Oligosaccharides

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1. Introduction

Oligosaccharides are traditionally defined as polymers of monosaccharides containing from two to ten residues. However, since the naturally occurring polysaccharides rarely contain less than 25-30 residues, it is possible to consider the range of polymers having between two and about 2-25 residues as oligosaccharides, particularly when considering the analytical methods involved. As oligosaccharides fall into the classification between monosaccharides and polysaccharides it is not surprising that the methods used for oligosaccharide analysis are extensions of those used for either monosaccharide or polysaccharide analysis. This chapter uses a slightly different format to that of Chapter 1 in order to give the maximum information, within the limitations of available space, on the modifications which have to be made in adapting the methods described in detail for the analysis of monosaccharides and polysaccharides. Rather than repeat descriptions of practical details etc. this chapter concentrates on the types of methods which can be applied and their relative merits and shortcomings.

As with monosaccharide analysis, the analysis of oligosaccharides can be divided into qualitative identification and quantitative determination but, in addition to determination of the number and type of component residues, the actual linkage between successive residues is an area not encountered in monosaccharide analysis. Due to the complexities of linkage analysis, discussion of the methodology is not described in this chapter but can be found in subsequent chapters

concerning the analysis of polysaccharides and carbohydrate-containing macromolecules. The traditional methods used for qualitative analysis: paper chromatography and thin-layer chromatography, (TLC), have largely been replaced by rapid quantitative techniques, but, for completeness, are described briefly. Methods for the separation and quantitation of mixtures of oligosaccharides and for the identification and quantitation of individual purified oligosaccharides are described. Rather than providing a listing of all methods which have been devised, only those which have become the more commonly used techniques are described.

2. Colorimetric methods

The colorimetric methods which have been devised are mainly used for the gross determination of total carbohydrate content or total reducing sugar content, although specific assay methods have been developed for the quantitation of an individual oligosaccharide from a mixture of compounds via the use of specific enzymes. Both total carbohydrate and reducing sugar contents are important features of oligosaccharide analysis since it is possible to obtain a value for the size of an oligosaccharide or, for a mixture of related oligosaccharides, a mean value for the ratio of total residues to terminal reducing residues. With the development of sugar syrups [1] methods for the characterization of these products have been developed with the various syrups being categorized by their dextrose (i.e. D-glucose) equivalent or DE value. Despite the shortcomings of defining mixtures of oligosaccharides in terms of this one parameter [2] it is still used as the principle method for characterizing oligosaccharide mixtures.

2.1 Total sugar assays

A number of assays have been developed which rely on the action of concentrated (or near concentrated) sulfuric acid causing hydrolysis of all glycosidic linkages and the subsequent dehydration of the monosaccharides released to give derivatives of furfural (e.g. hexoses produce 5-hydroxy-methyl furfuraldehyde). The dehydration products react with a number of compounds such as L-cysteine [3], phenol [4], orcinol [5], and anthrone [6] to give coloured products. Whilst total sugar concentrations are readily obtained for homoglycans, care must be taken in interpreting the results obtained with heteroglycans due to the different colour intensities produced by different monosaccharides. Practical details for the performance of the phenol assay can be found in Chapter 1, *Protocol 2*.

Protocol 1. Orcinol-sulfuric acid assay

Sensitivity: 0-20 µg carbohydrate in 200 µl ; Final volume: 1.0 ml

Reagent

Ice-cold orcinol (recrystallized from benzene) dissolved in concentrated acid (2 g/litre). This reagent should be prepared fresh each day but can be stored at 4°C for up to one week.

Method

1. To precooled (to 4°C) samples, standards, and controls (200 µl) add 800 µl of reagent with care. Mix well.
2. Heat the solutions at 80°C for 15 mm and cool rapidly to room temperature.
3. Determine the absorbance at 420 nm.

Note: The original method [4] suggested that absorbances should be determined at 510 nm but the use of 420 nm results in an increased sensitivity (by a factor of two) and a reduced interference from uronic acids and deoxy sugars.

2.2 Reducing sugar assays

Traditionally the Lane and Eynon [7] assay was used to determine the content of reducing sugars in a sample and still is the method of choice in some industrial applications. The method involves the reaction of reducing sugars with alkaline cupric salts to give cuprous oxide which can be monitored titrimetrically to give the concentration of various reducing sugars by reference to standard tables. Further modifications to this assay have eliminated the use of tables [8].

A far more convenient assay is that which involves the reaction with alkaline 3,5-dinitrosalicylic acid originally devised by Bernfeld [9] and for which practical details are given in Chapter 1, *Protocol 3*. The major disadvantages of this assay are its insensitivity at low carbohydrate concentrations [10] and some workers report different responses obtained for equimolar amounts of oligosaccharides [11]. For this reason the less convenient Nelson-Somogyi [12] assay is preferred in some instances [13]. This assay is based on the reduction of cupric salts to cuprous salts which further reduce the arsenomolybdate complex to molybdenum blue, the intensity of which is determined spectrophotometrically. It is however less sensitive than the 3,5-dinitrosalicylic acid assay. The assay of reducing groups in oligosaccharide homologues with 2,2'-bicinchonite has been recently proposed [14].

A number of other assays have been reported such as those using alkaline ferricyanide [15] or alkaline picric acid [16]. The reaction based on the production of coloured soluble formazan salt from the dye tetrazolium blue [17], as a result of an essential solvent extraction stage, can be applied to samples containing particulate matter.

Protocol 2. Tetrazolium blue assay

Sensitivity: 0-10 µg glucose equivalent in 100 µl ; Final volume: 1.0 ml

Reagent

Add 3 vol. of 0.3 M NaOH to 1 vol. of an aqueous suspension of tetrazolium blue (1% w/v) and stir until completely dissolved. Dilute with 5 vol. distilled water and store at 5°C in the dark.

Method

1. To samples, standards, and controls (100 µl) add 900 µl of reagent and mix well.
2. Heat the solution at 100°C for 30 sec and cool rapidly to room temperature.
3. Add 1 ml of toluene and shake until no more colour is extracted from the aqueous layer.
4. Determine the absorbance at 570 nm.

Note: The sensitivity of the assay can be adjusted to accommodate small or large colour changes by halving or doubling the volume of toluene used for the extraction stage. Extended heating of reaction mixture results in alkaline hydrolysis of oligosaccharides and increased absorbance values.

2.3 Automated assay system

One of the major uses of colorimetric assays is the monitoring of chromatographic columns. Traditionally this was done by collecting fractions of effluent stream and performing a series of manual assays. To a large extent this has now been replaced by either non-specific detectors (see Section 5.6) or automated assay systems of various degrees of specificity. The pioneering work on assay automation was performed using air segmented systems based on glass tubing of approximately 1.6-2.4 mm i.d. and Technicon AAI type equipment. This allows mixing of reagents via peristaltic pumps equipped with tubing of different sizes and construction to facilitate accurate mixing of the majority of reagents including certain organic solvents and concentrated minerals acids. Increasingly the assay systems now being devised use non-segmented flow-through narrow bore (0.5 mm i.d. and smaller) Teflon tubing but such systems require pumps capable of pumping solvents and corrosive liquids at the higher pressures resulting from the use of narrow bore tubing.

Several systems have been devised for the detection of reducing sugars (see Chapter 1) but these are only applicable to the detection of the smaller oligosaccharides due to the response being related to the molar concentration of the oligosaccharide and not the concentration by weight. Since the response of maltohexaose is only 18% of that obtained for the same weight of D-glucose, reducing sugar assays are never used for the detection of oligosaccharides above hexasaccharides, and ideally are best suited to the detection of disaccharides and trisaccharides. The automated 3,5-dinitro salicylic acid assay is shown in *Figure 1* by way of an example.

