

Evaluation of the Temperature Influence on Retention for HILIC of Glucose Oligomers Labeled with 2-Aminobenzamide: Retention Thermodynamics and Practical Influence on Separation of Glycans

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Overview

Purpose: Improve the understanding of retention mechanisms of glycans labeled with 2 aminobenzamide (2-AB) in amide columns.

Methods: 2-AB-glucose oligomers were separated in isocratic and gradient modes at various temperatures.

Results: Selectivity and peak broadening improve at high temperature.

Introduction

Separation of glycans with amide columns is widely used in glycomics and in biopharmaceutical analysis. Better understanding of the retention mechanism is beneficial for the development of future stationary phases and for optimum method development.

In this work we carried out an initial evaluation of the retention characteristic of linear glucose oligomers labeled with 2-AB. Additionally, based on the collected information, we provide some indication on how to improve the method resolving power without compromising on analysis time.

Methods

Liquid Chromatography

System: Thermo Scientific™ Dionex™ UltiMate™ 3000 BioRS UHPLC system equipped with low pressure gradient pump LGP-3400RS, fluorescence detector FLD-3400RS, and pre-column heater.

Mobile Phases: A- ammonium formate 0.1M pH 4.5. B- acetonitrile

Detector Setting: Ex. 320 nm, Em 420 nm, sensitivity 8, Filter wheel 370 nm, data collection rate 2Hz, response time 4 sec

Column: Thermo Scientific™ Accucore™ 150-Amide-HILIC 2.6 μm, 2.1 × 250 mm

Sample Preparation

Dextran ladder standard was labelled in house with 2-AB according to standard procedure. Final concentration was 0.1 μg/μL in acetonitrile/ammonium formate buffer = 9/1 v/v

Chromatography Data System

Thermo Scientific™ Dionex™ Chromeleon™ Chromatography Data System Software Version 7.2

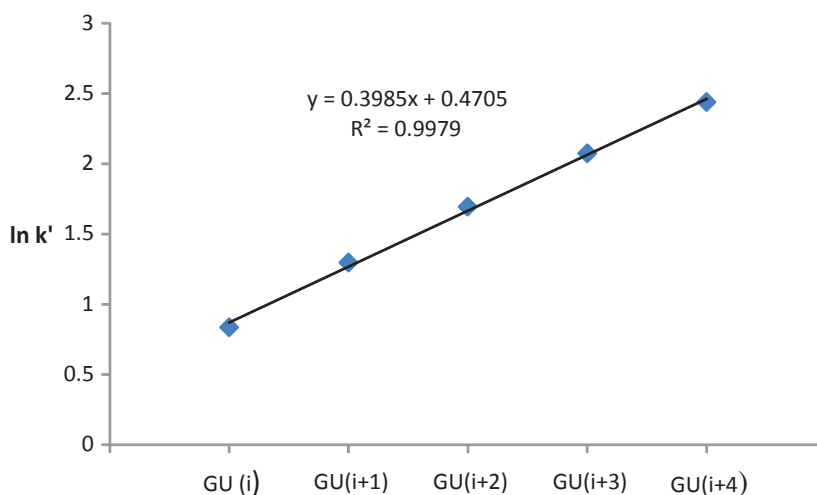
Results

Contribution of Additional Glucose Units to Retention

The plots logarithm of retention factor ($\ln k'$) vs Glucose Units (GU) at a given temperature and mobile phase composition can be interpolated by linear equation. This behavior is typically found for homologous series. Therefore, the relative importance of the 2-AB group appears negligible relative to the carbohydrate chain.

$$\text{slope} = \frac{(\ln k'_{GU(i+1)} - \ln k'_{GU(i)})}{GU(i+1) - GU(i)} = \ln(\alpha(GU))$$

FIGURE 1. Example of $\ln(k')$ vs GU plot. Isocratic elution at 66% B and 20 °C; flow rate 0.4 mL/min.



The slope of plots such as the one in Figure 1 represents the natural logarithm of the selectivity between the oligomer $GU(i+1)$ and oligomer $GU(i)$.

