Chapter 1.3
Separation and Purification of Carbohydrates

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1 Introduction

The separation and purification of carbohydrates is a broad field encompassing molecules having substantially different physical properties (see Chap. 1.2) requiring a wide variety of analytical and separation methods. This chapter is divided into three sections based on the following physical properties of the carbohydrates: unprotected/neutral; unprotected/charged; and protected (Fig. 1). This chapter will focus on preparative methods that are scalable and those that permit the preparation of sufficient carbohydrate material for biological evaluation. Several books have recently been published on micro-analytical methods in carbohydrate chemistry and glycobiology [1, 2, 3, 4, 5, 6]. These analytical methods often focus more on detection than on separation. The current chapter will only briefly describe analytical methods critical for following preparative separations. The scalability (mg to kg) of each method will be discussed together with important considerations and pertinent examples relating to each method.

2 Unprotected Neutral Carbohydrates

Although the isolations of unprotected neutral carbohydrates present substantial challenges in both separation and detection, the procedures offer some advantages for the removal of contaminating materials. As a rule, the difficulty of a separation is very dependent on both the amount and properties of the desired compound and the contaminants in the starting mixture. For example, the separation of L-rhamnose from D-glucose would necessitate a completely different strategy than the separation of L-rhamnose from a fermentation broth containing proteins, amino acids, and salts. In this chapter, examples will be selected that correspond to separation problems most frequently encountered by chemists working in the fields of glycobiology, carbohydrate biochemistry, and synthetic carbohydrate chemistry.

2.1 Isolation and Removal of Impurities

Many neutral carbohydrates are natural products obtained from complex biological systems such as plant and animal tissues or microbial fermentation.

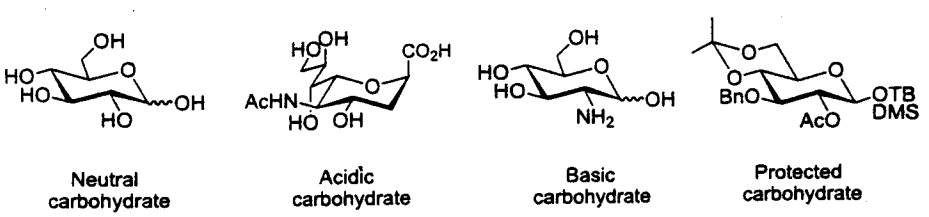

Fig. 1. Typical structures of unprotected neutral, acidic, and basic monosaccharides and a differentially protected monosaccharide

These neutral carbohydrates are often isolated as glycoconjugates containing fatty acid moieties (glycolipids [7, 8]) or peptides/proteins (glycopeptides/glycoproteins [8]). The unique properties of the glycoconjugate can be helpful in an initial purification step to isolate the desired carbohydrate [2]. A glycoprotein can usually be recovered from a complex biological sample by applying standard techniques for the isolation and purification of proteins [9, 10]. Often a simple step, such as precipitation through the addition of trichloroacetic acid, ammonium sulfate, polyethylene glycol, or water-miscible organic solvents is sufficient [2, 11]. A glycopeptide can be recovered from neutral (uncharged) species through its binding to a mixed-bed ion-exchange resin [1, 2, 10]. (If the sample contains substantial quantities of salt, a desalting step may be required). For glycolipid isolation from a biological sample, solvent extraction or adsorption on a hydrophobic resin such as polystyrene may be employed [2, 7]. Such initial separation steps, using a unique property of a glycoconjugate, are often scalable (mg to kg) and can markedly simplify subsequent purification, by eliminating difficult steps to remove contaminants, such as undesired neutral carbohydrates.

Once a glycoconjugate is recovered from a complex biological mixture, the non-sugar component can be removed either chemically, through hydrolysis, hydrazinolysis, β-elimination, or enzymatically (using an appropriate hydrolase such as *N*-glycanase, *O*-glycanase, ceramidase, etc.) [1, 2, 3, 4, 10].

