I. Monosaccharides

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1. Introduction

Monosaccharides exhibit a great variety of structural types with vastly different chemical and physical properties. In contrast to the several methods available for the extensive analysis of the other major class of biological molecules, the amino acids, there is no single method which is suitable for the quantitative or qualitative analysis of all monosaccharides. The method of choice will depend on a number of factors including the accuracy required and resources available. There are two techniques available for the quantitative analysis of mixtures of monosaccharides, high performance liquid chromatography (HPLC) and gas-liquid chromatography (GLC). If only a qualitative analysis is needed, either paper chromatography (PC) or thin-layer chromatography (TLC) may be used. Single monosaccharides may be identified by

means of mass spectrometry (MS), infra-red spectroscopy (IR), or proton or carbon-~3 nuclear magnetic resonance (^{1}H-NMR, ^{13}C-NMR), and quantified by use of specific colorimetric or enzymatic assays. An extensive and growing literature exists which describes methods within these techniques. In this chapter a number of these methods have been chosen and described. They encompass the most commonly occurring analytical circumstances but several volumes would be needed if a fully comprehensive text was to be presented.

2. Extraction

Often the sugars may be in a solution that can be analysed directly (e.g. after 3cid hydrolysis) or are easily extracted with water. Sometimes, however, the material is more intractable and an extraction and/or clean-up is necessary. Ethanol/water (80% v/v) has proved to be a good general purpose extractant for monosaccharides in which proteins, polysaccharides, and many oligosaccharides are insoluble. Such monosaccharide solutions are stable. Difficult extractions may be made by refluxing the finely divided material for about an hour, but care must be taken to avoid any unwanted hydrolysis. Where the samples are particularly complex (e.g. many solid foodstuffs), aqueous extracts can be clarified of proteins, other soluble macromolecules, and finely suspended or emulsified solids by use of Carrez reagent *(Protocol 1)*.

Protocol 1. Carrez reagent

Reagents

A 85 mM Potassium ferrocyanide. Dissolve 3.6 g $K_4[Fe(CN)_6].3H_20$ in 100 ml solution.

B 0.25 M Zinc sulfate. Dissolve 7.2 g $ZnSO_4{\times}7H_20$ in 100 ml solution.

Method

To the sample, add 5% (v/v) of each of reagents A and B sequentially followed by 10% (v/v) of 0.1 M NaOH (0.4 g/100 ml), shaking well between each addition. Note the final volume and filter.

3. Colorimetric assays

A number of colorimetric assays are presented for the main classes of monosaccharides, followed by an important specific enzymatic assay. A standard format has been chosen to describe these assays. The sensitivity describes the range over which the assay is fairly linear with a maximum absorbance of about 1.0 OD unit for a 1 cm path length cuvette. Distilled or deionized water is used throughout for aqueous solutions. The final volume, for spectrophotometric measurement, has normally been kept within the 1-2 ml range to allow microanalytical cuvettes to be used, but larger volumes can be arranged by increasing the sample and reagent volumes in proportion. In general, the protocols should be followed in as reproducible a manner as possible. Standards should always be run to check that the protocol is delivering the appropriate sensitivity and the spectrophotometer is functioning correctly.

The non-stoichiometric constant volume titration of Lane and Eynon [1] is still used as a standard procedure for reducing sugar in the food industry. It requires about 100 mg reducing sugar (~14 mM) and considerable skill and experience. It is not recommended except where required, by legislation or standard practice, in food analysis.

Protocol 2. The general phenol-sulfuric acid assay for carbohydrate [2]

• Sensitivity: ~ 1-60 µg glucose in 200 µl • Final volume: 1.4 ml (~ 30 µM -2 mM)[a]

Method

1. Prepare the reagent by dissolving phenol in water (5% *w/v*).[b]
2. Mix samples, standards, and control solutions (200 µl containing up to 100 µg carbohydrate) with 200 µl of phenol reagent.
3. Add 1.0 ml of concentrated sulfuric acid rapidly and directly to the solution surface without allowing it to touch the sides of the tube.[c]
4. Leave the solutions undisturbed for 10 mm before shaking vigorously.
5. Determine the absorbances at 490 nm after a further 30 mm.

[a] Aldoses, ketoses, and alduronic acids respond to different degrees. Protein, cysteine, non-carbohydrate reducing agents, heavy metal ions, and azide interfere with this assay. However, it remains useful as a rapid non-specific method for the detection of neutral carbohydrate in column eluates, and is also applicable to solids containing carbohydrate, such as cereal flours, so long as all particles are milled to less than 50 µm diameter. The cysteine-sulfuric acid assay [3] may be used where a sixfold increase in sensitivity is required.

[b] This reagent is stable indefinitely.

[c] The reproducibility of this assay is strongly dependent on the manner of the addition of the sulfuric acid.

Protocol 3. The dinitrosalicylic acid assay for reducing sugar [4]

• Sensitivity: ~500 µg glucose in 100 µl (0.3-30 mM)[a] • Final volume: 1.1 ml

1. Prepare the reagent by dissolving 0.25 g of 3,5-dinitrosalicyclic acid and 75 g sodium potassium tartrate (Rochelle salt) in 50 ml of 2 M NaOH (4 g NaOH in 50 ml water) and dilute to 250 ml with water.[b]
2. To samples, standards, and controls (100 µl) add 1.0 ml of the reagent. Mix well.

3. Heat the mixtures at 100°C for 10 mm.
4. After rapid cooling to room temperature, determine the absorbance at 570 nm.

[a] This assay does not cause detectable inversion of sucrose. Dissolved molecular oxygen interferes with this assay. This may be overcome either by purging the assay solutions with nitrogen or helium prior to the assay. or by the addition of a fixed known small amount of glucose (20 μg) to all samples, in order to raise the total reducing sugar concentration above a critically low value. Non-carbohydrate reducing agents also interfere with this assay. Some metal ions (e.g. manganous, cobalt (II), and calcium) may increase the assay response. The neocuproine (5,6) or Nelson-Somogyi assays (see *Protocol* 4) may be used where up to a 500-fold increase in sensitivity is required.

[b] The reagent is sensitive to CO_2. It is stable for several weeks if stored purged with helium or nitrogen, otherwise it should be freshly prepared.

Protocol 4. The Nelson-Somogyi method for reducing sugars [7]

• Sensitivity: ~ 10-100 μg glucose in 1 ml • Final volume: 6.0 ml[b] (~ 60-600 μM)

Reagents

A Dissolve 15 g of sodium potassium tartrate and 30 g of anhydrous Na_2CO_3 in about 300 ml water. Add 20 g $NaHCO_3$. Dissolve 180 g of anhydrous Na_2SO_4 in 500 ml boiling water and cool. Mix the two solutions and make up to 1 litre with water.[a]

B Dissolve 5 g $CuSO_4.5H_20$ and 45 g anhydrous Na_2SO_4 in water and make up to 250 ml.[a]

C. Mix reagents A (4 vol.) and B (1 vol.) just before use.

D Dissolve 25 g ammonium molybdate in 450 ml water. carefully add 21 ml concentrated H_2S0_4 with stirring. Dissolve 3 g $Na_2HAsO_4.7H_20$ in 25 ml water and add to the molybdate solution. Incubate for 24-28 h at 37°C and store in a brown glass-stoppered bottle. *a* Just before use this reagent should be diluted with 2 vol. of 0.75 M H_2S0_4 (4 ml concentrated H_2S0_4 in 100 ml solution).

Method

1. Mix the samples, standards, and control solutions (1.0 ml) containing up to 800 nmol reducing sugar with 1.0 ml of reagent C in small stoppered test-tubes.
2. Heat at 100°C for 15 mm. Cool the solution rapidly to room temperature.
3. Add reagent D (1.0 ml) and mix well.
4. Add 3.0 ml water, mix, and measure the absorbance at 520 nm.

a The reagents are stable in their concentrated unmixed forms.

b Care should be taken to minimize reoxidation by air (see *Protocol 3).*

Protocol 5. Ferric-orcinol assay for pentose [8]

• Sensitivity: 0.2-20 μg xylose in 200 μl Final volume: 1.6 ml (~ 7-700 μM)[a]

Reagents

A Trichloroacetic acid solution in water (10% *w/v).*[b]

B Freshly prepared solution of ferric ammonium sulfate (1.15% w/v) and orcinol (0.2% w/v) in 9.6 M HCI (made by diluting five parts concentrated HCI with one part water).

Method

1. Mix the samples, standards, and control solutions (200 μl) containing up to 40 μg pentose with 200 μl of reagent A.
2. Heat at 100°C for 15 mm. Cool the solution rapidly to room temperature.
3. Add reagent B (1.2 ml) and mix well.
4. Re-heat the solution at 100°C for a further 20 mm.
5. Cool the solution to room temperature and determine the absorbance at 660 nm.

[a] Hexoses interfere in this assay but can be accounted for by additionally determining the absorbance at 520 nm at which wavelength they have a strong absorbance. It is recommended that both hexose and pentose standards are used where the hexose content of the samples might be considerable (see Section 3.2).

[b] This is stable indefinitely.

Protocol 6. The phenol-boric acid-sulfuric acid assay for ketose [9]

• Sensitivity: ~ 0.1-9 μg fructose in 100 μl • Final volume: 2.0 ml (~ 30-500 μM)[a]

Method

1. Prepare the reagent by dissolving 2.5 g phenol (recrystallized from methanol and ethanol) in 50 ml water. Add 1.0 ml of acetone drop-wise with constant stirring over a period of 10 mm and stir the mixture for a further 10 mm at room temperature. Dissolve 2.0 g of boric acid in the mixture.[b]
2. Mix the samples, standards, and controls (100 μl) with 0.5 ml of reagent and then rapidly add 1.4 ml of concentrated sulfuric acid directly to the surface, avoiding the sides of the tubes.
3. After thorough mixing, leave the solutions for 5 mm at room temperature.
4. Incubate at 37°C for 1 h.
5. Determine the absorbance at 568

[a] Different ketoses give differing absorbances in this assay. Interferences from non-ketose carbohydrate is slight (<

1%) to non-existent. The reproducibility of this assay is strongly dependent on the manner of the addition of the sulfuric acid.

[b] The reagent is stable for at least two weeks at 4°C.

Protocol 7. The Morgan-Elson assay for hexosamine [10]

• Sensitivity: ~ 0.06-6 µg 2-acetamido-2-deoxy-D-glucose in 250 µl (~ 1-110 µM)[a] • Final volume: 1.8 ml

Reagents

A Dissolve 6.1 g of dipotassium tetraborate tetrahydrate in 80 ml of water and make up to 100 ml with water.

B Add 1.5 ml of water to 11 ml of concentrated HCl. Add a further 87.5 ml of glacial acetic acid and dissolve 10 g of 4-(N,N-dimethylamino)benzaldehyde in this mixture.[b] Dilute 10 ml to 100 ml with glacial acetic acid immediately prior to use.

Method

1. Add samples, standards, and controls (250 µl) to 50 µl of reagent A.
2. Heat each mixture at 100°C for 3 mm.
3. After cooling rapidly to room temperature, add 1.5 ml of reagent B, washing down any condensate formed.
4. Incubate the samples at 37°C for 20 mm.
5. After cooling to room temperature, determine the absorbance at 585 nm.

