Bioethanol Production Monitoring using Ion Exclusion HPLC with Rezex™ ROA Column

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Introduction
The dramatic increase in the price of fossil fuels as well as growing concerns over greenhouse gas emissions has led to an enormous interest in ethanol production from biological sources.1 While there is considerable interest in generating ethanol from cellulose based sources, the majority of current bioethanol manufacturing is focused on generating ethanol from starch based foodstuff such as corn or wheat. In the United States alone, there are 141 plants in operation as of 2007 with an additional 72 planned in the next two years.2

The current process for generating ethanol from biological sources relies on the use of amylase enzymes to break down complex starches in foodstuff to simple sugars, followed by yeast fermentation to convert the sugar to ethanol. Regular monitoring of the process by HPLC allows operators to track breakdown of starches to simple sugars as well as monitor ethanol production after yeast has been introduced. In addition, organic acids are also monitored in the HPLC run, allowing operators to assess if microbial contamination is affecting the fermentation process and, if additional remediation steps such as antibiotic addition are necessary to maximize ethanol yield.3

The HPLC method used for bioethanol monitoring, ion exclusion chromatography, is a method that uses several different separation modes (gel filtration, ion exchange, and reversed phase) to separate compounds of interest. This separation method has been around for over 20 years, yet still remains the most popular method for fermentation monitoring due to the ability to separate different classes of compounds (sugars, organic acids, and alcohols) all in one chromatographic separation.4 The method is a simple and rugged isocratic method using a dilute acid mobile phase, however some sample preparation of the fermentation broth is required to ensure good chromatographic performance and reasonable column lifetime.

In addition to the growing number of ethanol plants, many plants are also expanding their operations by adding additional fermenters. Such additions require increasing throughput of the current analytical methods for fermentation monitoring as groups wish to monitor more fermentation runs with existing HPLC equipment. Because the current ion exclusion HPLC method is a multimodal separation, there are significant limitations on increasing throughput of the method. However, some minor changes can be implemented that can reduce the analysis time by up to 50 % (from 24 minute to 12 minute run time). In this technical note the basic principles of the ion exclusion HPLC analysis will be discussed. Sample preparation methods to ensure proper sample cleanup and minimize instrument down time will be reviewed.

Materials and Methods
Analyses were performed using a Shimadzu LC-20AT LC system (Shimadzu Scientific Instruments, Columbia, MD, USA) equipped with a SIL-10AF autosampler, degasser, and a RID-10A RI detector; data was collected using CLASS-VP Version 7 Software. Various dimensions of Rezex ROA columns (Phenomenex Inc., Torrance, CA) were used (150 x 7.8 mm, 300 x 7.8 mm). Guard columns were SecurityGuard™ Carbo-H+ 4 x 3.0 mm cartridges (Phenomenex, Torrance, CA, USA). Aqueous mobile phase (0.005 N Sulfuric Acid in water) was purchased from CHATA (Mt. Collins CO, USA). Several samples from various fermentation timepoints were generously provided from ICM (Colwich, KS, USA). Ethanol HPLC testing standard was obtained from Midland Scientific (Omaha, NE, USA).

Crude samples from fermentation timepoints were filtered using a 0.20 µm Phenex™-RC syringe tip filter (Phenomenex, Torrance, CA). Filtered aliquots of 10 µL were injected on HPLC operating at a flow rate of 0.6 mL/min and the HPLC column was heated to 65 °C. Run time was 24 minutes for the 300 mm length column and 14 minutes for the 150 mm length column. SecurityGuard cartridges were regularly changed every 100 runs or whenever static backpressure increased 10 % above initial backpressure values. 50 % methanol was used in the autosampler needle wash to avoid bacterial contamination.
Results and Discussion

HPLC run of a fermentation standard using the Rezex ROA 300 x 7.8 mm column is shown in figure 1. Note that the early eluting peaks (Dp4, Dp3, Maltose, Glucose) represent the different degrees of polymerization of the various saccharides present in the sample. Monitoring of these peaks during early time points of the fermentation run gives operators a good indication as to the progression of the various amylases used to break down starches to simple sugars, and dictate when yeast is added to the fermentor to start generating ethanol. The later eluting peaks (Lactic acid, Glycerol, Acetic acid, Ethanol) represent the organic acids and alcohols generated during the fermentation. Monitoring of these peaks gives an operator an indication as to the fermentation endpoint as well as indicate when bacterial contamination is severe enough to warrant addition of an antibiotic to limit bacterial by-products that may inhibit ethanol production.

