13 Analysis of Prebiotic Oligosaccharides

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13.1 Introduction

Carbohydrates and more specifically prebiotics, are complex mixtures of isomers with different degrees of polymerization (DP), monosaccharide units and/or glycosidic linkages. Many efforts are focused on the search for new products and the determination of their biological activity. However, the study of their chemical structure is fundamental to both acquire a basic knowledge of the carbohydrate and to increase the understanding of the mechanisms for their metabolic effect.

Both the identification of their constituents (qualitative) and the determination of their concentrations (quantitative) are the aims of an analytical process. Selection of an appropriate analytical technique, sample preparation (purification, fractionation, etc.) and optimization of the methodology are necessary for determining prebiotic structure. These steps are clearly dependent on the analytes of interest and the type of sample.

There are two main groups of analytical techniques used for the analysis of prebiotics: separation and spectroscopic techniques. Separation techniques (chromatographic and electrophoretic) give rise to the resolution of the constituents of a sample allowing the obtainment of quantitative information; however, the structural knowledge afforded is usually limited. Spectroscopic techniques are frequently necessary to provide detailed structural data of an isolated compound or a simple mixture. Combination of several techniques is often necessary to achieve all the required information about composition of complex mixtures.

Detailed information about the different analytical techniques required for the characterization of prebiotics as well as the state of the art of their applications has been included in this chapter. Although colorimetric methods such as determination of total carbohydrate or reducing sugar contents are still in use for oligosaccharide characterization, the separation techniques such as planar

chromatography, gas chromatography (GC), high performance liquid chromatography (HPLC) and capillary electrophoresis (CE), which provide qualitative and quantitative information of independent oligosaccharides, are the most widely used and therefore the main aim of this section. These techniques can be coupled to spectroscopic instruments in order to obtain structural information. Moreover, nuclear magnetic resonance (NMR) and mass spectrometry (MS) are directly used for prebiotic structural analysis. These techniques have experienced exceptional advances in recent years, although application to prebiotics is still in progress.

13.2 Analytical Techniques

13.2.1 Planar Chromatography

This term was proposed in 1983 by the Chromatographic Society. It covers both paper (PC) and thin-layer chromatography (TLC). PC was one of the earliest chromatographic techniques used for carbohydrate analysis, but at present it is scarcely utilised and mainly combined with other techniques to give supporting information.

A combination of PC, HPLC and high performance anion exchange chromatography (HPAEC) was used for the isolation of two octasaccharides, two dodecasaccharides and a tridecasaccharide from human milk (Haeuwfievre et al., 1993). Paper chromatography of milk oligosaccharides was used to purify some fractions eluted from an anion exchanger; migration time was 5 days (Guerardel et al., 1999). It has also been used to isolate the transglycosylation product of sucrose with beta-glycosidase from recombinant *Sulfolobus shibatae*, which was identified as α-D-gal*p*-(1→6)-α-D-gluc*p*-β-D-fructofuranoside (Park et al., 2005).

Planar chromatography also covers modern techniques derived from TLC such as HPTLC (High Performance TLC), OPTLC (Over Pressured TLC) and UTLC (Ultra TLC). These techniques are relatively low-cost, easy to perform and they display simultaneously in the chromatogram the overall components present in the sample.

Especially for saving time, thin-layer plates replaced paper, but the earliest methods have evolved and new modes have been introduced. At present, TLC is a well established technique which offers several additional advantages: it is relatively cheap, automated, allows satisfactory quantification and it can even be coupled to spectroscopic techniques such as MS.

13.2.1.1 Pretreatment of Samples

As TLC plates are not reused, the careful pretreatments necessary to keep the integrity of HPLC columns can be omitted. A carbohydrate solution not excessively turbid can be applied to plates, only avoiding the presence (in high amounts) of those compounds which can interfere the elution of analytes, such as proteins, lipids, certain salts, amines or acids.

13.2.1.2 Sorbents and Eluents

The preferred sorbents for carbohydrates are based on silica gel. This substance basically retains solutes by adsorption; separation thus occurs by solubility. In order to introduce new interaction mechanisms, different approaches have been proposed. Impregnation with inorganic salts allows modulation of the separation through complex formation: boric acid, sodium acetate, sodium bisulphite and phosphate buffers have been used for this purpose. Silica can also be functionalised with different organic groups in order to work in reverse-phase mode: amine and diol groups are preferred for carbohydrate analysis. Amino plates can be buffered with phosphates in order to avoid the reaction of the amino groups with the free carbonyls of the sugars.

Elution is carried out with aqueous mixtures of alcohols (methanol, ethanol, isopropanol, butanol); minor amounts of less-polar solvents (acetonitrile, ethyl acetate, acetone) are frequently added depending on the mixture to separate.

Although classic TLC is carried out in isocratic mode, at present it is feasible to use elution gradients by means of AMD (automatic mode development) which allows the formation of step-to-step gradients. AMD is performed by means of commercial equipment which allows a careful control of the process; in brief, the plate is eluted for a short time with the starting solvent mixture, then the solvent is removed and the layer is dried under vacuum; finally, another run starts in the same direction with another solvent of lower elution strength than that used before, and so on. In this way, a stepwise elution gradient is formed. Resolution is improved since spots are focused through the successive elution steps, which affords very narrow bands.

The introduction of HPTLC has improved both resolution and quantitative measurements. This technique is based on the use of special plates which have been prepared with very thin and uniform particles of silica (5–6 μm average) which allows shorter migration distances (3–6 cm) and reduced elution times

(3–20 min). Resolution can be improved by the use of plates with spherical particles such as LiChrospher™ (Merck).

OPTLC is based on a pressured chamber in which the vapor phase above the sorbent is almost eliminated. The eluent is pushed through the layer by a pump; continuous development can be performed.

UTLC was introduced in 2002 and it is based on monolithic structures created on the plate without particles; the sorbent layer is a continuous bed about 10 μm thickness containing macropores (1–2 μm) and mesopores (3–4 nm) (Hauck and Schulz, 2003). This geometry allows faster separations (1–6 min), lower limits of detection and reduced analyte and solvent volumes, but resolution has not yet been totally optimized. This technique seems to be promising for carbohydrate analysis, although applications need to be developed.

13.2.1.3 Visualization and Quantification

There is a broad range of choices for visualization of sugar spots. Both spraying and dipping modes have been used, although the latter is preferred at present, especially with high-performance methods. Chromogenic reagents based on amines and strong acids such as diphenylamine-aniline-phosphoric acid in acetone (Martinez-Castro and Olano, 1981; Reiffova and Nemcova, 2006), urea-phosphoric acid (Bonnett et al., 1997) and *N*-(1-naphthyl)ethylenediamine dihydrochloride dissolved in sulfuric acid-methanol (Bounias, 1980) have been sprayed on silica gel plates. This latter reagent has been shown to be very sensitive (50 ng). 4-Aminobenzoic acid has been used for visualization on diol plates, whereas α-naftol has been used for amino plates. *In situ* reaction of sugars with the amino groups of amino layers also produces visualization, by simply heating the plate at about 170°C; sugars appeared as white-blue fluorescent spots under UV light at 365 nm.

13.2.1.4 Applications

Table 13.1 summarizes some applications of TLC to the analysis of prebiotic carbohydrates.

Chromatographic analysis of lactulose was revised in 1987 (Martínez-Castro et al., 1987). A TLC method was devised for analysis of lactulose in milk using silica gel plates charged with borate to form complexes allowing separation of lactulose from lactose (Martinez-Castro and Olano, 1981).

Table 13.1

TLC applications for the analysis of prebiotics (*Cont'd* p. 470)

Analytes	Bed	Elution system	Visualization	Quantification	Reference
Lactulose in milk	Silica gel G + 0.03M H_3BO_3	ACN:water (5:2)	1 g diphenylamine + 1 mL aniline + 50 mL H_3PO_4 + 50 mL acetone	Semiquantitative detection limit: 0.02% lactulose	Martínez-Castro and Olano (1981)
14 Sugars in biol fluids; maltodextrines in infant formulas	Cellulose	Threefold development (a) butanol/EtOH/w (3: 2: 1) (b) pyridine/ethyl acetate/ AcH/w (5: 5: 3: 1)	3 g $AgNO_3$ + 12 mL+ (a) 500 mL acetone (b) 50 ml of 10N NaOH in 450 mL EtOH	None	Bosch-Reig et al. (1992)
Oligosaccharides in beer	Amino + 0.4M KH_2PO_4	AMD linear gradient ACN: acetone (1:1)/w 40–20% in 15 steps	Thermal "*in situ*" reaction	Scanner in fluorescence mode at 366 nm	Brandolini et al. (1995)
FOS in biological fluids	Silica gel F254 + 0.02M Na Ac	Butanol:ethanol:w (5:3:2)	Diphenylamine-aniline-H_3PO_4 in acetone	Reflectance densitometry at 370 nm	Reiffova et al. (2006)
Microbiologically-produced Dextrans	Silica gel F	Three ascents EtAc: ACN: w propanol (2:7:5.5:5)	0.2%N(1-naphtyl) ethylendiamine HCl in MeOH with 3% H_2SO_4	Scanner in reflectance mode	Coté and Leathers (2005)
Hydrolyzates of polysaccharides from Linghzi	HPTLC silica gel 60 and HPTLC silica gel 50,000	AMD with 2 solutions: propanol:w (6:4) and propanol:w (83:17) in 7 steps	0.5 g 4-aminobenzoic acid, 9 mL AcH, 10 mL w, 0.5 mL H_3PO_4	Scanned at 365 in absorption mode	Di et al. (2003)
FOS in plants	Silica gel	Two developments in butanol:2-propanol: w (3:12:4)	Urea + H_3PO_4	Qualitative purposes	Bonnett et al. (1997)

Table 13.1

Analytes	Bed	Elution system	Visualization	Quantification	Reference
Oligosaccharides in molasses and artichoke leaves	Diol HPTLC	AMD with 3 solutions: ACN: acetone (1.1)/w (85/15), (94/6) and (95/5) in 9 steps	2 g 4-aminobenzoic acid + 36 mL AcH, 40 mL w, 2 mL H_3PO_4 + 120 mL acetone	Scanning in both fluorescence and absorbance (nanomol)	Vaccari et al. (2001)
Xylooligosaccharides	Microcristalline cellulose	EtOAc:AcOH:w (3:2:1)	Aniline + H Phthalate	Qualitative purposes	Katapodis et al. (2003)
Arabinoxylo-oligosachharides	Silica gel 60	Butanol:EtOH:w (3.2.2)	2% orcinol in (EtOH:H_2SO_4: w 8:1:1)	Qualitative purposes	Rantanen et al. (2007)
Dietary fiber	Silica gel	n-propanol:EtOH:w (7:1:2)	5% H_2SO_4 in MeOH		Mc Cleary and Rossiter (2004)
Human milk oligosaccharides	HPTLC silica	Butanol:AcH:w (2.5:1:1) two developments	0.1% orcinol in 20% H_2SO_4	Qualitative purposes	Kunz et al. (1996)

FOS, fructooligosaccharides; *ACN*, acetonitrile; w, water; *EtOH*, ethanol; *MeOH*, methanol

Classic TLC has been used to analyze different mixtures of oligosaccharides. Fructans of different plants from the family *Poaceae* were separated using two successive developments, allowing the separation of fructans belonging to both 2→1 and 2→6 series from DP3 to DP10 (Bonnett et al., 1997). TLC has been successfully used to monitor the formation of dextrans and isomaltooligosaccharides by the action of glucansucrases from *Leuconostoc* (Côté and Leathers, 2005), as well as xylooligosaccharides (XOS) (Katapodis et al., 2003) and arabinooligosaccharides (AROS) (Rantanen et al., 2007) formed by the action of different xylanases.

Prebiotics have been monitored in different parts of the intestinal tract (jejunum, ileum and colon) of monogastric animals by a simple TLC method (Reiffova and Nemcova, 2006).

HPTLC with AMD achieved a good separation and quantification of several oligosaccharides in molasses (Vaccari et al., 2001). This technique was also shown to be useful for the study of fingerprint profiles of hydrolyzates of polysaccharides from some important and popular Chinese medicinal mushrooms commonly known as Lingzhi (Di et al., 2003).

Human milk oligosaccharides (HMOS) are excessively complex to be directly separated by HPTLC. Nevertheless, this technique is very useful for the analysis of fractions obtained by preparative techniques, as carried out by Kunz et al. (1996).

13.2.1.5 Coupling with MS

This field was revised by Wilson in 1999. TLC was firstly used as a preparative technique for MS, thus working off-line: spots can be easily cut, solvent-extracted and injected into the ion source of the mass spectrometer (St-Hilaire et al., 1998). Nevertheless, many efforts have been directed to achieve an effective coupling, analyzing spots on the plate (Wilson, 1999). First couplings were carried out using fast atom bombardment (FAB) and liquid secondary ion (LSI). The spot was cut from an aluminum plate and attached to the MS probe. Interfaces based on motorised probes have been designed, where a strip of the plate can be slowly moved through the ion source, enabling the analysis of all spots from a lane to give a true chromatogram.

At present the more common techniques are those based on laser desorption, as MALDI (Matrix-Assisted Laser Desorption/Ionization) and SALDI (Surface-Assisted Laser Desorption/Ionization); in both cases the time of flight (ToF) analyzer is a good option, as it will be further seen.

As an example of coupling with MS, native milk oligosaccharides were separated on 10 × 10 silica gel plates and developed in *n*-butanol/acetic/water (110/45/45). MALDI-ToF was selected as MS technique. Glycerol was used as matrix, with an infrared laser for MALDI and an orthogonal ToF (o-ToF) for achieving high mass accuracy, allowing a straightforward method with a detection limit of ~10 pmol of individual compounds (Dreisewerd et al., 2006).

13.2.2 Liquid Chromatography

Liquid Chromatography (LC) is a separation technique which uses a liquid as mobile phase. Although HPLC is at present generally utilized for the analysis of prebiotic carbohydrates, the use of traditional open columns packed with ion exchange resins, carbon-celite or size exclusion gels as stationary phases is still widely practiced. Although analysis of oligosaccharides (such as malto-, isomalto-, gentio- and levan oligosaccharide series; Kennedy et al., 1989) has been carried out by traditional LC open columns coupled to mainly refractive index (RI) detectors, this technique has been mainly focused on preparative purposes. The collection of fractions of homologous oligosaccharides (different molecular weights or monosaccharide units) is in many cases, a required step for their further characterization by other analytical techniques such as MS or NMR.

13.2.3 High Performance Liquid Chromatography

HPLC is one of the most widespread techniques for oligosaccharide analysis, both for analytical and preparative purposes. A high number of methodologies for qualitative and quantitative characterization of prebiotic carbohydrates have been developed using different operation modes and detectors.

13.2.3.1 Sample Preparation

Analysis by HPLC commonly requires sample preparation methods to remove interfering compounds or impurities; the analysis of prebiotic oligosaccharides is not an exception. These methodologies are mainly based on dilution, liquid-liquid or liquid-solid extraction and filtration steps (Sanz and Martinez-Castro, 2007). Nevertheless, derivatization is in some cases necessary, mainly to enhance sensitivity in the detection during analysis.

There is a wide variety of derivatization reagents for oligosaccharides; the state the art in the preparation of derivatives being included in different reviews dedicated to sample preparation or chromatographic analysis of carbohydrates (Lamari et al., 2003; Sanz and Martinez-Castro, 2007), therefore, only a summary is mentioned here.

Most methods are based on the condensation of a carbonyl group in carbohydrates with primary amines to give a Schiff base which is then reduced to a N-substituted glycosil amine. The primary amine has to posses the desired chromophore or fluorophore substituent, usually an aromatic ring. Reductive amination has been carried out with 2-aminopyridine, different trisulphonates, esters of p-aminobenzoic acid, 2-aminoacridone, etc. (Sanz and Martinez-Castro, 2007).

Acetylation reactions of oligosaccharides overcome problems of solubility in organic solvents, whereas perbenzoylated derivatives improve the chromatographic properties on reverse phase columns (Kennedy and Pagliuca, 1994).

13.2.3.2 Chromatographic Columns

Chromatographic columns used for HPLC carbohydrate analysis can be divided according to the composition of their stationary phases and their dimensions and design.

13.2.3.2.1 Stationary Phase Composition

Both reverse phase and cation exchange chromatography have been the most common HPLC modes utilized for carbohydrate analysis till Rocklin and Pohl (1983) suggested the use of HPAEC for this aim. Most of the stationary phases used in these modes are available for both analytical and preparative purposes; this section being focused on the analysis of prebiotic oligosaccharides.