Based on the assumption that the 2-AB-glucose oligomers behave as series of homologous (*i.e.* 2-AB does not influence retention), the variation of Gibbs Free Energy related to the transfer from mobile to stationary phase, when one glucose monomer is added to the oligomer, can be calculated by the following formula:

$$\Delta G^0(GU) = -RT \ln(\alpha(GU))$$

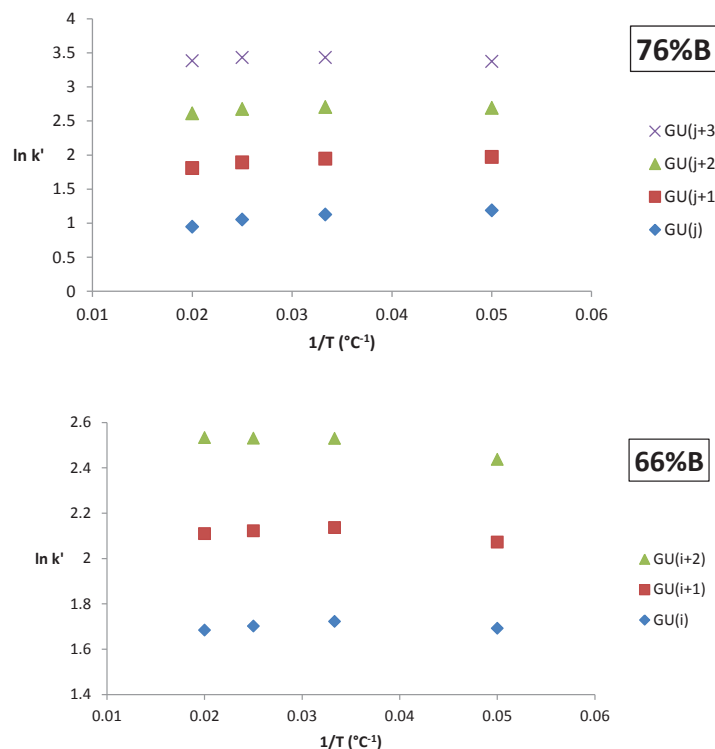
T (°C)	%B	ΔG^0 (J/mol)
20	80	-8.6E+03
30	80	-9.1E+03
40	80	-9.2E+03
50	80	-9.8E+03
20	76	-1.8E+03
30	76	-1.9E+03
40	76	-2.1E+03
50	76	-2.2E+03
20	66	-9.7E+02
30	66	-1.1E+03
40	66	-1.1E+03
50	66	-1.1E+03

Table 1. Estimated contribution to Gibbs Free Energy involved in the transition of one monomer (glucose) unit from mobile to stationary phase, at different temperatures and isocratic conditions. Flow rate 0.4 mL/min.

According to the Gibbs free energy values of Table 1, the selectivity between glucose oligomers differing by one monomer unit, is more favorable at high temperature. The observation applies to mobile phase with high organic content (80 %B and 76 %B), whereas at lower organic content (66 %B) the same behavior is not clearly visible.

The increased selectivity at high temperature can also be observed in the Van't Hoff plots of Figure 2, where the retention factor differences between 2-AB- labeled glucose oligomers are more pronounced at high temperatures

FIGURE 2. Van't Hoff plots for 2-AB-glucose linear oligomers obtained at 76% and 66 % organic. Flow rate 0.4 mL/min.



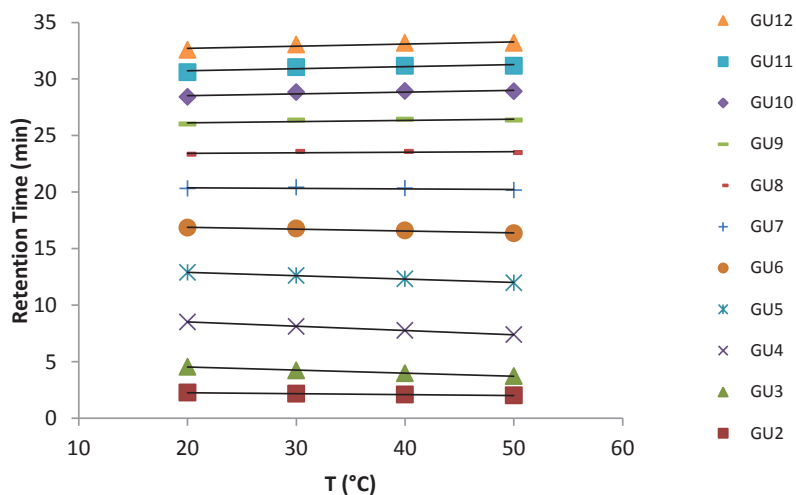
The Van't Hoff plots of Figure 2 are not linear. This observation indicates a change of the retention thermodynamics with temperature. Negative slopes and maxima are observed.