[a] 2-Acetamido-2-deoxy-D-galactose gives only one third the response of 2-acetamido-2-deoxy-D-glucose in this assay. Free amino hexoses may be *N*-acetylated prior to this assay by the addition of one part of freshly prepared 1.5% (v/v) acetic anhydride in acetone to eight parts of the aqueous solution and leaving for 5 mm at room temperature.

[b] This solution may be stored for several weeks.

Protocol 8. The carbazole assay for uronic acids [11]

• Sensitivity: ~ 0.2-20 µg D-galacturonic acid in 250 µl (~ 4-400 µM)• Final volume: 1.8 ml

Reagents

A Dissolve 0.9 g of sodium tetraborate decahydrate in 10 ml of water and add 90 ml of ice-cold 98% concentrated sulfuric acid carefully to form a layer. Leave undisturbed overnight to mix without excessive heat production. Check it is thoroughly mixed and at room temperature before use.[b]

B Dissolve 100 mg of carbazole (recrystallized from ethanol) in 100 ml of absolute ethanol.[b]

Method

1. Cool the samples, standards, and controls (250 µl) in an ice-bath.
2. Carefully add ice-cold reagent A (1.5 ml) with mixing and cooling in the ice-bath.
3. Heat the mixtures at 100°C for 10 mm.
4. Cool rapidly in the ice-bath.
5. Add 50 µl of reagent B and mix well.
6. Re-heat at 100°C for 15 mm.
7. Cool rapidly to room temperature and determine the absorbance at 525 nm.

[a] Neutral carbohydrates interfere with this assay to a greater (10% on a molar basis for hexoses) or lesser extent (2% on a molar basis for 6-deoxyhexoses). However interference can be reduced by use of appropriate controls as non-uronic acid carbohydrates give significantly different absorption spectra. Cysteine and other thiols increase the response of the assay but large amounts of protein may depress the colour development. Different uronic acids give different responses in this assay.

[b] These reagents are stable indefinitely if refrigerated.

Protocol 9. The Warren assay for sialic acid [12]

• Sensitivity: 0.08-8 µg N-acetylneuraminic acid in 80 µl (~ 3-300 µM)[a] • Final volume. 1.0 ml

Reagents

A Dissolve 4.278 g of sodium metaperiodate in 4.0 ml water. Add 58 ml of concentrated orthophosphoric acid and make up to 100 ml with water.[b]

B Dissolve 10 g of sodium arsenite, 7.1 g of sodium sulfate, and 10 mg potassium iodide in 0.1 M sulfuric acid (made by carefully diluting 5.7 ml concentrated sulfuric acid to one litre with water) to a total volume of 100 ml.[b]

C Dissolve 1.2 g of 2-thiobarbituric acid and 14.2 g of sodium sulfate in water to a total volume of 200 ml.[c]

D Redistilled cyclohexanone. [d]

Method

1. To the samples, standards, and controls (80 µl) add 40 µl reagent A and mix well. Leave at room temperature for 20 mm.
2. Add 400 µl reagent B and then shake the tubes vigorously to expel the yellow coloured iodine. Leave for a further 5 mm at room temperature.
3. Add 1.2 ml reagent C, shake the tubes, stopper them, and heat at 100°C for 15 mm.
4. Cool rapidly to room temperature.

5. Extract the chromophore into 1.0 ml of reagent D by vigorous shaking.
6. Centrifuge the solutions using a bench centrifuge for a few minutes in order to properly separate the two layers.
7. Determine the absorbance of the upper cyclohexanone layer of 549 nm.

[a] DNA, 2-deoxy-o-ribose. and substances producing malondialdehyde on period-ate oxidation interfere in this assay. This may be circumvented by additionally determining the absorbance at 532 nm and calculating from the resultant data (see Section 3.21. L-Fucose reduces the expected absorbance of this assay. Methoxyneuraminic acid and some acetylated neuraminic acids give no colour in this assay. The interfering O-acetyl groups may be removed by alkaline hydrolysis (0.1 M NaOH, 30 min, 37°C) followed by neutralization with 0.2 M HCl. Alternatively the resorcinol-HCl assay [13] may be used if the presence of any of these is suspected. The assay is less specific than the Warren assay, however, and is not recommended for general use.

[b] This reagent is stable indefinitely.

[c] This reagent is stable for several weeks but eventually forms a yellow precipitate which indicates the need for its renewal.

[d] This is stable for several months until noticeably discoloured.

3.1 Enzymatic methods

Enzymatic methods utilize the specificity of enzymes to pick out their substrates from mixtures, and are ideal for the analysis of known carbohydrates in complex mixtures, such as clinical samples and foodstuffs. The methods used are generally variations of that used for glucose *(Protocol 10).*

Protocol 10. The hexokinase/dehydrogenase assay for glucose [14]

• Sensitivity: 0.4-40 µg glucose in 100 µl • Final volume: 1.4 ml (20 µM -2 mM)[a]

Reagents[b]

A 0.33 M Triethanolamine, 4.3 mM Mg^{2+}. Dissolve 6.0 g triethanolamine hydrochloride and 0.11 g $MgSO_4.7H_20$ in 80 ml water. Adjust the pH to 7.6 with concentrated NaOH solution (20% w/v) and make up the solution to 100 ml with water.[c]

B 5.5 mM NADP. Dissolve NADP (disodium salt, 4.3 mg/ml) in distilled water.

C 35 mM ATP, 0.26 M $NaHCO_3$. Dissolve ATP (disodium salt hydrate, 22 mg/ml) and $NaHCO_3$ (22 mg/ml) in water.[c]

D 3.2 M Ammonium sulfate. Add 0.6 g ammonium sulfate to 1 ml of water and allow to dissolve.[d]

E Dissolve hexokinase (ATP: D-hexose-6-phosphotransferase, EC 2.7.1.1 *ex.* yeast, 280 U/ml, 2 mg/ml) and glucose-6-phosphate dehydrogenase (D-glucose-6-phosphate: NADP 1-oxidoreductase, EC 1.1.1.49, *ex.* yeast, 140 U/ml, 1 mg/ml) in solution *D.*[c]

Method

1. Add each sample and standard solution (100 µl) to a mixture containing 1.0 ml buffer solution A, 100 µl reagent B, and 100 µl reagent C. Mix well.
2. Start the reaction with 100 µl of enzyme solution F. The control solutions lack enzyme and so should consist of sample solution (100 µl) plus reagents A (1.1 ml), B (100 µl), and C (100 µl).
3. After further mixing, incubate the solutions at 37°C for 30 mm.
4. Cool and determine the absorbance at 340 nm. The reaction should have stopped at this stage. However, check whether there is a significant change (> 5%) in absorbance at 340 nm after a further 30 mm incubation at 37°C. If so, check the reagents and/or extend the incubation time.

[a] The sensitivity of this spectrophotometric assay may be increased 10-100-fold (0.2-20 µM) by using the fluorescence change rather than the absorption change. The excitation wavelength is 340 nm and the fluorescence is emitted at about 465 nm. This improvement in sensitivity is achieved at an extra cost in the care needed for the assay. All solutions should be dust-free and all glassware scrupulously cleaned. The cuvettes should be of low fluorescence glass or quartz and temperature-equilibrated before the determinations are made. Because of the higher background variability, this method is best chosen only when the additional sensitivity over the spectrophotometric assay is essential.

[b] Some of these reagents are available in a kit form from manufacturers such as Boehringer Mannheim GmbH.

[c] This solution is stable for a month at 4°C.

[d] This solution is stable.

Protocol 10 can be adapted for the determination of fructose and/or mannose in the presence or absence of glucose. Determine fructose by the addition of 100 µl phosphoglucose isomerase (D-glucose-6-phosphate ketol-isomerase, EC 5.3.1.9, *ex.* yeast, 65 U/ml, ~0.2 mg/ml in buffer solution A) to the reaction mixture after the glucose content has been determined. Mix this solution, incubate at 37°C for 30 minutes, cool, and re-measure the absorbance at 340 nm. Determine mannose in a similar manner subsequent to the addition of 100 µl phosphomannose isomerase (D-mannose-6-phosphate ketol-isomerase EC 5.3.1.8, *ex.* yeast, 60 U/ml, 1.0 mg/ml in buffer solution A). The sensitivities for fructose and mannose are similar to those for glucose. Starch and sucrose may also be determined [14] after enzymatic conversion to monosaccharides by glucoamylase or invertase respectively. As the enzymes are optimally active at substantially lower pH than hexokinase it is recommended that a separate procedure be adopted whereby the hydrolysed and unhydrolysed

samples are analysed in the above assay. Similar assay systems may be set-up for the determination of other carbohydrates where the appropriate enzyme is available, for example L-fucose using fucose dehydrogenase [15] and D-galactose using β-D-galactose dehydrogenase [16]. In these cases the buffer solution A should be replaced by a buffer appropriate to the determination and reagents B, C, D, and E will all probably be different. The principle of the assay however will remain as the change in absorbance at 340 nm due to the formation of the reduced dinucleotide.

3.2 Example calculations

A mixture of two components (A, B) can simply be determined spectrophotometrically if each component absorbs maximally at different wavelengths (X nm and Y nm).

(a) Using a suitable range of standard concentrations for both components separately determine the absorbance produced by each at both wavelengths (i.e. component A at concentration C_A gives absorbance ΔA_X at wavelength X nm and ΔA_Y at Y nm. Similarly component B at concentration C_B gives absorbance ΔB_X at X nm and ΔB_Y at Y nm).

(b) Determine the absorbance difference between blank and unknown sample S at the same wavelengths X (ΔS_X) and Y (ΔS_Y).

(c) The concentration of A in the sample is $\tilde{N}_A \times \frac{\Delta S_X \times \Delta B_Y - \Delta S_Y \times \Delta B_X}{\Delta A_X \times \Delta B_Y - \Delta A_Y \times \Delta B_X}$

The concentration of B in the sample is $\tilde{N}_B \times \frac{\Delta S_X \times \Delta A_Y - \Delta S_Y \times \Delta A_X}{\Delta B_X \times \Delta A_Y - \Delta B_Y \times \Delta A_X}$

For example, for the pentose assay *(Protocol 5)* the absorbance maxima X and Y are 660 nm and 520 nm, respectively. The absorbance of 20 µg xylose (A) at these maxima are $\Delta A_X = 1.0$ and $\Delta A_Y = 0.25$, whereas 180 µg of glucose (B) gives $\Delta B_X = 0.25$ and $\Delta B_Y = 0.60$.

4. Thin-layer chromatography [17-19]

4.1 Experimental approach

Thin-layer chromatography is a simple and rapid technique that is very useful for the preliminary examination of carbohydrate mixtures and as a screening technique for multiple samples. A number of solid supports have been used with a vast number of solvents and detection methods for separating a wide range of carbohydrates. Adding to the difficulty of choosing a system for recommendation is the fact that workers in this field never seem to choose the same range of standard sugars to calibrate and compare their systems. It is clear, however, that no single system is available that will separate all possible combinations of carbohydrates. The best plan is to try likely systems until a suitable one is found.

