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High-performance liquid chromatographic determination of neutral and amino monosaccharides by ultraviolet and fluorescence detection of sugar 9-fluorenylmethoxycarbonyl hydrazones and 9-fluorenylmethoxycarbonyl amino sugars at picomole and sub-picomole levels

Renen Zhang*
Institute of Chemistry, Academia Sinica, Beijing 100080 (China)

Zhidan Zhang
Beijing Institute of Pharmaceutical Industries, Beijing (China)

Guoquan Liu
Institute of Chemistry, Academia Sinica, Beijing 100080 (China)

Yuji Hidaka and Yasutrugu Shimonishi
Institute for Protein Research, Osaka University, Osaka (Japan)

ABSTRACT

Two fluorescence labelling agents, 9-fluorenylmethoxycarbonyl (Fmoc) hydrazide and 9-fluorenylmethyl chloroformate, were used for the determination of neutral and amino monosaccharides, respectively, in glycoproteins. The derivatives were separated on a Cosmosil 5 C_{18} column by isocratic elution with acetonitrile–water (30:70) and determined by using fluorimetric and ultraviolet detectors. The detection limits of sugar Fmoc-hydrazones and Fmoc-amino sugars were 0.05–0.4 pmol with fluorimetric detection at 270 nm (excitation) and 320 nm (emission) and 1–3 pmol for ultraviolet detection at 263 nm. The hydrolysis conditions of glycoproteins were studied and sugar components of transferrin and fetuin were determined.

INTRODUCTION

The compositional determination of the oligosaccharide moieties of glycoproteins is of basic importance in structural studies of these compounds. A number of methods have been proposed for determining monosaccharides derived from glycoproteins, including gas chromatographic [1,2] and liquid chromatographic methods [3]. More recently, high-performance liquid chromatographic methods have been developed, involving either precolumn derivatiza-

* Corresponding author.

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tion to produce a chromophore or fluorophore [4–6] and no derivatization method [7].

We have previously reported a new fluorescence reagent, 9-fluorenylmethoxycarbonyl hydrazine (Fmoc-hydrazine), for labelling sugars [8]. However, the two important acetyl amino sugars, N-acetylgalactosamine (GalNAc) and N-acetylglucosamine (GlcNAc), have not been studied with this reagent. In this paper we describe the application of two fluorescence labelling reagents with identical fluorophores, Fmoc-hydrazine to derivative GalNAc and GlcNAc and 9-fluorenylmethyl chloroformate (Fmoc-Cl) to derivatize galactosamine (GalN) and glucosamine (GlcN), and the separation and determination of neutral and amino sugars commonly occurring in glycoproteins by reversed-phase isocratic HPLC.

EXPERIMENTAL

Apparatus and reagents

Two HPLC systems were employed: a Tosoh CCPE liquid chromatograph with a Tosoh UV-8081 spectrophotometer and a Hitachi F-1050 spectrofluorimeter, and a Hitachi 635-A liquid chromatograph with a Hitachi 635-10LC spectrofluorimeter. The sample valves were 5 and 10 μl, respectively.

Most separations were performed on reversed-phase columns: Cosmosil 5 C_{18} (150 × 4.6 mm I.D., particle size 5 μm), TSK gel C_{18} (250 × 2 mm I.D., particle size 5 μm) and Nova-Pak C_{18} (250 × 2 mm I.D., particle size 5 μm).

Fmoc-Cl, saccharide standards and glycoprotein standards were obtained from Sigma (St. Louis, MO, USA), hydrazine monohydrate from Tokyo Kasei (Tokyo, Japan), trifluoroacetic acid from Fisher Scientific (Fair Lawn, NJ, USA) and HPLC-grade acetonitrile from the Institute of Pharmacy, Academia Sinica (Shanghai, China). Redistilled, deionized water was used to prepare the mobile phases. Saccharide standards were dissolved in ethanol to give 0.2 m*M* solutions and stored at 4°C.

