HILIC Separation of Carbohydrates using BEH Amide Particle Technology

by Kevin Jenkins, Waters Corporation, 34 Maple Street, Milford, MA U.S.A. 01757.

Separation scientists are challenged to develop carbohydrate assays that are both rapid and direct. Even ‘simple’ sugars and carbohydrates present separation challenges because of their structural and chemical differences due to chirality, anomer formation and their presence in a broad range of sample types [1-6]. For complex carbohydrates that are built upon the diverse arrangement of the simple sugar monomers, the separation challenge is seemingly unlimited by the scope and the diversity of monomer sugar units within the carbohydrate moiety. The separation mechanisms used are equally diverse and many approaches deal with complex interactions such as hydrophilic interaction, size-exclusion, ion-exclusion, and hydrophobic retention [6-10].

By taking advantage of the unique properties of the sugar molecule and structural characteristics of its functional groups, retention mechanisms can be chosen that improve carbohydrate retention and selectivity. Traditional HPLC approaches use cationic stationary phases, with calcium-based resins being the most common, and refractive index (RI) detection for quantification. However, phases such as Pb²⁺, and Ag²⁺ resins are commercially available that allow unique selectivity compared to calcium-based resins. These diverse column choices not only add complexity to the assay, but column lifetime from these columns is often limited in a routine setting.

As an alternative, silica-based amino phases are commonly used to separate sugars through weak ion-exchange interactions [9-12]. At higher mobile phase pH ranges, the weakly acidic nature of the hydroxyl groups have the potential to interact with the amino functionality on the sorbent, however, the stability of the chromatographic phase is greatly reduced [12]. For this reason, hydrophilic interaction chromatography (HILIC) separation mechanisms have been used extensively as an alternative separation mode, spurring the development of zwitterionic chromatographic phases [8,9]. In solution, reducing sugars such as the common dietary sugars galactose, glucose and fructose can freely mutarotate. The mutarotation creates equilibrium between their respective α and β anomers that creates a major challenge for the separation. At low pH, this mutarotation between the two forms is relatively slow in comparison to the mass transfer kinetics of the chromatographic separation, which allows the ability to separate the two anomic forms. In practice, this resolution is undesirable as it can result in multiple peaks for one analyte, which complicates both quantification and identification. Partial separation is just as deleterious because of peak broadening and tailing leading to lower separation efficiency for complex sample matrices [8].

There are many different techniques to collapse reducing sugar anomers into single quantifiable chromatographic peaks. Silica-based alkylamine phases are commonly chosen to separate mono- and di-saccharides but suffer from poor column lifetimes [9-12]. The amine functionality on the bonded phase creates a local high pH environment that is advantageous for collapsing the sugar anomers by increasing the kinetics of interconversion between α and β forms [9]. This effectively creates one chromatographic peak that greatly simplifies the analysis. However, the disadvantage of the localised alkaline environment, which is created by the aminopropyl ligand, is that the stationary phase rapidly self-deteriorates by dissolving the silica particle to which the ligand is bonded. This results in substantial column bleed, loss of performance and ultimately short column lifetimes.

Polymer-based amino columns overcome this issue by increasing the stability of the packed bed under alkaline conditions. Unfortunately, separation efficiency is reduced compared to its silica-based counterparts but this can often be overcome.
by increasing the column temperature [9]. The increase in column temperature improves the separation in two ways: the mass transfer of the analyte between the mobile phase and stationary phase increases, thus increasing column efficiency; and, the increased kinetics of the sugar mutarotation facilitates the collapse of the sugar anomers into a single chromatographic peak. The often overlooked disadvantage of this approach is the Schiff base reaction that occurs inside the column that will eventually reduce column lifetime. The chemical reaction and formation of the Schiff base is a consequence of the opening of the ring structure as the reducing sugar interconverts between anomeric forms. This mechanism (Figure 1) allows the reducing sugar to chemically bond with the highly reactive amine group on the stationary phase. This can have the effect of reducing column lifetime, reducing the number of chromatographic sites available over time and in extreme cases, loss of reducing sugar peaks as the sugars are sequestered by the stationary phase. An example of this can be shown in Figure 2, where the Schiff base formation significantly reduces the recovery of sugars using amine-based columns for the separation. This reaction is highly dependent on temperature and as the temperature of the separation was increased the amine-based columns showed considerable loss in analyte recovery due to imine formation. By preventing this reaction mechanism from occurring, such as the case with an amide-based stationary phase, imine formation was eliminated and full recovery of the reducing sugars was achieved with improved separation efficiency at elevated temperatures [8]. Carbohydrates are found in natural products, which include foods, beverages, and biological samples and the impact of these sample matrix constituents can further impede quantification [3,4,6]. Removing the salts and other polar matrix components that are found in these samples can be very difficult since the interferences and the carbohydrates of interest are both very polar. The impact of polar matrix components on the quality of a sugar separation for a sample containing high salt concentration is shown in Figure 3. In extreme cases, like the polymer-based amino column shown in