The ionic impurities present in a sample containing a free (unconjugated) neutral carbohydrate are most frequently removed by passing the sample over a mixed-bed ion-exchange resin [2, 10]. Care must be taken to ensure that the resin has sufficient capacity to handle the charged contaminants, and that the sample does not contain large amounts of salt that adversely influence ion-exchange chromatography. In such cases, a sample should either be dialyzed or desalted on a column [2].

A mixed-bed ion-exchange resin will bind all charged contaminating species and the desired neutral carbohydrate will be eluted in the void volume of the column. Similarly, hydrophobic contaminants can be conveniently removed from the desired hydrophilic neutral carbohydrate by the use of a hydrophobic resin such as porous polystyrene beads [2]. These beads will bind hydrophobic contaminants tightly, again permitting unimpeded elution of the neutral hydrophilic carbohydrate from the column. Both of these passive (i.e., non-target molecule interacting) chromatography steps are scalable and can be used to isolate mg to kg quantities of carbohydrates. At this stage in the purification, only neutral hydrophilic contaminants should remain with the target compounds.

2.2 Precipitation and Crystallization

Once the ionic and hydrophobic contaminants have been removed from a biological sample, it is often useful to recover the neutral carbohydrate target from solution by precipitation or crystallization [12]. Neutral carbohydrates, particularly oligosaccharides and polysaccharides, have limited solubility in mixtures

of aqueous/organic solvents such as aqueous ethanol or acetone. Typically, the concentration of organic solvent is slowly increased until the cloud point (the concentration at which a clear solution becomes cloudy) is reached. The sample is then cooled to 4 °C and left covered overnight in an explosion-proof refrigerator. This method can result in the formation of crystals or an amorphous precipitate that can be recovered through filtration or centrifugation. If crystals or a precipitate are not formed, additional cold organic solvent can be added to induce precipitation, or the solvent (both aqueous and organic) can be removed through rotary evaporation to recover the carbohydrate sample. Precipitation and crystallization steps are scalable (mg to kg). Monosaccharides and disaccharides are frequently crystallized while higher oligosaccharides and polysaccharides are recovered as precipitates. At this stage in the purification, a sample that has crystallized might only require a recrystallization step to afford a pure product. However, samples that either precipitate or fail to crystallize typically require additional purification steps.

2.3 Low Pressure Chromatographic Purification

Low pressure chromatography includes gel permeation, ion-exchange, partition, and affinity chromatography. Gel permeation chromatography (GPC), frequently used for the purification of neutral carbohydrates [2, 10], is generally a low resolution method capable of handling only small quantities (mg to g) of samples. Carbohydrates eluting from the column may be detected by a non-specific, neutral sugar test such as the phenol-sulfuric acid or anthrone assay [3, 13]. One approach is to use GPC to fractionate a glycan while it is still conjugated. Thus a glycoprotein [2], for example, will behave like the corresponding macromolecule, in this case a protein, eluting early at the column's void volume. Using *N*-glycanase the small glycan is then released from its core protein. Upon refractionation on GPC, this glycan would then elute in either the column's included volume or the total volume. Thus, the first fractionation removes contaminants having low and intermediate molecular size, while the second fractionation removes contaminants having high molecular size. Very low molecular weight contaminants such as salts are most often removed using a desalting column, which permits only very small molecules (e.g., salts) to enter its included volume [1, 2]. Recently, controlled pore dialysis membranes having molecular weight cut-off (MWCO) values of 500 or 1000 have been used in place of GPC for desalting [1, 2].

