Technical Note 147

UHPLC Analysis of 2-AB-labeled Dextran Ladder and Assignment of Glucose Units to Unknown Glycans

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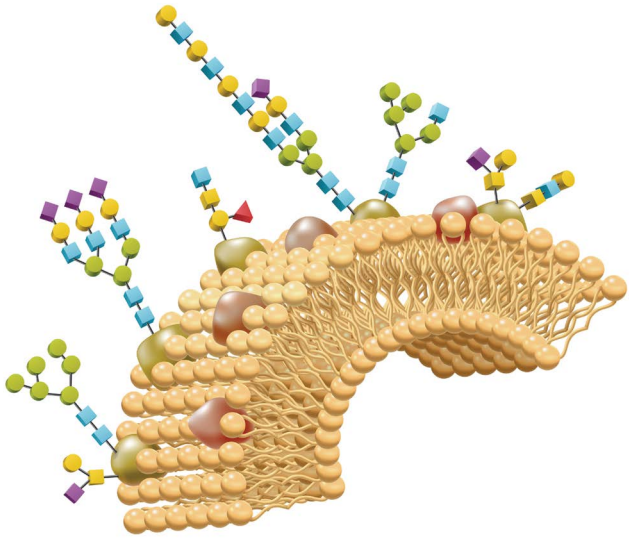
Key Words
Chromeleon 7.2 CDS, HILIC, Biomolecules, Dextran, UltiMate 3000 BioRS, Accucore

Introduction

Analysis of glycans labeled with 2-aminobenzamide (2-AB) by hydrophilic interaction liquid chromatography (HILIC) is a common and effective technique used in the field of glycomics and bio-pharmaceutical analysis. HILIC separation of 2-AB-glycans is usually accomplished with amide-bonded stationary phases and the labeled glycans can be detected by fluorescence with excellent sensitivity. The labeling occurs at the reducing end of the oligosaccharide, which ensures each glycan will react with only one 2-AB molecule, making this approach also suitable for quantitative analysis.

The hydrophilicity of a 2-AB glycan increases with the molecular weight, hence with the number of carbohydrate monomers. Because the main retention mechanism of 2-AB-glycans with amide columns is hydrophilic interaction, larger glycans will be more retained than smaller ones. A typical example is the retention of linear glucose oligomers, where the retention time increases with the number of monomeric units.

The number of monomers in linear oligomers of glucose is often expressed as Glucose Units (GU). The dependence of the logarithm of GU on the retention time can be described by a polynomial equation. Normally a 5th order equation is considered sufficiently informative to describe this relationship. The calibration curve is obtained by the analysis of mixture of linear glucose oligomers obtained by the partial hydrolysis of dextran, which is normally referred to as a dextran ladder. It is custom to use this calibration equation to express the relative retention time of any 2-AB-glycan in terms of GU values. The GU value is glycan-specific, and can be stored in libraries; those libraries can be used for structure assignment of unknown glycans, without the need of mass spectrometry analysis. With this approach, once potential structures are identified, they are confirmed with a series of exoglycosidase digestions followed by HPLC re-analysis of the digested glycans.



In any workflow that relies on GU measurement, high retention time precision is mandatory in order to obtain a reliable calibration curve, and to correctly assign GU values to the unknown glycans afterward.

In this paper we propose a solution for assignment of GU values to unknown glycans using HILIC separation and fluorescence detection. The solution includes:

- Thermo Scientific™ Dionex™ UltiMate™ 3000 BioRS system for high retention time precision analysis
- Thermo Scientific Accucore 150-Amide-HILIC 2.6 µm column, 2.1 × 150 mm, for high efficiency separation of 2-AB-glycans
- Thermo Scientific™ Dionex™ Chromeleon™ 7.2 Chromatography Data System (CDS) software, that facilitates the construction of the calibration curve followed by assignment of GU units to unknown 2-AB-glycans

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Goal

Provide a solution for analysis of 2-AB-glycans based on the GU assignment approach.

Experimental

Preparation of 2-AB Glucose Linear Oligomers Standard (Dextran Ladder)

Prepare the reaction mixture as following. First mix 50 µL acetic acid with 350 µL dimethylsulfoxide. Then dissolve 5 mg of 2-aminobenzamide and 6 mg of sodium cyanoborohydride in 100 µL of the acetic acid/DMSO mixture. Dissolve 200 µg of dextran ladder in the reaction mixture and keep at 65 °C for 3 h. Afterward store the solution at –20 °C. A post-reaction cleaning procedure is not mandatory. Before injection, dilute the sample 20 fold in 9/1 acetonitrile/ammonium formate buffer. Adjust the concentration and pH to 0.1M and pH 4.5.

