

Oligosaccharide analysis by graphitized carbon liquid chromatography–mass spectrometry

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Abstract Structural analysis of complex mixtures of oligosaccharides using tandem mass spectrometry is regularly complicated by the presence of a multitude of structural isomers. Detailed structural analysis is, therefore, often achieved by combining oligosaccharide separation by HPLC with online electrospray ionization and mass spectrometric detection. A very popular and promising method for analysis of oligosaccharides, which is covered by this review, is graphitized carbon HPLC–ESI-MS. The oligosaccharides may be applied in native or reduced form, after labeling with a fluorescent tag, or in the permethylated form. Elution can be accomplished by aqueous organic solvent mixtures containing low concentrations of acids or volatile buffers; this enables online ESI-MS analysis in positive-ion or negative-ion mode. Importantly, graphitized carbon HPLC is often able to resolve many glycan isomers, which may then be analyzed individually by tandem mass spectrometry for structure elucidation. While graphitized carbon HPLC–MS for glycan analysis is still only applied by a limited number of groups, more users are expected to apply this method when databases which support structural assignment become available.

Keywords Glycan · Graphitized carbon · Mass spectrometry

Abbreviations

Endo H Endoglycosidase H
Fuc Fucose
Hex Hexose



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HexNAc	<i>N</i> -Acetylhexosamine
HexU	Hexuronic acid
HILIC	Hydrophilic-interaction chromatography
Man	Mannose
NANA	<i>N</i> -Acetylneuraminic acid
PNGase F	<i>N</i> -Glycosidase F
TFA	Trifluoroacetic acid

Introduction

Nature is full of complex oligosaccharides and polysaccharides. Most organisms express a multitude of glycans, for example *N*-linked and *O*-linked glycans on proteins, the glycanic moieties of glycosylphosphatidylinositol (GPI)-anchors which are attached to proteins, and glycans on ceramide carriers (glycosphingolipids). They often cover the surface of organisms and cells and are involved in interaction, recognition, and defense. Detailed characterization of the glycan moieties is therefore often crucial for a molecular understanding of various biological processes.

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Another field of oligosaccharide analysis is the glycosylation analysis of recombinantly expressed glycoproteins which are prepared for therapeutic purposes [1, 2]. Glycosylation of therapeutic glycoproteins has to be controlled as it may influence the stability and efficacy of a drug and may lead to undesirable anti-glycan immune responses [1]. Moreover, plant polysaccharides such as cellulose, starch, and inulin, mammalian glycan polymers like glycosaminoglycans and glycogen, and bacterial polysaccharides are often enzymatically or chemically degraded to facilitate analysis at the oligosaccharide level. Most of these oligosaccharide preparations are rather complex, and, in order to obtain detailed structural information, several analytical approaches such as NMR and mass spectrometry can be used.

Mass spectrometric analysis may be performed on native oligosaccharides in positive-ion or negative-ion mode [3]. Alternatively, glycans may be derivatized, the most widely used approach being permethylation followed by mass spectrometric analysis by MALDI or ESI in positive-ion mode. Further structural information may be obtained by tandem mass spectrometry of native glycans as sodium adducts in positive-ion mode or as deprotonated species in negative-ion mode. Both techniques result in cross-ring cleavages providing linkage information [3, 4]. Mass spectrometric fragmentation of permethylated glycans enables deduction of linkages and also differentiates between terminal, subterminal, and branching residues [3].

Oligosaccharides may be directly analyzed using NMR or mass spectrometry, or in conjunction with a separation method such as HPLC or CE. Various separation techniques have been used for oligosaccharide analysis (Table 1), often in conjunction with radioactive labeling and scintillation counting, fluorescence/UV detection, and mass spectrometry. Introductory literature on these separation techniques is summarized in Table 1. This review will cover the separation of oligosaccharides both in their native and derivatized forms by graphitized carbon chromatography, with a focus on mass spectrometric detection. The most interesting feature of graphitized carbon liquid chromatography for oligosaccharide analysis is its efficacy in

separating isomeric structures, which is particularly valuable in conjunction with (tandem) mass spectrometric analysis, as this enables a very detailed characterization of complex oligosaccharide samples, which can hardly be achieved by mass spectrometry alone. One major objective of this review is, therefore, to show the extent to which graphitized carbon chromatographic systems are able to separate isomeric oligosaccharides. Further attention will be paid to the choice of solvents, ionization mode (negative or positive), and available or desirable tools for structure assignment.

A non-exhaustive overview of the literature on oligosaccharide separations on porous graphitized carbon is summarized in Table 2. Most oligosaccharide analyses by carbon HPLC have been performed on *N*-glycans and *O*-glycans (Table 2), predominantly of mammalian origin. Some publications have dealt with analysis of milk oligosaccharides (Table 2), and other publications have been dedicated to the analysis of plant polysaccharides (hexose polymers) [5] and oligosaccharides and sugar phosphates [6]. Another, rather promising, field of application of graphitized carbon is analysis of enzymatic degradation products of glycosaminoglycans [7, 8].

Retention principle and stationary phases

Several publications in the early nineties established graphitized carbon as a stationary phase for HPLC of oligosaccharides and glycopeptides with small peptide moieties [20–25]. Recently, the analysis of glycopeptides using porous graphitized carbon in an LC–MS chip was described [26]. Native reducing-end and reduced oligosaccharides are strongly retained by graphitized carbon stationary phases but tend to be not retained or hardly retained on C_{18} reversed-phase materials. Graphitized carbon undergoes both hydrophobic and polar interactions with oligosaccharides [25]. Ionic interactions also contribute to oligosaccharide retention [27, 28]. Clearly, additional studies will be necessary to provide a better understanding of oligosaccharide retention on graphitized carbon stationary phases.

