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Recent Liquid Chromatographic Approaches and Developments for the Separation and Purification of Carbohydrates

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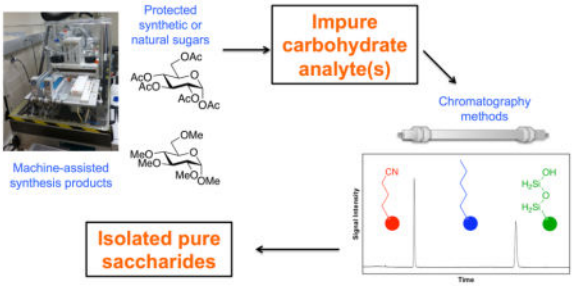
Abstract

Carbohydate purification remains a bottleneck in securing analytical standards from natural sources or by chemical or enzymatic synthesis. This review highlights the scope and remaining limitations of recent approaches and methods development in liquid chromatography for robust and higher-throughput carbohydrate separation and isolation.

Graphical Abstract

This review highlights current techniques for carbohydrate purification and identifies research graph



Introduction

Carbohydrates remain one of the most challenging biomolecules to analyze; the very structural heterogeneity of glycans that gives them such interesting biological and materials properties also renders them difficult to attain in pure form. Unlike peptides, carbohydrates vary more based on differences in the spatial orientations of atoms than on differences in functional groups (Figure 1). These subtle (and numerous) variations complicate the development of general, high-throughput analytical methods for carbohydrate analysis and isolation and thereby also make authentic carbohydrate analytical standards hard to procure through synthetic chemistry or from natural sources. This challenge was explicitly highlighted in a 2012 report by the United States National Academy of Sciences (NAS)[1], which called to “develop the technology to purify, identify, and determine the structures of all the important glycoproteins, glycolipids and polysaccharides in any biological sample [and] develop agreed upon criteria for what constitutes the acceptable level of structural

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detail and purity." For many immunology or therapeutic experiments, this "acceptable level of purity" requires reaching ≥99.5% purity[2–4]. For other applications, this extremely high level of purity may not always be necessary, with lower levels being adequate[2–4]. Given this clear cry for high quality purification and separation techniques for carbohydrates, this brief review aims to spark the interest of creative minds both within and outside of the carbohydrate chemistry community to collectively help solve the still evident shortcomings in the field.

To provide a context for consideration of the remaining struggles in glycan purification, this review will focus first on current methods to procure analytical standards by liquid chromatographic approaches for the separation and purification of oligosaccharides—glycans with ≤10 monosaccharide constituents—in the protected, partially protected, and deprotected/native stages. Protecting groups have historically been very valuable in blocking the many reactive hydroxyl and other functionalities of carbohydrates to alter their physical properties and control their reactivity; therefore, these modified forms are also very important targets for purification. Size-exclusion (gel filtration/gel permeation) chromatography[5,6] and glycoprotein analysis[7–12] lie outside the scope of this review. The wide variety of detectors that can be coupled to liquid chromatography will only be touched on briefly; instead, insight on the amenability of these LC-based techniques to facile coupling with other analytical and synthetic techniques will be discussed.

Thus, we will begin with an overview of the various state-of-the-art chromatographic modes presently available for carbohydrate purification with an emphasis on how chromatographic parameters—as well as the wide breadth of available stationary phases—play a tremendous role in the achievement of desired separations. Special attention will be given to various derivatization approaches as well as protecting group chemistry and how they are related to chromatographic separations of carbohydrates. Next, the emerging field of multidimensional chromatography will be discussed. Whereas chromatographic separations can exist as standalone techniques, their amenability to the continued development of technology that permits automated or machine-assisted oligosaccharide synthesis[13–22] will also be underscored. Other applications of significant analytical novelty published in the last three years will also be discussed throughout. Although the emphasis is on current methods in oligosaccharide purification, we will also attempt to highlight gaps in capabilities to inspire the development of the new tools needed to fully realize the NAS report goals.

Analytical toolbox of chromatographic modes

Liquid chromatography relies on mixtures dissolved in a fluid (the mobile phase) passing through a solid stationary phase with differential affinities for the mixture components. With the growing diversity of available stationary phases for liquid chromatography, the focus here will be on those that are great starting points for methods development for carbohydrate purification. Several of the common reversed- and normal-phase supports that are commercially available have applications to carbohydrates in both their protected and unprotected forms (Figure 2). Throughout this section, additional details will be provided about specific applications that have utilized these chromatographic supports and about

information on the less-commonly used stationary phases in carbohydrate purification and separation.

Reversed-phase chromatography

In this separation mode, a non-polar, hydrophobic, stationary phase with a polar mobile phase is used, where analytes are eluted based on increased hydrophobicity. Reversed-phase columns remain one of the most popular types of stationary phases in the analysis of various types of biomolecules, especially with such a diverse selection of column chemistries whose chromatographic conditions can be tailored to the separation of a given analyte mixture. Given the incredible complexity of carbohydrates in most natural sources, chemical and enzymatic synthesis is an increasingly viable method to obtain specific carbohydrate structures. Monosaccharide building blocks undergo glycosylation to form larger saccharide structures either through the use of specific enzymes or through a series of chemical steps that require protection and deprotection of certain functional groups in order to control the regioselectivity and stereoselectivity of the outcome. Commonly used protecting groups, such as acetyl, benzyl, and benzoyl groups, are hydrophobic, thus increasing the hydrophobicity of the entire saccharide structure. For these reasons, reversed-phase chromatography is a logical choice for the purification of protected carbohydrate compounds. Stationary/mobile phase choices as well as selected applications are described below.

For carbohydrate analytes, several possibilities exist in terms of suitable reversed-phase column chemistries to achieve high-level purification and desired separation of these synthesized compounds at the protected stage. Arguably the most common of the available hydrophobic stationary phases are alkyl-linked supports that can range from methyl (C1) to octadecyl (C18). For purification of protected or partially protected oligosaccharides, C5 and C18 alkyl-linked supports are most commonly employed with typically a water/acetonitrile mobile phase[23–28].

One of the major drawbacks of the commonly used alkyl-linked reversed-phase packings is their poor retention of highly polar and ionizable analytes[29]. In an effort to overcome these drawbacks, porous graphitized carbon (PGC) packings have been tested. These PGC supports elute analytes based on their polarity (more polar compounds will be more strongly retained), rather than based on hydrophobicity, which is the primary driving force in the elution profile of alkyl-linked supports[29]. A more comprehensive discussion on PGC supports is available[30], as is a more exhaustive discussion on comparisons between PGC supports versus reversed-phase and HILIC ones for glycan analysis[31]. Since PGC columns remain attractive for polar analytes, these packings have seen use in the separation of glycans isolated from mammalian sources [32,33] and have applications in the separation of other carbohydrates such as underivatized oligosaccharide anomers in vegetable matrices[34], cello-oligosaccharides[35,36] and fructan mixtures[37]. While these PGC packings remain somewhat unexplored for carbohydrate purification, a few drawbacks associated with these supports have come to light, including the loss of retention/variability in retention times[31,38] and poor stability of the packing material under acidic mobile phase conditions[39]. Clearly the appeal of such PGC packings to potentially be used in the separation of polar analytes

and/or difficult to resolve isomers remains attractive to carbohydrate chemists; however, additional work is still necessary to bring these types of stationary phases to the same level of reliability and robustness as their alkyl-linked support counterparts.

Whereas alkyl-linked supports remain the most commonly used ones for the purification of protected/partially protected carbohydrates, the less commonly employed phenyl-alkyl columns provide a viable alternative. As compared to alkyl-linked supports that separate analytes primarily based on hydrophobicity, phenyl-alkyl supports can also separate analytes based on π-π interactions[40–42]. Despite the prevalence in synthetic carbohydrate chemistry of benzyl and acyl protecting groups containing π-bonds, phenyl supports have seen surprisingly little exploration for the purification of protected carbohydrates; however, these phenyl columns can provide increased separation as compared to their alkyl-linked counterparts[43]. The use of methanol rather than the more common acetonitrile as the organic modifier proves particularly valuable when these phenyl-modified columns are operated in reversed-phase mode. Acetonitrile, with its two π-bonds, likely suppresses the necessary π-π interactions between π-bond containing analytes and the stationary phase, whereas methanol, lacking π-bonds, will better allow π-π interactions between the analyte and the stationary phase [40–42]. A methanol/water mixture therefore is a good starting point for the mobile phase in the development of methods using phenyl columns in reversed-phase mode for the purification of protected or partially protected oligosaccharides containing π-bonds.