Alkyl-Bonded Silica Phases Among the alkylated silica-based stationary phases, those of octadecyl-coated (C_{18}) sorbents are the most commonly utilized, this columns being useful for the separation of oligosaccharides with different DP. Moreover, columns can present different percentages of bonded alkyl chains which could show a wide effect on carbohydrate resolution. The operation mode used for these columns is the reverse phase (RP)-HPLC where the non-polar ligands are covalently bound to a solid support and the mobile phase is mainly composed by aqueous solutions moderately polar. The retention mechanism is based on the interaction of the packing with polar materials; the

most polar compounds elute first whereas those with lower polarity are more retained. The use of low temperatures for the elution improves the resolution (Kennedy and Pagliuca, 1994). General aspects of underivatised and derivatised carbohydrate analysis by RP-HPLC have been reviewed by El-Rassi (2002).

Water is the most common mobile phase chosen for underivatised carbohydrates since these compounds require high surface tension to achieve an appropriate resolution. Nevertheless, gradients with organic solvents are used, although problems of solubility of oligosaccharides can arise.

Aminoalkyl-bonded Silica Gel Phases Aminoalkyl-modified silica gel columns provide good resolution; however, their stability is low and can be easily degraded. The most common ones for oligosaccharide analysis are aminopropyl-bonded columns, although primary and secondary diamines and secondary and tertiary amines can be also found. Non-polar organic solvents or aqueous organic mixtures are used as mobile phases.

Different mechanisms have been proposed for this chromatography such as partition or hydrogen bonding (Herbreteau, 1992). Oligosaccharides are eluted in order of increasing their molecular weight. Carbohydrates up to DP15 can be separated by this technique, although solubility problems can be found for oligosaccharides of high molecular weights. Nevertheless, if a diamine or a polyamine is added to the eluent a dynamic equilibrium is formed and oligosaccharides up to DP25 can be separated (Kennedy and Pagliuca, 1994). In these cases a presaturation column has to be placed before the injector to avoid dissolution of the analytical column packing.

Many researchers have used amino columns to analyze fructooligosaccharides (FOS) of different DP using acetonitrile:water (75:25) as mobile phase (Sangeetha et al., 2005), however, resolution is not as good as that obtained for mono- and disaccharides and solubility problems appear (Herbreteau, 1992). Moreover, the formation of Schiff bases between reducing sugars and amino groups can reduce the lifetime of these columns.

Cyclodextrin-bonded Phases The use of cyclodextrin-based columns for the separation of neutral prebiotic carbohydrates such as those derived from xylan, inulin or mannan, has been also proposed as a substitution of aminoalkyl modified silica gel columns. These columns are particularly useful for the analysis of oligosaccharides since the retention of these compounds is mainly based on the hydrogen bonding interactions of oligosaccharide hydroxyl groups

with the stationary phase which allows the separation of the different molecular weights. Mobile phases are normally constituted by different percentages of acetonitrile and water. Carbohydrates elutes in order of increasing DP (Herbreteau, 1992).

Other Polar Bonded Phases Several stationary phases with highly polar sorbents such as cyano, hydroxyl, diol, derivatives of poly(succinimide), sulfoalkylbetaine, etc. have been also used for carbohydrate analysis (Ikegami et al., 2008). Analysis on these columns has in common that retention increases with the hydrophilicity of the stationary phase and the analytes and with decreasing hydrophilicity of solvents from mobile phase. All of them are therefore grouped under the acronym HILIC (hydrophilic interaction chromatography). The first generations of HILIC were based on the amino-silica stationary phases and mixtures of acetonitrile:water mobile phases that has been previously described. HILIC belongs to normal phase liquid chromatographic (NPLC) modes with the hydrophilic stationary phase but with the mobile phase replaced by an aqueous/organic mixture (typically acetonitrile in water or a volatile buffer). It is very useful for the separation of polar compounds such as oligosaccharides. The hydrophilic groups of the stationary phase attract water molecules from the mobile phase to form water-enriched layers. The chromatographic mechanism is therefore mainly based on partition equilibrium between both mobile and stationary phases facilitated by the aqueous layers.

Graphitized Carbon Phases Graphitized carbon columns (GCC) were developed as an alternative to RP columns for the analysis of polar compounds (Koizumi, 2002). Their mechanism is based on the unspecific adsorption of polar compounds such as carbohydrates and interaction is enhanced with increasing molecular size. The effect of temperature is not drastic, although high temperatures can produce an increase in the retention due to the higher adsorptive activity of carbon. Eluents for mobile phases include high percentages of organic modifiers such as acetonitrile with no ion-pairing agents; these eluents being compatible to MS detectors.

Size Exclusion Phases As it has been indicated before, size exclusion chromatography (SEC) is widely utilized in its classical form with open columns. Nevertheless, the use of size exclusion for HPLC (HPSEC) is also commonly applied, although its inability to separate linkage isomers has limited its development.

Oligosaccharides are eluting in order of decreasing molecular size from a stationary phase constituted by cross-linked polysaccharide or polyacrylamide. These packing material are available with a range of pore volumes; separation depends on the ratio of their molecular dimensions and the average diameter of the pores (Churms, 1996). Mobile phases should be carefully chosen to avoid all types of interaction, such as electrostatic interactions; acetate buffers or pyridinium acetates being used among others.

Cation Exchange Phases Cation exchange resins are composed by cross-linked polystyrene and silica-based ion exchangers such as calcium or silver. Oligosaccharides up to DP8 for calcium columns and DP12 for silver columns can be separated (Kennedy and Pagliuca, 1994). Carbohydrates elute in order of decreasing molecular size and the chromatographic mechanism is based on both the size exclusion and ligand-exchange.

These phases can show different disadvantages such as compressibility of the gel matrix, efficiency losses when flow rate is increased, the need of high temperature operation (85°C) and extended analysis times (Kennedy and Pagliuca, 1994). The use of H^+ columns and 0.01M sulfuric acid as mobile phase which allows the regeneration of the H^+ reduces the losses of efficiency.

Anion Exchange Phases The advent of HPAEC in 1983 (Rocklin and Pohl, 1983) for carbohydrate analysis notably improved knowledge about oligosaccharide composition of a wide variety of products. Carbohydrates are negatively charged at high pH (pH $>$ 13) and oligo- and polysaccharides up to DP50 can be separated by anion-exchange chromatography using NaOH as mobile phase; amylopectins up to DP80 have even been separated by this technique (Hanashiro et al., 1996). A gradient of increasing concentration of sodium acetate is normally used to help elution of oligosaccharides. Stationary phases are composed of polymeric, non-porous, MicroBead™ pellicular resins such as polystyrene/divinylbenzene or ethylvinylbenzene/divinylbenzene substrates agglomerated with Microbead™ quaternary amine functionalized latex, which are highly resistant to high pHs. CarboPac PA100 and more recently, CarboPac PA200 are columns mainly designed for oligosaccharide separation, although CarboPac PA1 and PA10 can be also used (Cardelle-Cobas et al., 2008; Splechna et al., 2006).

Carbohydrate elution takes place with increasing the molecular weight for oligosaccharides with the same glycosidic linkage, nevertheless, this order can

change when families of oligosaccharides with different linkage variants are mixed (i.e., isomaltohexaose elutes before maltotriose; Morales et al., 2006). The combination of different effects (charge, molecular size, sugar composition and glycosidic linkages) is implied in the chromatographic separation (Gohlke and Blanchard, 2008). This retention behavior is one of the disadvantages of this technique which requires the use of standards for the identification of complex mixtures of carbohydrates with different DP and glycosidic linkages. Nevertheless, this chromatographic technique coupled to a pulse amperometric detector (PAD), as it will be shown later, presents significant advantages: fast analysis, samples do not require a previous derivatization, low to sub-picomole sensitivity, high resolution, etc.

13.2.3.2.2 Column Dimensions and Design

Recently, there is a trend to develop miniaturized systems which allow reduced solvent consumption and disposal, fast analysis and increased sensitivity. Downscaling of the column dimensions to the capillary- or nano-scale has shown several advantages over the conventional chromatography for carbohydrate analysis. Glycans at femtomole level can be analyzed without derivatization. This miniaturization has been carried out for graphitized carbon stationary phases (Ninonuevo et al., 2005), normal and reverse phases (Wuhrer et al., 2005) and HPAE columns (Bruggink et al., 2005a).

Conventional HPLC phases of between 3 and 10 μm diameter of particles are commonly used for oligosaccharide analysis; 3 μm silica columns (such as amino-bonded silica phases) have been demonstrated to improve the analysis of glucooligosaccharides up to a DP of 30–35 (Herbreteau, 1992). Columns with smaller diameter of particles (sub-2 μm) have recently been introduced in the market and allow faster separations without resolution losses.

The use of columns with small particle diameter and column length produces an increase in pressure. In order to solve this backpressure problem, Ultra Performance Liquid Chromatography (UPLC) systems are employed. However, applications to oligosaccharides are scarce.

Fast separation can be also achieved using monolithic columns constituted by highly porous materials with a network of interconnecting channels. These columns allow the use of very fast flows: a mixture can be resolved at a flow of 9 mL min^{-1} reducing the elution time between 5 and 10 times. A wide range of underivatized or derivatized carbohydrates (mono- and oligosaccharides) can be successfully separated with this kind of columns achieving higher

column efficiencies than with particle-packed columns (Ikegami et al., 2006; Ikegami et al., 2008).

13.2.3.3 Detectors

Not only is the separation of oligosaccharides a problem in HPLC due to their similar structures, but also to achieve a sensitive detection can be a difficult task. Carbohydrates itself do not contain a specific chromophore or fluorophore, being necessary to fall back on universal detectors such as RI detectors, or on electrochemical ones. Nevertheless, ultraviolet (UV) detectors for both derivatised and non derivatised carbohydrates or fluorimetric detectors for derivatised ones are also used.

Refractive Index Detector

RI detectors are the most common detectors used for carbohydrate analysis although a lack of sensitivity is normally associated to them. Their main drawback arises from their dependence on temperature and mobile phase composition changes. Therefore, these detectors are commonly utilized with isocratic mode or, if gradients of mobile phases are used, solvents with the same refractive index must be used (Davies and Hounsell, 1996). These detectors are mainly used in the mM-μM concentration range.

UV Detector

UV detectors at low wavelengths (below 210 nm) show similar sensitivity to RI detectors however, they allow changes in temperature and gradient elution. As has been indicated, different methods to introduce chromogenic groups in saccharide molecules have been proposed. However, post-column derivatization (not considered as sample preparation) also improves UV detection. Carbohydrates can be labeled with different reagents such as tetrazolium blue or cyanoacetamide. Hase (2002) reports a table with all the possible reagents used for post-column derivatization in HPLC.

Fluorometric Detector

Postcolumn derivatization of carbohydrates with fluorescent labels such as 2-aminopyridine or 2-aminobenzoic acid allows their detection in subpicomolar concentrations (Gohlke and Blanchard, 2008). However, most of postcolumn derivatization methods have been applied to monosaccharide analysis, while only few works have been reported about oligosaccharide analysis.

Light Scattering Detectors

Evaporative light scattering detectors (ELSD) utilize a spray which atomizes the column effluent into small droplets. These droplets are evaporated and the solutes as fine particulate matter are suspended in the atomizing gas. These particles diffuse the light originated from a monochromatic or polychromatic source. These detectors are universal, more sensitive than RI and are compatible with elution gradients (Herbreteau, 1992).

Liquid light scattering detectors differ from ELSD in that they respond to the light scattered by a polymer or large molecular weight substance present in the column eluent. The high intensity light source is achieved by the use of a laser. There are two forms of the detector: Low angle laser light scattering (LALLS) and multiple angle laser light scattering (MALLS) which provide an appropriate sensitivity and baseline stability. They have commonly been applied to SEC. Light from a laser is scattered to different degrees by the concentration and size of the analyte passing through the cell flow. The intensity of scattered light is highest at low scattering angles and also increases with the molecular weight of the carbohydrate (Davies and Hounsell, 1996). However, laser light scattering can sometimes give confusing results because of molecule-molecule interactions and associations of oligosaccharides with high DP.

Pulse Amperometric Detectors

PADs are commonly coupled to HPAEC and allow the detection of non-derivatised carbohydrates at very low picomole levels. This detection provides a high selectivity; only compounds oxidizable at the selected voltages being detected. PAD is composed by a working electrode of Au or Pt, a stainless steel auxiliary electrode and a reference electrode of Ag/AgCl or H_2. The Au electrode is able to catalyze the oxidation reactions and is the best choice for detection of carbohydrates. Carbohydrates are detected by measuring the electrical current generated by their oxidation at the surface of the working electrode at the selected potential (E_1). Next, the voltage is increased (E_2) to oxidize the gold detector which causes desorption of the carbohydrate oxidation products. Finally, the potential is lowered ($E_{3;}$ negative potential) to reduce the electrode surface for the next pulse. The three potentials are applied for fixed times. More recently, this potential sequence has been modified, because although good results can be achieved the gold electrode surface is gradually lost which affects reproducibility (Rohrer, 2003). Similar to the previous method, the first potential is applied to oxidise the carbohydrate on the surface of the gold electrode. However, the second potential is in this case a reductive potential to clean the working

electrode surface, whereas the third short potential reactivates the electrode oxidizing its surface. A fourth potential is necessary to achieve the initial conditions of the Au surface.

Mass Detectors

The use of MS detectors coupled to HPLC systems has considerably enriched the field of carbohydrate analysis. MS detectors have been commonly utilised with alkyl- and aminoalkyl-bonded phases, although other columns such as GCC can be also used. Currently, MS (both quadrupole (Q) and ion trap (IT) MS; Bruggink et al., 2005a,b) are also being coupled to HPAEC using automated systems for neutralization and removal of eluent salts to be compatible with electrospray ionization (ESI) MS requirements. These systems also allow the collection of different desalted fractions which are suitable for further enzymatic and chemical digestions or NMR and chromatographic analysis. A splitter to divide the effluent to the PAD and a MS detector is installed after the column and a membrane-desalting device placed before the MS system convert the sodium hydroxide into water and sodium acetate into acetic acid. Lithium or sodium chlorides are added after the membrane desalter to enhance the MS signal by the formation of lithium or sodium adducts of carbohydrates (Bruggink et al., 2005a,b). These couplings are a great advantage for carbohydrate analysis allowing the acquisition of information related not only to carbohydrate retention, but also to structural characteristics.

Electrospray ionization (ESI) is the most common ionization source coupled to HPLC for the analysis of carbohydrates. Analytes (which are in an ionic state) and eluent from HPLC (which is highly volatile) are sprayed at atmospheric pressure from a needle which is subjected to a high potential (3,000–5,000 V) giving rise to small droplets. Desolvation of ions is assisted by a heated inert gas (N_2). As the solvent evaporates, the ionic charges of analytes are closer and repel each other, breaking up the droplets. The ions free from solvents are focused in the analyzer: Q, IT, Q-ToF, etc.

13.2.3.4 Multidimensional HPLC

Multidimensional HPLC has been used to mainly separate fluorescently-labeled oligosaccharides. Combinations of amine-bonded phases which can perform as both hydrophilic interaction media and as an anion exchange

phase and RP-columns have been applied for this purpose (Gohlke and Blanchard, 2008).

13.2.3.5 Applications

The number of applications of HPLC to analyze prebiotic oligosaccharides in recent years is huge and only some of them can be mentioned in this chapter, although it is good to consider that most of the methods here reported for common prebiotic oligosaccharides [FOS and galactooligosaccharides (GOS)] can be applied to the analysis of different carbohydrate sources. *Table 13.2* summarizes some of the applications described below.

The most recent applications of prebiotic analysis have been developed for HPAEC-PAD. This technique is widely used to both determine the oligosaccharide composition of prebiotic carbohydrates and to study the degradation patterns of oligosaccharides during fermentation assays to evaluate their prebiotic effect. Different methodologies, mainly based in the use of eluents indicated above (NaOH and NaOAc) and Carbo-Pac PA100 or Carbo-Pac PA200 columns, have been developed by many researchers who tried to achieve the optimum separation depending on the prebiotic source to be analyzed.

Fructooligosaccharides and Inulin

Sangeetha et al. (2005) reviewed the different methods reported in the literature for the analysis of FOS, most of them being based on the use of polar-bonded phases or resin based ion exchange columns coupled to RI detectors and HPAEC-PAD analyses.

There are many reports on the analysis of FOS by RP-HPLC coupled to a diode array detector at a wavelength of 190 nm (Grizard and Barthomeuf, 1999) or to a RI detector (Mujoo and Ng, 2003) using a C18 column and water as mobile phase. Chromatographic profiles showed coelution of glucose and fructose, whereas kestose, nystose and fructofuranosylnystose appeared as separated peaks.