Almost all the work summarized in Table 2 was performed using Hypercarb graphitized carbon columns. Particles of 5 μm have mostly been applied, although the 7- μm material was used in some studies. From the early nineties until 2001, columns of 4.6 mm and 2.1 mm diameter were used. Miniaturization began with some publications in 2002, which described the use of capillary-scale [29–32] and nano-scale [33, 34] graphitized carbon HPLC. The use of smaller columns generally increased sensitivity, as pointed out by Karlsson et al., who observed a marked increase in sensitivity in nano-scale graphitized

Table 1 A selection of oligosaccharide separation methods

Method	Ref.
Reversed-phase chromatography	[9–11]
Graphitized carbon chromatography	This review, [11]
High-pH ion-exchange chromatography	[12, 13]
Hydrophilic-interaction liquid chromatography	[2, 11, 14]
Capillary electrophoresis	[15, 16]
(Capillary) gel electrophoresis	[11, 17]
Lectin affinity chromatography	[18, 19]

Table 2 Graphitized carbon HPLC for oligosaccharide analysis

Column and flow	Solvents	pH	Samples	Separation	Detection	Ionization mode	Ref.
Hypercarb 5 μm ; 4.6 mm \times 100 mm (1000 $\mu\text{L min}^{-1}$)	10 mmol L ⁻¹ ammonia; 50% acetonitrile in 10 mmol L ⁻¹ ammonia; between 40 °C and 70 °C	Basic	Endo H-released <i>N</i> -glycans and chitin oligosaccharides	Without addition of ammonia separation of anomers was observed	UV absorbance (210 nm)		[25]
Hypercarb 5 μm ; 2.1 mm \times 150 mm (120 $\mu\text{L min}^{-1}$);	5 mmol L ⁻¹ ammonium formate; acetonitrile	9.3	<i>O</i> -glycans after reduction	Resolution of up to six isomers of sulfated <i>O</i> -glycans	ESI-MS	–	[37]
Hypercarb 5 μm ; 2.1 mm \times 100 mm (200 $\mu\text{L min}^{-1}$)	5 mmol L ⁻¹ ammonium acetate; 50% acetonitrile in 5 mmol L ⁻¹ ammonium acetate	8.5	<i>N</i> -glycans after reduction; aliquots subjected to exoglycosidase treatments, followed by graphitized carbon SPE	Isomer separation of sialylated triantennary and tetraantennary <i>N</i> -glycans; separation of 10 isomers of composition Hex7HexNAc6NANA2	UV absorbance (206 nm); ESI-MS	–	[38, 39]
Hypercarb 7 μm ; 0.25 mm \times 100 mm (4 $\mu\text{L min}^{-1}$)	10 mmol L ⁻¹ ammonia or 25 mmol L ⁻¹ ammonium formate; acetonitrile	9.5	Reducing <i>N</i> -glycans and reduced <i>O</i> -glycans	Separation of Hex2HexNAc4 isomers	ESI-MS	–	[29]
Hypercarb 5 μm ; 0.32 mm \times 150 mm (6 $\mu\text{L min}^{-1}$)	10 mmol L ⁻¹ ammonium bicarbonate; acetonitrile	Basic	<i>N</i> -glycans; <i>O</i> -glycans after reduction		ESI-MS	–	[30, 40]
Hypercarb 5 μm ; 0.3 mm \times 100 mm (6 $\mu\text{L min}^{-1}$); 0.15 mm \times 100 mm (0.5 $\mu\text{L min}^{-1}$)	10 mmol L ⁻¹ ammonium bicarbonate; 80% acetonitrile in 10 mmol L ⁻¹ ammonium bicarbonate	Basic	Reduced <i>N</i> -glycans and <i>O</i> -glycans after desalting with graphitized carbon micro SPE	Separation of various isomeric, fucosylated <i>O</i> -glycans; 4 isomeric core 4 <i>O</i> -glycans of composition Hex2HexNAc3dHex1 were separated.	ESI-MS	–	[31, 32, 35, 41– 43]
Hypercarb 5 μm ; 0.2 mm \times 75 mm (0.4 $\mu\text{L min}^{-1}$)	0.04% ammonia in water; acetonitrile	Basic	<i>O</i> -glycans from MUC1 after reduction	Separation of various isomeric <i>O</i> -glycans	ESI-MS	–	[44]
Hypercarb; 0.3 mm \times 100 mm (4–7 $\mu\text{L min}^{-1}$)	10 mmol L ⁻¹ ammonium bicarbonate; 80% acetonitrile in 10 mmol L ⁻¹ ammonium bicarbonate	Basic	Enzymatic digests of glycosaminoglycans, often with reduction and cation exchange desalting	Separation of various isomeric, sulfated disaccharide units	ESI-MS	–	[7, 45]
ProteCol column with carbon clad zirconia; 0.15 \times 100 mm (0.5 $\mu\text{L min}^{-1}$)	10 mmol L ⁻¹ ammonium bicarbonate; 80% acetonitrile in 10 mmol L ⁻¹ ammonium bicarbonate	Basic	<i>O</i> -glycans after reduction		ESI-MS	–	[36]
Hypercarb; 0.2 mm \times 150 mm (2–3 $\mu\text{L min}^{-1}$)	5 mmol L ⁻¹ ammonium acetate; 50% acetonitrile in 5 mmol L ⁻¹ ammonium acetate	8.5	Monosaccharides and <i>N</i> -glycans labeled with 2- aminopyridine	Resolution of various isomeric hybrid-type and complex biantennary structures, including five isomers of composition Hex5HexNAc4NANA1	ESI-MS	+ and –	[46]
Hypercarb 5 μm ; 0.2 mm \times 150 mm (2 $\mu\text{L min}^{-1}$)	2% acetonitrile in 5 mmol L ⁻¹ ammonium acetate; 80% acetonitrile in 5 mmol L ⁻¹ ammonium acetate	9.6	<i>N</i> -glycans after reduction	Separation of Man7-D1 from Man7-D3 and other isomer separations	ESI-MS, proton adducts;	+ and alternating + and –	[47, 48]
Hypercarb 5 μm ; 0.2 mm \times 150 mm (2 $\mu\text{L min}^{-1}$)	2% acetonitrile in 5 mmol L ⁻¹ ammonium acetate; 80% acetonitrile in 5 mmol L ⁻¹ ammonium acetate	8.5	PNGase F-released <i>N</i> -glycans after reduction and graphitized carbon SPE	Separation of various isomers	ESI-MS, proton and sodium adducts	+	[49]