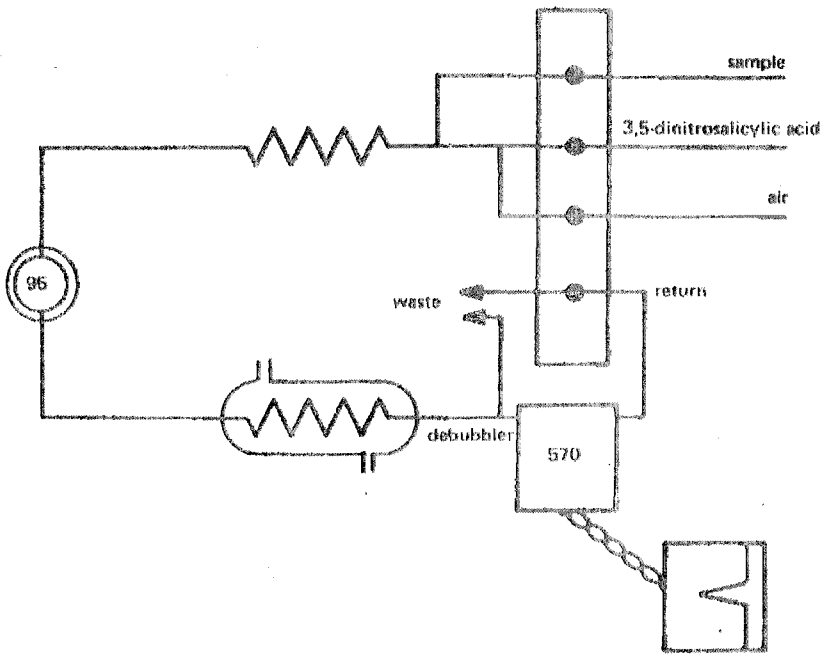


Figure 1. The automated 3,5-dinitrosalicylic acid assay. (DNS reagent consisting of 1.0 g of 3,5-dinitrosalicylic acid and 300 g of potassium sodium tartrate, per litre of 0.4 M NaOH 0.73 ml/mm; sample, 0-3 mg D-glucose equivalent/ml water or buffer, 0.20 ml/mm; air, 0.73 ml/mm; waste, 0.73 ml/mm.)

The detection of total sugar content relies on the use of reagents such as concentrated acid to hydrolyse the oligosaccharides to monosaccharides and to produce a suitable chromogen (e.g. 5-hydroxymethyl furfuraldehyde) and consequently the development in suitable automated assay systems has not received the same degree of attention. However systems have been devised for the estimation of total sugar content based on the L-cysteine [18] (see *Figure 2*) or orcinol assays [19] for neutral oligosaccharides and the carbazole [18] assay for uronic acid-containing oligosaccharides using segmented [18] or non-segmented [19] flow systems. The combination of such systems and chromatographic columns has led to the development of fully automated oligosaccharide analysers based on ion-exchange [19] or gel permeation [20] chromatography. Oligosaccharides were labelled with a solution containing 5% 4-aminobenzoic acid hydrazide in 0.5 M HCl with a 2.4 M NaOH solution in a ratio of 1:2 (v/v) [21]. This method was used as post-column reaction pumping the reagent in a ratio of 1:2 (v/v) to a HPLC column eluent and detecting the complex at 400 nm. The reaction conditions (reaction time 200 sec and reaction temperature 105°C) were optimized, giving a detection limit of 20-50 pmol for mono- and oligosaccharides [22].

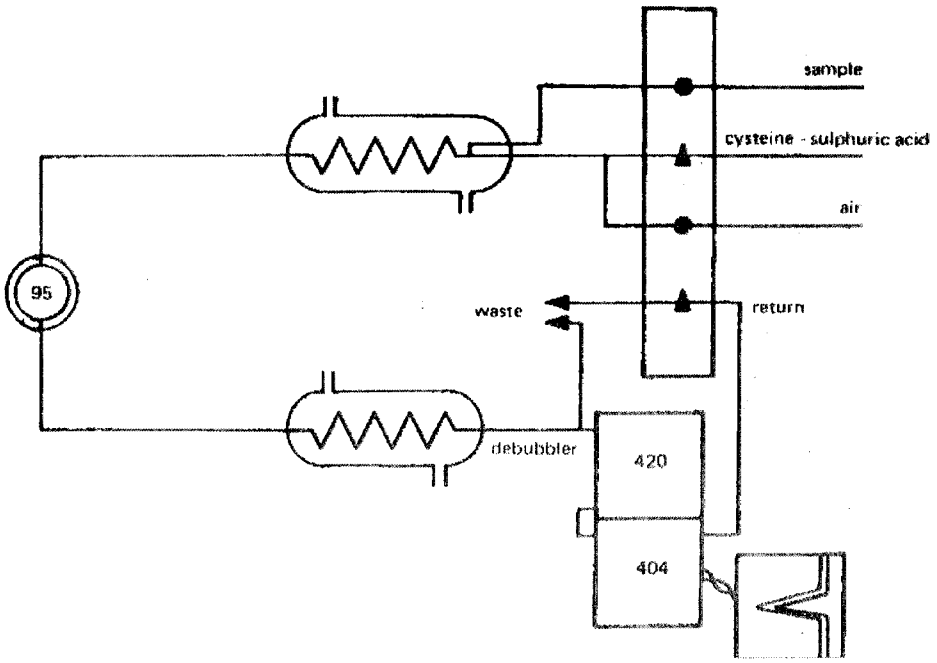


Figure 2. The automated l cystein-sulfuric acid assay. (L-Cysteine hydrochloride reagent, 700 mg/ml 86% sulfuric acid, 0.53 ml/mm; sample, 0-50 µg/ml water or buffer, 0.1 ml/mm; air, 0.23 ml/mm; waste 0.3? ml/mm.)

3. Thin-layer chromatography

TLC is not widely used for oligosaccharide analysis, but can be useful as a rapid technique for monitoring, for example, the hydrolysis of starch to give a particular oligosaccharide spectrum. It is also useful when it is not possible to perform more complex studies, as for example during field studies. The technique is essentially qualitative in its execution, although semi-quantitative data can be obtained using scanning densitometric analysis of the visualized chromatogram. Such results must however be interpreted with care since the degree of colour produced by the various visualization techniques is affected by parameters such as temperature and duration of heating, coverage of spray reagent, traces of salt or eluants, etc. all of which can vary on a single chromatogram as well as between different chromatograms.

Two systems which can be used to provide initial data on the relative abundance of the lower members of homologous series of oligosaccharides are described briefly. Both use the multiple-ascents technique, whereby the chromatogram is eluted in the normal manner, dried, and re-eluted with the same eluant a number of times, in order to improve the resolution between components. Full practical details for analysis can be found in Chapter 1.

