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Compositional analysis of glycoproteins

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1. Introduction

Once a protein has been purified to homogeneity (or radiochemical purity in the case of metabolically-labelled molecules), it is of interest to know if it is a glycoprotein. Several indirect methods can be used to detect the presence of sugars on the protein (e.g. lectin blotting, periodate oxidation/biotin hydrazide coupling, metabolic-labelling with radioactive monosaccharides). This chapter concerns itself with the next step: obtaining an accurate compositional analysis of a glycoprotein in terms of the molar ratios of the various monosaccharides present on it. In some cases, the techniques described in this chapter may serve as the first indication that oligosaccharides are indeed present on a protein. This chapter is primarily focussed on glycoproteins from animal sources, which tend to have a restricted number of types of monosaccharides: D-galactose, D-mannose, D-glucose, L-fucose, D-xylose, *N*-acetyl-D-glucosamine, and *N*-acetyl-D-galactosamine, uronic acids, and non-ulosonic acids (sialic acids).

2. Choice of techniques

A variety of techniques are presented in this chapter, which have varying degrees of sensitivity, specificity, and ease of performance. The choice of the techniques to be used in a given case often rests upon the most practical consideration: the amount of material available, and the portion that can be spared for carbohydrate analysis. The reader may follow the information in *Table 1*, and the algorithm presented in *Figure 1* to choose the techniques to be used. In general, it is recommended that the more labile components such as sialic acids and monosaccharide substitutions be analysed on separate aliquots from those used for the harsher methods for hexoses, hexosamines, and uronic acids. Multiple techniques may be needed to come to firm conclusions. Once this is done, plans can be made for more detailed structural analyses, such as those described in the following chapters.

Table 1. Choice of techniques to be used for compositional analysis of glycoproteins

	Amount of purified protein available for analysis		
Type of sugars	**> 10 nmol**	**1–10 nmol**	**< 1 nmol**
Total sugars	Phenol–sulfuric assay	GLC-FID GLC/MS	HPAEC/PAD
Sialic acids (total)	Ferric-orcinol assay	TBA assay Spectrophotometric	TBA assay HPLC
Types of sialic acids		Amino column HPLC or HPAEC/PAD	DMB derivatives/ HPLC
Fucose		GLC/FID GLC/MS	HPAEC/PAD
Hexoses	Phenol–sulfuric assay	GLC-FID GLC/MS	HPAEC/PAD
Hexosamines	MBTH assay	GLC-FID GLC/MS	HPAEC/PAD
Uronic acids	MHB assay	GLC-FID GLC/MS	HPAEC/PAD

This table assumes that the protein in question has about 5–10 moles of each monosaccharide per mole of protein, and that ~10% of the available material can be spared for compositional analysis. It also assumes that the instrumentation available is of average sensitivity and that assays will need to be done at least in duplicate.

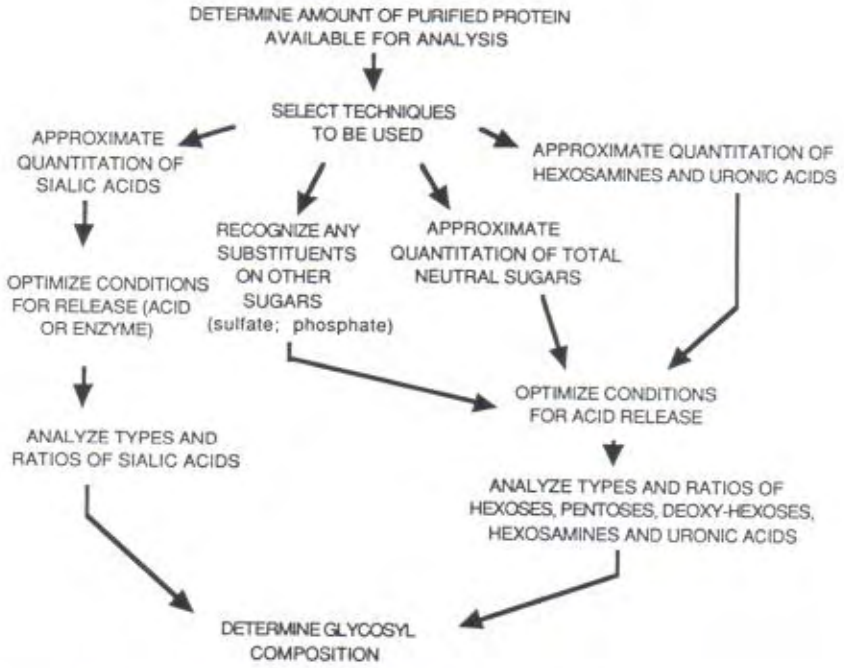

Figure 1. Algorithm for compositional analysis of glycoproteins.

3. Determination of total carbohydrate content by colorimetric methods

What is the approximate percentage of total sugars? What type of sugars are present? If sufficient material is available, an approximation of carbohydrate content can be obtained with a set of simple colormetric assays. Some reagents, and a spectrophotometer are the only materials required, and the protocols can be optimized in any laboratory to obtain good reproducibility. In all cases, it is advisable to run controls using buffers, protein, lipids, or other substances present or suspected to be present in the sample.

3.1 Analysis of native glycoproteins or glycopeptides

The main types of monosaccharides present in glycoproteins can be determined while they are still glycosidically bound. Such assays, although not very sensitive (lowest limit of detection ~5–10 nmol, 1–2 μg) overcome the problems arising from the different conditions required for complete release of glycosidic linkages (see Section 4.1).

A direct total sugar assay like Dubois's phenol–sulfuric acid assay (2) (see reference 1 for protocol) confirms the presence of glycosylation and gives an idea of the amount of glycoprotein required for glycosyl compositional analysis. When low values are obtained, the presence of high proportions of hexosamines or glycosyluronic acids (that do not react or give poor responses in the previous assay) can be directly checked using *Protocol 1* for amino sugars, or the methahydroxybiphenyl assay (MHB) for uronic acids (4) (see reference, 2 for protocol). When glycosyluronic acids are a small proportion of the total sugars, the accuracy of the latter can be improved by adding a small amount of sulfamate to the reaction (5). The determination of total bound sialic acids at this step (*Protocol 2*) is optional, since simple methods exist for more sensitive detection (*Protocol 4*).

The before-mentioned reactions are suggested because of their relative specificity. Others are available (1), and can be chosen according to personal preferences, provided their specificity, interferences, and differences in responses for different monosaccharides are considered.

3.1.1 Benzothiazolone hydrazone assay for bound amino sugars

Glucosamine and galactosamine are the only hexosamines detected as components of animal glycoproteins, generally occurring in the *N*-acetylated form. Most of the methods reported for the determination of hexosamines or *N*-acetylhexosamines are based on the procedures proposed by Elson and Morgan (see reference 1 for historical background). While several modifications have been developed, the formation of chromophore in every case requires

the free amino sugar (*N*-acetylated or not). However, quantitative liberation of amino sugars is difficult because of their extreme stability to strong acid conditions (see Section 4.1.2, *iii*). Thus, before quantitative hydrolysis is achieved, the rate of destruction of the sugar equals the rate of additional release.

Bound hexosamines, *N*-acetylated or not, can be determined after deamination by reacting with 3-methyl-2-benzothiazolinone hydrazone hydrochloride (MBTH) under mild acidic conditions (6). The method is highly specific for amino sugars. Complete cleavage of the glycosidic linkage is not required for the reaction with MBTH, and it is possible to use 0.5 M HCl to obtain complete de-*N*-acetylation. Glucosamine and galactosamine give identical colour yields.

Protocol 1. Direct benzothiazolone assay for detection of bound amino sugars

Materials

- Spectrophotometer and 1.0 ml cuvettes
- 13 × 100 mm Pyrex test-tubes with Teflon-lined screw cap
- water-bath (37 °C)
- HCl (A.C.S. reagent, Fisher Scientific A144SI-212)
- sodium nitrite (FW 69; Sigma S-2252)
- ammonium sulfamate (MW 114.12; A.C.S. reagent, Eastman–Kodak, 117 4531)
- 3-methyl-2-benzothiazolinone hydrazone hydrochloride (MW 215.70; 98%, Eastman–Kodak 119 8696)
- ferric chloride (FW 270.32; lump, certified A.C.S., Fisher Scientific 188–500)
- glucosamine hydrochloride (Sigma G-4875) or *N*-acetyl-D-glucosamine (Sigma A-8625).

Method

1. Prepare the reagents:
   - A—2.5% (w/v) sodium nitrate (2.5 g in 100 ml of water)
   - B—12.5% (w/v) ammonium sulfamate (6.25 g in 50 ml of water)
   - C—0.25% (w/v) MBTH[a] (125 mg in 500 ml of water)
   - D—0.5% (w/v) ferric chloride (0.83 g $FeCl_3.6H_2O$ in 100 ml water)

2. Prepare duplicates containing 5–30 nmol of standard, each sample, and a blank, in small glass test-tubes with Teflon-lined screw caps. Bring the total volume to 200 µl with a final HCl concentration of 0.5 M. Vortex.
3. Heat at 110 °C for 2 h.
4. Cool the samples to room temperature in a water-bath.
5. Add 400 µl of reagent A, in a fume hood, and vortex. Allow to stand at room temperature for 15 min.
6. Add 200 µl of reagent B, vortex, and stand at room temperature for 5 min.
7. Allow the nitrogen oxides (brownish fumes) to dissipate.
8. Add 200 µl of reagent C, and vortex. Incubate at 37 °C for 30 min.
9. Add 200 µl of reagent D, and incubate at 37 °C for an additional 5 min.
10. Cool the samples to room temperature and carefully transfer the solutions to cuvettes.
11. Read the absorbance at 650 nm, against the blank.
12. Prepare the standard curve (absorbance versus nanomol of hexosamine) using the average value for each concentration.
13. Determine the amount of hexosamine in the sample by reference to the standard curve.

[a] Must be prepared fresh weekly.

3.1.2 Ferric-orcinol assay for sialic acids

Glycosidically-bound and free sialic acids form a blue-purple chromophore, soluble in organic solvents, when heated with orcinol/Fe^{2+} (Bial reagent) in concentrated HCl (7). The assay is accurate and reproducible, and the minimum detection limit is about five nanomol (1.5 µg). Determination of free sialic acids is best achieved by the more sensitive TBA assay (see *Protocol 4*). Free or glycosidically bound hexoses, pentoses, and uronic acids can interfere in the assay. Therefore, an error must be expected when analysing biological materials that, purified or not, have a high content of these sugars. Nevertheless, this assay provides a simple way to verify the presence of sialic acids. A more accurate determination can be carried out later, after release and purification using *Protocols 6* or *7*. *N*-acetylneuraminic acid is used as a standard. However, it should be noted that the extinction coefficient is different for *N*-glycolylneuraminic acids. Any *O*-acylated sialic acids will have the same extinction coefficients as their parent molecules since, under the strong acidic conditions, all ester groups are rapidly hydrolysed.

Protocol 2. Direct ferric-orcinol assay for detection of total bound sialic acids

Materials

- spectrophotometer and 1.0 ml cuvettes
- heating block or boiling water-bath
- table-top centrifuge
- glass-stoppered, thick-walled, 16 × 125 mm Pyrex glass tubes
- hydrochloric acid (11.6 M; A.C.S. reagent, Fisher Scientific A144SI-212)
- ferric chloride (FW 270.32; lump, certified A.C.S., Fisher Scientific 188–500)
- orcinol (5-methylresorcinol, MW 124.16; Eastman–Kodak 113 7801)
- amyl alcohol (Mallinckrodt 2996)
- *N*-acetylneuraminic acid (MW 309.3; Boehringer–Mannheim 101–931)

Method

1. Prepare the orcinol reagent (Bial reagent) by dissolving 0.2 g of orcinol in 81.4 ml cooled, concentrated HCl. Add 2 ml of 1% (w/v) aqueous ferric chloride solution, and take to 100 ml with water. This reagent is stable for one week, stored at 4 °C.
2. Prepare a 1 mM (309 μg/ml) solution of standard Neu5Ac in water. This solution must be stored frozen.
3. Prepare duplicate tubes containing 5–40 nmol of Neu5Ac standard and the samples in 200 μl of water, as well as duplicate blanks.
4. Add 200 μl of Bial reagent, vortex, and cover with glass marbles.
5. Heat at 100 °C for 15 min.
6. Cool under tap water.
7. Add 1 ml of amyl alcohol. Vortex well and place on ice for 5 min.
8. Centrifuge (at 1000 *g*) for 3 min.
9. Transfer the upper phase to the spectrophotometer cuvettes, and read the absorbance at 570 nm, against the blank.
10. Prepare the standard curve (absorbance versus nanomol of Neu5Ac) using the average values of the standard solutions.
11. Determine the amount of sialic acid in the sample (expressed as Neu5Ac) by reference to the standard curve.

3.2 Colorimetric assays for released saccharides

3.2.1 Reducing sugar assay

The Mopper–Gindler assay (8) is the most sensitive method to date to measure free reducing terminii in sugars. Confident detection is achieved with less than three nanomol of mono-, oligo-, or polysaccharides. Buffering salts, sulfuric acid, sodium hydroxide, hydrochloric acid, and acetic acid do not interfere. It is also possible to work in the presence of borate ions if the pH is adjusted (9). The expected reducing sugar for the type of sample under analysis is used as standard. However, linear responses may not be obtained with increasing size of the oligosaccharide. Interference caused by proteins occurs at low level (about 1:50 w/w).