Several critical issues need to be addressed in selecting a GPC packing material. The exclusion limits are generally presented for proteins and peptides and often do not correlate well to carbohydrates, which appear to be somewhat larger than proteins of equivalent molecular weight. While Sephadex, Sepharose, and agarose have excellent performance characteristics, they are composed of carbohydrates and can leach small amounts of neutral sugars, contaminating samples. This is of particular concern in fractionating sub-milligram amounts

of sample, and so for these separations, synthetic polyacrylamide or polyvinyl alcohol gels are often used. The column capacity (volume) depends primarily on sample size. A sample should be dissolved at a concentration exhibiting low viscosity and a volume corresponding to <10% of the column volume. The addition of salt to the aqueous eluent often prevents interaction of the neutral carbohydrate with the supporting matrix. Neutral sugars can also be fractionated by ion-exchange chromatography, eluting in order of decreasing molecular size. This method is particularly suited to separations involving large amounts of solute [3, 14]. Glycolipids are often purified by partition chromatography on columns or on plates by thin layer chromatography (TLC) [2, 6]. Lectin affinity chromatography of neutral carbohydrates is a powerful tool for the fractionation of glycans on a wide variety of commercially available, immobilized lectin supports [1, 2, 3]. For example, glycans terminating in mannose can be purified by binding to immobilized ConA-Sepharose and eluted with α-methylmannoside, which can be removed from the glycan by dialysis or by using a GPC column. Serial lectin chromatography is also possible using multiple lectin columns to fractionate complex glycan mixtures [1, 4].

2.4 High Pressure Chromatographic Purification and Analysis

GPC using high pressure liquid chromatography (HPLC) is often applied to the microanalysis (μg to mg) of underivatized neutral glycans. Detection usually relies on low (<200 nm) ultraviolet absorbance, refractive index, viscosity, and post-column derivatization. At pH 13, the hydroxy groups of neutral carbohydrates can be ionized, enabling their fractionation by anion-exchange HPLC on a pellicular resin-based, base-stable packing material. Detection relies on a pulsed amperimetric detector (PAD) and both a high resolution separation and a high detection sensitivity is observed [2, 4]. Fractions can be collected and basic eluent (usually NaOH) neutralized to perform preparative (mg scale) separations [4]. One limitation of this method is the base lability of many neutral oligosaccharides, leading to β-elimination, peeling, and epimerization [1, 15].

2.5 Derivatization

The two major goals in derivatizing a neutral carbohydrate are to improve its detection and to facilitate its separation. Derivatives having improved detection include UV active chromophores, and radio and fluorescence labels [1, 2, 3]. The most common method of introducing these labels is through either reduction or reductive amination [1, 2, 3, 4]. Although the yields of these reactions are usually nearly quantitative, a major limitation is that the neutral oligosaccharide must be a reducing sugar. The introduction of a hydrophobic label, a charge or an affinity ligand can facilitate glycan purification [4, 5]. In most cases the labeling chemistry alters the glycan structure making it impossible to recover the glycan

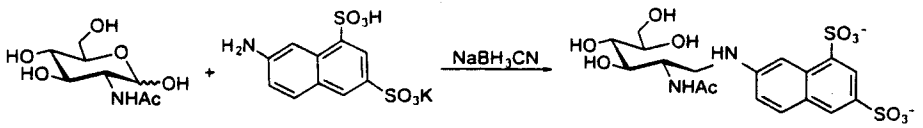

Fig. 2. Reductive amination of *N*-acetylglycosamine with a fluorescent dye proceeding through an intermediate Schiff base

in its natural state. However, reversible labeling of neutral carbohydrates is possible, and this facilitates separation and analysis while allowing the recovery of the natural product at the conclusion of the fractionation [16].

2.6
HPLC Purification and Analysis of Derivatized Neutral Carbohydrates

Carbohydrates derivatized with a hydrophobic label through reductive amination can be fractionated by reversed-phase HPLC. Reductive amination with charged fluorescent or UV active labels results in derivatives that can be fractionated using ion-exchange HPLC [4, 5]. By using semi-preparative HPLC columns these separations can be used to purify multi milligram quantities of glycans. Microanalysis of glycans labeled with charge- containing tags can also be performed on gel and capillary electrophoresis [4, 5, 6, 17, 18]. Biotinylated (or biotin-aminopyridinylated) glycans can be separated either on the basis of their hydrophobic properties by reversed-phase HPLC or by affinity chromatography on avidin columns [5].