Figure 2: The impact of Schiff base formation on the quantification of 1 mg/mL reducing sugar standards. Conditions: Column 1: YMC-Pak Polyamine II, 5 µm, 4.6 mm x 150 mm; Injection Volume: 10 µL; Mobile Phase: 75% acetonitrile/25% water (v/v); Flow Rate: 1.4 mL/min. Column 2: ACQUITY UPLC BEH Amide, 1.7 µm, 2.1 mm x 150 mm; Injection Volume: 2 µL; Mobile Phase: 75% acetonitrile/25% water (v/v); Flow Rate: 0.29 mL/min. Temperature as indicated in the figure. Peak Identification: 1) fructose; 2) glucose; 3) sucrose; 4) maltose; 5) lactose. All standards are 1 mg/mL prepared in mobile phase.

Figure 3: The effect of matrix constituents using amino-based columns for sugar separations. The charged surface of the amine-based columns created unwanted retention of the matrix constituents in the sample (shown as asterisked peaks in the chromatograms), which created problems for accurate quantification. Conditions: Column 1: Nova-Pak NH3, 4 µm, 4.6 mm x 150 mm; Column 2: YMC-Pak Polyamine II, 5 µm, 4.6 mm x 150 mm; Column 3: apHera Amino, 5 µm, 4.6 mm x 150 mm; Injection Volume: 10 µL; Mobile Phase: 75% acetonitrile/25% water (v/v); Flow Rate: 1.4 mL/min; Temperature: 35°C.
Figure 3, matrix coelution with the fructose peak can make quantification ineffective without further sample preparation. In this case, the weakly cationic surface of the amino phase, which was advantageous for sugar retention, has retained unwanted ionic interferences from the sample as well. When a more neutral ligand was chosen along with an alkaline mobile phase additive, many of the polar cationic and anionic interferences lost retention and eluted in the column void volume.

An example of this approach is shown in Figure 4. The triethylamine (TEA) mobile phase additive was used for the separation to provide the high pH environment, which helped maintain good peak shape. The neutral BEH amide ligand was effectively used to reduce the ionic interactions and decreased the chromatographic impact of polar matrix constituents in the sample. For this application, the high pH stability of the hybrid amide particle provided significant advantages over the silica-based materials without decreasing column lifetime.

This paper describes a HILIC approach for carbohydrate analysis using an organofunctional amide stationary phase that has been developed to overcome many of the separation challenges that are experienced with silica-based column choices. Methods that use amide-derived phases minimise the effect of salt interferences, anomer mutarotation, and Schiff base formation at elevated temperatures [8]. Further to this, detection methods are migrating away from traditional refractive index instrumentation to mass spectrometry (MS) and evaporative light scattering (ELS) detection, which allows the analyst to take advantage of the ability to operate under gradient mobile phase conditions and to greatly improve their ability to quantify complex sample types.

Experimental

Carbohydrate standards were prepared at a concentration of 1 mg/mL in 50/50 (v/v) acetonitrile/water. All beer samples were prepared and diluted to 50/50 (v/v) with acetonitrile unless otherwise noted in the text. All dissolved or diluted samples were filtered using a 0.45 µm polyvinylidene fluoride (PVDF) syringe filter (Acrodisc, Pall Life Sciences). Fructose, glucose, sucrose, lactose, maltose, and maltooligosaccharides are commercially available reagents (99+ % purity) that were purchased through Sigma-Aldrich. HPLC grade acetonitrile (Optima Purity Grade, Fisher Scientific) was used without further preparation. Water was purified.
using a Milli-Q System (EMD Millipore) with a cation-exchange cartridge.

All separations were performed on an ACQUITY UPLC System (Waters Corporation) equipped with evaporative light scattering detection. A variety of column types were used for the sugar separations. For the alkylamine columns that were packed with 4 µm and 5 µm particles [YMC-Pak Polyamine II, 5 µm, 4.6 mm x 150 mm (YMC America, Inc); aPherA Amino, 5 µm, 4.6 mm x 150 mm (Sigma-Aldrich Corporation); Nova-Pak NH2, 4 µm, 4.6 mm x 150 mm (Waters Corporation)] the samples were chromatographed using an isocratic mobile phase (75% acetonitrile/25% water (v/v)) at a flow rate of 1.4 mL/min. The column temperature was maintained at 35°C. The injection volume was 10 µL.

