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Introduction

The term “saccharide” generally refers to simple saccharides, oligosaccharides, and polysaccharides. Simple saccharides include monosaccharides and disaccharides, excluding sugar alcohols. See Figure 1 below for a simple breakdown.

![Figure 1: The carbohydrate family breakdown](image)

In this text, organic acids are considered weakly acidic substances containing carboxyl groups (carboxylic acid groups) in their molecular structure. Many of the organic acids discussed do not fully dissociate in water, a factor that is considered for separation.

Samples often contain a mixture of saccharides and/or organic acids. To achieve the desired resolution a chromatographer can utilize columns which employ a combination of separation modes.

Reducing Sugars

The two main modes of separation for low molecular weight saccharide analysis are ligand exchange mode and hydrophilic interaction liquid chromatography (HILIC). For the chromatographer encountering monosaccharide analysis for the first time, these separations can present new challenges. The most prevalent challenge of analyzing monosaccharides is that they have the ability to tautomerize between cyclic anomers. This can make detection difficult from the split peaks and lack of an active chromophore in the UV wavelengths. Different conditions can be employed when utilizing HILIC or ligand exchange modes to resolve issues with anomer separation and several alternative universal detectors are available for saccharide detection.
HILIC – The HILICpak and Ashipak Series

HILIC is a relatively new member of partition chromatography. It is considered a part of normal phase because of the high polarity of the packing surface. The base material can be either silica or polymer with various polar functional groups including amide, amino, diol, and cyano added. Compared to normal mode, the eluents used for HILIC separations are similar to those used in reversed phase (RP); a mixture of water and acetonitrile. Hydrophilic compounds that were “too polar” to be retained by RP can be analyzed under HILIC without the need for normal phase solvents. HILIC has been found applicable for the separation of carbohydrates, especially saccharides, which are hydrophilic.

Advantages of polymer based HILIC:
- Analysis under moderate and basic conditions (pH 2-12 and room temperature)
- Sharp, near-symmetric peaks can be obtained for a wide variety of saccharides
- Accurate quantitative determination can be made
- A wide range of eluents, such as various buffer solutions, alkaline solutions, or acidic solutions can be used
- Alkaline washing of columns is possible

Reducing saccharides can adopt various cyclic structures, as well as a linear structure. Two tautomers, α-type and β-type, are produced when the carbonyl carbon atoms become asymmetric. Figure 2 left depicts the relation between α-type and β-type is called anomer.

When the conversion rate between tautomers is low, α and β anomers are separated by the column causing the peak tops to split or widen. (Figure 2 right)

Two methods are available to prevent anomer separation during analysis are:
- Analysis at high temperature (Ligand Exchange)
- Analysis under strong alkaline conditions (HILIC)

\[ \text{Cyclic Saccharides} \]

<table>
<thead>
<tr>
<th>Stereoisomers: Anomers α and β</th>
</tr>
</thead>
<tbody>
<tr>
<td>α-D-glucose</td>
</tr>
<tr>
<td>D-glucose</td>
</tr>
<tr>
<td>β-D-glucose</td>
</tr>
</tbody>
</table>

\[ \text{Figure 2: Saccharides and tautomerization} \]
With HILIC columns, saccharides elute in order of increasing polarity due to the function of normal phase chromatography. Typically, various ratios of acetonitrile and water are used as the eluent. When the mixing ratio of acetonitrile is increased, the polarity of the eluent becomes lower. This results in a stronger interaction between saccharides and the column and a larger elution volume. Please see Figure 6 for a discussion on why elevated temperatures are not necessary when utilizing a Shodex HILIC columns to avoid anomer separation.
HILICpak VG-50 4D shows high recovery ratio of reducing sugars, such as mannose (Figure 4). Silica-based amino columns show low recovery ratios of certain reducing sugars due to the interaction of the primary amino group with the carbonyl group of reducing sugar. The formation of a Schiff base causes reducing sugars to be adsorbed to the packing material. Functional group loss leads to a decrease in theoretical plates.

The VG series features a substituted amino with a hydrophilic group to avoid Schiff base formation and increase recovery. The Schiff base may form with various functional groups on the packing material within the column. Figure 5 shows a schematic of the formation of a Schiff base.
NH2P-50 and VG columns have weak alkaline amino functional groups, enabling saccharides to be analyzed without causing separation of anomers even at room temperature.