3
Unprotected Charged Oligosaccharides

Charged glycoconjugates are frequently associated with important biological or pharmacological activities, and are therefore important targets for glycan purification. While the presence of charged groups simplify certain aspects of purification (i.e., permitting the application of ion-exchange chromatography), they complicate others such as removal of charged protein contaminants and labeling chemistry.

Most charged oligosaccharides found in nature are anionic, containing carboxylate, phospho, or sulfo groups. Anionic glycans are found in all forms of glycoconjugates, glycolipids, glycoproteins, and proteoglycans. While cationic glycans are less frequently observed than anionic glycans, the methods for their separation are similar (relying on different matrices and elution conditions) to those described for the fractionation of anionic glycans.

The purification of charged oligosaccharides can often be followed using specialized assays or detection methods. For example, uronic acids and sialic acids can be detected through the use of specific colorimetric assays [2, 3] while acidic sugars can also be measured non-specifically using conductivity detection [19].

3.1 Isolation and Removal of Impurities

The purification of charged glycans usually begins with their recovery in the glycoconjugate form (i.e., glycolipids through hydrophobic interaction, reversed-phase chromatography or TLC, and glycoproteins through precipitation or GPC) [2]. This first step often eliminates many of the contaminants in the mixture. After purification of a glycoconjugate, the glycan is then released from its carrier by enzymatic (i.e., *N*-glycanase, ceramidase, etc.) or chemical means (i.e., hydrazinolysis or β-elimination, etc.) [1, 2, 3, 4, 10]. The carrier (i.e., lipid or protein) can then be removed by repeating the recovery steps described above and residual reagents (i.e., hydrazine or base) and salts are removed by dialysis or by GPC using a desalting column.

3.2 Extraction and Precipitation

Large-scale (mg to kg) recovery of charged carbohydrates is often possible using solid-phase extraction techniques or through precipitation [3, 11]. Oligosaccharides containing a small number of charged groups, in the form of carboxylate, phospho, or sulfo groups can often be recovered through batch addition of an ion-exchange resin to a desalted sample to selectively bind negatively charged components. After washing the resin with water, release of carboxylated sugars (i.e., sialic acid-containing glycans) can be accomplished by washing with formic acid [2]. The wash is then neutralized with ammonium hydroxide and desalted by lyophilization, dialysis, or using a GPC column. Acidic glycans containing sulfo or phospho groups are typically recovered by washing the resin with sodium chloride and desalting by dialysis or by GPC.

It is often possible to precipitate anionic polysaccharides through the addition of solvent, hydrophobic amine, soluble polyamine, or a metal [2, 10]. For example, the polysulfated heparin polysaccharide can be precipitated by the addition of methanol, cetylpyridinum chloride, polybrene or protamine, or barium. The polycarboxylated alginic acid can be gelled by the addition of calcium hydroxide [13]. Following precipitation, the residue can often be recovered by centrifugation, redissolved in sodium chloride solution, and the precipitant and salt removed by dialysis.

3.3 Derivatization

Acidic carbohydrates are sometimes difficult to derivatize owing to stability problems and their charged character. Sialic acid-containing glycans are susceptible to loss of sialic acid even under relatively mild conditions [6]. In addition, derivatization by reductive amination [20] is often restricted to neutral labels because acidic labels are repelled while basic labels are bound by anionic carbo-

hydrates. Occasionally it is possible to mask these undesirable charge interactions by using hydrophobic ion-pairing agents or high salt concentrations. Direct derivatization of carboxyl groups is also possible by amidation following carbodiimide activation [18]. As with the labeling of neutral oligosaccharides, the two goals for labeling acidic oligosaccharides are to improve detection and separation properties. Derivatization reactions are generally not reversible [16] and lead to the isolation of a derivatized glycan in place of the natural product needed for biological evaluation.