Kieselgel G has been used to separate the individual members of a series of non-reducing oligofructosides, up to a hexasaccharide, derived from plant extracts by applying the mixture in 70% ethanol and eluting the chromatogram with chloroform/acetic acid/water (3:3.5:0.5) at ambient temperature with three ascents being used for optimum resolution [23]. Individual members of the series of oligosaccharides derived from the enzymatic hydrolysis of starch, up to maltodecaose, can be separated using silica gel G and an eluent of butanol/ethanol/water (5:3:2) with three ascents for optimum resolution [24] (see *Figure 3).*

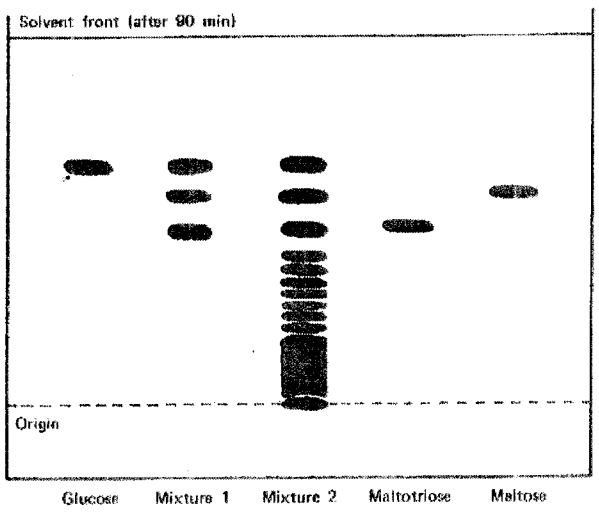


Figure 3. TLC separation of standard maltooligosaccharides on silica gel G using butanol/ethanol/water (5:3:2) eluent. (Individual components, 4 mg/ml, 5 µl loaded; mixture 1 = D-glucose, maltose, and maltotriose, 2 mg each/ml, 10 µl loaded; mixture 2 = starch hydrolysate 50 mg/ml, 5 µl loaded.)

Detection of components on a thin-layer chromatogram can readily be achieved using a non-selective charring technique by heating the chromatogram after spraying it with a reagent prepared from concentrated sulfuric acid in ethanol (1:9). This method can be improved such that increased contrast and sensitivity is obtained if ceric sulfate (3% w/v) is added to the sulfuric acid/ethanol spray [24]. Selective detection can be obtained using spray reagents containing diphenylamine aridine phosphate [25] or o-anisaldehyde [26] which give different colours with different carbohydrates and thereby assist in identifying overlapping or unresolved peaks. The use of reagents such as tetrazolium blue and its

derivatives which only visualize reducing sugars, are of little value for oligosaccharide analysis due to the diminishing response with increasing molecular weight of oligosaccharides.

Amino-bonded high performance TLC silica plates, and pyridine/ethyl acetate/acetic acid/water (6:2:1:3) as eluent, has been used for directly immunostaining of free oligosaccharides [27].

4. Low pressure column chromatography

The distinction between low pressure (traditional) column chromatography and high performance liquid chromatography (HPLC) is not as clear cut as many workers would have you believe, with definitions being based on particle size (25 μm is a typical division) or the capital cost of equipment. Consequently some of the discussion in this section can equally apply to Section 5 and vice versa. Low pressure column chromatography is typified by the use of compressible column packings which require the use of low pumping pressures (hydrostatic pressure of peristaltic pumps frequently being used) and extended analysis times in the region of 2-18 hours. Detection is frequently performed using automated assay systems (Section 2.3) or by manual assay following collection of eluate fractions. Many of the historical developments and applications with respect to oligosaccharide analysis have been reviewed [28].

4.1 Ion-exchange chromatography

4.1.1 Types of systems available

Ion-exchange chromatography has almost universally replaced the traditional adsorption chromatography based on charcoal-celite mixtures developed in the early 1950s which was capable of separating series of oligosaccharides up to a degree of polymerization (DP) of 8-10 using ethanol or butanol gradients in water as eluent [29]. The use of ion-exchange resins is preferred due to their increased selectivity, lower operational back pressures, increased reproducibility, and the ability to use eluents which do not decrease the solubility of the higher oligosaccharides as is the case with mixtures of alcohols in water.

Anion-exchange resins, most commonly in the carbonate or bicarbonate form, although chloride and hydroxyl forms can be used, will fractionate oligosaccharides in order of decreasing molecular size. The use of water as eluent allows fractionation of neutral sugars up to DP about 6 but the possibility of interconversion of terminal residues under the effects of the basic ion-exchange resins has prevented a full exploitation of the method. The use of acetic acid, sodium acetate, formic acid, or lithium chloride eluents allows the fractionation of acid-containing oligosaccharides such as uronic acid- or aldonic acid-containing oligosaccharides with, for example, up to DP 14 being fractionated for the series of xylonic acids prepared from a birch xylan hydrolysate using Dowex 1-X2.

Cation-exchange resins have also been used for separation of oligosaccharides in an attempt to overcome the effects of sugar interconversions by anion-exchange resins. Cation-exchange resins in the lithium, barium, or potassium form have been used to give results similar to those obtained with anion-exchange resins but with the aid of less complex eluents. The effect of the counterion is considerable and the correct choice is essential for optimum resolution of a given oligosaccharide mixture [30].

Use of water/alcohol mixtures to elute ion-exchange columns results in a partition mechanism being responsible for the separation, with equilibria being established between the phase within the resin and the bulk solution. With anion-exchange resins there is a higher proportion of water within the resin than in the bulk solution which favours retention of the higher oligosaccharides such as a reversal of elution order can be achieved compared to anion-exchange chromatography using water as sole eluent, with monosaccharides eluting before disaccharides and the higher oligosaccharides following. This results in incomplete recovery of some mixtures of higher oligosaccharides and restriction of the method to mixtures containing DP less than 10. The most common resin used is the sulfate form of styrene-divinylbenzene resins [31] although quaternary ammonium salts derived from polymeric carbohydrate resins (i.e. cross-linked dextran) can be used [32]. The use of cationic resins in this partition-type mode is of little use in oligosaccharide analysis due to the high alcohol concentrations required for elution. This restricts the analysis to disaccharides only.

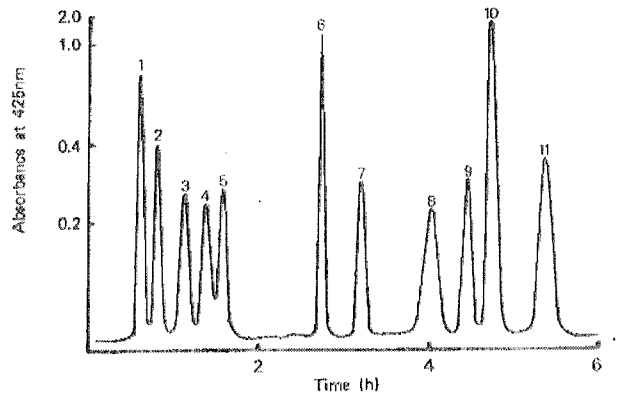
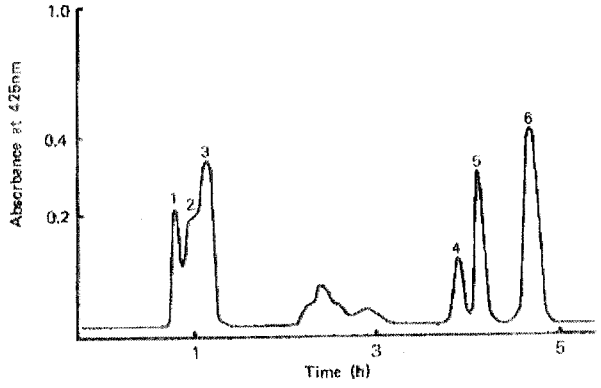


Figure 4. Ion-exchange chromatography of monosaccharides and disaccharides using Jeol LC-R 3 ion-exchange resin (1, sucrose; 2, cellobiose; 3, maltose; 4, lactose; 5, rhamnose; 6, ribose; 7, mannose; 8, fucose; 9, galactose; 10, xylose; 11, glucose).

If borate buffers are used to form complexes with carbohydrates the different affinities of the negatively charged complexes for the ion-exchange resins can be exploited as a means of separating oligosaccharides as an extension to the normal ion-exchange chromatographic separation of monosaccharides [19] (see *Figure* 4). The extended analysis times



required and the alkaline conditions can cause interconversion of the terminal residues of reducing sugars. By alteration of the composition of the eluting buffers the system is ideally suited for separation of families of structurally related disaccharides and trisaccharides such that structural features of, for example, reversion products of enzymically degraded starch can be identified [33] (see *Figure 5*).