There are two solid supports which have proved themselves to be particularly useful, microcrystalline cellulose and silica gel. Cellulose separates essentially by liquid-liquid partition. The sugar is distributed between the mobile phase and the cellulose-bound water complex, dependent upon the solubility of the sugar in the eluent and the ease with which it can enter the structures of the complex and/or solid support. This latter ability is determined by its size and steric configuration. Generally cellulose TLC has the same chromatographic characteristics as paper with the advantages that elution times are shorter and the sensitivity enhanced.

Silica gel separates in a similar manner but with an additional adsorption component. Often inorganic salts (e.g. phosphate, bisulfite, or citrate) are impregnated into the gel by wetting with the salt solution followed by thorough drying after the plates have been coated, or by inclusion in the slurry solvent. In these cases, the selectivity of the inorganic salt greatly influences the carbohydrate separation and is, in turn, determined by its concentration and ionic form. In use, a gradient of salt is formed up the plate according to the composition of the eluent.

Thin-layer plates can either be prepared in the laboratory or purchased ready-coated with cellulose, silica gel, or impregnated silica gel as the solid support. Plates prepared in the laboratory should be thoroughly dried in an oven at 100°C and stored in a desiccator until used. In general, precoated plates are to be preferred to home-made plates as they give excellent reproducibility, a higher sensitivity to detection reagents, and because of their bonded strength, they allow multiple elutions and reagent applications without their surface breaking up. Simple, inexpensive, and rapid preliminary investigation of a system is possible by use of coated microscope slides. Clean dry slides are coated by dipping in a slurry of the chromatographic material, dried, and run in a covered beaker.

The choice of a suitable solvent for TLC is not easy unless a very simple sugar mixture is anticipated. The initial choice should lie between the solvent systems suggested below (see *Table I)* but if all of these prove unsatisfactory there is an abundance of choice in the literature [17-19]. It should be noted, however, that even under optimal conditions a maximum of about ten carbohydrates may be separated in a one-dimensional run, increasing to about 20 if a suitable two-dimensional system is appropriate. Carbohydrates with relative R_fs closer than about 5% cannot normally be resolved unless a discriminating detection system is used. Solvents are generally binary, tertiary, or quaternary and always include an aqueous solution, usually between 10 and 20% by volume. Small changes in the composition of such mixtures may have large and possibly unpredictable effects on the relative movement of the carbohydrate (R_f) and the efficiency of the separations; for example, the elution order of glucose, mannose, and galactose may change. Therefore, R_f or $R_{glucose}$ values

should not be regarded as constant parameters, especially between laboratories. They may vary with temperature, humidity, coating batch, coating method, and thickness, any pretreatment, and chromatographic tank size. They should be established in the system under scrutiny and literature values should be used for guidance purposes only. As a general rule, a carbohydrate has a higher R_f if it is more hydrophobic or of lower molecular weight. There are many apparent exceptions to this rule however.

Table 1. Retention data[a] and sample visualization[b]

Monosaccharide	TLC (Solvent[c])					PC	Visualization	
	(a)	(b)	(c)	(d)	(e)	(f)	11A	11B
D-Xylose	1.32	-[d]	1.34	1.70	1.54	1.79	Faint grey blue	Brown
D-Ribose	1.56	-	1.49	1.95	1.46	2.14	Faint grey blue	Light blue
L-Arabinose	1.24	-	1.30	1.45	1.15	1.36	Faint grey blue	Light blue
D-Glucose	1.00	1.00	1.00	1.00	1.00	1.00	Blue grey	Violet
D-Galactose	1.24	0.89	0.93	0.87	0.73	0.80	Blue grey	Blue
D-Fructose	-	-	1.21	1.26	1.12	1.38	Light red	Purple/red
D-Mannose	1.20	1.13	1.17	1.15	1.22	1.43	Blue grey	Blue
L-Fucose	-	1.34	1.44	1.83	1.59	1.96	Olive intense blue	Pink
D-Glucosamine	-	0.72	-	0.0	0.0	-	Pale grey	Grey
D-Galactosamine	-	0.55	-	0.0	0.0	-	Pale grey	Grey
Reference	[17]	[20]	[18,19]	[21][e]	[22][e]	[23]	[17][e]	[17][e,f]

[a] Values given are $R_{glucose}$.
[b] Colours using silica gel TLC after incubation for the times indicated in *Protocol 11*.
[c] See Section 4.1 for details.
[d] Not reported.
[e] Chaplin, M. F. (1993). Unpublished results.

Given below is a list of solvent systems for use in TLC and PC.

(a) Ethyl acetate/pyridine/water: mix 100 ml of ethyl acetate with 35 ml of pyridine and 25 ml of water. This solvent is suitable for the TLC and PC analysis of hexoses, deoxyhexoses, and some disaccharides on cellulose using three successive developments.

(b) Butanol/pyridine/0.1 M HCl: mix 50 ml of n-butanol with 30 ml of pyridine and 20 ml 0.1 M HCl (made up by adding 1.0 ml of concentrated UCI to 114 ml of water). This solvent is suitable for TLC and PC use on cellulose in order to separate monosaccharides derived from the acid hydrolysis of glycoproteins, i.e. galactose, mannose, fucose, glucosamine, and galactosamine.

(c) Formic acid/ethyl methyl ketone/tert-butanol/water: mix 30 ml of formic acid, 60 ml of ethyl methyl ketone (2-butanone), 80 ml of tert-butanol, and 30 ml of water. This solvent is suitable for TLC and PC use on cellulose for the analysis of carbohydrates derived from plant extracts, including uronic acids. D-Arabinose may be distinguished from its L-isomer.

(d) Acetonitrile/water: mix 85 ml acetonitrile (HPLC grade) with 15 ml water. This solvent is suitable for use, employing three successive developments, on silica plates that have been pretreated by spraying with 0.1 M sodium metabisulfite (1.9 g/100 ml), followed by drying and spraying with 0.009 M sodium citrate/citric acid buffer pH 4.8 (159 mg trisodium citrate dihydrate + 76 mg citric acid monohydrate in 100 ml), finally drying at 100°C for 1 h and keeping desiccated until use.

(e) 2-propanol/acetone/0.1 M lactic acid: dissolve 148 mg of lactic acid in 20 ml of water. Add 40 ml of 2-propanol and 40 ml of acetone. This solvent system is recommended for use on phosphate impregnated silica gel plates. The system separates many of the more common constituents found in the clinical analysis of urine and plasma (e.g. glucose, galactose, and mannose). The impregnated plates are obtained by either:
   i. buying phosphate-activated precoated silica gel plates
   ii preparing the coating silica gel slurry in *0.5* M NaH_2PO_4 or KH_2PO_4 (dissolve 19.5 g of $NaH_2PO_4.2H_20$ in 250 ml of water)
   iii spray dampening precoated plates in 0.5 M NaH_2PO_4 followed by drying.

(f) n-Butanol/pyridine/water: mix 100 ml of n-butanol with 30 ml of pyridine and 30 ml of water. This solvent is suitable for the PC. analysis of carbohydrates derived from glycoproteins. A run takes about 25 hours at room temperature. The chromatogram should be dried in a fume cupboard for about 4 hours (until only a faint odour of n-butanol remains) before use of detection reagents.

(g) n-Butanol/n-propanol/0.1 M HCl: mix 25 ml of n-butanol with 50 ml of n-propanol and 25 ml of 0.1 M HCl. This solvent is suitable for the PC and TLC analysis, on cellulose, of sialic acids derived from glycoproteins.

4.2 Detection methods

There has been a large number of spray reagents described in the literature for the detection of carbohydrates. Two are

suggested here *(Protocol 11A* and B, see *Table I)* which cover most analyses. They give different colours with different carbohydrates and are useful in identifying components in mixtures and overlapping unresolved carbohydrates. The third reagent *(Protocol 11C)* may be used for dipping silica HPTLC plates prior to densitometric analysis. The colour of background and samples produced by these detection systems is dependent on both the temperature and the duration of the heating period. This might affect the selectivity of the reaction. Additionally trace amounts of eluent persisting in the chromatographic layer may affect both the colour and sensitivity of the reactions. Care should be taken with precoated plates to avoid destroying the coating by overheating during colour development.

It is possible to quantify material separated by TLC. Dipping of the plates give a much more uniform colour development than spraying. Direct densitometric assessment of the sprayed plates is possible but, due to difficulties arising from spot irregularity, perhaps should only be used with samples applied as bands in one-dimensional TLC. In addition, the spray detection techniques give poor reproducibility of quantitative results even on the same plate. It is best to locate the bands by use of co-chromatographed standards, identified by spray detecting parts of the TLC plates, while protecting other parts by shielding with a glass plate. The samples may then be scraped off from the unsprayed part of the plate, eluted, and analysed by a specific colorimetric assay. Care must be taken that different impurities in standard or sample do not cause a difference in R_f. The dipping method largely avoids this problem. Plates are dipped at uniform pace into and out of the reagent (24 sec), avoiding pauses which may cause tide-marks. Dipping must be carried out in such a way that the sugars are not lost from the plates; e.g. by leaving in a wet state for too long allowing spots to diffuse away. Instrumentation is being developed for the mass spectrometric scanning of TLC plates [24].

Protocol 11. Detection reagents for use in TLC and PC

A. *Diphenylamine/aniline/phosphoric acid*

1. Prepare the spray reagent solutions:
   - dissolve 4 g of diphenylamine in 80 ml of acetone and make up to 100 ml with more acetone
   - add 4 ml of aniline to 96 ml of acetone and mix well
   - 20 ml of 85% ortho-phosphoric acid

   Mix the three solutions just prior to use.
2. Spray the plate, air dry, and then heat at 100°C for 10 mm. The colour appears after 2-4 min. This spray reagent can be used for aldoses, ketoses, deoxysugars, oligosaccharides, and uronic acids and may be used on both cellulose and silica thin-layer plates. It gives a wide variation of colours with different carbohydrates, aldoses producing blue grey spots whereas ketoses give light red spots. The sensitivity is about 1 µg.
3. For the PC dip reagent, dissolve 0.15 g of diphenylamine in 25 ml of ethyl acetate. Add 0.8 ml of aniline and 75 ml of ethyl acetate. Finally add 1.0 ml of water and 10 ml of concentrated ortho-phosphoric acid. The reagent should be made up immediately before use.
4. Dip the dry chromatograms through the reagent, dry, and heat at 95-100°C until the background is faintly grey. This is a good general purpose reagent giving similar results to the spray reagent.

B. *Naphthoresorcinol/ethanol/sulfuric acid*

1. Prepare solution A by dissolving 0.2 g of naphthoresorcinol (naphthalene-1,3-diol) in 100 ml of 95% ethanol. Diphenylamine (0.4 g) may be added in order to reduce the background coloration.
2. For the spray reagent, carefully add 4 ml of concentrated sulfuric acid to 96 ml of solution A just prior to use.
3. Spray the plate and then heat at 100-150°C for 5 mm.
4. This spray reagent can be used for aldoses, ketoses, uronic acids, deoxysugars, glycosides, and oligosaccharides. It is recommended for use only on silica thin-layer plates. It gives a wide variation of distinctive colours with different carbohydrates; aldoses producing blue or violet spots, ketoses producing pink or red spots, and uronic acids producing characteristic blue spots. The sensitivity is between 0.1 µg (L-sorbose) and 4 µg (o-glucose).