Protocol 3. Direct assay for reducing sugars (Mopper–Gindler)

Materials

- spectrophotometer and 1.0 ml cuvettes
- 16 × 115 mm Pyrex test-tubes
- dispenser
- heating block
- disodium 2,2′-bicinchoninate (4,4′-dicarboxy-2,2′-biquinoline, disodium salt; FW 388.3; Sigma D-8284)
- anhydrous sodium carbonate (FW 105.99; A.C.S. reagent, Fisher Scientific S-263)
- aspartic acid (FW 133.1; Sigma A-9256)[a]
- copper sulfate ($CuSO_4.5H_2O$ FW 249.6; A.C.S. reagent, Aldrich 20, 919–8)
- ethanol (100%)
- *N*-acetyl-D-glucosamine hydrochloride (see *Protocol 2*)

Method

1. Prepare the reagents:
   (a) A—dissolve 1.5 g of disodium 2,2′-bicinchoninate in 1 litre of water. Add 71.6 g of anhydrous sodium carbonate, while stirring. Bring up to 1.15 litres with water[b].
   (b) B—dissolve 3.5 g of aspartic acid, and 5.0 g of anydrous sodium carbonate in 100 ml of water. Shake to dissolve (produces foam). Dissolve 1.09 g of copper sulfate in 40 ml of water. Mix the two

Protocol 3. *Continued*

solutions, and bring up to 150 ml with water. Temporary fluffy blue precipitate may appear[c].

(c) C—mix 23 ml of reagent A and 1 ml of reagent B with 6 ml of 100% ethanol. Store in brown bottle. Stand for 2 h before use[d].

2. Prepare duplicates containing 2–25 nmol of standard, and each sample in 300 µl of water. prepare also a blank, and a control containing as much BSA as protein is expected in the samples.
3. Add 700 µl of reagent C to each tube, vortex, and cover with glass marbles.
4. Heat at 80 °C for 30 min. Cool to room temperature.
5. Transfer the solutions to cuvettes and read the absorbance at 560 nm, against the blank.
6. Prepare a standard curve (absorbance versus nanomol reducing sugar).
7. Determine the amount of reducing sugar in each sample by reference to the standard curve.

[a] Improvement of the sensitivity has been reported when aspartic acid is replaced by serine (9).
[b] The solution is stable for several months at room temperature if stored in a dark bottle.
[c] The solution is stable for several months at room temperature.
[d] The solution must be prepared every two weeks (or earlier if background colour increases).

3.2.2 TBA (2-thiobarbituric acid) assay for free sialic acids

The original assay described by Warren (10) and Aminoff (11) is based upon the formation of an adduct between TBA and the periodate oxidation product of free, unsubstituted sialic acids. The procedure has been modified over the years, to maximize sensitivity and specificity and to minimize interference. The most useful modifidations have been the following.

(a) Increase in the acid concentration in the arsenite reagent to avoid subsequent reaction failure.

(b) Prior de-*O*-acetylation to eliminate interference with release and reaction by *O*-acetylesters. An increase in colour yield after de-*O*-acetylation can be taken as indication of the presence of *O*-acetylation.

(c) Addition of BHT (butylated hydroxytoluene) during acid or enzyme release to minimize lipid peroxidation and formation of interfering malondialdehyde.

(d) Use of reverse-phase HPLC to separate away interfering chromophores and to improve sensitivity (12).

Prior de-*O*-acetylation prevents interference caused by *O*-acetylesters in the colour yield. For unknown reasons, the colour yield also varies according

to the substitution at the five position, in the approximate ratio of 1:0.8:2.0 for Neu5Ac:Neu5Gc:KDO (and presumably KDN). Rarer substitutions, e.g. *O*-methyl groups cause varying degrees of loss of reactivity in comparison to Neu5Ac. For working purposes, Neu5Ac is initially used as a standard. Once the approximate amount of sialic acid in the sample is known, more detailed analysis of the types of sialic acids can proceed (see Section 4.1.2, *ii* and 4.1.4).

Protocol 4. Thiobarbituric acid assay for sialic acids with spectrophotometric or HPLC detection

Materials

- spectrophotometer and 1.0 ml cuvettes
- sodium hydroxide (FW 40.00; anhydrous pellets, Sigma S-5881)
- concentrated sulfuric acid (A.C.S. reagent, Fisher Scientific A300S-500)
- sodium *meta*-periodate (FW 213.9; A.C.S. reagent, Sigma S-1147)
- butylated hydroxytoluene (BHT, 2,6-di-*tert*-butyl-4-hydroxyanisole, FW 220.4; Sigma B-1378).
- sodium arsenite (FW 129.0; 90%, Sigma S-7400)
- sodium sulfate (certified A.C.S. reagent, anhydrous, Fisher Scientific S-421)
- 2-thiobarbituric acid (4,6-dihydroxypyrimidine-2-thiol, FW 144.1; 98%, Sigma T-5500)
- cyclohexanone (Sigma C-8390), for spectrophotometric detection

For HPLC detection:

- versapack C18 HPLC column (250 × 4.1 mm, particle size, 10 μm), with a C18 guard column
- deionized water (passed through a five-stage Milli-Q Plus system, Millipore Corp.)
- sodium perchlorate (FW 122.44; 99%, Aldrich 20, 842–6)
- *ortho*-phosphoric acid (85%, A.C.S. reagent, Aldrich 21, 510–4)
- methanol (HPLC grade, Fisher Scientific A452–4)
- acetonitrile (HPLC grade, Fisher Scientific A998–4)

A. *Preparation of reagents*

1. Periodate reagent—dissolve 4.3 g of sodium *meta*-periodate in 4 ml water. Add 58 ml of concentrated *ortho*-phosphoric acid, and make up to 100 ml. Store at 4 °C in a dark bottle covered with foil (stable indefinitely).

Protocol 4. *Continued*

2. Arsenite reagent—dissolve 10.0 g of sodium arsenite and 7.1 g sodium sulfate in 0.2 M sulfuric acid and make up to 100 ml in the same acid. Store at room temperature (stable indefinitely).
3. TBA reagent—dissolve 1.2 g of 2-thiobarbituric acid and 14.2 g sodium sulfate in water (complete dissolution is obtained by dropwise addition of 1 M NaOH solution) and make up to 200 ml. Store at room temperature (stable almost indefinitely—even when a yellow precipitate appears, the reagent can still be used, avoiding the particulate material).

B. *De-O-acetylation of sialic acids (optional)*

1. Mix sample (40 µl) with 40 µl of 0.2 M NaOH in the bottom of a clean glass tube.
2. Incubate at 37 °C for 30 min.
3. For subsequent enzymatic release, neutralize with 20 µl of 0.2 M H_2SO_4 (check pH on a blank). Alternatively for acid release, acidify with 20 µl of 0.9 M H_2SO_4 (0.1 M excess acid final).

C. *Release of sialic acids*

1. Enzymatic release. Adjust buffer and treat with enzyme(s) as described in Section 4.1.4. An aliquot of the final reaction must be adjusted back to 0.1 M excess acid in 100 µl prior to proceeding further. Avoid formate containing buffers.
2. Acid release. Add one part per 100 of 1% BHT in ethanol. Heat at 80 °C for 1 h in 0.1 M excess acid (cover the tube with a clean glass marble to minimize evaporation).

D. *Formation of chromophore*

1. Cool sample to room temperature. Add 25 µl of periodate reagent carefully to the bottom of the tube. Avoid touching the sides of the tube. Vortex at low speed to mix. Leave at room temperature for 20 min.
2. Add 250 µl of arsenite reagent along the rim of the tube so that it flows down the sides of the tube. When a brown colour begins to develop, vortex vigorously (failure to neutralize all traces of periodate reagent will abrogate further reaction).
3. Leave for 5 min at room temperature (samples can be stored overnight at 4 °C without losses).
4. Add 1 ml of TBA reagent, mix well, and heat at 100 °C for 15 min.

E. *Detection of chromophore*

1. For spectrophotometric detection, add 1 ml of cyclohexanone (the

volume can be reduced depending upon the size of the cuvettes available), vortex well to extract the chromophore, and chill on ice for 2–3 min (colour fades). Centrifuge (at 1000 *g*) at room temperature for 5 min. Read the absorbance at 549 nm (sialic acid chromophore absorbance max) and at 532 nm (interfering chromophore absorbance max) against blank samples treated in identical fashion.

2. For HPLC detection, total volumes of sample and reagents can be decreased proportionately to further improve sensitivity. Prepare phosphoric acid–perchlorate buffer stock (2.35 ml of 85% phosphoric acid, and 28.1 g sodium perchlorate made up to 1 litre in HPLC grade water). Equilibrate a C18 HPLC column eluting at 1 ml/min of 2:3:5 (v/v/v) with water:MeOH:phosphoric acid–perchlorate buffer stock. Centrifuge the reaction mixtures (at 1000 *g*) for 5 min, and inject 10–400 µl of the supernant on to the column. The eluate is monitored for absorbance at 549 nm. The ratio of water to methanol may have to be adjusted for each particular C18 column to allow the chromophore peak to elute between 6–10 min, away from the breakthrough peak. The isocratic run must be continued until the interfering chromophore elutes completely (between 12–20 min). Between batches of runs, the column should be washed thoroughly with 50% CH_3CN or 50% MeOH.

F. *Calculation of results*

Run all samples in triplicate and/or at multiple concentrations, and compare to a set of standards handled under identical conditions.

1. Spectrophotometric detection (working range 1–10 nmol). Correct the value for A_{549} (sialic acid chromophore absorbance max) for overlap with the interfering chromophore A_{532} using the formula: $(0.9A_{549} - 0.3A_{532})$. Correct the standards in similar fashion. The correction becomes less accurate if A_{532} is greater than $0.7 \times A_{549}$, and the result practically meaningless if A_{532} is greater than $1.5 \times A_{549}$.
2. With HPLC detection (working range 10 pmol to 20 nmol) the interfering chromophore is completely separated. Relate the peak area of the early eluting peak to the amount of starting material in comparison to identically handled standards.

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4. Determination of the molar ratio of monosaccharides

Having confirmed the presence of carbohydrate in a glycoprotein, the next step is to obtain a precise molar ratio of its monosaccharide constituents. This information helps to predict the type of oligosaccharide present and in

determining the approach to be used for detailed structural characterization. In some cases, it can also provide the first clues to the presence of a new type of sugar chain. The analysis involves:

(a) Release of the individual monosaccharides.

(b) Analysis (fractionation and quantitation) of the mixtures of monosaccharides by:

   i. high performance anion exchange chromatography with pulsed amperometric detection (HPAEC-PAD), or

   ii. gas–liquid chromatography with flame ionization detection (GLC-FID).

(c) Identification of the individual monosaccharides by gas-liquid chromatography–mass spectrometry (GLC-MS).

With the GLC approach, a derivatization step is always required to produce volatile compounds for analysis. Most HPLC methods do not require derivatization. Many different methods have been developed for each of these approaches, each one with its own advantages and disadvantages. This chapter will focus on reliable methods that have been proven to be most widely applicable.

4.1 Release of monosaccharides

The way in which individual monosaccharides are released and processed depends on the methods available for their analysis and identification. If HPLC is going to be used for the characterization and quantitation of the monosaccharides, the glycoproteins need to be acid hydrolysed. Free glycoses can be separated using a pellicular resin anion exchange column and detected by pulsed amperometry (see *Protocol 14*).

When a GLC approach is taken, and the glycoprotein contains amino sugars and/or uronic acids, methanolysis followed by trimethylsilylation of hydroxyl and carboxyl groups is the method of choice. If the glycoprotein does not contain amino sugars or uronic acids, or only neutral sugars need to be measured, acid hydrolysis followed by reduction and peracetylation is preferred.

When an HPLC approach is taken, neutral sugars, hexosamines, and uronic acids can be directly analysed after strong acid hydrolysis. When sialic acids are involved, they have to be analysed separately, submitting an aliquot of the sample to mild acid conditions. Sialic acids are then purified and analysed without derivatization (*Protocols 15* or *16*), or after derivatization (*Protocol 18*) by HPLC, or eventually submitted to FAB-MS (13) to determine their composition. In some cases, enzymatic release of sialic acids (see Section 4.1.4) may be chosen to preserve the substitutions.

Of course, there are instances where ambiguous results will be obtained. If

a glycoprotein produces peaks with unexpected retention times (in HPLC or GLC) not comparable with any of the known standards, mass spectrometry has to be employed to identify these products.

The selection of methods also depends in the amount of carbohydrate available (see *Table 1*). When less than five micrograms of carbohydrates are processed, it is advisable to use silanized glassware to achieve maximum recovery. In every case, the use of extremely well cleaned glassware is recommended (see *Protocol 5*).

4.1.1 Problems with the quantitative estimation when different types of monosaccharides are present

A precise quantitative ratio of the monosaccharides in a glycoprotein is very difficult to obtain. The problem arises from the fact that all glycosidic bonds have to be split, while avoiding destruction of the liberated monosaccharides. The susceptibility to acid hydrolysis, as well as the stability of the released unit, is different for each monosaccharide; pyranosides and furanosides hydrolyse at different rates; the presence of substituents affect the rate of hydrolysis; α and β anomers have different rates of hydrolysis, and so on. Therefore, the perfect protocol does not exist, and optimum conditions have to be experimentally determined for each sample. The starting point can be based on information available for closely related glycoproteins. In some cases, more than one set of conditions may be required to achieve a complete analysis.