Further studies are required to elucidate the retention mechanism of glucose oligomers with amide phases, and to explain behavior such as those of Figure 2.

Gradient Separation of Linear Glucose Oligomers at Different Temperature: Effects on Retention

The retention of linear glucose oligomers with gradient mode is in agreement with the behavior observed for isocratic elution: selectivity increases at high temperature. Figure 3 shows that smaller oligomers (up to GU6-GU7) are less retained at high temperature, whereas longer oligomers are more retained at high temperature.

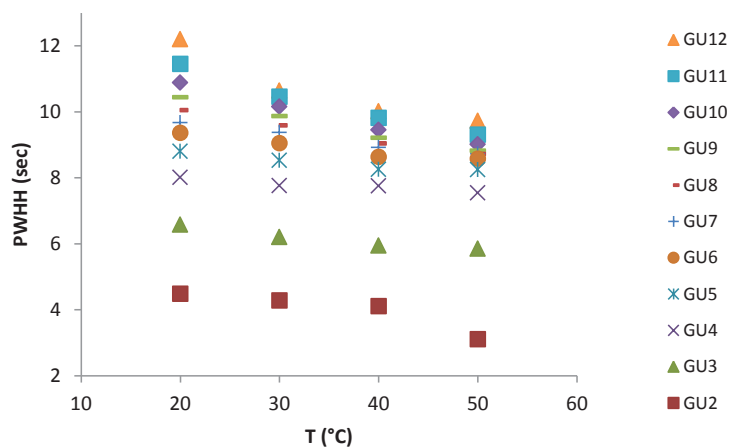
FIGURE 3. Retention time of linear oligomers at different temperatures. Gradient 80 to 50% acetonitrile in 40 minutes. Flow rate: 0.4 mL/min.



Gradient Separation of Linear Glucose Oligomers at Different Temperature: Effects on Peak Broadening

Besides influencing the retention thermodynamics, temperature affects the mass transfer rate between mobile phase and stationary phase. Peak dispersion generated by mass transfer is lower at higher temperature, and consequently peaks are narrower. This behavior, extremely common in any liquid chromatography technique, is observed for all oligomers between GU2 and GU12.

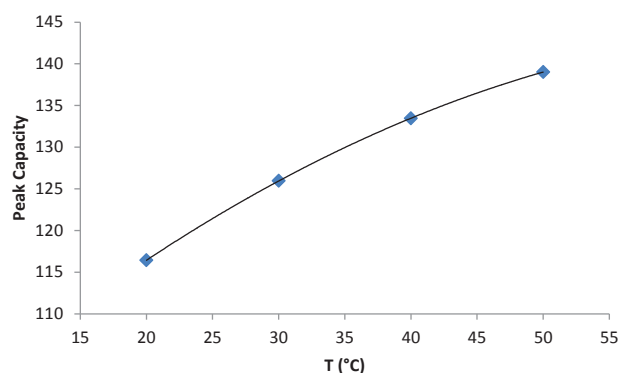
FIGURE 4. Peak Width at Half Height (PWHH) of linear oligomers at different temperatures. Gradient 80 to 50% acetonitrile in 40 minutes. Flow rate 0.4 mL/min.



Gradient separation of linear glucose oligomers at different temperatures. Practical consequences on peak capacity

It was observed that the retention window of 2-AB glucose linear oligomers can be enlarged significantly (Figure 3) by increasing temperature. Additionally all peaks become sharper when increasing temperature (Figure 4). Both effects contribute to increase of peak capacity. Figure 5 shows that peak capacity increases of about 16% from 20 °C to 50 °C. The increase of capacity occurred without changing the analysis time.

FIGURE 5. Peak capacity at different temperature for separation of GU2 to GU12 labeled linear oligomers. Gradient 80 to 50% acetonitrile in 40 minutes. Flow rate: 0.4 mL/min.



This work was performed with linear glucose oligomers; however it is expected that similar conclusions can be reached for branched 2-AB-glycans. Further experiments are required to confirm this hypothesis.

Conclusion

- Isocratic retention at a given temperature of 2-AB-labeled linear glucose oligomers can be approximated to the behavior of homologous series
- Gibbs Free Energy measurements and Van't Hoff plots indicate that isocratic selectivity improves at high temperature
- Selectivity for gradient separations is improved at high temperature
- Peaks of labeled linear glucose oligomers are narrower at high temperature
- The peak capacity of 2-AB-glycan separations increases with temperature

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