C. *Ceric sulfate/sulfuric acid*

1. Mix 5 ml of 0.1 M ceric sulfate with 100 ml of 15% (v/v) sulfuric acid. This can be used as a dip reagent for silica HPTLC plates.
2. Heat rapidly to 120°C for 15 mm to develop the colour.
3. Hexoses, deoxyhexoses, and pentoses appear as blue grey, brown, or pinkish-grey spots, respectively, on a white background. The spots can be quantified by densitometric scanning at 440 nm. Gentle brushing of the plates with a camel hair brush immediately prior to dipping provides a cleaner background by removing small dust particles that otherwise char. The sensitivity is between about 1 µg (hexoses and pentoses) and 10 µg (hexosamines).

4.3 General thin-layer chromatographic method

For one-dimensional development, samples and standard mixtures (made by dissolving 0.05-2 mg dry component in 0.5 ml water plus 0.5 ml isopropanol) are applied, via a drawn-out capillary tube, as short streaks (1-2 cm long) 1.0 cm from the edge to be dipped in the eluent and parallel to that edge. Specialist spray applicators may be used. The volume applied should depend on the sensitivity of the detection system and the estimated concentration of the carbohydrate mixture. If more than one application is necessary, the previous application should first be dried well by a gentle draught of warm

(not hot) air from a hand-held hair dryer. After sample application, the dry plates are placed in chromatographic tanks to which the elution solution has already been placed sufficient in advance to saturate its atmosphere. Filter paper placed along one side of the tank will help this process. Given a choice, the coated face of the plate should be pointed towards the eluent rather than away from it. The top should be placed on the tank and the chromatogram developed at room temperature. This usually takes about 2# mm/cm. If a multiple run is necessary (e.g. using a cellulose plate in ethyl acetate/pyridine/ water (solvent system (a)) for hexoses), the plate should be removed and dried thoroughly in a stream of warm air before running again, in order to avoid streaking and diffuse spots. Each run should be slightly longer than the previous one.

If a two-dimensional development is to be attempted, the sample is applied as a spot to a corner position on the plate 1.0 cm from each side. The second run will be at right angles to the first in a significantly different solvent. The plate should be thoroughly dried in a stream of warm air before the second run is commenced. A difficulty with this method concerns the standards since they have to be included with the unknown sample and co-chromatographed. Urea is reported to be a useful internal standard as it is generally well separated from carbohydrates but can be identified with the naphthoresorcinol reagent *(Protocol 11B).* The plates should be thoroughly dried in a stream of warm air before using the spray reagents for detecting the sugars. It is suggested that a commercial aerosol spray gun be used, spraying into a fume cupboard.

HPTLC precoated plates are now available from a number of suppliers. These have a much narrower particle size and particle size range enabling an improved resolution at higher efficiency. In addition more samples may be applied to each plate due to the small degree of spreading and, hence, spot size. The smoother, more homogeneous, media gives improved reproducibility and samples may be quantified by densitometry in a far more satisfactory manner than standard TLC plates. Some of these plates also come with a concentration zone for improved sample application. The major and only drawback to the use of these plates is their relatively high cost (>£3 per 10 x 10 cm plate, in 1993).

5. Paper chromatography [25]

Paper chromatography has similar characteristics to TLC on cellulose. It is mainly a partition process with the possibility of some adsorption. It is a heap and simple method and easier to use, on a preparative scale than TLC. The use of dip reagents for visualizing the spots *(Protocol 11A,* step 4, see *Table* I) is also an easier and more uniform process than spraying. Although the solvents, developed for cellulose-coated TLC may be used in paper chromatography, some systems have been developed particularly for the paper medium. One such solvent system is n-butanol/pyridine/water (see Section 4.1, solvent system (f)). In general, the preferred conditions are descending chromatography on Whatman No.1 paper. Multiple or continuous development may be used. For this latter technique pinking shears are used on the bottom end of the paper to ensure a uniform flow of the solvent. Thicker paper (e.g. Whatman No.17) can be used for preparative separations of up to about 0.5 grams, bands being visualized by using a dip reagent on a copy of the chromatogram "blotted" onto thin chromatographic paper (e.g. Whatman No. 1). Chromatography paper is most often used straight from the pack but pre-washing (e.g. with 0.25% (w/v) hydroxyquinoline in 8% (w/v) acetic acid or sequentially with 0.1 M HCI, water, and chloroform/methanol (2:1 v/v)) may be beneficial. In either case the paper should be well equilibrated in the solvent vapours for several hours before use. For reproducible R_fs the chromatographic runs should be performed under constant temperature conditions in cold (slower running) or warm (faster running) rooms. Many a run has been spoilt by running overnight in a laboratory with no overnight heating.

6. High performance liquid chromatography

The separation and quantification of the components in mixtures of monosaccharides forms an important part of carbohydrate analysis. There are two main methods available, HPLC and GLC, which both deliver quantitative data where standards are available. The choice between these two depends on a number of factors in addition to personal preferences and prejudice. One clearly important factor is the available resources and expertise. Both methods need fairly expensive equipment and are facilitated by helpful and experienced operators. Experience in running these systems can only be built up over a fairly long period through a number of mishaps and, therefore, it is best if it is gained second-hand. As a broad generalization, HPLC is preferred for the analysis of simple monosaccharide mixtures, oligosaccharide analysis and purification, whereas GLC can be used on very complex monosaccharide mixtures. There are, however, protocols available for separating and analysing most mixtures of carbohydrates by either method. Advances in both methods, but particularly HPLC, are constantly being made and reported in the literature (e.g. in *Analytical Biochemistry* and the *Journal of Chromatography)* and by the column manufacturers and suppliers. This section describes the practical use of HPLC for carbohydrate analysis and Section 7 describes GLC.

6.1 Experimental approach

There are a number of different HPLC columns and processes for separating carbohydrates which depend on different chemical and physical properties for resolution (see [26,27] and *Table 2).* This is a somewhat confusing state of affairs but no single method is useful over the entire range of possible separations. Advice and literature from prospective column suppliers. concerning the separation required, should be sought before any column is bought.

Table 2. Typical conditions for the HPLC of monosaccharides

Column type	Mobile phase	Separation mechanism	Typical analysis	Commercial analytical columns
Anion exchange (quaternary ammonium)	(a) Sodium hydroxide	Anion exchange	Alditols, monosaccharides	Dionex CarboPac MA1 (0.4 x 25) Dionex CarboPac PA1 (0.4 x 25)
	(b) Acetate buffers		Sialic acids, uronic acids	Bio-Rad Aminex A-29 (0.9 x 99)
Anion exchange (amino-propylsilane bonded silica, OH-form)	Acetonitrile/water	H-bonding between hydroxyls and amines	Monosaccharides, oligosaccharides	Waters Carbohydrate Analysis (0.39 x 30) Supelco Supercosil LC-NH_2 (0.46 x 25) Whatman Partisil 10 PAC (0.4 x 25)[c] Varian MicroPak AX-5 (0.4 x 30) Merck Lichrosorb NH_2 (0.4 x 25) Alltech Carbohydrate 1[d]
Cation exchange (sulfonate, H^+ form[a])	(a) Citrate buffers (b) Acetonitrile/water	Cation exchange Ion-moderated partition	Hexosamines Glycoprotein-derived carbohydrates, uronic acids, lactones	Pierce PC-6A (0.9 x 30) Bio-Rad Aminex HPX-87H (0.78 x 30)
Cation exchange (sulfonate, Ca^{2+} form[a])	Water	Ion-moderated partition	Alditols, mono-saccharides, and oligosaccharides	Waters Sugar-Pak I (0.65 x 30) Bio-Rad Aminex HPX-87C (0.4 x 25)
Cation exchange (sulfonate, Pb^{2+} form[a])	Water	Ion-moderated partition	Pentoses and hexoses	Bio-Rad Aminex HPXE7P (0.78 x 30) Bio-Rad Fast Carbohydrate (0.78 x 10)
Cation exchange (sulfonate, Ag^+ form[a])	Water	Ion-moderated partition	Monosaccharides, oligosaccharides	Bio-Rad Aminex HPX-42A (0.78 x30) Bio-Rad Aminex HPX-65A (0.78 x30)
Silica, straight phase	(a) Acetonitrile/water	Polar interactions	Prederivatized carbohydrates	Whatman Partisil O (0.46 x 25) Merck (LiChrosorb Si60 (0.4 x 25)
	(b) Acetonitrile/water + diaminoalkanes	H-bonding between hydroxyls and amines	Monosaccharides, oligosaccharides	Waters µPorosil (0.39 x 30)
Silica, reverse phase	Acetonitrile/water	Hydrophobic interactions	Prederivatized carbohydrates, oligosaccharides	Waters µBondakpak C_{18} (0.39 x 30) Merck LiChrosorb RP-18 (0.4 x 25) Waters Radialpak C_{18} (0.4 x 30) Supelco Supelcosil LC-18 (0.45 x 25) DuPont Zorbax ODS (0.46 x 25) Toso Haas TSK ODS-120-T (0.46 x 25)

[a] Bonded to polymerized styreneldivinylbenzene (PS-DVB).
[b] Ethylvinyl benzene/divinylbenzene pellicular non-porous.
[c] Contains cyano and secondary amine groups (2:1); resistant to Schiff base formation.
[d] Cross-linked amino-propyl phase; resistant to hydrolysis.

Sulfonated polymeric columns containing metal-loaded cation exchangers. at moderately high temperatures (~85°C), or amino-bonded silica columns operating at around ambient temperature are popular. The cation exchangers act by ion-moderated partition, forming linkages to the sugar hydroxyl groups, and size exclusion. The variations in resolution between columns containing the different metal counterions (Ag^+, Ca^{2+}, and Pb^{2+}) are due to the different modes of complex formation. Elution is roughly in order of increasing affinity but decreasing molecular weight; the resolution normally' increasing with the temperature as long as the ion-exchange resin is stable. Of these cation exchangers, the H+ form is suited for carbohydrate mixtures including acids and the separation of amino sugars, the Ca^{2+} form is the most popular general purpose column which is used for corn sugars and syrups and at low temperatures (1.5°C) separates a- and fi-anomers, the Pb^{2+} form is used for mixed hexoses and pentoses such as those released from cellulosic materials, and the Ag^+ form is most useful where oligosaccharides also need to be separated from monosaccharides. It is essential to remove Na^+ and K ions (e.g. by prior de-ashing ion-exchange or use of a suitable guard column) from samples when using these metal-loaded columns or they will exchange so reducing the efficiency of the separations. Where particularly high levels of Na^+ might be expected (e.g. molasses) a Na^+-loaded column may be useful, so avoiding the de-ashing.

