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Determination of the concentrations of oligosaccharides, complex type carbohydrates, and glycoproteins using the phenol–sulfuric acid method †

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ABSTRACT

The concentrations of methyl glycosides, oligosaccharides, glycopeptides, and glycoproteins can be accurately determined by using calibration curves composed of the appropriate monosaccharide(s) obtained with a modified version of the colorimetric phenol–sulfuric acid method. Calibration curves of μg sugar vs. 490 nm for Man, Glc, or Gal are shown to provide reliable determinations (typically ±3–4%) of corresponding methyl glycosides and linear and branched-chain oligosaccharides containing the corresponding reactive hexose residue. For complex oligosaccharides containing a known mixture of reactive hexose units, the appropriate mixture of monosaccharides are shown to provide equally accurate calibration curves for concentration determinations. In the case of the soybean agglutinin, which is a tetramer possessing one Man9 oligomannose-type chain per subunit, the protein concentration was determined from the Man calibration curve which agreed with that obtained from the molar extinction coefficient of the protein.

INTRODUCTION

The phenol–sulfuric acid method described by Dubois et al.[1] has been used for some time as a colorimetric test for determining the concentrations of reducing pentoses and hexoses in solution, and, in conjunction with paper chromatography, the composition of oligosaccharides and polysaccharides. Different hexoses treated in this manner were shown to have different molar absorbance values at 490 nm. Oligosaccharides and polysaccharides were also shown to produce linear absorbance profiles of μg sugar vs. 490 nm which differed in their slopes and also from the slope of the corresponding free monosaccharides[1]. (This is due, in part, to

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differences in the unit mass of the free monosaccharide from its chemical form in these larger molecules.) Determination of the concentrations of different monosaccharides derived from the hydrolysis of polysaccharides, using paper chromatography and the phenol–sulfuric acid method[2] as well as other colorimetric methods, have been described (compare ref 3). Estimations of the so-called neutral sugar content of glycoproteins have also been made using the phenol–sulfuric acid method directly (compare ref 4). However, it has not been shown that the concentrations of oligosaccharides, in particular, those containing more than one kind of sugar residue, and those derived from glycoproteins can be accurately determined by any colorimetric method.

For several years now, we have been investigating the interactions of monosaccharides, oligosaccharides, and glycoproteins with lectins using a variety of biophysical and immunochemical techniques[5–10]. In the course of these studies it has been necessary to determine accurately the concentration of carbohydrates, especially in recent titration microcalorimetry experiments in which the concentration of the titrating saccharide must be known with high accuracy[11].

In the present study, we provide evidence that the concentration of methyl glycosides and linear and branched-chain oligosaccharides may be reliably determined by using a modification of the phenol–sulfuric acid technique together with appropriate calibration curves. We also demonstrate that the reactive hexose(s) concentration of a glycoprotein can also be accurately determined using this method.

EXPERIMENTAL

Materials and methods.—Analytical grade (96.4%) H_2SO_4 (Baker Analyzed) was obtained from the Fisher Chemical Company. A 5% aq phenol solution was prepared by adding 5 g of redistilled reagent-grade phenol (Aldrich Chemical Company) to 95 g of glass-distilled water. Soybean agglutinin (SBA) was purified according to Bhattacharyya et al.[12]. Protein concentration of SBA was determined spectrophotometrically using $A^{1\%,1\,\mathrm{cm}}$ as 12.8 at 280 nm and expressed in terms of subunit (M_r, 30000)[13]. Anhydrous forms of Glc, Man, Gal, and Fuc, methyl α-glucopyranoside, methyl α-mannopyranoside were obtained from Sigma Chemical Co. Man (anhydrous)[14] was obtained from Mann Research Laboratories, Inc. Maltose (monohydrate) (**5**) and maltotriose (monohydrate) (**6**) were obtained from Sigma Chemical Co. α-Man-(1 → 2)-Man (**2**), α-Man-(1 → 3)-Man (monohydrate) (**3**), α-Man-(1 → 6)-Man (monohydrate) (**4**), α-Man-(1 → 6)[α-Man-(1 → 3)]-Man (monohydrate) (**8**), mannopentaose (**10**), *N*-acetyllactosamine (monohydrate) (**1**), and GlcNAc-(1 → 2)-Man (**7**) (Fig. 1) were obtained from Dextra Laboratories Ltd, UK. Oligosaccharides **9** and **11** were obtained from Dr. Hans Lonn, Bio Carb, Sweden and Dr. Martin Haraldsson, University of Stockholm, respectively, as gifts. All of the sugars were of the D configuration except Fuc which was of the L configuration. The structures and purity of the oligosaccharides were determined