HPAEC-PAD analysis of fractionated inulin allows the separation of different molecular weight fructooligosaccharides (G-F_n; α-D-gluc*p*-[β-D-fruct*f*]$_{n-1}$-D-fructofuranoside) and inulooligosaccharides (F-F_n; β-D-fruct*p*-[α-D-fruct*f*]$_{n-1}$-α-D-fructofuranoside). Assuming that retention time in HPAEC-PAD of a homologous series of carbohydrates increases with increasing DP, the assignment of these carbohydrates can be feasible. However, the coexistence of both series

Table 13.2

HPLC applications for the analysis of prebiotics

Prebiotic	Chromatographic column	Detector	Mobile phase	Reference
FOS in onion	CarboPac PA1	PAD	NaOH and CH_3CO_2Na	Kaack et al. (2004)
FOS from levan	Cation exchange (Ca^{2+})	RI	Water	Kennedy et al. (1989)
FOS	CarboPac PA200	MS	NaOH and CH_3CO_2Na	Bruggink et al. (2005a)
FOS in nutraceutical and functional foods	CarboPac PA100 CarboPac PA10	PAD	NaOH and CH_3CO_2Na	Corradini (2002)
GOS	CarboPac PA10	PAD	NaOH and CH_3CO_2Na	Cardelle-Cobas et al. (2008)
GOS	Cation exchange (Ca^{2+})	RI	Water	Goulas et al. (2007)
	CarboPac PA1	PAD	NaOH and CH_3CO_2Na	
Human milk oligosaccharides	On chip GCC	o-ToF	Formic acid in acetonitrile/water	Ninonuevo et al. (2005)
Soybean oligosaccharides	HPSEC	RI	$NaNO_3$ with NaN_3	Giannoccaro et al. (2008)
	CarboPac PA10	PAD	NaOH and CH_3CO_2Na	
Sucrose derived oligosaccharides	Amino bonded	ELSD	Methanol: acetonitrile:water	Yin et al. (2006)
XOS	Cation exchange (Na^+)	RI	Water	Ohara et al. (2006)
XOS	Cation exchange (Ca^{2+})	RI	5 mM H_2SO_4 in water	Moura et al. (2008)
Lactulose	Amino bonded	RI	Acetonitrile:water	Paseephol et al. (2008)
FOS and glucooligosaccharides	C18	RI	0.1% trifluoroacetic acid (v/v) in water	Rousseau et al. (2005)

($G\text{-}F_n$ and $F\text{-}F_n$) makes their identification more difficult and coelution of some oligosaccharides can be observed. Schütz et al. (2006) investigated the chromatographic profile of inulin up to carbohydrates of DP79 in artichoke heads and dandelion roots by this technique, although quantitative analysis was only carried out for glucose, fructose, sucrose, kestose, nystose and

fructofuranosylnystose. The lack of higher molecular weight standards is one the limitations of this analysis. Ronkart et al. (2007) developed a method to obtain F-F_n standards and to identify them in a complex inulin chromatogram. F-F_n standards were isolated by semi-preparative HPSEC from inulin from globe artichoke treated with endo-inulinase, and analyzed by HPAEC-PAD. Coelution problems of some G-F_n and F-F_n oligosaccharides after HPAEC-PAD analysis can be solved by the use of a coupled ESI MS detector which allows the unveiling of both series by the extraction of the ion chromatograms at the appropriate m/z ratios (Bruggink et al., 2005a,b). *Figure 13.1a* shows the extracted ion chromatograms of FOS up to DP13 obtained by HPAEC-MS using a CarboPac PA200 capillary column (Bruggink et al., 2005a), the F-F_n series being more retained than G-F_n series. A MS spectrum of $[GF_4 + Na]^+$ is also shown in *Figure 13.1b*. As can be seen in *Figures 13.1c* and *13.1d*, MS/MS spectra of the two series (i.e., for DP5 variants) showed similar fragmentation patterns with different relative intensities of m/z ions.

Wang et al. (1999) quantitatively analyzed FOS from different food matrices and compared these results to those obtained by MALDI-ToF. The PAD response was different depending on DP and oligosaccharide linkage; the use of specific standards being necessary to avoid overestimated results.

Different authors have shown the suitability of HPAEC-PAD to evaluate fermentation properties of FOS. Hartemink et al. (1997) observed a different HPAEC degradation pattern of FOS for the different strains assayed (*Ent. cloacae, E. coli, Salm. infantis* and *Sh. flexneri,* among others). Moreover, depending on the FOS source employed, different behavior was observed; i.e., using Profeed P95 (from Nutreco, Boxeer, The Netherlands) as a substrate, *Ent. Clocae and Salm. infantis* produced very little degradation of FOS, however these bacteria showed degradation, mainly of F-F_n series, using Raftilose P95 (from Orafti). Corradini et al. (2004) optimised a gradient elution program using water, 0.6 M sodium hydroxide and 0.5 M sodium acetate as eluents to selectively separate glucose, fructose, sucrose and fructans with DP from 3 to 60 in microbial cultures, obtaining good resolution during the whole chromatogram. This method allowed the evaluation of FOS and inulin consumption by pure cultures of *Bifidobacterium* spp. and by fecal cultures.

Galactooligosaccharides

GOS are produced by transgalactosylation reactions catalyzed by β-galactosidases using mainly lactose as substrate. As consequence of these reactions, a large variety of structures can be obtained (different glycosidic bonds and

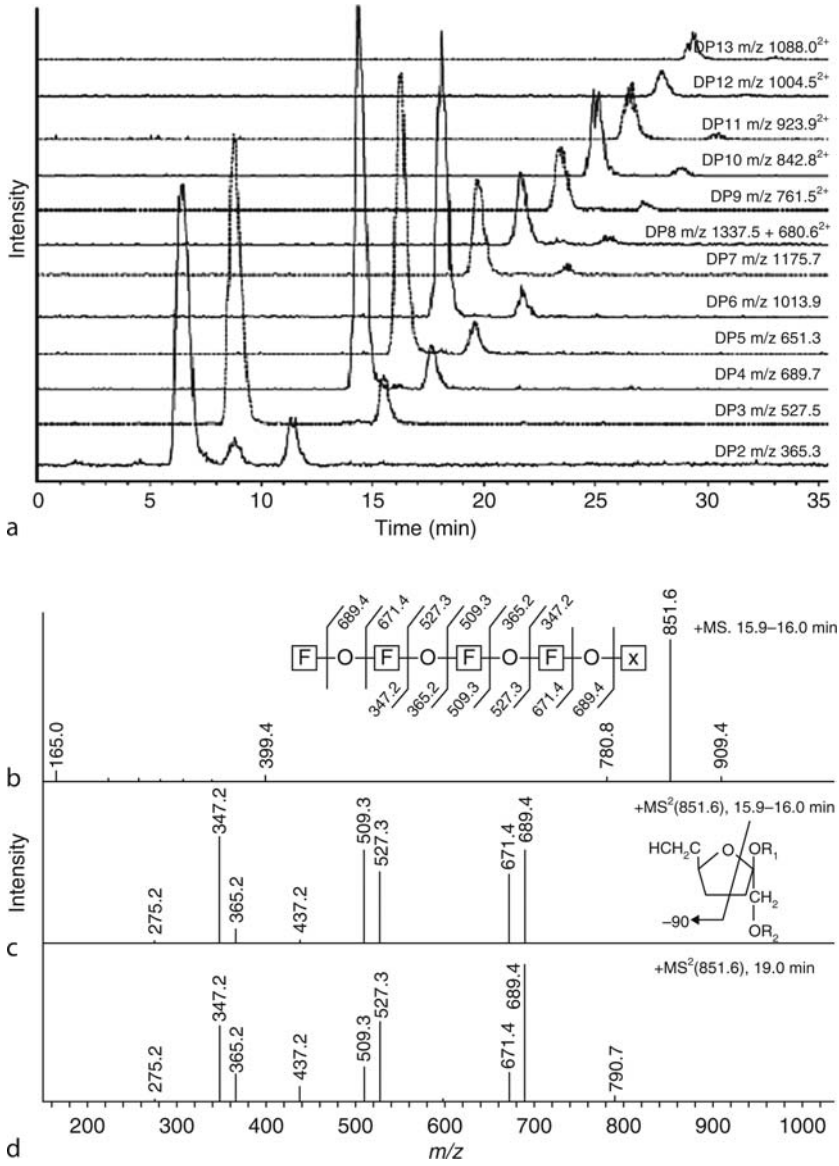

Figure 13.1

HPAEC-on-line-MS analysis of FOS. Extracted ion chromatograms for fructan oligosaccharides of various degrees of polymerization (DP) detected as sodium adducts by capillary HPAEC-on-line-MS (a). Mass spectra of the two isobaric sodium adducts of DP5 fructans: Part (b) shows the MS spectrum of [GF_4 + Na]+; parts (c) and (d) are the MS2 spectra with m/z 851.6 as precursor ion, where (c) represents GF_4 and (d) F_5. In the fragmentation scheme, F stands for fructofuranosyl and X is glucopyranosyl or fructopyranosyl, R_1 and R_2 stand for the rest part of the oligosaccharide chain and R_2 can also be a H. From Bruggink et al. (2005a) with permission from Elsevier.

oligosaccharides of different molecular weights are formed depending on the enzymatic source and the reaction conditions used). Therefore, it is necessary to use high resolution methods to distinguish between structural isomers of oligosaccharides consisting of monosaccharides linked together in various anomeric and positional configurations.

Splechtna et al. (2006) analyzed the composition of GOS (mainly mono-, di- and trisaccharides) by HPAEC-PAD using a Carbopac PA-1 column, although identification of all of the structures could not be completely achieved. Moreover, coelution of some carbohydrates (i.e., glucose and galactose; lactose and allolactose) was observed. These coelution problems have been avoided using a modified method with a CarboPac PA-10 column (Cardelle-Cobas et al., 2008; Martínez-Villaluenga et al., 2008a). Goulas et al. (2007) used a cation exchange column (Ca^{2+}) at 85°C with water as mobile phase and RI detector to determine the synthesis and purification of GOS. Under these conditions, two not well-resolved chromatographic peaks were obtained for oligosaccharides of DP higher than three, whereas disaccharides eluted as one peak and glucose and galactose appeared as separated peaks. HPAEC-PAD with a CarboPac PA1 allowed the separation of the disaccharides obtained in these samples.

Figure 13.2 shows an HPAEC-PAD profile of GOS before (*Figure 13.2a*) and after (*Figure 13.2b*) removal of mono- and disaccharides using activated charcoal (Sanz et al., 2007). The use of a CarboPac PA-100 column allowed the separation of oligosaccharides up to DP7, although a complete resolution of these carbohydrates was not achieved. The lack of standards was the main disadvantage for identification and quantification purposes, necessitating the isolation of oligosaccharides followed by ESI-MS analysis to determine their molecular weights.

Other Prebiotics

Several methods have been also developed for the analysis of other oligosaccharides such as xylooligosaccharides (XOS; Ohara et al., 2006), soybean oligosaccharides (Giannoccaro et al., 2008), lactulose (Paseephol et al., 2008) or glucooligosaccharides (Rousseaua et al., 2005), some of them only tentatively considered as prebiotics. Giannoccaro et al. (2008) have compared two methods using HPSEC-RI (using two analytical Shodex OHpak SB 802HQ columns) and HPAEC-PAD (CarboPac PA10) to analyze soybean sugars. Although both systems gave reproducible results, HPAEC-PAD was more sensitive, faster, and with higher resolution than the HPSEC-RI method.

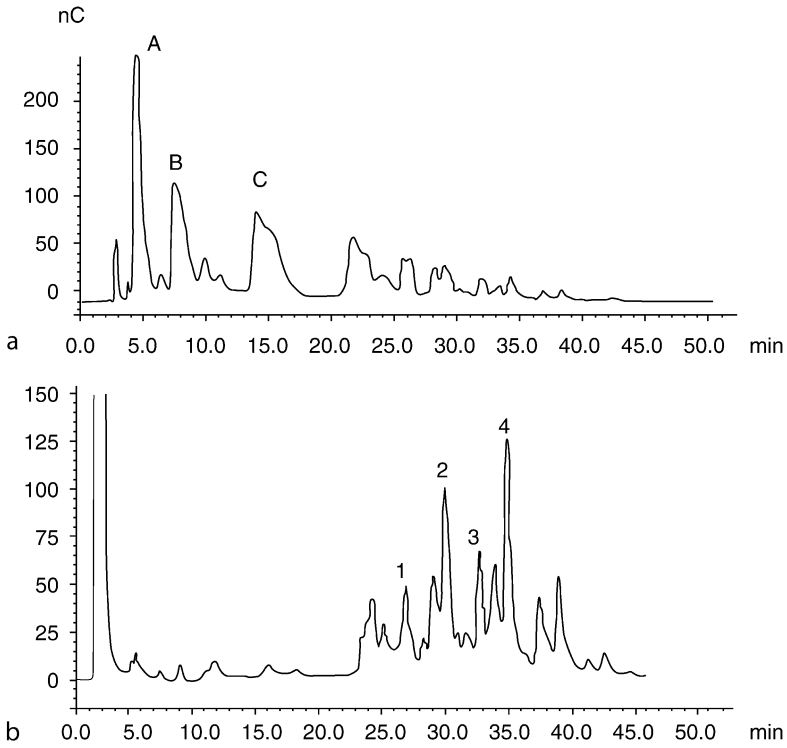

Figure 13.2

HPAEC-PAD analysis of GOS. HPAEC-PAD profiles of: (a) GOS, (b) GOS previously treated with activated charcoal. (A) glucose and galactose, (B) lactose, and (C) unknown disaccharides. From Sanz et al. (2007) with permission from ACS publications.

GCC have recently been used for the separation of carbohydrates in a wide range of applications including coupling to ESI MS. Robust and rapid separations were achieved with these methods. Separation of human milk oligosaccharides has been carried out using on-chip GCC (Ninonuevo et al., 2005) coupled to a MS detector with o-ToF which allows isomeric detection. Consumption of these oligosaccharides by intestinal bacteria has also been evaluated by Ninonuevo et al. (2007) using GCC and UV detection (206 nm) obtaining different chromatographic profiles for the bacteria studied.

Recently, the use of HPLC-ESI MS has allowed the identification of 19 peptides glycated with GOS from 2 to 7 hexose units which prebiotic potential is being studied (Moreno et al., 2008).

13.2.4 Gas Chromatography

Since its first application to carbohydrate analysis by Langer et al. (1958), GC has seen widespread use for sugar determination as it is a relatively cheap, simple and powerful analytical technique. Higher oligosaccharides in foods and diets are often present at low concentrations, thus, the high resolving power, sensitivity and selectivity of GC results is extremely advantageous. The potential of this technique for carbohydrate determination was achieved with the development of capillary columns and their coupling to mass spectrometric detectors; identification and quantification of many prebiotic oligosaccharides as well as structural studies can be performed.

13.2.4.1 Sample Preparation

Purification/Fractionation

As oligosaccharides usually appear in complex matrices, a purification step is required before their analysis as with HPLC determinations. This procedure is often carried out to discard insoluble material, lipids and proteins, desalt the sample or remove impurities.

Soluble carbohydrates in foods are usually extracted with ethanolic or methanolic solutions: oligosaccharides up to DP6 are easily soluble in these solvents while other interfering substances are not, being discarded by filtration or centrifugation. Although these methods are still in use on standard protocols and regulations, other modern procedures such as membrane filtration have been introduced for more complex mixtures.

In those cases where the study of a specific carbohydrate or a group of carbohydrates is required, a fractionation step can be also necessary. This procedure provides an enrichment of the samples in carbohydrates and purifies them before their chromatographic analysis. As an example, nanofiltration, yeast (*Saccharomyces cerevisiae*) treatment, and adsorption onto activated charcoal were used by Sanz et al. (2005) prior to GC analysis in order to remove honey monosaccharides and study the potential prebiotic effect of its oligosaccharides.

Recent methods for the selective extraction of lactulose from a mixture with lactose have been developed by accelerated solvent extraction (ASE; Ruiz-Matute et al., 2007) and supercritical fluid extraction (SFE; Montañés et al., 2007), which allowed the obtainment of a high purity lactulose fraction, using rapid processes with low solvent consumption.