Amino-bonded silica columns use an acetonitrile/water mobile phase and separate by hydrophobic and polar interactions and partition between the acetonitrile-rich mobile phase and the water-enriched stationary phase. Separation processes involving partition may make use of a number of different types of stationary phase: amino-derivatized anion-exchange resins, cation-exchange resins (H^+ form, see ref. 28 and *Figure 1),* and derivatized silica. Monosaccharides may be separated in all these processes using aqueous acetonitrile solvents. The amino-derivatized columns tend to lose their resolving power quicker than average due to stripping of the aminopropyl ligand and Schiff base formation. A similar

chromatographic separation is achievable using an underivatized silica solid phase plus a diaminoalkane coeluent with the aqueous acetonitrile. Anion-exchange chromatography, at high pH using strongly basic anion-exchange resins, is a reproducible method which is rapidly becoming preferred *(Figure 2)*. Carboxylic acids and polyhydroxyl compounds, such as the carbohydrates, are negatively charged under such conditions of high pH and are separated by anion exchange. Although base-catalysed rearrangements were originally thought to be a possible drawback to this method, they have been shown not to occur under the conditions used. Derivatized carbohydrates may be analysed by standard techniques using normal-phase or reverse-phase silica columns at ambient temperatures *(Table 2)*.

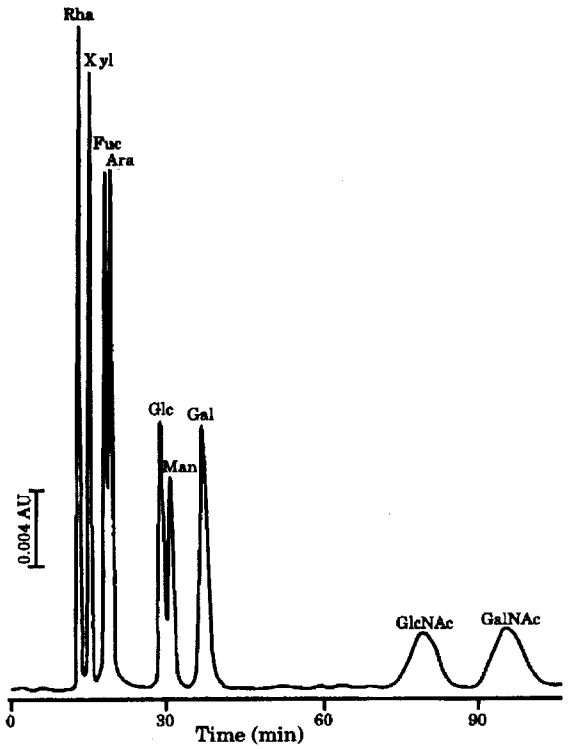

Figure 1. Analysis of an equimolar mixture of aldoses and N-acetylhexosamines.
5 nmol each of a mixture of L-rhamnose, o-xylose, L-fucose, L-arabinose, D-glucose, D-mannose. D-galactose N-acetyl-D-glucosamine and N-acetyl-D-galactosamine were dissolved in 20 µl water/acetonitrile (8:92 v/v) and applied to a Shodex DC-613 column (4 µl mm i.d. × 25cm). The column was a highly cross-linked sulfonated polystyrene resin in the H^+ form which separates by partition chromatography. It was run isocratically at 30°C, 0.6 ml/mm, using aqueous 92% (v/v) acetonitrile with post-column detection by UV 280 nm absorbance after reaction with 2-cyanoacetamide/borate *(Protocol 12)*. This system can be used for the analysis of carbohydrates derived from glycoproteins. The amino sugars produced by hydrolysis must be reacetylated (see *Protocol 13, step* 6) before analysis. N-Acetyl- and N-glycollylneuraminic acids may be analysed (as N-acylmannosamines) after hydrolysis and conversion using N-acetylneuraminidase (0.5 U) and N-acetylneuraminate pyruvatelyase (0.3 U, 1 h 37°C, 200 µg glycoprotein in 800 µl 0.06 M phosphate buffer pH 7.0). From ref. 28 with permission.

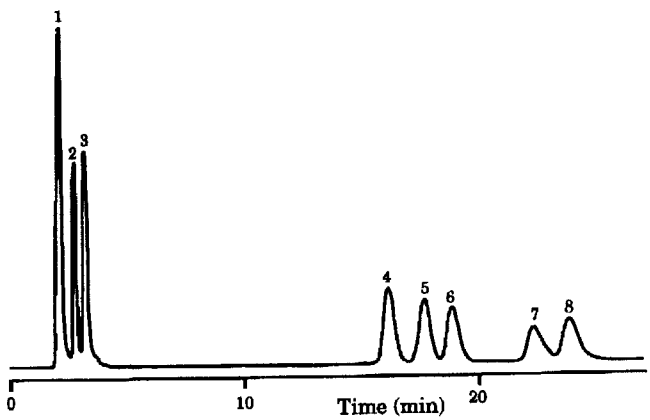

Figure 2. Separation of monosaccharides by anion-exchange HPLC on a Dionex CarboPac PA1 column eluted with 26 mM NaOH. Detection was by PAD after post-column addition of 0.5 M NaOH. The sugars (1 nmol each) are: 1, myo-inositol plus glycerol; 2, D-arabitol; 3, D-sorbitol; 4, D-galactose; 5, D-glucose; 6, D-mannose; 7, D-fructose; 8, D-ribose. From ref. 37 with permission.

Optimization of the conditions for the HPLC separation of a number of known carbohydrates is a straightforward if somewhat lengthy and tedious process. The variation of elution times of all the components with the separation parameters (e.g. temperature, acetonitrile concentration, buffer pH) must be determined. The optimum conditions may then be determined by computer analysis of the resultant curves or by visual inspection of the curves generated for the resolution of all pairs of components against the separation parameter chosen (see ref. 28 for further details).

The columns used in HPLC are usually bought ready-packed. They may be packed in-house, however, with a saving in cost and a possible increase in efficiency. The best method appears to be slurry-packing. The calculated amount of the packing material is dispersed in the slurry medium (often water) by use of an ultrasonic bath and thoroughly degassed. The slurry is then pressed into the column by use of the expected elution solution at a high enough pressure to achieve at least twice the analytical flow rate. In use, the columns gradually deteriorate. This process of deterioration can be reduced by careful attention to procedure. All solvents to be passed through the column should be as pure as possible and have

been filtered through a 0.5 μm filter, to avoid plugging of the frits by fine particles, and thoroughly degassed, preferably by use of helium bubbled through air stones. Where possible, microbial growth inhibitor (e.g. 0.01% (w/v) sodium azide) should be present n all aqueous phases. Samples applied to columns should also be free from particulate matter and other gross contaminants (e.g. proteins). The analytical column should, where feasible, be protected from contamination use of a short 'guard' column containing the same type of resin (although possibly with a larger bead size). It should also be used only within the manufacturer's recommended range of pH and solvent. This is particularly .important for silica-based stationary phases which dissolve readily at alkaline pH. At the end of each analytical session the column and all eluent delivery lines should be rinsed through with pure solvent (e.g. water) especially if salt solutions were being used. Where the column is constantly being removed from the HPLC apparatus it should be stored carefully and not dropped. Even with all these precautions some alkylated silica columns have a fairly short life of as little as three months due to particle dissolution and loss of the bonded phase. If, however, the analytical method fails suddenly, suspect the sample preparation before the column.

Table 3. Pre-column derivatization of carbohydrates for use in C_{18} reverse-phase HPLC

Carbohydrate	Reagent	Sensitivity[a]	Reference
Reducing sugars	(a) Aminopyridine	10	[30]
	(b) Dansyl hydrazine	10	[31]
Monosaccharides	Benzoyl chloride	10[b]	[32]
Sialic acids	1,2-Diamino-4,5-methylenedioxybenzene	3	[33]
Glycoprotein hexoses and hexosamines	Reductive amination/ Phenylisothiocyanate	20	[34]
Amino sugars	Reduction/o-phthalaldehyde	20	[35]

[a] pmol sample, as determined by fluorescence; about 10% applied to chromatographic column.
[b] By UV absorbance.

Amino sugars can be released by hydrolysis from glycoproteins (4 M HCl, 4 hours, 100°C, under nitrogen) and determined by use of an amino acid analyser with ninhydrin detection or after derivatization *(Tables 2-4)*.

Table 4. Post-column detection of specific classes of carbohydrates

Carbohydrate	Reagent	Sensitivity[a]	Reference
Carbohydrates, sugar alcohols	(a) Ammoniacal cupric sulfate	3	[36]
	(b) 0.5 M NaOH	0.01[b]	[37]
Reducing sugars	Tetrazolium blue	1	[8]
Amino sugars	(a) o-Phthalaldehyde	0.04[c]	[38]
	(b) Ninhydrin	1	[8, 39]
Sialic acids	300 mM NaOH	0.2[b]	[40]

[a] nmol; by UV/colorimetric detection, except where indicated.
[b] Pulsed amperometric detection.
[c] Fluorimetric assay.

6.2 Detection methods

6.2.1 DIRECT DETECTION

The direct detection of carbohydrates is not straightforward as they do not absorb light in the normal UV or visible range and have no fluorescence. Monosaccharides, however, do absorb at wavelengths in the far-UV. The absorption maxima are at about 188 nm but, due to noise in the detection signal below 190 nm, detection is normally performed at wavelengths between 192 nm and 200 nm. The response, depending largely on the freedom of the carbonyl group, differs between the monosaccharides, being six times higher for D-fructose (~2 μg in 20 μl, 0.6 mM) than D-glucose; the response of most other carbohydrates lying between these extremes. Analyses are restricted to solvents which do not absorb significantly. The response is linear over a wide range but the absolute response depends on other parameters such as the solvent purity, the reference cell, and the spectrophotometer's condition. Detection in this range requires an expensive instrument and a very pure solvent. Acetonitrile/water mixtures are often used. A reliable supplier of non-absorbing HPLC grade acetonitrile should be sought as this has often been found to be the critical component and it is not easy or practicable to purify in-house.

Another detection method of general applicability makes use of changes in refractivity. The refractive index detector is inherently as sensitive as absorbance measurement in the far-UV but, in practice, is at least ten times less sensitive due to the background noise caused by pump pulsations and temperature fluctuations. The method is generally applicable to

carbohydrate detection but is not suitable for separations involving gradient elution. The sensitivity of detection varies between carbohydrates and with the solvent composition but is generally linear.

Pulsed amperometric detection (PAD) is an electrochemical method [29] for detecting carbohydrates that is rapidly proving most popular and has reduced the need for post-column detection, by other means. It is a very sensitive technique (~1 ng glucose) which makes use of the electrochemical oxidation, at a gold electrode poised at positive potential, of the carbohydrates made anionic at about pH 13. The subsequent pulsing of the electrode with high positive and negative potentials (which provides the name for the device) being necessary in order to clean the electrode surface. This detection method is usually used after anion-exchange chromatography on quaternary ammonium resins using NaOH as eluent, but may also be used after post-column reaction of column eluates produced by other methods; particularly if base-labile groups (e.g. O-acetyl) groups are present. It is very important to ensure that the NaOH contains minimal carbonate on preparation and by degassing with helium gas and storing under helium. A metal-free chromatography system must be used if strong alkali is employed.