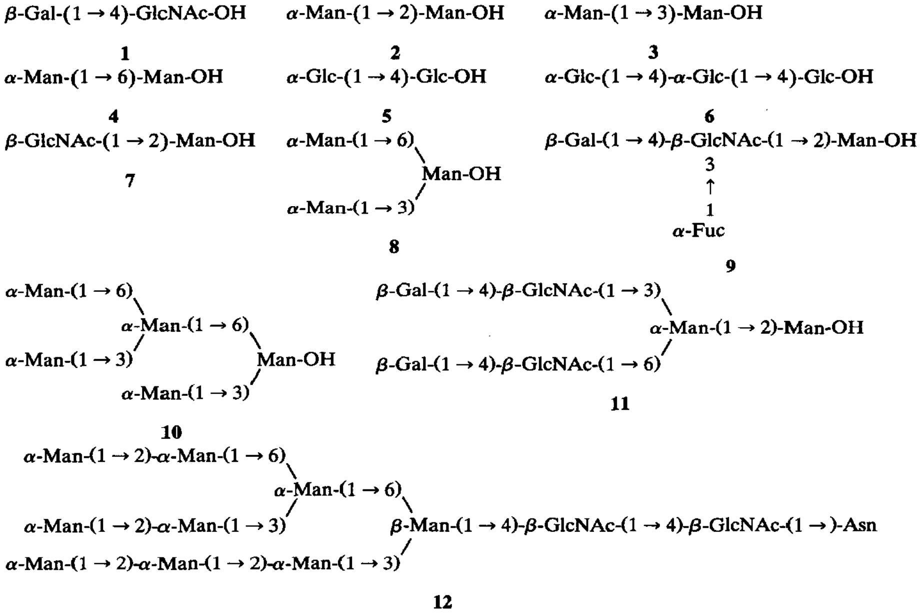

Fig. 1. Structures of oligosaccharides **1**–**11** and glycopeptide **12**. Man, Glc, Gal, Fuc, and Asn denote mannose, glucose, galactose, fucose, and asparagine, respectively.

by ^{1}H NMR at 500 MHz.

Colorimetric analysis.—The method is that described by Dubois et al.[1] with some modifications. To 0.5 mL of sugar solution (5–50 μg), 0.5 mL of 5% phenol solution was added and mixed. Then 2.5 mL concentrated H_2SO_4 was added directly into the solution within 1–2 s by means of a spring-loaded syringe (4 mL Veripet Manostat syringe, Teflon Tip, VWR Scientific). The mixture was then vortexed, and allowed to stand for 30 min at room temperature. Readings were taken at 490 nm against a blank prepared by substituting distilled water for the sugar solution. A Gilford model 084 spectrophotometer was used for the absorbance measurements at 490 nm.

RESULTS

The oligosaccharides used are depicted in Fig. 1. The gravimetrically determined amounts of mono- and oligo-saccharides used in the assays accounted for the presence of hydrated water in the compounds, where known[14]. Stock solutions of weighed carbohydrates were diluted to different volumes which were subjected to the phenol–sulfuric acid treatment. Comparisons between the amounts of free monosaccharides calculated in the diluted samples and the colorimetrically determined amounts are shown in Table I.