An even less-used column chemistry for reversed-phase chromatography relies on fluorine substitution, especially in the form of pentafluorophenyl (PFP) supports. As compared to the phenyl ring with their electron-rich ring faces, these perfluoro-substituted phenyl supports have relatively electron-poor ring faces and electron-rich edges and thereby create an alternative affinity surface for analyte interactions[40–42]. Interestingly, these fluoro-substituted supports can also isolate analytes based on hydrogen bonding, thus they show some utility in the separation of polar analytes [40–42]. As with phenyl supports, these perfluoro-phenyl rings also separate analytes based on π-π interactions; therefore, methanol again proves to be a superior organic modifier as compared to acetonitrile, especially for the purification of protected/partially protected carbohydrates. Recently, these phenyl/perfluoro-phenyl supports proved far superior to alkyl-linked supports in the purification of various globally protected carbohydrates[43]. The development of additional modified-phenyl and other hydrophobic supports could further tune separations capabilities compared to existing column packings. Additionally, important attention must be paid in regard to solubility and the choice of sample diluent in reversed-phase chromatography of protected carbohydrates. Since most mobile phases consist of mixtures of organic modifiers—such as methanol or acetonitrile—with water, the design of synthetic routes should perhaps account for the potential solubility of protected intermediates or products in these mobile phases to avoid the risk of on-column precipitation. Whereas fluorous tags at the anomeric position have been previously used in carbohydrate synthesis[20–2], the development of other anomeric tags or an overall increase in the number of free hydroxyl groups in the molecule so as to increase its hydrophilicity could allow for larger amounts of water to be used in the mobile phase to increase retention under reversed-phase conditions. Such steps would be especially important to decrease the cost and time for purification of protected carbohydrates in preparative-scale quantities via reversed-phase chromatography.

For synthetic, protected, carbohydrates, high-level purification of carbohydrates reaching levels ≥99.5% purity is certainly attainable with reversed-phase packings, especially when special attention is paid to the role of various stationary and mobile phases on separation mechanisms.

Normal-phase and hydrophilic interaction liquid chromatography (HILIC)

For native carbohydrate analytes—ones that are either fully deprotected as the final synthetic step or obtained as unprotected from natural sources, and thus extremely polar—normal-phase chromatography and/or HILIC are suitable stationary phase choices for liquid chromatography. As discussed above, PGC packings have also shown promise in the separation of polar analytes, but this discussion was included in the reversed-phase section since these PGC supports are more commonly compared side-by-side to traditional alkyl-linked supports. Fortunately, a direct comparison between HILIC, PGC, and reversed-phase supports has been carried out with glycans associated with monoclonal antibodies (mAb)[31] (Figure 3). It was observed that HILIC supports offered the greatest selectivity and thus provide an excellent chromatographic method for *N*-glycan separation. Reversed-phase packings offered less selectivity for glycan analysis, especially since only the fluorescent aromatic label on the anomeric position of the glycans would interact well with the stationary phase[31]. However, these authors hypothesize that reversed-phase supports could be used for quality control in glycan analysis, such as for the determination of various specific glycosylations such as fucosylation or sialylation[31]. PGC supports offer increased retention of glycans as compared to traditional reversed-phase ones, and thus provide a potential alternative to HILIC packings in the separation of multiantennary sialic acid linkage isomers[31]. However, these PGC supports still suffer from poor reproducibility and robustness, which greatly limits their applicability in glycan separation[31].

In normal-phase chromatography, a polar, hydrophilic, stationary phase with a less-polar, non-aqueous, mobile phase is used. Silica supports remain the most widely employed in normal-phase chromatography, especially for protected carbohydrates; however, completely deprotected or natively unprotected saccharides exhibit poor solubility in the commonly used mobile phase solvents (typically 100% organic solvents such as ethyl acetate and hexane), thereby rendering silica supports less than ideal.

One way to overcome these solubility issues in traditional normal-phase chromatography is to turn to hydrophilic interaction liquid chromatography, or HILIC, coined by Dr. Andrew Alpert in 1990[44] as was discussed in the above mAb glycan case. In HILIC, an aprotic solvent such as acetonitrile embracing small amounts of water is used as the mobile phase and compounds are eluted based on increasing hydrophilicity[45–47]. Although the retention mechanism of HILIC is still not fully understood, electrostatic interactions and adsorption, as well as liquid partitioning, are likely involved[45–47]. Clearly, retention of an analyte is related to the specific mobile phase, the functional groups present on the analyte, and the stationary phase column chemistry[45–47]. Moreover, HILIC-based stationary phases offer high selectivity and increased compatibility with mass spectrometry (MS) as compared to other normal-phase supports[46].

When ionic rather than neutral carbohydrates are the analytes of interest, precise control of buffer salt concentration and pH of the mobile phase is required to increase the analyte-stationary phase interactions[46]. Additional information on the role of mobile phase composition and stationary phase on the retention and selectivity of analytes in HILIC has been previously discussed[45–48.] In terms of the effect of sample diluent on peak shape and retention in HILIC, it has been demonstrated that the lowest amount of water used in the sample diluent provides the best peak shapes[48]. Matrix effects in HILIC versus reversed-phase chromatography have been explored, but, as expected, they are largely dependent on the analyte of interest and perhaps, more importantly, on the process used to obtain the analyte from natural, or other, sources[49]. Interestingly, it has been observed that sugar aldoses have increased detection at low column temperatures under HILIC[50]. Whereas this temperature effect is a column-dependent phenomenon, this interesting observation illustrates the importance of verifying the thermal stability of carbohydrates under any type of retention mode.

With the growing number of applications in the analysis of carbohydrates from non-mammalian sources comes the need for high-throughput and robust separations techniques, especially ones that do not rely on extensive derivatization. One such study demonstrated the simultaneous separation of iminosugars (a sugar analog where nitrogen replaces the ring oxygen) and other low molecular weight carbohydrates in *Aglaonema* extracts[51] through a HILIC-MS/MS assay. Moreover, this work laid the groundwork for starting point experimental conditions, such as column temperature, mobile phase strength, and mobile phase additives, to provide reproducible data in terms of peak width, symmetry, and resolution[51]. This work presents a useful starting point for HILIC method development in the separation of low molecular weight carbohydrates, and great interest remains in how non-nitrogen-containing carbohydrates behave under these same conditions.

Not only have HILIC column supports shown promise in the separation and purification of carbohydrates, but these supports themselves have been modified with carbohydrates[52]. For example, silica materials can be modified with maltose to create a “click-maltose” HILIC support that can separate hydrophilic compounds such as galacto/fructo-oligosaccharides[52]. Through a similar notion that modified HILIC silica supports could result in superior separation of carbohydrates as compared to unmodified HILIC supports, silicon oxynitride (SiON) was evaluated as a potential packing material in glycoside separations[53]. In this study it was observed that the hydrophilicity of this silicon oxynitride stationary phase increased with increased buffer salt concentration in the mobile phase[53]. As compared to traditional bare silica supports, this SiON column exhibited excellent column selectivity, separation efficiency, and retention for mono-, di-, and tri-saccharides[53]. Based on this information, other such modified HILIC supports should certainly be explored and further developed.

HILIC supports have also been used for the analysis of underivatized monosaccharides released from mammalian glycoproteins through amide column packing with an MS detector[54]. It remains to be seen if this approach can be used to separate monosaccharides from non-mammalian sources, especially ones that contain some of the less common aldohexose isomers, such as allose, altrose, gulose, idose, and talose.