Table 2 (continued)

Column and flow	Solvents	pH	Samples	Separation	Detection	Ionization mode	Ref.
Hypercarb 5 μm ; 0.2 mm \times 150 mm (2 $\mu\text{L min}^{-1}$)	2% acetonitrile in 5 mmol L ⁻¹ ammonium acetate; 80% acetonitrile in 5 mmol L ⁻¹ ammonium acetate	9.6	<i>N</i> -glycans after labeling with 2-aminopyridine and graphitized carbon SPE		ESI-MS, sodium adducts	+	[50]
Hypercarb 5 μm ; 4.6 mm \times 100 mm (1000 $\mu\text{L min}^{-1}$)	0.01% ammonia; 50% acetonitrile containing 0.01% ammonia	Basic	Endo H-released <i>N</i> -glycans after reduction	Complete isomer separation of many oligomannosidic <i>N</i> -glycans of ribonuclease B	UV absorbance (206 nm); ESI-MS, proton adducts	+	[41, 51]
Hypercarb 5 μm ; 3 mm \times 50 mm; 1 mm \times 100 mm; 0.32 mm \times 100 mm; 0.075 mm \times 70 mm	100 mmol L ⁻¹ ammonium acetate; acetonitrile	8.0	Reducing <i>N</i> -glycans and reduced <i>O</i> -glycans		ESI-MS, proton adducts;	+	[33]
Hypercarb 5 μm ; 1 mm \times 100 mm (50 $\mu\text{L min}^{-1}$)	2% acetonitrile in 5 mmol L ⁻¹ ammonium acetate; 80% acetonitrile in 5 mmol L ⁻¹ ammonium acetate	9.6	<i>N</i> -glycans after reduction;	Isomer separation	ESI-MS, proton adducts	+	[52]
Hypercarb 5 μm ; 2.1 mm \times 100 mm (250 $\mu\text{L min}^{-1}$)	Water; acetonitrile	Neutral	<i>O</i> -glycans after reduction	Resolution of various isomers, including four structures of composition Hex2HexNAc2dHex2.	MALDI-FT -ICR-MS	+ and -	[53, 54]
Hypercarb 5 μm ; 4.6 \times 100 mm (600 $\mu\text{L min}^{-1}$); 0.1 mm \times 100 mm (150 nL min ⁻¹)	Ternary gradient of water, acetonitrile, and isopropanol	Neutral	Wheat oligosaccharides after reduction;	Resolution of many isomers of Hex3 to Hex16	ESI-MS, sodium adducts	+	[5]
Alltech, home packed column, <70 μm ; 0.5 mm \times 100 mm (5 $\mu\text{L min}^{-1}$)	Isocratic, 40% acetonitrile in water	Neutral	<i>N</i> -glycans from ovalbumin and human plasma		ESI-MS, proton and sodium adducts	+ and -	[55]
Hypercarb 5 μm ; 2.1 mm \times 100 mm (200 $\mu\text{L min}^{-1}$)	Water; acetonitrile	Neutral	Milk oligosaccharides after labeling with <i>N,N</i> - dimethylbenzylamine;	Separation of isomers lacto- <i>N</i> -fucospentaose I and II	ESI-MS, permanent charge on label	+	[56]
Hypercarb 5 μm ; 4.6 mm \times 100 mm (1000 $\mu\text{L min}^{-1}$)	65 mmol L ⁻¹ ammonium formate; acetonitrile	3.0	<i>N</i> -glycans after reduction; use of two internal chromatographic standards;	Separation of many isomers of biantennary and hybrid-type <i>N</i> -glycans, with and without sialylation and core-fucosylation	ESI-MS, proton adducts	+	[57–59]
Hypercarb 5 μm on-chip column; 0.075 mm \times 0.050 mm \times 50 mm (300 nL min ⁻¹)	0.1% formic acid; 90% acetonitrile with 0.1% formic acid	Acidic	<i>O</i> -glycans and milk oligosaccharides after reduction;	Baseline separation of many isomers	ESI-MS, proton, sodium, and ammonium adducts	+	[60]
Hypercarb 5 μm ; 4.6 mm \times 100 mm (1000 $\mu\text{L min}^{-1}$)	0.05% TFA; 40% acetonitrile in 0.05% TFA	Acidic	Endo H-released <i>N</i> -glycans after reduction;	Separation of two Man6 structural isomers	UV absorbance (206 nm); MALDI-TOF -MS; NMR	+	[61]
Hypercarb 5 μm ; 2 mm \times 50 mm; 0.32 mm \times 100 mm	0.05% formic acid; acetonitrile– isopropanol (1:1, v/v) with 0.05% formic acid	Acidic	<i>N</i> -glycans after permethylation;	Partial isomer separation of oligomannosidic <i>N</i> -glycans of ribonuclease B; separation of isomers with core vs. antenna fucosylation	ESI-MS, sodium adducts	+	[62]

Table 2 (continued)

Column and flow	Solvents	pH	Samples	Separation	Detection	Ionization mode	Ref.
Hypercarb 5 μm ; .32 mm \times 100 mm	0.1% formic acid; 0.1% formic acid in acetonitrile; 28 $^{\circ}\text{C}$	Acidic	Chondroitinase ABC digest of glycosaminoglycans;	Separation of four structural isomers of composition HexU1HexNAc1sulfate1	ESI-MS	–	[8]
Hypercarb 5 μm ; 4.6 mm \times 100 mm (600 $\mu\text{L min}^{-1}$)	Ternary gradient of water, acetonitrile, and 15% formic acid	Acidic	Soluble sugars and sugar phosphates from plant tissue;	Separation of trehalose, sucrose, and maltose	ESI-MS	–	[6]

Hex, hexose; HexNAc, *N*-acetylhexosamine; dHex, deoxyhexose; Man, mannose; NANA, *N*-acetylneuraminic acid

carbon HPLC compared with capillary scale graphitized carbon HPLC [35]. Only very recently, a carbon-clad zirconia column (ProteCol; nano-scale) was reported by Karlsson and Thomsson [36] as a stationary phase with properties similar to Hypercarb.

