Carbohydrate analysis

a practical approach

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ublished in the
ractical Approach Series
eries editors: D.Rickwood and B.D.Hames

IRL PRESS
rd · Washington DC

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IRL PRESS
Oxford · Washington DC

IRL Press Limited,
P.O. Box 1,
Eynsham,
Oxford OX8 1JJ,
England

British Library Cataloguing in Publication Data

Carbohydrate analysis: a practical approach.
–(The Practical approach series)
1. Food–Analysis 2. Carbohydrates–Analysis
I. Chaplin,M.F. II. Kennedy,John F. (John Frederick) III. Series
641.1'3 TX553.C28

ISBN 0-947946-44-6 (softbound)
ISBN 0-947946-68-3 (hardbound)

Printed in England by Information Printing, Oxford.

Preface

The involvement of carbohydrates in biological processes has greatly fuelled the current interest in this diverse range of molecules. This has resulted in a vast literature covering numerous analytical methods for these carbohydrates. The size of the literature arises in part from the large number of classes of carbohydrates – macromolecular and monomolecular – basic, neutral and acidic – derivatized and underivatized – and the wide range of presentation of carbohydrates in everyday life – biosynthesis or chemicosynthesis – structural or gelatinous – edible or unmetabolizable. Superimpose upon just these selected aspects the notion of primary, secondary, tertiary and quaternary structure and the fact that the number of ways of covalently joining two carbohydrate monomers is at least one order of magnitude greater than the similar joining of two amino acids, then it is clear that analysis of the field of carbohydrates is, and must be, vast. For this reason, experts in particular areas of the field have been asked to contribute to this book – without them it would not have been possible.

It is not always obvious to the researcher or analyser which method, or even physical technique, is most appropriate to a particular investigation. This book has been produced in order to answer the need for a handbook of laboratory protocols in this field. It gives details of the approach needed to analyse a wide variety of carbohydrates and carbohydrate-containing molecules. We have attempted to show how particular analytical problems should be tackled, describing the most suitable, well tried and trusted methods in exact practical detail.

Chapters are arranged on the basis of carbohydrate moiety to facilitate choice of analytical method for specific applications. Thus the chapters act as a ready source of reference at the initial planning stage for the choice of approach to situations where carbohydrate analysis is required.

We are indebted to our co-authors, who have made this book a useful source of reference for carbohydrate analytical protocols which should find active and practical use within the laboratory.

Martin Chaplin and John F.Kennedy

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Abbreviations

c.d.	circular dichroism
CIAE	crossed immuno-affino electrophoresis
DEAE	diethylaminoethyl
DMSO	dimethylsulphoxide
d.p.	degree of polymerization
DPO	diphenyloxazole
EDTA	ethylenediaminetetraacetic acid
e.i.-m.s.	electron impact mass spectrometry
f.a.b.-m.s.	fast atom bombardment mass spectrometry
GAG	glycosaminoglycan
g.l.c.	gas-liquid chromatography
g.p.c.	gel permeation chromatography
HA	hyaluronic acid
HABR	hyaluronic acid binding region
h.p.l.c.	high performance liquid chromatography
i.r.	infra-red spectroscopy
LP	link protein
m.s.	mass spectroscopy
NEM	*N*-ethylmaleimide
n.m.r.	nuclear magnetic resonance
PBS	phosphate-buffered saline
p.c.	paper chromatography
PMSF	phenylmethylsulphonyl fluoride
SDS	sodium dodecylsulphate
SDS-PAGE	polyacrylamide gel electrophoresis in the presence of SDS
TEMED	*N,N,N′,N′*-tetramethylethylenediamine
TFA	trifluoroacetic acid and trifluoroacetates
t.l.c.	thin-layer chromatography
WCOT	wall coated open tubular

Contents

CHAPTER 1

Monosaccharides

MARTIN F.CHAPLIN

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 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 (h.p.l.c.) and gas-liquid chromatography (g.l.c.). If only a qualitative analysis is needed, either paper chromatography (p.c.) or thin-layer chromatography (t.l.c.) may be used. Single monosaccharides may be identified by means of mass spectrometry (m.s.), infra-red spectroscopy (i.r.) or proton or carbon-13 nuclear magnetic resonance (^{1}H-n.m.r., ^{13}C-n.m.r.), and quantitated 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. COLORIMETRIC ASSAY

A number of colorimetric assays are presented for the main classes of monosaccharides, to be 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 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 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.

2.1 **Hexose**

2.1.1 *L-Cysteine sulphuric acid assay* (1)

Sensitivity: ~0.2−20 μg glucose in 200 μl (~5−500 μM).

Final volume: 1.2 ml.

Reagents:

(A) Ice-cold 86% v/v sulphuric acid, prepared by the very careful addition of 860 ml good quality concentrated sulphuric acid to 140 ml of water. This addition generates considerable heat and should be carried out within a fume cupboard (using protective glasses and clothing) at least the day before the solution is to be used. The reagent is stable if kept dust-free in a stoppered containers.

