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Systematic Evaluation of Hydrophilic Interaction Liquid Chromatography Stationary Phases for Oligonucleotide Characterization by LC/MS

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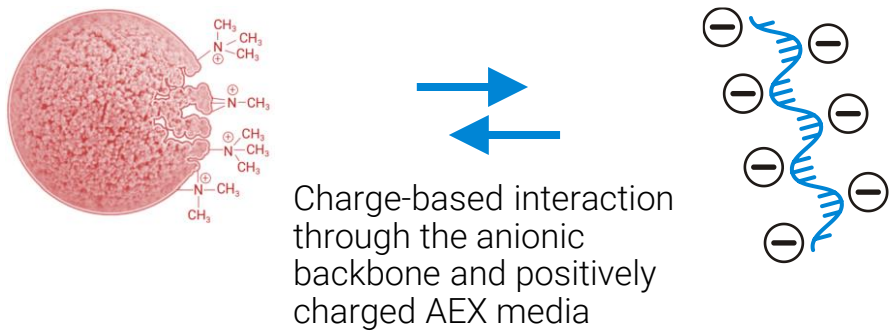
Introduction

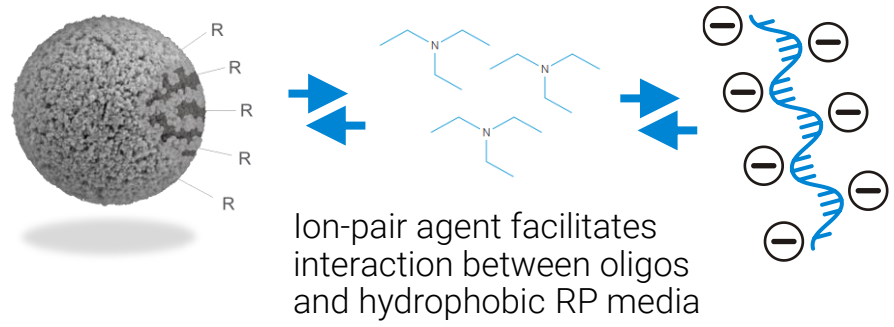
- Ion-pairing reversed-phase chromatography (IP-RPLC) coupled to MS represents the most common analytical method for oligonucleotide analysis.
- However, alternative separation methods are desired as alkylamine ion-pair reagents force users to have dedicated instruments.
- While ion-exchange chromatography (IEX) represents a viable alternative technique due to its excellent selectivity for oligos based on their length, it is not preferred due to mobile phase incompatibility with MS detection.
- Hydrophilic interaction chromatographic (HILIC) is a valuable alternative to IP-RPLC and IEX as HILIC mobile phases are compatible with MS and provides flexibility in instrument-use.
- This work highlights the utility of HILIC for oligonucleotide analysis and critical parameters that need to be considered to optimize LC/MS performance.

Illustration of Different Stationary Phases' Suitability for Oligonucleotide Separation

IEX



IP-RP



HILIC

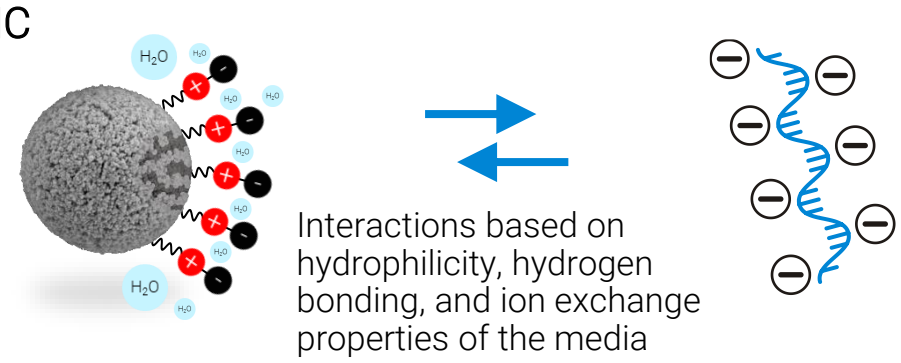


Figure 1. Understanding the retention mechanisms of IEX, IP-RP, and HILIC chromatography.