The basic conditions speed the tautomerization and result in a single peak. Although amide columns have acrylamide groups introduced, analysis has to be made at high temperature because the acrylamide group is not alkaline.

**Figure 6: Anomers and basic conditions**

**Phosphorylated Saccharide Analysis (VT-50)**

**Sample**
- Glucose-6-Phosphate
- Fructose-6-Phosphate
- Glucose-1-Phosphate
- Fructose-1-Phosphate

**Conditions**
- Eluent: 20/80 A:25mM HCOONH4, B:CH3CN
- Flow rate: 0.2 mL/min
- Detector: ESI-MS SIM (-)
- Temp: 40 C

**Figure 7: Phosphorylated sugars (glucose-6-phosphate, fructose-6-phosphate, glucose-1-phosphate, and fructose-1-phosphate) analysis on the VT-50 2D**
The VT-50 series is packed in peek housing, a modification to improve peak shape when analyzing phosphorylated sugars. Stainless steel housing can retain phosphorylated analytes, leading to peak tailing. Figure 4 utilized a MS detector as in-vivo phosphorylated analytes are often present in very low concentrations but the conditions can be used for several detectors including RI, ELSD, and corona CAD.

Shodex HILICpak Columns

- **NH2P Series**: A polyvinyl alcohol base with amino functional groups is a durable option for most sugar analysis

- **VG series**: A polyvinyl alcohol base with modified tertiary amino groups to prevent the absorption of certain reducing sugars due to Schiff base formation.

- **VT series**: A polyvinyl alcohol base with quaternary ammonium functional groups and PEEK housing allows highly sensitive analysis of phosphorylated sugars and anionic substances.

- **VN series**: A diol functionalized polyvinyl alcohol specifically designed for analysis of oligosaccharides.

- **VC series**: A Carboxyl functionalized polyvinyl alcohol column designed for analysis of compounds with amino or ammonium groups.
Detection of Saccharides

The most commonly used approach for detecting saccharides is the use of the differential refractive index (RI) detector, which is based on the refractive index differences between sample components and a mobile phase. Although the RI detector is highly versatile, it exhibits low selectivity and is not suitable for gradient elution. Another drawback of RI detection is that it has slightly lower sensitivity than other types of detectors. When high selectivity and high sensitivity are required, the ultraviolet absorption (UV) detector and the fluorescence (FL) detector are effective. However, saccharides must be derivatized because they lack a structure for absorbing UV or emitting fluorescence. Other available methods of detection include the mass spectrum (MS) analyzer, the electrochemical (EC) detector which electrochemically detects ionized saccharides under alkaline conditions, the evaporation light scattering (ELS) detector which detects saccharides by evaporating an eluent and an irradiating laser, and charged aerosol detector (CAD).

Ligand Exchange – Shodex SUGAR series

A strong feature of the Shodex SUGAR series columns is the ligand exchange mechanism of separation. Ligand exchange refers to a mode of separation based on the interaction (ligand exchange potential) between hydroxyl groups and metal ions to form a complex. Saccharides typically consist of a 5-membered (furanose) or 6-membered (pyranose) ring structure, containing a large number of hydroxyl groups. These hydroxyl groups bind together either equatorially or axially with respect to the carbon plane.

The conformation of the hydroxyl groups differs between various saccharides. Figure 8 depicts the relationship between hydroxyl group conformation and counter ion interaction. In Figure 8 (a), three hydroxyl groups form a complex with the metal ion. In Figure 8 (b), two hydroxyl groups form a complex with the metal ion due to the hydroxyl group conformation. Hence, ligand exchange potential is higher for (a) than for (b). The complex formation potential also differs depending on the kind of metal ion.
Figure 10: Glucose, sorbose, fructose, and gulose were analyzed on the SP0810 under ligand exchange.

Glucose has four stereocenter carbons, resulting in 8 named stereoisomers, each with their own enantiomers. As seen in Figure 9, glucose and its stereoisomer gulose, can be separated by their ligand exchange potential. Differing from HILIC, ligand exchange utilizes elevated temperatures to prevent enantiomer separation.

The Elevated temperate increases the rate of tautermization, collapsing the split peak seen in Row A. Row B displays the peak splitting that can occur without high temperature.
Oligosaccharides

Commonly considered to be composed of 3-10 monosaccharides, oligosaccharides are the smallest of the carbohydrate polymers. As with many of the samples discussed, there are several ways to approach oligosaccharide separations.

