Application Note

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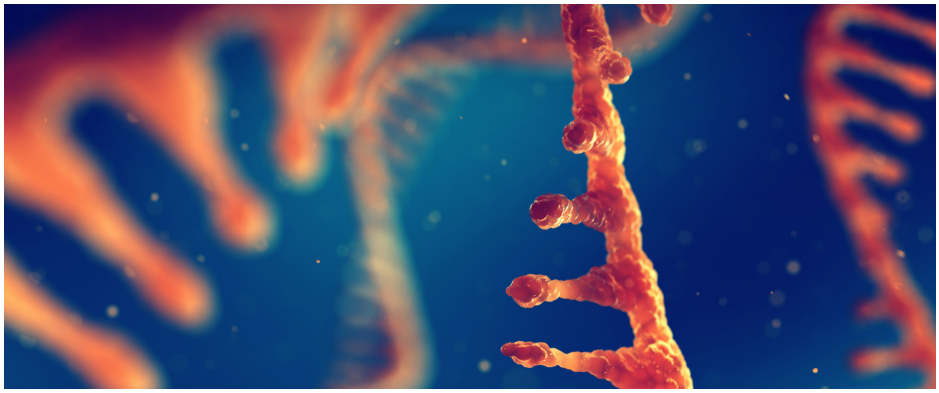
Evaluating HILIC Stationary Phases for Oligonucleotide Separation by LC/MS



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Abstract

Hydrophilic interaction chromatography (HILIC) represents a versatile and effective alternative to ion-pairing reversed-phase liquid chromatography (IP-RPLC) for analyzing oligonucleotides. In this study, five different HILIC stationary phases were comprehensively evaluated and optimized to determine their suitability and complementarity for oligonucleotide analysis using liquid chromatography/mass spectrometry (LC/MS). Key factors for optimizing retention and detection include column selectivity and the pH of the mobile phase. Further studies were then carried out to characterize heavily modified oligonucleotides with the optimized HILIC-LC/MS method.

Introduction

IP-RPLC coupled with MS represents the most common analytical method for oligonucleotide analysis.[1-3] However, alternative separation methods are needed because alkylamine ion-pair reagents require dedicated instruments. While ion-exchange chromatography (IEX) represents a viable alternative technique due to excellent selectivity for oligonucleotides based on their length, it is not preferred due to mobile phase incompatibility with MS detection. HILIC is a valuable alternative to both IP-RPLC and IEX as HILIC mobile phases are compatible with MS and offer flexibility in instrument use. This work highlights the utility of HILIC for oligonucleotide analysis and the critical parameters that need to be considered to optimize LC/MS performance.

Experimental

Reagents and supplies

- DNA ladder standard (part number 5190-9029)
- RNA resolution standard (part number 5190-9028)
- Polypropylene vials (part number 5188-2788)
- Blue snap caps (part number 5182-3458)

Sample preparation

Oligonucleotide standards covering DNA and RNA sequences ranging from 14- to 40-mer in length were selected for HILIC stationary phase screening and HILIC liquid chromatography/quadrupole time of flight (LC/Q-TOF) MS method development. A pharmaceutically relevant 18-mer antisense oligonucleotide (ASO) was then used to validate the optimized HILIC-MS methods. All samples were resuspended in nuclease-free water and stored at –20 °C. The samples were then transferred to polypropylene vials and kept in a temperature-controlled autosampler for up to 2 days before analysis. The sequences and modifications for the 14-, 17-, 20-, and 21-mer RNA and the 18-mer ASO can be found in a previously published application note.[4]

HILIC columns

Five HILIC columns from Agilent were evaluated in this study: InfinityLab Poroshell 120 HILIC (P120 HILIC), InfinityLab Poroshell 120 HILIC-OH5, InfinityLab Poroshell 120 HILIC-Z, AdvanceBio Amide HILIC, and AdvanceBio Glycan Mapping. The Glycan Mapping column features a neutral amide stationary phase, while the Amide HILIC column has a mixed-mode HILIC stationary phase with both amide and ion-exchange functionalities. All columns contain 2.7 µm superficially porous particles, except for the Amide HILIC column, which has 1.8 µm fully porous particles. A summary of the columns used is provided in Table 1.