Derivatization

Due to the polar nature of carbohydrates, a derivatization step previous to GC analysis is required. Classical methods are based on the substitution of the polar groups in order to increase their volatility. Acetates, methyl ethers, trifluoroacetates and trimethylsilyl ethers have been the most common derivatives used for carbohydrate determination (Knapp, 1979). Among them, trimethylsilyl derivatives are the most popular, since they present good volatility and stability characteristics. Trimethylsilylation has been recognized as a quantitative rapid derivatization method for a wide range of carbohydrates and related compounds including aldoses, ketoses, aminosugars, alditols, inositols as well as oligosaccharides up to DP4 (Brobst and Lott, 1966; Sweeley et al., 1963).

However, these derivatives give multiple peaks corresponding to the different anomeric forms of carbohydrates. Even though high-resolution capillary columns can adequately resolve complex mixtures, multiple peaks may cause interferences for qualitative identification and quantitative measurement.

Sometimes, the multiple peaks obtained are not considered a disadvantage as they serve as a "fingerprint" for each sugar, aiding their identification. Nevertheless, other derivatives are preferred for analyses of mixtures containing many sugars. Usually the anomeric carbon is modified in order to reduce this effect. Several attempts have been made for this purpose, among them, some possibilities are to: (1) convert the free carbonyl group into an oxime using hydroxylamine chlorohydrate or to an *O*-methyloxime using *O*-methylhydroxylamine chlorohydrate; (2) reduce the aldehyde with sodium borohydride to the corresponding alditol or (3) convert the aldehyde into an oxime and then dehydrate into a nitrile.

Trimethylsilyl oximes can be easily obtained by a two step derivatization procedure (oximation and silylation). They have been widely used for the GC analysis of many oligosaccharides since they produce only two peaks corresponding to the syn (*E*) and anti (*Z*) forms for reducing sugars, and only one peak for non reducing carbohydrates, the derivatives formed having satisfactory GC properties (Molnar-Perl and Horváth, 1997; Sanz et al., 2002).

Alditol acetate derivatives have also been used for sugar GC analysis due to their stability and the simplicity of the resulting chromatograms. The reduction of aldoses to alditols and their conversion to alditol acetates simplifies the chromatograms by producing only one peak for each aldose. Abazia et al. (2003) used these derivatives for the simultaneous GC measurement of lactulose and other sugars in urine. Sugars were reduced with sodium borohydride and acetic acid/methanol 1:9 (v/v) was added to remove the boric acid. Then acetylation was performed by the

addition of dry pyridine and acetic anhydride. Although these derivatives show a high chemical stability and low cost of reagents, they also present some disadvantages. On reduction, ketoses yield a mixture of two sugar alcohols (i.e., fructose produces glucitol and mannitol) and thus, give two chromatographic peaks. In some cases, a significant loss of information may occur in the reduction step as some aldoses and ketoses produce the same alditol and cannot be differentiated (i.e., glucose and fructose both produce glucitol). Although significant improvements have recently been made simplifying the derivatization procedure, some common versions of this method still require tedious evaporations to remove the borate before acetylation. The alditol acetate derivatization has been widely used for monomer analysis of macromolecules by GC (Fox et al., 1989).

An alternative to eliminate the anomeric center is the conversion of sugars to their aldononitrile acetates derivatives. They give a unique peak for every sugar but they cannot be applied for ketoses (Ye et al., 2006). These derivatives have been applied for structural analysis of gums and food samples (McGinnis and Biermann, 1989).

Structural Analysis

Structural analysis of complex carbohydrates requires the characterization of monomer composition and anomeric configuration, as well as the determination of the sequence of monosaccharide residues, branch position, functional groups and glycosidic linkages. The elucidation of structural chemistry of complex carbohydrates requires sophisticated instrumentation such as mass spectrometry (MS) or nuclear magnetic resonance (NMR), but the additional information that GC-MS data provides is essential for carbohydrate characterization.

GC-MS has been applied for either the determination of composition and sequence of oligosaccharides released by partial depolymerization after being converted into proper volatile derivatives or for sugar monomer analysis after complete hydrolysis and derivatization. Partial degradation of polysaccharides to oligosaccharides is achieved by means of enzymatic or mild acidic hydrolysis. The use of acids implies the optimization of conditions to achieve maximum cleavage to oligosaccharides and minimum decomposition of the liberated mono- and/or oligosaccharides.

Methylation analysis is the most widely used method for determining linkage structure of prebiotic oligosaccharides by GC. It basically consists of the following steps: Firstly, the free hydroxyl groups of polymerized sugars are completely methylated, forming their correspondent methyl ethers. Then,

hydrolysis of the polymer is performed releasing the free hydroxyl groups in those places in which previously there were glycosidic linkages. Finally, these hydroxyl groups are converted into more volatile compounds, the most common derivatives being alditol or, aldonononitrile acetates. These samples are analyzed by GC-MS in order to ascertain the original linkages and to obtain quantitative linkage information on complex polysaccharides.

The major drawback of standard methylation analysis is that in certain cases the carbon involved in the cyclic hemiacetal of the monosaccharide is not distinguished from linked positions after hydrolysis of the permethylated polysaccharide. As an alternative, the reductive-cleavage method, which yields partially methylated anhydroalditols while retaining the ring structure, has been successfully used to investigate the structure of different prebiotic oligosaccharides. Rolf and Gray (1984) studied the suitability of the reductive-cleavage method for the study of the linkage positions in D-fructofuranosyl residues of D-fructans of different sources. The same derivatization method was used by Stumm and Baltes (1997) for the structural determination of polydextrose. Previous to methylation, ultrafiltration was applied in order to yield fractions free from monomeric residues. Carbohydrate analysis was performed by GC and GC-MS using both electronic impact (EI) and chemical ionization (CI). EI MS was used to confirm the identity of the carbohydrate derivatives while CI MS with ammonia as reagent gas was useful for the determination of the molecular weight. This method resulted to be useful for the elucidation of the degree of branching, position of the linkages and the type of monomeric compounds involved in the studied samples.

Many novel prebiotic oligosaccharides synthesized either by the use of microbial cells or enzymes have been also characterized by methylation analysis. The determination of carbohydrate structures of neofructo-oligosaccharides produced by *P. citrinum* (Hayashi et al., 2000), oligosaccharides formed by a fructosyltransferase purified from asparagus (Yamamori et al., 2002), and oligosaccharides synthesized by glucosyl transfer from β-D-glucose-1-phosphate to raffinose and stachyose using *T. brockii* kojibiose phosphorylase (Okada et al., 2003), are some examples of the application of methylation analysis.

13.2.4.2 Columns and Stationary Phases

The most significant improvement in GC separation was achieved with the advances in capillary column technology. Although many separations of prebiotic

oligosaccharides have been carried out on packed columns (Farhadi et al., 2003; Karoutis et al., 1992), the use of capillary columns involves an increment on resolution while the analysis time is decreased. The most common liquid stationary phases used for carbohydrate analysis by GC are those based on polysiloxanes (also called "silicones") since they present good thermal stability and high permeability towards solutes. A wide polarity range can be found and depends on the percentage of polar phenyl or cyanopropyl groups in the siloxane chain, the most apolar being 100% methylsilicone while 100% cyanopropyl silicone is the most polar. For high-temperature separations, phases based on a carborane skeleton have been proposed (Joye and Hoebergs, 2000).

Dimensions of capillary columns used for carbohydrate GC analysis can vary in the range of: 1–50 m (length); 0.1–0.5 mm (diameter), and 0.02–2 μm (df).

It has been demonstrated that oligosaccharides with up to 11 monosaccharide units can be analyzed using capillary columns with ultrathin films (<0.05 μm) of thermostable bonded stationary phases at high temperatures (Carlsson et al., 1992).

13.2.4.3 Chromatographic Conditions

As carbohydrates usually appear as a mixture of mono- and oligosaccharides, programed temperature is convenient during the chromatographic run, so that each compound can be analyzed under adequate conditions. The temperature commonly used for carbohydrate analysis range from 60 to 330°C.

GC and high temperature gas chromatography (HT-GC) have been used for carbohydrate analysis by many authors (Joye and Hoebergs, 2000; Karoutis et al., 1992; Montilla et al., 2006). The main differences between these techniques are their operating conditions: GC uses temperatures below 360–370°C whereas HT-GC works above them, but requires special apparatus, high temperature capillary columns and heat resistant fittings for the analysis. Carlsson et al. (1992) developed a HT-GC method for the determination of high oligosaccharides: the temperature program was increased from 40 to 400°C with a linear temperature program of 20°C/min. A conventional GC procedure for the determination of high DP oligosaccharides was developed by Montilla et al. (2006). A maximum temperature program of 360°C allowed the determination of oligosaccharides up to DP 7 in different foodstuff.

High carrier gas velocities and columns coated with thin layers of stationary phase can help to minimize the time spend through the column and, consequently,

reduce analysis time. The use of helium as a carrier gas further decreases analysis time without any substantial loss in column efficiency.

13.2.4.4 Detectors

Flame ionization detection (FID) is the most frequently used for GC analysis of carbohydrates since it possesses good sensitivity for organic products. However, the identification by GC always requires the use of standard compounds, and only few of them are commercially available. The coupling of a MS detector to a gas chromatograph contributes to the identification and quantification of carbohydrates, especially in those cases where complex mixtures of the same DP have to be determined.

Most of GC–MS applications utilize capillary GC with Q MS and EI ionization due to its relatively low cost, high sensitivity, high information content, and the ready availability of commercial instruments. CI with ammonia as reagent gas has also been used in order to confirm the molecular weight in methylation analysis (Stumm and Baltes, 1997).

Certain carbohydrates with the same DP and only differing in the position of their hydroxyl groups show similar mass spectra. In those cases, the combination of retention information provided by GC analysis and spectral data from MS is necessary for carbohydrate identification, although it is not always enough for a proper characterization.

Spectral information of different carbohydrate derivatives such as alditol acetates (Fox et al., 1989), trimethylsilyl oximes (Molnárl-Perl and Horváth, 1997; Sanz et al., 2002), trimethylsilyl O-methyloximes (Laine and Sweeley, 1973) and methyl acetates (Stumm and Baltes, 1997) can be found in literature.

13.2.4.5 Applications

GC applications for prebiotic carbohydrate analysis have been mainly focused on both direct analysis of oligosaccharides with a previous derivatization procedure and methylation analysis for their structural determination.

Many references can be found about the analysis of lactulose by gas chromatography. Lactulose is a disaccharide obtained through lactose isomerization (through Lobry de Bruyn-Alberda Van Ekenstein rearrangement). It is not digested in the small intestine and is fermented by the colonic flora. Some examples are summarized in ◉ *Table 13.3*. Farhadi et al. (2003) developed two

gas chromatographic methods using a packed and a capillary column respectively, for the simultaneous quantitation of urinary lactulose and other carbohydrates (sucrose and mannitol). Columns and chromatographic conditions are summarized in *Table 13.3*. The capillary method was more sensitive, accurate and reproducible for lactulose determination. Moreover, it permitted the use of smaller volumes of urine in the analysis and did not require pretreatment of the samples.

A method was developed for the determination of lactulose in milk (Montilla et al., 2005). This method gave good chromatographic resolution, as well as precise and reproducible results when applied to commercial milk samples submitted to heat treatments of different intensity. In addition, this method provided a good separation among sucrose, lactulose and lactose peaks which allowed the suitable quantification of lactulose in samples containing a high concentration of sucrose.

The degree of polymerization as well as the presence of branches are important in inulin, since they affect to their functionality. Short chain inulin carbohydrates should be separated from their long chain analogues for prebiotic uses. Lopez-Molina et al. (2005) characterized artichoke inulin and demonstrated its health-promoting prebiotic effects. The extraction of artichoke inulin involved several physical steps (see *Table 13.3*); hydrolysis of inulin was also performed. GC-MS analysis of their trimethylsilyl derivatives confirmed that fructose was the main monosaccharide unit in artichoke inulin and its degradation by inulinase indicated that it contained the expected β-2,1-fructan bonds.

Packed column GC was used by Sosulski et al. (1982) for the analysis of oligosaccharides in legumes but long analysis time and poor reproducibility for larger oligosaccharides were the major drawbacks of the method. Karoutis et al. (1992) optimized a methodology by GC for the analysis of raffinose, stachyose and verbascose. Different analytical parameters were assayed: carrier flow-rate, split ratio and nature of derivatization agent (trimethylimidazole or N-methyl-bis (trifluoroacetamide).

Joye and Hoebregs (2000) developed a method for the quantitative determination of oligofructose in foods. The use of high temperature chromatography with an Al-clad capillary column and oven temperatures up to 440°C allowed the determination of carbohydrates up to DP9 in complex matrices in only one chromatographic run. *Figures 13.3a* and *13.3b* show the chromatogram obtained for Raftilose P95 X (Orafti) and an enzymatically synthesized FOS (Actilight®). Malto-, isomalto- and galactooligosaccharides were also analyzed by this method to exclude possible interferences from other sugar compounds. *Figure 13.3c* shows the GC profile of GOS as an example. This method was very accurate and reproducible for the study of these carbohydrates.

Table 13.3

GC applications for the analysis of prebiotics (*Cont'd* p. 496)

Carbohydrates	Derivatives	Pretreatment	Column and dimensions	Cromatographic conditions	References
Lactulose and other sugars in urine	Alditol acetates	Urine purification with Dowex mixed bed resin	ZB-1 capillary column (30 m × 0.25 mm ID)	T programme: 230–300°C Carrier gas: Nitrogen	Abazia et al. (2003)
Lactulose and other sugars in urine	TMSO	Centrifugation and filtration	Glass column packed with 3% SE-30 on 80/100 chromosorb WHP (6-ft × 2 mm ID)	T programme: 220–274°C Carrier gas: Nitrogen	Farhadi et al. (2003)
		None	DB1 capillary column (15 m × 0.53 mm ID × 1.5 μm)	T programme: 220–274°C Carrier gas: Helium	
Lactulose from a sugar mixture in urine	TMSO	None	DB-1701 capillary column (30 m × 0.25 mm ID × 0.25 μm)	T programme: 180–250°C Carrier gas: Helium	Farhadi et al. (2006)
Lactulose in dairy products	TMS	Methanol to remove proteins and fats	SPB-17 capillary column (30 m × 0.32 mm ID × 0.25 μm)	T programme: 235–270°C Carrier gas: Nitrogen	Montilla et al. (2005)
Lactulose from a mixture with lactose	TMS	PLE extraction	SPB-17 capillary column (30 m × 0.25 mm ID × 0.25 μm)	T programme: 250–270°C Carrier gas: Nitrogen	Ruiz-Matute et al. (2007)

Inulin from artichoke	Complete hydrolysis with formic acid and TMS	Aqueous extraction, ultrafiltration, precipitation by ionic-exchange chromatography, low temperature precipitation, centrifugation and lyophilization	HP-5MS capillary column (30 m × 0.25 mm ID)	T programme: 250–280°C Carrier gas: Helium	Lopez-Molina et al. (2005)
FOS, GOS, malto- and isomaltooligosaccharides in food products	TMSO	Extraction with water Foods containing fats: hexane and centrifugation	Al-clad capillary column coated with 5% phenyl polycarborane-siloxane (6 m × 0.25 mm ID)	T programme: 105–440°C Carrier gas: Helium	Joye and Hoebregs (2000)
Oligosaccharides (DP up to 7) in foods (FOS and GOS)	TMSO	Dilution with methanol and centrifugation	CP-SIL 5CB capillary column (8 m × 0.25 mm ID × 0.25 μm)	T programme: 130–360°C Carrier gas: Nitrogen	Montilla et al. (2006)
		Diafiltration of FOS	HT5 capillary column (12 m × 0.32 mm ID × 0.1 μm)	T programme: 130–440°C Carrier gas: Nitrogen	
Soybean oligosaccharides (raffinose, stachyose, verbascose and maltooligosaccharides)	Methyl alditols	Water extraction and clean up with chloroform/ethanol	GC: HT SE-54 capillary column (25 m × 0.32 mm ID × 0.05 μm)	T program (GC): 40–400°C T program (GC-MS): 70–390°C	Carlsson et al. (1992)
			GC-MS: PS264 (10 m × 0.25 mm ID × 0.02 μm)	Carrier gas: Helium	