One notable complication in the separation of completely deprotected or natively unprotected carbohydrates is that the anomeric (C1) hydroxyl group exists in a mutarotation/anomerization equilibrium between alpha- and beta-anomers[55]. This mutarotation leads to broader chromatographic peaks since one peak will contain these interconverting α/β anomers[55]. Increased temperature and the addition of triethylamine have been shown to limit the peak broadening caused by mutarotation[23]. One recent development to alleviate this α/β anomerization was through the development of a hydrolytically stable bidentate urea-type HILIC column packing support[56]. This bidentate urea-type support offered better peak shape and improved separation efficiency compared to commercially available HILIC columns[52]. Additionally, the free amino groups on the stationary phase resulted in reproducible peaks shapes and no peak splitting from α/β saccharide anomerization[56]. Although this specific bidentate urea-type support is not yet commercially available, these positive results, as they relate to better separation efficiency as compared to existing HILIC supports, should prompt manufacturers to look at commercializing this packing.

Another solution to dealing with the complexity generated by this mutarotation/anomerization equilibrium is to alter the carbohydrates themselves. The reduction of saccharides with sodium borohydride to their sugar alcohol counterparts can eliminate α/β anomers by producing straight chain polyols. However, this reduction greatly limits the amount of information that can be obtained from the original sample and, of course, no longer makes isolation of the original carbohydrate possible. For example, the reduction of the 24 possible hexoses results in only 10 different hexitols. Work is still needed to find other experimental methods to provide sharper peaks in the purification of completely deprotected/natively unprotected carbohydrates.

HILIC is not as easily coupled to non-destructive detectors, because native/unprotected carbohydrates do not possess good chromophores; HILIC's overall utility for strict purification purposes is therefore somewhat limited. Nonetheless, the technique can easily be coupled to mass spectrometry to provide high-level quality control applications.

High-performance anion-exchange chromatography (HPAEC)

One of the drawbacks associated with liquid chromatographic detection of completely deprotected/native carbohydrates is the fact that some form of derivatization is usually required to increase sensitivity of detection and provide better separation by increasing the structural differences amongst analytes or altering their overall retention through the installation of hydrophilic and/or hydrophobic functional groups[57]. One alternative in overcoming these shortcomings is high-performance anion-exchange chromatography, usually coupled with pulsed amperometric detection (HPAEC-PAD)[58,59]. HPAEC-PAD separates anions, either in their natural state or ionized at high pH values[58,59]. For example, D-glucose has a pKa of 12.28 and thus when the pH is above this value, D-glucose will exist in equilibrium with its oxyanion[59]. Thus, a stationary phase unlike most LC column packings is needed that is compatible with such high pH values in the hydroxide mobile phases[55] and, for isolation and use of the purified compound, removal of the base is needed before base-mediated reactions start to degrade the carbohydrate.

While HPAEC-PAD has been more widely applied for the separation of *N*-linked glycoproteins[60,61], the technique has also been used for the separation of aldopentoses and aldohexoses on a polystyrene-based copolymer and diamine stationary phase with a sodium hydroxide mobile phase[62], for monosaccharide analysis in complex polysaccharides such as glycosaminoglycans, alginate, fucoidan, and glycans[57], and for the analysis of biomass sugars and galacturonic acid[63]. In such complex polysaccharide samples, repeated chromatographic runs—such as two-dimensional approaches that will be highlighted later on in this review—would be necessary to achieve high-level purity whereas less complicated sample matrices can be much more easily purified.

While PAD is the common detector applied for HPAEC, since carbohydrates do not naturally possess a chromophore, it has been possible to couple HPAEC to a UV detector without derivatization. Under highly alkaline conditions, a photochemical reaction occurs during the detection window that converts the carbohydrate into a malonenolate UV-absorbing species at 266 nm[64]. This form of direct UV detection of small saccharides shows promise as an alternative method of detection for HPAEC[64].

Whereas HPAEC shows great promise in the separation of small, deprotected/native carbohydrates, the method suffers due to the difficulty of coupling it to electrospray ionization- mass spectrometry (ESI-MS) because of the high NaOH concentrations required in the mobile phase[60]. Desalting with a carbohydrate membrane can help overcome this drawback by the neutralization of hydroxide to water by hydronium ions and removal of sodium ions into the cathode via diffusion[60]. Clearly, more such desalting membranes will need to be developed to make HPAEC robust and high-throughput in its amenability to mass spectrometry, especially as compared to HILIC and reversed-phase chromatographic modes.

One recent promising method to overcome these desalting issues—at least with compounds that are already negatively charged at neutral pH—is through the use of a volatile, ammonium carbonate buffer applied in cetyltrimethylammonium strong anion exchange chromatography for heparin oligosaccharide separation[64]. Since ammonium carbonate is volatile, it can be removed by evaporation, thereby alleviating any need for unnecessary desalting steps that could lead to potential sample loss as well as poorer sensitivity when coupled to ESI-MS[65]. Efforts to improve the amenability of HPAEC-PAD to ESI-MS analysis has led to the design of a sheath liquid interface with a volatile ammonium salt buffer added to the column effluent[66]. This interface resulted in greater ionization efficiency of mono/di-saccharides for the analysis of neutral sugars in pectin samples that were separated with HPAEC[66]. In another study, 13 monosaccharides from polysaccharide from *Arthrospira* (*Spirulina) platensis* (both neutral ones and acidic, carboxy, sugars that are already negatively charged at neutral pH) were simultaneously separated through a gradient elution of sodium hydroxide and sodium acetate with HPAEC coupled to both PAD and MS detectors[67]. Although separation of the neutral monosaccharides can be increased when a lower pH is used for the alkaline eluent since both neutral and acidic, carboxylated, sugars were analyzed, it was found that a higher concentration of NaOH was necessary for the desired separation[67].

HPAEC will always have utility for the purification of acidic (or neutral at high pH) carbohydrates, especially when coupled with a non-destructive detector such as a pulsed amperometric one. At present, there have been no studies to determine if HPAEC-PAD has the necessary sensitivity for ≥ 99.5% high-level purity.

Other

Whereas the aforementioned discussion of chromatographic modes has focused primarily on the use of commercially available, or partially modified, packing supports to separate diastereomers or other sugars, some work has also been carried out in chiral separations related to carbohydrates and on the use of saccharides themselves as mobile phase modifiers or stationary-phase supports. Recently, a saccharide-based stationary phase was created by the reaction of dextran with a carbonyl diimidazole (CDI)-activated silica cross-linker[68]. This new dextran support exhibited similar retention behavior as HILIC columns as well as high column stability for the separation of neutral mono-, di-, and tri-saccharides as well as various nucleic acids under mobile phase conditions of 80% acetonitrile and 20% water using an evaporative light scattering detector[68]. In a similar study from the same laboratory, an ionic-bonded cellulose stationary phase was prepared by the immobilization of cationic cellulose on sulfonated silica[69]. This packing support was utilized for the selective enrichment of glycopeptides—a potentially interesting contribution to the challenging field of glycoproteomics and separation of glycopolymers[69]. Support modifications are not limited to the sugars themselves, but can include the proteins called lectins that bind to carbohydrates. In regard to the field of glycoproteomics, for example, glycan affinity chromatography is able to analyze the interactions between lectins and glycans/glycoconjugates[70]. Specifically, a lectin affinity chromatography-mass spectrometry platform allows for the characterization of glycan-lectin binding affinities to “capture” various glycome species.

Whereas these partially modified stationary phases have mainly been used in a proof-of-concept format, they certainly provide an intriguing platform for the development of future chromatographic supports.

One of the challenges associated with the purification/separation of biomolecules and particularly carbohydrates is that they can exist as D/L enantiomers, which are unresolvable on the achiral stationary phases that are commonly used. The 3-point Pirkle rule[71] for chiral recognition clarifies why chiral stationary phases are required to permit such enantioseparations. One example of chiral recognition is through the design of saccharide-based stationary phases through the immobilization of neutral polysaccharides (amylose and cellulose) on silica supports for the separation of chiral pairs of acidic, basic, and bifunctional analytes in reversed-phase mode, such as ibuprofen, β-blockers, diuretics, and amphetamine[72]. Although these chiral stationary phases have yet to be applied to carbohydrate-related separations, they illustrate the use of carbohydrates as functionalized particles on stationary phases for enantioseparations.