Influence of solvents

The most frequently used gradients are binary. Usually, component A is water containing a low concentration of volatile acid (formic acid, acetic acid, or trifluoroacetic acid), volatile base (ammonia), or volatile buffer (ammonium formate, ammonium acetate, or ammonium bicarbonate) (Table 2). Component A may also contain up to a few percent of acetonitrile. Component B is either acetonitrile or a water–acetonitrile mixture that may contain some volatile acid or buffer. Some ionic strength in the mobile phase components is necessary for separation of charged compounds including sialylated or sulfated oligosaccharides or oligosaccharides with a charged aglycone. The importance of specific additives to mobile phases has been shown by Packer et al. [28], who described the sequential elution of neutral and acidic oligosaccharides from graphitized carbon SPE using 25% acetonitrile and 25% acetonitrile containing 0.05% trifluoroacetic acid, respectively. The influence of ionic strength, pH, and temperature on graphitized carbon HPLC separations has only recently been studied in detail. Pabst and Altmann [27] showed that trisialylated and tetrasialylated *N*-glycans were eluted with good peak shape by use of 65 mmol L⁻¹ ammonium formate (pH 3.0) and acetonitrile as mobile phase components in graphitized carbon HPLC. When ionic strength is reduced while pH is kept constant, peaks of these sialylated *N*-glycans become broader and elute later. Notably, when 0.1% formic acid and acetonitrile were used as mobile phase components, the trisialylated and tetrasialylated *N*-glycans were not eluted [27]. Regarding the influence of pH on retention, the authors observed an

increase in retention for sialylated glycans at lower pH whereas neutral glycans were found to be hardly affected by pH changes. Notably, higher temperatures lead to an increase in oligosaccharide retention in graphitized carbon HPLC [27].

Detection methods

Upon separation of oligosaccharides using graphitized carbon stationary phases, both UV absorbance and mass spectrometric detection have been performed. While mass spectrometry may enable direct identification of compounds, identification using UV absorbance detection can only be performed indirectly. Mass spectrometric detection is mostly performed by electrospray ionization, though some reports describe off-line detection by MALDI-TOF-MS and MALDI-FT-ICR-MS (Table 1). Both positive-ion mode and negative-ion mode ESI are often used, and in many cases data-dependent tandem mass spectrometry using collision-induced dissociation (CID) is performed. Seven of the publications presented in Table 2 use acidic mobile phases, four use neutral mobile phases (mostly unbuffered), and 26 publications present graphitized carbon LC-MS results with (slightly) basic mobile phases (pH 8 or higher). Interestingly, there seems to be some correlation between mobile-phase pH and detection mode—five of the seven publications with acidic mobile phases use positive-ion mode mass spectrometric detection, and only two publications present mass spectrometry in negative-ion mode. For the 26 graphitized carbon LC-MS studies performed at high pH, the situation is reversed—most use negative-ion mode mass spectrometry (19 publications) and nine apply positive-mode ionization. Thus, there seems to be some correlation between basic mobile phases and negative-ion mode. Notably, the ionization mode has an influence on the relative ionization efficacies of neutral versus acidic *N*-glycan alditols [47]: whereas oligomannosidic *N*-glycans were major components in positive-ion

mode HPLC–ESI–MS, in negative-ion mode HPLC–ESI–MS they were ionized much less efficiently than multiply sialylated complex-type *N*-glycans (Fig. 1). This is in agreement with recent findings of Pabst and Altmann, who observed strong promotion of negative-ion mode ionization of both neutral and acidic glycans with increasing acetonitrile concentration; this may, in part, explain the intense signals obtained in negative-ion mode for multiply sialylated, late-eluting *N*-glycans [27]. The influence of charges (in particular sialylation) of oligosaccharide alditols on their ionization efficacy has also been demonstrated in a recent multi-institutional comparison of glycosylation profiling methods [41].

Isomer separation and its aid in structure elucidation

As mentioned in the Introduction, one of the most desirable features of an LC–MS method for detailed oligosaccharide analysis is the separation of isomers. Moreover, the remarkable isomer-separation power of graphitized carbon has repeatedly been demonstrated in more recent studies (Table 1). Robinson et al. have reported the separation of multiple isomers of hexose polymers of compositions Hex3 to Hex16, without providing structural details for the various isomers [5]. Likewise, Ninonueva et al. have described separation of multiple isomers of milk oligosaccharides of various compositions [60]. For m/z 1246.5, for example, they detected seven isomers, for which no structures were given [60].

Several other studies by Karlsson, Schulz, Packer, and coworkers have likewise described the occurrence of isomers in various *O*-glycan preparations [7, 29–32, 35, 36, 40, 42, 43, 45] (Table 2). In a study from 2004, for example, Karlsson et al. described the separation and tandem mass spectrometric characterization of complex *O*-glycan mixtures of human MUC5B, comprising four well-separated structural isomers of reduced oligosaccharides of composition Hex1HexNAc3dHex1 (Fig. 2) [43]. The isomers (a) and (b) with blood group H type 2 epitopes were characterized by intense Z-ions representing loss of the 3-substituent of the innermost GalNAcitol. Together with the ⁴A cleavage of the innermost GalNAcitol, this enables assignment of the deoxyhexose (fucose) to the 3-branch (a) and 6-branch (b) of the *O*-glycans. Tandem mass spectra also enabled distinction between a blood group H type 2 epitope (a) and a blood group H type 1 epitope (c) on the 3-branch of the *O*-glycan on the basis of a ^{0,2}A cross-ring cleavage of the 4-substituted HexNAc (a), whilst the 3-substituted HexNAc (c) does not exhibit this cross-ring cleavage. Moreover, a structure with additional branching was observed, resulting in a Lewis X/A unit (d). This species was characterized by the lack of the ion at m/z 772,

which indicates that the fucose is not attached to galactose but to the HexNAc, resulting in a branched structure. In conclusion, this example shows the usefulness of the combination of high-resolution graphitized carbon HPLC with (negative-ion mode) tandem mass spectrometry [43].