Figure 5. Ion-exchange chromatography of starch-derived oligosaccharides showing structural features enhanced by borate ion complexation using Jeol LC-R-3 resin (1, panose; 2, maltotriose; 3, maltose; 4, isopanose; 5, isomaltose; 6, D-glucose).

Protocol 3. Ion-exchange column chromatography

1. Convert the ion-exchange resin to the desired form and solvate following the manufacturers' recommendations.
2. Fill a glass column (diameter in the range of 0.6-1.0 cm, and length 10-30 cm) drawn to a neck at the lower end and containing a small plug of glass wool with buffer. Pour the resin in and allow it to settle.
   Note: The use of proprietary columns and fittings such as the Omnifit® range (Omnifit Ltd.) greatly assists in the production of a chromatographic column which can be pumped under increased pressure or which flows under hydrostatic pressure.
3. Pump the resin with buffer or eluent until a stable packed bed is obtained.
4. Introduce the sample onto the column by use of proprietary system injector. Alternatively it can be introduced by removal of the eluent head from above the resin, allowing the sample to enter the resin (with the aid of air pressure from a syringe attached via a rubber bung to the top of the column if necessary), washing the sample into the resin with a small amount of eluent, and replacing the eluent head.
5. Pump the column with eluent and monitor either by automated assay (Section 2.3) or by collection of fractions and manual assay (Section 2.1).

4.2 Gel permeation chromatography

Gel permeation chromatography (GPC) is defined as the separation of compounds according to their molecular weight, or more correctly their hydrodynamic volume. It has been developed into a very useful technique for oligosaccharide analysis. Tridimensional cross-linked gels, based on dextran (e.g. Sephadex G-10 or G-25 from Pharmacia) or polyacrylamide (e.g. Bio-Gel P-2, P-4, P-6 from Bio-Rad Laboratories) have the degree of cross-linking controlled such that the resultant pores are too small to allow high molecular weight species to penetrate. They are excluded from the gel and therefore elute in a volume equal to the void (dead) volume (V_0) of the column. Very small molecules penetrate the gel freely and are eluted in a volume (V_T) equal to the sum of the void volume and the volume of the solvent within the gel matrix available to small molecules (i.e. the pore volume, V_p). Thus

$$V_T = V_0 + V_p$$

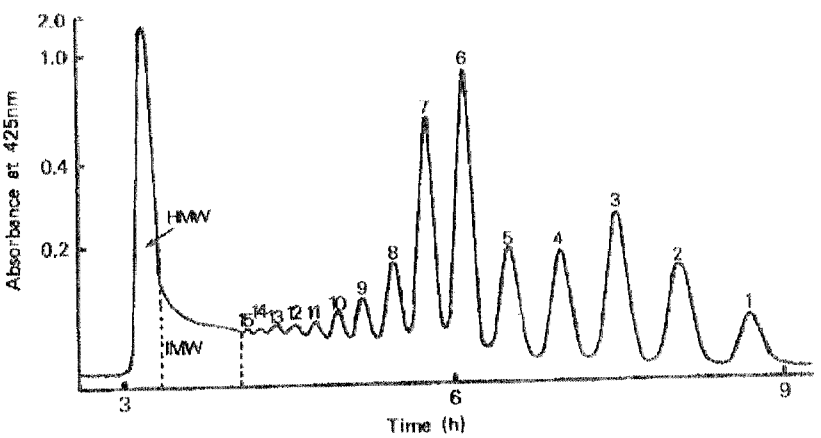


Figure 6. Gel permeation chromatographic fractionation of starch-derived oligosaccharides on Bio-Gel P-2. (1,2,3 etc., D-glucose, maltose+isomaltose, D-glucotrisaccharides, etc., IMW, intermediate molecular weight material DP 15 to approximately 20; HMW, high molecular weight above DP approximately 20.)

Molecules of intermediate size can penetrate some of the pores of the gel matrix and the degree of penetration, which is related to the distribution of pore sizes and the hydrodynamic volume of these intermediate size molecules is reflected in the elution volume (Ve) which lies within the range V_0 and V_T (see *Figure 6*). The distribution of pore sizes within the gel matrix is such that over a considerable range of elution volumes the relationship:

$V_e \alpha$ log (molecular weight)

can be applied. At the extremities of the range of elution volume this relationship does not apply, but it is still possible to obtain some data provided a calibration curve (see *Figure 7*) is constructed to cover the range of elution volumes involved, using materials having similar known hydrodynamic volumes. Examples have been published [34,35] which show that homologous series of oligosaccharides have different gradients for their calibration curves due to the different hydrodynamic volumes for oligosaccharides having the same molecular weight. Therefore it is important to exercise care in interpreting the molecular weight of an unknown oligosaccharide when using a gel permeation column which has been calibrated with, for example, the maltooligosaccharide series (derived from the controlled hydrolysis of amylose) or the cello-oligosaccharide series (as shown in *Figure 7*).

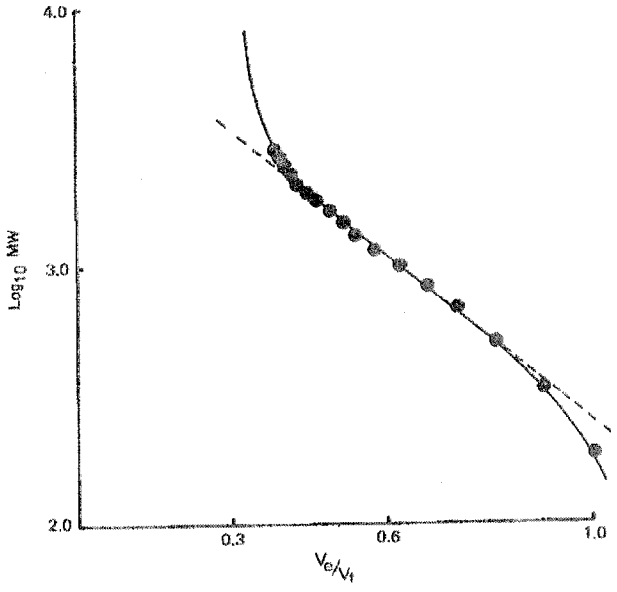


Figure 7. Calibration curve for Bio-Gel P-2 derived from the cellooligosaccharide series obtained by the controlled hydrolysis of cellulose.

In an ideal system the only effects which contribute to the separation of oligosaccharides are steric effects but it has now become well-recognized that elution volumes greater than unity can be obtained for both carbohydrate-[36] and polyacrylamide-[18] based permeation supports. It is recommended that eluents containing, for example, 0.1 M NaCl should be used to prevent adsorption phenomena affecting the separation. The use of polyacrylamide-based gels is also recommended for optimum resolution at high temperatures (e.g. 65°C) [20] or when samples of biological origin are used [18] in order to prevent elution of extraneous carbohydrate material due to disruption of the gel matrix. Such a system can be operated using automated assay detection systems for up to one year with no loss of resolution [20].

The use of oligosaccharide fractionation by gel permeation is now recommended as a superior method for the characterization of starch hydrolysates [2] particularly in the determination of dextrose equivalent [37] based on the actual composition of oligosaccharides present rather than on a single gross determination (see Section 2).

Gel permeation gives very good results for both analytical and preparative separations [38] and has been frequently combined with HPLC [39]. The effective size of oligosaccharides from glycoprotein, expressed in glucose units using Bio-Gel P-4 (< 400 mesh), has been summarized [40].

4.3 Affinity chromatography

Affinity chromatography is a unique separation method which allows the isolation of particular molecules according to the biospecific interaction with an immobilized ligand. This technique, using immobilized carbohydrate-binding proteins (i.e. lectins [41] and antibodies [42]) permits separation of carbohydrate complex mixtures, based on the stereochemistry [43]. The elution is performed with buffers in controlled temperature (water-jacked columns are often used).