Instead of modification of the stationary phase to create a chiral environment, derivatization of the carbohydrate moiety can be done to convert the coeluting enantiomers into potentially separable diastereomers. This concept was recently demonstrated via the derivatization of

aldopentoses and aldohexoses with L-tryptophanamide and the use of butylboronic acid as a complexation agent in reversed-phase chromatography[73]. In a similar study, various deoxyhexose, hexose, and pentose enantiomers were prepared as their arylthiocarbamate derivatives to allow for chiral separation[74]. Of course, for these methods to be useful for purification of the carbohydrates, these derivatization processes need to be easily reversible —an area that is not yet well explored.

Although not as robust as other supports, chiral columns are increasingly becoming available commercially. The use of a Chiralpak AD-H column for the separation of both monosaccharide anomers and enantiomers is an example[51]. Clearly more work still needs to be done in the field of carbohydrate enantioseparations. Additionally, carbohydrates in the form of macrocylic glycopeptides or cyclodextrins have been successfully applied as chiral mobile phase additives in enantioseparations as highlighted in a 2013 book chapter[75].

Whereas the above experiments represent quite interesting advancements in regard to the use of carbohydrates as both mobile phase additives and/or stationary-phase supports, a novel analytical problem—such as the separation of multi-antennary sialic acid *N*-glycan isomers —will likely need to be solved before these projects gain more notice in the liquid chromatography community. In addition, if carbohydrate-based mobile phase additives can be useful in the enantioseparations of mono/oligo-saccharides still needs to be determined. Chiral liquid chromatography also largely remains an unexplored field for the enantiomeric purification/separation of carbohydrates. Future developments in packing supports could allow for much more robust separations. For example, ideas for potential packing supports could arise from mining the literature for sugar-binding host/guest complexes[76–79] or for mass spectrometry-based techniques that have shown potential for individual chiral discrimination of monosaccharides through the formation of gas-phase, chiral, non-covalent complexes[80–82]. These metal-amino acid-based ligand combinations—or host/guest complexes—could potentially be linked to silica or other supports to form chiral stationary phases to permit enantioseparations of a wider range of small saccharide analytes.

Although limited in their present utility, these described chiral separations are a starting point for future developments and applications toward high-level purifications of enantiomers.

Multidimensional and recycling liquid chromatography

Multidimensional chromatography has the potential to overcome some of the limitations of single chromatography runs discussed above; this review will focus on two forms: two dimensional-liquid chromatography (2D-LC) and alternate-pump recycling-high performance liquid chromatography (R-HPLC). Both of these multidimensional chromatographic techniques can be applied to carbohydrates obtained via synthesis and those from natural sources. In the latter case, carbohydrates extracted from natural sources are often part of complex mixtures in the form of glycopolymers, oligosaccharides, and monosaccharides. Such an analyte mixture that is both complex, and large in quantity, in its nature is well suited for 2D-LC applications[83]. In contrast, synthetic routes are usually targeted to form a single carbohydrate compound as the desired final product. Thus, in

carbohydrate synthesis, the analyte mixture will usually be more constrained as compared to that from carbohydrates obtained from natural sources. However, the analyte mixture from a synthetic scheme—especially as the multistep processes involved in machine-assisted or automated oligosaccharide synthesis become more common—can potentially be more difficult to resolve, as a result of the various isomeric mixtures (anomers, diastereomers, regioisomers) that are so closely related in their structure. For analyte mixtures that are small in quantity, but difficult to resolve, R-HPLC is as a rule the more suitable multidimensional chromatographic technique. Rules, of course, can sometimes be broken in specific cases. Depending on the particular application, 2D-LC can also be well suited for the purification of carbohydrates in targeted syntheses and R-HPLC can be utilized for purification of complex mixtures of carbohydrates from natural sources, although these applications are beyond the scope of this review.

Two dimensional-liquid chromatography (2D-LC)

2D-LC is a burgeoning area in liquid chromatography, as evidenced by this more comprehensive recent tutorial[83]. 2D-LC applications are specifically tailored to when a single column LC (1D-LC) experiment is unable to separate a large analyte set, such as the complex mixtures obtained from biological sources[84]. Since each type of stationary phase has a slightly different selectivity, by coupling various such column supports in series, more analytes can be separated than would be possible with only a single column[83]. However, parameter optimization in 2D-LC remains much more challenging than in its 1-D counterpart [85,86]. Since different column chemistries are employed, an optimization of conditions for the first dimension may result in suboptimal conditions in the second dimension[85,86]. Specifically, orthogonality in 2D-LC refers to when the two dimensions, or separations, are both independent and complementary to one another, so as to provide as many possible separation mechanisms as possible[87,88]. Two specific parameters that must receive special attention, as they relate to orthogonality, in 2D-LC method optimization are mobile phase complementarity between both dimensions and sample dilution issues that may result in detection issues[85]. A recent review[85] is a good starting point for optimizing separation conditions in 2D-LC.

Presently, 2D-LC applications for carbohydrates have been limited to the analysis of charged low molecular weight heparins[89] and various neutral mono/oligo-saccharides[90,91]. Briefly, the separation of low molecular weight heparins was made possible by the utilization of size exclusion chromatography in the first dimension, followed by ion-pairing reversed phase in the second dimension, coupled to a time-of-flight mass spectrometry detector[89]. Since heparin is a charged polymer, the phases used in both dimensions provided great orthogonality, and such a 2D-LC approach has potential for quality control purification of such low molecular weight molecules [89] as well as related glycosaminoglycans.

In the case of small neutral sugar separations, various mono- and oligosaccharides obtained from milk powder were profiled via a reversed-phase C4 column in the first dimension and a HILIC NH_2 column in the second dimension, with a refractive index detector[90]. In another study that also analyzed mono/oligo-saccharides from human plasma, a C18 reversed-phase column was applied in the first dimension with a carbohydrate Pb^{2+} ion exchange column in

the second dimension and coupled to mass spectrometry[91]. From these studies it was observed that complicated sample preparation procedures prior to analysis by common LC-MS methods were minimized. Additionally sample throughput and robustness—both techniques were amenable to thousands of samples—are other benefits of these described methods[90,91]. However, if such 2D-LC techniques can hold up to the wide variety of possible saccharide constituents, such as those from non-mammalian biological sources, remains to be seen.

A non-carbohydrate application that is worth mentioning, especially since it uses HILIC and reversed-phase chromatography—both commonly used in carbohydrate purification, was the analysis of phenolic acids and flavonoids[92]. There, a new monolithic (made from a continual portion of silica or other material, as opposed to traditional packed particle columns) sulfobetaine polymethacrylate capillary HILIC column was used in the first dimension with various reversed-phases in the second dimension[92]. This 2D-LC setup (HILIC-RP) with the new monolithic HILIC columns provides better orthogonality as compared to when commercially available HILIC (diol, amide, etc) columns are applied[92]. We envision that such new HILIC columns in the first dimension, especially with phenyl hexyl or PFP columns in the second dimension, can have great use for the purification of partially-protected carbohydrates that are synthesized in a one-pot fashion or of carbohydrates obtained from natural sources that are then derivatized with a hydrophobic, aromatic, group at the anomeric position to increase π-π interactions between analyte and stationary phase in the second dimension. Reversed-phase packings remain the most common choice as the second dimension in 2D-LC systems, especially because of their amenability to mass spectrometric detection, as compared to ion exchange packings[93]. Whereas reversed-phases are not typically used as both dimensions in 2D-LC, a recent review[93] discusses methods on how to increase the peak capacity in RP-RP systems.

With the wide breadth of now available column chemistry supports—such as ion exchange to separate molecules based on charge state, HILIC to separate analytes according to functional groups, and reversed-phase to separate compounds based on hydrophobicity and π-π interactions—the development of fast and robust 2D-LC methods for the purification of carbohydrates, especially those obtained in complex mixtures from natural sources, is increasingly feasible. The yet unexplored application between chromatography runs of recent advances in solvent exchanges required for more complex flow chemistry/continuous processing synthetic schemes could also potentially greatly expand the practical dimensional space in multidimensional chromatography.