3.4 Chromatographic Purification and Analysis

The major chromatographic method for the separation of acidic carbohydrates is anion-exchange chromatography. Carboxylated carbohydrates are most often fractionated using a weak anion-exchange (WAX) while sulfo and phospho group-containing glycans are frequently fractionated on a strong anion-exchange (SAX) [2, 5]. Both low pressure chromatography and HPLC are frequently used and these separations are scalable (mg to kg). Elution from a WAX column often relies on a stepwise or gradient change, in which the pH is decreased below that of the pKa of the carboxyl group (~3–4). Elution from a WAX or SAX column can also be accomplished using a salt wash (either stepwise or gradient). Salts such as sodium phosphate are often preferred, as sodium chloride requires the use of corrosion-resistant titanium or polymer-based pumps [2]. Conductivity detection is also possible if sodium carbonate is used as eluent and the sodium cation is exchanged with a proton by a post-column suppressor [19]. Alternative detection methods include UV absorbance, fluorescence (of derivatized sugars), and post-column derivatization [2, 3, 4, 5, 6].

3.5 Electrophoresis

Electrophoresis has become a very popular method for the high resolution analysis of acidic carbohydrates. Agarose gel electrophoresis, while sometimes still used in the analysis of acidic polysaccharides, is being displaced by the use of higher resolution polyacrylamide gel electrophoresis (PAGE). Visualization is possible through the use of dye-based staining, silver staining, and direct observation of a fluorescent oligosaccharide by UV transillumination [5, 21]. PAGE of acidic carbohydrates has also been used preparatively (μg to mg scale). Electroelution of bands (prior to staining) onto positively charged nylon membrane is followed by recovery by washing the membrane with salt followed by desalting [22]. Gel-based carbohydrate analysis can also be performed in a 2-dimensional format [23, 24]. Typically, the first dimension is affinity electrophoresis, using a lectin or other carbohydrate binding protein, and the second dimension is a size-based separation.

Capillary electrophoresis is becoming increasingly popular for the microanalysis of anionic carbohydrates [18].

Detection of underivatized oligosaccharides is possible based on low UV absorbance. Alternatively, high sensitivity detection is possible for derivatized acidic carbohydrates containing a chromophore or fluorophore. Polyanionic polysaccharides, such as heparin, can be detected by complexation with Cu^{2+} present in the electrophoresis buffer, which eliminates the requirement of derivatization for high sensitivity detection [25].

4 Protected Carbohydrates

Synthetic carbohydrate chemists encounter an entirely different set of chemical and physical properties than do natural products chemists and biochemists when working with carbohydrates. The properties of a fully protected carbohydrate more closely resemble those of a carbocyclic natural product rather than an unprotected carbohydrate. However, partially protected carbohydrates or ones containing charged groups such as carboxylate, phospho, or sulfo groups often pose a separation challenge to the synthetic carbohydrate chemist. Finally, once the synthetic carbohydrate chemist has completed his/her task, then deprotection of the target compound for the biochemist, biologist, or pharmacologist results in a deprotected product that requires the same separation methods as described above.

4.1 Extractive Work-up

Following complete protection of a carbohydrate through peracetylation, perbenzylation, persilylation, etc., the derivative formed is often isolated from hydrophilic reagents and salts by extractive work-up. While this method can be used on a large scale (kg), it is also useful for the removal of contaminants from small quantities (ng) of carbohydrates isolated from natural products prior to mass spectrometric analysis. A wide variety of water immiscible, volatile, organic solvents is useful in extractive work-up offering a major opportunity to extensively purify a carbohydrate following its protection. Problems can, however, be encountered when not all the hydroxy groups are protected or when charged groups are present. Such molecules may be partially soluble in water, or surface active, making phase separation difficult and thus complicating the extraction.