HILIC

At their simplest, oligosaccharides are repeating units of a single monosaccharide with no branching. Under HILIC conditions, the elution order of simple is determined by the length of the chain.

![Hydrolyzed Dextran Via HILIC Analysis](image)

As seen in Figure 12, the longer the oligosaccharides are retained longer by the polar HILIC phase. Each additional sugar unit increases the overall polarity of the chain.

Oligosaccharides by Size Exclusion Chromatography

Size exclusion was developed for large polymers but extends to oligosaccharides. As seen below in Figure 13, the largest chain oligosaccharide elutes first.
The order of elution using a KS-802 column (a combination of SEC and ligand exchange) is reversed from HILIC separations. The higher degree of polymerization (DP) prevents the oligosaccharide from travelling as deep into the pore relative to lower DP oligosaccharides.

**Polysaccharides**

**Size Exclusion Chromatography**

Monosaccharides, linked together by glycosidic bonds, can form a variety of long polymer chains grouped under the term polysaccharides. When utilizing SEC it is important to remember that the elution pattern of an analyte correlates to the volume it occupies within the solvent. The choice of appropriate standards with similar behavior the sample is imperative to achieve accurate molecular weight determination.

![Figure 14: Molecular weight compared to volume.](image)
As illustrated in Figure 14, a branched or ionic polysaccharide may occupy the same volume in solution as a straight chained polysaccharide with a molecular weight that is several orders magnitude smaller.

Ionic and other interactions between the polysaccharide and the column packing material can also affect the elution. Techniques to suppress these interactions include the addition of salts and are displayed in Figure 16. Dextran sulfate was analyzed on a mixed bed column (Shodex OHpak SB-806M HQ) to determine the molecular weight using size exclusion chromatography.

![Dextran Sulfate With A Mixed-Bed Column (SB-806M HQ)](image)

**Figure 15: Size exclusion chromatography analysis of dextran sulfate in 0.1 M NaCl aq. on a OHpak SB-806M HQ column.**

The SB-800 series offers single bed columns with sharp pore size distribution for high resolution within a specific molecular weight range (Shodex reports these ranges using the straight chain polysaccharide pullulan). Additionally, featured in Figure 15 is the SB-806M, is an example of a mixed bed column that adds versatility. With a mixture of different pore sizes, the estimated molecular weight range is extended, making these columns ideal when for unknown molecular weight ranges or samples with analytes that have a large variance in molecular weight, eliminating the need to run multiple columns in series.

**Hurdles with Size Exclusion Chromatography**

For the analysis of polysaccharides such as starch, which do not dissolve easily in water, samples can be dissolved in DMSO before SEC analysis with an aqueous an eluent. However, this can be unsatisfactory as undissolved components were left undetected. If a mixed eluent of DMSO/DMF containing salts is used, the solubility of starch is improved allowing a more accurate SEC analysis. There are some cases in which samples have ionic functional groups such as carboxyl...
groups. An addition of a salt to the eluent allows SEC analysis without interference from the ionic groups. Below in Figure 16 an analysis of corn starch was prepared by adding DMSO followed by heating the sample to 120 °C to achieve dissolution. The OHpak SB-806 HQ, a column for aqueous SEC column was used in Figure 16.

![Addition of LiBr to Eluent for Corn Starch Analysis (SB 806 HQ)](image)

**Figure 16:** The addition of LiBr to achieve complete corn starch dissolution followed by SEC analysis on OHpak SB-806M HQ.

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**SEC with Light Scattering Detection**

Refractive index detection has and continues to be an important detector for large polysaccharide analysis but new techniques, such as light scattering, offer analysts complementary and additional information about their molecules. Light scattering (LS) provides measurements for molecular characteristics such as molar mass. Shodex has optimized several series for light scattering through controlled column bleed, allowing the user to gain more information on their sample.
The absolute molecular weight of heparin was determined by OHpak LB-806M, an aqueous SEC column (Figure 17). The LB-800 series are ideal for multi angle light scattering (MALS) detectors. By controlling the column bleed, the baseline noise has been minimized, enabling MALS detectors to detect low molecular weight compounds which are difficult to be detected by conventional columns (Figure 18).
Organic acids
Ion exclusion, reverse phase, and HILIC are applicable to organic acid separations. Each has its unique conditions and driving mechanisms.