Experimental

Tanaka Test

All HILIC columns were screened with the Tanaka test to characterize the critical interactions of HILIC separation[1]. These characteristics included hydrophobicity, hydrophilicity, shape/steric selectivity, hydrogen bonding, ion exchange, and acidic-basic nature of the stationary phase. The selectivity values were calculated based on the relative retention of key analyte pairs[2, 3].

LC/MS Analysis

Oligo standards and samples were separated using Agilent's HILIC columns on a LC system coupled on-line with a 6545XT Advance Bio LC/Q-TOF.

Instruments and Supplies

Agilent HPLC Columns (2.1x 150 mm):

- InfinityLab Poroshell 120 HILIC
- InfinityLab Poroshell 120 HILIC-OH5
- InfinityLab Poroshell 120 HILIC-Z
- AdvanceBio Glycan Mapping column
- AdvanceBio Amide HILIC column

Agilent LC and LC/MS Instruments:

- 1260 Infinity Bioinert Quat Pump LC System
- 1260 Infinity Diode Array Detector
- 1290 Infinity Thermostatted Column Compartment
- 6545 XT AdvanceBio LC/ Q-TOF

Mobile Phase:

- 100 mM ammonium acetate stock solutions were made in water and adjusted to pH 4.4 or pH 9 with either acetic acid or ammonium hydroxide.
- Mobile phase A = 10% (100 mM stock solution)/ 90% water.
- Mobile phase B = 10% (100 mM stock solution)/ 90% acetonitrile.

Oligonucleotide Samples

- Agilent Oligo Ladder Standard
- Agilent RNA Resolution Standard
- Modified oligos were custom ordered from IDT.