4.2 Crystallization

Protected carbohydrates are often oils, amorphous solids, or glasses. Occasionally, crystallization of these molecules is possible, thereby aiding in the purification of the protected carbohydrate. A survey of the synthetic carbohydrate liter-

ature suggests that <10% of all protected carbohydrates afford crystalline products. Recrystallization (mg to kg scale) would then lead to a pure sample often suitable for X-ray crystallographic analysis.

4.3 Chromatographic Separation

The chromatographic separation of protected carbohydrates generally depends on partition chromatography. The solvents used are organic solvents when the carbohydrate is fully protected and aqueous/organic solvents (or hydrogen bonding polar organic solvents) when the carbohydrate is only partially protected or contains unprotected charged groups. While column packing material usually is silica or alumina, specialized packing materials such as C_{18}-silica, ion-exchange resins, or organic solvent compatible GPC resins (i.e., Sephadex LH-20) have also been used to separate protected carbohydrates.

4.3.1 *Low Pressure and Flash Column Chromatography*

Low pressure and flash column chromatography are the most commonly used methods for the separation and purification of protected carbohydrates. Thin layer chromatography is most frequently used for monitoring the progress of reactions of protected carbohydrates. The most commonly used solid phase is silica with common solvent systems including chloroform/methanol, hexane/ethyl acetate, or dichloromethane/acetone, etc. Visualization can rely on a specialized reagent for carbohydrate staining such as Von's reagent [26] or general methods such as iodine or sulfuric acid charring. TLC is also frequently used to follow column-based purifications although it is often also possible to assay fractions by UV absorbance when UV-active protecting groups are used. Small-scale (1–10 mg) purification of synthetically prepared, derivatized oligosaccharides is required prior to submission for elemental analysis and often relies on small, low pressure columns. Large-scale (10 mg–10 g) purification, however, generally makes use of flash columns typically run under nitrogen gas pressure. Choice of solid support and solvent system is usually based on TLC resolution.

4.3.2 *High Pressure Liquid Chromatography*

Occasionally a low pressure or flash column separation provides insufficient resolution between two protected carbohydrate components isolated from a reaction mixture. Often these components are diastereomers, or a mixture of α- and β-anomeric forms. In such cases, HPLC may be advantageous. The packing material is composed of substantially smaller particle size (3–10 μm) than a flash column (35–63 μm) and therefore offers improved resolution. In addition, a wide range of different column packing materials is also available (including

chiral supports) often making it possible to separate a mixture of closely related components. In spite of the availability of analytical, semi-preparative, and preparative HPLC columns, the scale (mg to g) of HPLC-based separations is almost always lower than that of flash column chromatography. In addition, because of the smaller volumes collected, it is more convenient to monitor the separation by an on-line UV absorbance detector, rather than by TLC.

4.3.3
Capillary Electrophoresis

Capillary electrophoresis (CE) has only recently been applied to monitor reactions in synthetic carbohydrate chemistry [27]. This approach is particularly useful for the microanalytical separation (ng to μg) of highly charged oligosaccharides that do not move on TLC. CE also offers ultra-sensitive detection and can be automated to directly monitor reactions.

4.3.4
Gas Chromatography

Gas chromatography is one of the most frequent methods applied to the microanalysis (ng to μg) of protected carbohydrates [2, 3, 28]. Resolution is quite high permitting the complete analysis of highly complex glycan mixtures. The requirement of sample volatility, however, limits the size and functionality of the carbohydrate that can be analyzed. Detection is by flame ionization or mass spectrometry.

5
Summary

The physical and chemical properties of carbohydrates (see Chap. 1.2) determine the methods that can be used for the separation and purification of these molecules. Additionally, many carbohydrates are derived from natural sources in a highly contaminated form or in very complex mixtures. Thus, specialized skills are required for the purification of these molecules.

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