Table 13.3

Carbohydrates	Derivatives	Pretreatment	Column and dimensions	Cromatographic conditions	References
Pea oligosaccharides (raffinose, stachyose, verbascose)	Trifluoroacetates and TMS	80% aqueous methanol and membrane filtration	DB5–60W capillary column (10 m × 0.32 mm ID × 0.25 μm)	T programme: 188–316°C and 80–250°C Split ratio: 1:50,1:100 and 1:150 Carrier gas: Helium	Karoutis et al. (1992)
Polydextrose	Methyl alditols	Ultrafiltration	SA-5 capillary column coated with 5% diphenyl-95% dimethylpolysiloxane (30 m × 0.25 mm ID)	EIMS: to prove the identity of the carbohydrate derivatives NH_3-CIMS: determination of the molecular weight	Stumm and Baltes (1997)
D-Fructofuranosyl residues of D-Fructans of different sources	Permethylated derivatives		10% SP2401 (1.83 m × 3.18 mm); (3.66 m × 3.18 mm); 3% OV-225 (2.68 m × 3.18 mm); SE-30 (1.83 m × 3.18 mm)	Different temperatures	Rolf and Gray (1984)
Honey oligosaccharides	TMSO	Nanofiltration, yeast (Saccharomyces cerevisiae) treatment, and Adsorption onto activated charcoal	DB1 capillary column (25 m × 0.25 mm ID × 0.25 μm)	T programme: 200–300°C Carrier gas: Nitrogen	Sanz et al. (2005)

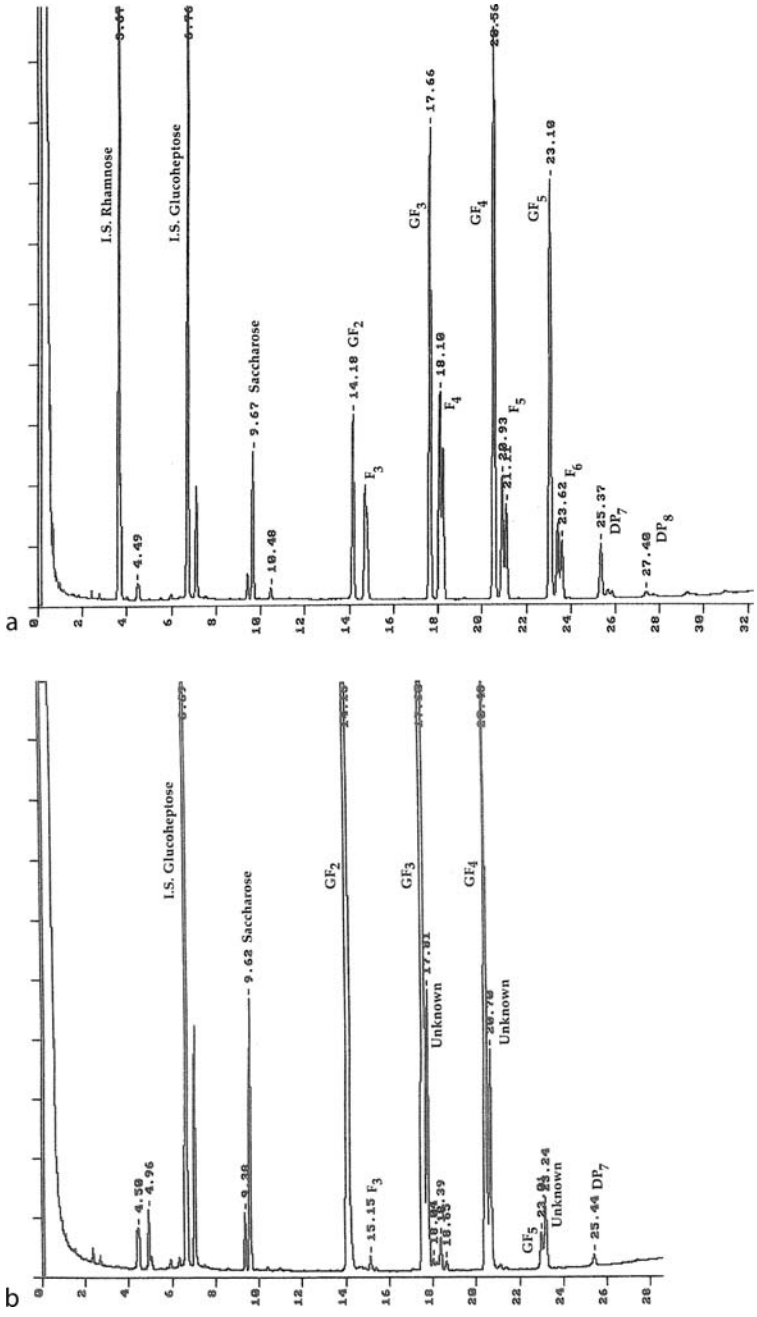

Figure 13.3 (Continued)

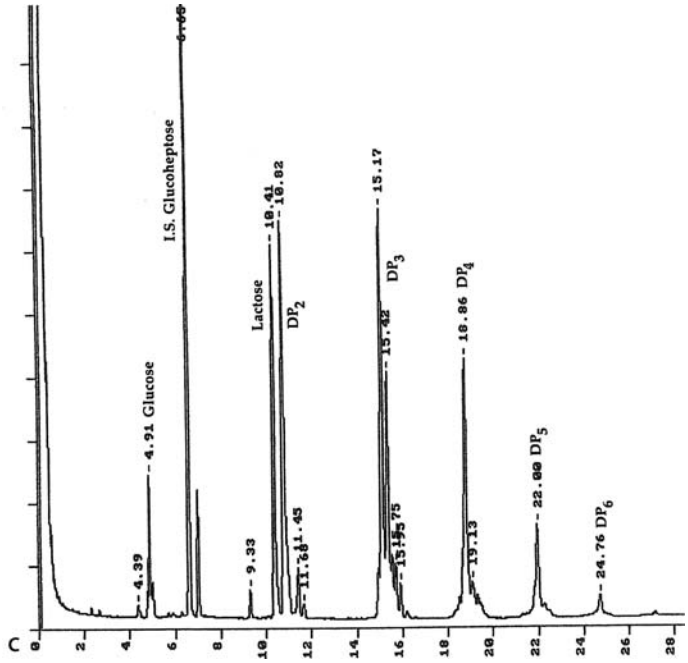

Figure 13.3

GC analysis of FOS and GOS. GC profile of Raftilose P95 X (a), Actilight (b) and galacto-oligosaccharides (c). From Joye and Hoebregs (2000) with permission from AOAC International.

Oligosaccharides up to DP7 were determined in foods and in various pure mixtures using conventional GC (Montilla et al., 2006). The accuracy, repeatability and reproducibility of the method were similar to the results obtained with HT-GC method from Joye and Hoebregs (2000).

Carlsson et al. (1992) developed an HT-GC methodology for the quantitative analysis of oligosaccharides in foods, diets and intestinal contents. Methylation was also performed for the identification of these oligosaccharides by GC-MS, which was able to analyze sugars up to 12 sugar units. Red lentils, soybeans, rapeseed, mung beans and chickpeas were found to contain considerable amounts of the raffinose family of oligosaccharides.

Multiple applications can be found for the structural analysis of oligosaccharides. As an example, the studies about structural determination of D-fructans (Rolf and Gray, 1984) and different oligosaccharides produced from alternansucrases (Côté and Sheng, 2006) can be pointed out. The potential prebiotic properties of these last oligosaccharides are still under study (Sanz et al., 2006).

13.2.5 Capillary Electrophoresis

The high speed of analysis, the minute amounts of analyte required and the high resolution make CE an attractive and powerful microanalytical technique to separate a wide range of charged and uncharged compounds. It is a suitable analytical tool for the analysis of foods and beverages and also has been successfully applied in other fields, such as biochemistry, biotechnology and clinical chemistry (Soga and Serwe, 2000). The advantages of CE over other traditional chromatographic methods include the extremely simple operation and the low consumption of sample and buffers (Bao and Newburg, 2008). The main drawback is the lack of sensitivity when low concentration levels are present.

13.2.5.1 Operation Modes

CE instrumentation includes a high voltage power supply (5–30 kV), buffer reservoirs, a narrow-diameter (50–100 μm) capillary, an automated sampler and detector. Separation is based on migration of compounds in narrow capillaries (length of 0.5–1.5 m) made of fused silica. The two ends of the capillary are immersed in two separated electrolyte reservoirs containing a high voltage electrode.

The mobility of analytes under an electric field depends on several factors, including the analyte charge (neutral, positive or negative), charge to mass ratio, buffer system (pH and ionic strength), presence of buffer additives (surfactants, ion-pairing agents, complexing agents), voltage applied, temperature inside of capillary, length and diameter of the capillary, and nature of the capillary wall (Bao and Newburg, 2008).

Samples can be directly analyzed with minimal sample preparation without a loss of separation performance. After detection of peaks, the hollow capillary is flushed with fresh buffer and is ready for the next injection (Soga and Serwe, 2000).

There are different operation modes of CE: capillary zone electrophoresis (CZE), capillary gel electrophoresis (CGE), micellar electrokinetic chromatography (MEKC), capillary isolectric focusing (CIEF) and capillary isotachophoresis (CITP). In all cases, the separation is achieved due to differences in migration of different solutes, on chosen electrolyte and capillary tube, under an applied electric field.

The most commonly used modes in the analysis of carbohydrates are CZE and MEKC. CZE is also called free solution electrophoresis, and the separation is based on the differences in the charge-to-mass ratio. MEKC is a pseudochromatographic mode of CE whereby separation and analysis of neutral molecules occurs through inclusion into micelle-forming detergents added to the electrophoretic medium (Cheung et al., 2007).

CE has emerged as an alternative to current analytical techniques for carbohydrates. However, carbohydrates analysis can present difficulties such as lack of electric charge and absence of chromophoric/fluorophoric groups in the analyte molecules. To overcome these limitations different procedures have been developed.

Taking into account that sugars are very weak acids (have high ionization constant, pK_a values of 12), different methodologies have been established to ionise them. Electrolyte systems based on borate complexation, metal complexation or highly alkaline pH (e.g., NaOH) have been used. The complexation of carbohydrates with borate-based electrolytes to impart the necessary charge for electrophoresis is the most widely used approach for CE separation of derivatized and underivatized sugars (E-l Rassi, 1999).

13.2.5.2 Detection

Derivatized Carbohydrates

Carbohydrates have high ionization constants (pK_a values of 12 or higher), and therefore they do not carry electrical charges at neutral pH. This, along with the fact that carbohydrates do not absorb UV light above 200 nm hinders its analysis by CE. To overcome both problems different procedures have been developed, such as derivatization with direct detection, using chromophore and fluorescent probes carriers of electrical charges, to facilitate detection via UV-VIS absorbance or laser-induced fluorescence (LIF). However, although derivatization methods lead to improve sensitivity and resolution, several drawbacks are often encountered, such as control problems due to a different reactivity of derivatizing reagents for analytes, formation of several adducts, etc. (Lee and Lin, 1996).

Similarly to HPLC, derivatization of reducing carbohydrates in CE is often performed by reductive amination, between the reducing end and an amino group of the tag reagent, using amines with strong chromophores or fluorophores such as 4-amino benzoic acid and its ethyl ester, 2-aminobenzoic acid, 4-aminobenzonitrile, 2-aminopyridine, etc. (Andersen et al., 2003). A large

variety of derivatization reagents have been suggested for carbohydrate analysis (Campa et al., 2006; Cortacero-Ramirez et al., 2004).

Underivatized Carbohydrates

Indirect Detection An alternative methodology that allows CE analysis of underivatized carbohydrates includes the use of highly alkaline electrolytes, to ionize and ensure an electrophoretic mobility of the saccharides towards the anode and make them suitable for indirect UV detection using chromophore compounds and modifiers (background electrolyte; BGE) to reverse the direction of electroosmotic flow inside the capillary and promote the co-migration of the analytes (Jager et al., 2007; Soga and Serwe, 2000).

Electrochemical Detection Ionization of carbohydrates at high pH values also allows CE analysis with electrochemical detection using copper or gold electrodes. It is an interesting approach because the hydroxyl groups can be partially ionized, which in turn permits their effective separation in CZE mode. Cao et al. (2004) described a simple, reliable and reproducible CE method using NaOH (50 mmol/L) as running buffer and amperometric detection to quantify mono- and disaccharides in rice flour. The detector is composed by an electrode cell system consisting of copper working electrodes, platinum auxiliary and reference electrodes. Likewise, Chu et al. (2005) developed a miniaturized CE method and amperometric detection, with an electrode of copper, to quantify carbohydrates in soft drinks.

Complexation of alternate hydroxyl groups with borate and electrochemical and amperometric detection is another alternative for CE analysis of carbohydrates without derivatization (Cheung et al., 2007).

Refractive Index Detection Refractive index detection has been successfully used in CE to separate carbohydrates considering that they do not possess chromophore groups in their structures. Although the detection limits are relatively low, this type of detector could be used as universal detector in CE of carbohydrates (El Rassi, 1999).

13.2.5.3 Coupling with MS

The coupling of CE–MS can provide important advantages in food analysis because of the combination of the high separation capabilities of CE and the

power of MS as identification and confirmation method (Simó et al., 2005). A detailed review has been written by Campa et al. (2006) which describes the advances in CE-MS of carbohydrates.

13.2.5.4 Applications

Besides the difficulties discussed above with the analysis of carbohydrates by CE, it is also necessary to consider the different structures resulting from heterogeneity in primary sequence, branching and the variety of structural isoforms of oligosaccharides. In order to address these challenges several studies have been focused on finding different separation methods. In this section a description of different CE methodologies found in the literature to analyze oligosaccharides is presented.

Human Milk Oligosaccharides (HMOS)

The difficulty of HMOS analysis is not only due to the low and variable (depending on lactational stage) content but also to the complexity of their structures. Therefore, it is necessary to arrange appropriate and powerful analytical methods to achieve efficient separation and quantification of oligosaccharides and their structural isomers.

Acidic oligosaccharides are not intrinsically strong chromophores, however they can absorb in the low UV range due to the aminoacyl moieties and sialic acid present in the structures of many HMOS (Bao and Newburg, 2008).

Underivatized HMOS

Shen et al. (2000) developed a very reproducible and sensitive CE method (fmol level) for underivatized acidic oligosaccharides with detection by UV absorbance at 205 nm. Eleven oligosaccharides of human milk, ranging from tri- to nonasaccharide (3′-sialyllactose, 6′-sialyllactose, 3′-sialyllactosamine, 6′-sialyllactosamine, disialyltetraose, 3′-sialyl-3-fucosyllactose, etc.) were resolved by MEKC. These oligosaccharides were detected in pooled human milk samples, from different donors, and comparison of oligosaccharides profiles revealed an extensive variation in the structural isomers of sialyllacto-N-tetraose. The running conditions were selected as the best compromise between resolution and running time.

The resolution of structurally similar oligosaccharides, especially those containing chemically labile sialic acid residues is a challenging problem. Shen et al. (2001) employed a CE method and UV detection (205 nm) to separate three sets of structural isomers of sialylated oligosaccharides in human milk and bovine

colostrum. They developed conditions for baseline resolution of specific sets of isomers within a 35 min run. Each set of structural isomers of sialylated oligosaccharides, 3'-silayllactose/6'sialyllactose, sialyllacto-N-tetraose-a (linear), -b (branched) and -c (linear), required a unique running buffer with respect to buffer type, concentration, pH, presence of organic modifiers, and surfactants.

Likewise, Bao et al. (2007) developed a novel method to quantify sialyloligosaccharides from human milk by MEKC and UV detection at 205 nm. As running buffer, they used aqueous 200 mM sodium phosphate (pH 7.05) containing 100 mM sodium dodecyl sulfate (SDS) mixed with 4% (v/v) methanol. The method describes new CE conditions that simultaneously resolve not only separation between pairs of structural isomers of HMOS, 3'-sialyllactose/6'sialyllactose and sialyllacto-N-tetraose-a, -b and -c, but also quantification of the 12 major sialyloligosaccharides of human milk in a single 35 min run. *Figure 13.4* shows a CE separation of sialyloligosaccharide standards and HMOS found in colostrum (b) and in human milk (c). The method allowed finding differences in sialyloligosaccharide concentrations between less and more mature milk from same donors. It is possible to define acidic oligosaccharide expression in milk as function of stage of lactation, genetic variation among lactating mothers, diet, diurnal variation, stress, disease, and geographic origins of a population.

Derivatized HMOS

Major neutral oligosaccharides from human milk, such as 2′-fucosyllactose (2′FL), 3′-fucosyllactose (3′FL), lacto-N-tetraose (LNT), lacto-N-fucopentaose I (LNFP I), lacto-N-fucopentaose II (LNFP II) and fucose, have been quantified by CE using LIF as detection system (λ_{exc} = 488 nm; λ_{em} = 520 nm). Oligosaccharides were derivatized via reductive amination with 2-aminoacridone (AMAC). The CE method allowed to resolve two sets of structural isomers, 2′FL/3′FL and LNFP I/LNFP II. This was rapid, sensitive (2 fmol) and reproducible, and required a simple sample preparation (Song et al., 2002).