Conditions required for a successful release of all the different monosaccharides in a glycoprotein vary to a great extent when hydrochloric or sulfuric acids are used. However, the range required for trifluoroacetic acid (TFA) is considerably narrower. Apart from the expected destruction of sialic acids, all other sugars including fucose, hexosamines, and uronic acids can be confidently determined after treatment with TFA under optimized conditions. TFA also has the advantage of being easily eliminated by evaporation without leaving any residue behind. Mild acid hydrolysis using acetic acid and/or enzymatic release is recommended for sialic acids (see Sections 4.1.2 *ii*, and 4.1.4).

Alternatively, methanolysis followed by derivatization and GLC can be used. All monosaccharides are stable to methanolysis, including sialic acids. However, sialic acids present further problems arising from the difficulty in their derivatization, and destruction of labile substituents during the release step (see Section 4.1.3).

It has to be kept in mind that, even when treating the standards in parallel they may not resemble the decomposition suffered by the glycosidically bound monosaccharides in the sample since in the latter case they spend less time as free glycoses. Therefore, a method that yields less decomposition is always preferred over using a correction factor.

4.1.2 Procedures based on acid catalysed hydrolysis

The different conditions used for the release of monosaccharides from glycoproteins, including a detailed analysis of the mechanisms of hydrolysis by acid of glycosides, have been reviewed (14). Hydrolysis procedures used prior to analysis by GC-MS were also reviewed by Kamerling and Vliegenthart (15). Destruction arising from the interaction between liberated sugars and amino acids can be minimized by using low concentrations of glycoprotein during the hydrolysis (< 1 mg/ml), and by excluding oxygen (displacing it with dry nitrogen or hydrolysing in a tube sealed under vacuum). A competing sugar, not present in the glycoprotein can also be added to reduce destruction (ribose has been used for this purpose). The use of high quality acids is important. Highly purified, deionized water should be used to dilute the acid since any trace of iron could lead to destruction of hexosamines.

i. Mild acid hydrolysis for release of fucose

Fucosyl residues are typically in non-reducing terminal positions, and are susceptible to acid hydrolysis under relatively mild conditions. However, specific sites of attachment can increase the stability of the glycosidic bond (e.g. a positively charged amino group obtained by de-*N*-acetylation of a *N*-acetylhexosamine residue will lead to a stable glycosidic linkage at the 3-position). Thus, it is necessary to determine for each specific glycoprotein the conditions required to obtain complete liberation with minimal destruction.

Most of the problems encountered when trying to determine the best hydrolysis conditions for fucose in a given glycoconjugate have been reviewed by Gottschalk (14). The protocol suggested here will cleave the glycosidic linkage of all terminal, non-reducing, fucosyl residues. The release will be complete at different times for different glycoconjugates. To obtain a total monosaccharide composition, including fucose, *Protocol 8* can be used.

Protocol 5. Procedure for the quantitative release of terminal non-reducing fucose

Materials

- small glass tubes or 3.5 ml glass vials with Teflon-lined screw caps
- heating block or oven
- nitrogen or vacuum evaporation system (Speed-Vac or shaker-evaporator)
- HCl (A.C.S. reagent, Fisher Scientific A144SI-212)
- methanol (anhydrous, 99%, Aldrich 32, 241–5)
- L-fucose (MW 164.2; Sigma F-2252)

Method

1. Prepare 0.05 M HCl in water.
2. Prepare a 10 mM solution of L-fucose in water. Use large volumes to minimize weighing errors.
3. Dry down three aliquots of the sample (containing at least 10 nmol of carbohydrates) in glass tubes or vials[a]. Dry down three 10 µl (10 nmol) aliquots of the aqueous solution of L-fucose.
4. Dissolve samples and standards in 0.05 M HCl (0.4 ml/mg of material), flush nitrogen, and cap the tubes. Heat at 100 °C for 2h, 4h, and 8h[b].
5. Evaporate the solution with a stream of nitrogen or under vacuum. Wash the residue twice with methanol followed by evaporation.
6. Store the dry hydrolysates in the freezer until they are analysed using *Protocol 14*, or derivatized (*Protocol 12*) and analysed using *Protocol 17*.

[a] Always use brand new pre-cleaned glass vials or clean the used ones by heating first in concentrated nitric acid and then in 6 M HCl at 50 °C for at least 3 h each. Rinse thoroughly with water, and finally with ethanol, and dry in an oven. When less than 5 µg of total carbohydrates are analysed, it is recommended to silanize the glass vials by incubating for 15 min at room temperature with a 2% solution of dichlorodimethylsilane in toluene. Decant the solution, and rinse successively with methanol, and hot distilled water, and allow to dry.

[b] When glass vials are used, nitrogen can be introduced through a needle inserted in the septum, and evacuated through a second needle. When a heating block is used, care must be taken that the transfer of heat to the tubes is adequate. This can be achieved by preparing a sand or metallic bath or by filling the wells with oil. It is also important that most of the tube or vial is covered (excluding the cap) to avoid distillation of the solvent on the upper part. When oil is used, contamination must be avoided.

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ii. Mild acid hydrolysis for release of sialic acids

More than 25 different kinds of modified sialic acids have now been reported in nature. Most of these arise from substitution of the parent molecule with a variety of different groups. These modifications have been shown to affect a wide spectrum of biological phenomena (16). Many studies of the sialic acids have failed to recognize the complexity of this family because conventional methods for the analysis of glyconjugates, such as methanolysis, hydrazinolysis, methylation analysis, and β-elimination result in the destruction of *O*-substituted species, especially *O*-acetylesters. The classical method for release of sialic acids from glycoconjugates (0.1 M H_2SO_4 at 80 °C for one hour) also results in extensive destruction of the *O*-acetyl groups. In addition, the presence of *O*-acetyl groups make the sialic acid molecule partially or completely resistant to release by all available bacterial and viral neuraminidases (see Section 4.1.4). Migration of *O*-acetyl groups from the seven or eight positions to the thermodynamically more stable nine position of the sialic acid exocyclic side chain can also occur, with $T_{\frac{1}{2}}$ ranging from minutes to hours depending on the pH and temperature.

The use of milder conditions (0.5 M HCOOH at 80 °C for one hour) allowed the release and identification of many previously undetected *O*-acetylated sialic acids. However, quantitative analysis was not possible due to significant destruction and incomplete release of *O*-acetylated species using formic acid (pH 2.1) for acid hydrolysis. Prolonged hydrolysis in 2 M acetic acid (pH 2.4–2.5) at 80 °C was found to achieve maximal release of *O*-acetylated sialic acids with minimal loss of *O*-acetyl groups (17). Hydrolysis times vary for different glycoproteins. Subsequent handling of the sialic acids hydrolysate was also designed to avoid migration or loss of *O*-acetyl groups (17). These methods have been successfully used to release and purify labile substituted sialic acids from different biological sources (13).

All the parameters (temperature, pH, concentrations) must be carefully controlled. pH values outside the range of 3.0–7.0 should be avoided. Strong basic ion exchange resins, (e.g. Dowex 1 or 2) must also be avoided. Samples must be freshly obtained, and be processed and analysed immediately. It is also critical that purified samples are kept dry at the lowest possible temperature (−70 °C, −80 °C) whenever a waiting period is required. However, even storage at very low temperature (−70 °C) for long enough time could result in the migration or loss of *O*-acetyl groups.

Careful use of *Protocol 6* will release, and conserve during the purification, most substituted sialic acids if they are present in the starting material. The only known exception will be the 4-*O*-acetylated species in which case *Protocol 7* should be used. Sialic acids obtained by these procedures can be analysed by HPAEC-PAD (*Protocol 15*), HPLC (*Protocol 16*), or direct

Table 2. Commercially available standards with sialic acids of different types and linkages

Standard glyconjugate	Type of molecule	Type(s) of linkages	Types of sialic acids	Approx. sialic acid content (nmol/mg)
Sialylactose (mixed isomers)	Oligosaccharide	α2→3 α2→6	Neu5Ac	1600
Fetuin	Glycoprotein (*N*- and *O*-linked)	α2→3 α2→6	Neu5Ac Neu5Gc(?)	280
Bovine Submaxillary Mucin	Mucin (*O*-linked)	α2→6	mono- and di-*O*-acetylated Neu5Ac and Neu5Gc	150–400[a]
Colominic acid	Polysaccharide	α2→8	Neu5Ac	3400
Mixed brain gangliosides	Glycolipid	α2→3 α2→8	Neu5Ac[b]	950

[a] Depending on the manufacturer.
[b] Commercial preparations of bovine brain gangliosides have been treated with base during purification, resulting in loss of *O*-acetylesters.

FAB-MS (13). The protocol described in this section can be used to obtain a mixture of Neu5Ac, Neu5Gc, some of their mono-, di-, and tri-*O*-acetylated derivatives from commercially available standards (see *Table 2*). This mixture of standards will become very useful when analysing the presence of sialic acid modifications in a glycoprotein. When the total content of sialic acid in a given sample is under one nanomole, it is still possible to determine the sialic acid composition by incorporating a fluorogenic tag. This method is described in *Protocols 13* and *18*. It should be pointed out that optimal methods for the release and purification of the rare forms of sialic acids, (e.g. *O*-methylated, *O*-sulfated, multiply modified) have not been adequately worked out.

Protocol 6. Procedure for the quantitative release and purification of sialic acids (excluding 4-*O* acetylated species)

Materials

- glass tubes with Teflon-lined screw caps
- heating block or oven
- lyophilizer, or shaker-evaporator
- small (0.5 × 10 cm) glass columns (can be replaced by glass wool fitted Pasteur pipettes)
- stirring plate and stirring bars
- dialysis tubing (1000 cut-off)
- Dowex 50 AG 1X8 (100–200 mesh, hydrogen form), Bio-Rad
- Dowex AG 3X4A (100–200 mesh, hydroxyl form), Bio-Rad
- sodium formate (certified A.C.S., Fisher S648–500)
- formic acid (certified A.C.S., Fisher A1188–4)
- acetic acid (HPLC grade, Fisher A35–500)
- butylated hydroxytoluene (BHT, 2,6-di-*tert*-butyl-4-hydroxyanisole, FW 220.4; Sigma B-1378)

A. *Preparation of the sample*

Crude biological samples should be thoroughly homogenized and dialysed overnight at 4 °C against a 100-fold excess of 10 mM sodium formate buffer, pH 5.5 (12 000 MW cut-off tubing). Dialysis is not necessary for relatively purified biological samples that do not contain low molecular weight contaminants. Store all the samples lyophilized, in the freezer[a].

B. *Determination of optimal conditions for release*

1. Determine the total sialic acid content of the material by performing the

Protocol 6. *Continued*

TBA assay after de-*O*-acetylation and hydrolysis in 0.1 M H_2SO_4 at 80 °C for 1 h (*Protocol 4*).

2. Transfer three aliquots of the sample containing ~10 nmol of sialic acids each to 5 ml glass tubes.
3. Add 10 M acetic acid for a final concentration of 2 M.
4. Add one part per 100 of 1% BHT in ethanol[b].
5. Heat at 80 °C for 3 h, 4 h, and 5 h, respectively (see footnote b, *Protocol 5*).
6. Lyophilize the hydrolysates, subject them to de-*O*-acetylation, and determine the total free sialic acid in each one by the TBA assay (*Protocol 4*). Compare the numbers obtained to determine the time required for maximal release of sialic acids.

C. *Release and purification of sialic acids*

1. Hydrolyse the lyophilized sample in 2 M acetic acid at 80 °C for the time determined in part *B*. Cool to room temperature.
2. Transfer the reaction mixture into dialysis tubing. Wash the hydrolysis tube twice with water and transfer the washings into the dialysis tubing. A MW cut-off of 1000 is sufficient to allow the passage of free sialic acids. Bigger pores may allow more contamination by permitting bigger molecules to dialyse out. Place the tubing in a container where the bag can be covered by approximately ten volumes of water relative to its content. Add a stirring bar, and place the container on a stirring plate at 4 °C, overnight[c]. When analysing pure samples, dialysis can be omitted.
3. Prepare a 1.5 ml column of Dowex 50 AG 1X8 in ice-cold water[d].
4. Apply the dialysate directly to the column[e]. Wash the tube with another millilitre of cold water, and apply to the column. Wash the column with 6 ml of cold water, and collect in a glass tube, on ice. Alternatively, the chromatography can be performed in the cold room.
5. Check the pH of the eluate with pH paper. If it is greater than three, go to step **6**. If it is lower, add 10 mM sodium formate buffer, pH 5.5 until the pH is greater than three.
6. Prepare a 1 ml column of Dowex 3X4A (formate form)[f] equilibrated in ice-cold 10 mM sodium formate buffer (pH 5.5) as above.
7. Load the sample on the column. Wash the column immediately with 7 ml of ice-cold 10 mM formic acid, and discard the washings.
8. Elute the sialic acids with 10 ml of ice-cold 1 M formic acid. Collect the eluate on ice in a 20 ml glass tube, and take it to dryness by lyophilization or using a shaker-evaporator with the temperature of the water-bath maintained below 37 °C.

9. The purified mixture of sialic acids thus obtained can be analysed by HPLC (see *Protocols 15* and *16*), derivatized and analysed by reverse-phase HPLC with fluorometric detection (using *Protocols 13* and *18*), or submitted to direct FAB-MS (13).