Ion exclusion mode
In organic acid analysis, strong cation exchange resin with sulfo-groups bound to the packing surface are used. Weak acids, such as organic acids, exhibit only partial dissociation when dissolved in water. In a dissociated state (negatively charged), the positive charge of the cation is neutralized and excluded by the negative charge of the sulfo-groups on the resin surface. There is no concern over organic acid adsorption into the resin. In the undissociated state ion exclusion does not occur; hydrophobic adsorption into the resin substrate occurs instead to allow organic acids to be retained by the resin (Figure 19).

![Figure 19: Basic ion exclusion diagram](image)

Organic acids are eluted in ascending order of pKa (descending order of acidity) and in descending order of polarity.
Figure 20: Succinic acid, lactic acid, formic acid, acetic acid, proplanic acid, isobutyric acid, n-butyric acid, isovaleric acid, and n-valeric acid was analyzed on the Shodex KC-811 column in 2mM HClO4.

When analyzing organic acids using RSpak KC-811, the elution time of organic acids with strong hydrophobicity can be shortened by adding 10% acetonitrile to the eluent.

Organic Acids by Reverse Phase

The reversed-phase mode is the most commonly used separation mode of HPLC. This mode is based on the hydrophobic interaction between the portion of low polarity of the packing and the portion of low polarity of the sample. Hence, elution occurs in descending order of polarity. A key to successful organic acid analysis in reversed-phase mode resides in thoroughly suppressing the dissociation of organic acids and reducing the polarity of the sample. Organic acids are generally reported to become non-dissociated when the eluent has a pH value lower than the pKa by 1.5.
Organic Acids via HILIC

While reverse phase organic acid separations rely on acidic conditions, HILIC separations use basic conditions to fully dissociate the organic acid. This increases the polarity of the acid and allows them to be retained by highly polar HILIC columns.

Figure 21: Glyoxylic acid, tartaric acid, malic acid, lactic acid, malonic acid, acetic acid, sOrganic Acids via Reversed Phase Analysis

Figure 22: Organic Acids Via HILIC Analysis
Organic Acid analysis is also commonly run in water rich environments, which is not convenient for LC/MS. HILIC analysis, via the VT-50, utilizes a large amount Acetonitrile in the eluent, allowing the user to take advantage of the sensitivity of Mass Spectroscopy (all samples in Figure 18 are 100 ppb).

**Artificial sweeteners**

**LC/MS Analysis of Various Sweeteners (NH2P-40 2D)**

Various sweeteners were analyzed using Asahipak NH2P-40 2D, a polymer-based amino column, with LC/MS/MS detection. Anion substances can be eluted under alkaline conditions, as alkaline conditions promote the deprotonation of hydroxyl groups. Alkaline conditions are suitable for high sensitive detection of the substances with hydroxyl groups such as saccharides under the negative mode.

**Complex samples**

**Mass spectroscopy + HILIC**

LC columns optimized for MS detection offer the versatility to look at many families of compounds with a single injection. Shodex has optimized its HILIC columns for highly sensitive detectors like MS and the ability to tolerate large pH ranges (2-13) adds enhanced capabilities.
Figure 23 displays examples of the analysis of saccharides on Shodex HILIC polymer columns. A large variety of compounds can be separated (including saccharides, organic acids, and amino acids) via ESI-MS simultaneously. Additionally, all Shodex polymer HILIC series are optimized for high sensitive detectors like Corona Charge Aerosol (CAD), Evaporative Light Scattering (ELSD) and LC/MS.
The SH1821 utilizes Size Exclusion Chromatography to separate the neutral and larger sugars while also separating organic acids through ion exclusion chromatography.
## Appendix

### Shodex Columns for Saccharide Analysis

<table>
<thead>
<tr>
<th>Product Code</th>
<th>Product Name</th>
<th>Counter Ion</th>
<th>Separation Mode(^1)</th>
<th>Exclusion Limit (Pullulan)</th>
<th>Theoretical Plate Number (TP/column)</th>
<th>Particle Size (μm)</th>
<th>ID x Length (mm)</th>
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</thead>
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<td>SUGAR SH1011</td>
<td>H(^+)</td>
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\(^1\): SEC (Size exclusion), IEX (Ion exclusion), LEX (Ligand exchange), NP (Normal phase)

### Shodex Columns for Polysaccharide Analysis

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<th>Product Code</th>
<th>Product Name</th>
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<th>Usable Organic Solvents (Max,%)</th>
<th>Theoretical Plate Number (TP/column)</th>
<th>Particle Size (μm)</th>
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\(^1\): See note above