Fractionation and structural analysis of oligosaccharides by means of immobilized lectin columns has been recently reviewed [44].

5. High performance liquid chromatography

HPLC, characterized by small particle sizes (< 25 μm), narrow bore columns, high inlet pressures, and short analysis times, uses many of the same separation principles described for low pressure column chromatography. HPLC can be considered a valid alternative to gas chromatography for quantitative and qualitative analysis. It is possible to inject the sample without a prior derivatization, obtaining in short time analysis a high resolved chromatogram. Since its inception in the mid 1970s the number of methods and types of packing material have expanded rapidly. More recently the bonded-phase columns represent the most used separation systems. Much of the earlier work on oligosaccharide analysis has been reviewed [45] and the theory, separation modes, and instrumentation described [46], and the developments are such that a number of the low pressure chromatographic systems are now being replaced, particularly in the food industry for the analysis of the simpler mixtures. Details of the experimental approach can be found in Chapter 1, Section 6.

5.1 Adsorption chromatography

Adsorption, or normal phase, chromatography relies on the surface hydroxyl groups of silica (and to a lesser extent alumina) which can interact with solutes and affect a separation on account of the different strengths of interaction

Alumina causes a strong adsorbment and its basic character can induce, in some cases, epimerization. The separation of neutral oligosaccharides cannot be carried out conveniently by this method although limited separations can be achieved in water [47] or using solvent mixtures [48]. The method is, however, well suited to the analysis of derivatives of oligosaccharides of low DP using non-aqueous eluents (see *Table 1)* particularly when ultrasensitive (10-100 ng level) detection is required (see Section 5.6).

Table 1. Derivatives used for the adsorption chromatography of oligosaccharides

Derivative	Eluent	Reference
Benzoate	Hexane/ethyl acetate (85:15)	49
4-Nitrobenzoate	Hexane/acetonitrile/chloroform (65:20:15)	50
Benzyloxime-perbenzoate	Hexane/dioxane (80:20)	51
Phenyldimethylsilyl ethers	Hexane/ethyl acetate (197:3)	52

5.2 Reversed-phase chromatography

Reversed-phase chromatography packings are characterized by hydrocarbon chains bonded to the surface of the silica matrix. Whilst chain lengths can range from 1 to 22 carbon atoms the most popular are the 18 carbon atom chain (octadecylsilyl silica), the eight carbon chain (octylsilyl silica), and the six carbon chain (hexylsilyl silica) [53]. The essential criterion responsible for separation is the interaction of the packing with polar materials. Using aqueous solutions or solvents of medium polarity the more polar species elute first and as the polarity decreases the more tightly bound less polar species are eluted. Using this kind of packing it is possible to separate both derivatized and underivatized sugars. Separations of underivatized oligosaccharides were obtained at room temperature, using pure water as eluent [54, 55]. Using low temperatures it is possible to achieve an improvement in the resolution. Broad peaks poorly resolved are obtained if anomers are present in the mixture. To overcome this shortcoming a derivatization is necessary by reducing the terminal aldehyde group to the alcohol or by reductive amination.

The use of high concentrations of organic solvents in the mixtures of water and solvent used as eluent gives rise to problems of solubility of oligosaccharides. Nevertheless oligosaccharides up to DP 30 contained in wood extracts have been fractionated in under 30 minutes using a gradient of 70-62.5% acetonitrate in water [56] although the separation between the first five members of the series (DP 1-5) was very poor. Use of fully acetylated oligosaccharides overcomes problems of solubility and has resulted in the fractionation of maltooligosaccharides up to DP 30 in about 150 minutes [57] using an exponential gradient of acetonitrile (10-70%) in water. Another advantage is the possibility after preparative HPLC to recover the original oligosaccharides by treatment with mild base allowing further analysis.

Perbenzoylated derivatives of high mannose-type oligosaccharides were used to improve the chromatographic properties on reverse-phase columns, using a 15 minutes linear gradient from acetonitrile/water (4:1 v/v) to pure acetonitrile [58].

5.3 Bonded-phase chromatography

By far the most frequently used systems for separation of oligosaccharides are those using chemically bonded phases which fractionate materials on the basis of their relative affinities for the mobile phase and the bonded phase. Oligosaccharides are eluted in order of increasing molecular weight. Supports containing a variety of cyano- or amino-bonded phases have been used in the past but the two most important types of column are those containing the aminopropyl-bonded phase [59] and hybrid phase of cyano- and amino-derivatives, i.e. the Partisil PAC column [60] with the aminopropyl-bonded column being regarded by some workers as the ultimate in column technology for oligosaccharide analysis [61].

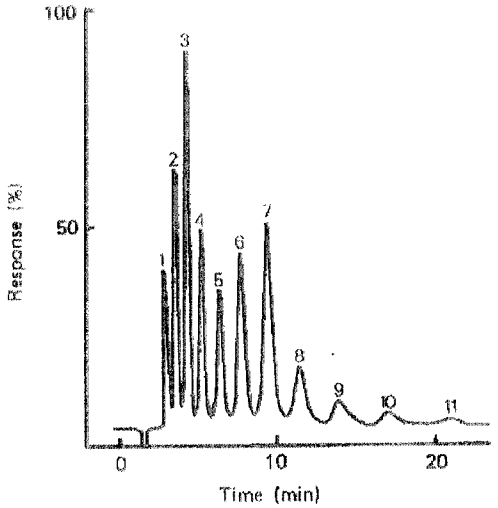


Figure 8. Separation of starch-derived oligosaccharides on a Spherisorb S5 NH_2 column (1,2,3, etc., refer to the DP of the oligosaccharide).

As eluent, nonpolar organic solvents or an aqueous/organic mixture (such as aqueous methanol or aqueous acetonitrile) are usually used. Separation of series of oligosaccharides from, for example, hydrolysed starch can readily be achieved with up to DP 10 being separated in 15-20 minutes using acetonitrile/ water eluents containing 35-40% water (see *Figure 8).* Increasing the water content to 45% can increase the number of detectable oligosaccharides up to about DP 15. However very high molecular weight materials cannot be analysed due to prolonged retention times and solubility problems in the acetonitrile/water eluents and for a full analysis separation by gel permeation of ion exchange is also required.

In some cases [45] interactions are possible between the reducing sugar and the amino group of the stationary phase. Prolonged use of bonded-amine columns is accompanied by a loss of performance through loss of the bonded phase, and to overcome this shortcoming and to introduce different degrees of selectivity into the system the use of *in situ* modification of the silica has been developed [62]. Instead of using an amine phase bonded to the silica support a diamine or polyamine is added to the eluent which results in a dynamic equilibrium between an amine phase coating the silica and that in the eluent. Separations are similar to those obtained using bonded-amine columns with separations of up to 20-25 being achieved in 45 minutes (see *Figure 9)* using eluents containing 50% water in acetonitrile to which the modifier has been added at the 0.01% (v/v) level. Even at this low modifier concentration it is imperative to use a presaturation column in the system prior to the injector to protect the analytical column packing from dissolution. Whilst diamine modifiers give the optimum resolution between oligosaccharides of different DP polyamine modifiers give improved selectivity and separation of oligosaccharides of the same DP such that the presence of, for example, isomaltose in starch derived oligosaccharides can be determined [61]. Recently stationary phases similar to the aminopropyl silica have been developed that give a higher packing stability [63, 64].