Instrumentation

- Dionex UltiMate 3000 BioRS UHPLC system equipped with:
  - LPG-3400RS pump (P/N 5040.0036)
  - FLD-3400RS fluorescence detector (P/N 5078-0020)
  - WPS-3000 TBRS (P/N 5841.0020)
  - TCC-3000RS (P/N 5730.0000)
- Chromeleon 7.2 CDS software

Conditions		
Column:	Accucore 150-Amide-HILIC 2.6 µm, 2.1 × 150 mm (P/N 16726-152130)	
Mobile Phases:	A. Ammonium formate 0.1 M pH 4.5 B. Acetonitrile	
Gradient:	Time (min)	%B
	–20	80
	0	80
	40	50
	45	50
Flow Rate:	0.4 mL/min	
Temperature:	40 °C (column compartment)	
Inj. Volume:	5 µL	
Inj. Wash Solvent:		
Fluorescence Det:	2 µL flow cell	
Configuration:	Ex. 320 nm, Em 420 nm, Sensitivity 8, Filter wheel 370 nm Data collection rate 2Hz, Response time 4 sec	

Chromeleon 7.2 CDS GU eWorkflow

eWorkflow™ is a Chromeleon 7.2 CDS software feature that combines all the elements required for a specific workflow to record and analyze the data. A dedicated eWorkflow for GU analysis has been created, which provides a predefined sequence structure, as well as the elements required for data processing (Figure 1). The Processing Method contains additional columns to assign GU values to the individual components in the calibration standard. These GU values are then used to create the calibration curve used for the automatic calculation of GU values for unknown samples in the Report Template.