(B) Freshly prepared solution of L-cysteine hydrochloride (700 mg/l) in reagent A.

Method

(i) To samples, standards and control solutions (200 μl) containing up to 20 μg neutral carbohydrate cautiously and reproducibly add 1.0 ml of reagent B, with immediate thorough mixing in an ice bath.

(ii) Heat at 100°C for 3 min in a glass stoppered test tube.

(iii) Rapidly cool the mixture to room temperature.

(iv) Determine the absorbance at 415 nm.

Comments. Assay tubes must be scrupulously clean for this assay as it measures both monomeric and polymeric neutral carbohydrate and is, therefore, interfered with by extraneous glucose in airborne dust. Deoxy sugars, heptoses, pentoses and uronic acids all produce colour in this assay with absorbance maxima in the range 380−430 nm. The stability of this colour varies between the carbohydrates and distinctive shifts in absorbance may be obtained on standing for 24−48 h. Azide, heavy metal ions and non-aldose reducing substances interfere in this assay. Mixtures of carbohydrates giving different absorbance maxima can be analysed by use of two standard determinations as outlined in Section 2.9.

2.1.2 *Phenol-sulphuric acid assay* (2)

Sensitivity: ~1−60 μg glucose in 200 μl (~30 μM−2 mM).

Final volume: 1.4 ml.

Reagents:

(A) Phenol dissolved in water (5% w/v). This solution is stable indefinitely.

(B) Concentrated sulphuric acid.

Method

(i) Mix samples, standards and control solutions (200 μl containing up to 100 μg carbohydrate) with 200 μl of reagent A.

(ii) Add 1.0 ml of reagent B rapidly and directly to the solution surface without touching the sides of the tube.

(iii) Leave the solutions undisturbed for 10 min before shaking vigorously.

(iv) Determine the absorbances at 490 nm after a further 30 min.

Comments. Other 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.

2.2 **Reducing sugar**

2.2.1 *Neocuproine assay* (3)

Sensitivity: ~20 ng–2 μg glucose in 200 μl (~0.5–50 μM).

Final volume: 2.0 ml.

Reagents:

(A) Dissolve 40 g of anhydrous sodium carbonate, 16 g of glycine and 450 mg cupric sulphate pentahydrate in 600 ml water and make up to 1 litre with more water. The solution is stable indefinitely.

(B) Dissolve 0.15 g neocuproine hydrochloride in 100 ml water. This solution is stable for months if stored in a dark bottle.

Method

(i) To samples, standards and controls, (200 μl) add 400 μl reagent A and 400 μl reagent B. Mix well.

(ii) Heat the solutions at 100°C for exactly 12 min and cool rapidly to room temperature.

(iii) Add 1.0 ml of water, washing down any condensate formed.

(iv) Mix well and determine the absorbance at 450 nm.

Comments. If a precipitate forms during the final cooling it may simply be redissolved by slight warming. Non-carbohydrate reducing agents interfere in this assay.

2.2.2 *Dinitrosalicylic acid assay* (4,5)

Sensitivity: ~5–500 μg glucose in 100 μl (~0.3–30 mM).

Final volume: 1.1 ml.

Reagent: Dissolve 0.25 g of 3,5-dinitrosalicylic acid and 75 g sodium potassium tartrate (Rochelle salt) in 50 ml 2 M sodium hydroxide (made by dissolving 4 g NaOH in 50 ml water) and dilute to 250 ml with water. This is stable for several weeks.

Method

(i) To samples, standards and controls (100 μl) add 1.0 ml of the reagent. Mix well.

(ii) Heat the mixtures at 100°C for 10 min.

(iii) After rapid cooling to room temperature, determine the absorbance at 570 nm.

Comments. 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 sugars 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.

2.3 **Pentose**

2.3.1 *Ferric-orcinol assay* (4)

Sensitivity: ~200 ng–20 μg xylose in 200 μl (~7–700 μM).

Final volume: 1.6 ml.

Reagents:

(A) Trichloroacetic acid solution in water (10% w/v). This is stable indefinitely.
(B) Freshly prepared solution of ferric ammonium sulphate (1.15% w/v) and orcinol (0.2% w/v) in 9.6 M hydrochloric acid (made by diluting five parts concentrated HCl with one part water).

Method

(i) Mix the samples, standards and control solutions (200 μl) containing up to 40 μg pentose with 200 μl of reagent A.
(ii) Heat at 100°C for 15 min.
(iii) Cool the solution rapidly to room temperature. Add reagent B (1.2 ml) and mix well.
(iv) Reheat the solution at 100°C for a further 20 min.
(v) Cool the solution to room temperature and determine the absorbance at 660 nm.

Comments. 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.

2.4 **Ketose**

2.4.1 *Phenol-boric acid-sulphuric acid assay* (6)

Sensitivity: ~0.1–9 μg fructose in 100 μl (~30–500 μM).

Final volume: 2.0 ml.