Alternate-pump recycling-high performance liquid chromatography (R-HPLC)

When the final analyte mixture to be purified is well understood in the number and nature of the possible compounds present, such as from a targeted organic synthesis process, the rigorous method development required by 2D-LC may be unnecessary for purification of the mixture. For example, in a targeted synthesis, the structures of the final desired product, as well as the possible undesired side products such as regio- or stereoisomers of the desired product or unreacted starting materials, can often be predicted. However, even with the analyte mixture being small in magnitude, the analytes can still be very challenging to purify

in a single column experiment, largely because of closely related compounds being present, such as α/β anomers and other unwanted isomers that can result from side reaction such as acyl group migrations.

One alternative to 2D-LC for such a targeted purification is alternate-pump recycling-high performance liquid chromatography (R-HPLC). Alternate-pump R-HPLC is a variant of multidimensional chromatography where an analyte is recycled between two identical column supports, in isocratic mode, that are connected via a 10-port switching valve. This alternate-pump design has been previously demonstrated to be superior to the direct pump design, where the analyte is pumped through the volume of the mobile-phase pump that results in unnecessary peak broadening[94]. From a simplified viewpoint, alternate-pump R-HPLC can be thought of as an infinitely long column coupled with a non-destructive detector that allows the separation of close structural relatives. The original reference contains additional useful background information on the theory behind this technique [94].

Figure 4 depicts how an alternate-pump R-HPLC setup along with valve actuation is configured for the purification with a pentafluorophenyl stationary phase of a synthetic protected carbohydrate obtained from a solution-phase automated synthesis platform. Detailed information on valve actuation as well as the ease of implementation of alternate-pump R-HPLC has been discussed previously[43,95]. With the growing need for carbohydrates as authentic analytical standards or as components of bioassay screens, high-level purification of synthetic compounds remains of the utmost important. Alternate-pump R-HPLC has successfully been applied for the separation of *N*-glycan isomers with the use of HILIC amide columns after the analytes were tagged with a bi-functional aromatic amine[95] to allow for ultraviolet detection and the creation of glycan arrays. After 53 effective columns, mannose-7 *N*-glycan isomers were successfully separated and purified from one another[95]. In another study that employed alternate-pump R-HPLC, it was determined that synthesized protected carbohydrates could be successfully purified to ≥99.5% purity[43] with a reversed-phase approach. It was observed that pentafluorophenyl supports were ideal for the purification of protected monosaccharides, whereas phenyl hexyl supports were well suited for the purification of protected di-/tri-saccharides[43]. Interestingly, both phenyl/fluoro-phenyl stationary phases were far superior in the purification of protected carbohydrates, especially when methanol was used as the organic modifier instead of acetonitrile to increase π-π interactions between analyte and stationary phase, as compared to the more commonly employed alkyl-linked, C5/C18, supports[43].

For such difficult purifications of closely related carbohydrate structures, such as unprotected *N*-glycan isomers[95] or protected carbohydrate anomers[43], alternate-pump R-HPLC shows great promise. Although adaptation of R-HPLC to a high-throughput format would be challenging, its ability to increase the number of effective columns to achieve high-level purification far outweighs the drawback of potentially lengthy run times. We envision that the future development of new stationary phases will only further aid the amount of possible applications for this technique since alternate-pump R-HPLC must use two identical columns with the same column chemistry and be operated in isocratic elution mode so that valve actuation times remain in sync. Additionally, the availability of

preparative-scale alternate-pump recycling chromatography instruments to allow bulk purification of synthesized compounds would also increase the utility of this technique.

Sample preparation and derivatization

As is evident from the above discussion, the inherent properties of the carbohydrate analyte —its charge state, complexity, etc.—often dictates the technique that can be most readily applied for its purification. Carbohydrates can be detected in their natural state without any further derivatization by their UV absorbance at low wavelengths (< 200 nm), evaporative light scattering, or their refractive index. However, due to their inherent structural similarities and usual lack of a conjugated system or chromophore, various chemical modification methods have been developed and applied to carbohydrate analysis for enhanced sensitivity. Additionally, derivatization is not only useful for better sensitivity in detection, such as through the installation of protecting groups that increase ionization efficiency when coupled to mass spectrometry, but also for increased retention such as by the installation of chemical tags that alter the hydrophilicity/hydrophobicity of the analytes to provide better separations and better interactions with the stationary-phase supports. Unfortunately, many of these derivatization techniques do not lend themselves to easy reversal and are therefore more applicable to identifying than to isolating a pure compound as discussed below.

Permethylation

Base-mediated permethylation—developed in the 1980s to boost the ionization efficiency of carbohydrate for mass spectrometry analysis—is amongst the most common carbohydrate derivatization reaction[96]. Permethylated products can be readily produced with over 98% yield within 10 minutes. This method has since been used widely in mass spectrometry-based glycomics studies[97,98]. Recent improvements in methylation reduce the potential of undesired oxidative degradation side reactions, by adding methyl iodide in the presence of excessive sodium hydroxide power in *N,N*-dimethylacetamide under non-anhydrous conditions, with a trace of water, and neutralization of the base before extraction[99,100]. Permethylation remains the preferred form of derivatization for glycan analysis with mass spectrometry, as it is both fast and easy, as well as provides stable products. Unfortunately, methyl ether removal is not trivial[101] and therefore this technique will likely remain limited to analysis rather than purification protocols.

Hydrolysis/alcoholysis

Derivatization of carbohydrates with alkyl groups can also be performed via hydrolysis (here termed alcoholysis), where the monosaccharide constituents, as opposed to the entire polysaccharide, are analyzed by mass spectrometry. Methanolysis has been the most popular version of alcoholysis, with uses in a wide range of biological samples[102,103]; this reaction can also be performed on monosaccharide analytes to prevent the anomerization/mutorotation problem discussed above. Recently, butanolysis has been identified as a better alternative to methanolysis for the quantitation of heparin sulfate from mucopolysaccharidosis patients via LC-MS/MS. Butanolysis derivatization was shown to improve the sensitivity by at least 70-fold compared to methanolysis as heparin sulfate

quantification was achieved in 5ul urine samples from patients[104]. While this acid-catalyzed butanolysis reaction does require higher temperatures than its methanolysis counterpart, it permits shorter reaction times. For this approach to be broadly practical, improvements in both reaction time and yield for such alcoholysis reactions need to be considered. Whereas a single alkyl group at the anomeric position removes the issue of mutarotation, the process of alcoholysis also increases the number of possible analytes to purify at least two-fold since now both alpha- and beta-alkyl-glycosides become present in the sample mixture (as well as the possibility of five-membered furanose structures in the case of some alkylated monosaccharides). Yields of the reverse reaction that regenerates the original desired compounds are also not yet quantitative.

Chemical tags

Various chemical tags have been synthesized and applied to carbohydrate analysis, such as isotopic labeling and UV/fluorescence derivatization. Isotopic labeling, for example, has seen most use in NMR experiments as well as to create distinct *m/z* fingerprints in mass spectrometry. UV-active tags, such as 4-aminobenzamide or 4-(2-aminoethyl)-aniline, are commonly used in reducing end modifications, with simple procedures and sample clean-up, to allow for the carbohydrate analyte to absorb at higher wavelengths[95,105]. UV tag selection remains of the utmost importance as such derivatization methods can readily improve ionization efficiency in mass spectrometry as well as eliminate interfering signals in low wavelengths. In the case of larger oligosaccharides, such a UV tag can be quite necessary so as to both allow absorbance as well as increase ionization efficiency; however, deprotection of such tags can be non-trivial[101] and creates a bottleneck in high-level analyte purification.

Fluorescent tags, such as 2-aminobenzoic acid and 1-phenyl-3-methyl-5-pyrazolone, have been commonly applied in HPLC-based analyses [106,107]. Recent advances in this area have shown that 2-pyridylfuran, as a newly developed fluorescent tag, is more promising in terms of separation ability and enhanced signal as compared to traditional tagging reagents[104]. A fluorescent pyridylfuran moiety was formed upon incubation of saccharide and 1,3-di(2-pyridyl)-1,3-propanedione overnight at 110 °C. D-Galactose pyridylfuran derivatives can be detected at femtomole levels, which are significantly lower than traditionally used fluorescent tags [108]. Such tags also permit faster separations, where monosaccharide derivatives can be resolved within 6 minutes with liquid chromatography. One drawback to this 2-pyridylfuran tagging approach is that the reaction opens the monosaccharide ring, which results in C-2 epimers having the exact same structure and thus remaining inseparable. This issue also remains problematic when bulky reducing end modifications may favor one dominant α/β anomeric configuration. Clearly, future derivatization approaches are needed to match the same level of increased sensitivity with current tags, but also to eliminate inseparable and/or tough to purify derivatives and challenging reversibility issues.