The complexity of *O*-glycan analyses by graphitized carbon LC–MS–MS can be further illustrated on the basis of the study by Schulz et al. [42]. More than 50 sputum mucin oligosaccharide structures were determined by mass spectrometry. Remarkably, many of these structures were part of groups of structural isomers present in the same sputum samples. While no separation data were shown in this study, the isomer separation power of graphitized carbon HPLC obviously contributed to the detailed characterization of the structures of these complex biological samples. Notably, the level of complexity caused by the differences in sialic acid numbers and attachment sites was only addressed in part, as the authors followed a strategy of analyzing two *O*-glycan pools for each sample, i.e. a neutral pool and a desialylated pool [42]. Moreover, in order to further reduce the complexity of the data sets obtained, the authors decided to include only those ions in the analysis which were above a 10% relative abundance cut-off of the most abundant ion. Structure elucidation was performed on the basis of negative-ion mode tandem mass spectrometric data using the GlycosidIQ (Proteome systems) software, and all assigned structures were manually confirmed [42].

A similar complexity of biological samples with separation of multiple isomers is often observed in *N*-glycan analysis by graphitized carbon LC–MS (Table 1), as shown in the following examples. Kawasaki et al. succeeded in differentiating three Man7 isomers from RNase B by Endo H-release of oligosaccharides, reduction, and graphitized carbon LC–MS with ESI–MS–MS of proton adducts [51]. They used commercial standard oligosaccharides for comparison of both elution positions and tandem mass spectra. When analyzing complex-type *N*-glycans Wilson et al. [40] were able to differentiate between antenna fucosylation and core fucosylation. Kawasaki et al. observed approximately 100 different glycan species from recombinantly expressed erythropoietin with various degrees of sialylation, acetylation, and sulfation. These glycan species subdivided into various clusters of structural isomers [39]. The authors described ten separated structural isomers of composition Hex7HexNAc6NANA2, which were not structurally elucidated. In various other publications [46–49, 52] the same group separated many isomeric complex-type and hybrid-type *N*-glycans, which, however, were not structurally elucidated in detail. Taken together, these studies demonstrate the enormous isomer separation power of graphitized carbon HPLC for *O*-glycans and *N*-glycans, which seems to exceed that of other oligosaccharide separation systems. However, most of the early studies did not succeed in using