Synthesis of Fmoc-hydrazine and derivatization of GalNAc and GlcNAc (Fig. 1)

The synthesis of Fmoc-hydrazine and the preparation of its monosaccharide derivatives were performed as described previously [8]. The Fmoc-hydrazones of GalNAC and GlcNAC were prepared in a 20 × 13 mm I.D. Teflon tube with a screw-cap. To 10 μl of an ethanol solution containing 1 nmol of GalNAc or GlcNAc were added 110 μl of 0.1% acetic acid in ethanol and 100 μl of Fmoc-hydrazine in acetonitrile. The molar ratio of sugar to Fmoc-hydrazine was 1:20 in standard sugar samples and 1:200 in hydrolysis samples of glycoproteins. The mixture was reacted at 65°C in a water-bath and then cooled to room temperature. The derivatized sugars were diluted progressively with acetonitrile–water (50:50) and injected directly on to the column.

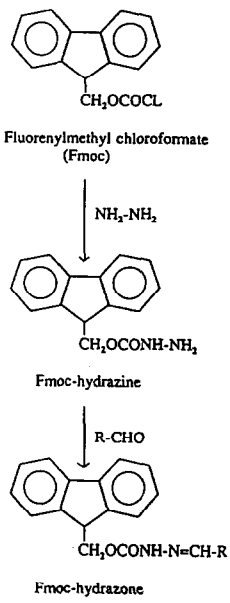

Fig. 1. Synthesis of Fmoc-hydrazine and its reaction with a carbonyl compound.

Hydrolysis of glycoproteins

Acid hydrolysis of proteins was carried out as follows. To each sample dissolved in 200 μl of water in a 20 × 13 mm I.D. screw-capped Teflon tube were added 200 μl of 4 *M* or 8 *M* TFA or 8 *M* HCl. The mixture was then heated on a boiling water-bath for 6 h. The tube was cooled and the contents dried with a stream of nitrogen. The hydrolysate was dissolved in 100 μl of ethanol and two aliquots were used for the derivatization of neutral and amino sugars.

HPLC conditions

Separations were performed on three C_{18} columns, Cosmosil, TSK gel and Nova-Pak, at ambient temperature by means of isocratic elution with 30% (v/v) aq. acetonitrile at a flow-rate of 1.0 ml/min for the Cosmosil column and 0.2 ml/min for the other two columns. The effects of the pH and composition of the mobile phase on the selectivity and efficiency of the Cosmosil 5 C_{18} column were determined by isocratic elution with either 30% (v/v) aq. acetonitrile, 30% (v/v) aq. acetonitrile containing 100 m*M* sodium acetate or 30% (v/v) aq. acetonitrile containing 100 m*M* acetic acid. The saccharide derivatives were determined using the fluorescence detector with excitation and emission at 270 and 320 nm, respectively. Ultraviolet detection at 263 nm was used for the determination of sugar derivatives.

RESULTS AND DISCUSSION

Derivatization of acetyl amino sugars

The reactivity of acetyl amino sugars with Fmoc-hydrazine is lower than that of other monosaccharides [8]. Hence the reaction time was extended to 6 h to obtain the maximum yield of derivatives (Fig. 2). This result indicated that the stability of sugar Fmoc-hydrazones was excellent during the reaction. This result is also different from that with Dns-hydrazine, used for labelling sugars [9], where the derivatization recovery decreased with extended reaction times after reaching the maximum yield.

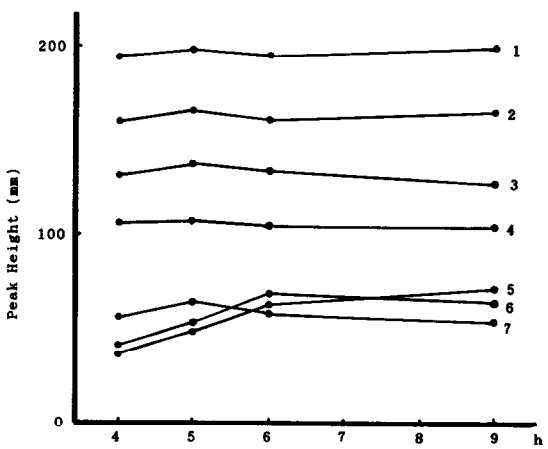

Fig. 2. Effect of reaction time on derivatization of sugar with Fmoc-hydrazine at 65°C. Chromatographic conditions: column, Cosmosil 5 C_{18} (150 × 4.6 mm I.D., particle size 5 μm); mobile phase, 30% (v/v) aq. acetonitrile; reaction medium, 0.1% acetic acid in ethanol. Molar ratio of saccharide to Fmoc-hydrazine = 1:20. Peaks: 1 = mannose (Man); 2 = glucose (Glc); 3 = xylose (Xyl); 4 = fucose (Fuc); 5 = GlcNAc; 6 = GalNAc; 7 = fructose (Fru).