Schmid et al. (2002) analyzed using MEKC free oligosaccharides from human milk. They used as derivatization agent various esters of aminobenzoic acid and sodium phosphate (20 mM), pH 7.0, and sodium dodecyl sulfate (SDS) (50 mM) as buffer. Oligosaccharide was detected by UV absorbance at 285 and 310 nm. Previous to their analysis, samples were submitted to a simple clean up of deproteination and defatting before derivatization of oligosaccharides. Major human milk oligosaccharides were detected (FL, LNT, LNFP, lacto-N-difucohexaose).

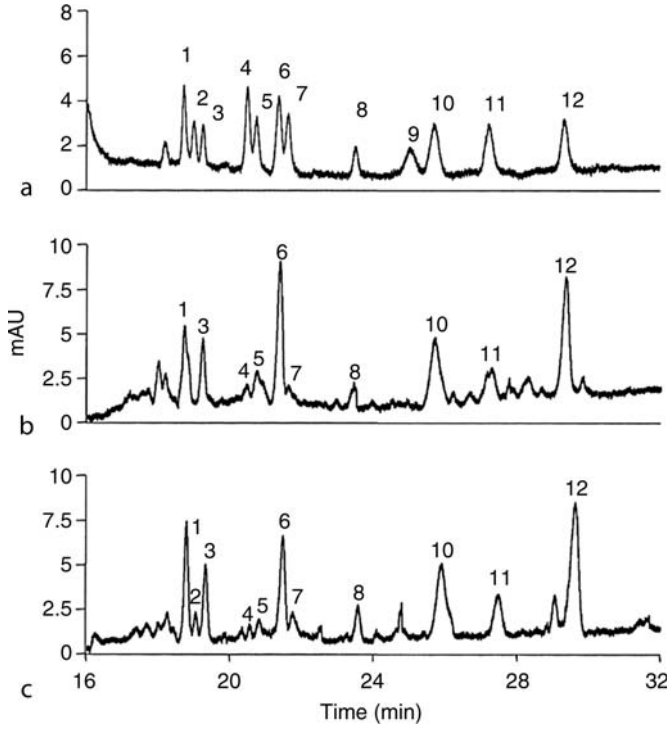

Figure 13.4
CE analysis of sialyloligosaccharides, colostrum sample and mature milk sample. Electrophoregrams of sialyloligosaccharides from a mixture of 12 standard sialyloligosaccharides (a), a colostrum sample (b) and a mature milk sample (c) from a mother in the Boston area. (1) MSMFLNnH (monosialyl, monofucosyllacto-N-neohexaose; (2) MSLNnH I (monosialyllacto-N-neohexaose); (3) MFMSLNH I (monofucosyl, monosialyllactose-N-hexaose); (4) SLNFP II (sialyllacto-N-fucopentaose); (5) SLNT b (sialyllacto-N-tetraose); (6) SLNT c; (7)SLNT a; (8) DSMFLNH (disialyl, monofucosyllacto-N-hexaose); (9) 3′-S-3FL (3′ sialyl-3-fucosyllactose); (10) -6′-SL (6′ sialyllactose); (11) 3′-SL (3′ sialyllactose); (12), DSLNT (disialyllacto-N-tetraose). From Bao et al. (2007) with permission from Elsevier.

Galactooligosaccharides

There is scarce literature on CE methods to analyze GOS. Petzelbauer et al. (2006) separated and quantified the major GOS obtained during lactose conversion at 70°C, catalyzed by β-galactosidases from the archea *Sulfolobus solfataricus* and *Pyrocccus furiosus*. Carbohydrates were analyzed using as running buffer phosphate pH 2.5, derivatized using an aminopyridine solution and detected by UV (240 nm). The authors identified two disaccharides β-D-Gal*p*-(1→3)-D-Glc and β-D-Gal*p*-(1→6)-D-Glc (allolactose); and two trisaccharides

β-D-Gal*p*-(1→3)-lactose and β-D-Gal*p*-(1→6)-D-lactose. As minor compound, β-D-Gal*p*-(1→6)-D-Gal was also identified.

Total GOS and di-, tri-, and tetrasaccharides, derived from lactose hydrolysis with β-galactosidases of *Lactobacillus reuteri* L103 and L461, have been also quantified by CE (Splechtna et al., 2006). The method includes a pre-column derivatization with 2-aminopyridine and detection of derivatives by UV-diode-array detector (DAD). The running buffer was 100 mM phosphoric acid, pH 2.5. The sugars eluted in groups depending on their degree of polymerization and the identified GOS were the same found by Petzelbauer et al. (2006); *Figure 13.5*

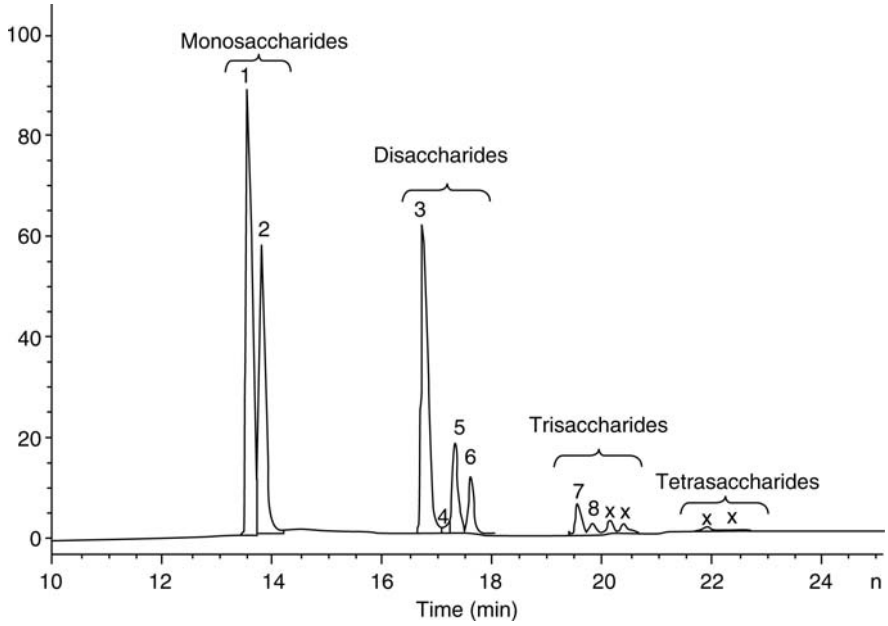

Figure 13.5

CE analysis of GOS. Separation and quantification by capillary electrophoresis of individual GOS produced during the lactose conversion catalyzed by L103 or L461 β-galactosidase. The sample presents a mixture of sugars obtained after the reaction of L103 β-Gal with 205 g/L lactose. The extent of substrate conversion is approximately 67%. The identified compounds are indicated: (1) glucose, (2) galactose, (3) lactose, (4) D-Gal*p*-(1→3)-D-Glc, (5) D-Gal*p*-(1→6)-D-Glc (allolactose) with D-Gal*p* D-Gal*p*-(1→3)-D-Gal, (6) D-Gal*p*-(1→6)-D-Gal, (7) D-Gal*p*-(1→6)-Lac, and (8) D-Gal*p*-(1→3)-D-Lac. Products marked with an x are minor components and were not identified. Peaks appearing at ~ 22 min are tetrasaccharides. From Splechtna et al. (2006) with permission from American Chemical Society.

shows the CE separation attained. Likewise, a quantification of the resulting GOS mixtures obtained from recombinant β-galactosidase of *Lactobacillus reuteri* on lactose hydrolysis has been performed by CE (Maischberger et al., 2007). A comparative study on GOS production was carried out using lactose solutions and whey permeate; and although the initial reaction rate was higher for the latter GOS, the yield was slightly lower.

Lactulose

CE can be used to separate lactulose from mixtures of carbohydrates (Vorndran et al., 1992). This allows a rapid and sensitive analysis of underivatized carbohydrates with indirect UV detection using 6 mM sorbic acid (pH 12.1) as both carrier electrolyte and chromophore. CE analysis has been used to detect carbohydrates in their original form by means of indirect photometry (Oefner et al., 1992). However, direct UV detection of saccharides derivatized pre-column with 2-aminopyridine, ethyl *p*-aminobenzoate or *p*-aminobenzoic acid, allowed a reproducible determination of aldoses and ketoses in fmol range (Oefner et al., 1992).

Underivatized lactulose along with other carbohydrates has been analyzed by CE and amperometric detection using a copper microelectrode. The separation of sugars has been performed in strongly alkaline solutions (LiOH, KOH and NaOH) at pH 13. Among the three studied reagents, the NaOH solution offered good resolution with a suitable time of analysis and it was employed as separation electrolyte. The method is simple, sensitive, and, relatively easy to implement (Colon et al., 1993).

Different CE methods have been used to determine lactulose content in heated milks; thus Guingamp et al. (1999), using an indirect UV detection at 254 nm and sorbate pH 12.04 as running buffer, evaluated heat load of commercial milks. Afterwards, Humbert et al. (2007), by improving milk sample preparation, they determined the lactulose content in pasteurized, indirect and direct UHT, and in-bottle sterilised milk by CE. Lactulose in milk samples was also measured by HPLC and an enzymatic method. The authors found a good correlation between the three methods.

Determination of lactulose along with mannitol is a highly sensitive test for the screening of the diseases that affect intestinal permeability. Paroni et al. (2006) set up a method by CE with indirect UV detection (254 nm) and sorbate, cetyltrimethylammonium bromide and LiOH as background electrolyte to estimate the lactulose-mannitol intestinal permeability in a cohort of patients with type I diabetes.

Fructooligosaccharides (FOS)

FOS are probably the most commonly used prebiotic fibers in the production of functional foods. An evaluation of prebiotic character of FOS has been carried out by Corradini et al. (2004) through short chain fatty acid (SCFA) measurement using CE.

α-Galactosides

The raffinose family of oligosaccharides (ROS) are composed of α-(1→6) galactosides bound to sucrose at C-6 of the glucose. By successive binding of one, two and three additional α-galactoside units to C-6 of the terminating galactose unit, the compounds stachyose, verbascose and ajugose are formed (Andersen et al., 2003). These oligosaccharides are decomposed in the large intestine causing unpleasant effects. However, these effects have been counterbalanced by an increasing interest in non digestible oligosaccharides as functional food ingredients.

Since α-galactosides are non-reducing oligosaccharides, borate complex formation seems to be a promising analytical methodology to analyze them. Arentoft et al. (1993) optimized a high performance capillary electrophoresis (HPCE) method to quantify ROS (raffinose, stachyose and verbascose) based on the formation of borate-carbohydrate complexes and UV detection at 195 nm. Pea seed samples were submitted to a simple extraction procedure and a purification step prior to the determination of individual oligosaccharides. This method could be adapted for the determination of other low-molecular-mass carbohydrates.

Frias et al. (1996) also quantified the ROS family by CZE using disodium tetraborate as running buffer; high-quality electrophoregrams were obtained due to a purification step of pea seeds samples, using Sep-Pak C_{18} cartridges. The ROS family was also quantified by HPAEC-PAD; both methods showed a good linearity and reproducibility and did not show significant differences.

Using indirect UV detection, Andersen et al. (2003) also quantified the ROS family by HPCE. The signal wavelength was set at 350 nm with a reference at 275 nm. As background electrolyte they used pyridine-2,6-dicarboxylic acids, sodium borate decahydrate ($Na_2B_4O_710H_2O$) and hexadecyltrimethylammonium bromide, adjusted to varying pH values (8.0–10.0). The method was applied for the quantification of α-galactosides in a lupine seed sample (*Lupinus angustifolius*) after extraction and purification.

Other Nondigestible Oligosaccharides

There are other oligosaccharides, the so-called "second generation of prebiotics" which could present new physical and chemical properties and different and more specific bioactivities (Joucla et al., 2004).

Homologous of glycoglucans of isomaltose and *Laminaria* have been analyzed by CZE using as running buffer 200 mM borate buffer (pH 9.5) and UV detection at 245 nm. Oligosaccharides were pre-column derivatized with 3-methyl-1-phenyl-2-pyrazolin-5-one (MPP). A good separation was achieved for each oligosaccharide series, which have various types of glycosidic linkages (Honda et al., 1991).

Oligosaccharides derived from partial hydrolysis of dextran were satisfactorily separated by HPCE using coated capillary with a copolymer of hydroxypropylcellulose and hydroxyethyl metacrylate. The running buffer was 100 mM tris-borate buffer, pH 8.8. Derivatization of oligosaccharides was performed using N-(4-aminobenzoyl)-L-glutamic acid and UV detection (Plocek and Novotny, 1997).

Also, efficient electrolyte systems for underivatized carbohydrates based on co-electroosmotic CE can be useful for the separation of derivatized counterparts. A selective separation of derivatized (reductive amination) carbohydrates (xylose, cellobiose, melibiose, maltotetraose, gentiobiose, etc.) using ethyl *p*-aminobenzoate or ethyl *p*-aminobenzonitrile can be obtained using as running buffer electrolyte borate and an organic solvent. Co-directional migration of the anionic analytes with the electroosmotic flow (EOF) was achieved by adding a cationic polymer (hexadimethrine) bromide (HDB). The carbohydrates were detected by UV (280 nm) and the method was applied to the analysis of carbohydrates of plant hydrolyzates (Nguyen et al., 1997).

A comparative study of chromophore response (CZE-UV) and electrochemical signal (HPAEC-PAD) of some model gluco-oligosaccharides (dextrans) with different DP has been carried out. UV detection was performed using 8-aminonaphtalene-1,3,6,-trisulphonic acid (ANTS) as chromophoric dye. Both methods provided similar response for DP 1,000 and 5,000 dextrans (Abballe et al., 2007).

CE with LIF (excitation at 488 nm and emission at 520 nm) and ESI-MS detection has been used to characterise gluco-oligosaccharide regioisomers synthesised by *Leuconostoc mesenteroides* NRRL B-512F with a DP ranging from 2 to 9 (Joucla et al., 2004). Resolution of APTS (9-aminopyrene-1,4,6-trisulfonate) derivatives of gluco-oligosaccharide regioisomers over a wide DP range is more appropriately performed with borate buffer systems (Joucla et al., 2004). The use of combined methods looks promising for profiling mixtures of gluco-oligosaccharides synthesised by glucansucrases.

Xyloglucans belong to the groups of hemicelluloses and are constituted by a β(1→4) linked glucan chain to which different short side chains are attached.

An unambiguous letter code is used for the nomenclature of each segment depending on the side chain, thus they can be classified in few types of structure: XXXG (X = xylose; G = glucose); XXGG and XXXGG. HPAEC-PAD, RP-HPLC and CE (LIF and ESI-MS detection) methods have been compared to determine xyloglucan structures in blackcurrants (Hilz et al., 2006). For CE xyloglucan oligosaccharides were labeled with APTS, separated on a polyvinyl alcohol (N-CHO) coated capillary and detected by LIF (λ_{exc} = 488 nm-λ_{em} = 520 nm). Before analysis, samples were submitted to different extraction steps and xyloglucan material was hydrolyzed with a specific endo-glucanase. The method allowed the identification the structures of xyloglucans as well as the quantification of the main oligomer as XLFG (L = galactose; F = fucose-galactose) present in black currant.

Structural isomers of short oligosaccharides have been also analyzed by CE. In this case, separation of oligosaccharides derived from maltose, cellobiose, xylobiose, and isomaltose has been performed using lithium acetate (pH 5) as running buffer. Oligosaccharides were derivatized using APTS and detected by LIF using wavelengths of excitation and emission of 488 and 520 nm, respectively. The method was applied to the analysis of structural isomers of short oligosaccharides in various plant substrates, and a baseline resolution of three different galactobioses isoforms β (1→4), α (1→4) and α (1→3) was obtained (Khandurina and Guttman, 2005).

13.2.6 Mass Spectrometry

The analysis of prebiotic carbohydrates by different analytical techniques coupled to MS has been reviewed in previous sections. However, a specific mention to this technique has to be done, considering the large number of reported applications where carbohydrates are analyzed directly by MS. Direct infusion ESI and MALDI are the most common ionization sources employed for this purpose. These techniques in combination with tandem MS analyzers have been used to solve structural problems of carbohydrates (Harvey, 1999). Although separation of isomeric oligosaccharides is not possible by MS, identification of their structures based on their different fragmentation patterns has been achieved. Nevertheless, it is not feasible yet to determine the structure of an oligosaccharide just by the study of these patterns. Comparisons to several reference carbohydrates is necessary. Moreover, isomeric oligosaccharides give rise to fragments at the same m/z values, and differences can be observed only in their abundances. Kurimoto et al. (2006) developed a quantitative procedure by quadrupole ion trap (QIT) to solve these problems. Detailed information about

MS of oligosaccharides can be obtained from an exhaustive review written by Zaia (2004), which includes the ionization methods and the mass analyzers generally used for oligosaccharide analysis.