6.2.2 PRE-COLUMN DERIVATIZATION

In order to increase the sensitivity of detection of carbohydrates, and allow the use of highly efficient normal or reverse-phase silica columns and gradient elution profiles, they may be derivatized to give light-absorbing or fluorescent materials before separation on the HPLC column. Pre-column derivatization riot only increases the carbohydrate sensitivity to detection but also substantially changes its chromatographic behaviour. Suitable procedures are given in *Table 3.*

6.2.3 POST-COLUMN DERIVATIZATION

In general, post-column derivatization should be the method of choice if a greater sensitivity than that achieved by direct detection is required, since using this approach the separation process can make full use of the differences between the carbohydrates without the addition of a number of groups with inritrinsically similar properties. Post-column derivatization is, in general, a straightforward technique requiring one or two additional pumps, a mixing coil of Teflon tubing, and a thermostatted bath. The derivatization process must be compatible with the mobile phase used. A generally applicable but simple and sensitive method is fully described below *(Protocol 12).* The assay uses non-corrosive reagents, shows good linearity, and is highly sensitive for aldoses, hexosamines, and alduronic acids. A number of other methods are given in *Table* 4. Quantification from the detector's response is made by direct comparison with standards (or standard curves). The amounts are usually proportional to the area of the peak in the detector output. This may be measured by use of an integrator or, more cheaply, by physically weighing on a balance the peak cut out from a photocopy of the trace. This method is particularly useful for overlapping peaks. If the system gives reproducible elution times, the peak heights are normally proportional to the amount of material for any given compound.

6.2.4 SIALIC AND URONIC ACIDS

Sialic [40] and uronic acids [41] can be separated using anion-exchange chromatography on strong anion-exchange resins using a pH gradient of acetate buffers and detected by PAD after post-column addition of NaOH.

6.2.5 INOSITOLS

The naturally occurring inositols can be separated on Ca^{2+} cation-exchange resins using deionized water at 50°C and detected by PAD after post-column addition of NaOH [42].

Protocol 12. Post-column detection using 2-cyanoacetamide [28,43]

• Sensitivity: photometric; 18 ng glucose in 20 µl (5 µM) • Sensitivity: fluorometric; 2 ng glucose in 20 µl (0.5 µM)

Reagents

A Dissolve 2-cyanoacetamide in methanol, decolorize with activated carbon, and recrystallize from methanol. Dissolve 1.0 g of the purified 2-cyanoacetamide in 100 ml of water. This solution may be stored in a refrigerator within a dark bottle for at least one month.

B Dissolve 31 g of boric acid in 600 ml of water. Make the solution pH 9.5 by the addition of 10% (w/v) potassium hydroxide and dilute with water to a final volume of 1 litre (0.5 M borate).

Method

1. Mix the column eluate (0.6 ml/mm; if a different elution rate is used, scale all the rates accordingly) successively with reagent A (0.5 ml/ mm) and reagent B (0.5 ml/mm) by use of Teflon Y-shaped joints.
2. Pass the mixture through an open tubular Teflon reaction coil (10 m x 0.5 mm i.d.) held at 100°C in a thermostatted bath containing glycerol (80 sec contact time) followed by an air-cooled Teflon cooling coil (1 m x 0.5 mm i.d.).
3. Determine the absorbance at 280 nm (274 nm maximum) or the fluorescence at 331 nm (excitation)/383 nm (emission).

   Note: The fluorescence is quenched by acetonitrile, if present in the eluate, but the absorbance is unaffected.

7. Gas-liquid chromatography

7.1 Experimental approach

GLC is preferred to HPLC in a number of instances. It is a sensitive technique, allowing the analysis of sub-nanomolar amounts of carbohydrates, and is generally less prone to interference (e.g. from salts and protein). Detection is usually by means of a flame ionization detector (FID) which responds to all carbohydrate related molecules over an extremely wide linear range. GLC separation is dependent upon the differential extractive distillation of the components in the mixture. It is fundamental to the technique, therefore, that volatile derivatives of the carbohydrates are prepared. There are two schools of thought concerning this derivatization. One is to produce a single derivative from each carbohydrate and the other allows several derivatives to be produced dependent upon the anomeric composition. The advantage of the former is that the chromatograms are simpler and the sensitivity may be marginally greater. However by producing more than one derivative in a well defined mass ratio, a recognizable fingerprint' can be obtained which is confirmatory for a particular component. This reduces the possibility of a chromatographic peak being incorrectly assigned to a carbohydrate when actually due to an unexpected impurity. It is not necessary to simplify analyses of complex mixtures to one peak/ component as highly efficient, fused-silica, wall coated open tubular (WCOT) columns are generally powerful enough (≥ 100 000 theoretical plates) to resolve a large number of components (> 30). The much better resolution (> tenfold) and speed (> fivefold) of WCOT columns, compared with packed bed columns, recommends them for routine analytical work; packed bed columns being rarely used these days. There is no substantial change in the relative elution positions between these two types of column if similar liquid phases are used *(Table 5)*. WCOT columns are more fragile and deteriorate more easily than packed bed columns. In general, this deterioration may be greatly reduced by the use of oxygen-free dry helium as the carrier gas. This helium should be passed through the appropriate gas purifiers before entering the column as oxygen has a particularly devastating effect on the column. If well treated, and run below the supplier's maximum operating temperature limit, the WCOT columns should last for over a year of continuous use, which is longer than most IIPLC columns last.

Table 5. Alternative liquid phases for the GLC of carbohydrates

Polarity	Typical composition	Alternative phases
Most nonpolar	Dimethyl silicone	SE-30, OV-1, BP-1, OV-101, CP-Sil 5CB
Nonpolar	5% Phenyl, methyl silicone	SE-52, SE-54, BP-5, CP-Sil 8CB
Intermediate polarity	50% Phenyl, methyl silicone	OV-17, BP-10
	Cyanopropyl, phenyl. methyl silicone	CP-Sil 19CB
Polar	Trifluoropropyl, methyl silicone	OV-202, OV-210
Most polar	Cyanopropyl silicone, or similar	OV-275, CP-Sil 88, Silar 9CP, BP-75, SP-2330

Internal standards are generally necessary for GLC analyses since it is difficult to inject a reproducible proportion of a sample into the column. Standards may be any similar compound which is not already present in the mixture to be analysed and which is clearly separable from the other components. Myo-inositol (mesoinositol) may be used, except for plant or membrane carbohydrate analyses where it is usually already present. An alternative internal standard is pentaerythritol. Glucose should be avoided as an internal standard, even if it is known not be expected in the samples, as it is a common, and annoying, contaminant.

7.2 Detection methods

Three GLC derivatization methods are described in *Protocols 13-15. Protocol 13,* involving trimethylsilylation, is a good, generally applicable, method whereas Protocol 14, involving acetylation, is recommended particularly for methoxy-derivatized monosaccharides derived from the methylation of more complex molecules (e.g. glycoproteins, polysaccharides, or glycolipids). Protocol 15, involving oxime formation gives only one peak per sugar, And is particularly useful where fructose is present. Quantification of the components in a mixture is by analysis of the peak areas (heights) as described for HPLC (Section 6.2.3).

Most monosaccharides give a mixture of isomers on trimethylsilyl derivatization (Table 6, Figure 3). The ratio of the peak areas is constant, however, and may be used to confirm small amounts of material having a low signal-to-noise ratio. Labile carbohydrates (e.g. N-acetylneuraminic acid) are not destroyed by this analytical process and it is therefore a good method for the analysis of all the carbohydrate moieties derived from glycoproteins. Fructose, however, is not analysable by this method and should be derivatized as the trimethylsilyl or oxime derivative of the free sugar, because the methylglycoside is not formed on methanolysis. In general, free sugars may be determined by GLC analysis, as above, after derivatization of the dry material (i.e. without any methanolysis or re-N-acetylation steps). The retention times, number of peaks, and relative peak heights will all be different to those obtained for the methylglycosides and should be determined separately. The [) and L configurations of neutral monosaccharides may be determined in a similar manner to methanolysis above but by the use of R(-)-2-butanol in place of methanol throughout. The trimethylsilylated (-)-2-

butylglycosides are separable on an 5E30 capillary column [44].

Table 6. GLC data of trimethylsilylated monosaccharides

Parent carbohydrate	Peak[a]	Time (min)[b]	Peak height[c]
(Solvent front)	1	1.00	
L Arabinose	2	4.82	0.271
	3	4.95	0.122
	6	5.36	0.024
D-Ribose	4	5.03	0.064
	5	5.26	0.317
L-Rhamnose	5	5.25	0.433
	6	5.36	0.046
L-Fucose	5	5.28	0.054
	7	5.52	0.266
	8	5.80	0.156
D-Xylose	9	6.10	0.377
	10	6.37	0.188
D-Glucuronic acid	11	7.14	0.069
	23	9.37	0.073
	24	9.49	0.240
D-Galacturonic acid	12	7.31	0.186
	13	7.90	0.056
	19	8.79	0.160
	20	8.90	0.051
D-Mannose	14	8.02	0.598
	16	8.42	0.042
D-Galactose	15	8.12	0.109
	17	8.54	0.382
	18	8.62	0.048
	21	8.97	0.153
D-Glucose	22	9.27	0.478
	25	9.59	0.201
N-Acetyl-D-glucosamine	-	10.57	0.031
	-	11.17	0.029
	28	11.70	0.359
N-Acetyl-D-galactosamine	-	10.45	0.031
	26	10.70	0.082
	27	11.35	0.257
Myo-inositol	29	12.24	1.000
N-Acetylneuraminic acid	30	15.35	0.200

[a] Refers to Figure 3.
[b] SD = 2 sec.
[c] SD = 3.5%; peak height is proportional to peak area under these conditions.

Protocol 13. Preparation of trimethylsilyl derivatives [45]

Reagents

A Dry methanol. It may be possible to purchase this. Otherwise methanol may be dried by refluxing 500 ml with 2.5 g of magnesium turnings and 0.1 g of iodine for 1 h, followed by distillation into a clean dry container.

B Anhydrous pyridine, bought or prepared by the addition of 30 g dry KOH pellets to 500 ml of pyridine. The mixture should be well stirred and allowed to settle for 24 h before use.

C Methanolic hydrogen chloride. This may be prepared by either of two methods:

(a) Bubble hydrogen chloride gas through a train of moisture traps, containing concentrated sulfuric acid, into dry methanol (reagent A) until the weight of the methanol solution increased by about 3%. Care should be taken over preventing the sulfuric acid traps sucking back when the process is finished. The methanolic HCl should be standardized to 0.625 M by the titration of an aliquot against standard 0.1 M NaOH using phenolphthalein as an indicator, and dilution with more dry methanol.

(b) Add 4.65 ml of good quality acetyl chloride carefully to 100 ml of dry methanol.