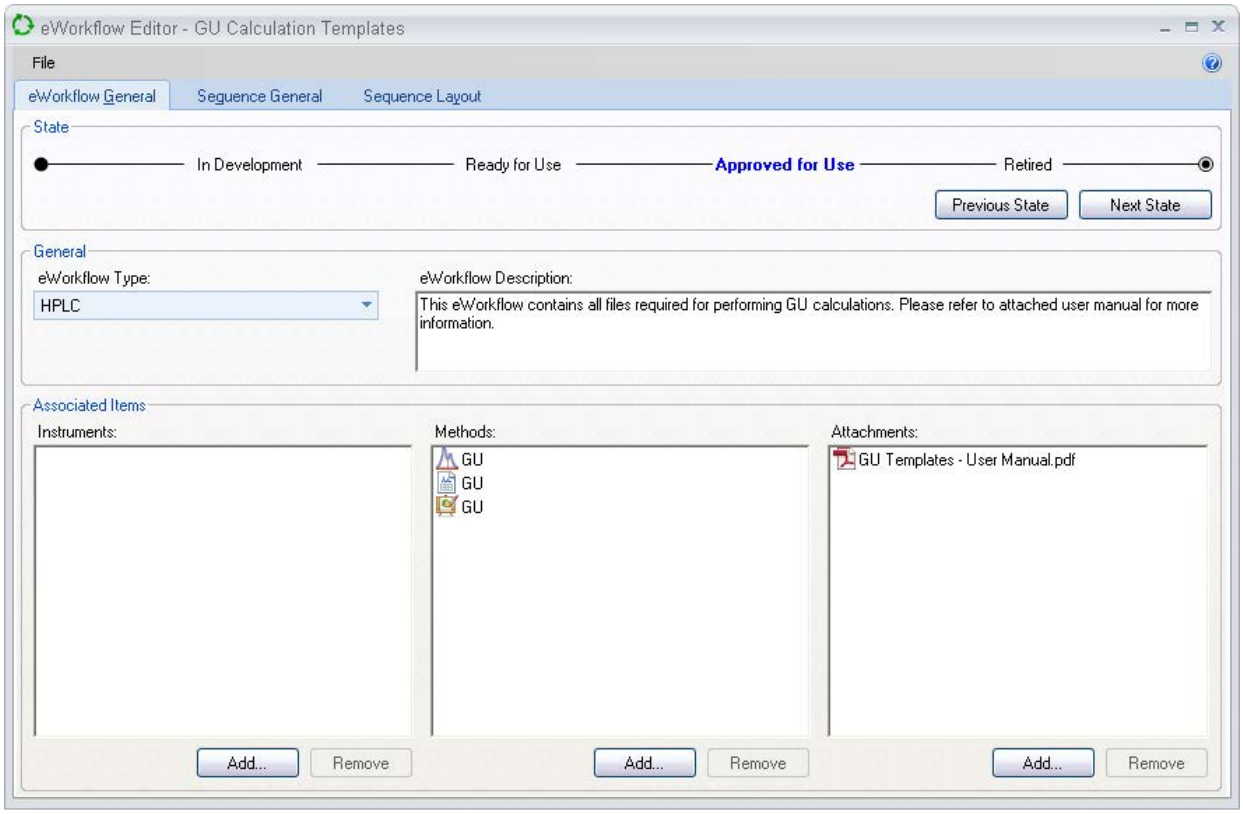


Figure 1. eWorkflow general page of the GU calculation template.

Results and Discussion

The sequence was created using the GU Calculation eWorkflow with 3 repeated injections of the calibration standard (the dextran ladder) and 2 repeated injections of the unknown sample. The raw data were processed using the Processing Method included in the eWorkflow. An example of separation of glucose ladder is visible in Figure 2. This Processing Method is designed in a generic way, containing a default component table with glucose units from 1 to 20 at arbitrary retention times. The actual retention time of the components of the 2-AB-glycans depends on the column type (dimension, particle size, vendor, etc.) and other chromatographic conditions, such as system dwell-volume for instance. Moreover, the peak width also depends on these variables; therefore the first step in data analysis is to assign correct windows to each GU value. Chromeleon 7.2 CDS software allows a quick method to adapt any peak retention time and corresponding window easily by a drag-and-drop mouse action in the chromatogram (Figure 3). Here the original processing method did not assign the correct GU value to Peak 4, because the expected retention time window is centered at 12.265 minutes (retention time window is 0.4 minutes). The actual retention time of peak 4 is 13.033 minutes, hence outside the peak window originally associated to GU5. By clicking and holding the red bar, the original peak window can be dragged (in this example to the right) above peak 4. This correction is then saved and will apply to all the data analysis in this sequence.

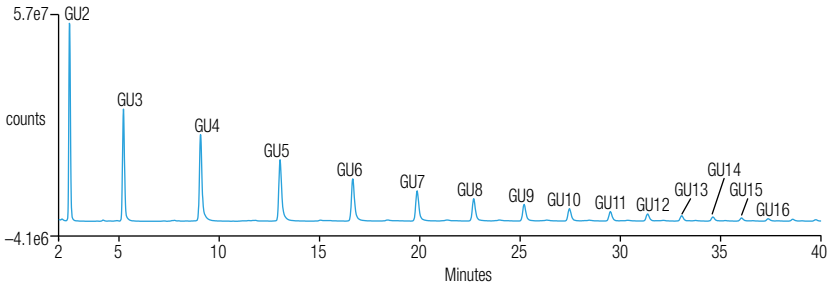


Figure 2. Chromatogram of a 2-AB-labeled dextran ladder with assigned GU values.