TABLE I

Comparisons (percentage error) of the gravimetrically and colorimetrically determined amounts of reactive hexose(s) in methyl glycosides, oligosaccharides and SBA using the phenol–sulfuric acid method [a]

Compound	Weighed amount free hexoses in each compound [b] (μg)	Wt calculated from calibration curve [c] (μg)	Error wrt weighed amount (%)	Average error (%)
Methyl α-D-glucopyranoside	40.0	39.5	1.2	1.8
	30.0	29.5	1.7	
	20.0	19.5	2.5	
Maltose (**5**)	46.0	44.5	3.3	3.0
	34.5	34.0	1.4	
	23.0	22.0	4.3	
Maltotriose (**6**)	43.8	43.0	1.8	3.1
	32.9	34.0	3.3	
	21.9	21.0	4.1	
Methyl α-D-mannopyranoside	38.9	38.5	1.0	2.3
	29.2	29.0	0.7	
	19.5	20.0	2.6	
	9.7	9.2	5.1	
(1 → 3)-Mannobiose (**3**)	42.0	41.0	2.4	3.1
	21.0	20.2	3.8	
(1 → 6)-Mannobiose (**4**)	42.5	42.0	2.3	3.5
	17.0	18.0	5.9	
(1 → 3)(1 → 6)-Mannotriose (**8**)	41.4	40.0	3.4	3.4
	31.0	30.2	2.6	
	20.7	19.7	4.8	
	10.3	10.0	2.9	
Mannopentaose (**10**)	42.6	41.0	3.7	1.9
	31.9	31.5	1.2	
	21.3	21.5	0.9	
(1 → 2)-Mannobiose (**2**)	40.0	33.0	17.5	18.1
	30.0	25.0	16.7	
	20.0	16.0	20.0	
SBA (**12**)	31.2	31.0	0.6	2.1
	25.0	25.5	2.0	
	18.7	18.0	3.7	
N-Acetyllactosamine (**1**)	18.8	17.7	5.8	4.6
	14.1	13.5	4.2	
	9.4	9.0	4.2	
	4.7	4.5	4.2	
Oligosaccharide **11**	25.5	25.0	2.0	2.7
	19.1	19.0	0.5	
	12.8	12.5	2.3	
	6.4	6.0	6.2	

TABLE I (continued)

Compound	Weighed amount free hexoses in each compound [b] (μg)	Wt calculated from calibration curve [c] (μg)	Error wrt weighed amount (%)	Average error (%)
Tetrasaccharide **9**	22.7	23.0	1.3	3.4
	15.1	14.5	4.0	
	7.6	8.0	5.0	
Disaccharide **7**	16.9	6.0	64.5	64.5
	12.7	4.5	64.5	

[a] For each carbohydrate, two to four dilutions of a stock solution of known concentration, prepared by weighing, were subjected to the phenol–sulfuric acid treatment. [b] The weight of reactive hexose(s) calculated in each compound. [c] Derived from the appropriate calibration curve for each compound.

The standard calibration curve for Glc is shown in Fig. 2. The reproducibility of the curve (and all such curves in the present study) is generally very good. The absorbance values for weighed amounts of maltose (**5**), maltotriose (**6**), and methyl α-glucopyranoside in terms of the amount of Glc in each compound (μg of Glc/3.5 mL) fit well on the standard curve. The absorbance values for weighed amounts of methyl α-mannopyranoside, 3,6-mannotriose (**8**), mannopentaose (**10**), (1 → 3)-mannobiose (**3**), and (1 → 6)-mannobiose (**4**) likewise fall on the standard curve of Man for the amount of Man in each compound (Fig. 3). The disaccharides (1 → 2)-mannobiose (**2**) and β-GlcNAc-(1 → 2)-Man (**7**) which are hygroscopic