Mobile phase preparation

LC/MS-grade acetonitrile and Milli-Q purified water were used for the LC/MS analysis. Detailed steps for solvent buffer preparation are shown in Table 2.

Table 1. List of HILIC stationary phases evaluated for oligonucleotide separation.

No.	Column	Pore Size (Å)	Dimensions (mm)	Particle Size (µm)	Surface Chemistry	pH Range	Pressure Rating (bar)	Maximum Temperature (°C)	Part Number
1	HILIC	120	2.1 × 150	2.7	Bare silica	0 to 8	600	–	693775-901
2	HILIC-OH5	120	2.1 × 150	2.7	Poly-hydroxy fructan	1 to 7	400	45	683775-601
3	HILIC-Z	100	2.1 × 150	2.7	Zwitterionic	2 to 12	600	80	683775-924
4	Glycan Map	120	2.1 × 150	2.7	Neutral amide	2 to 7	600	40	683775-913
5	Amide HILIC	300	2.1 × 150	1.8	Mixed mode amide	2 to 7	1,200	80	859750-91

Table 2. Mobile phase preparation for LC/MS analysis.

Method	10x Stock Buffer	Mobile Phase A	Mobile Phase B
High pH Method	100 mM ammonium acetate, pH 9.0, adjusted with ammonium hydroxide	100 mL of stock buffer was mixed with 900 mL of water	100 mL of stock buffer was mixed with 900 mL of ACN
Neutral pH Method	100 mM ammonium acetate, pH 6.8		
Low pH Method	100 mM ammonium acetate, pH 4.4, adjusted with acetic acid		

Instrumentation

LC/MS analysis was performed using an Agilent 1290 Infinity II LC system coupled to an Agilent 6545XT AdvanceBio LC/Q-TOF with an Agilent Jet Stream Electrospray source. The LC consisted of:

- Agilent 1290 Infinity II high-speed pump (G7120A)
- Agilent 1290 Infinity II multisampler with thermostat (G7167B)
- Agilent 1290 Infinity II multicolumn thermostat (MCT) (G7116B)
- Agilent 1290 Infinity II diode array detector (DAD) (G7117A)

Dynamic mass axis correction was achieved by continuous infusion of a reference mass solution. Tables 3 and 4 summarize the optimized LC and the MS conditions. Data acquisition and analysis was done using the Agilent MassHunter software suite.

Table 3. LC parameters for 18-mer ASO analysis.

Agilent 1290 Infinity II LC System		
Column	Agilent InfinityLab Poroshell 120 HILIC-Z, 2.1 mm × 150 mm, 2.7 µm (part number 683775-924)	Agilent AdvanceBio Glycan Mapping 120Å, 2.1 mm × 150 mm, 2.7 µm (part number 683775-913)
Solvent	A) 10 mM ammonium acetate in water, pH 9.0 B) 10 mM ammonium acetate in water/ACN 10:90 (v:v), pH 9.0	A) 10 mM ammonium acetate in water, pH 6.8 B) 10 mM ammonium acetate in water/ACN 10:90 (v:v), pH 6.8
Gradient	Time (min) %B 0.00 80 2.00 80 9.00 60 11.0 60 12.0 80 17.0 80	
Column Temperature	30 °C	
Flow Rate	0.25 mL/min	
Autosampler Temperature	8 °C	

Table 4. MS parameters for 18-mer ASO analysis.

Agilent 6S45XT AdvanceBio LC/Q-TOF	
Ionization Mode	Dual AJS ESI
Ionization Polarity	Negative
Gas Temperature	275 °C
Drying Gas	12 L/ min
Nebulizer Pressure	35 psi
Sheath Gas temperature	350 °C
Sheath Gas Flow	12 L/min
Capillary Voltage	3,500 V
Nozzle Voltage	2,000 V
Fragmentor	175 V
Skimmer	65 V
Octopole 1 RF Voltage	750 V
Acquisition Range	*m/z* 300 to 3,200
MS Acquisition rate	1 spectrum/s
Reference Mass	*m/z* 980.01637500

Results and discussion

To assess the utility of different HILIC stationary phases for oligonucleotide separation, the columns were evaluated with a mobile phase containing 10 mM ammonium acetate buffers at pH 4.4, 6.8, and 9.0. The varying mobile phase compositions could change the stationary phase selectivity, thereby altering oligonucleotide separation. Moreover, mobile phase buffer pH may influence the electrospray ionization of oligonucleotides.[5] Understanding and balancing the impact of buffer choice on retention, selectivity, and ionization efficiency is critical when developing optimal LC/MS methods.