Desalting

Affinity-based desalting methods have been widely used for the analysis of more hydrophobic macromolecules such as proteins, peptides, and nucleic acids. For carbohydrate analysis, desalting is a crucial sample preparation step before mass spectrometry and high-

performance liquid chromatography analysis to eliminate any potential salt interference. Desalting of larger carbohydrate samples, such as glycans, can be done with commercial reversed-phase-based desalting membranes with a relatively high recovery ratio. In the case of smaller carbohydrates such as monosaccharides and disaccharides, desalting has been quite challenging due to their smaller size and hydrophilicity. Commercial reversed-phase and normal-phase desalting membrane tips have been shown to only recover less than 10% of monosaccharides released from enzymatic hydrolysis[109]. This lack of efficient desalting truly limits the potential for absolute quantification of monosaccharides after enzymatic treatment. With the increased development of carbohydrate chemoenzymatic synthesis and glycan sequencing protocols, the awareness of a need for a better monosaccharide desalting methods should be raised among the carbohydrate and separations communities. Additionally, future work could be envisioned in the development of more methods using mass spectrometry-friendly salts, such as ammonium acetate, for example, in enzymatic hydrolysis experiments. Clearly, other avenues to circumvent these desalting issues need to be developed to further the purification of mono- and disaccharides.

Ease of coupling to other techniques

High-performance liquid chromatography can be effectively coupled to other analytical techniques due to its exceptional separation ability and non-destructive nature. Among various analytical techniques, mass spectrometry is most commonly used to complement HPLC. Other useful techniques include ion mobility spectrometry, nuclear magnetic resonance spectroscopy, and surface plasmon resonance [110,111].

LC-MS

LC-MS based methods have been widely used in the fields of proteomics and glycomics[112]. In glycomics studies, liquid chromatography has the ability to separate complex glycan isomers, making it possible for subsequent mass spectrometry analysis to identify thousands of glycans from natural sources. This traditional LC-MS approach has contributed greatly, for example, in recent studies of cancer-related biomarker identification[113]. Unfortunately, absolute quantification of analytes remains challenging through mass spectrometry. A recent flow split design[114] combines both a UV-detector and electrospray ionization ion trap mass spectrometry for the quantification of various carbohydrates. Termed “LC-UV-ESI-MS/MS”, this method also utilizes a 96-well plate format for rapid carbohydrate hydrolysis and derivatization. In combining UV-detection and mass spectrometry, this method provides a way for absolute carbohydrate quantification that has potential in sequencing applications. The utility for carbohydrate purification strategies is still unclear.

LC-IM-MS

Ion mobility spectrometry is a powerful tool for the gas-phase separation of carbohydrates, either as a standalone technique[115,116] or via coupling with other analytical techniques[117]. A recent study has shown that non-derivatized glycans can be separated through a reversed-phase column on a liquid chromatography-ion mobility-mass spectrometry (LC-IM-MS) instrument[118]. The addition of ion mobility to traditional LC-MS setups provides an added dimension of analyte separation, in the gas-phase, as well as an increase in overall sensitivity

(Figure 5). This LC-IM-MS approach is able to analyze underivatized glycans, which removes any need for derivatization. Despite the increased separation power of this new platform, there still remains the drawback of IM-MS where multiple drift time features may indicate the presence of two ion conformations rather than two distinct compounds [115,119,120,121]. Future work is ongoing in ion mobility spectrometry instrumentation, such as structures for lossless ion manipulations (SLIM) devices that can provide increased resolution and improved structural characterization[122]. These methods could quickly identify the success of other carbohydrate purification protocols.

Amenability to synthetic routes

Carbohydrate synthesis remains challenging, not in the least because of the bottleneck in available high-quality separations protocols. Oligosaccharide synthesis usually requires synthetic routes that consist of orthogonal protection with repeated protection and deprotection steps[123]. In terms of separation of intermediates between reaction steps, flash chromatography is commonly used for economic reasons and its ability to process in large scale. However, flash chromatography purification and NMR identification is limited in its nature when carbohydrates are needed at levels of >95% purity[43]. Certain limitations do exist for HPLC purification of synthetic carbohydrates, such as compound solubility, process scale, and instrument expense compared to flash chromatography. Single HPLC runs are also not guaranteed to provide material at the >99.5% purity require for immunological studies. That said, HPLC is used for purification of final synthesized products intended for either authentic analytical standards or biological activity experiments[124] where less stringent purity criteria are acceptable.

Coupling to automated or machine-assisted carbohydrate synthesis

Decades ago, automated synthesis methods were developed for macromolecules such as peptides and nucleic acids, resulting in a rapid expansion of structure/function studies of these classes of biomolecules. As for carbohydrates, that lack a natural synthetic template, automated synthesis remains difficult. Only with the advent of C18 supports did automated peptide synthesis become accepted as a commonplace strategy for peptide access[125–129]. Unfortunately, no similar consensus yet exists for purification protocols of materials coming from automated synthesis platforms[20–22,130–137.] Solubility issues of synthesized carbohydrates in mobile phases, the wide variety of possible isomers present through various protection and deprotection stages, and the variety of machine-assisted synthesis platforms complicates the development of such standard operating protocols for products of machine-assisted synthesis.

To date, bench top flash chromatography followed by NMR or HRMS identification has been the norm for the characterization of synthesized carbohydrates, but provides no information on purity[138]. For purity-sensitive applications of synthetic carbohydrates, such as biological microarrays and immunological studies, HPLC/rHPLC allows for quality control and high-level purity of final compounds. Perhaps the greatest current limitation for a wide application of HPLC purification to carbohydrate synthesis is its scalability, as efficient gram scale purification remains challenging. As machine-assisted/automated

oligosaccharide synthesis methods become more common, the future development of accessible, affordable and scalable LC-based purification methods along with a wider breadth of stationary-phase supports will be needed.

Conclusions

While applications of machine-assistance including flow chemistry techniques to carbohydrate synthesis via chemical and enzymatic methods promise the rapid development of carbohydrate studies, one of the greatest bottlenecks remains the lack of high-throughput and robust purification techniques that demonstrably scale. Current choices of a chromatographic method for the purification of a carbohydrate(s) of interest are still limited and many questions remain (Figure 6). Additionally, Table 1 provides a starting point for the glycobiologist/carbohydrate chemist, where each chromatographic method from Figure 6 is briefly summarized and a few selected applications references are given to provide further background information. We hope that this flow chart summary and review stimulates the creative minds of synthetic and analytical chemists alike, including those who have perhaps not before considered tackling the complexities of saccharides. Clearly, the area of 2D-LC promises many advances in the next decade as it pertains to carbohydrate purification. Additionally, the ever-growing analysis toolbox will need to be tailored in its complementarity to existing, as well as future, liquid chromatography-based assays. Clearly, this present time represents one of tremendous excitement as many carbohydrate purification and separation challenges still remain unsolved, but their huge impact on biology is also quite clear.

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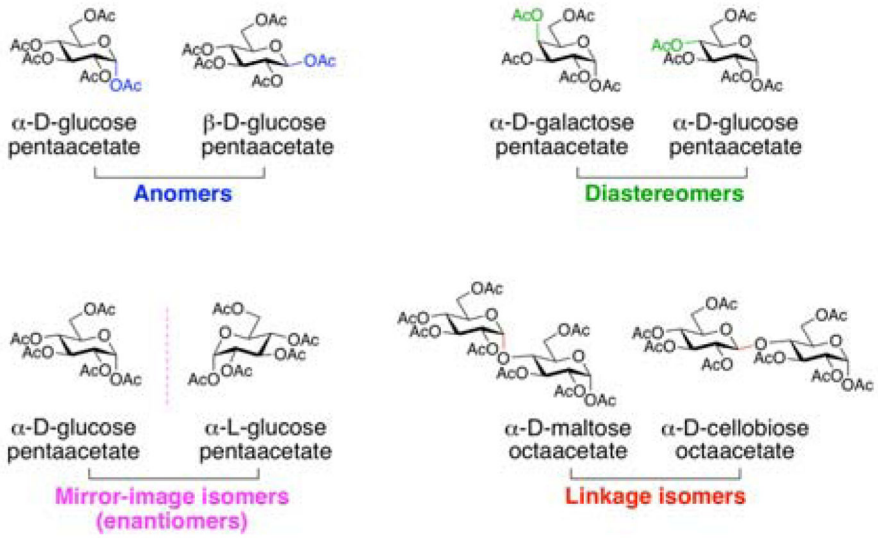

Figure 1.