Optimization of chromatographic conditions

The separation of monosaccharides that occur most commonly in proteins on the three C_{18} columns was investigated and the results are given in Table I. The capacities and elution orders of the sugar derivatives on the Cosmosil 5 C_{18} and TSK gel C_{18} columns are similar. However, on the Nova-Pak C_{18} column the k' values of sugar derivatives are relatively small, with that of galactose being the smallest. The selectivity of the sugar derivatives examined is better on the Cosmosil 5 C_{18} column than on the other two columns. On TSK gel C_{18} and Nova-Pak C_{18} columns Gal, Glc and Man–Gal sugar derivatives cannot be separated.

The effects of the acidity of the mobile phase on the selectivity of the Cosmosil 5 C_{18} column were examined. Fig. 3 shows that same elution order of sugar derivatives were obtained at pH 3–7 with the best selectivity at pH 6.5.

The effects of mobile phase composition on the column selectivity and efficiency are shown in Fig. 4. The selectivity of the sugar derivatives examined is better with acetonitrile–water (70:30) than the other two mobile phases. Fig. 4 shows the significant effect of the composition of the mobile phase on the column efficiency for sugar derivatives. The efficiencies of the Cosmosil 5 C_{18} column obtained with (C) 30% (v/v) aq. acetonitrile and (B) 30% (v/v) aq. acetonitrile containing 100 m*M* sodium acetate (pH 6.5) are similar and 2.5 times higher than when using (A) 30% (v/v) aq. acetonitrile containing 100 m*M* acetic acid; its efficiencies (*N*) [10] determined with the Xyl derivative were 9347, 9429 and 3627 with mobile phases A, B and C, respectively. The separation of the sugar derivatives on Cosmosil 5 C_{18} with 30% (v/v) aq. acetonitrile is shown in Fig. 5 and indicates that it is possible to obtain a good separation of sugar Fmoc-hydrazones on a reversed-phase column under isocratic conditions by selecting a suitable column and optimizing the composition of the mobile phase. Compared with gradient elution

TABLE I

CAPACITY FACTORS (k') AND SELECTIVITIES OF SUGAR DERIVATIVES ON VARIOUS C_{18} COLUMNS

Chromatographic conditions as in Fig. 5. The hold-up time (t_0) was determined by injecting a solution of potassium nitrate with UV detection at 254 nm.

Sugar	Cosmosil 5 C_{18}		TSK gel C_{18}		Nova-Pak C_{18}	
	k'	Selectivity	k'	Selectivity	k'	Selectivity
GalNAc	2.94		2.86		1.79	
		1.08		1.07		1.03
GlcNAc	3.17		3.07		1.85	
		1.03		1.10		1.04
Man	3.27		3.40		1.93	
		1.06		1.06		1.01
Gal	3.45		3.84		1.77	
		1.11		1.01		1.03
Glc	5.00		4.83		2.84	
		1.25		1.21		1.28
Xyl	6.28		6.09		3.64	
		1.27		1.28		
Fuc	8.07		7.77			

for the separation of monosaccharides, isocratic elution not only avoids the necessity for re-equilibration of the column for each subsequent sample injection but also improves the baseline stability, reproducibility and ease of operation. Our experiments showed that the reproducibility of the retention time of sugar derivatives was in the range 0.6–1.2% (R.S.D., $n = 25$).

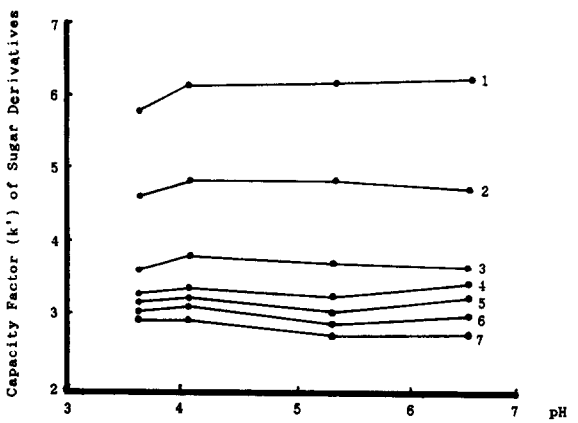

Fig. 3. Effect of pH of mobile phase on selectivity. Peaks: 1 = Xyl; 2 = Fru; 3 = Glc; 4 = Gal; 5 = Man; 6 = GlcNAc; 7 = GalNAc. Chromatographic conditions as in Fig. 2 except the mobile phase was 30% (v/v) aq. acetonitrile containing 100 mM sodium acetate.