To ensure a fair comparison across all columns, the same gradient slope (30% B change in 10 minutes, 3% B/min) was used. In general, the columns performed better at pH 6.8 than the acidic pH 4.4 condition (Figure 1). This is understandable given the benefits of elevated pH for oligonucleotide analysis because it promotes conditions that result in better retention, resolution, selectivity, and ionization for IP-RP.[6] Moreover, the Glycan Mapping column yielded the best selectivity in resolving the varied oligonucleotides of different sequence lengths, followed by the HILIC-Z, then the HILIC-OH5 columns.

The P120 HILIC bare silica column showed poor selectivity for the DNA oligonucleotides, while the Amide HILIC column results were unexpected, as changes to the mobile phase composition did not yield any detectable oligonucleotide peak. This may be due to the ion-exchange properties of the mix-mode column that are reflective of the structural complexity of the oligonucleotides as well as the positional location of the ion exchange functionality on the different bonded HILIC phases. Further mobile phase and gradient optimizations such as increase in salt concentrations are required to elute the oligonucleotides off the column.

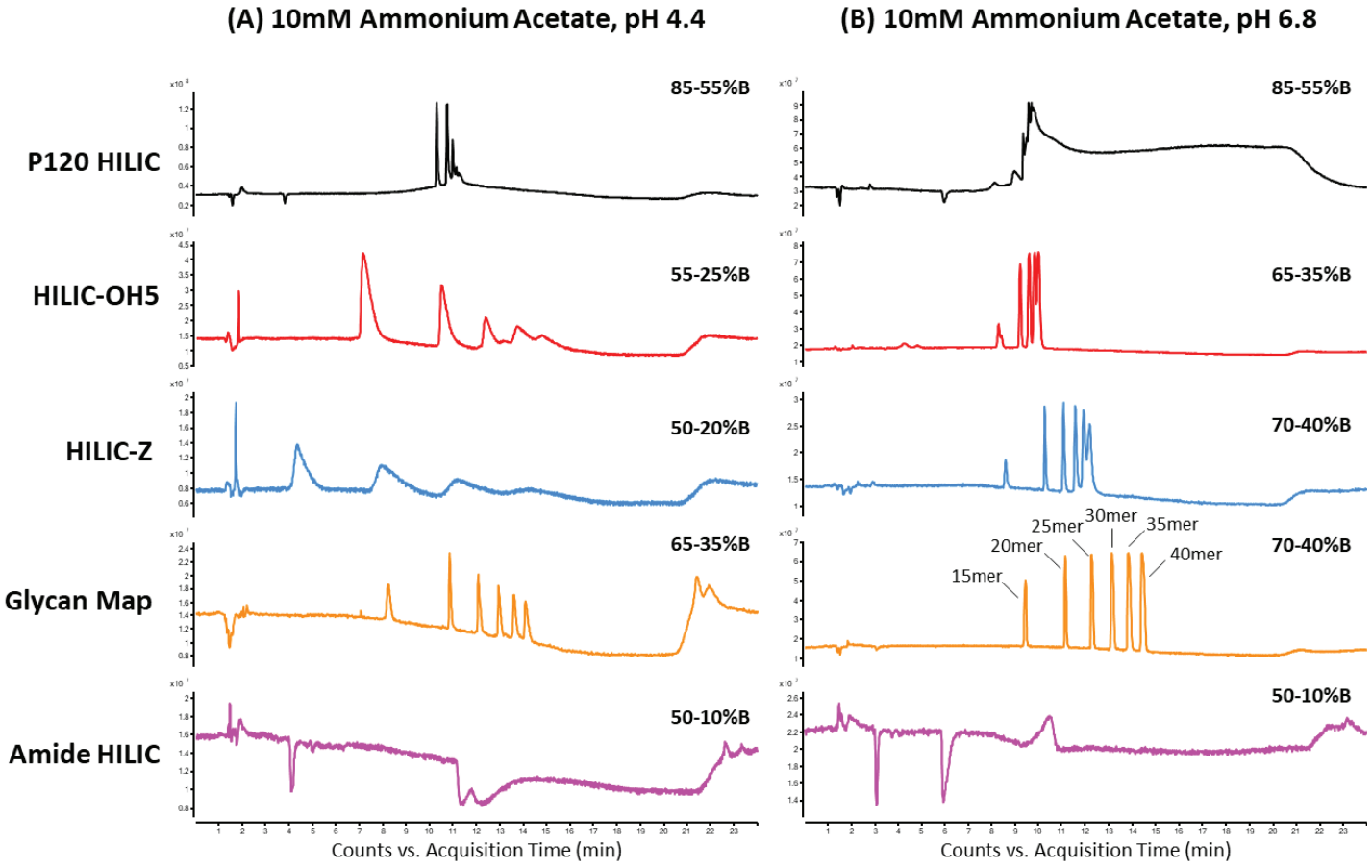