[a] Keep in mind that crude biological samples might contain *O*-acetylesterases, and they could cause the de-*O*-acetylation of sialic acids while they are isolated. These enzymes can be inactivated by treating the sample with 1 mM final concentration of diisopropyl fluorophosphate (DFP; prepare a 100 mM stock solution in isopropanol) on ice, in a fume hood, for 15 min. *Caution*: DFP is extremely toxic, study safety recommendations carefully.

[b] This retards lipid peroxidation.

[c] When total volumes are small, the dialysis tubing can be placed inside a test-tube provided it is tied with knots instead of plastic clamps. Assume a 10% loss of sialic acids in this step if equilibrium is achieved in the dialysis using ten volumes of water.

[d] The resin must be extensively washed with water before use.

[e] When dealing with larger volumes, the dialysate can be lyophilized, dissolved in a small volume of water, and loaded on to the Dowex 50 AG 1X8 column. The content of the dialysis tubing can also be frozen and lyophilized, and reserved for other analyses. It contains the desialylated glycoproteins under study.

[f] Wash the Dowex AG 3X4A (hydroxyl form) resin extensively with water. Convert it to the formate form by equilibrating in three volumes of 1 M formic acid. Let stand for 15 min. Wash with 10 mM sodium formate (pH 5.5) until effluent pH is stable at 5.5.

Protocol 7. Procedure for the release and purification of 4-*O*-acetylated sialic acids

- all materials and reagents required in *Protocol 6* with the exception of acetic acid

A. *Preparation of the sample.*

See *Protocol 6* part A.

B. *Release and purification of sialic acids*

1. Follow *Protocol 6*, part B, step **1.**

2. Adjust the pH of the dialysed sample to 2.0 by dropwise addition of concentrated formic acid.

3. Heat at 70 °C for 1 h (see footnote b, *Protocol 5*). Cool to room temperature, and centrifuge.

4. Dialyse the supernatant against ten volumes of water (1000 MW cut-off tubing) at 4 °C, and lyophilize (see footnote c, *Protocol 6*).

5. Treat the residue twice more under the same conditions, recovering the hydrolysis products after each step as indicated in step **4** (see footnote e, *Protocol 6*).

6. Pool the lyophilized diffusates and purify the mixture of sialic acids by ion exchange chromotagraphy following steps **3–8**, part C, *Protocol 6*.

Protocol 7. *Continued*

7. The purified mixture of sialic acids thus obtained can be subjected to analysis by HPLC (see *Protocols 15* and *16*), derivatized and analysed by reverse-phase HPLC with fluorometric detection (using *Protocols 13* and *18*), or submitted to direct FAB-MS (13).

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iii. *Strong acid hydrolysis for release of hexoses, deoxyhexoses, pentoses, hexosamines, and glycosyluronic acids*

Hydrolysis of glycosidic linkages involving hexoses require more vigorous conditions than the ones used for fucose or sialic acids. Typically, hydrochloric acid (1 or 2 M, 100 °C, 1–6 h), sulfuric acid (0.1–2 M, 100 °C, 4–12 h), or trifluoroacetic acid (2 M, 121 °C, 1–2 h) have been used for this purpose in different conditions depending on the substrate. Provided that the most labile species, such as sialic acids, are quantitated separately (*Protocols 6* and *7*), the most severe problem encountered when determining the monosaccharide composition is the incomplete release of 2-acetamido sugars and glycosyluronic acids. Depending on the origin of the glycoprotein under study, the presence of these sugars can be predicted. In the case of acetamido sugars, if *N*-deacetylation occurs first, the stability of the glycosidic linkage is increased. Therefore, the conditions must be such that the hydrolysis of the glycosidic linkage is substantially faster. The glycosidic linkage of glycosyluronic acids is particularly resistant to acid hydrolysis, and further prevention of their complete release arises when the adjacent sugar in the chain is a *N*-sulfated or *N*-acetylated hexosamine. This problem is routinely encountered in glycosaminoglycan chains, where quantitative release requires a previous reduction of the carboxylic acid. On the other hand, liberated glycosyluronic acids are very susceptible to degradation, particularly by decarboxylation. However, analysis of proteoglycans is beyond the scope of this chapter and we refer the reader to reference 18 for specific methods. In most cases release with 2 M TFA will produce as good recovery of fucose as that with 0.05 M HCl.

When enough material is available, several conditions can be tried, checking for recovery and reproducibility. In many cases this is not possible, and the use of 2 M TFA for one hour at 121 °C is recommended.

Protocol 8. Quantitative release of hexoses, deoxyhexoses, pentoses, hexosamines, and glycosyluronic acids

Materials

- all materials required for *Protocol 5*
- D-(+)-glucose (anhydrous, mixed anomers, Sigma G-8270)

- D-(+) galactose (crystalline, Sigma G-0750)
- L-fucose (MW 164.2; Sigma F-2252)
- D-(+)-mannose (crystalline, Sigma M-4625)
- D-(+)-xylose (99%, Sigma X-1500)
- glucosamine hydrochloride (Sigma G-4875)
- D-glucuronic acid (crystalline, Sigma G-52690)
- D-galacturonic acid (monohydrate, crystalline, Sigma G-2125)

Method

1. Dry down three aliquots of the sample (containing 5–50 μg of total sugars) in glass tubes or vials. For GLC analysis add 1–10 μg of internal standard[a].
2. Prepare three aliquots of a mixture of all the neutral monosaccharide standards for GLC of alditol acetates, or neutral sugars, hexosamines, and glycosyluronic acids for HPAEC-PAD, in equimolar amounts[b]. For GLC analysis, also add the internal standard.
3. Prepare three blanks in order to address possible contaminants. An adequate blank is a material that has been processed exactly as the sample was (e.g. a pool of fractions from a chromatography step that showed only background response to the method used for monitoring). This is especially useful when very small amounts of material are processed since the limit of sensitivity will be determined by the accumulated contaminants (coming from Sephadex columns, dirty glassware, etc.).
4. Dissolve the three aliquots of each sample in 100–500 μl of: (a) 4 M TFA, (b) and (c) 2 M TFA, flush with nitrogen, and cap the tubes. Heat (a) and (b) at 100 °C for 4 h and (c) at 121 °C for 1 h[c].
5. Evaporate the solution with a stream of nitrogen or under vacuum. Wash the residue twice with methanol followed by evaporation.
6. Keep the dry hydrolysates in the freezer until they are analysed by HPAEC-PAD using *Protocol 14*, or derivatized (*Protocol 12*) and analysed by GLC using *Protocol 17*.
7. Compare the recovery of each monosaccharide using the different conditions and decide upon the most suitable hydrolysis for the sample under study. Repeat the analysis using such conditions and check for reproducibility.

[a] The internal standard should not occur naturally in the samples under study. Monosaccharide alditols, that yield only one peak in the GC run, are adequate. See also footnote a, *Protocol 5*.

Protocol 8. *Continued*

[b] When HPLC or GC are used as detection methods, the time involved in the analysis of each sample is quite long. Preparation of a mixture of the adequate standards in equimolar amounts helps in reducing the analysis time since all the required monosaccharide standards are analysed in one run. On the other hand, when identification of one given component is doubtful (when no MS is available), the co-injection of one aliquot of the sample with one aliquot of the suspected standard gives an answer about the presence or absence of that particular monosaccharide. Therefore, processing individual standards in parallel can become helpful.

[c] When glycosyluronic acids are detected a time course is recommended.

4.1.3 Procedures based on methanolysis

Cleavage of all glycosidic linkages by methanolysis is very effective, and causes less destruction of the monosaccharides than acid hydrolysis. Monosaccharides are converted to their methylglycosides, and glycosyluronic acids to their methylesters methylglycosides. The *N*-acetyl group of amino sugars are completely cleaved by extensive methanolysis, and it is advisable to incorporate a re-*N*-acetylation step to avoid the production of additional peaks in the chromatogram. A drawback to the reacetylation step is the production of some extent of *O*-acetylation (in some primary hydroxyl groups), but a short additional treatment with methanolic HCl eliminates these groups. Due to anomerization, each monosaccharide produces several methylglycosides (α and β anomers of the pyranosidic and furanosidic forms), which yields a characteristic pattern of peaks in the GC run. Thus, when the sample contains several monosaccharides, the pattern is quite complex because of the superimposed position of some peaks, making quantitation difficult. Nevertheless, by processing each of the individual expected monosaccharides in parallel, running each one separately in the same conditions, and determining the contribution of each peak to the total, an accurate quantitation can be achieved.

Different conditions have been used for the methanolysis of carbohydrates in glycoproteins using methanolic HCl, including 0.5 M at 65 °C for 16 h (19), and 0.75 M at 80 °C for 3 h (20). Simultaneous analysis of neutral monosaccharides, hexosamines, octulosonic acids, and glycosyluronic acids after methanolysis with 1 M HCl at 85 °C for 24 h has been reported. Nevertheless, the cleavage of the uronic acid glycosidic linkage may be incomplete in some cases, and several glycosyluronic acids produce a certain percentage of 3,6-lactones. The glycosidic linkage of sialic acids is cleaved with good yield, and special conditions to minimize destruction have been worked out. However, most *O*-acetylesters and the *N*-acyl group are eliminated.

The linkage between *N*-acetylglucosamine and asparagine is hardly cleaved by methanolysis, and this fact has to be considered when quantitation is required. Dehydration of monosaccharides can also occur to a small extent.

Protocol 9. Quantitative release of neutral monosaccharides, hexosamines, and glycosyluronic acids by methanolysis

Materials

- glass Reacti-Vials (1.5 ml) with Teflon-lined screw cap
- heating block or oven
- nitrogen or vacuum evaporation system (Speed-Vac, shaker-evaporator)
- methanol (anhydrous, 99%, Aldrich 32, 241–5; store in a desiccator)
- HCl (A.C.S. reagent, Fisher Scientific A144SI-212)
- anhydrous pyridine (silylation grade, Pierce 27530; store in a desiccator)
- acetyl chloride (FW 78.50; 98%, Aldrich 11, 418–9)
- phosphorous pentoxide (Fisher Scientific A245–500)
- monosaccharide standards (see *Protocol 8*)

Method

1. Place the sample, containing 5–50 μg of total sugars in a Reacti-Vial. Add the adequate internal standard (1–10 μg) and lyophilize (see footnote a, *Protocol 8*).
2. Prepare a set of the necessary standards (50 nmol), each one containing the same internal standard used for the sample (5 nmol). Also prepare a mixture of all the expected monosaccharides with internal standard.
3. Dry the lyophilized samples overnight in a vacuum desiccator over P_2O_5.
4. Prepare methanolic HCl by carefully adding acetyl chloride to anydrous methanol in the following proportions[a]:

	Concentration	Acetyl chloride	Anhydrous methanol
A	0.50 M	3.50 volumes	100 volumes
B	1.00 M	7.00 volumes	100 volumes

5. Dissolve the residue in 200 μl of methanolic HCl and cap the vials.
6. Heat at 65 °C for 16 h when using reagent A, and at 85 °C for 24 h when using reagent B[b]. Check the screw caps for tightness after heating for 15 min. Vortex and continue heating.
7. Dry down using a stream of nitrogen at room temperature.
8. Add 100 μl of dry pyridine, and vortex to resuspend the residue. Add 100 μl of acetic anhydride, vortex for 1 min, and evaporate to dryness under a stream of nitrogen.

Protocol 9. *Continued*

9. Dissolve the residue in 200 µl of 0.5 M methanolic HCl, and heat at 65 °C for 1 h.
10. Dry down under a stream of nitrogen, and further in a vacuum desiccator over P_2O_5, overnight.
11. Submit the dry residues to derivatization, *Protocol 11*.

[a] Prepare a large enough volume to reduce pipetting errors, but only in the range required for the experiment since it can not be stored. Use care, since this reaction is very exothermic.

[b] Reagent B is preferred when glycosyluronic acids are present. Alternatively, a first step of acid hydrolysis using 200 µl of 2 M TFA for 1 h at 121 °C may be incorporated. TFA is evaporated under a stream of nitrogen, and samples washed twice with methanol and evaporated again. This step assures complete recovery of hexosamines and glycosyluronic acids, but destruction of sialic acids occur. Proceed afterwards to step **4**.

4.1.4 Enzymatic release of sialic acids with sialidases (neuraminidases)

The more sensitive and specific assays for sialic acids require that they be first released from glycosidic linkage to the oligosaccharide (see *Protocol 4*). While careful acid hydrolysis can be used for this purpose (see *Protocols 6* and *7*), partial destruction of modifications and/or incomplete release can be a problem. Further, it is often desirable to leave the underlying glycoconjugate in intact form after release of sialic acids. In these cases, it may be better to use a commercially available sialidase (a term preferred to 'neuraminidase') to release the sialic acids prior to purification and analysis. However, not all sialidases can release all forms of sialic acids from all types of linkages (see also Chapter 4a of this volume).

There is a remarkable degree of confusion in the literature about the specificities of the various sialidases for different substituted sialic acids and for different linkages. Results from experiments done on a few model compounds have been extrapolated to related molecules and linkages without adequate justification. Thus, there are many erroneous statements about the matter in the conventional literature and in the commercial information packages provided with sialidases. *Table 3* summarizes information based upon our experience and that of others we have consulted. In using sialidases for analytical release, the following caveats should be noted.