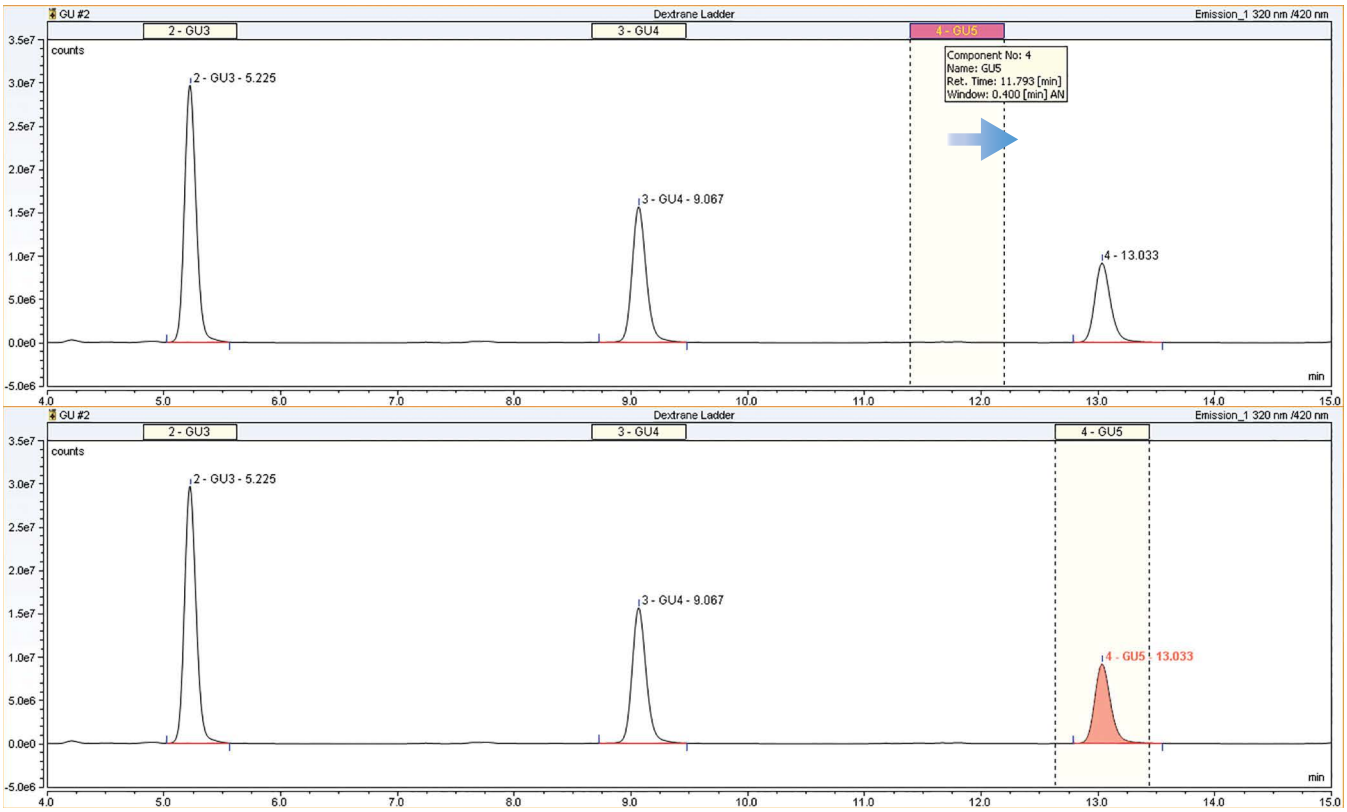


Figure 3. Easy changing of the processing method by drag-and-drop mouse action.

Data obtained from the triplicate dextran ladder injection are used to create the calibration plot displaying the relation of the Retention Time versus ln(GU) (Figure 4); the data points are automatically interpolated with a 5th order polynomial equation, defined in the Processing Method. The calibration curve will be used to assign the GU values to unknown peaks. The report sheet with GU value assignment is given in Figure 5, where the GU value for all peaks is given. These values can be used for assignment of putative structure by matching GU values to existing databases.

GU Calibration

General Information

Sequence:	GU_sample-evaluation
Sequence Directory:	Projects\0034 - Glycan (GU Analysis) with CM7