Analysis of amino sugars

GalNAc and GlcNAc released from glycoproteins were de-N-acetylated to form the corresponding GalN and GlcN. The de-N-acetylation is quantitative on reaction with 2 M TFA at

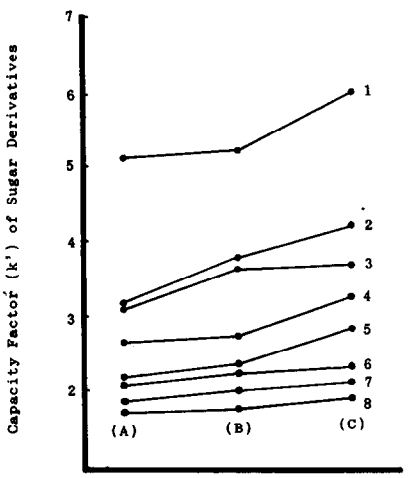

Fig. 4. Effect of the composition of the mobile phase on selectivity. Mobile phase: (A) 30% (v/v) aq. acetonitrile containing 100 mM acetic acid; (B) 30% (v/v) aq. acetonitrile containing 100 mM sodium acetate (pH 6.5); (C) 30% (v/v) aq. acetonitrile. Peaks: 1 = Xyl; 2 = Fru; 3 = Sor; 4 = Glc; 5 = Gal; 6 = Man; 7 = GlcNAc; 8 = GalNAc.

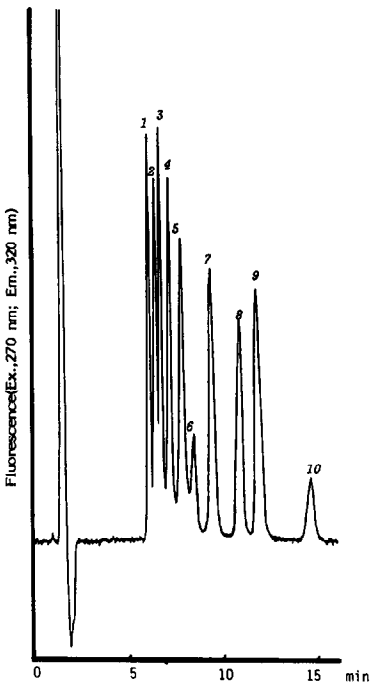

Fig. 5. RP-HPLC of sugar Fmoc-hydrazones. Chromatographic conditions: column, Cosmosil 5 C_{18} (150 × 4.6 mm I.D., particle size 5 μm); mobile phase, 30% (v/v) aq. acetonitrile; fluorescence detection. Peaks: 1 = GlaNAc; 2 = GlcNAc; 3 = Man; 4 = Gal; 5 = Glc; 6 = sorbose; 7 = Fru; 8 = ribose; 9 = Xyl; 10 = Fuc. Column efficiency (N) = 62 000 plates/m (Xyl).

100°C for 2 h [7]. This result indicates that the analysis of N-acetylated amino sugars of glycoproteins can be performed by determining amino sugars or re-N-acetylation followed by derivatization with Fmoc-hydrazine. However, the reactivity of GalN and GlcN with Fmoc-hydrazine was very poor, with low yields of hydrazones, under the conditions described. Re-N-acetylation of hexosamines was difficult to achieve quantitatively.

Fmoc-Cl has been used as a fluorescence labelling agent for amino acids [11] and polyamines [12]. It can react quantitatively with amino groups within a few minutes. The results show that both Fmoc-GalN and Fmoc-GlcN exist in two anomeric forms. Their capacity factors and separations are shown in Table II and Fig. 6. GalN(II) and GlcN(II) cannot be separated using the present method, but GalN(I) and GlcN(I) can be separated. The ratio of the

TABLE II

CAPACITY FACTORS (k') OF FMOC-AMINO SUGARS

Chromatographic conditions as in Fig. 5. The hold-up time (t_0) was determined as in Table I.

Sugar	k'
GalN(I)	1.55
GalN(II)	2.71
GlcN(I)	1.95
GlcN(II)	2.71

capacity factors of anomers can be used for identification of hexosamines.