Chromatographic Separations for the Tanaka Test

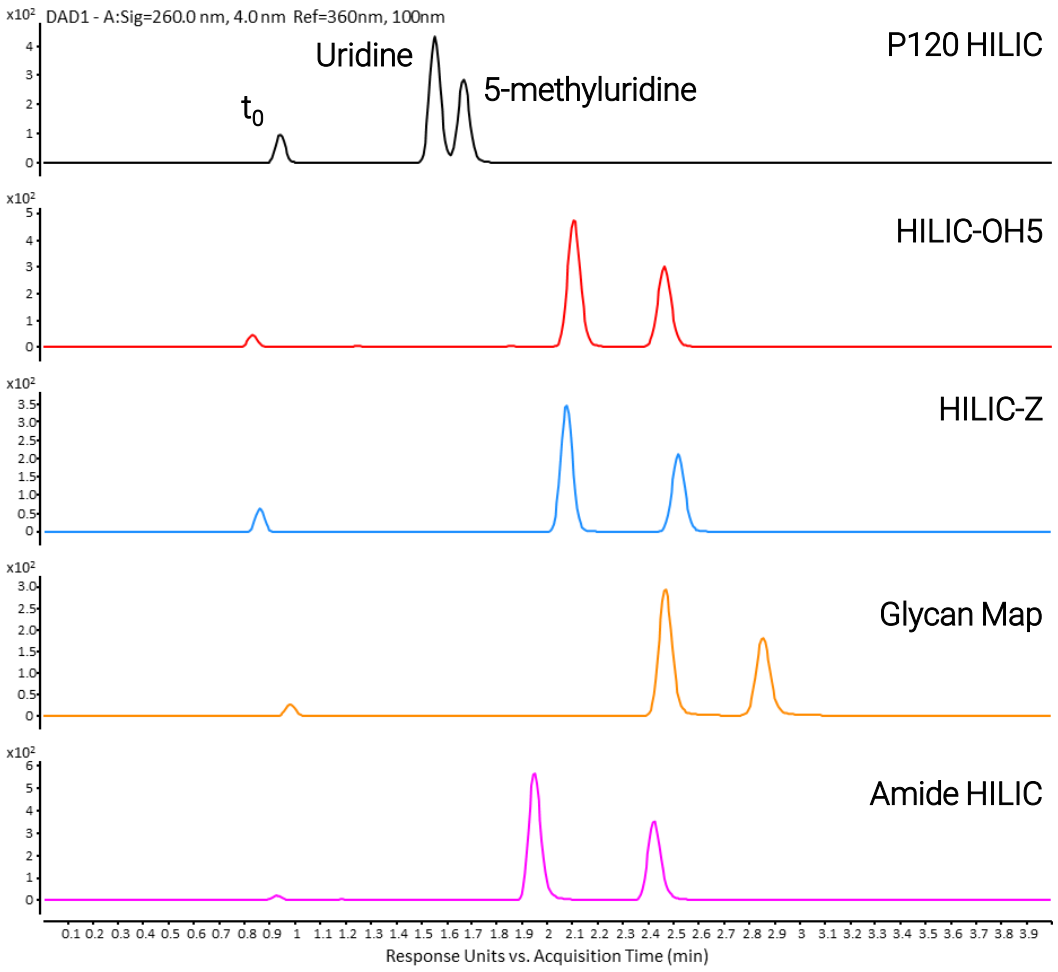


Figure 2. Separation of key analyte pairs to determine attributes that participate in the HILIC retention mechanism. The example shown illustrates the retentiveness of the HILIC stationary phases that can be estimated based on the retention factor (k) of uridine relative to 5-methyluridine.