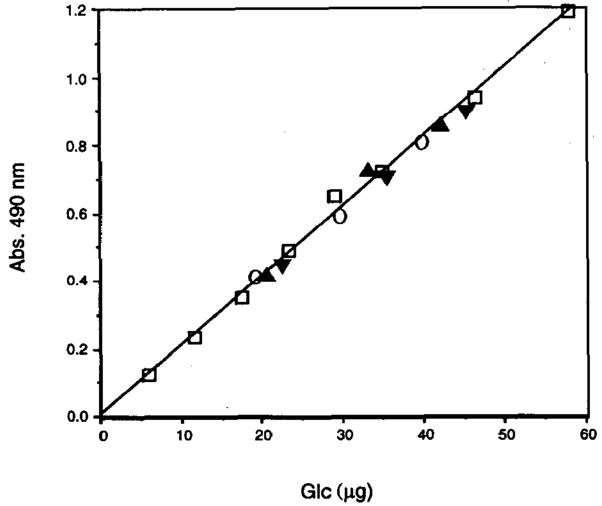

Fig. 2. Calibration curve for Glc (□). The absorbance values at 490 nm vs. calculated weight of Glc in **5** (▼), **6** (▲), and methyl α-glucopyranoside (○) at different dilutions of the respective stock solutions using the phenol–sulfuric acid method are shown.

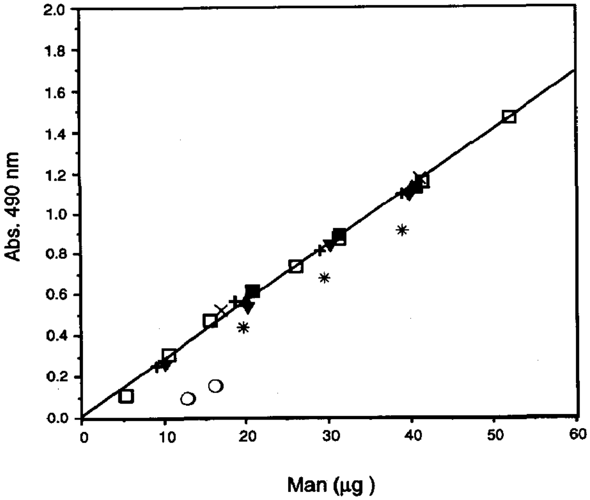

Fig. 3. Calibration curve for Man (□). Weighed amounts of the saccharides were converted into μg/3.5 mL Man. The absorbance values at 490 nm vs. calculated weights of Man in weighed amounts of **8** (▼), **10** (■), **3** (▲), **4** (×), methyl α-mannopyranoside (+), **2** (*), and **7** (○) at different dilutions of the respective stock solutions using the phenol–sulfuric acid method are shown.

show lower absorbance values on the standard curve of Man than predicted from their weighed amounts (Fig. 3).

SBA has four similar subunits with each subunit possessing one Man9-oligomannose type chain (**12**)[15,16]. The concentration of Man in the protein solution was calculated from the extinction coefficient of SBA and an excellent fit of absorbance of SBA solution at the calculated weight of Man is observed on the Man standard curve (Fig. 4).

The absorbance values of *N*-acetyllactosamine (**1**) fall on the standard curve of Gal when the weight of Gal present in the disaccharide is calculated (Fig. 5). GlcNAc in the concentration range tested did not give rise to a change in absorbance at 490 nm, which is a result known for 2-amino hexoses.

Complex oligosaccharide (**11**) has equimolar amounts of Gal and Man as reactive hexose units in the molecule. A standard curve consisting of equimolar amounts of Gal and Man is shown in Fig. 6. Conversion of the weight of **11** into the weight of a 1:1 mixture of Gal and Man and dilution of the stock solution of **11** to different amounts of the oligosaccharide produced absorbance values that fall on the standard curve (Fig. 6).

The absorbance values of diluted solutions of complex tetrasaccharide **9**, which contains Gal, Fuc, and Man in equimolar amounts, fall on a standard curve of an equimolar mixture of the monosaccharides after conversion of the weights of the complex oligosaccharide into those of the constituent neutral sugars (Fig. 7).