D Trimethylsilylation reagent. This may be purchased, ready mixed and ampouled (e.g. Tri-Sil® Reagent from Pierce), but can be prepared by mixing dry pyridine (10 vol.), with hexamethyldisilazane (2 vol.), and trimethylchlorosilane (1

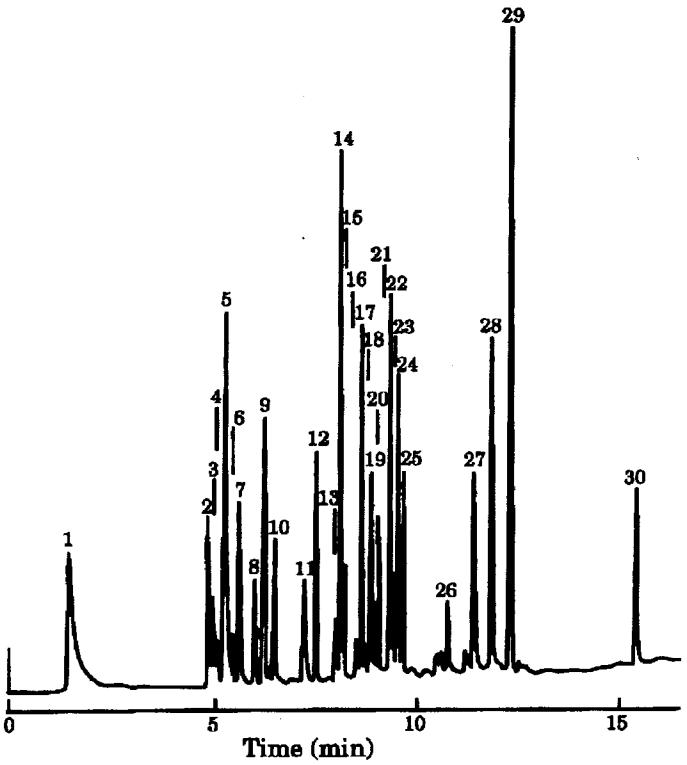

Figure 3. GLC of trimethylsilylated monosaccharides. Approximately 0.5 nmol of each monosaccharide was derivatized (see *Protocol 13).* The peaks are identified in *Table 6.* From ref. 45 with permission.

vol.). It is best stored in sealed or serum-capped ampoules under nitrogen. An alternative reagent for use on carbohydrate syrups can be prepared by mixing dry pyridine (10 vol.), with hexamethyldisilazane (9 vol.), and good quality trifluoroacetic acid (1 vol.). This may be used in the presence of up to 5.0 mg water/ml reagent if the carbohydrate content is fairly high (~1 mg/ml reagent).

Method

A dry cabinet (glove box) should be used throughout for all reagent manipulations.

1. Dry the samples containing glycoproteins (~0.5-100 µg), polysaccharide or monosaccharide mixtures, or standards (~0.1-100 nmol) together with myo-inositol (~100 ng; or other suitable internal standard) over P_2O_5, under vacuum, in 100 µl or 300 µl Reacti-Vials (screw topped, serum-capped vials, e.g. as supplied by Pierce. Note that crystalline standard N-acetylneuraminic acid contains acetic acid of crystallization which prevents the use of P_2O_5 for drying since it causes decomposition).
2. Add a mixture of dry methanolic HCl (4 vol.) and methyl acetate (1 vol., total volume of about 40-200 µl depending on the expected carbohydrate content). if reagent C(b) is used (see above), which already contains some methyl acetate, the ratio of methanolic HCl to additional methyl acetate should be 5:1 (v/v). Seal the tube by means of the Teflon-lined septa in the screw caps and mix thoroughly be use of a vortex mixer.
3. Incubate the tubes at 70°C in an oven for about 16 h. Check the screw caps for tightness after the first 10 min of this incubation and vortex mix the tubes again before replacing in the oven. Only very occasionally will this system of sealing fail (~1 in 50), usually due to use of a cracked or chipped tube.
4. After cooling, add redistilled 2-methyl-2-propanol (t-butyl alcohol, 20% v/v).
5. Vortex mix the samples and evaporate to dryness using a stream of dry nitrogen (oxygen-free grade) at room temperature.
6. Add 50 µl of dry methanol, 5 µl of pyridine, and 5 µl of acetic anhydride successively with intermediate mixing in order to re-*N*-acetylate the amino sugars (twice these volumes should be used if more than 50 nmol of amino sugars is present; fatty acid methyl esters may be removed, if suspected, by extraction with hexane).
7. Leave the solutions at room temperature for 15 min before evaporating to near dryness by use of a stream of dry nitrogen (oxygen-free grade).
8. Completely dry the residue using gentle vacuum over P_2O_5.
9. After this thorough drying stage, add the silylation reagent (reagent D, ~20-100 µl depending on the carbohydrate content), vortex mix the tubes for 30 sec, and leave for 1 h at room temperature. The derivatized samples should be analysed the same day.
10. Re-evaporate using the dry nitrogen stream and immediately dissolve each sample in 14-50 µl of redistilled hexane.
11. Apply some or all of this sample to the capillary column. The set-up recommended is a fused-silica WCOT column (25 m x 0.32 mm i.d.) coated with CP Sil 5 liquid phase (see *Table 5* for alternative liquid phases; any other reasonably equivalent fused-silica column is suitable), using an all-glass solid injector (moving needle, Chrompack,

previously inactivated by reaction with the silylation reagent Rejuv 8; supplied by Supelco, but any similar product is suitable). Other injection techniques may be used but this method has the advantage that all the sample is injected onto the column without excessive solvent.

12. Perform the analyses using dry oxygen4ree helium as carrier gas and a temperature program (140°C for 2 min, then increasing at 8°C/min up to 240°C). A higher final temperature improves the analysis but tends to cause the more rapid deterioration of the shrinkable Teflon link between the injector and the column.

Protocol 14. Preparation of alditol acetate derivatives [46, 47]

Reagents

A Dry dimethyl sulfoxide, prepared by storage of material, from a previously unopened bottle, over molecular sieve type 4A. Care should be taken not to disturb any fine sediment that may be present when removing aliquots for use.

B Dissolve 2 g of sodium borohydride in 100 ml of anhydrous dimethyl sulfoxide (reagent A) at 100°C. This reagent is stable if kept dry at 4°C. If deuterium is to be inserted (see *Table* 7) then $NaBD_4$ should be used in place of the $NaBH_4$; no special precautions are needed.

Method

1. Dissolve the samples and standards containing up to about 500 µg of the monosaccharides in 0.1 ml of 1 M ammonia solution (prepared by diluting 3 ml of concentrated ammonia solution, of sp. gr. 0.88, with 50 ml of water).
2. Reduce the carbohydrates by the addition of 1.0 ml of reagent B followed by incubation at 40°C for 90 min.[a]
1. After reduction, decompose the excess sodium borohydride by the addition of 0.1 ml of glacial acetic acid.
2. The sample can be cleaned somewhat of contaminants, such as surfactants and lipids, at this stage by selective extraction with *n*-heptane (2 ml) from an equal volume of sample dissolved in acetonitrile/water (9:1 v/v), keeping the bottom aqueous acetonitrile layer, followed by rotary evaporation.
3. Peracetylate the reduced monosaccharides by the addition of 0.2 ml of 1-methylimidazole (catalyst), followed by 1.0 ml of acetic anhydride, and thorough mixing.
4. After 10 min at room temperature, add 2 ml of water to decompose the excess acetic anhydride.
5. After cooling, extract the peracetylated carbohydrates into the lower layer formed after vortex mixing the aqueous solution with 1 ml of dichloromethane. This layer may be removed via a Pasteur pipette and stored in a septum-capped vial at -20°C until analysis on a polar OV-275, or similar, column *(Table 7, Figure* 4, see ref. 48).

[a] Some carbohydrate pairs are reduced to the same alditol and cannot be resolved by this method, e.g. lyxose/arabinose, glucose/gulose, and altrose/talose. In addition, ketoses are not reduced stereospecifically and give rise to isomeric hexitols, e.g. D-fructose gives D-glucitol and D-mannitol. and D-sorbose gives L-glucitol and D-iditol.

Protocol 15. Preparation of oxime derivatives

Reagent

Dissolve 2.5 g hydroxylamine hydrochloride in 100 ml redistilled pyridine.

Method

1. Dry the samples and standards containing up to about 5 mg of the monosaccharides in a screw topped, Teflon-capped vial. Add 500 µl of the hydroxylamine reagent,[a] cap, and heat for 30 min at 70-75°C.
2. Cool to room temperature, add 500 µl of hexamethyldisilazane, and mix.
3. Add 50 µl anhydrous trifluoroacetic acid, cap, vortex mix, and leave at room temperature for 30 min to allow the white precipitate to settle.
4. Add 500 µl N-trimethylsilylimidazole, cap, vortex mix, and leave at room temperature for a further 30 min.
5. Apply some or all of this sample to an intermediate polarity capillary column, such as OV-17.

[a] Where sucrose is thought to be present, add 27 µl dimethylaminoethanol to avoid its slight, but detectable, hydrolysis.

7.2.1 INOSITOL ANALYSIS

The naturally occurring inositols can be simply separated by GLC as hexakis-O-trimethylsilyl derivatives. The trimethylsilyl derivatives may be produced by the silylation reagent used for monosaccharide derivatization *(Protocol 13)* but allowing the reaction to proceed for 48 hours due to the slowness with which neo-inositol reacts [49].

8. Mass spectrometry

Electron impact mass spectrometry (EI-MS) is an extremely powerful analytical technique and is usually used in conjunction with GLC (as GLC-MS) [50]. Together they have become the fastest technique for the analysis of complex mixtures using two sets of data, the retention times and the mass spectra. EI-MS is based on the positive ionization of molecules due to bombardment with a beam of electrons. As carbohydrates are normally converted into volatile derivatives before analysis, the linkage of MS to GLC. which also requires volatile derivatives, is clearly beneficial. The mass spectra from monosaccharide derivatives consist of primary fragments formed from the initial cleavage of the molecular ion plus many secondary fragments due to the elimination of neutral molecules from these primary fragments. The fragmentation pattern may be used for identification by comparison with spectra from known materials or by deduction from the known cleavage and elimination probabilities [51]. In general, the molecular ion of a derivatized

carbohydrate is not seen and large primary fragments tend to eliminate neutral molecules quite readily. Most of the usable spectra are, therefore, below about 300 m/z. Care should also be taken not to over-rely on the absolute or relative peak heights in the spectra as these may vary considerably from day to day, depending on the precise conditions used. However if care is taken, and suitable peaks are chosen, a reproducibility of better than ± 10% is possible. One further drawback to the use of mass spectrometry is that there is generally little difference between isomers. This is easily overcome by the additional use of the retention data. GLC-MS is a particularly powerful technique for the analysis of partially methylated alditol acetates *(Figure 4, Table 7)* especially as known standards are not always available. The mass spectra may be analysed with reference to a few simple rules (see also Figure 5).