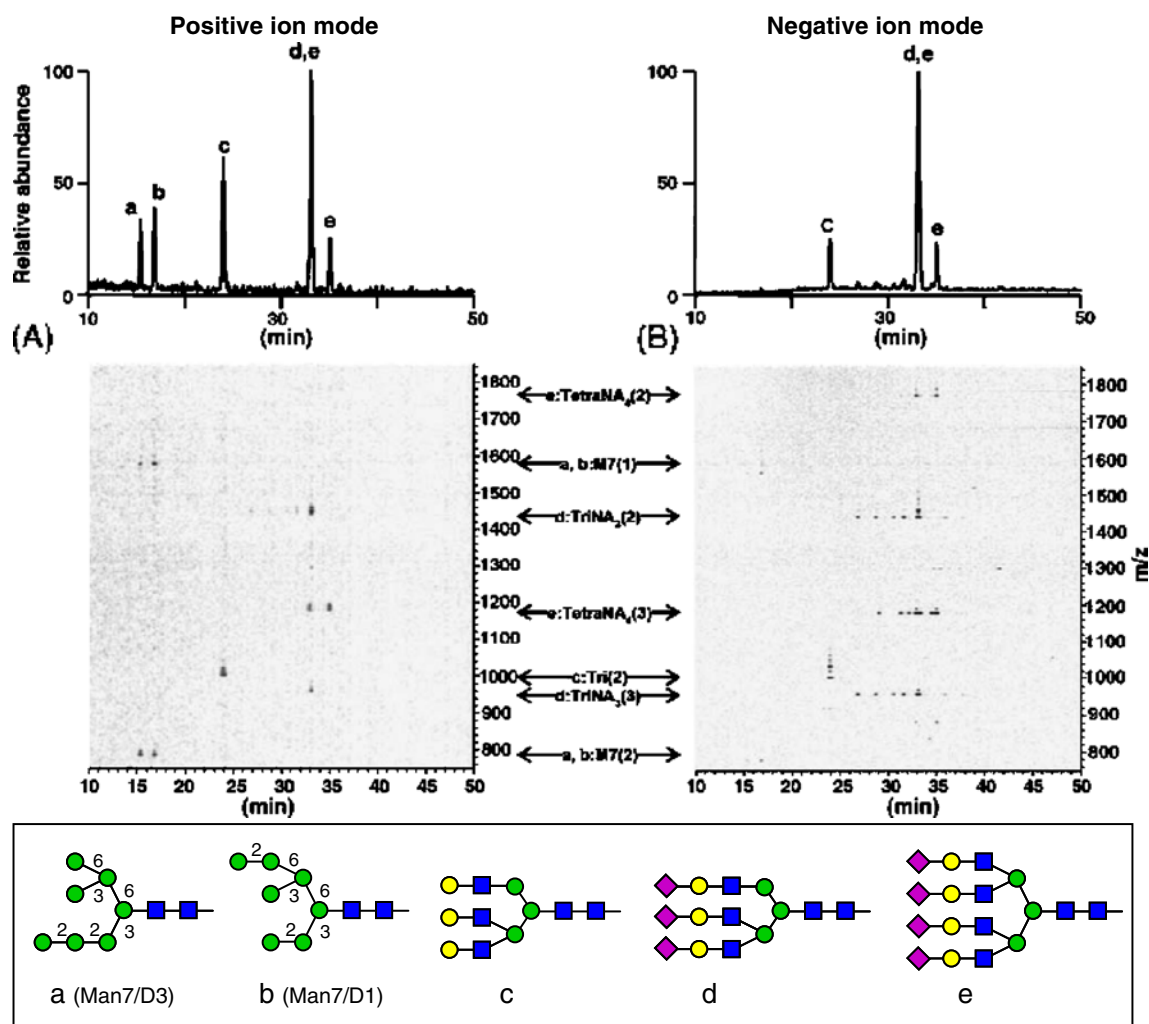


Fig. 1 Analysis of an oligosaccharide mixture by alternating positive/negative-ion mode ESI-FT-ICR-MS. Total ion chromatograms (*top*) and two-dimensional displays (*bottom*) are shown for positive-ion mode (a) and negative-ion mode (b). Numbers in parentheses after the abbreviation of the model oligosaccharides refer to the charge state.

The structural schemes of the analyzed oligosaccharides (a to e) use the following key: *green circle*, mannose; *blue square*, *N*-acetylglucosamine; *yellow circle*, galactose; *purple diamond*, sialic acid. Reproduced from Ref. [47], with permission

these complex LC–MS datasets for differential structural analysis at the isomer level [39, 46–49, 52]. Thomsson et al. have compared the isomer separation power of an amine-bonded HILIC column and a graphitized carbon column using sulfated mucin oligosaccharide alditols [37]. Isomeric compounds coeluting on one column could be separated on the other, as indicated by the different numbers of isomers per molecular composition separated in each system. Thus, although graphitized carbon and HILIC exhibited similar isomer separation power, the combined use of the two methods appears advantageous [37], as these are orthogonal separation techniques.

Interpretation of the tandem mass spectrometric data obtained for oligosaccharide species that may—even after separation by graphitized carbon LC—still represent mixtures of isomers is currently supported by software tools

such as Glycopeakfinder and Glycoworkbench (<http://www.EuroCarbDB.org>) [63, 64]. In another attempt to facilitate assignment of the structures of various isomers of complex-type and hybrid-type *N*-glycan alditols separated by graphitized carbon HPLC, Altmann group has recently started an initiative to determine standardized retention times of structural isomers in porous graphitized carbon separations. To this end, they prepared oligosaccharide standards by purification from natural sources and by synthesis using recombinantly expressed glycosyl transferases. Retention times were standardized using internal oligosaccharide standards. *N*-glycans from biological samples were structurally assigned on the basis of specific combinations of retention time and mass, with the option of using MS–MS data as additional identifier [58]. Some results for neutral, biantennary *N*-glycans are shown in

Fig. 3 [58]. This method was used for characterization of the structure of butyrylcholine esterase [57] and various IgGs [59]. While setting up the database for this approach is very tedious, this method provides a unique chance to obtain structural details for complex glycan mixtures in a single LC–MS run. However, the general availability of this method is restricted, because the oligosaccharide standards and the database are not publicly available.

Separation of reducing glycans

While most graphitized carbon HPLC separations have been performed on reduced oligosaccharides or fluorescently labeled glycans, some analyses have been performed using reducing end glycans. These glycans may occur in two different anomeric configurations, which can be separated by graphitized carbon HPLC. In most situations such a separation of anomers is not desirable. Fan et al. [25] have shown that the anomers can be separated at neutral pH (Fig. 4a). Separation of the anomers was—at least partially—suppressed by choosing a high pH for the mobile phases which stimulates mutarotation (Fig. 4). Chromatographic resolution was much less at alkaline pH than under neutral conditions, which may indicate that mutarotation was still too slow to suppress anomer separation completely, resulting in peak broadening. It is advisable to avoid any complications from anomer separation by subjecting oligosaccharide samples to reduction or reductive amination prior to graphitized carbon HPLC.

Separation of permethylated glycans

Recently, a graphitized carbon method for separation of permethylated glycans was presented by Costello and coworkers [62]. Partial isomer separation for oligomannosidic *N*-glycans was achieved using this method, while most graphitized carbon separation procedures for native glycans feature complete isomer resolution. This is the first LC–MS method which has been shown to separate isomers of permethylated oligosaccharide alditols. The method allows the online acquisition of tandem mass spectra of sodium adducts of permethylated glycans. The resulting spectra enable discrimination between terminal and internal structural elements and the deduction of branching points. Moreover, linkage information is obtained, because of the occurrence of specific patterns of cross-ring cleavage. Three isomeric *O*-glycan structures of composition Hex3-HexNAc1 of *Caenorhabditis elegans* were successfully characterized using this separation procedure. Notably, multistage tandem mass spectrometry (MSⁿ) of sodium adducts of permethylated glycans allows an even more

detailed structure characterization. The resulting branched fragmentation paths may comprise dozens of fragmentation spectra, and the integration of this information enables discrimination between structural isomers [65, 66]. Analysis of complex oligosaccharide mixtures in this manner requires in-depth knowledge for interpretation of the spectra, but software tools to support the interpretation of the spectra for glycan structure elucidation are currently under development [67–69]. Naturally, such MSⁿ characterization is rather time-consuming and would require the collection and off-line analysis of the graphitized carbon-separated, permethylated glycans.

Graphitized carbon SPE in sample preparation

Graphitized carbon is widely used for glycan purification and desalting, following the procedure described by Packer et al. [28]. Oligosaccharide samples in aqueous solutions are applied to a graphitized carbon solid phase-extraction cartridge. The cartridge is washed with water, and neutral glycans are eluted with 25% acetonitrile, followed by elution of acidic glycans with 25% acetonitrile containing 0.05% TFA. Graphitized carbon SPE is widely used for sample preparation for MALDI-MS [70–73].

