms obtained from gum arabic and gum karaya are shown in Fig. 5.

Quantitative analysis was carried out. As expected from previous studies on other solutes[18,20], the calibration graphs obtained with four methyl glycosides showed a sigmoidal response of the detector in the concentration range studied (1–15 mg per 100 ml) (Fig. 6). Plots of peak height *versus* sample concentration in double logarithmic coordinates are linear (Fig. 7) with a similar slope for all the solutes of 1.277 ± 5.9%, indicating a similar mode of response. The regression data are given in Table II. The data obtained from three quantitative analysis of ovalbumin were in agreement with the literature values[4,8] as we obtained 23.8 and 21.5 μg per mg of glycoprotein for mannose and N-acetylglucosamine, respectively (with relative standard deviations less than 5%). Methanolysis of a non-glycosylated protein, bovine serum albumin, was performed and showed no interference in the sugar chromatogram.

The method described is very simple as it requires only a single-step metha-

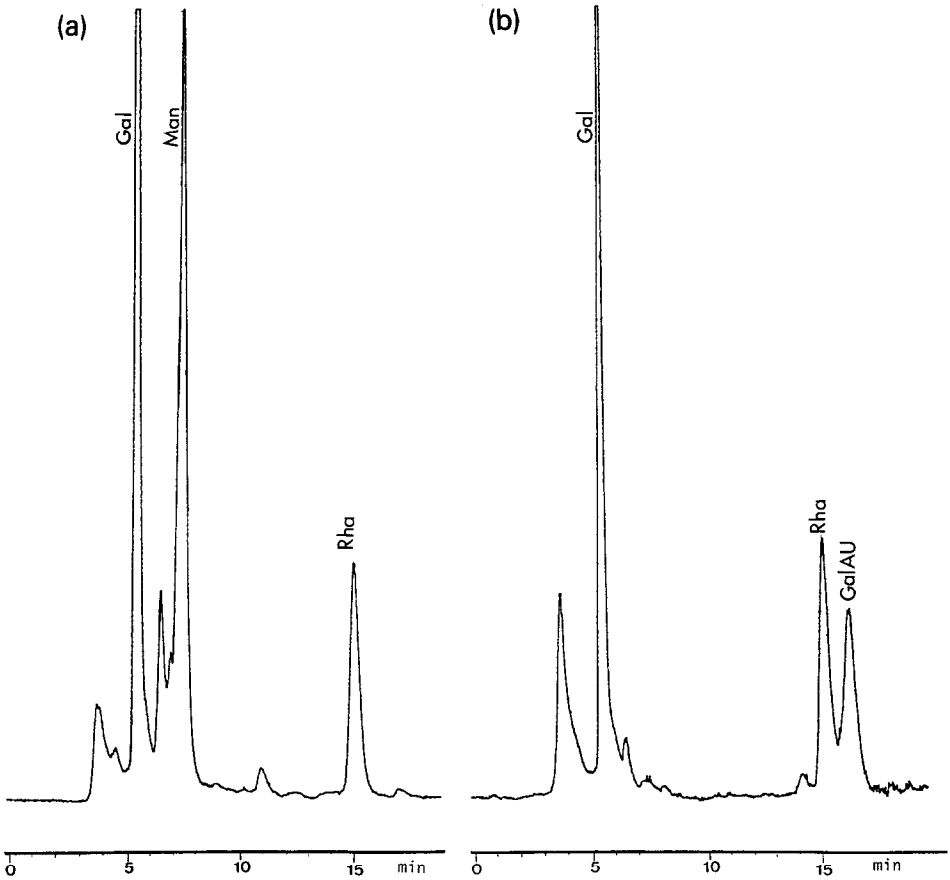

Fig. 5. Analyses of (a) gum karaya (b) gum arabic. Conditions as in Fig. 4. GalAU = galacturonic acid.

nolysis at 70°C followed by a chromatographic separation of the derivatives within 16 min. All carbohydrate moieties, including N-acetylamino sugars, contained in glycoproteins can be determined from a single hydrolysate. Moreover, the method can be applied to a wide range of glycoconjugates, including polysaccharides. This analysis