(a) The relative rates of different enzymes against various linkage-types are of little consequence in most instances, because the enzymes are always being used in excess. Important exceptions are:

i. The Newcastle Disease virus neuraminidase cleaves only α2→3 and α2→8 linkages.

Table 3. Susceptibility of various types of sialic acids to commercially available sialidases

Sialic acid		Relative susceptibility to sialidase from:			
Type	**Linkage(s)**	***Vibrio* cholerae**	***Clostridium perfringens***	***Arthrobacter ureafaciens***	**Newcastle disease virus**
Neu5Ac	α2→3	++++	++++	++	++++
	α2→6	+++	+++	++++	R
	α2→8	++	++	+++	+++
Neu5Gc	α2→3	++++	++++	++	++++
	α2→6	+++	+++	++++	R
	α2→8	++	++	+++	+++
7(9) mono-*O*-acetyl	α2→3	++	++++	++	++++
	α2→6	++	+++	++++	R
	α2→8	+	++	+++	+++
4-mono-*O*-acetyl	α2→3	R	R	R	R
	α2→6	R	R	R	R
	α2→8	R	R	R	R
7(8)9-di-*O*-acetyl	α2→3	R?	R?	++	?
	α2→6	R?	R?	++++	R
	α2→8	R?	R?	+++	?
7,8,9-tri-*O*-acetyl	α2→3	R?	R?	++?	?
	α2→6	R?	R?	++?	R
	α2→8	R?	R?	++?	?
O-methyl	α2→3	?	?	?	?
O-sulfate	α2→6				
Combinations	α2→8				
Periodate-oxidized	α2→3	+	++	R?	?
	α2→6	+	++	R?	
NANA-7(8)	α2→8	?	?	R?	

R = practically resistant.

ii. The *Vibrio cholerae* enzyme does not cleave the α2→3-linked 'internal' sialic acids of extended gangliosides, (e.g. G_{M1}).

iii. The α2→8 linkages of colominic acid and B-series gangliosides have a tendency to form lactones in acidic conditions, resulting in resistance to sialidase release. Repeated treatments with intermediate neutralization of pH may be required for complete release.

(b) Substitutions of the sialic acids have variable effects upon release, depending upon the enzyme used. The decrease in rate commonly seen with the *N*-glycolyl modification is not practically relevant in using any of the enzymes. The decrease in rates seen for the 9- or 7-mono-*O*-acetyl substitutions are also not practically relevant, except for the *Vibrio* enzyme, and particularly with gangliosides. On the other hand, the 4-mono-*O*-acetyl substitution results in complete resistance to all known

sialidases. The effects of di- and tri-*O*-acetyl substitutions have not yet been carefully studied, and it is not safe to extrapolate from data on mono-*O*-acetylated molecules. Likewise, combinations of substitutions, (e.g. *N*-glycolyl and *O*-acetyl) have not been studied carefully. Finally, there is no information on the rarer types of sialic acids, (e.g. *O*-methyl and *O*-sulfate substituted).

(c) Sialidases (neuraminidases) from microbial sources are preferred for analytical studies, because of their relative stability and commercial availability in phenotypically pure form. The choice of enzymes to be used may depend upon the type of sialic acid(s) expected. Sometimes mixtures of enzymes, or sequential treatment with various enzymes are helpful.

(d) Whenever possible, control incubations (minus enzyme and minus substrate) must be studied in parallel to be sure that the sialic acids detected were indeed released from the sample being studied. This is particularly important when ultra-sensitive methods (such as DMB derivatization, followed by HPLC with fluorometric detection, see Section 4.2.3) are employed. This method is so sensitive that 'environmental contamination' with sialic acids can become an issue.

Protocol 10. Release of sialic acids by sialidases

Materials

- buffer: 1 M sodium acetate stock solution (pH according to the enzyme being used)
- sialidase: stock solution of 1–10 mU/µl in 100 mM acetate buffer
- 100 mM calcium acetate stock solution (for *Vibrio* enzyme only)
- standard glycoconjugate with similar types of sialic acids (see *Table 2*)
- *Arthrobacter ureafasciens* neuraminidase (Calbiochem 480714)
- *Clostridium perfringens* neuraminidase (Type X, Sigma N-2133)
- *Vibro cholerae* neuraminidase (Sigma N-6514)

Method

1. Dissolve the oligosaccharides or glycoconjugates in a final concentration of 100 mM sodium acetate (with 1 mM calcium acetate, 100 mM NaCl final for *Vibrio cholerae* enzyme only).
2. Prepare control incubations (minus enzyme and minus substrate) in parallel.
3. Add 1–20 mU enzyme (~1 mU per nmol expected to be removed) and mix well.

4. Incubate for 3–4 h at 37 °C.
5. Check an aliquot for release of sialic acids after de-*O*-acetylation by the TBA method (see *Protocol 4*). For radiolabelled sialic acids, do a quick dialysis (5 h) and use the radioactivity in the dialysate as indication of release.
6. Purify the released sialic acids by dialysis and sequential Dowex resin chromatography as described in *Protocol 6*, part *C*.
7. Analyse released sialic acids by direct HPLC (*Protocols 15* and *16*), or HPLC after derivatization (*Protocols 13* and *18*).
8. Compare with release, yield, and composition from a standard glycoconjugate of similar type (see *Table 2*).
9. Also compare release, yield, and composition with mild acid hydrolysis (*Protocol 6* or *7*).

4.2 Derivatization of glycoses and glycosides

In order to analyse the mixtures of glycoses or methylglycosides by gas-liquid chromatography, it is necessary to prepare volatile derivatives. Alditol acetates are the most widely used derivatives for monosaccharides obtained by acid hydrolysis, while trimethylsilyl ethers of methylglycosides or methylesters–methylglycosides are the preferred derivatives for methanolysis products. These are the derivatives of the choice for any glycoprotein derived sugar because of the amount of information available, particularly regarding their mass spectrometric patterns. Other volatile compounds, such as aldononitrile acetates and trifluoroacetylated alditols, have been successfully employed. These methods are reviewed elsewhere (15).

4.2.1 Trimethylsilyl derivatives

The content of neutral sugars, amino sugars, and glycosyluronic acids can be simultaneously determined by this method. Mixtures of methylglycosides have been silylated using different reagents and procedures (15). The most widely used reagent is the mixture of pyridine, hexamethyldisilazane, and trimethylchlorosilane 5:1:1 (v/v/v), followed by an incubation that ranges from 15 minutes at room temperature to 20 minutes at 80 °C.

Sialic acids have also been analysed as trimethylsilyl esters, trimethylsilyl ethers, or methylesters, trimethylsilyl ethers. Although conditions for the preparation of these two types of derivatives have been worked out to preserve labile natural substituents such as *O*-acyl groups (16), the derivatization of sialic acids is frequently incomplete. Further problems are encountered during the chromatographic separation since the high temperatures required for the elution of these molecules result in partial destruction of the

labile substituents. Therefore, this approach to sialic acids has been replaced by HPLC methods that do not require derivatization (*Protocols 15* and *16*) or direct FAB-MS (13).

Protocol 11. Preparation of trimethylsilyl ethers of methylglycosides of neutral monosaccharides, and hexosamines, and methylesters methylglycosides of glycosyluronic acids

Materials

- nitrogen
- Tri-Sil (Pierce 48999J or 49001J)
- hexane (95%, HPLC grade; Aldrich 27, 050–4)

Methods

1. Dry the residue obtained in *Protocol 9* overnight in a vacuum desiccator over P_2O_5. Process samples and all required standards in parallel[a].
2. Suspend in 100 µl of Tri-Sil, and cap the vials.
3. Incubate at room temperature for 30 min.
4. Evaporate the silylating reagent under a stream of dry nitrogen.
5. Dissolve the derivatives in hexane (0.5 ml), and centrifuge (at 1000 *g*) to separate the insoluble salts.
6. Transfer the supernatant to a clean Reacti-Vial, and evaporate under dry nitrogen as before.
7. Dissolve the residue in hexane (5–50 µl, depending on the amount of starting material), and use 1 µl of this solution for GLC analysis (see *Protocol 17*).

[a] Silylated samples must be immediately analysed. See also footnote a, *Protocol 5*.

4.2.2 Acetylesters of alditols

When only neutral sugars need to be analysed, the most common derivatization method is the conversion of the free monosaccharides into their alditol acetates. Each monosaccharide produces a single derivative, giving a simple chromatogram that can be easily interpreted and quantitated. The total time of analysis is also shorter since all required standards can be processed as a mixture, requiring only one GLC run.

The procedure involves the reduction of the glycoses to alditols with sodium borohydride, elimination of the excess of hydride with acid, elimination of the boric acid produced as trimethylborate by co-evaporation with acidified methanol, and peracetylation of all free hydroxyl groups. Different protocols to achieve this last step have been developed (15, 22, 23).

It is possible to simply use the sodium acetate produced in the reaction as the catalyst, and achieve complete acetylation by heating with acetic anhydride. Since left over borate could inhibit the acetylation, some protocols add pyridine to drive the reaction.

Protocol 12. Preparation of acetylesters of alditols

Materials

- ammonium hydroxide (Fisher A669S)
- sodium borohydride (FW 37.8; Sigma S-9125, must be stored ina desiccator)
- acetic acid (FW 60.05; 99.99%, Aldrich 33, 882–6)
- methanol (anhydrous, 99%, Aldrich 32, 241–5)
- acetic anhydride (99%, Alrich 11, 004–3)
- chloroform (anhydrous, 99%, Aldrich 28, 830–6)
- acetone (99.9%, HPLC grade, Aldrich 27, 072–5)
- nitrogen evaporation unit (Reacti-Vap Evaporator, 18780 Z; Reacti-Therm heating module, 18870 Z; Reacti-Block b-1, 18802 Z, or S-1, 18816 Z, Pierce)
- heating block or oven

Method

1. Add 250 µl of a solution of 1 M NH_4OH containing 10 mg/ml of sodium borohydride to the samples and the mixture of neutral sugar standards obtained in *Protocol 8*. After 10 min, check for persistence of microbubbles in the reaction mixture. If these can not be seen, add a few micrograms of solid sodium borohydride[a].
2. Incubate at room temperature for 2 h[b].
3. Add glacial acetic acid dropwise until no further bubbling is observed[c].
4. Add 250 µl of 1% acetic acid in methanol to each tube and evaporate to dryness using a nitrogen evaporation unit or under vacuum using a Speed-Vac system, with the heating element set at 40 °C. Repeat four more times[d].
5. Add 200 µl of acetic anhydride, vortex, and cap the tubes.
6. Heat at 100 °C for 3 h (mix after the first 15 min of heating to make sure that the solid residue is suspended). Let cool to room temperature.
7. Add 200 µl of toluene, and evaporate to dryness at 40 °C using a shaker-evaporator or Speed-Vac system. Add toluene as needed to dry completely[e].

Protocol 12. *Continued*

8. Add 0.5 ml of water and 0.5 ml of chloroform to each tube, cap, and vortex well[f]. Centrifuge briefly to clearly separate the two phases.
9. Take out the water layer with a Pasteur pipette, and wash the organic layer with water twice again.
10. Transfer the chloroform layer to a clean tube or Reacti-Vial and dry down under nitrogen at room temperature.
11. Dissolve in 5–50 µl of acetone, depending on the amount of starting material, and use 1 µl for GLC analysis (*Protocol 17*).

[a] It is important that no residue of acid, from the hydrolysis, is present or the hydride will be rapidly destroyed. If excessive bubbling occurs when adding the borohydride, add methanol and evaporate two or three times. Then repeat the reduction.

[b] This reaction can be prolonged up to overnight according to the time schedule.

[c] Care must be taken to avoid losing sample with the bubbling since the reaction is violent. This problem may be circumvented by using a 20% (v/v) solution of acetic acid in methanol.

[d] Complete removal of borate is indicated by colourlesss crystals, as compared with white powder previously present. When a nitrogen evaporation unit is employed, care must be taken not to blow off the solid residue, particularly in the final steps.

[e] Toluene helps to eliminate the reagents by forming an azeotrope.

[f] Salts will be extracted into the water layer, leaving the peracetylated alditols in the organic layer.

4.2.3 Fluorescent derivatives of sialic acids

When only picomol amounts of sialic acids are available (as determined by *Protocol 4*), the method of choice to characterize the individual components of the mixture is the preparation of fluorescent derivatives. Different fluorogenic reagents for α-keto acids were tested, and 1,2-diamino-4,5-methylenedioxybenzene dihydrochloride (DMB) was found to give the best response (24). Mixtures of free sialic acids obtained using *Protocols 6*, *7*, or *10* can be directly reacted with the fluorogenic reagent without the need for further purification.

The reaction of sialic acids with DMB is highly specific under controlled conditions (other α-keto acids possibly present in biological samples give very weak responses and/or give very delayed retention times under the recommended conditions for the analysis). Most of the known, naturally occurring, substituted sialic acids can be derivatized and separated (25). The exceptions are the 2,3-dehydrosialic acids as well as glycosides that do not have the α-keto group available for the reaction. The mixture of derivatives is separated by HPLC (see *Protocol 18*).

When *O*-acetylesters are present, the identity of the peaks can be confirmed by repeating the analysis after base treatment as indicated in *Protocol 4*.