Figure 1. HILIC-MS analysis of 15-, 20-, 25-, 30-, 35-, and 40-mer DNA samples using HILIC stationary phases with varying chemical properties with 10 mM ammonium acetate buffer at (A) pH 4.4 and (B) pH 6.8. The gradient used for each column is listed in the top right corner of each chromatogram. The gradient was modified to improve peak resolution and adjust for retention time shifts when switching the pH of the mobile phase.

Given the improved performance of the oligonucleotides going from acidic to neutral pH, the impact of elevated pH was further explored with the HILIC-Z column because of its stability at higher pH.[7] The oligonucleotide sample was analyzed with the HILIC-Z column at pH 6.8 and pH 9.0 (Figure 2). At pH 6.8, the HILIC-Z column yielded slightly broader peaks than pH 9.0 with lower resolution between the 35- and 40-mer oligonucleotides. At pH 9.0, the oligonucleotide peaks were noticeably sharper. Baseline separation could be achieved by flattening the gradient from 3 to 2% B/min, with the potential to use an even shallower gradient to further resolve the oligonucleotides.

Based on the results shown in Figure 2, it was decided to continue the downstream analysis with the Glycan Mapping column at pH 6.8 and the HILIC-Z column at pH 9.0. RNA samples composed of 14-, 17-, 20- and 21-mer lengths were analyzed on the two columns (Figure 3). Importantly, the 20- and 21-mer RNA samples were resolved with both columns, where separation of the n–1 oligonucleotide pair represents a critical attribute that is closely monitored for the incomplete synthesis of an oligonucleotide.[8,9]