TABLE II

REGRESSION DATA FOR LOGARITHMIC PLOTS OF DETECTOR RESPONSE AGAINST CONCENTRATION OF METHYL GLYCOSIDES

Methyl glycoside	*Correlation coefficient*	*Slope*	*Intercept*
D-Galactose	0.997	1.273	4.54
D-Mannose	0.994	1.251	4.27
N-Acetyl-D-Glucosamine	0.997	1.203	4.08
L-Fucose	0.994	1.380	3.71

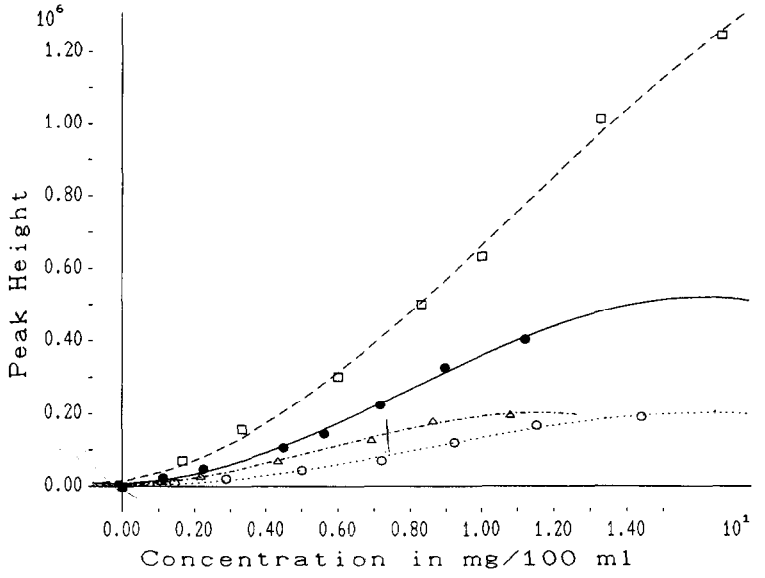

Fig. 6. Calibration graphs with the light-scattering detector for four methylglycosides. Values are means of three consecutives injections. □ = Methyl-α-D-galactopyranoside; ● = methyl-α-D-mannopyranoside; ○ = methyl-α-L-fucopyranoside; △ = methyl-α-D-N-acetylglucosamine.

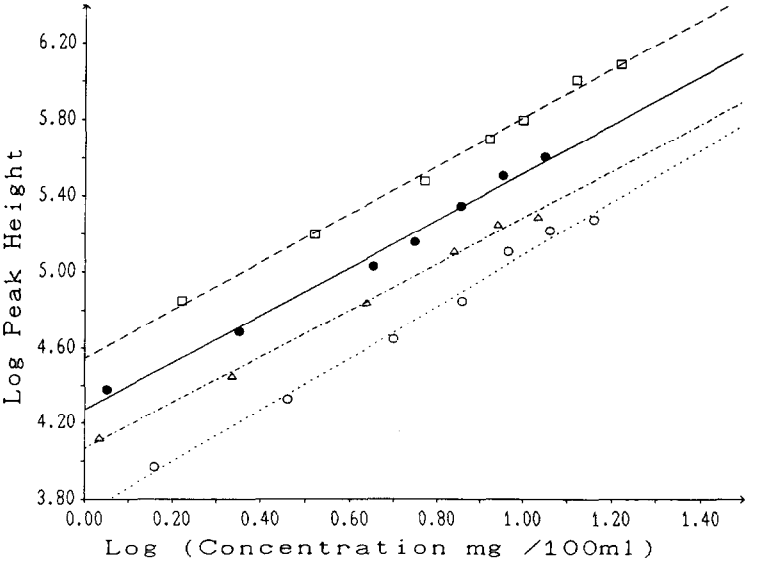

Fig. 7. Log–log plot of the light-scattering detector response against concentration of methyl glycoside. □ = Methyl-α-D-galactopyranoside; ● = methyl-α-D-mannopyranosides; ○ = methyl-α-L-fucopyranoside; △ = methyl-α-D-N-acetylglucosamine.

is interesting because of the main chromatographic peak obtained for most compounds. The linear response of the detector obtained in double logarithmic coordinates permitted quantitative analysis.

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