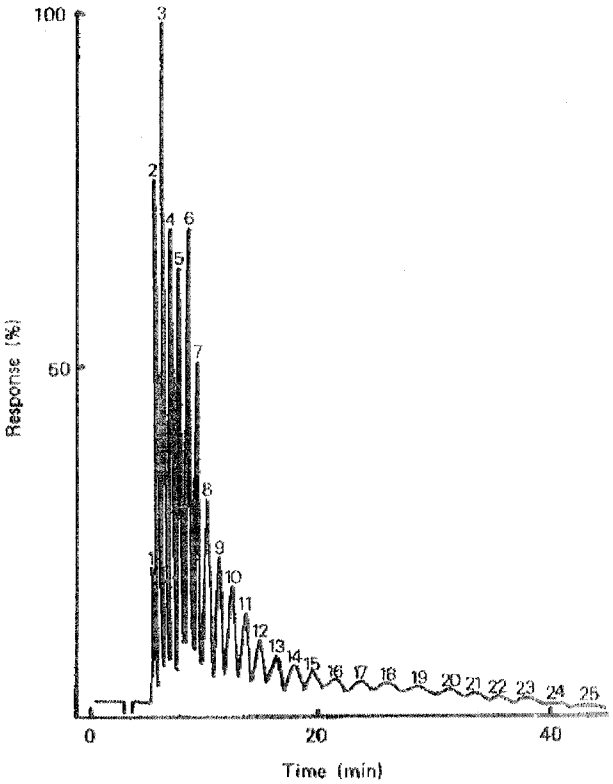


Figure 9. Separation of starch-derived oligosaccharides on an *in situ* modified silica column using 1,4-diaminobutane (0.01% v/v) as modifier (1,2,3, etc., refer to the DP of the oligosaccharide).

5.4 Ion-exchange chromatography

High performance ion-exchange chromatography utilizes the same mechanisms for separation described in Section 4.1.

Anion-exchange HPLC has been recently developed performing an exceptional resolving power for complex oligosaccharides. These analyses are carried out at high pH coupled with pulsed amperometric detection (PAD) (see Section 5.8), allowing separation of oligo- and polysaccharides up to DP ≥ 50 (see *Figure 10)* [65]. The separation depends on the molecular size, sugar composition, and kind of linkages between the monosaccharide units.

Analysis of linear and branched glucose oligosaccharides was investigated [66]. Anion-exchange chromatography has also been used to separate a variety of positional isomers of neutral, sialylated, and phosphorylated oligosaccharides in a wide range of pH values (pH ~4-13) [67,68]. A very interesting application is given by the possibility of injecting crude enzyme digests of complex oligosaccharides [69]. This method does not damage the phase and permits a quick and easy monitoring of the enzymatic digestion course and to isolate the reaction intermediates. Anion-exchange chromatography has been used recently to analyse oligosaccharides contained in complex biological samples [22,70].

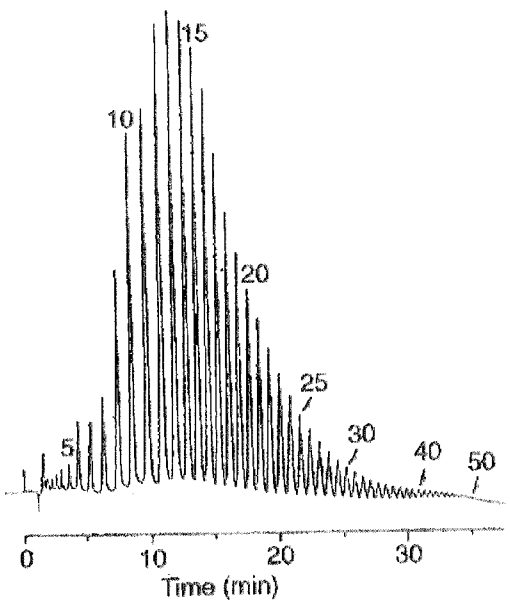


Figure 10. Chromatogram of (1→4)-α-D-glucans (short chain amylose EX-1 (DP ≈ 17)).
The number on each peak indicates its DP. Chromatographic conditions: column, HPIC-AS6 (250 mm x 4 mm i.d.); eluent and gradient program as shown in *Table 1;* flow rate, 1 ml/min; detector, PAD II; meter scale, 10 K nA; temperature, ambient.

The separation of oligosaccharides by cation-exchange chromatography is based on the size exclusion and ligand-exchange mechanism. Most cation-exchange resins are prepared from cross-linked polystyrene and silica-based ion exchangers. The use of 4% and 8% cross-linked cation-exchange resins in the calcium [71,72] or silver [73] form have been used to provide a rapid separation of oligosaccharides of up to DP 6-8 for calcium counterions [72] or DP 8-12 for silver counterions [73] depending on the size of the chromatographic column and the time of analysis. The major drawbacks to the use of such systems include the compressibility of the gel matrix (depending on the amount of cross-linking), extended analysis times, efficiency losses of the order to *50%* for a doubling of the flow rate, the need for high temperature (85°C) operation, and the need for specialized regeneration and re-packing of contaminated columns. However these drawbacks are offset by the ability to obtain a total analysis of material applied to the column (see *Figure 11)* and; the use of water as the only eluent [74]. Another way to overcome the losses in efficiency is the use of 2% cross-linked stationary phase (H^+ form) with low flow rates and very low back pressure [75]. In this case the size exclusion mechanism is predominant. The use of 0.01 M sulfuric acid enables the constant regeneration of the H^+ form stationary phase. The effects of the temperature and the flow rate on the resolution has been investigated [76]. The size fractionation of oligosaccharides up to DP 20 has been performed using a cation-exchange resin column (Na^+ form) using water/acetonitrile as mobile phase, in the presence of variable concentrations of sodium acetate or triethylammonium buffer [77]. The separation efficiency has been tested at different temperatures and mobile phase pH.

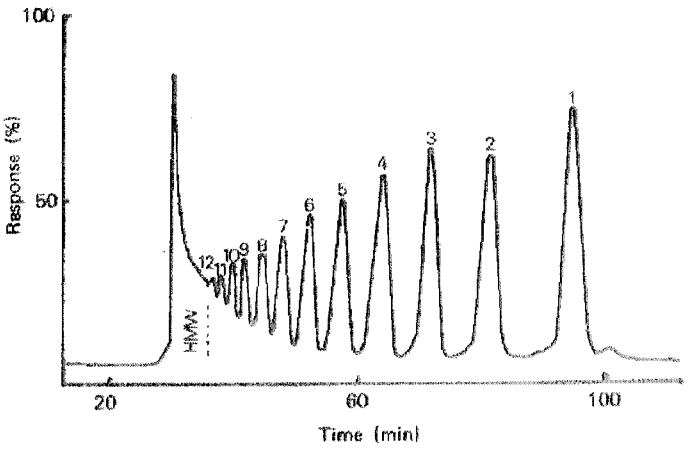


Figure 11. Separation of starch-derived oligosaccharides by high performance ion-exchange chromatography using 4% cross-linked cation-exchange resin with silver counterions (1,2,3, etc. refer to the DP of the oligosaccharide, HMW, high molecular weight material above DP 12).

5.5 Gel permeation chromatography

The development of non-compressible matrices for GPC analysis of water soluble materials has not reached the degree of sophistication available for the organic eluent compatible matrices. Consequently there has been no direct replacement for the cross-linked polysaccharide or polyacrylamide materials used for traditional gel permeation analysis of oligosaccharides. Some advances have, however, been made with the development of silica matrices deactivated by the chemical bonding of an organic ether stationary phase to provide a hydrophilic surface. The currently available materials have fractionation ranges which extend down to molecular weights of about 2000 (i.e. DP 10-12) whilst unmodified silica with 6 nm pore size can extend the fractionation range down to molecular weights of about 1000 (i.e. DP 5-6). Even with the modified materials adsorption effects are present and elution with ionic buffers (0.1 M) is recommended within the pH range 2-7. Products of starch hydrolysis has been separated using 0.15 M NaCl as the mobile phase [78]. Oligosaccharides up to DP 6 from partially degraded hyaluronic acid have been resolved but a complicated three-column arrangement was required [79].