As a variant of this procedure, several publications from the group of Packer and Karlsson describe graphitized carbon microcolumn desalting [7, 30, 31]. Five microlitres of graphitized carbon is suspended in 50% methanol and added to a C-18 ZipTip (Millipore). The column is washed with 90% acetonitrile, 0.5% TFA, the sample is applied to the “column”, desalted with 3 × 25 μL 0.5% TFA, and eluted with 3 × 25 μL 40% acetonitrile. The eluate is dried and reconstituted with water for LC–MS analysis.

Conclusion

Graphitized carbon HPLC efficiently separates oligosaccharides — in both reduced and reductively aminated forms — and shows excellent compatibility with mass spectrometric detection. It shares these features with HILIC HPLC [2]. Moreover, like HILIC, graphitized carbon HPLC shows excellent performance in the separation of isomeric structures, as shown by Thomsson et al. [37], who emphasized the complementary separation principles of the two stationary phases. While both HILIC and graphitized carbon HPLC have undergone miniaturization [2, 35], only limited improvements in the carbon stationary phases have been obtained. HILIC, however has seen the advent of zwitterionic stationary phases [2], 3-μm amide-functionalized silica particles [74], and monolithic materials [75] with vastly improved separation power. A similar development

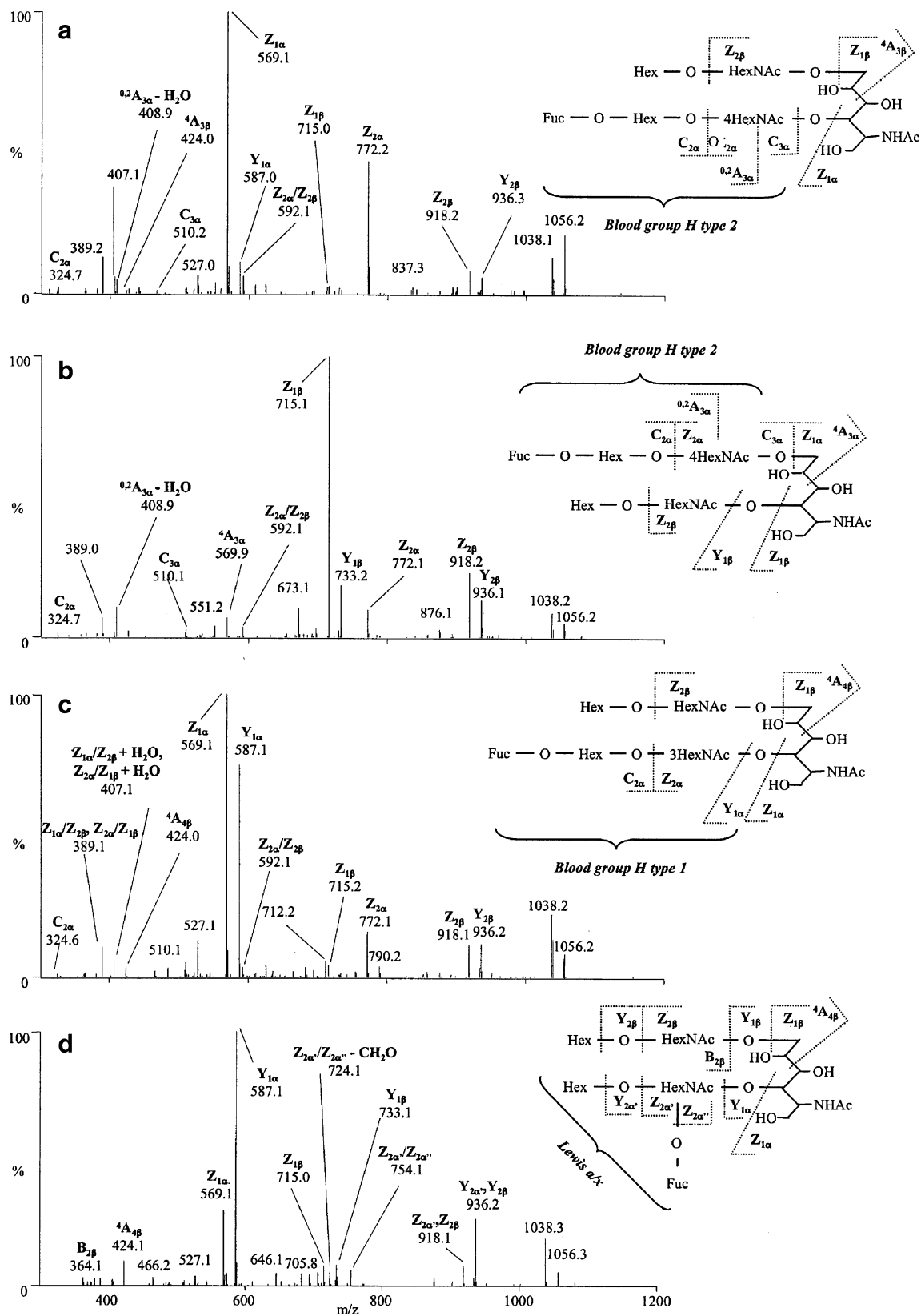


Fig. 2 LC–MS² spectra and assigned structures of four isomeric Core 4 *O*-linked oligosaccharide alditols with [M – H][–] ion of *m/z* 1098 corresponding to composition Hex₂HexNAc₃Hex₁ prepared from

human MUC5B; (a) retention time 19.1 min, (b) retention time 18.1 min, (c) retention time 16.2 min, and (d) retention time 14.9 min. Reproduced from Ref. [43], with permission

Fig. 3 Graphitized carbon HPLC–ESI–MS of neutral *N*-glycans. In panels **a–c**, extracted ion chromatograms are shown for *m/z* 822.3. Panel **a** shows the separation of the four isomers of diantennary *N*-glycans together with two hybrid-type structures of the same mass (*m/z* 822.8). Panel **b** shows the result for desialylated fibrin *N*-glycan. Panel **c** is an HPLC–ESI–MS result for α -Gal-containing glycans with a total of five hexose residues. In panel **d**, a combined extracted-ion chromatogram is shown for Man5Gn (*m/z* 720.8) and for glycans containing one or two α -Gal residues linked to A4A4 (*m/z* 903.3 and 984.4, respectively). Reproduced from Ref. [58], with permission