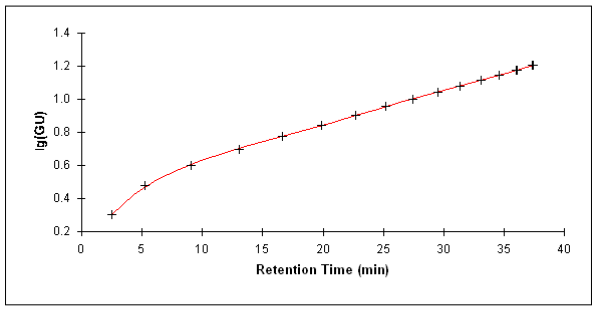
Calibration Settings

Number of Calibration Points:	45
Calibration Type:	Poly5

Calibration Coefficients

C0	C1	C2	C3	C4	C5
0.0669	0.1146	- 0.0092	0.0004	0.0000	0.0000

Calibration Plot



Calibration Points

Peak Number	Peak Name	GU	Log(GU)	RT (Repl. 1) [min]	RT (Repl. 2) [min]	RT (Repl. 3) [min]	RT (Repl. 4) [min]
1	GU2	2	0.301	2.533	2.533	2.533	n.a.
2	GU3	3	0.477	5.225	5.225	5.217	n.a.
3	GU4	4	0.602	9.067	9.067	9.058	n.a.
4	GU5	5	0.699	13.033	13.033	13.025	n.a.
5	GU6	6	0.778	16.658	16.658	16.650	n.a.
6	GU7	7	0.845	19.867	19.867	19.850	n.a.
7	GU8	8	0.903	22.683	22.683	22.675	n.a.
8	GU9	9	0.954	25.200	25.192	25.192	n.a.
9	GU10	10	1.000	27.458	27.450	27.450	n.a.
10	GU11	11	1.041	29.500	29.492	29.492	n.a.
11	GU12	12	1.079	31.358	31.350	31.342	n.a.
12	GU13	13	1.114	33.058	33.042	33.042	n.a.
13	GU14	14	1.146	34.608	34.600	34.600	n.a.
14	GU15	15	1.176	36.042	36.033	36.025	n.a.
15	GU16	16	1.204	37.367	37.350	37.358	n.a.

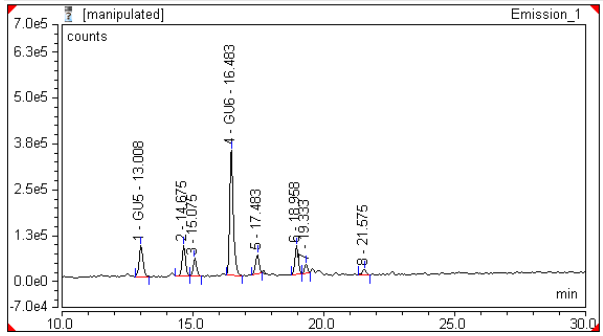
Figure 4. View of the Calibration tab of the Report Designer: 5th order calibration curve obtained from the repeated injections for the dextran ladder standard.

Sample Report

Sample Details

Sample Name:	Sample	Processing Method:	GU
Sequence:	GU	Start Time:	2013-08-16 13:46
Directory:	GU\Demo Data	Injection Volume:	20.0
Sample Type:	Unknown	Channel:	Emission_1
Instrument Method:			

Chromatogram



Peak Results

No.	Peakname	Ret. Time min	GU
1	GU5	13.008	5.027
2		14.675	5.436
3		15.075	5.537
4	GU6	16.483	5.911
5		17.483	6.194
6		18.958	6.644
7		19.333	6.765
8		21.575	7.550

Figure 5. Peak results for the unknown sample of 2-AB-*N*-glycans. The column on the right displays the GU values calculated for the unknown peaks.

Because data from multiple runs is correlated in the analysis, excellent retention time precision is extremely important for accurate and reliable conclusions. As can be observed in Table 1, the retention time precision throughout the experiments was excellent. The peak shape and width provided by the Accucore 150-Amide-HILIC column were excellent as well with peak width at half height between 5 and 10 sec. The resulting high resolution separations allow confident assignment of GU values to closely eluting peaks. For instance, peaks numbers 6 and 7 of Figure 5 were base-line resolved and had accurate GU value assignment in spite of eluting close to each other.

Table 1. Retention time and retention time precision of 3 reported injections of dextran hydrolysate ladder.

	Retention Time (Min)			RSD(%)
GU	Injection #1	Injection #2	Injection #3	
2	2.533	2.533	2.533	0.00
3	5.225	5.225	5.217	0.09
4	9.067	9.067	9.058	0.05
5	13.033	13.033	13.025	0.04
6	16.658	16.658	16.650	0.03
7	19.867	19.867	19.850	0.05
8	22.683	22.683	22.675	0.02
9	25.200	25.192	25.192	0.02
10	27.458	27.450	27.450	0.02
11	29.500	29.492	29.492	0.02

Conclusion

The eWorkflow in Chromeleon 7.2 CDS software offers a user-friendly approach to data handling for the assignment of GU values to unknown 2-AB-glycans. The general Processing Method included in the eWorkflow can be easily adapted to suit the demands of any laboratory. The high-precision data generated with the Dionex UltiMate 3000 BioRS system, in synergy with the Accucore 150 Amide HILIC column, allowed unambiguous GU assignment. Finally, the high resolution obtained with Accucore 150 Amide-HILIC, allowed accurate GU assignment even to close eluting peaks.