Depiction of the structural complexity of carbohydrate isomers as their peracetylated derivatives.

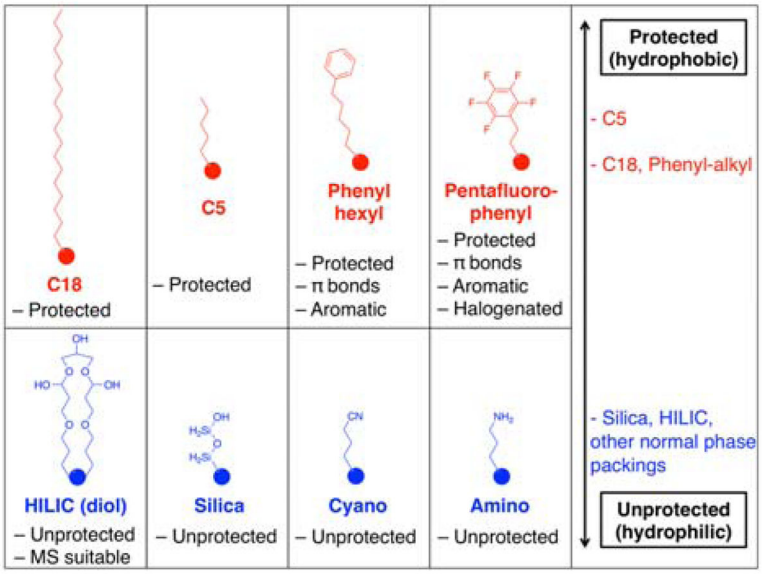

Figure 2.

Representation of common stationary phases used for purification and separation of protected and deprotected/natively unprotected carbohydrates.

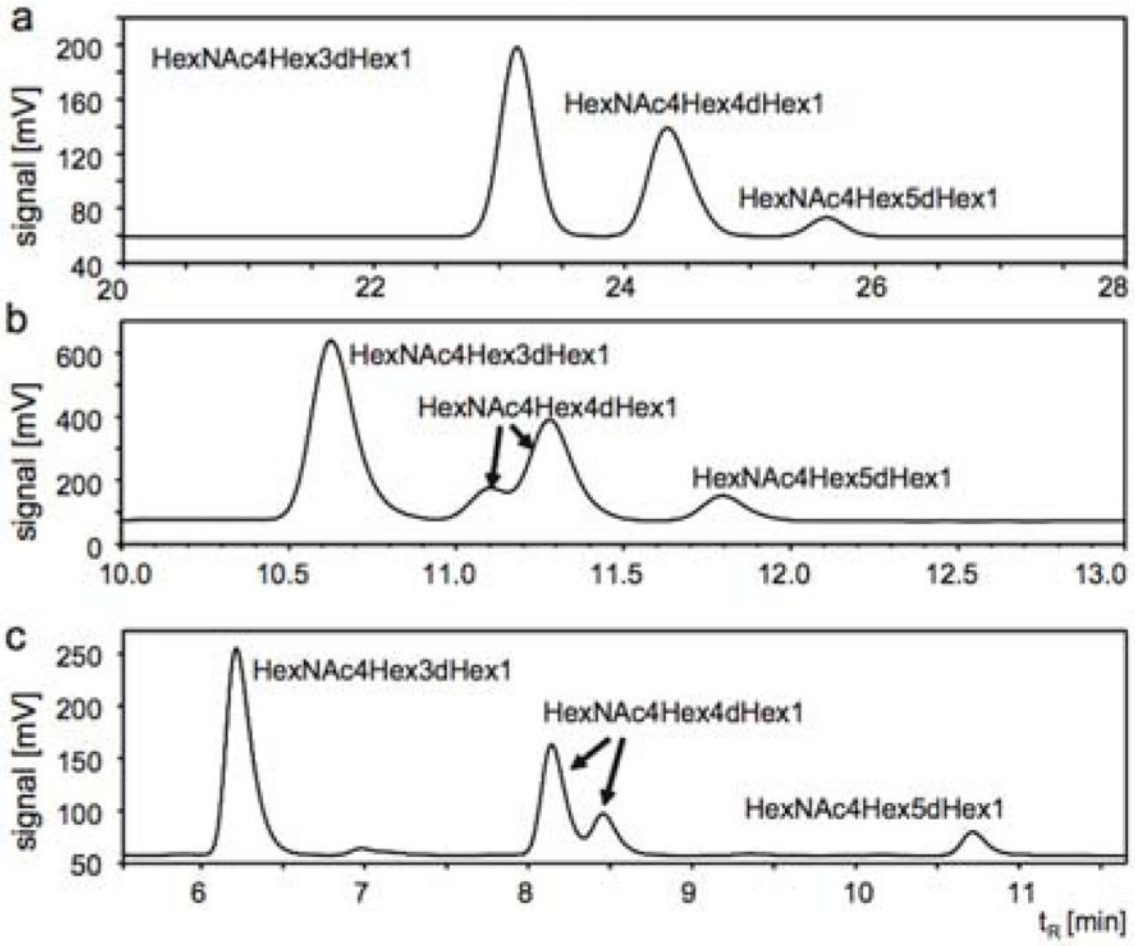

Figure 3.

Comparison of stationary phases for the separation of mAb glycans ($HexNAc_4Hex_3dHex_1$, $HexNAc_4Hex_4dHex_1$ (two isomers), and $HexNAc_4Hex_5dHex_1$). A) C18 reversed-phase. B) PGC. C) HILIC. As can be seen, the $HexNAc_4Hex_4dHex_1$ isomers are unable to be separated with C18 reversed-phase packing supports. Reprinted from *J. Chromatogr. A*, **1218**, M. Melmer, T. Stangler, A. Premstaller, and W. Lindner, "Comparison of hydrophilic-interaction, reversed-phase and porous graphitic carbon chromatography for glycan analysis", Pages 118–123, Copyright (2011), with permission from Elsevier.