Protocol 13. Preparation of fluorescent derivatives of sialic acids

Materials

- plastic conical tubes (Eppendorf)
- heating block
- acetic acid (HPLC grade, Fisher A35-500)
- 1,2-diamino-4,5-methylenedioxybenzene dihydrochloride (FW 225.1; Sigma D-4784)
- β-mercaptoethanol (MW 78.13; Bio-Rad 161–0710)
- sodium hydrosulfite (FW 174.1; 80%, Sigma S-1256)
- *N*-acetylneuraminic acid (Neu5Ac, MW 309.3; Boehringer–Mannheim 101–931)
- *N*-glycolylneuraminic acid (Neu5Gc, MW 325.3; Sigma G-2755)
- mixture of sialic acids from bovine submaxillary mucin (BSM), prepared by *Protocol 6* is recommended

Method

1. Prepare the fluorogenic reagent in an Eppendorf tube:
   - 1.575 mg of DMB (final concentration 7 mM)
   - 80.4 µl of glacial acetic acid (final concentration 1.4 M)
   - 52.8 µl of β-ME
   - 72 µl of a 0.25 M (43.5 mg/ml) solution of sodium hydrosulfite in water

   Cover the tube with aluminium foil because the reagent is light-sensitive. Can be stored at 4 °C overnight.
2. Transfer 5–1000 pmol of free sialic acids to an Eppendorf tube, and dry down. Prepare 1 nmol of each standard in similar tubes.
3. Dissolve the samples in 5 µl of water.
4. Add 25 µl of DMB reagent and wrap each tube in aluminium foil.
5. Heat at 50 °C for 2.5 h.
6. Remove and place on ice.
7. Use 10 µl of the reaction mixture for HPLC analysis (*Protocol 18*)[a].

[a] Analysis should be done as soon as possible, but the DMB derivatives can be kept overnight at 4 °C.

4.3 Qualitative and quantitative analysis

The most widely used methods today for the compositional analysis of the carbohydrate moiety of glycoproteins are: HPAEC-PAD, and GLC-MS.

Both can detect all the possible known monosaccharides. With the HPAEC-PAD system it is possible to analyse qualitatively and quantitatively a mixture of free monosaccharides containing as little as 50 picomol of each individual component. GLC-MS analysis requires at least 500 picomol of each component, but the use of special techniques such as selected ion monitoring (SIM) can reduce this value to 50–100 picomol. With GLC methods, the use of internal standards improves the quantitation since the injection of reproducible proportions of the sample into the column is not easy.

4.3.1 Free monosaccharides: high performance anion exchange chromatography with pulsed amperometric detection (HPAEC-PAD) and other HPLC techniques

Different HPLC systems have been used for the analysis of free glycoses obtained by acid hydrolysis (1), but resolution between individual monosaccharides and sensitivity were poor. The technique became popular for the analysis of glycoproteins (and other glycoconjugates) available in limited amounts from biological origin, only when pellicular resin anion exchange columns and the pulse amperometric detector were developed. Neutral saccharides are analysed at high pH (12–14) at which they are partially or completely ionized. Separation is achieved by adjusting the eluent pH. Anionic saccharides can be separated by sodium acetate/sodium hydroxide gradients. Therefore, with an adequate change in the elution conditions, this system not only permits the compositional analysis of free monosaccharide mixtures, but also the analysis of oligo and polysaccharides (for a review see reference 26).

Pulsed amperometric detection utilizes a repeating sequence of three applied potentials applied for specific durations. The resulting total current is the sum of:

- the carbohydrate oxidation current
- the current due to the charging of the electrode surface, and
- the current caused by the oxidation of the gold electrode

The current is only measured during the first applied potential, and has been shown to be linear over more than four and one-half orders of magnitude. Molecules with similar size and structure are reported to have similar response factors. On the other hand, substituents on hydroxyl groups are expected to affect the sensitivity of detection. The detector works only at high pH. Thus, when base-labile species that are separated without the use of base in the eluent are analysed, it is necessary to add alkali to the effluent before it enters the PAD cell.

An isocratic elution with sodium hydroxide (3–15 mM, the optimal concentration varies from column to column) allows for the separation of all known neutral sugars, and hexosamines. Different sodium acetate gradients

have been employed for the analysis of acidic monosaccharides, and mixtures of these and other sugars. The system is also useful for sialic acids, with a limit of detection in this case of about 20 picomol. However, when gradients that end in high concentrations of acetate (above 100 mM) are used, the slow re-equilibration of the column with low concentrations of hydroxide and acetate results in lower retention times and impaired separation. Resolution of some pairs of monosaccharides (Gal-GlcN; Xyl-Man) during the first isocratic step is extremely sensitive to minor changes in solvent composition. Therefore, extended equilibration periods between runs are necessary to obtain reproducible results. A shorter delay between runs, resulting in a non-fully equilibrated column that still achieves adequate separation for a set of monosaccharides, can produce repeatable results if an automatic sample injector with constant intervals between injections is employed.

Since only 1% of the sample is oxidized in the electrode, the system can be used preparatively without the need for a bypass. When analysing base-labile sialic acids, it is not possible to use this method because the post-column addition of base required for detection will cause the saponification of the *O*-acetylesters. However, the pellicular resin column can be used for fractionation and the eluate routed to a fraction collector. After monitoring the fractions by the TBA assay (*Protocol 4*), they can be pooled and desalted. The method proved to be the best HPLC technique available for preparative fractionation of mixtures of sialic acids containing labile substituents. After removal of the low amount of acetate, required for separation using the HPAEC column, by Dowex 50 (H^+) chromatography, an excellent recovery ($> 90\%$) of acetylated species is possible (25).

Another useful system for the analysis of free sialic acids is the amine adsorption/ion suppression HPLC. It is based upon the hydrogen bonding between the hydroxyl groups of saccharides and the amine functions of the stationary phase. To fractionate anionic molecules, phosphate is added to the mobile phase to suppress the ionic effect, while retaining hydrogen bonding. Good separations are obtained in isocratic mode with a mixture of acetonitrile:water:0.25 M monobasic sodium phosphate at 1 ml/min. The relative proportion of solvents can be varied according to the kind of sialic acids to be separated. It is necessary to maintain a minimum concentration of 10% phosphate buffer with a maximum working percentage of acetonitrile of 72%, above which the phosphate starts precipitating. Chromatography is monitored by absorption at 200 nm, which requires extreme purity of the samples and reagents. Residual amounts of purification reagents produce an absorption peak close to the void volume. The lower limit of confident detection is two nanomol, and the detector responses are equal for all sialic acids.

The two HPLC systems described here, and others used for the analysis of sialic acids are analysed in detail in reference 25.

Protocol 14. Qualitative and quantitative analysis of free monosaccharides by HPAEC-PAD[a]

Materials

- CarboPac PA-1 column (250 × 4 mm)
- CarboPak PA guard (3 × 25 mm)
- QIC reciprocating single piston pump (Dionex Corp.)
- Iochrom pulsed amperometric detector (PADII, Dionex Corp.)
- helium
- nitrogen
- NaOH (certified 50% solution, with less than 0.1% sodium carbonate, Fisher SS254–500)
- sodium acetate ($CH_3COONa.3H_2O$, FW 136.08; certified A.C.S., Fisher S209–500)
- deionized water (passed through a five-stage Milli-Q Plus system, Millipore Corp.)
- filtering unit with 0.45 μm nylon 66 membranes (Alltech Associates, Inc.)

A. *Neutral monosaccharides and hexosamines*

1. Filter 6 litres of Milli-Q water, transfer 2 litres to bottle No. 1, 1995 ml to bottle No. 2, and 1896 ml to bottle No. 3. Connect the sparge lines and degas for 15 min.
2. Prepare 50 mM and 1 M sodium hydroxide solutions by adding 5.2 ml and 104 ml of the 50% sodium hydroxide solution to bottles No. 2, and No. 3, respectively. Degas for another 15 min. Transfer the necessary volume of the 1 M solution to the reservoir for post-column addition, and cap the container.
3. Disconnect the sparge lines, cap the bottles, and pressurize bottles No. 1 and No. 2 to 6–8 psi.
4. Run 100% of each solvent through the lines to remove any bubbles.
5. Connect the CarboPak PA-1 (4 × 250 mm) column with the guard column.
6. Set up an isocratic run, with a flow rate of 1.0 ml/min, from the pump control unit[b].

	%				
Time (min)	1	2	3	4	5[c]
0	94	6	0	0	0
0.1	94	6	0	0	1
30	94	6	0	0	1

7. Start the system and wait until the stable back pressure is attained (usual working pressures are between 1200–1500 psi, with the maximum allowable pressure for the column being 2000 psi).
8. Enter the settings for the PAD dectector (or PED detector working in PAD mode).

Applied potential	Voltage (mV)	Time (msec)
E1	0.15	720
E2	0.70	120
E3	−0.30	60

Response time: 1 sec
Applied potential range: 1
Output range: 300 nA

9. Pressurize the post-column reservoir to deliver 0.3–0.4 ml/min of the 1 M NaOH solution (helium pressure should be around 32 psi that results in a 360 mM sodium hydroxide concentration at the electrode). Check the amount delivered by measuring the total flow out of the PAD cell with a small graduated cylinder.
10. Make sure that there are no bubbles inside the reference electrode.
11. Turn on the PAD detector and wait 15 min for stabilization, then press the 'Auto Offset' control.
12. Check the baseline using an integrator, and do not proceed until it is stable. Due to the sensitivity of the detector, the adequate integrator settings are: attenuation = 1024, and peak threshold = 10 000.
13. Inject 10 µl of the mixture of standards obtained in *Protocol 8* (containing 250 pmol each of: Fuc, Ara, GalN, GlcN, Gal, Glc, Xyl, and Man) or in *Protocol 5*, (250 pmol Fuc).
14. Control the resolution between monosaccharides. If it is not good (resolution of Gal/GlcN and Xyl/Man are usually critical, and inversion of the relative order of the components in the pair can be observed), change the concentration of base (when retention times are too short, less base is required). The optimum NaOH concentration needs to be re-checked each time a new batch of 50 mM NaOH solution is prepared. The total running time is below 30 min.
15. When good resolution and adequate retention times are achieved, inject the samples obtained in *Protocols 5* or *8*.
16. Change the working range (to 100 nA or 1 µA) if necessary (depending on the response obtained for the samples). Wait until a good baseline is obtained again before re-injecting.
17. Quantitate the standard run, and with these data (area per nanomol or

Protocol 14. *Continued*

picomol of each monosaccharide) calculate the nanomol or picomol of each component in the sample.

18. If resolution is lost, and retention times are too short, wash the column for 30 min with 100% No. 3 (1 M sodium hydroxide). Wash extensively with water, and return to the working eluent. Wait for the stabilization of the baseline in the running conditions and check the performance of the column by injecting 10 µl of the mixture of standards. If this is not enough to recover performance, flush the column with 1 M HCl for 30 min at 1.0 ml/min. Wash extensively with water, and flush with 1 M NaOH for 30 min at the same flow. Wash extensively with water, re-equilibrate with the working solvent, and check again.

B. *Neutral monosaccharides, hexosamines, and glycosyluronic acids*

1. Prepare the following solutions:

(a) Bottle No. 1 (1 M sodium hydroxide). Transfer 1896 ml of filtered water, degas for 15 min. Add 104 ml of 50% sodium hydroxide. Transfer the required volume for the post-column reservoir, and keep the remaining capped after flushing with helium. Must be prepared fresh weekly. Put another bottle in this place and fill it with water. Connect the sparge line, and degas for 15 min.

(b) Bottle No. 2 (25 mM sodium hydroxide and 0.25 mM sodium acetate). Weight 68 mg of sodium acetate and dissolve in water, taking the solution to 1997 ml in a graduated cylinder. Filter and transfer to bottle No. 2, connect the sparge line, and degas for 15 min. Add 2.6 ml of 50% sodium hydroxide and continue degasing for 15 min.

(c) Bottle No. 3 (200 mM sodium hydroxide and 300 mM sodium acetate). Weigh 82 g of sodium acetate and dissolve in water, taking the solution to 1979 ml in a graduated cylinder. Filter and transfer to bottle No. 3, connect the sparge line, and degas for 15 min. Add 20.8 ml of 50% sodium hydroxide and continue degasing for 15 min.

(d) Bottle No. 4 (125 mM sodium hydroxide and 10 mM sodium acetate). Weigh 2.7 g of sodium acetate and dissolve in water, taking the solution to 1987 ml in a graduated cylinder. Connect the sparge line and degas for 15 min. Add 13 ml of 50% sodium hydroxide and continue degasing for 15 min.

2. Disconnect the sparge lines, cap the bottles, and pressurize to 6–8 psi.

3. Follow steps **4** and **5**, part A.

4. Set up the following gradient with a flow rate of 1.0 ml/min, from the pump control unit[b].

	%				
Time (min)	1	2	3	4	5[c]
0	89	11	0	0	0
0.1	89	11	0	0	1
20	89	11	0	0	1
50	39	11	50	0	1
60	39	11	50	0	0
63	0	0	0	100	0
70	0	0	0	100	0
73	89	11	0	0	0
90	89	11	0	0	0
RESET (manually) 100 next injection					

5. Follow steps **7** to **12**, part A.
6. Inject 10 µl of the mixture of standards obtained in *Protocol 8* containing 250 pmol each of the before mentioned neutral monosaccharides, hexosamines, and the required glycosyluronic acids[d].
7. Perform quantitation as indicated in step **17**, part A.