Characterizing HILIC Columns with the Tanaka Test

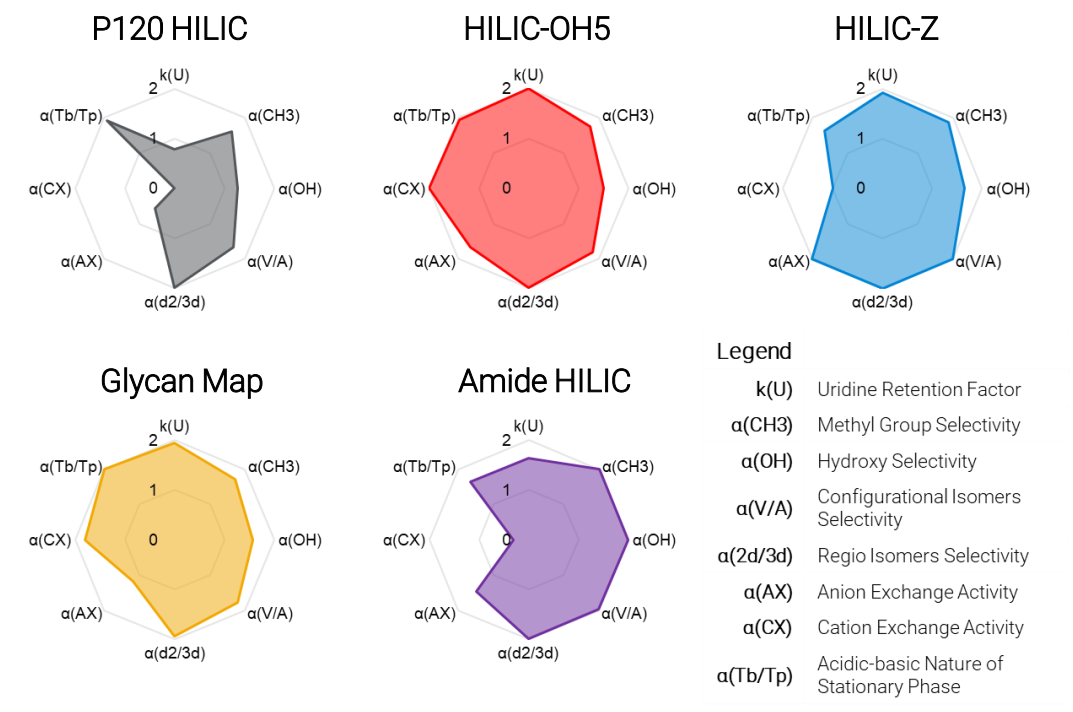
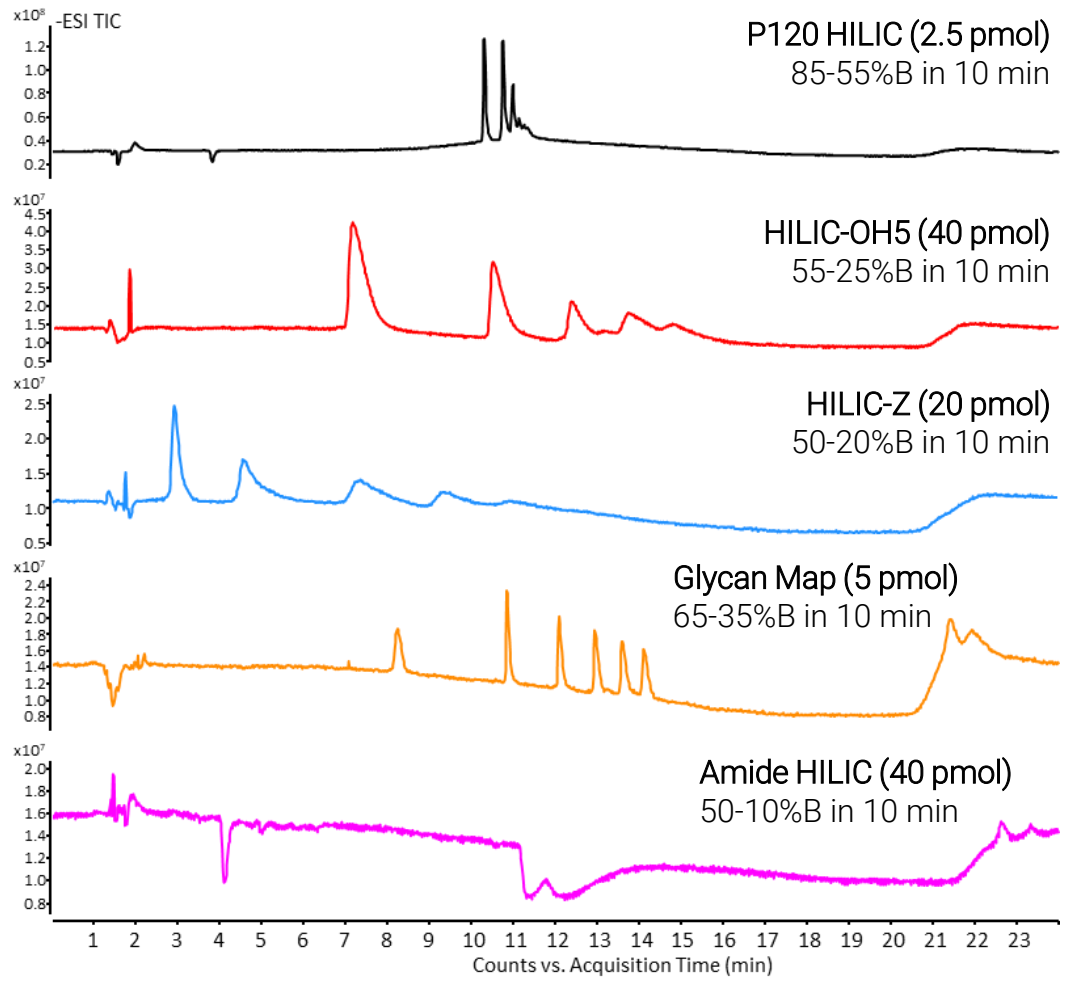


Figure 3. Radar plots for five stationary phases of different chemistries. Each attribute was normalized to each parameter's maximum value at 2.0, to distinguish each columns' features.