The amide-based columns were used at elevated mobile phase pH to collapse the sugar anomers. The isocratic mobile used for the XBridge BEH Amide, 5 µm, 4.6 mm x 150 mm (Waters Corporation) was 75% acetonitrile/25% water with 0.1% TEA (v/v/v) at a flow rate of 1.4 mL/min. The column temperature was maintained at 35°C. The injection volume was 10 µL. To increase separation efficiency, an ACQUITY UPLC BEH Amide, 1.7 µm, 2.1 mm x 100 mm column was used with gradient mobile phase conditions. The mobile phase gradient was a 10 minute gradient from 75% acetonitrile/25% water/0.2% TEA (v/v/v) to 45% acetonitrile/55% water/0.2% TEA (v/v/v) running at a flow rate of 0.13 mL/min. The column temperature was maintained at 35°C. The injection volume was 1.3 µL.

**Results and Discussion**

Some important application areas for carbohydrate analysis include characterising of raw materials, in-process sample monitoring, and quality control of finished products. Each of these outlined areas can present unique challenges that can hinder the chromatographic separation, final analysis and interpretation of the sample data. As a model of the approach, a beer fermentation process was monitored to determine yeast activity, fermentation, and product comparison for quality control.

Beer production can be divided into three major processes. The first critical step is the liquid extraction of the complex carbohydrates and starches from the malted barley. Many of these complex carbohydrates impart the flavour characteristics and mouthfeel that is characteristic of the particular beer style that is desired. The majority of the complex carbohydrates are converted into simple sugars, primarily maltose, through enzymatic digestion. This initial starch conversion controls, to some degree, the strength of the final product because the yeast to make alcohol converts the fermentable sugar content in the mash. Once the fermentation is complete, the beer is allowed to condition prior to final packaging. In a commercial setting, strict control of each of these major steps ensures consistent product quality.

Efficiency of the enzymatic conversion is critical to the maltose production. The enzymes in the malted barley require optimum temperature and time for complete sugar conversion. Too little time limits this conversion, while too much time can create oxidation by-products without the benefit of improving fermentable sugar yield. For the example shown, the maltose concentration reaches its maximum within 90 minutes. The additional processing steps shown stabilise the sugar content in the extracted wort prior to fermentation. The fermentation was completed in less than 3 days. Figure 5 shows a visual summary of the sampling points that were monitored during the production process, while Figure 6 summarises the results of the process to show the extraction, sugar conversion and fermentation of a typical ale-style beer.

Due to the complexity of the sample matrix, gradient separation conditions were used to separate the simple yeast-digestible sugars from the more complex carbohydrates found in beer. Figure 7 demonstrates the separation of different beer styles based on their carbohydrate profile. The chromatographic conditions for these separations are as follows: ACQUITY UPLC BEH Amide, 1.7 µm, 2.1 mm x 100 mm; Injection Volume: 1.3 µL; Mobile Phase: 10 minute gradient from 75% acetonitrile/25% water/0.2% TEA (v/v/v) to 45% acetonitrile/55% water/0.2% TEA (v/v/v); Flow Rate: 0.13 mL/min; Temperature: 35°C. The beer samples were directly injected into the chromatograph without further dilution or sample preparation. Peak Identification: 1) fructose; 2) glucose; 3) sucrose; 4) maltose; 5) maltotriose. The standards were prepared in mobile phase at the indicated concentration.
in the sample. Traditional chromatographic methods for carbohydrate analysis utilise refractive index detection, which is neither sensitive nor compatible with gradient elution chromatography. For the beer samples shown, evaporative light scattering detection was used, which provides a more universal and sensitive detection technique compared to RI detection. The main advantage of RI detection is its wide linear response; however, the increased sensitivity of ELS detection with the compatibility gradient elution provides a well-suited approach for complex assays, such as the beer example shown.

As a follow-up to this study, several beer styles were analysed based on their unique carbohydrate profile. A chromatographic comparison is shown in Figure 7 that provides the mono-, di-, and tri-saccharide content of different beer styles. As expected, the concentration of the monosaccharides in the final product is small due to their consumption during fermentation. However, the more complex carbohydrates that were non-fermentable remain to impart unique profiles that are indicative of the particular beer style. Figure 8 shows, in more detail, the gain adjusted chromatogram of the trace level sugar content in a stout style beer sample.

**Conclusion**

There are many different methods to chromatographically separate carbohydrates including HILIC, ion/ligand exchange, ion chromatography, size exclusion and combinations of these various separation modes. Each of these separation mechanisms comes with certain advantages and disadvantages. Modern chromatographic phases, such as the hybrid bonded amide ligands can be used with modern chromatographic detection techniques such as UV, ELS, fluorescence and mass spectrometry to allow direct carbohydrate quantification. The methods demonstrated in this paper do not require sample derivatisation, post-column additive addition, or difficult sample processing procedures to provide cycle times suitable for high throughput analysis. The BEH amide chemistry maintains a neutral charge state under the recommended high pH mobile phase conditions. This eliminates salts co-eluting with the carbohydrates of interest to provide higher confidence in the chromatographic results.

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**References**