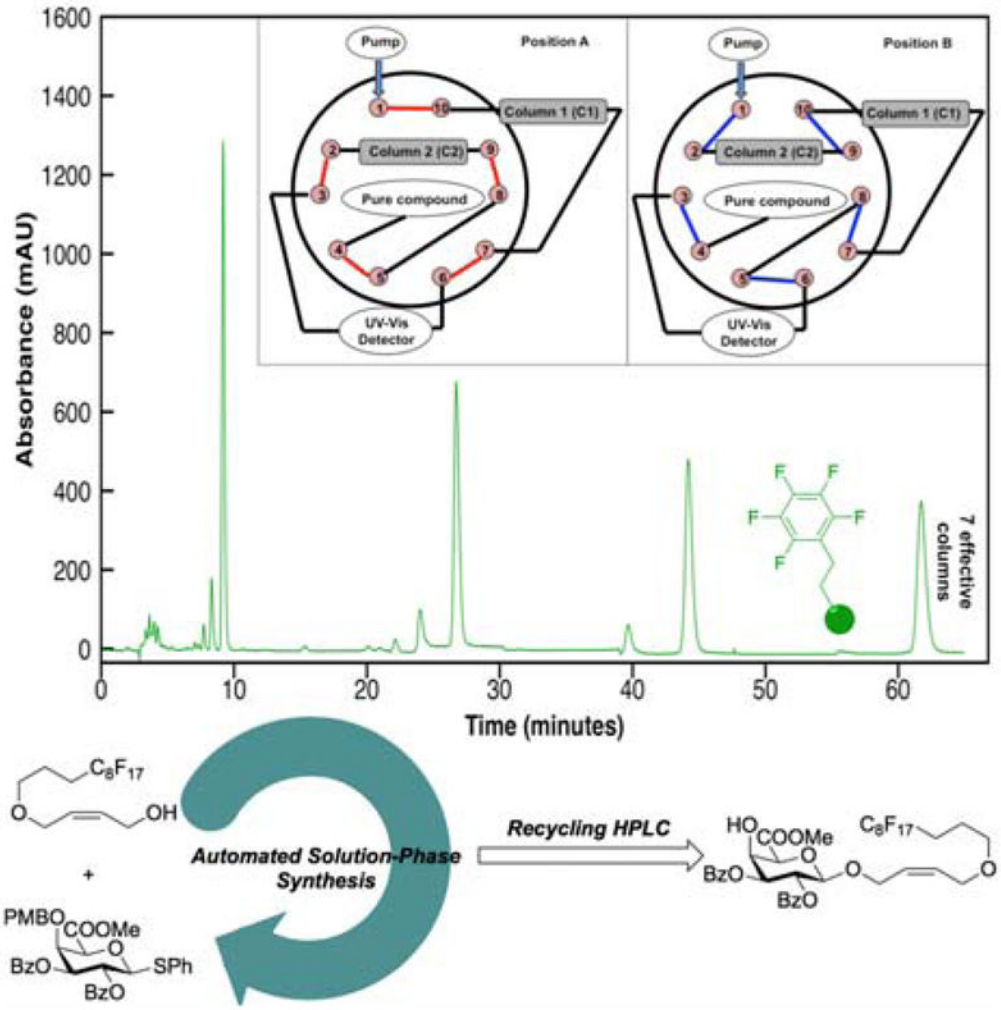

Figure 4.

Schematic of 10-port switching valve used in alternate-pump recycling HPLC. Purification, from its undesired anomer/other impurities, of a protected carbohydrate compound synthesized via machine-assisted synthesis is shown after seven effective columns with a pentafluorophenyl stationary phase. Adapted from Ref. 43 with permission from The Royal Society of Chemistry.

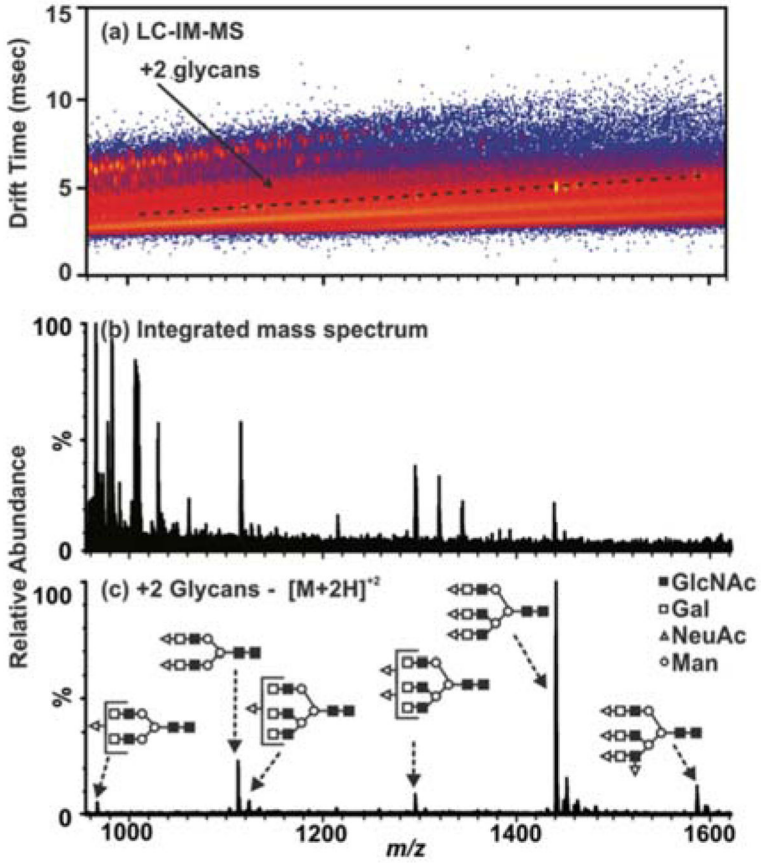

Figure 5.

LC-IM-MS figure of bovine fetuin glycans released via PNGaseF. (a) Two dimensional IM-MS spectrum of released glycans. (b) Integrated mass spectrum. (c) Extracted mass spectrum. Reproduced from Ref. 118 with permission from The Royal Society of Chemistry.

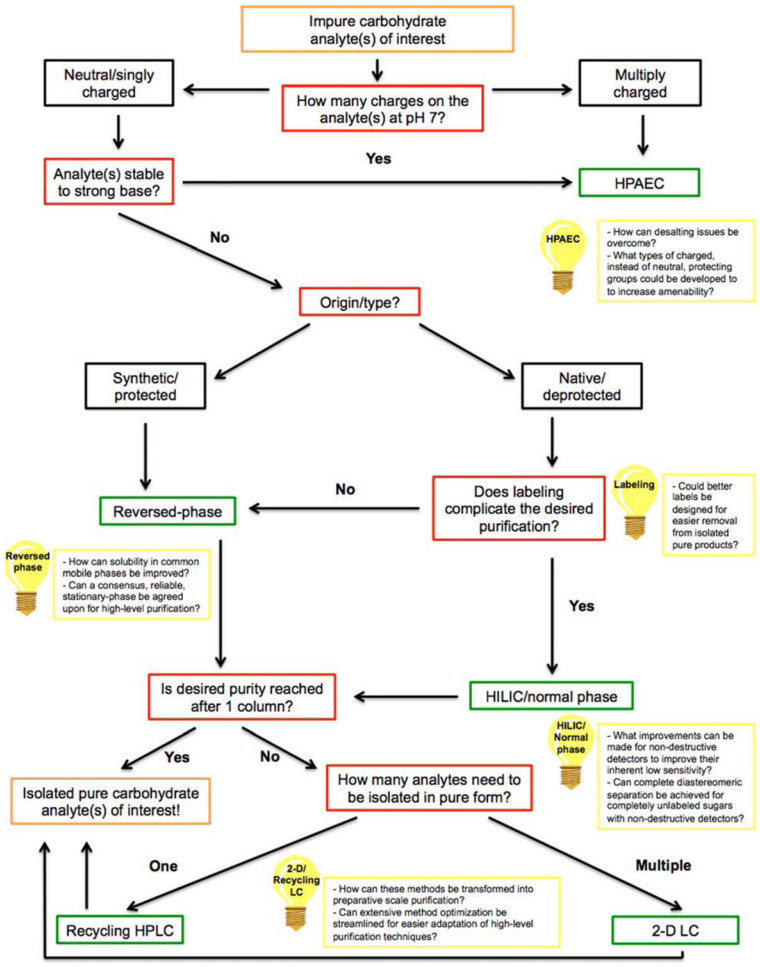

Figure 6.

Flow chart summary of purification methods for carbohydrate analytes with non-destructive detectors.

Table 1

A brief summary of the chromatographic methods that are highlighted in the flow chart from Figure 6. All applications presented in this table were performed with commercially available modified-silica supports. Selected applications, with their corresponding references, are given.

Chromatographic method	Potential applications
Reversed-phase	Synthesized protected saccharides; hydrophobic analytes; phenyl-based supports with methanol-containing mobile phase are ideal for p-bond containing analytes as compared to alkyl-linked supports (C5/C18) [23–28, 40–42]
Normal-phase/HILIC	Native/unprotected saccharides; highly polar mono/oligo-saccharides with amine or diol-based supports [31, 51–54]
HPAEC	Native/unprotected carbohydrates that are negatively charged (either natively acidic or neutral ones that are made acidic at high pH) [57–64]
Recycling HPLC	Fluorescently labeled *N*-glycan isomers with HILIC-amide supports [95]; protected mono/oligo-saccharide anomers with phenyl-based reversed-phase supports [43]
2D-LC	Low molecular weight oligosaccharides; charged and neutral saccharide analytes with combinations of reversed-phase, size-exclusion, and anion-exchange supports [83–91]