Sensitivity and linearity of response

Sugar Fmoc-hydrazones and Fmoc-amino sugars possess intense fluorescence and ultraviolet absorption. The optimum wavelengths were 270 nm for excitation and 320 nm for emission, and 263 nm for ultraviolet adsorption. The detection limits of sugar Fmoc-hydrazones and Fmoc-amino sugars were determined with a signal-to-noise ratio of 3 (see Table III). Fig. 7 shows the separation of sugar Fmoc-hydrazones

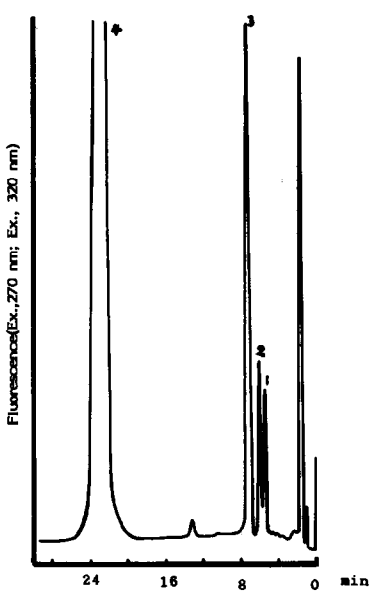

Fig. 6. RP-HPLC of Fmoc-amino sugars. Peaks: 1 = GalN(I), 2 = GlcN(I); 3 = GalN(II) + GlcN(II); 4 = hydrolysate of Fmoc-Cl. Chromatographic conditions as in Fig. 5.

TABLE III

DETERMINATION LIMITS OF SUGAR FMOC-HYDRAZONES AND FMOC-HEXOSAMINES

Chromatographic conditions as in Fig. 6.

Sugar	Determination limit (pmol)	
	Fluorescence detection (λ_{ex} 270 nm, λ_{em} 320 nm)	Ultraviolet detection (263 nm)
GalNAc	0.2	2
GlcNAc	0.2	2
Man	0.05	1
Gal	0.07	1
Glc	0.1	1
Fru	0.4	1
Xyl	0.1	1
Fuc	0.2	3
GlcN[a]	0.07	
GalN[a]	0.1	

[a] Fmoc-hexosamines.

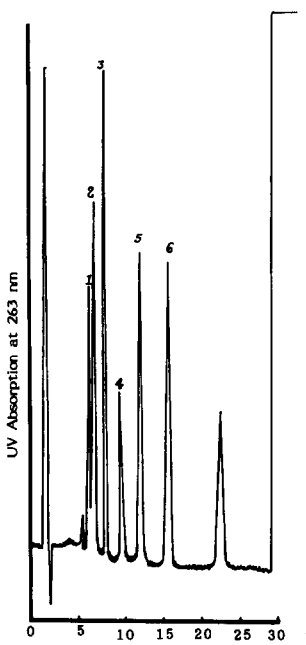

Fig. 7. Separation of sugar Fmoc-hydrazones. Peaks: 1 = GlNAc (50 pmol); 2 = Man (30 pmol); 3 = Glc (40 pmol); 4 = Fru (90 pmol); 5 = Xyl (80 pmol); 6 = Fuc (80 pmol). Chromatographic conditions as in Fig. 5, except ultraviolet detection at 263 nm.

in the picomole range with UV detection at 263 nm.

The linearity of the detector response was investigated by injection of progressively diluted samples of sugar Fmoc-hydrazones and Fmoc-amino sugars. The response was linear over the range 10–100 pmol. The relative responses of sugar Fmoc-hydrazones and Fmoc-amino sugars, normalized to GlcN in terms to peak height, are GlcN:Gal:Glc:Man = 1.00:0.98:0.69:0.48.