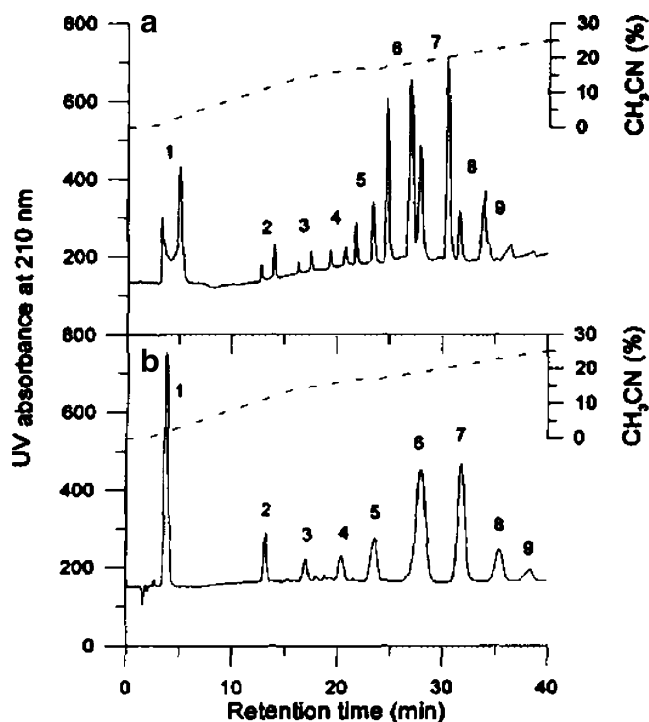
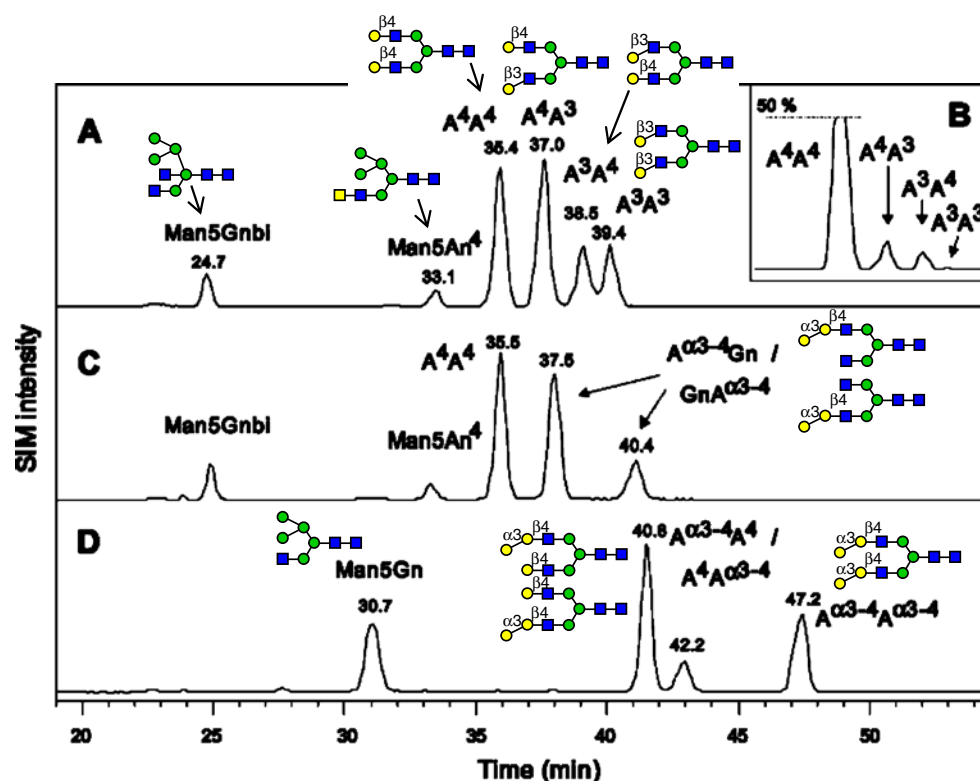


Fig. 4 Separation of chito-oligosaccharides by graphitized carbon HPLC. The numbers above the peaks give the degree of polymerization. The column was kept at 50 °C. (a) Elution with water and acetonitrile; (b) elution with 10 mmol L⁻¹ ammonia and acetonitrile containing 10 mmol L⁻¹ ammonia. Reproduced from Ref. [25], with permission

of new graphitized carbon stationary phases is desirable and will hopefully lead to an even better analytical performance with regard to (isomer) separation and speed.

Interestingly, graphitized carbon HPLC is hardly used with fluorescence detection of reductively aminated oligosaccharides, in contrast with HILIC, reverse phase-HPLC, and capillary electrophoresis, which are often used for separation and fluorescence detection of reductively aminated glycans. This reflects the fact that graphitized carbon HPLC is hitherto only used by a limited number of research groups in the field of mass spectrometry, whereas graphitized carbon SPE is widely and successfully applied within the field of glycan analysis. Because of its excellent analytical performance, however, we expect that glycan analysis by graphitized carbon HPLC with fluorescence and/or mass spectrometric detection will soon find broader acceptance both in the biotechnological industry and in academia.

Next to de-novo structure analysis, the matching of retention times and (tandem) mass spectrometric data are expected to be important future steps in the development of graphitized carbon HPLC–MS, and first steps in this direction have been taken in the field of *N*-glycan analysis [58]. A successful database approach for analysis of the structures of oligosaccharides is the GlycoBase (<http://glycobase.nibr.ie/>) established by Rudd for identification of aminobenzamide (AB)-labeled glycans separated by

HILIC [76], using primarily fluorescence detection in combination with exoglycosidase treatment. Similarly, the availability of standards and online tools for matching of retention times, masses, and possibly tandem mass spectrometric data will be important steps in the development of broadly applicable graphitized carbon LC–MS methods for analysis of oligosaccharides from various biological sources.

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