[a] This system can be used for the analysis of radioactive samples routing the column effluent to a flow detector, or to a fraction collector. In the latter case, each fraction is counted in a β-counter after addition of scintillation cocktail.

[b] Do not use settings below 5% in the pump control unit because they can produce a considerable error in the final concentration.

[c] This controls the opening/closing of the injection valve.

[d] A continuously drifting baseline from the beginning of the gradient may be obtained. In this case, add sodium acetate to the post-column reservoir (500 mM) and run the standards to determine if the detector response is maintained.

Protocol 15. Qualitative and quantitative analysis of free sialic acids by HPAEC-PAD[a]

Materials

- all materials required for *Protocol 14*
- acetic acid (glacial, HPLC grade, Fisher A35–500)
- *N*-acetylneuraminic acid (Neu5Ac, MW 309.3; Boehringer–Mannheim 101–931)
- *N*-glycolylneuraminic acid (Neu5Gc, MW 325.3; Sigma G-2755)
- mixture of sialic acids from BSM (obtained by *Protocol 6*) is recommended

Method

1. Filter 2 litres of Milli-Q water, transfer to bottle No. 1, connect the sparge line, and degas for 15 min.

Protocol 15. *Continued*

2. Prepare the following solutions.
   (a) Bottle No. 1 (1 M sodium hydroxide): as indicated in step **2**, *Protocol 14*, part A.
   (b) Bottle No. 2 (5 mM sodium acetate). Weigh 0.68 g of sodium acetate and dissolve in water, taking the solution to 1 litre. Filter and transfer to bottle No. 2, connect the sparge line, and degas for 15 min.
   (c) Bottle No. 3 (5 mM acetic acid). Prepare 1 litre of 5 mM acetic acid in water and filter. Transfer to bottle No. 3, connect the sparge line, and degas for 15 min.
3. Disconnect the sparge lines, cap the bottles, and pressurize bottles to 6–8 psi.
4. Follow steps **4** and **5**, *Protocol 14*, part A.
5. Set up a two-step run, with a flow rate of 1.0 ml/min, from the pump control unit[b].

	%				
Time (min)	1	2	3	4	5[c]
0	0	100	0	0	0
0.1	0	100	0	0	1
5	0	100	0	0	1
35	0	50	50	0	1
36	0	0	100	0	1
46	0	0	100	0	1

6. Start the system and wait until a stable back pressure is attained (usual working pressures are between 950–1400 psi, with the maximum allowable pressure for the column being 2000 psi).
7. Follow steps **9** to **12**, *Protocol 14*, part A.
8. Inject 10 µl of a water solution containing 5 nmol of Neu5Ac, working in the 300 nA range.
9. Change the range to 100 nA, and wait until a good baseline is obtained again, before re-injecting. Inject 500 pmol of Neu5Ac in the same volume.
10. If a standard mixture containing several sialic acids is available, inject 10 µl containing 500 pmol each, and check the resolution. The total running time is below 30 min.
11. When good resolution and adequate retention times are achieved, inject the samples obtained in *Protocols 6*, *7*, or *10*. Compare the relative elution times with those listed in *Table 4*.

12. Characterization of individual peaks can be achieved by repeating the analysis after saponification of *O*-acetyl groups (see *Protocol 4*).

13. Determine the response obtained for standard Neu5Ac (area per picomol) and calculate the molar ratio of sialic acids in the mixture considering the following response factors[d].

Sialic acid	Response factor
Neu5Ac	1.00
Neu5Gc	1.15
Neu5,9Ac_2	0.46
Neu5,7(8),9Ac_3	0.45

[a] See footnote a, *Protocol 14*.
[b] For preparative purposes, a 9 × 250 mm column at a flow rate of 5 ml/min is used. See also footnote b, *Protocol 14*.
[c] This controls the opening/closing of the injection valve.
[d] The detector response for other sialic acids has not been determined, but lower response factors for more highly substituted species have to be expected.

Table 4. Relative elution times of sialic acids found in glycoproteins in different HPLC systems

Sialic acid	Relative elution times		
	HPAE (*Protocol 15*)	Amino column (*Protocol 16*[a])	TSK-ODS (*Protocol 18*)
Neu5Ac	1.00	1.00	1.00
Neu5Gc	1.17	1.5	0.84
Neu5,7Ac_2	0.74	0.36	1.06
Neu5,9Ac_2	0.95	0.35	1.57
Neu4,5Ac_2	0.76	0.39	1.68
Neu5,7(8),9Ac_3	0.74	0.23	1.90
Neu5,7,8,9Ac_4	0.62	[b]	1.98
Neu5Gc9Ac	1.06	0.49	1.20
Neu4Ac5Gc	0.86	0.54	1.53

[a] Using conditions B.
[b] Elutes close to the salt peak.

Protocol 16. Qualitative and quantitative analysis of free sialic acids by amine adsorption/ion supression HPLC with UV detection[a]

Materials

- MicroPak AX-5 (300 × 7.8 mm i.d.; particle size, 9 μm; Varian)
- guard column, Micropak AX–5 (4 cm × 4 mm)
- Spectra-Physics 8700 or 8700XR pump

Protocol 16. *Continued*

- SP-8440 UV-visible detector, Spectra-physics
- helium
- acetonitrile (HPLC grade, Fisher A998–4)
- monobasic sodium phosphate (anhydrous, MW 120.0; reagent grade, Sigma S-0751)
- deionized water (passed through a five-stage Milli-Q Plus system, Millipore Corp.)
- filtering unit with 0.45 μm nylon 66 membranes (Alltech Associates, Inc.)
- *N*-acetylneuraminic acid (Neu5Ac, MW 309.3; Boehringer–Mannheim 101–931)
- *N*-glycolylneuraminic acid (Neu5Gc, MW 325.3; Sigma G-2755)
- mixture of sialic acids from BSM (obtained by *Protocol 6*) is recommended.

Method

1. Turn on the UV detector. Set the wavelength to 200 nm.
2. Prepare 1 litre of 0.25 M monobasic sodium phosphate (30 g in 1 litre of water) in Milli-Q water and filter.
3. Fill the bottles in the HPLC system with:
   (a) acetonitrile
   (b) filtered Milli-Q water
   (c) filtered 0.25 M monobasic sodium phosphate, and degas for 15 min.
4. Run 100% of each solvent through the lines to eliminate any bubbles. *Caution*: do not allow direct mixing of stock acetonitrile and sodium phosphate, to avoid precipitation of salts in the lines.
5. Set up the isocratic elution conditions in the pump control unit. Condition A is used when only separation of Neu5Ac and Neu5Gc is required. Condition B is used when a mixture containing substituted species will be analysed.

	Conditions	Flow (ml/min)	%	
		1	2	3
A	1	64	26	10
B	1	72	18	10

6. Start running the solvent and then connect the MicroPak AX-5 column with the guard column.
7. Wait until the back pressure stabilizes, and check the baseline using the following settings for the integrator: attenuation = 8; peak threshold = 100. Wait until a stable baseline is obtained.

8. Inject 10 µl (10 nmol) of Neu5Ac in water. Check the sensitivity and adjust the integrator settings as required. Repeat if necessary[b].
9. Inject 10 µl (10 nmol each) of a mixture of Neu5Ac and Neu5Gc standards (or a mixture containing substituted standards if available, when using conditions B). Check for resolution, and adjust the elution conditions if required[c].
10. Inject the samples obtained in *Protocol 6*, *7*, or *10*. Adjust the integrator settings as required, and repeat if necessary.
11. Determine the percentage of each component considering equal detector responses.
12. Confirm the present of *O*-acetylated species by repeating the analysis after de-*O*-acetylation as indicated in *Protocol 4*. Compare the relative retention times to those listed in *Table 4*.
13. Wash the column with 100% water for 30 min. Run 100% acetonitrile for 10 min. Disconnect the column. *Caution*: never stop the flow if the lines contain phosphate, they may get clogged. Also wash the filter and the line coming from the reservoir after using any salt.
14. If the column loses resolution and retention is reduced, it can be cleaned by running 0.5 M phosphoric acid for at least 1 h and washing extensively with water before changing to the working buffer. More complete regeneration of the column can be achieved by injecting 3-aminopropyltriethoxysilane (3 × 1 ml) while running the column in 100% acetonitrile, washing extensively with the same solvent, and then with water and 0.5 M phosphate before use. This treatment restores the amino groups on the resin. The performance of the column is fully restored after this treatment; however, the retention times may not be identical.

[a] This system can be used for the analysis of radioactive samples if the column effluent is routed to a flow detector, or to a fraction collector. When a flow detector is used, the appropriate ratio of scintillation fluid to be used has to be checked because of the quenching effect of acetonitrile. When fractions are collected and 72% acetonitrile is used, it is necessary to dry them down, redissolve in water, and then count in a β-counter after addition of scintillation cocktail. When 64% acetonitrile is used, the addition of one volume of water followed by ten volumes of scintillation cocktail yields good sensitivity.

[b] It is possible to work in attenuation range 4 to detect 5 nmol of Neu5Ac, but the background increases considerably.

[c] *Caution*: remember that sodium phosphate will start precipitating above 72% acetonitrile. Condition B indicates the maximum compatible percentages of acetonitrile and phosphate.

4.3.2 Derivatized monosaccharides and alditols

i. Gas–liquid chromatography (GLC) with flame ionization detection (FID) or GLC-mass spectrometry (GLC-MS)

Analysis of volatile monosaccharide derivatives is achieved by gas-liquid chromatography. As indicated before, a known amount of an internal

standard (monosaccharide that does not occur naturally) is added to each glycoprotein at the beginning of its processing. Losses during the preparation of the sample for GLC will be proportionally the same for each component of the sample and for the internal standard. Thus, the ratio analyte/internal standard will remain the same, although the absolute amounts of both could be lower at the end.

When a flame ionization detector is used, initial characterization of individual peaks is done by comparison with standards run in the same conditions. The retention times of each standard relative to the component used as internal standard in the sample are calculated and compared with the relative retention times of each peak in the chromatogram of the sample. Typical elution profiles as well as relative retention times in the different columns, using different conditions can be found in the literature (15, and references therein). If the assignment of some peaks remains uncertain, a co-injection with the suspected monosaccharide allows confirmation. Quantitative analysis is performed by determining the detector responses for individual monosaccharides relative to the internal standard (known amounts are injected and the areas determined). As mentioned before, a characteristic pattern is obtained for each monosaccharide in the case of trimethylsilylated methylglycosides. Doing individual runs of each monosaccharide, containing an internal standard, it is possible to calculate the percentage of the total monosaccharide that each one of the peaks represents. The quantitation of the sample is done by choosing one non-superimposed peak for each monosaccharide and considering how much of the compound this area represents. Relative and absolute molar monosaccharide content in the sample can be calculated. Generally, a minimum of 100 ng of each component are required for confident detection. Molecular weights of the monosaccharides found in glycoproteins, their alditols and methylglycosides, and their volatile derivatives used in the protocols included in this chapter are listed in *Table 5*.

When a mass spectrometer is connected to the GLC, identification of each peak is achieved by the combination of the EI spectrum and the relative retention times. The EI spectrum of the different stereoisomers within each group of alditol acetates (hexitols, pentitols, etc.) is similar, and the relative retention times allow identification of the particular monosaccharide. The base peak is always m/z 43 ($CH_3-CO=^+$). In the mass spectra of trimethylsilylated methylglycosides, stereochemical differences do not appear but it is possible to differentiate between pyranose and furanose ring forms in one type of monosaccharide. Molecular ions are not regularly observed unless an ion trap detector (ITD) is used. This type of mass spectrometer also has the advantage of a ten-fold increase in sensitivity over the quadrupole instruments (27). Extensive information about the EI fragmentation patterns of alditol acetates and trimethylsilylated methylglycosides is available in the literature (15, and references therein).

Total ion currents are processed to obtain the composite signal for all

Table 5. Molecular weights of the monosaccharides found in glycoproteins and their volatile derivatives

Monosaccharide	Molecular weight				
	Aldoses	Alditols	Acetylated alditols	Methyl-glycosides and methyl-esters methyl-glycosides	TMS ethers of methy-glycosides and methyl-esters methyl-glycosides
Hexoses	180	182	434	194	482
Pentoses	150	152	362	164	380
Deoxyhexoses	164	166	376	178	394
Hexosamines	179	181	431	193	481
N-acetyl-hexosamines	221	223	473	235	523
Hexosyluronic acids	194	n.a.	n.a.	222	438

n.a. = not applicable.

carbohydrate derived fragments and this signal is used for quantitation. Relative response factors for this detector (the mass spectrometer) are also determined with standards processed in the same manner, and quantitative analysis achieved similarly. If the mass spectrometer is used in the scanning mode, 100–500 nanograms of each component are required for confident detection. However, if the mass spectrometer is equipped with software that allows scanning of selected ions, 100–500 nanograms of each component are sufficient. Quadrupole mass spectrometers are ideally suited for this mode of operation called 'selected ion monitoring' (SIM), but it is also feasible for magnetic sector instruments. A detailed explanation of its principle can be found in reference 27.