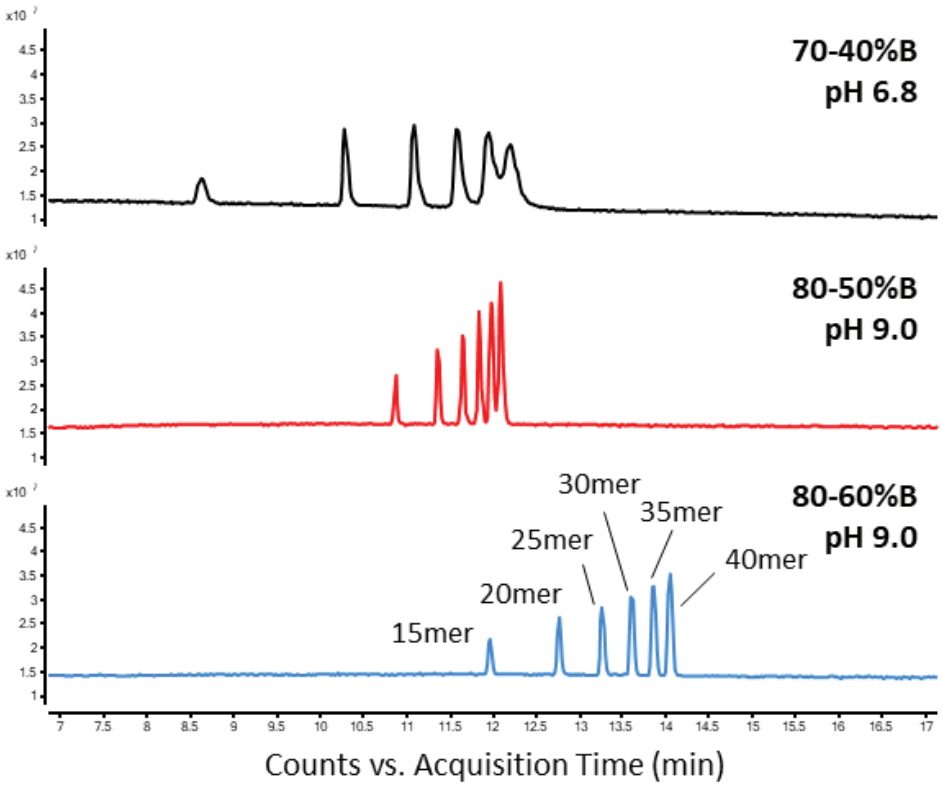


Figure 2. HILIC separation of 15-, 20-, 25-, 30-, 35-, and 40-mer DNA using an Agilent HILIC-Z column at pH 6.8 and pH 9.

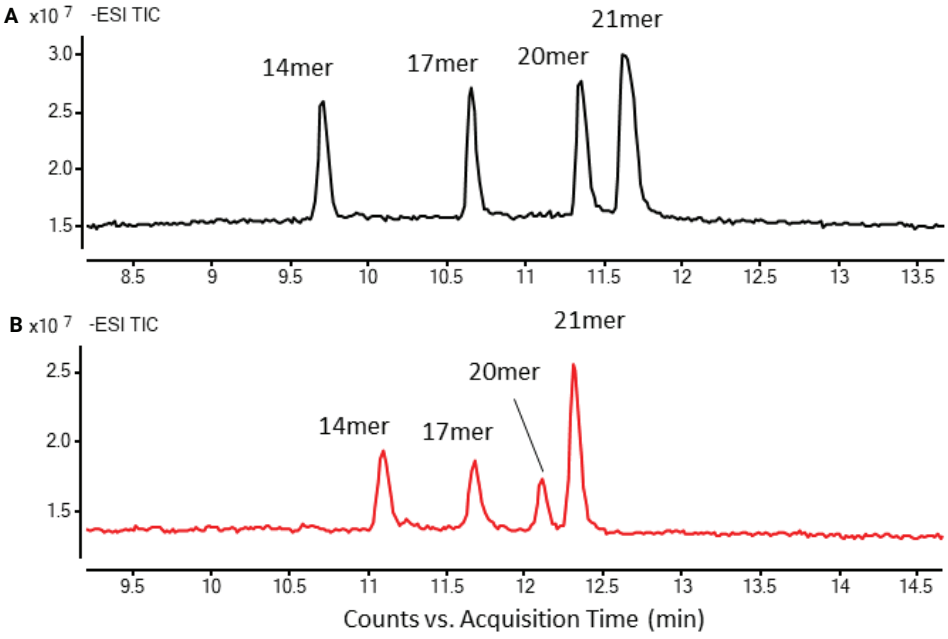


Figure 3. HILIC separation of 14-, 17-, 20-, 21-mer RNA using an Agilent Glycan Mapping column at pH 6.8 (A) and an Agilent HILIC-Z column at pH 9 (B).

A closer examination of the mass spectra revealed a wider charge state distribution for the oligonucleotides analyzed with mobile phase buffer at pH 6.8 than that at pH 9.0. Specifically, a range of 4- to 8- charge states were observed for the 21-mer RNA at pH 6.8 (Figure 4A). In contrast, a range of 4- to 6- charge states were observed for the RNA oligonucleotides at pH 9.0 (Figure 4B). As the length of the oligonucleotide sequence increases, the ability to detect higher charge states becomes necessary for detection within the mass range limitations of the mass spectrometer. Furthermore, a broader range of detectable charge states lends more confidence in the target analyte identification.