Evaluating HILIC Stationary Phases for Oligo Separation

(A) 10mM Ammonium Acetate, pH 4.4



(B) 10 mM Ammonium Acetate, pH 6.8

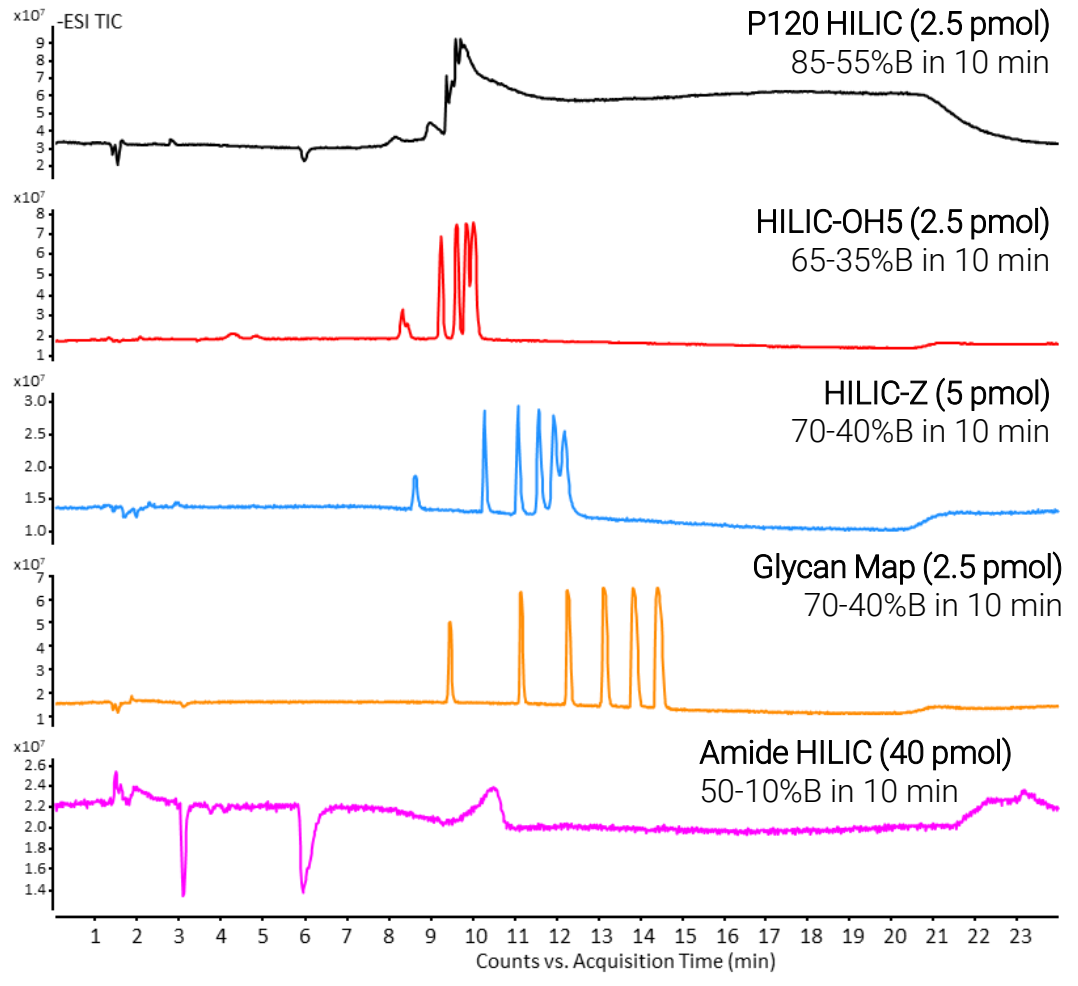
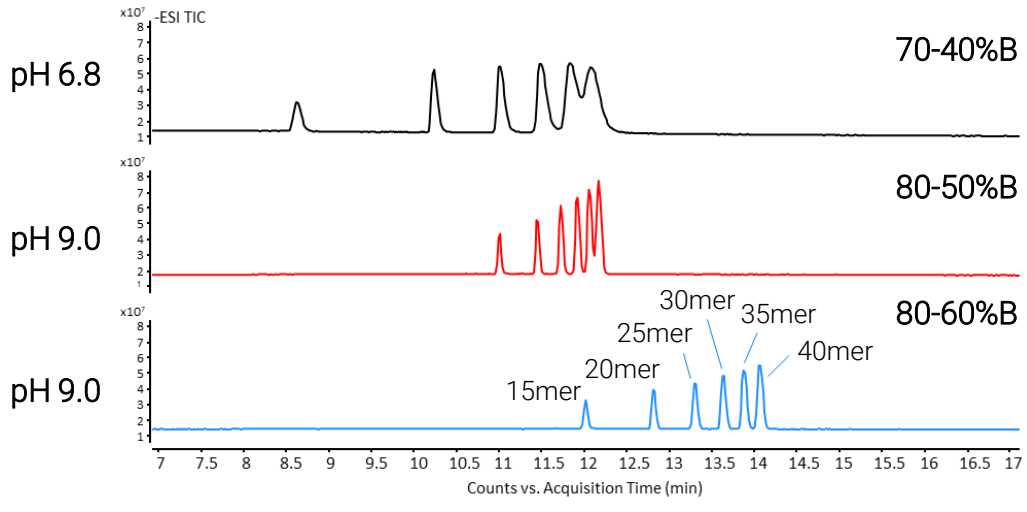