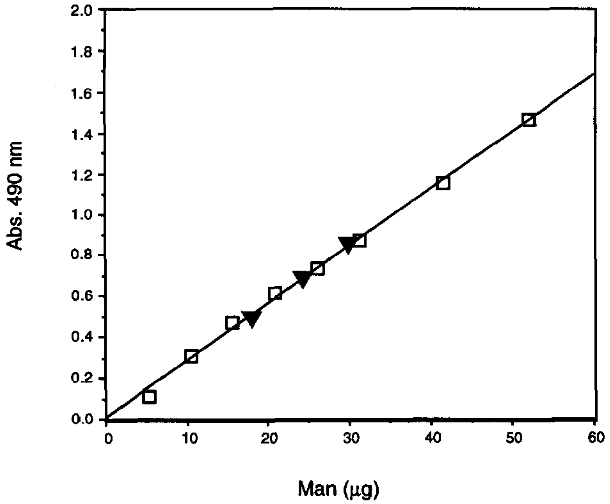

Fig. 4. Calibration curve for Man (□). Absorbances at 490 nm vs. calculated weights of Man in the soybean agglutinin (▼) at different dilutions of the glycoprotein using the phenol–sulfuric acid method are shown.

Table I shows the agreement between the phenol–sulfuric acid method vs. the gravimetric method for determining the concentrations of the glycosides, oligosaccharides, and glycoprotein in the present study. The method is generally accurate to ±3–4%, with a maximum average error of 5%.

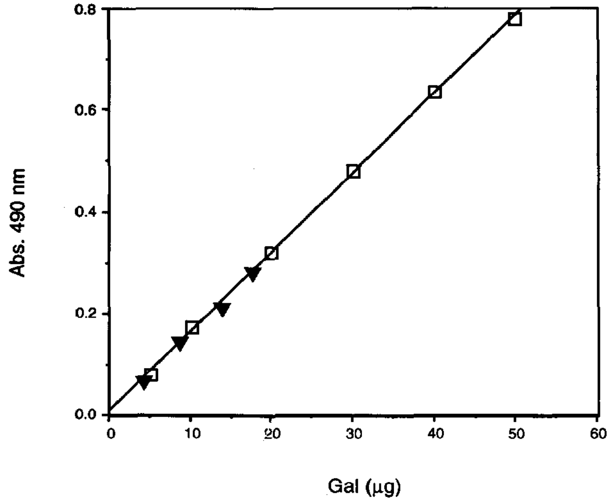

Fig. 5. Calibration curve for Gal (□). Absorbances at 490 nm vs. calculated weights of Gal in **1** (▼) at different dilutions of a stock solution using the phenol–sulfuric acid method are shown.

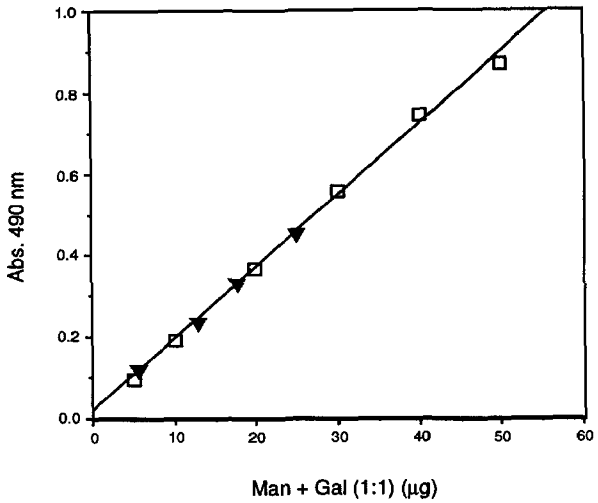

Fig. 6. Mixed calibration curve for Gal and Man in equimolar amounts (□). Absorbances at 490 nm vs. calculated weights of the 1:1 Gal-Man mixture in 11 (▼) at different dilutions of a stock solution of the oligosaccharide using the phenol–sulfuric acid method are shown.