13.2.6.1 Electrospray Ionization

Samples are introduced by direct infusion into the ESI ion source. As indicated above, different analyzers can be coupled to ESI, Q and IT being the most common ones. Nevertheless, in the last years tandem analyzers such as QToF, QIT or triple quadrupole (QqQ) are gaining a significant acceptance and becoming more widespread. While ESI produces a soft ionization the quasi-molecular ion which allows to determine the molecular weight of the analyte, its coupling to IT, QIT, QToF, QqQ or Fourier-transform ion cyclotron resonance (FT-ICR), provides higher structural information by the generation of MS/MS and MS^n. Collision-Induced Dissociation (CID) is the most common method of fragmentation, although other methods such as electron transfer dissociation (ETD), electron capture dissociation (ECD) and infrared multiphoton dissociation (IRMPD), can be applied. In CID the precursor ion is submitted to repeated collisions with a gas and product ions are formed, whereas with IRMPD, photon energy is imparted on both precursor and product ions, resulting in a higher fragmentation (Seipert et al., 2008). ECD and ETD induce fragmentation of positive ions by electron transfer. IT and FT-ICR allow a higher control over CID operation, the possibility of obtaining MS^n and low energy reactions, while QqQ and Q-ToF produce more fragmentation from CID and less operator control, and only MS/MS can be performed.

Table 13.4 shows some recent applications of ESI MS to the analysis of oligosaccharides.

XOS have been analyzed by ESI MS both on positive and negative modes. Whereas the positive ESI MS allowed the identification of neutral and acidic XOS, the negative mode results in simpler MS (Reis et al., 2003a) since there is a lower adduct formation and only acidic XOS ions appear. Isomeric structures of a mixture of arabinoxylooligosaccharides (AXOS) have been also differentiated by analysing their permethylated derivatives by ESI-IT MS upon CID (Matamoros-Fernández et al., 2003); however, the direct analysis of these oligosaccharides using a Q-TOF or IT did not allow the distinction between linear and branched structures.

Table 13.4

Some direct infusion ESI-MS applications for the analysis of prebiotics

Oligosaccharides	Treatment of the sample	Analyzer	Ionization method	Reference
AXOS	Permethylation	IT	CID	Matamoros-Fernández et al. (2003, 2004)
CEOS, MOS, XOS	Addition of ammonium acetate or alkali metal salts	Q-IT FT-ICR	CID	Pasanen et al. (2007)
MOS, CEOS, IMOS	Pyridylamination	Q-IT	CID	Kurimoto et al. (2006)
Sulfated human milk oligosaccharides	Pyridylamination	QqQ	–	Guerardel et al. (1999)
Human milk oligosaccharides	–	Q-ToF	CID	Kogelberg et al. (2004)
XOS		Q-ToF	CID	Reis et al. (2003a)

Fragmentation of cello- (CEOS), malto- (MOS) and xylooligosaccharides (XOS) has been recently studied by ESI MS coupled to QIT and FT-ICR (Pasanen et al., 2007). The effect of different precursor ion types (deprotonated, protonated, ammoniated and alkali metal cationized precursors) and carbohydrate structure (α or β configuration and presence of hexose or pentose units) on the fragmentation of these carbohydrates in CID was evaluated. As an example, *Figure 13.6* shows the scheme of fragmentation of a trisaccharide (*Figure 13.6a*) and the different spectra obtained for deprotonated cellopentaose (*Figure 13.6b*), maltopentaose (*Figure 13.6c*) and xylopentaose (*Figure 13.6d*). Both CEOS and MOS showed similar fragments (A, B and C), however A fragments were more abundant in CEOS. In contrast, the behavior of XOS was completely different; A fragments were the most abundant, and the intensity ratios of C fragments were clearly different from those of CEOS and MOS. In this example, the different fragmentation observed depended both on the anomeric configurations of the glycosidic linkage and on the presence of monosaccharide units. Results of this work also confirmed that the structural information obtained from the CID of oligosaccharides was dependent on the precursor ion type.

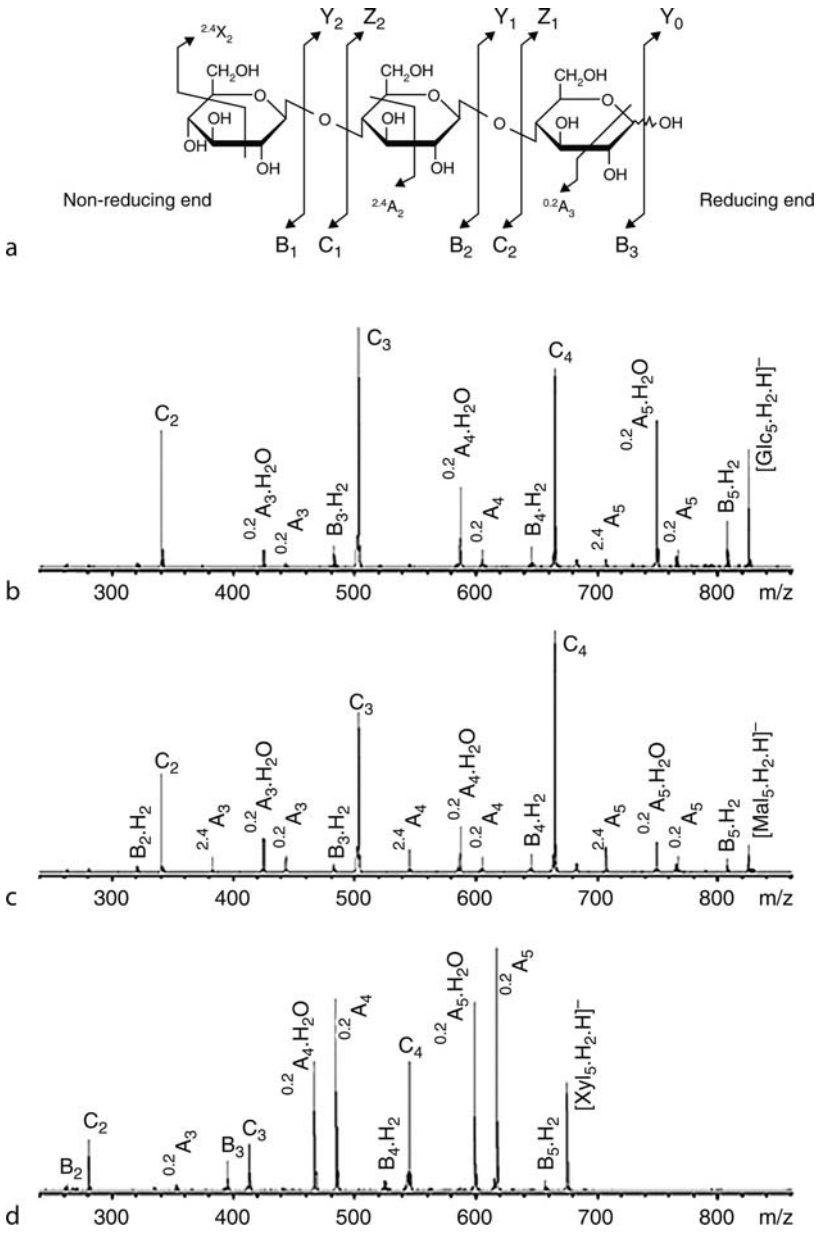

Figure 13.6

CID ESI MS analysis of cellopentaose, maltopentaose, and xylopentaose. Nomenclature for oligosaccharide fragments illustrated for cellotriose (a). CID spectra for deprotonated (b) cellopentaose, (c) maltopentaose, and (d) xylopentaose at a fragmentation amplitude of 0.4 V. From Pasanen et al. (2007) with permission from Elsevier.

13.2.6.2 Matrix-Assisted Laser Desorption/Ionization

An extensive and well written review about the application of MALDI MS to carbohydrate analysis which covers the period 1991–1998 has been reported by Harvey (1999). Although in this section some basic points will be referred to the mentioned study, most recent applications of prebiotic analysis will be discussed.

In MALDI, the sample is mixed with a matrix, allowed to crystallize by evaporation of the solvent and submitted to the laser whose energy is absorbed by the matrix and transferred to the carbohydrate which is ionized.

Nitrogen lasers emitting in the UV at 337 nm are those most commonly used for MALDI analysis, although other lasers that emit in the infrared (IR) have been also assayed.

It has been observed that ionization efficiency by MALDI is similar for neutral carbohydrates of different molecular weights, while efficiency of ESI decreases for carbohydrates of higher DPs (Harvey, 1999). The high sensitivity of MALDI allows the detection of oligosaccharides at picomole levels (Morelle and Michalski, 2005).

MALDI is generally coupled to a time of flight (ToF) analyzer resulting in high sensitivity, because most ions generated by the laser are recorded by the detector (Harvey, 1999), although couplings to other analyzers such as IT (Qin et al., 1996), FT-ICR (Carroll et al., 1996) ToF/ToF (Spina et al., 2004) or Q-ToF (Morelle and Michalski, 2005) have been also described. These couplings provide high-mass accuracy, high resolution, and the possibility of performing multiple methods of tandem MS which can be used to obtain higher structural and complementary compositional information. MALDI-FT-ICR has been recently used to evaluate the consumption of human milk oligosaccharides (Ninonuevo et al., 2007) and FOS (Seipert et al., 2008) by intestinal bacteria.

Harvey (1999) in his review exhaustively explained the different matrices that can be used for the MALDI analysis of free neutral carbohydrates, free acidic carbohydrates, sulfated carbohydrates and glycoproteins. Most recognized prebiotics are neutral carbohydrates, which are commonly analyzed by MALDI using 2,5-dihydroxybenzoic acid (DHB), although other matrices such as 2′,4′,6′-trihydroxyacetophenone (THAP) or mixtures of DHB with different compounds have been also proposed by several authors to obtain finer crystals. One of the problems of DHB matrix is the appearance of multiple matrix peaks at low masses which could interfere with low molecular weight carbohydrates. Therefore, molecular weight of target oligosaccharides should be considered when selecting

a matrix. A good repeatability of spot-to-spot or sample-to-sample, a good crystallization and the production of a spectrum with good signal to noise ratio and good resolution should be also be taken into account for matrix selection (Wang et al., 1999).

Qualitative analyses of prebiotic carbohydrates by MALDI-ToF have been widely carried out (Huisman et al., 2001; Lopez et al., 2003; Reis et al., 2003b; Sanz et al., 2006), however quantitative applications are less common (Wang et al., 1999) since they present difficulties associated to a poor shot-to-shot repeatability and to the crystal's lack of homogeneity. Quantification is normally carried out using an internal standard calibration method and it is desirable in order to obtain single alkali ion adduct peaks to gain peak intensity. Wang et al. (1999) quantitatively analyzed FOS using γ-cyclodextrin as internal standard and KCl to obtain single potassium adduct peaks. However, one of the drawbacks of this technique is the variability of ionization regarding the sample. The medium can show a significant effect on MALDI analysis (Reiffová et al., 2007). A previously proposed method to analyze FOS was used by Wang et al. (1999) to determine FOS content in food extract. Nevertheless, these extracts (i.e., from red onions) suppressed the ions produced from the internal standard although FOS could be detected. Therefore, internal standard should be selected depending on the sample to be analyzed, however, commercial standards are scarce and most of them (such as maltodextrins) posses similar molecular weight to the analytes giving the same response. Seipert et al. (2008) used deuterated reduced maltoheptaose to distinguish its masses from those of FOS with the same molecular weight.

Different comparative studies of prebiotic analyses by HPAEC-PAD and MALDI have been carried out. HPAEC-PAD has been found to be more sensitive in terms of detection limits than MALDI for the analysis of FOS and allowed the separation of linear and branched oligosaccharides. However, MALDI was a faster method, more tolerant to impurities and produced more correct molecular assignments, and was probably in general more accurate for quantitative determinations (Wang et al., 1999).

Table 13.5 shows as an example of some MALDI qualitative and quantitative studies of prebiotic oligosaccharides described in the literature.

13.2.7 Nuclear Magnetic Resonance Spectroscopy

For a long time, NMR has significantly contributed to the knowledge of the structure and conformation of carbohydrates. This technique is especially

Table 13.5

Some examples of MALDI analysis of prebiotics

Prebiotic	Analyzer	Selected matrix	Salts	Analyzes	Calibration	Reference
FOS	ToF	THAP with acetone	0.01 M KCl	Qualitative and quantitative	i.s.	Wang et al. (1999)
FOS and inulin	FT-ICR	0.4 M DHB	0.01 mM NaCl	Qualitative and quantitative	i.s.	Seipert et al. (2008)
Human milk oligosaccharides	FT-ICR	0.4 M DHB	0.01 M NaCl	Qualitative and quantitative	e.s. and i.s.	Ninonuevo et al. (2007)
Human milk oligosaccharides	ToF/ToF	10 mg mL^{-1} DHB	–	Qualitative (CID)	–	Spina et al. (2004)
FOS	ToF	10 mg mL^{-1} DHB	1 mg mL^{-1} CH_3COONa (only for standards)	Semi-quantitative	e.s.	Reiffová et al. (2007)
Fructans	ToF	DHB	–	Qualitative	–	Lopez et al. (2003)
Arabinogalactans	ToF	9 mg mL^{-1} DHB + 3 mg mL^{-1} 1-hydroxy-isoquinoline in water: acetonitrile (70:30)	–	Qualitative	–	Huisman et al. (2001)
XOS				Qualitative	–	Reis et al. (2003b)
XOS	ToF	1% DHB in methanol	–	Qualitative	–	Cano and Palet (2007)
GEOS	ToF	DHB in acetonitrile	–	Qualitative	–	Sanz et al. (2006)
Alternansucrase acceptor products	ToF	DHB in acetonitrile	–	Qualitative	–	Côté and Sheng (2006)

useful for detailed structural analysis of pure products, either in solution or in solid state, but it has been also applied to the study of mixtures of oligosaccharides.

NMR relies on the magnetic properties of the atomic nuclei (spin). The most used nuclei in carbohydrate chemistry are ^{1}H and ^{13}C. NMR operates on a time-scale slower than other spectroscopic techniques (such as IR or UV). This is not a big drawback, since NMR is usually used to elucidate structures, and not as a routine control technique.

13.2.7.1 Sample Preparation

This subject has been described by Bock and Pedersen (1983) and the main features are summarized below.

Samples can be dissolved in deuterium oxide (D_2O) and deuterated solvents, such as dimethylsulfoxide (Me_2SO-d_6), pyridine (Py-d_6) or chloroform (DCl_3). The less polar solvents are usually selected for low-molecular weight sugars, whereas water is necessary for oligosaccharides. It should be taken into account that solvent-induced shifts are low for neutral oligosaccharides when working with ^{13}C-NMR, whereas the effect on ^{1}H-NMR spectra is high. The effect of solvent is always important in acidic or basic carbohydrates. Concentration should be adjusted to avoid high-viscosity solutions, which can cause signal broadenings. Sample clean-up is recommended to suppress soluble paramagnetic impurities.

13.2.7.2 Reference Compounds

The most used reference signals for chemical shifts measurements of carbohydrates are probably tetramethylsilane and acetone; nevertheless, other compounds as DSS (sodium salt of 2,2-dimethyl-2-silapentane-5-sulfonic acid) and TSP ([2,2,3,3-d_4]-3-(trimethylsilyl)-propanoic acid sodium salt) are also used.

13.2.7.3 Methodology

Classic studies about NMR of carbohydrates were published by Vliegenthart et al. (1983) and Rathbone (1985). Whereas the assignment of ^{1}H signals is relatively easy in pure products (specially the anomeric proton), the high amount of

protons when several tautomeric forms and various oligosaccharides are present makes difficult the assignment of all signals. Thus, it becomes necessary to use ^{13}C spectra and different methods including 1D and 2D homo- and heteronuclear experiments, correlation spectroscopy (COSY) including total correlation spectroscopy (TOCSY) and nuclear Overhauser effect spectroscopy (NOESY), etc. The number of different NMR experiments which have been described to elucidate oligosaccharide structures is really high. Experiments such as HSQC (heteronuclear single quantum correlation) and HMQC (heteronuclear multiple quantum correlation) which correlate the chemical shift of proton with the chemical shift of the directly bonded carbon, and HMBC (heteronuclear multiple bond correlation) which uses two or three bonds couplings, are frequently used to assign signals from complex oligosaccharides. A recent review describes several NMR methods for analyzing the structure of oligosaccharides, including assignment of all H-1 NMR signals, NOE experiments, and modification of pulse programs (Kajihara and Sato, 2003).