Figure 4. HILIC-LC/MS analysis of 15, 20, 25, 30, 35, and 40 mer DNA standard using HILIC stationary phases with varying chemical properties at (A) pH 4.4 and (B) pH 6.8.

Results and Discussion

Chromatographic Separation of Oligos at high pH

(A) DNA Oligo Standard



(B) RNA Resolution Standard

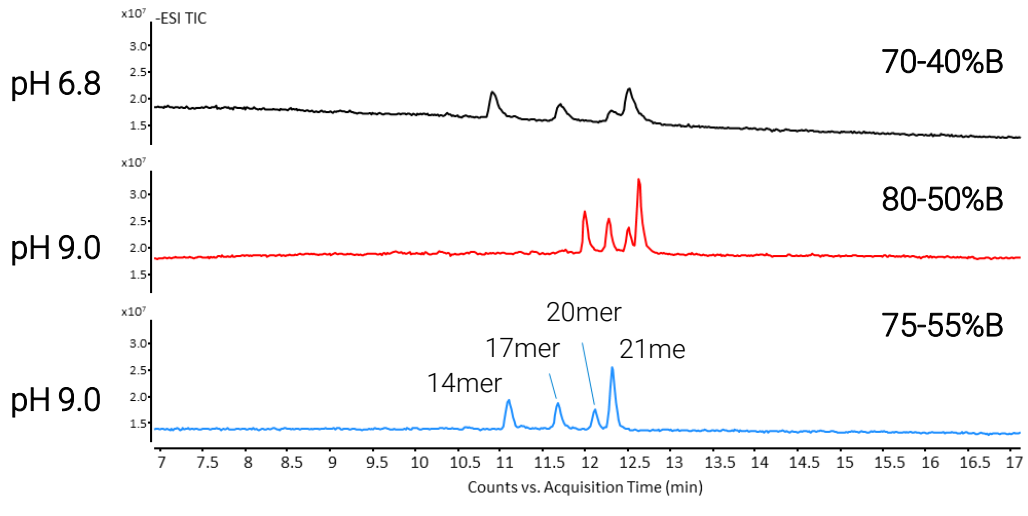


Figure 5. HILIC-LC/MS analysis of (A) DNA and (B) RNA oligo standards at pH 6.8 and pH 9.0 with the HILIC-Z column. The gradient was modified to improve peak resolution and adjust for retention time shifts when switching the pH of the mobile phase.