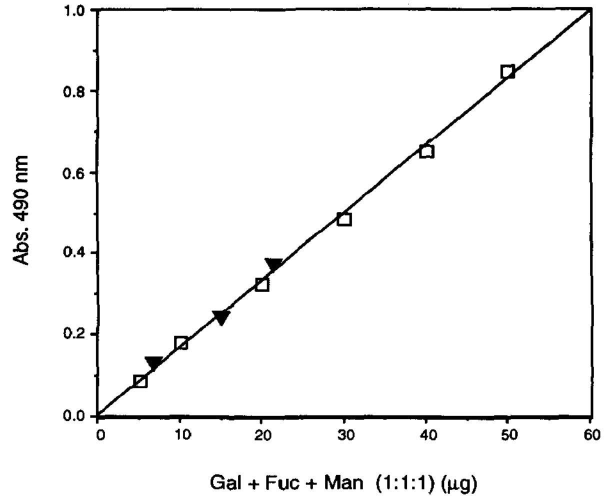

Fig. 7. Mixed calibration curve for Gal, Fuc, and Man in equimolar amounts (□). Absorbances at 490 nm versus calculated weights of equimolar mixtures of Gal-Fuc-Man in 9 (▼) at different dilutions of a stock solution of the tetrasaccharide using the phenol–sulfuric acid method are shown.

DISCUSSION

Upon rapid addition of concentrated sulfuric acid to aqueous phenol solutions containing glycosides, oligosaccharides, polysaccharides, and glycoproteins, an orange–yellow color is generated which is characteristic of the reactions of phenol with pentoses and hexoses in strongly acid medium[1]. In the original study, the concentrated sulfuric acid was delivered to the phenol solution from a pipet in 10–20 s. The present study shows that *rapid* injection (1–2 s) of concentrated sulfuric acid into aqueous phenol solutions generates enough heat under the high acidity conditions to completely hydrolyze the various glycosidic bonds of the carbohydrates in Fig. 1 and quantitatively convert the reactive hexoses into stable phenol adduct(s) which gives rise to absorbance at 490 nm. Thus, the method together with appropriate calibration curves can be used to determine the concentrations of mono- and oligo-saccharides as well as glycoproteins if the number of reactive hexose (and pentose) units are known in these molecules.

For example, methyl α-glucopyranoside and the α-(1 → 4)-Glc di- and tri-saccharides, maltose (**5**) and maltotriose (**6**), give one, two, and three moles of Glc, respectively, per mole of each carbohydrate. Determination of their absorbance values at 490 nm and conversion of the weight of each carbohydrate into the weight of Glc in each compound shows that they fall on the Glc standard curve (Fig. 2). The average errors are 1.8, 3.0, and 3.1%, respectively (Table I). This demonstrates that the reaction conditions are sufficiently strong to quantitatively convert the methyl glycoside as well as the di- and tri-saccharides with α-(1 → 4) glycosidic bonds into the corresponding phenol adduct(s) in each compound.

Similarily, methyl α-mannopyranoside gives one mole of Man and the Man α-(1 → 3) and α-(1 → 6) disaccharides (**3** and **4**) give two moles of Man, respectively. When the amounts of these oligosaccharides are converted to the amounts of constituent Man, the absorbances of the methyl glycoside and oligosaccharides fall on the standard curve for Man (Fig. 3). Furthermore, when trimannoside (**8**), which is present in all *N*-linked carbohydrates, and mannopentaose (**10**), which represents the *N*-linked Man5 oligomannose type carbohydrate moiety, are converted to three and five moles of constituent Man, respectively, the absorbances of the oligosaccharides fit well on the calibration curve of Man (Fig. 3). The errors range from 2–5% in comparing the weighed amounts with the colorimetrically determined amounts for the saccharides. This demonstrates that the reaction conditions are sufficiently strong to quantitatively convert the methyl glycoside as well as the di-, tri-, and penta-saccharides with α-(1 → 3) and α-(1 → 6) glycosidic bonds into the corresponding phenol adduct(s) in each compound.

Hygroscopic oligosaccharides such as α-Man-(1 → 2)-Man (**2**) and β-GlcNAc-(1 → 2)-Man (**7**) fall well below the Man standard curve (Fig. 3) which is consistent with the difficulties of weighing hygroscopic compounds in air. The amounts of these disaccharides in solution determined from the Man standard curve is 18.1 and 64.5% less than that obtained by weighing (Table I). This points out the necessity of applying a method such as the phenol–sulfuric acid technique for measuring the actual concentrations of saccharides such as these in solution.