While the standard in figure 1 may show the idealized separation of compounds, a more typical example of different timepoints run on the 300 x 7.8 mm column is shown in figure 2. Early in the fermentation, the oligosaccharide and saccharide peaks can be so abundant that resolution between components is reduced; later in the run, as sugars are converted into ethanol, resolution of the saccharides is more in line with what is seen in the HPLC standard. Conversely, early in the process, ethanol, glycerol, and organic acid peaks often are below detection limits and increase as fermentation progresses.

Resolution of key components will also tend to decrease over time as sample contaminants build up on the column; key to maintaining resolution and increasing column lifetime is using a guard column system like the SecurityGuard guard cartridge system. Regular replacement of the guard cartridge is indicated when a significant decrease in resolution or increase in system backpressure is noted. Filtration of sample timepoints with a syringe tip filter can increase the lifetime of both the guard and analytical column, as well as reduce chromatographic interferences due to particulates.

As some groups have increased the scale of their ethanol production and may now be operating several fermentors in parallel, there is an interest in reducing the analysis time for fermentation monitoring. Since the ion exclusion HPLC run is isocratic, few parameters can be employed to reduce run time. Mobile phase and temperature modifications have little effect on the critical oligosaccharide peak separations early in the chromatogram. Flow rate can be increased slightly to reduce run time, however one must factor in the maximum backpressure of the Rezex HPLC columns (600 psi) as well as reduced lifetime when columns are run close to their maximum backpressure.

One method for reducing HPLC run time is by reducing the length of the Rezex column used. An example is shown in Figure 3 where the fermentation standard is run on a 150 x 7.8 mm (half the length of the typical 300 mm column used). As expected, the run time using a shorter column is significantly reduced from 24 to 13 minutes. If one looks closely, while the resolution of the late eluting organic acid and alcohol peaks are acceptable, the early eluting oligosaccharide and saccharide peaks are significantly reduced. Since the separation of oligosaccharides is based primarily on a gel filtration mechanism, there is a limitation on how much the column length can be shortened and still maintain resolution of key saccharide peaks. Examples of a late timepoint for a fermentation run using the 150 x 7.8 mm is shown in Figure 4. Quantitation of the Dp4⁺ and Dp3 peaks at early timepoints may be compromised due to poor resolution of these abundant peaks. However, in later timepoints the reduced levels of the Dp4⁺ and Dp3 peaks may allow for accurate quantitation.

While the analysis of early timepoints from a fermentation run may need the increased resolving power of the longer 300 x 7.8 mm Rezex ROA column, it may be practical to use a 150 x 7.8 mm Rezex ROA column for later timepoints, where saccharide peaks are smaller. This is most practical in a larger operation where multiple fermentors are used and multiple HPLCs might be used for monitoring. Alternatively, for sites where only one HPLC is used, a column-switching valve might be employed to switch to the shorter column later in a fermentation run.

Conclusions

The standard HPLC method used for fermentation monitoring of bioethanol production is a simple yet powerful method for monitoring saccharides, organic acids, and alcohols generated during production. Simple steps such as using guard columns and filtering samples can greatly increase the reliability of the method as well as improve column lifetime (thus reducing analysis cost). For larger operations, shorter columns can be used in some circumstances to reduce analysis time for monitoring; potentially reducing the need for additional analytical equipment.

References
2. American Coalition for Ethanol; www.ethanol.org

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