Spectral Deconvolution of HILIC-LC/MS Runs

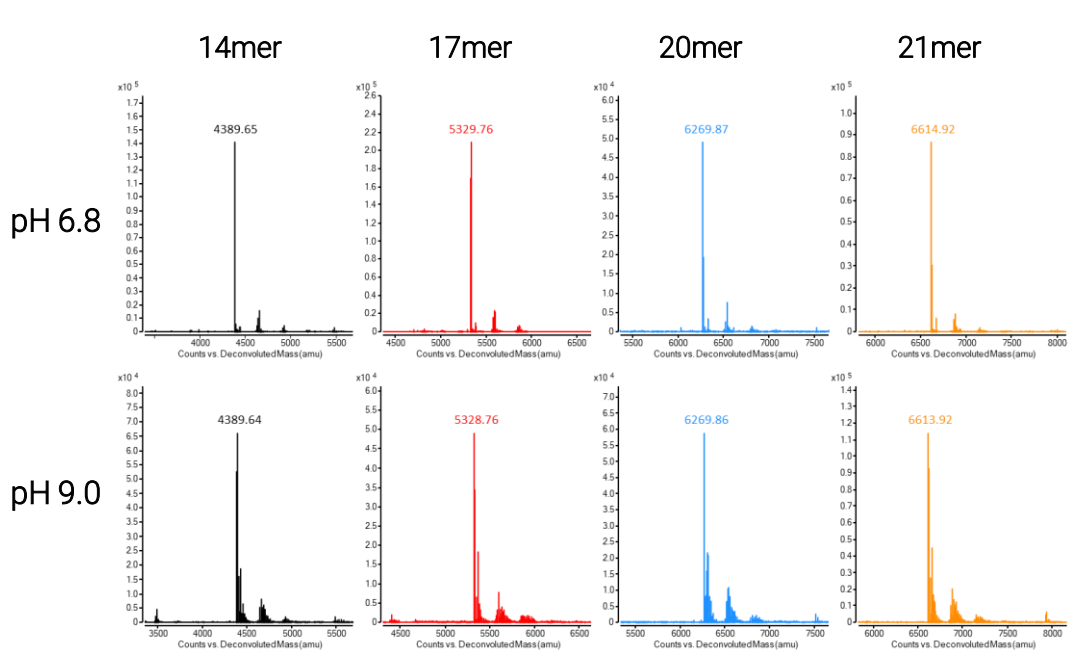


Figure 6. Higher levels of sodium and potassium adducts were observed when using pH 9 mobile phase vs. pH 6.8.

HILIC-LC/MS Analysis of Modified Antisense Oligos

(A) Antisense Oligo (ASO) Sequence

ASO #1	5'- U*/i2MOErC/*U*U*/i2MOErG/*T*T*/i2MOErA/* /i2MOErC/*/i2MOErA/*/i2MOErT/*/i2MOErG/*/i2MOErA/*/i2MOErA/*/i2MOErA/*/i2MOErU/*/i2MOErC/*/i2MOErC/*/i2MOErC/*C -3'
ASO #2	5'- U*/i2MOErC/*/i2MOErA/*/i2MOErC/*U*U*U*/i2MOErC/* /i2MOErA/* U*/i2MOErA/*/ i2MOErA/* U*/i2MOErG/*C*U* /i2MOErG/*G -3'

(B) Chromatographic Separation

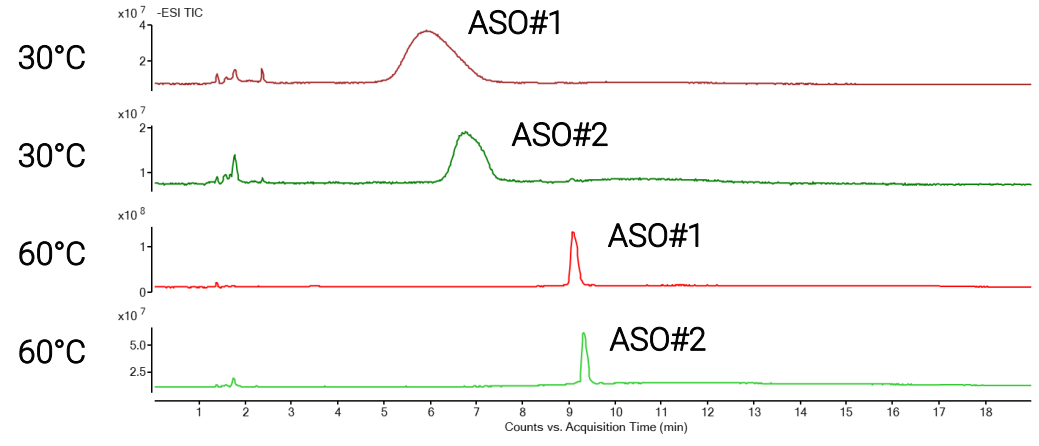


Figure 7. HILIC-LC/MS Analysis of ASOs using the HILIC-Z column at pH 9. Increasing column compartment temperature yielded sharper, symmetrical peak shapes.

Conclusions

- Optimizing mobile phase pH and column temperature can yield better chromatographic performance for oligonucleotides.
- HILIC serves as an attractive approach for the analytical characterization of heavily modified oligonucleotides.
- We plan to further optimize the mobile phase buffer, MS source conditions, and other experimental parameters in future studies.

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