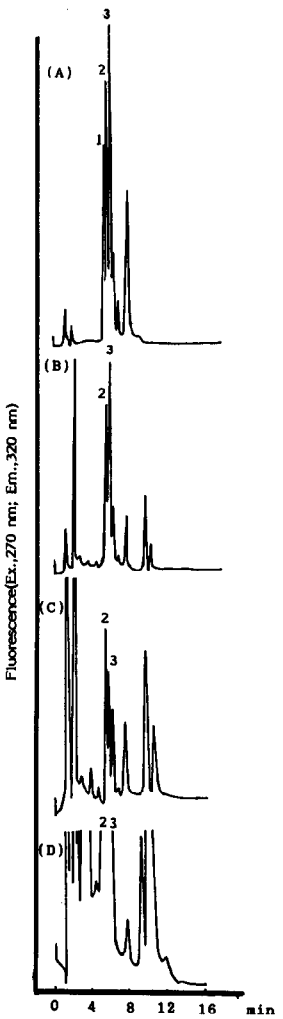

Fig. 8. Chromatograms of Man and Gal obtained from transferrin hydrolysate. Chromatograms: (A) Fmoc-hydrazones of GlcNAc, Man and Gal; (B) hydrolysate with 2 *M* TFA at 100°C for 6 h; (C) hydrolysate with 4 *M* TFA at 100°C for 6 h; (D) hydrolysate with 4 *M* HCl at 100°C for 6 h. Peaks: 1 = GlcNAc; 2 = Man; 3 = Gal. Chromatographic conditions as in Fig. 5.

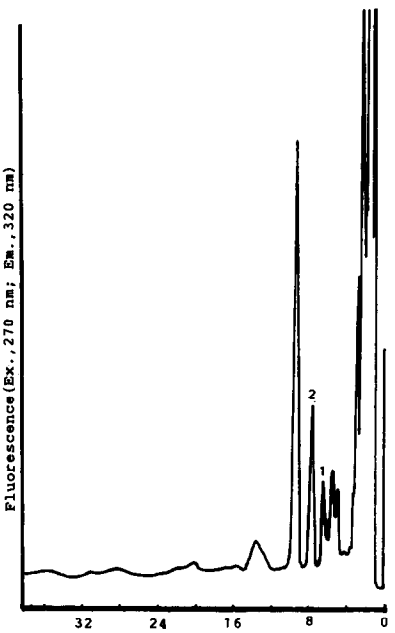

Fig. 9. Analysis of GlcN of transferrin. Sample amount injected, 57 ng protein. Hydrolysis conditions as in Fig. 8B. Chromatographic conditions as in Fig. 5. Peaks: 1 = GlcN(I); 2 = GlcN(II).

Analysis of the monosaccharide components of glycoproteins

Fig. 8 shows chromatograms for the standard samples of Glc, Man and Gal (Fig. 8A) and the hydrolysate of transferrin under different hydrolytic conditions. In Fig. 8B the peak of GlcNAc of the hydrolysate of transferrin disappears, which indicates that the de-N-acetylation of GlcNAc of the hydrolysate of transferrin is complete with 2 *M* TFA at 100°C for 6 h. The determination of the de-N-acetylated product of GlcNAc, GlcN, is shown in Fig. 9. The result indicates that the determination of GlcN is not affected by the presence of amino acids in the hydrolysate. Fig. 8C shows that the recovery of Gal is reduced by nearly half although the recovery of Man was not changed compared with the result in Fig. 8B. Fig. 8D shows that the peak positions of Gal and Glc were covered by the hydrolysate. These results indicate that the hydrolysis conditions used in Fig. 8C and D cannot be used for the determination of the monosaccharides of glycoproteins. The results of the determination of both neutral and amino sugar composition of fetuin and transferrin are shown in Table IV and the results with 2 *M* TFA at 100°C for 6 h agreed with the values reported previously [12,13].

CONCLUSIONS

Two fluorescence labelling reagents, Fmoc-hydrazine and Fmoc-Cl, can be used for the determination of neutral and amino sugars occurring most commonly in glycoproteins by means of reversed-phase isocratic HPLC with fluorescence and ultraviolet detection under identical hydrolysis and chromatographic conditions in the picomole and sub-picomole range.

TABLE IV

MONOSACCHARIDE COMPOSITION OF TRANSFERRIN AND FETUIN GLYCOPROTEINS

Sample	Saccharide	Reported value[a]	This study[a]		
			2 *M* TFA, 100°C, 6 h	4 *M* TFA, 100°C, 6 h	4 *M* HCl, 100°C, 6 h
Transferrin	Gal	1[b]	1	1	
	Man	1.26[b]	1.48	0.88	
	GlcNAc	1.70[b]	1.67		
Fetuin	Gal	1[c]	1	1	1
	Man	0.82[c]	0.88	1.5	0.4

[a] The results are given as molar ratios normalized to galactose set to 1.
[b] Ref. 13.
[c] Ref. 5.

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