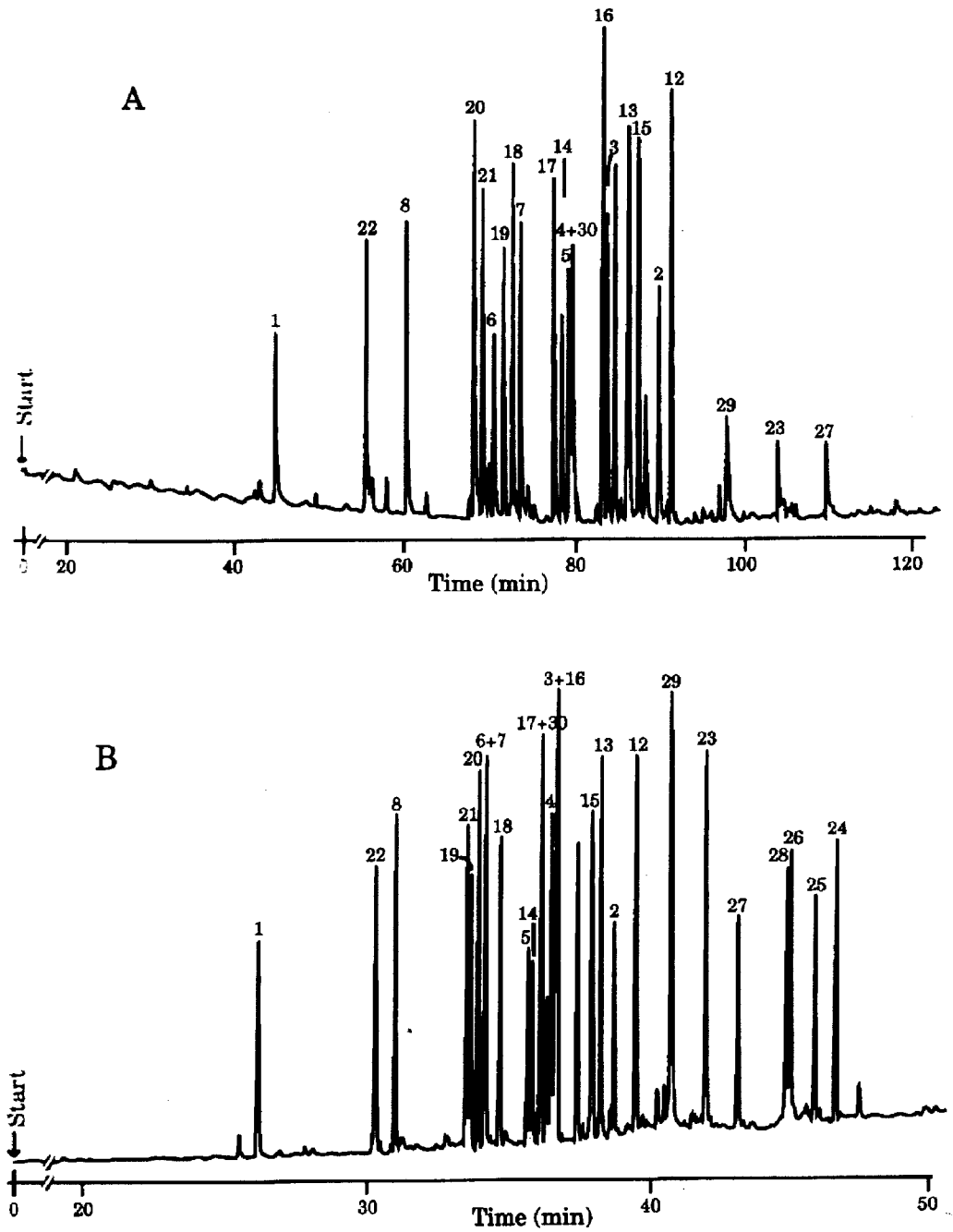

Figure 4. GLC of peracetylated hexitols and methyl hexitols. 25-100 pmol of each of 32 O-methylated hexitol and 2-deoxy-2-(N-methyl)acetamidohexitol acetates, most of which are commonly found during the methylation analysis of N-glycosidically linked glycoprotein oligosaccharides were analysed by (A) capillary GLC using polar (22 m x 0.25 mm i.d. WCOT column containing Silar 9CP) and (B) nonpolar (85 m x 0.25 mm i.d. WCOT column containing OV 101) liquid phases. The peaks are identified in *Table 7*. The analytical conditions were (A) 20 mm at 100°C then raised to 230°C at 1°C/min. and (B) 100°C to 240°C at 3°C/mm. From ref. 48 with permission.

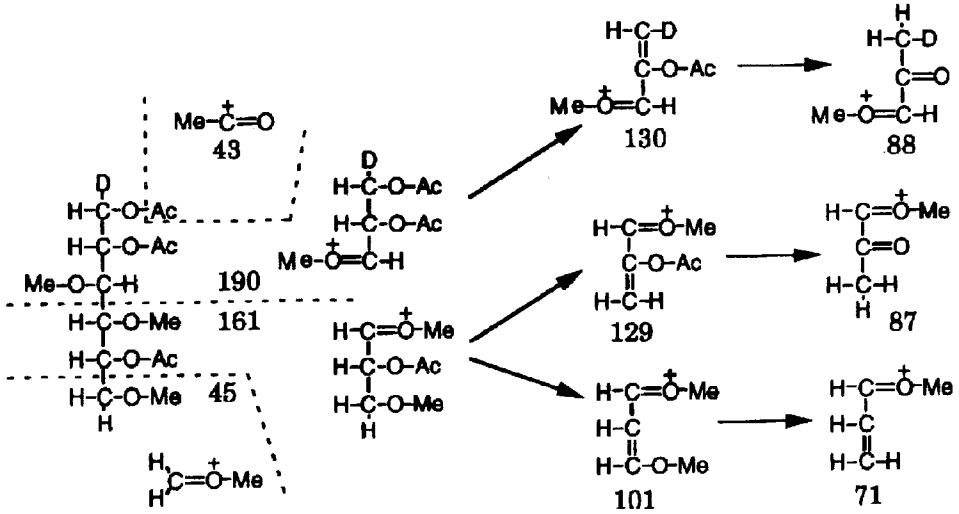

Figure 5. The fragmentation pattern in mass spectrometry of 1,2,5-triacetyl-3,4,6-tri-O-methyl-D-glucitol. The initial cleavage pattern giving about 43% m/z 43, 27% m/z 161 (including 12% m/z 129, 5% m/z 87, 3% m/z 101, and 2% m/z 71 secondary cleavage products), 18% m/z 190 (including 10% m/z 130 and 6% m/z 88 secondary cleavage products), and 12% m/z 45.

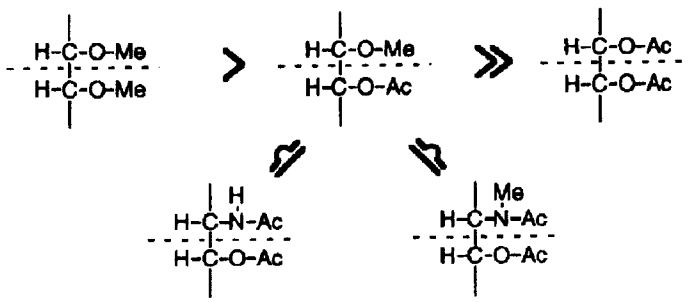

Figure 6. The relative ease of cleavage, in mass spectrometry between carbon atoms in peracetylated methylated alditols and amino alditols. The unmethylated amino group often arises due to under-methylation.

(a) Fission prefers to take place next to the methoxyl or N-acetyl groups rather than the O-acetyl groups, with the positive ion stabilized by the methoxyl or N-acetyl grouping *(Figure 6).*

(b) Secondary fragments are derived from the primary fragment by β-elimination of methanol (mol. wt. 32) or acetic acid (mol. wt. 60) from the carbon atom two-removed from the formal charge on the carbonium ion. Some fission of acetic acid from the α-carbon atom is detected where the β-carbon is methoxylated (see *Figure* 5). Additional fission of ketene (mol. wt. 42) and, more rarely, formaldehyde (mol. wt. 30) also occurs, (e.g. fragments such as R-$CH{=}N^{+}(CH_3)Ac$ immediately eliminate ketene to form R-$CH{=}N^{+}HCH_3$).

(c) Large primary fragments eliminate neutral molecules more readily than small primary fragments. A base fragment at m/z 43 ($CH_3C^{+}{=}O$) is normally found.

There is no significant difference between isomeric alditols having the same substitution pattern, alditols from 2,3- and 3,4-di-O-methyl pentoses or from 3- and 4-O-methyl hexoses would be indistinguishable unless sodium borodeuteride was used in the reduction step.

The trimethylsilyl derivatives of alditols, monosaccharides, or methylglycosides do not give molecular ions but weak $(M\text{-}15)^{+}$ ions may be seen in aldoses. They cleave between carbon atoms carrying trimethylsilyl ether or methoxyl groups to form the primary fragments. The secondary fragments are formed from these by the consecutive elimination or trimethylsilylbydroxide Me_3SiOH, mol. wt. 90) molecules to give ions such as $(M\text{-}15\text{-}90)^{+}$. A base fragment at m/z 73 (Me_3Si^{+}) is normally found. The mass spectra of the trimethylsilyl derivatives of cyclic carbohydrates are complicated because the initial cleavage does not reduce the m/z of the resulting fragment; secondary cleavages must occur and these produce fragments at m/z 147 ($Me_3Si\text{-}O^{+}{=}SiMe_2$) from pairs of derivatized hydroxyl groups, 204 [$(Me_3SiO\text{-}CH)_2^{+}$] from vicinal hydroxyl groups, and 217 ($Me_3SiO\text{-}CH{=}CH\text{-}CH\text{-}OSiMe_3$), and 305 [$Me_3SiO\text{-}CH{=}C(\text{-}OSiMe_3)\text{-}C^{+}H\text{-}OSiMe_3$] from three neighboring hydroxyl groups, in addition to the base peak at m/z 73 and a peak at m/z 191 [$(Me_3SiO)_2C^{+}H$] due to the straight chain form at C-1. The ratio of these five peaks may be used to distinguish between carbohydrates if reproducible analytical conditions are possible. In particular, for aldohexoses, aldopentoses, and 6-deoxyaldohexoses, the pyranoid forms give an m/z 204/217 ratio of greater than one (~ 1 to > 1 for aldopentoses), whereas the furanoid forms

show a ratio of less than one. An intense ion at m/z 173 (Me_3SiO-CH-C^+H-NHCOMe) is seen in the spectra of the TMS ethers of the N-acetylhexosamines.

9 Infra-red spectroscopy

Infra-red spectroscopy is mainly of use in monosaccharide analysis for the confirmation of the identity of a molecule by comparison with a published standard spectrum or the spectra of known material. About one milligram of pure dry carbohydrate is needed for an analysis although a smaller amount may give useful spectra. The preferred method makes use of salt discs made by the intimate mixture of about one milligram of carbohydrate with 300 milligrams of pure dry KBr followed by pressing into a disc. The whole IR spectrum (4000-650 cm^{-1}) should be used for comparison [52].
Fourier transform IR spectroscopy (FTIR) may be used for the on-line quantitative analysis of relatively simple sugar solutions, such as soft drinks by use of a matrix of absorbances within the mid-infrared range (4000-400 cm^{-1}). The sugar concentrations may be determined from matrix constants previously deduced from standard mixtures [53].

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* Indicates a modification of the cited method.

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