Protocol 17. Qualitative and quantitative analysis of derivatized methylglycosides and alditols by GLC

Materials

- 5% DB-5 fused silica capillary column (0.25 mm × 30 m, J&B, Scientific)[a]
- Hewlett-Packard 5840 A gas-chromatograph with a dual flame detector and a Hewlett-Packard 5840 A GC terminal, or
- Finnigan 4510 gas-chromatography-quadrupole mass spectrometer with an INCOS 200 data system
- helium (research grade)

Protocol 17. *Continued*

- hydrogen (for FID detector only)
- air (for FID detector only)

A. *GLC with flame ionization detector (FID)*

1. Connect the capillary column and start running the carrier gas at a flow rate of 1.5 ml/min. Set the make-up gas flow rate of 35 ml/min, and the split ratio to 10:1[b].
2. Set the oven temperature to 50 °C, the injector-port temperature to 150 °C, and the detector temperature to 250 °C, and wait for stabilization.
3. Open the air and hydrogen and adjust the flows. Ignite the detector. Check the background.
4. Set up the appropriate temperature programme.
   (a) Alditol acetates. Start at 50 °C, keep this temperature for 2 min, and then go from 50 °C to 150 °C at 20 °C/min (5 min), and from 150 °C to 250 °C at 4 °C/min (25 min), keeping this temperature for 5 min. Total running time = 37 min[c].
   (b) Trimethylsilyl ethers of methylglycosides. Start at 50 °C, keep this temperature for 3 min, and then go from 50 °C to 170 °C at 20 °C/min (6 min), and from 170 °C to 250 °C at 6 °C/min (13.3 min), keeping for 5.7 min at 250 °C. Total running time = 30 min.
5. Inject 1 µl of the mixture of standards prepared in *Protocol 11* or *12*. Adjust the settings of the integrator and repeat as necessary to obtain all the peaks on scale.
6. Determine the retention times of each peak relative to the internal standard.
7. Inject the standards (containing internal standard), one at a time, to assign the peaks and determine the response factors. In the case of TMS derivatives, also determine the contribution of each peak to the total (%), and select the peak of each monosaccharide to be used for quantitation.

$$R = \frac{\text{nanomol internal standard} \times \text{area monosaccharide}}{\text{nanomol monosaccharide} \times \text{area internal standard}}$$

8. Inject 1 µl of the sample. Repeat as needed to obtain a good profile.
9. Tentatively assign the peaks by comparison of the relative retention times with those of the standards. If a discrepancy arises, co-inject the sample with the individual standard. The questioned peak will be separated from the standard if it is not the same compound.

10. Determine the molar percentage of individual components by reference to the internal standard.

$$\text{nmoles sugar/}\mu\text{g sample} = \frac{\text{area under monosaccharide peak} \times \text{nmoles internal standard}}{\text{area under internal standard peak} \times R \times \mu\text{g of sample}}$$

B. *GLC-MS* (fragmentation patterns for each monosaccharide derivative can be found in reference 15, and references therein)

1. Set the oven temperature to 50 °C, the injector-port temperature to 200 °C, the ion source temperature to 300 °C, and the interface temperature to 250 °C.

2. Calibrate the mass spectrometer using perfluorokerosen (PFK).

3. Set up the mass spectrometer to scan over the m/z range of 40–600, with a scan time of 0.95 sec (0.05 sec interscan time), at 70 eV.

4. Set up the adequate temperature programme as indicated in part A, step **4**.

5. Inject 1 µl of the mixture of standards prepared in *Protocol 11* or *12*. Repeat as necessary to obtain all the peaks on scale. As in the case of FID detector, the injection can be done in split or splitless modes depending on the amount of sample available.

6. Calculate the relative retention times and determine the response factors using the areas under the composite signal profile as indicated in step **7**, part A.

7. Inject 1 µl of the samples. Repeat as needed to obtain a good profile.

8. Identify the components by a combination of the relative retention times and mass spectra.

9. Determine the molar percentage of individual components in each run by reference to the internal standard as indicated in step **10**, part A.

[a] Several other types of stationary phases can be used for the analysis of acetylated alditols: CP-Sil 5 WCOT, SP-1000, Silar 10C, OV-1, SE-54, 5% DB-5, DB-1, Carbowax 20M, SE-30, OV-101, OV-275, etc. In the less polar phases, amino sugar alditols can also be analysed. Other phases used for the analysis of trimethylsilylated glycosides and methylesters methylglycosides are: CP-Sil 5 WCOT, 5% DB-5, DB-1, SE-30, OV-101, etc. Conditions to be used vary from one to the other. Typical chromatograms and relative retention times can be found in reference 15, and references therein.

[b] When less than 25 µg of total carbohydrates are processed, a splitless injection is required.

[c] These conditions can also be used to analyse mixtures of partially methylated, partially acetylated alditols when doing glycosyl linkage analysis. See Chapter 4A.

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ii. High performance liquid chromatography with fluorometric detection

As indicated before, the HPLC analysis of DMB derivatives of sialic acids with fluorometric detection permits the characterization of the individual

components of the mixture with only picomol amounts. The separation is achieved using a reverse-phase TSK ODS-120T column eluted with acetonitrile: methanol:water at room temperature. Fluorescence of the eluate is monitored at an excitation wavelength of 373 nm and an emission wavelength of 448 nm.

Confident detection and quantitation is possible with 2.5 pmol of any sialic acid. To maintain column performance it is advisable to keep the injection volume of DMB reagent as low as possible. Evidence obtained using radioactive labelled sialic acids indicates a probable different degree of derivatization of molecules with different substitutions (25). Therefore, it is possible to accurately quantitize a given peak when the corresponding standard is available, and quantitation of substituted species by comparison with Neu5Ac is only approximate.

Protocol 18. Qualitative and quantitative analysis of DMB derivatives of sialic acids by HPLC with fluorometric detection[a]

Materials

- TSK Gel ODS-120T column (250 × 4.6 mm i.d.; particle size, 5 μm; TosoHass)
- guard cartridge TSK gel ODS-120T (1.5 cm × 3.2 mm)
- Spectra-Physics 8700 or 8700XR pump
- FD-300 dual monochromator fluorescence detector (2 μl flow-cell; Spectro Vision, Inc.)
- helium
- acetonitrile (HPLC grade)
- methanol (HPLC grade, Fisher A452–4)
- deionized water (passed through a five-stage Milli-Q Plus system, Millipore Corp.)
- filtering unit with 0.45 μm nylon 66 membranes (Alltech Associates, Inc.)

Method

1. Turn on the fluorometer. Set the emission wavelength to 448 nm, the excitation wavelength to 373 nm, the high voltage at 900, the range at 500, and the response time at 0.5 sec.
2. Fill the bottles of the HPLC system with:
   - acetonitrile
   - 50% methanol
   - filtered Milli-Q water, and degas for 15 min.
3. Run each individual solvent through the lines to eliminate any bubbles.
4. Elute with CH_3:50% MeOH:H_2O using a linear gradient from 7:14:79 to

11:14:75 (v/v/v) in 30 min. Maintain the final ratio of solvent for an additional 10 min, and return to the starting ratio using another linear gradient over a 20 min period. Wait for an additional 10 min before injecting the next sample.

5. Start running the solvent at 0.3 ml/min, and then connect the column with the guard column. Increase the flow 0.1 ml/min at a time, waiting for a stable back pressure between steps, until 0.9 ml/min flow is attained.
6. Check the baseline using the following settings for the integrator: attenuation = 1024; peak threshold = 10 000. Wait until a stable baseline is obtained.
7. Inject 100 pmol of DMB-derivatized Neu5Ac obtained in *Protocol 13*. Check the sensitivity and adjust the integrator settings as required. Repeat if necessary.
8. Inject 100 pmol each, of a mixture of DMB derivatized Neu5Ac and Neu5Gc standards (or a mixture containing substituted standards if available). Check for resolution, and adjust the elution conditions if required.
9. Inject the samples obtained in *Protocol 13*. Adjust the integrator settings as required, and repeat to obtain all peaks in scale. Calculate the retention time of each peak relative to Neu5Ac, and compare with those listed in *Table 4*.
10. Determine the units of area per picomol for Neu5Ac and/or Neu5Gc, and assuming equal detector responses, calculate the number of picomol represented by each of the peaks afforded by the samples.
11. Corroborate the presence of the *O*-acetylated species (see *Table 5* for relative retention times) by collecting each peak, lyophilizing, submitting to de-*O*-acetylation as indicated in *Protocol 4*, and to re-chromatography. Alternatively, another aliquot of the starting material can be submitted to de-*O*-acetylation prior to derivatization. In this case, all peaks corresponding to *O*-acetylated species will collapse into the peak of their parent molecule (Neu5Ac or Neu5Gc).
12. Wash the column with 50% methanol for 30 min at a flow of 0.5 ml/min at the end of the day. When resolution becomes poor, the column can be cleaned by running in reverse flow at 0.5 ml/min with a gradient from 10% to 100% acetonitrile in 0.05% trifluoroacetic acid over 1 h. The column must then be extensively washed with water at 0.5 ml/min, turned around, and equilibrated with 50% methanol.

[a] This system can be used for the analysis of radioactive samples. In this case, the column effluent can be routed to a flow detector, or to a fraction collector. The appropriate ratio of scintillation fluid needs to be checked because of the quenching effect of acetonitrile.

5. Compositional analysis of radiolabelled glycoproteins

When monosaccharide precursors are used for metabolic-labelling of glycoproteins, the label introduced into the oligosaccharides can be utilized to study their nature and structure (28, 29). The labelled glycoconjugate of interest needs to be purified only to 'radiochemical purity' and not to absolute homogeneity. The different monosaccharide precursors can be interconverted to varying extents into other sugars (see *Table 6*). With prolonged labelling, some can also enter into other cellullar components. The composition of labelled monosaccharides can be ascertained following enzymatic release or acid hydrolysis, utilizing several different systems of paper chromatography, thin layer chromatography, or HPLC (see *Protocols 14–16*, and *18* and references 28 and 30). Details regarding some of these systems are presented elsewhere in this chapter and volume. It should be kept in mind that certain techniques (particularly those utilizing derivatization) cannot be uniformly applied to radiolabelled monosaccharides.

Table 6. Entry of commonly used radiolabelled precursors into cellular monosaccharides

	Labelled precursor used				
Entry of label into	**2[^{3}H]Man**	**6[^{3}H]Gal**	**6[^{3}H]GlcNH$_2$**	**6[^{3}H]ManNAc**	**6[^{3}H]GalNAc**
[^{3}H]Man	+++	+/–	+/–	+/–	+/–
[^{3}H]Fuc	+++	+/–	+/–	+/–	+/–
[^{3}H]Gal	—	++++	+/–	+/–	+/–
[^{3}H]Glc	—	+++	+/–	+/–	+/–
[^{3}H]GlcNAc	—	+/–	++++	++	+++
[^{3}H]GalNAc	—	+/–	+++	+	++++
[^{3}H]Sia	—	—	++	++++	+/–
[^{3}H]GluA/ IdUA	—	—	—	—	—

6. Problems arising from the presence of substituents (acetate, sulfate, phosphate) or unexpected sugars

The presence of labile *O*-acetylesters in the sialic acids linked to glycoproteins is well documented in the literature (16, 17, 31). Their detection and quantification is particularly difficult and requires very careful manipulation. Methods for the analysis of *O*-acetylated sialic acids have been extensively

described in Sections 4.1.2 *ii*, 4.1.4, 4.3.1, and 4.3.2. *ii*. To the best of our knowledge, other *O*-acetylated sugars have not been conclusively demonstrated in glycoproteins from animal sources.

Sulfate esters are unstable in acidic conditions and are completely lost when acid hydrolysis or methanolysis are performed in all the conditions previously described. Their replacement by an acyl group (acetyl, deuteroacetyl, propionyl) has been attempted for further FAB-MS analysis (32). Initial detection can be achieved using the rhodizonate assay (33) when sufficient material is available (minimum amount of sulfate detected two nanomol), or by HPLC on an anion exchange column detected by conductivity (a few picograms can be detected) (34).

Phosphatases allows detection of change in the behaviour of the substituted glycoprotein that confirms the presence of phosphate esters. Organic phosphate can be determined using the assay described by Ames and Dubin (35), or by HPLC on an anion exchange column detected by conductivity (as in the case of sulfate, a few picograms can be detected) (34). Sugars phosphates usually obtained as salts can be converted to the corresponding free acids by passing the aqueous solution of the sugar through a Dowex 50 (hydrogen form) column, eluted with water. Adequate derivatives for GLC-FID or GLC-MS are obtained by esterification of the phosphoric acid with diazomethane (36), followed by trimethylsilylation as indicated for the rest of the sugars.

The presence of sulfates and phosphates is conveniently addressed by metabolic-labelling and analysis of the radiolabelled glycoproteins. Although quantitation is not possible, conclusive indication of the presence of such substituents and indications of their location can be obtained.

It has to be kept in mind that, the presence of any substituent may change the behaviour of the parent molecule in such a way that, for instance, it becomes resistant to enzymes (see Section 4.1.4) or presents a different susceptibility towards acid hydrolysis. Therefore, besides the problem encountered with an accurate determination of the content of a particular substituent when this is detected, the problem of an underestimation of the parent molecule may arise. Once again, the application of more than one method will reduce the possibility of error.

Only a few of the many possible stereoisomers of 6- and 9-carbon monosaccharides, bearing a few types of substituents, in a few positions, have been found as constituents of animal glycoproteins. The possibility of many more that may remain undiscovered can not be ruled out. It is wise to keep an eye open for such unexpected possibilities.

Acknowledgements

The authors are indebted to Sandra Diaz, Bradley Hayes, and Karin Norgad for helpful discussions.

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