Water-compatible hydroxylated polyether-based matrices have been developed (for example the TSK PW and TSK HW series) which overcome some of the disadvantages of silica-based materials and have fractionation ranges which are comparable to the cross-linked polysaccharide and polyacrylamide gels [80]. Such materials are less rigid than the silica-based materials and therefore require lower operating pressures. Their lower selectivity compared to silica-based matrices is offset by their increased stability towards alkaline pH (up to pH 12). Whilst analysis times are of the order of one third to one tenth that of traditional low pressure gel permeation analysis the separations are inferior to those currently obtainable by ion-exchange chromatography (Section 5.4) and consequently little emphasis is placed on high performance gel permeation chromatography for oligosaccharide fractionation.

5.6 Affinity HPLC

The affinity principle (see Section 4.3) has been used in HPLC to separate oligosaccharide molecules [81,82]. The ligand is covalently bonded with a 10 µm macroporous silica matrix. This improvement provides better resolution and shorter analysis times. Varying the temperature it is possible to obtain excellent chromatographic separation.

5.7 Preparative HPLC

The HPLC wide bore columns (2.0-2.5 cm i.d.) have been used to isolate milligram to gram quantities of maltooligosaccharides from starch hydrolysates [83]. Very good results have been achieved using reverse-phase, bonded, and cation-exchange (H^+ and Ag^+ form) packings. In order to obtain retention times for each oligosaccharide, comparable

to those obtained with the analytical columns, the flow rates for the preparative column (F_p) have been calculated using the following equation:

$$F_p = F_a[\text{i.d.p/i.d.a}]^2 L_p/L_a$$

where F_a is the analytical flow rate, i.d.p and i.d.a are the inner diameters of the preparative and analytical columns, and L_p and L_a are the column lengths.

5.8 Detection

Detection of oligosaccharides eluting from HPLC columns is the biggest challenge and the weakest link in the analysis of oligosaccharides. For underivatized oligosaccharides the most common detectors are: refractive index, UV, and electrochemical detectors. Non-specific detectors such as the refractive index detector are used routinely [64,74,76,83]. Ultra-sensitive refractive index detectors have detection limits of less than 200 nanograms of monosaccharide [83]. A severe restriction is the high sensitivity to the temperature changes and to the mobile phase composition. It is necessary to use isocratic elution (i.e. single non-gradient eluents). Low wavelength UV detection (below 210 nm) [54] has been shown to have comparable sensitivity whilst allowing the use of limited gradient elution [84]. Practically insensitive to the temperature and mobile phase changes (if the solvents used do not adsorb in the UV).

Electrochemical detection by means of a pulsed amperometric detector (PAD) has been considerably developed in the last few years, coupled in particular with anion-exchange [69,70] and affinity [81,82] HPLC. This technique is very sensitive (detection of 10-100 pmol) [67]. A complete study of linear and branched glucose oligosaccharides using anion-exchange HPLC and PAD has been recently carried out looking at the variation of detector response at different degrees of polymerization [85]. An electrochemical quantification of underivatized oligosaccharides has also been performed [68]. The main limit of PAD is the necessity to operate at relatively high pH, and that can influence the choice of the separation conditions.

A mass detector has been developed which allows gradient elution to be performed and has been used for monosaccharide analysis [86] but the use of high water contents in the eluants needed for oligosaccharide analysis results in instability of the evaporation system which is an integral part of the detection system.

The problem of removing all traces of eluent after chromatographic separation are being resolved (e.g. termospray interface) and are allowing the mass spectrometer to be developed as an ultra-sensitive HPLC detector [87] which could have many applications in oligosaccharide analysis [53].

To obtain an improved sensitivity pre- or post-column derivatizations have been developed. Aromatic or heterocyclic substituents are added to the oligosaccharide structure to allow the UV or fluorescence detection. Oligosaccharides can be labelled with 2-aminopyridine (UV and fluorescent absorber) by reductive amination [77,88], or with ethyl 4-aminobenzoate UV absorber) [63,89]. This last derivative allows the quantification of picomolar amounts of oligosaccharides [58]. Another alternative method in which the reducing carbohydrates are labelled for UV detection with 1-(4-methoxy)phenyl-3-methyl-5-pyrazolone allows sensitivity close to the subnanomole level [90].

Oligosaccharides have been ^{3}H-labelled at the reducing terminal by reduction with $NaBH_4$ for radioactivity monitoring [91]. The reduction conditions has been studied [92] to obtain the best detector limit (0.3 pmol).

Post-column derivatization labels the saccharides with a chromophore (see Section 2) for variable wavelength or fluorescence detectors. Whilst the use of post-column reaction systems have been developed for monosaccharide analysis (see Chapter 1) such systems have a limited applicability to oligosaccharide analysis due to the reliance on reducing sugar assays. Attempts to use strong cation-exchange resins (in the protonated form) to hydrolyse the glycosidic bonds in oligosaccharides to give complete conversion to monosaccharides after chromatographic separation have been reported [93] with the resulting monosaccharides being detected as reducing compounds. The method of post-column reaction for reducing carbohydrates, based on 4-aminobenzoylhydrazide followed by variable wavelength detection at 400 nm (see Section 2.3), has been compared with the PAD [22]. Post-column reactions has been developed in order to detect reducing and non-reducing sugars at trace levels [94].

A wide number of detection systems have been recently reviewed [45].

6. Gas chromatography

Despite the expansion of HPLC techniques, gas chromatography (HGLC) still continues to have a place in oligosaccharide analysis for both structural studies, to determine the component monosaccharide residues [95] and position of inter-residue glycosidic bonds (see Chapter 3), and the routine analysis and quantitation of oligosaccharides. Whilst the determination of oligosaccharides by GLC is normally restricted to the analysis of disaccharides as an extension of monosaccharide analysis (see Chapter 1) methods have been reported for the fractionation of oligosaccharides up to DP 6-7 [96,97].

The efficacy of packed column GLC analysis has been improved using the high resolution as chromatography (HRGC), with higher resolution and reproducibility. The analysis times are shorter and the chromatograms show sharp peaks for the larger oligosaccharides too. The use of high-temperature columns and more volatile derivatives has extended separation of permethylated isomaltooligosaccharides up to DP 10 with a sensitivity of one nanogram (< 1 pmol) [98, 99]. The effect of GLC analysis parameters (i.e. carrier flow rate, split ratio, and nature of the derivatizing agent) on the

separation of legume oligosaccharides on HRGC was recently studied [100]. Using long elution times and temperatures higher than 400°C the thermal degradation of saccharides occurs [98,99].

The choice between HPLC and GLC is not clear cut with GLC being at least ten times more sensitive and producing shorter separation times than HPLC techniques. To offset these advantages the additional time required to prepare the sample and produce the required derivative (see Chapter 1) with no partially derivatized contaminants makes single analyses very unattractive. The final decision as to whether to use HPLC or GLC frequently depends not on the relative merits of the method but on the nature of a particular sample [101]. Typical samples which are better suited to GLC analysis are those containing trace amounts of oligosaccharides as in plant tissues or where the low DP oligosaccharides are present in small amounts relative to high contents of disaccharides and monosaccharides such as found in high DE glucose syrups [96] or adulterated honey [102].