Reagents:

(A) Dissolve 2.5 g phenol (recrystallized from methanol and ethanol) in 50 ml water. Add 1.0 ml of acetone dropwise with constant stirring over a period of 10 min and stir the mixture for a further 10 min at room temperature. Dissolve 2.0 g of boric acid in the mixture. The reagent is stable for at least 2 weeks at 4°C.
(B) Concentrated sulphuric acid.

Method

(i) Mix the samples, standards and controls (100 μl) with 0.5 ml of reagent A and then rapidly add 1.4 ml of reagent B directly to the surface, avoiding the sides of the tubes.
(ii) After thorough mixing, leave the solutions for 5 min at room temperature.
(iii) Incubate at 37°C for 1 h.
(iv) Determine the absorbance at 568 nm.

Comments. 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 sulphuric acid.

2.5 **Hexosamine**

2.5.1 *Morgan-Elson assay* (7)

Sensitivity: ~60 ng–6 μg 2-acetamido-2-deoxy-D-glucose in 250 μl (~1–110 μM).

Final volume: 1.8 ml.

Reagents:

(A) Dissolve 6.1 g of di-potassium 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 hydrochloric acid. Add a further 87.5 ml of glacial acetic acid and dissolve 10 g of 4-(*N*,*N*-dimethylamino)-benzaldehyde in this mixture. This solution may be stored for several weeks. Dilute 10 ml to 100 ml with galacial acetic acid immediately prior to use.

Method

(i) Add samples, standards and controls (250 μl) to 50 μl of reagent A.
(ii) Heat each mixture at 100°C for 3 min.
(iii) After cooling rapidly to room temperature, add 1.5 ml of reagent B, washing down any condensate formed.
(iv) Incubate the samples at 37°C for 20 min.
(v) After cooling to room temperature, determine the absorbances at 585 nm.

Comments. 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 min at room temperature.

2.6 **Uronic acid**

2.6.1 *Carbazole assay* (8)

Sensitivity: ~200 ng–20 μg D-glucurono-6,3-lactone in 250 μl (~4–400 μM).

Final volume: 1.8 ml.

Reagents:

(A) Dissolve 0.95 g of sodium tetraborate decahydrate in 2.0 ml of hot water and add 98 ml of ice-cold concentrated sulphuric acid carefully with stirring. This reagent is stable indefinitely if refrigerated.

(B) Dissolve 125 mg of carbazole (recrystallized from ethanol) in 100 ml of absolute ethanol to give a stable reagent.

Method

(i) Cool the samples, standards and controls (250 μl) in an ice bath.
(ii) Add ice-cold reagent A (1.5 ml) with mixing and cooling in the ice bath.
(iii) Heat the mixtures at 100°C for 10 min.
(iv) Cool rapidly in the ice-bath.
(v) Add 50 μl of reagent B and mix well.
(vi) Reheat at 100°C for 15 min.
(vii) Cool rapidly to room temperature and determine the absorbance at 525 nm.

Comments. Neutral carbohydrates interfere with this assay to a greater (~10% on a molar basis for hexoses) or lesser extent (~1% on a molar basis for pentoses). However both types of interference can be eliminated by use of appropriate controls as they 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.

2.7 **Sialic acid**

2.7.1 Warren assay (9)

Sensitivity: 80 ng−8 μg *N*-acetyl neuraminic acid in 80 μl (3−300 μM).

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. This reagent is stable indefinitely.

(B) Dissolve 10 g of sodium arsenite and 7.1 g of sodium sulphate in 0.1 M sulphuric acid (made by carefully diluting 5.7 ml concentrated sulphuric acid to 1 litre with water) to a total volume of 100 ml. The solution is stable indefinitely.

(C) Dissolve 1.2 g of 2-thiobarbituric acid and 14.2 g of sodium sulphate in water to a total volume of 200 ml. This reagent is stable for several weeks but eventually forms a yellow precipitate which indicates the need for its renewal.

(D) Redistilled cyclohexanone. This is stable for several months or until noticeably discoloured.

Method

(i) To the samples, standards and controls (80 μl) add 40 μl reagent A and mix well.

(ii) Leave at room temperature for 20 min.

(iii) Add 400 μl reagent B and then shake the tubes vigorously to expel the yellow-coloured iodine.

(iv) Leave for a further 5 min at room temperature.

(v) Add 1.2 ml reagent C, shake the tubes, stopper them and heat at 100°C for 15 min.

(vi) Cool rapidly to room temperature.

(vii) Extract the chromophore into 1.0 ml of reagent D by vigorous shaking. Centrifuge the solutions using a bench centrifuge for a few minutes in order to properly separate the two layers.

(viii) Determine the absorbance of the upper cyclohexanone layer at 549 nm.

Comments. DNA, 2-deoxy-D-ribose and substances producing malondialdehyde on periodate 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 2.9). L-Fucose reduces the expected absorbance of this assay. Methoxyneuraminic acid and some acetylated neuraminic acids give no colour in this assay and the resorcinol-HCl assay (10) should 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.

2.8 **Enzymatic methods**

2.8.1 *Hexokinase/dehydrogeanse assay* (11)

Sensitivity: 400 ng−40 μg glucose in 100 μl (20 μM−2 mM).