The modified phenol–sulfuric method can also be used to determine the concentration of a glycoprotein in solution from a determination of its carbohydrate concentration. Thus, in the present work the 490 nm absorbance for SBA matches the Man standard curve when the weight of protein is converted into the weight of Man present as Man9 oligomannose type chains (**12**) (four chains per molecule) (Fig. 4)[15,16]. Thus, if the composition of the carbohydrate chains of a glycoprotein are known, the appropriate calibration curve can be used to determine its concentration. Further, if the concentration of the glycoprotein is known from its protein extinction coefficient, then a calibraton curve can be used to determine if a full complement of sugar chains is present, as in, for example, recombinant glycoproteins.

The present procedure also can be used to determine the concentration of oligosaccharides with both reactive and non-reactive hexoses. For example, *N*-acetyllactosamine (**1**) gives one mole of Gal which is reactive in the assay and one mole of GlcNAc which is not reactive. The results shown in Fig. 5 and Table I indicate that hydrolysis of the disaccharide occurs to give quantitative conversion to the Gal–phenol adduct(s). The error is 4.6% between the weighed amount and the colorimetrically determined amount of Gal present in **1**.

Complex-type oligosaccharides with two different reactive hexoses, such as **11**, may also be determined by the present method. Oligosaccharide **11** contains two moles of Gal and two moles of Man. A calibration curve prepared of equal amounts of Gal and Man shows that the absorbance values for **11** fall directly on the line after converting the oligosaccharide to the amounts of the two monosaccharides in the molecule (Fig. 6). Thus, the concentrations of oligosaccharides with two different reactive hexoses can be determined in this manner.

The concentrations of oligosaccharides with three different reactive hexoses including Fuc can similarily be determined. Tetrasaccharide **9**, which contains[17] the Lewis^x blood group antigen, β-Gal-(1 → 4)[α-Fuc-(1 → 3)]-GlcNAc, contains one mole each of Gal, Fuc, and Man as reactive sugars. Thus, when the amount of tetrasaccharide is converted to the total amount of the constituent reactive hexoses, the absorbance of the oligosaccharide falls at the calculated amount(s) on the standard curve, prepared using Gal, Fuc, and Man in equimolar amounts (Fig. 7).

It should be emphasized that in using the appropriate calibration curve for determining the concentration of a saccharide, the effective molecular weight of the oligosaccharide is the sum of the molecular weights of the monosaccharides in the molecule. For example, in the case of maltose (**5**) the effective molecular weight is $2 \times 180 = 360$ in determining its concentration from the Glc calibration curve. For maltotriose (**6**), the effective molecular weight is $3 \times 180 = 540$ in using the Glc calibration curve. For tetrasaccharide **9**, the effective molecular weight is 2×180 (Gal and Man) + 164 (Fuc) = 524 in using the Gal-Man-Fuc calibration curve. Thus, the number of μg/3.5 mL of sugar derived from the calibration curves is divided by the effective molecular weights of the respective saccharides to obtain their concentrations.

CONCLUSIONS

When the exact carbohydrate composition of a glycoside, oligosaccharide or glycoprotein is known, the presently described phenol-sulfuric acid method can be used to obtain accurate determinations of their concentrations using suitable standardization curves of the constituent monosaccharide(s). In addition to the periodate-resorcinol method for the quantitation of sialic acids[18], the present method can be used to estimate the concentrations of sialic acid containing oligosaccharides and glycopeptides since sialic acid residues do not give rise to a colored product. Instead, the reactive hexose (and pentose) residues in these compounds can be used to determine their concentrations. The present method is especially applicable for determination of the concentrations of hygroscopic saccharides which are difficult to accurately weigh. These results thus provide a simple technique for determining the concentrations of a variety of glycosides, oligosaccharides, and glycoconjugates in solution.

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