7. Supercritical fluid chromatography

Supercritical fluid chromatography (SFC) is a relatively new technique which combines the advantages of GLC and HPLC. In fact, using CO_2 as the mobile phase above its critical temperature, it is possible to analyse high molecular weight or thermolabile compounds. Another great advantage of this technique is the compatibility with universal detectors for organic compounds (e.g. flame ionization detector). The possibility of SFC-MS (mass spectrometry) coupling has been discussed [103]. Small internal diameter capillary columns are used (50-100 µm) with a stationary film (similar to the bonded-phase GLC columns) of 0.05-0.02 µm. The low solubility of carbohydrates in the CO_2 fluid is the biggest shortcoming. For this reason derivatization is necessary (trimethylsilyl or permethyl derivatization) [104-106]. Silylated maltooligosaccharides can be separated by SFC from DP 1 to beyond 20 [104] in pressure programmed conditions. In this case double peaks, due to the two anomers at each DP value have been observed. Resolution decreases in the course of the program due to increased mobile phase pressure.

8. Capillary electrophoresis

Capillary electrophoresis (CE) is a powerful new technique that provides short time and high efficiency separations of complex mixtures of ions. This is due to the application of electrophoretic separation to a fused silica capillary. The two ends of the capillary are immersed in two separated electrolyte reservoirs containing a high voltage electrode. Variable parameters to improve the separation are the buffer composition and the pH.

Detection is usually performed with on-line systems (UV or fluorometric detectors) and derivatization of the oligosaccharides is necessary. It is very important that the sample components possess charged moieties in their structures. For this reason the neutral oligosaccharides are converted to primary amines by reductive amination. This derivatization followed by the reaction with a fluorogenic reagent (3-(4-carboxybenzoyl)-2-quinolinecarbox aldehyde or 3-benzoyl-2 naphthaldehyde) has extended the detection limit down to the attomole level [107]. The capillary zone electrophoresis (CZE) is the simplest form of CE. The capillary is only filled with the buffer solution (phosphate or borate buffer). The separation is due to variations in the molecular size/electric charge ratios of the mixture components. Direct CZE separates the oligosaccharide derivatives according to the increasing degree of polymerization up to 20 (see *Figure 12)* but cannot distinguish oligosaccharides having the same DP CZE as borate complexes [108] provides a separation according to the structural differences of the outer monosaccharide residues. The two systems used to analyse the same sample are demonstrated to be complementary to each other [109].

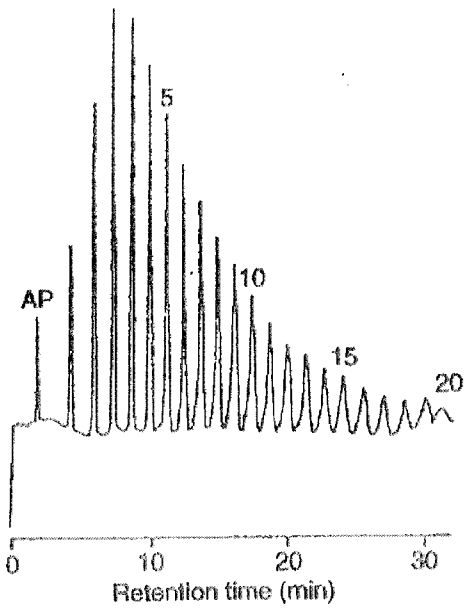


Figure 12. Separation of reductively pyridylaminated isomaltooligosaccharides by direct CZE. Capillary, fused silica coated with polyacrylamide (Bio-Rad, 25 µm i.d., 20 cm); carrier, 100 mM phosphate buffer (pH 2.5); applied voltage, 8kV; detection, UV absorption at 240 nm. AP, 2-aminopyridine (excess reagent).

9. Mass spectrometry

While mass spectrometry has traditionally been of major use when coupled to GLC columns (more recent is the possibility to interface HPLC and SFC to mass spectrometers) to provide structural information on oligosaccharides and polysaccharides (see Chapter 3) the development of several new ionization techniques now allows the direct analysis of underivatized oligosaccharides [110]. Field desorbtion, in which ions are produced by thin wire emitters on which the

sample is deposited, is a very soft ionization technique capable of producing molecular ions or simplified spectra [111]. Other soft ionization techniques, that has been recently developed, are fast atom bombardment (FAB) and liquid secondary ionization (LSI). Fast atom bombardment (FAB), produces ions via bombardment of the sample with high energy rare gas atoms. It direct coupling with micro HPLC for oligosaccharide analysis has been studied [112]. Structural information can be obtained in addition to molecular weight for underivatized oligosaccharides of DP up to 20 and beyond. Analysis by FAB of neutral underivatized oligosaccharides showed a low sensitivity (1-50 nmol of sample required) [113].

Also liquid secondary ionization mass spectrometry has been used to analyse native neutral homoglycan [114]. This technique has shown many advantages like, short measuring time and direct access to molecular masses and high sensitivity. These characteristics are maintained when derivatized oligosaccharides are submitted to LSI mass spectrometry with a detection limit of 100 fmol for maltopentaose derivatized by reductive amination using phosphatidylethanolamine dipalmitoate) [115].

10. Nuclear magnetic resonance spectroscopy

Nuclear magnetic resonance (NMR) spectroscopy has been developed into a very useful non-destruction technique for the determination of oligosaccharide structures.

^{13}C-NMR not only gives information on the anomeric configuration on the carbohydrate residues but also provides information on the composition of component monosaccharides, their sequence, and the overall conformation of the molecule (see Chapter 5). Methylated and charged agarose oligo-saccharides have been analysed by ^{13}C-NMR [116]. The primary analysis of the structures of mannooligosaccharides has been carried out [117].

^{1}H-NMR whilst being a more sensitive technique, does not provide as much structural information due to incomplete separation of the proton resonance signals obtained for carbohydrate molecules. It can, however, be used to quantitate individual oligosaccharide contents in situations where the unique selectivity of the method overcomes interference problems encountered when using other spectroscopic methods of great sensitivity but inferior selectivity. Determination of substituent groups on carbohydrate residues can also be made without the problems encountered with the traditional degradation techniques [118]. Recently ^{1}H-NMR has been used to characterize enzymically derived oligosaccharides [119,120].

The degree of polymerization of reducing oligosaccharides can be deter-mined by using ^{2}H-NMR [121].

Another recent development which allows the complete structural determination of oligosaccharides is two-dimensional nuclear magnetic resonance (2D NMR) [122]. Some peculiar problems (hidden resonance) can be in this way resolved. 2D NMR can be homonuclear [123,124] (same nuclear types ^{1}H-^{1}H) or heteronuclear [125] (different nuclear types ^{1}H-^{13}C). Combining the data of the 2D signals, unambiguous information can be obtained about the type of residue and its position in the carbohydrate chain, also without derivatization or reducing of the reducing end. This method allows the study of oligosaccharide mixtures [126].

It is outside the scope of this chapter to describe the various methods available for recording spectra and the methods by which spectra can be interpreted. These subjects have been reviewed in depth with particular emphasis being given to oligosaccharide analysis [127,128].

With the advent of Fourier transform techniques development is underway to prepare on-line HPLC detectors [129] which will provide valuable structural information on components in mixtures rather than the more normal determination of net properties of a component. This non-destructive detector will allow its use in conjunction with other detectors or fraction collectors if preparative scale chromatography is used.

11. Infra-red spectroscopy

Infra-red spectra of carbohydrates are complex and the method is usually restricted to the identification of specific structural features, particularly when monitoring of chemical reactions is required with interpretation of spectra normally comparison with published spectra or spectra obtained for a standard reference compound. The advent of infra-red spectroscopy has led to the development of analysers capable of detecting total carbohydrate contents in aqueous solutions and complex mixtures and formulations [130,131]. By choice of selected resonance frequencies it is possible to introduce a degree of selectivity into the determination such that the concentration of D-glucose in starch suspensions or specific oligosaccharides such as lactose can be measured.

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