13.2.7.4 Applications

A series of interesting applications, covering different oligosaccharides with prebiotic properties have been summarized in *Table 13.6*. Saccharides from DP2 (lactulose) and DP3 (kestoses) to higher DP have been included.

Although basic NMR data of many pure sugars, including mono- di- and trisaccharides have been published in the 1980s, fully assigned highly resolved spectra of some prebiotic sugars have been recently reported: lactulose in solution (Mayer et al., 2004) and in crystalline state (Jeffrey et al., 1992); the three natural kestoses (Calub et al., 1999; Liu et al., 1991). α-D-Gal*p*-(1→6)-β-D-Gal*p*-(1→4)-β-D-fructose (three tautomers) and β-D- Gal*p*-(1→4)-D-fructose-(1→1)-β-D-Gal*p* (three tautomers) resulting from enzymatic transgalactosylation during lactulose hydrolysis by the galactosidase from *K. lactis* have also been characterized (Martínez-Villaluenga et al., 2008b). Twelve novel non-reducing oligosaccharides from DP3 to DP6, namely [β-D-Gal*p*-(1→4)]$_n$-α-D-Glc*p*-(1→1)-β-D-Gal*p*[-(4→1)- β-D-Gal*p*]$_m$, with n, m = (1, 2, 3, or 4) and β-D-Gal*p*-(1→2)-α-D-Glc*p*-(1→1)-β-D-Gal*p* were characterized in a mixture produced by β-galactosidase using lactose as a substrate (Fransen et al., 1998). Although the mixture was fractionated, several oligosaccharides were found in the same fraction and several experiments were necessary to achieve complete characterization. A detailed description of signal assignments of two trisaccharides and four tetrasaccharides

Table 13.6

NMR applications for the analysis of prebiotics (*Cont'd* p. 519)

Analyte	Experiments	Solvent	Reference compound	Reference
Related-kestose oligosaccharides in plants	^{1}H, ^{13}C	D_3HCl	Tetramethylsilane	Forshyte et al. (1990)
1-Kestose	2D homonuclear and heteronuclear	D_2O	Acetone	Calub et al. (1990)
6-Kestose, neokestose	2D homonuclear and heteronuclear	D_2O	Acetone	Liu et al. (1991)
Crystalline lactulose	^{13}C CPMAS	–	Adamantane	Jeffrey et al. (1992)
5 Trisaccharides in goat colostrum	^{1}H, ^{13}C, several 2D experiments	D_2O	Expressed relative to DSS, but actually measured by reference to acetone	Urashima et al. (1994)
12 non-reducing oligosaccharides	^{1}H, ^{13}C, several 2D experiments	D_2O	Acetone or acetate for ^{1}H Ext. glucose for ^{13}C	Fransen et al. (1998)
XOS	^{1}H, ^{13}C, several 2D experiments	D_2O	Acetone	Nishimura et al. (1998)
3 Sulfated OS in human milk	^{13}C and ^{1}H	D_2O	Expressed by reference to DSS, but actually measured by reference to acetone	Guerardel et al. (1999)
5 FOS from *Asparagus*	1D ^{1}H and ^{13}C, several 2D experiments	D_2O	TSP	Fukusi et al. (2000)
Neo-FOS produced by a *Penicillium citrinum*	^{13}C	D_2O	Tetramethylsilane	Hayashi et al. (2000)
Kojioligosaccharides	^{13}C	D_2O	TSP	Chaen et al. (2001)
Lactulose	^{1}H, several 1D and 2D experiments		Tetramethylsilane	Mayer et al. (2004)
Inulin-type FOS from *Matricaria maritima*	2D ^{1}H, ^{1}H DQF-COSY/TOCSY and ^{1}H, ^{13}C HMQC/HMBC	D_2O	TSP	Cerantola et al. (2004)
Neutral oligosaccharides from human milk	^{1}H, several 2D experiments	D_2O	Acetone	Kogelberg et al. (2004)

Table 13.6

Analyte	Experiments	Solvent	Reference compound	Reference
Novel oligosaccharides from raffinose and stachyose	^{1}H and ^{13}C 2D-NMR including COSY, HSQC, HSQCTOCSY, HMBC and other	D_2O	TSP and 1,4-dioxane	Takahashi et al. (2005)
Oligosaccharides produced by alternansucrase	^{1}H, ^{13}C, several 2D experiments	D_2O	Acetone	Coté and Sheng (2006)
Cyclic isomaltooligosaccharides	^{13}C	D_2O	DSS	Funane et al. (2007)
IMOS	^{1}H	D_2O		Ao et al. (2007)
GOS from cyanobacterium *Nostoc commune*	^{1}H	D_2O	Acetone	Wienecke et al. (2007)
2 Trisaccharides derived from lactulose	^{1}H, ^{13}C, several 1D and 2D experiments	D_2O	Tetramethylsilane	Martinez-Villaluenga et al. (2008b)
FOS produced by *Aspergillus*	^{1}H, ^{13}C and 2D HMQCT	D_2O	Tetramethylsilane	Mabel et al. (2008)

isolated from *Asparagus* through several NMR experiments has been published by Fukushi et al. (2000).

Among the oligosaccharides with prebiotic properties, natural fructans (FOS) with the two series of inulin and levan with linkages 2→1 and 2→6 respectively, have probably been the most studied (Cerantola et al., 2004; Hayashi et al., 2000).

Most oligosaccharides obtained by enzymatic synthesis such as kojioligosaccharides (Chaen et al., 2001), cycloisomaltooligosaccharides (Funane et al., 2007) are mixtures of similar saccharides with a definite glycosidic linkage and different DP, thus the NMR signal assignment can be performed by comparison with published data.

Three xylooligosaccharides with general structure [*O*-α-D-Glc*p*-(1→2)]$_n$-*O*-α-D-Xyl*p*-(1→2)-β-D-Fru*f* (n = 1,2,3) required several techniques for structural characterisation. The ^{1}H and ^{13}C NMR signals of each saccharide were assigned using two dimension (2D)-NMR including COSY, HSQC, HSQC-TOCSY and HMBC (Takahashi et al., 2007). Similar techniques were applied to the structural

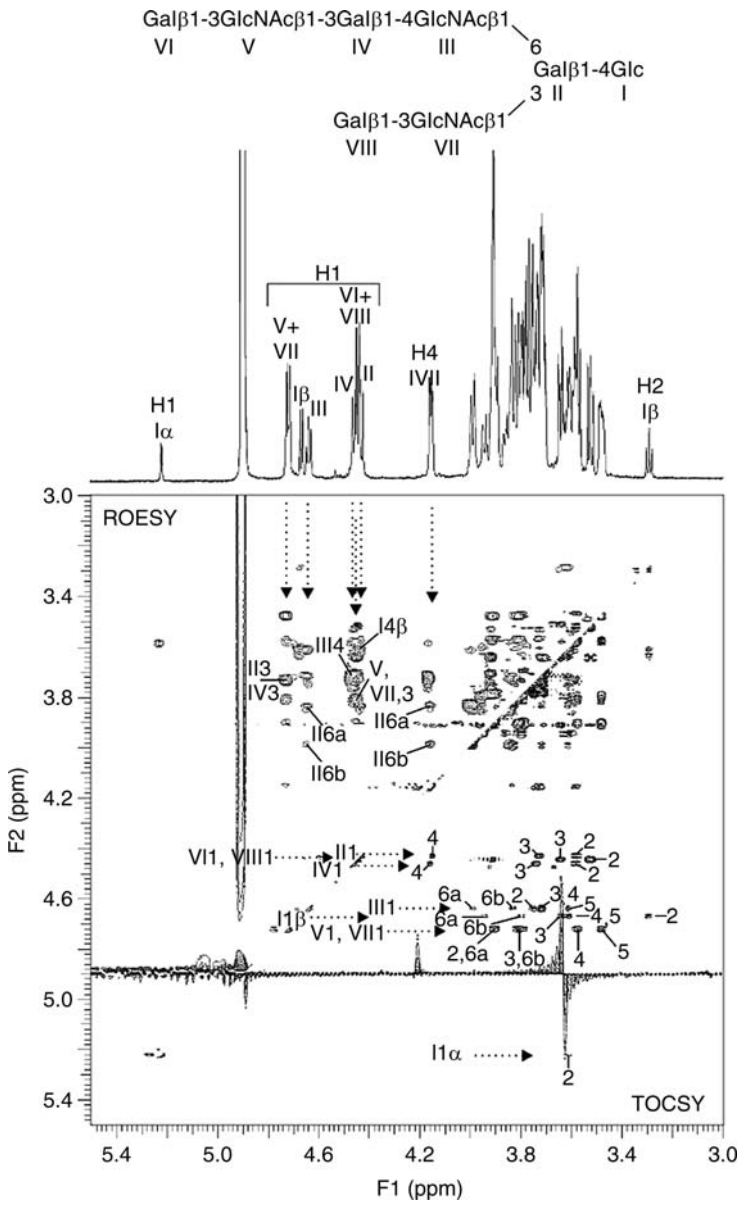

Figure 13.7

^{1}D and ^{2}D ^{1}H NMR spectra of iso-lacto-N-octaose. ^{1}D and ^{2}D ^{1}H NMR spectra (800 MHz) of iso-lacto-N-octaose, region 5.5–3.0 ppm at 15°C. Upper trace, ^{1}H NMR spectrum; top-left half, 300-ms ROESY spectrum and bottom-right half, 140-ms TOCSY spectrum. The structure is shown at the top, depicting the residue labeling. From Kogelberg et al. (2004) with permission from FEBs.

characterization of six novel oligosaccharides (one tetra-, two penta-, two hexa- and one hepta-saccharide) synthesized by glucosyl transfer from β-D-glucose-1-phosphate to raffinose or stachyose by the action of *Thermoanaerobacter brockii* kojibiose phosphorylase (Takahashi et al., 2005).

A special case is that of alternansucrase, which produces oligosaccharides with alternating α-(1→3) and α-(1→6) linkages. When this enzyme was incubated with maltose, one pentasaccharide, two hexasaccharides and one heptasaccharide were isolated as main products, with general structure [α-D-Glc_p-(1→6)-α-D-Glc_p-(1→3)]$_x$-α-D-Glc (Coté and Sheng, 2006). Experiments (gradient-enhanced band-selective HSQC and HSQC–TOCSY and gradient-enhanced band-selective HMBC) were performed at 27°C for the lower DP products and at 50°C for the higher DP ones.

Milk oligosaccharides are extremely complex: about 150–200 oligosaccharides (neutral and acidic) have been described in human milk, whereas a smaller number exist in ruminants' milk. As an example of NMR application to oligosaccharides in goat's milk, four neutral trisaccharides were characterized in goat colostrum: α-L-Fuc*p*-(l→2)-β-D-Gal*p*-(l→4)-Glc, α-D-Gal*p*-(1→3)-β-D-Gal*p*-(1→4)-Glc, β-D-Gal*p*-(l→3)-β-D-Gal*p*-(l→4)-Glc, and β-D-Gal*p*-(1→6)-β-D-Gal*p*-(1→4)-D-Glc (Urashima et al., 1994); NMR spectra were assigned by comparison with those of other previously described oligosaccharides and by several experiments, such as double quantum filtered correlation (DQF-COSY), 1D TOCSY and (^{1}H, ^{13}C) shift correlation.

Human milk neutral oligosaccharides contain galactose, *N*-acetylglucosamine, fucose, and lactose; several anionic oligosaccharides containing *N*-acetylneuraminic acid also exist; a lactose unit at the reducing end is also frequently found (Mehra and Kelly, 2006) but other substituents are possible. As an example, three sulfated oligosaccharides have been analyzed using 2D homonuclear COSY and HMQC (Guerardel et al., 1999). The structural elucidation of very complex milk oligosaccharides has been undertaken by combining two techniques: ESI-MS for determining the branching pattern and ^{1}H NMR for sequence assignment (Kogelberg et al., 2004); *Figure 13.7* shows some spectra of *iso*-lacto-*N*-octaose.

13.3 Summary

- Despite advances in analytical techniques in recent years there is still a lack of accurate and precise methods to characterize and quantify prebiotic oligosaccharides which frequently consist of complex mixtures with similar structural characteristics.

- Traditional analyses such as methylation, TLC or open chromatographic columns with RI detectors are still commonly used. The lack of standards and the similar structure of oligosaccharides are the main drawbacks to achieve a truthful qualitative and quantitative result.
- The selection of the most appropriate methodology for the analysis of prebiotics mainly depends on the nature of the carbohydrate mixture: the complexity of the sample, range of expected molecular weight, etc. No one protocol is able to cover all the possible cases.
- While GC has commonly been used to determine the composition of low molecular weight carbohydrates, oligosaccharides with high DPs are mainly characterised by HPLC.
- Considering the complexity of prebiotic samples their analyses require the use of different techniques, which are combined to obtain useful information.
- HPLC, CE or GC are used for the separation and isolation of the different constituents; methylation analysis and NMR to determine their structures; MS for studying their molecular weight and/or tandem MS systems to complement the structural information.

List of Abbreviations

2′FL	2′-fucosyllactose
2D	two dimensions
3′FL	3′-fucosyllactose
AMAC	2-aminoacridone
AMD	automatic mode development
ANTS	8-aminonaphtalene-1,3,6,-trisulphonic acid
APTS	9-aminopyrene-1,4,6-trisulfonate
ASE	accelerated solvent extraction
AXOS	arabinoxylooligosaccharides
BGE	background electrolyte
CE	capillary electrophoresis
CEOS	cellooligosaccharides
CGE	capillary gel electrophoresis
CI	chemical ionization
CID	collision-induced dissociation
CIEF	capillary isolectric focusing
CITP	capillary isotachophoresis

COSY	correlation spectroscopy (COSY)
CZE	capillary zone electrophoresis
DAD	diode-array detector
DHB	2,5-dihydroxybenzoic acid
DP	degree of polymerization
DQF-COSY	double-quantum-filtered correlation spectroscopy
DSS	2,2-dimethyl-2-silapentane-5-sulfonic acid sodium salt
ECD	electron capture dissociation
EI	electronic impact
ELSD	evaporative light scattering detectors
EOF	electroosmotic flow
ESI	electrospray ionization
ETD	electron transfer dissociation
FAB	fast atom bombardment
FID	flame ionization detector
FOS	fructooligosaccharides
FT-ICR	fourier-transform ion cyclotron resonance
GC	gas chromatography
GCC	graphitized carbon columns
GOS	galactooligosaccharides
HDB	hexadimethrine bromide
HILIC	hydrophilic interaction chromatographic
HMBC	heteronuclear multiple bond correlation
HMOS	human milk oligosaccharides
HMQC	heteronuclear multiple quantum correlation
HPAEC	high performance anion exchange chromatography
HPCE	high performance capillary electrophoresis
HPLC	high performance liquid chromatography
HPSEC	high performance size exclusion chromatography
HPTLC	high performance TLC
HSQC	heteronuclear single quantum correlation
HT-GC	high temperature gas chromatography
IMOS	isomaltooligosaccharides
IR	infrared
IRMPD	infrared multiphoton dissociation
IT	ion trap
LALLS	low angle laser light scattering
LC	liquid chromatography

LIF	laser-induced fluorescence
LNFP I	lacto-N-fucopentaose I
LNFP II	lacto-N-fucopentaose II
LNT	lacto-N-tetraose
LSI	liquid secondary ion
MALDI	matrix assisted laser desorption/ionization
MALLS	multiple angle laser light scattering
MEKC	micellar electrokinetic chromatography
MOS	maltooligosaccharides
MPP	3-methyl-1-phenyl-2-pyrazolin-5-one
MS	mass spectrometry
NMR	nuclear magnetic resonance
NOESY	nuclear overhauser effect spectroscopy
NPLC	normal phase liquid chromatography
OPTLC	over pressured TLC
o-ToF	orthogonal ToF
PAD	pulse amperometric detector
PC	paper chromatography
Q	quadrupole
QIT	quadrupole ion trap
QqQ	triple quadrupole
RI	refractive index
ROS	raffinose oligosaccharides
RP	reverse phase
SALDI	surface assisted laser desorption/ionization
SCFA	short chain fatty acid
SDS	sodium dodecyl sulfate
SEC	size exclusion chromatography
SFE	supercritical fluid extraction
THAP	2',4',6'-trihydroxyacetophenone
TLC	thin layer chromatography
TOCSY	total correlation spectroscopy
ToF	time of flight
TSP	[2,2,3,3-d_4]-3-(trimethylsilyl)-propanoic acid sodium salt
UPLC	ultra performance liquid chromatography
UTLC	ultra TLC
UV	ultraviolet
XOS	xylooligosaccharides

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