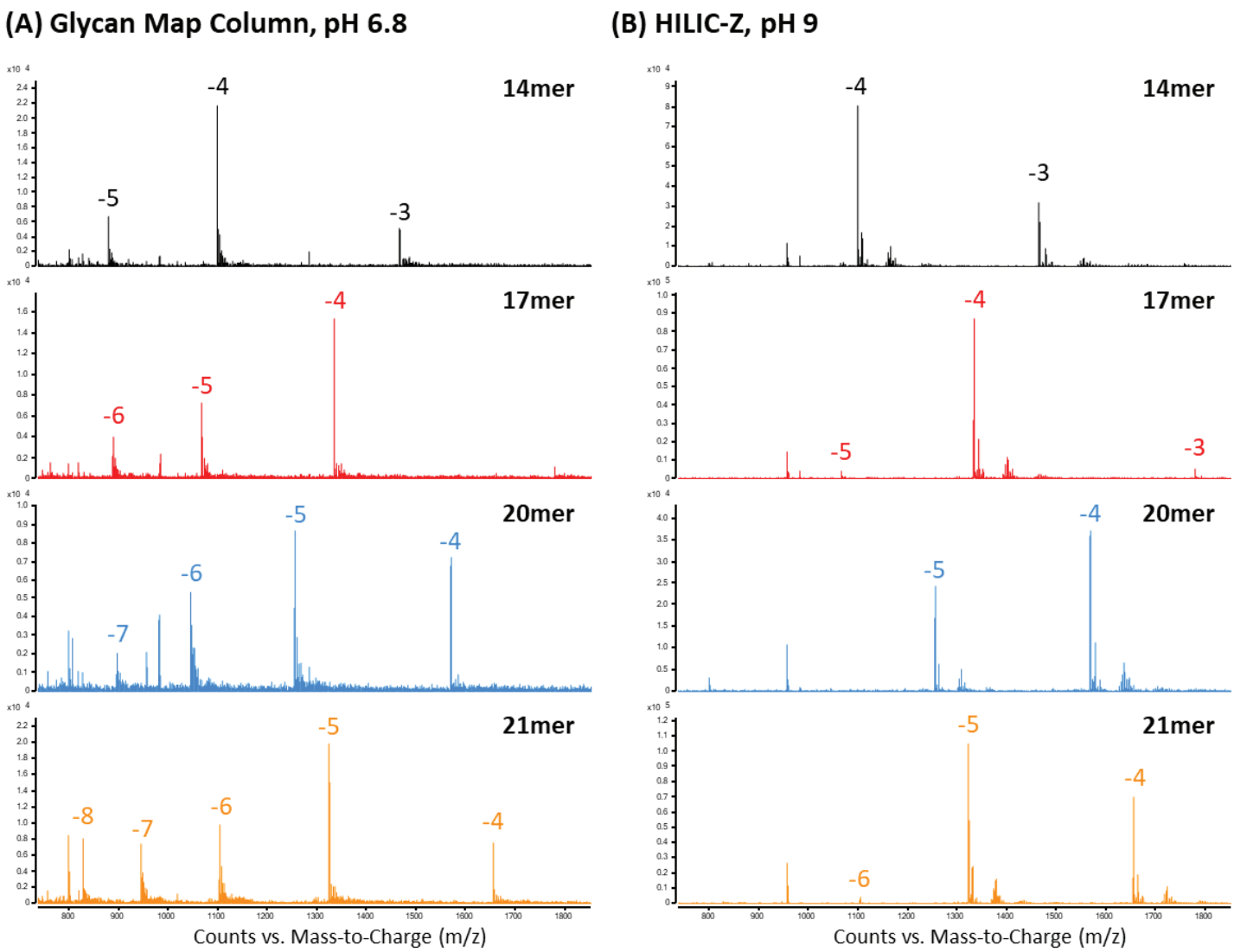


Figure 4. Full scan MS1 mass spectra for 14-, 17-, 20-, 21-mer RNA.

In addition to optimizing the chromatographic conditions for the columns, it is equally important to determine the method compatibility to yield an optimal MS signal response. This optimization allows users to confidently identify the peaks detected by LC/MS. This is demonstrated in Figure 5, where the spectral deconvolution of the mass spectra confirmes the masses for the 14-, 17-, 20-, and 21-mer RNA.

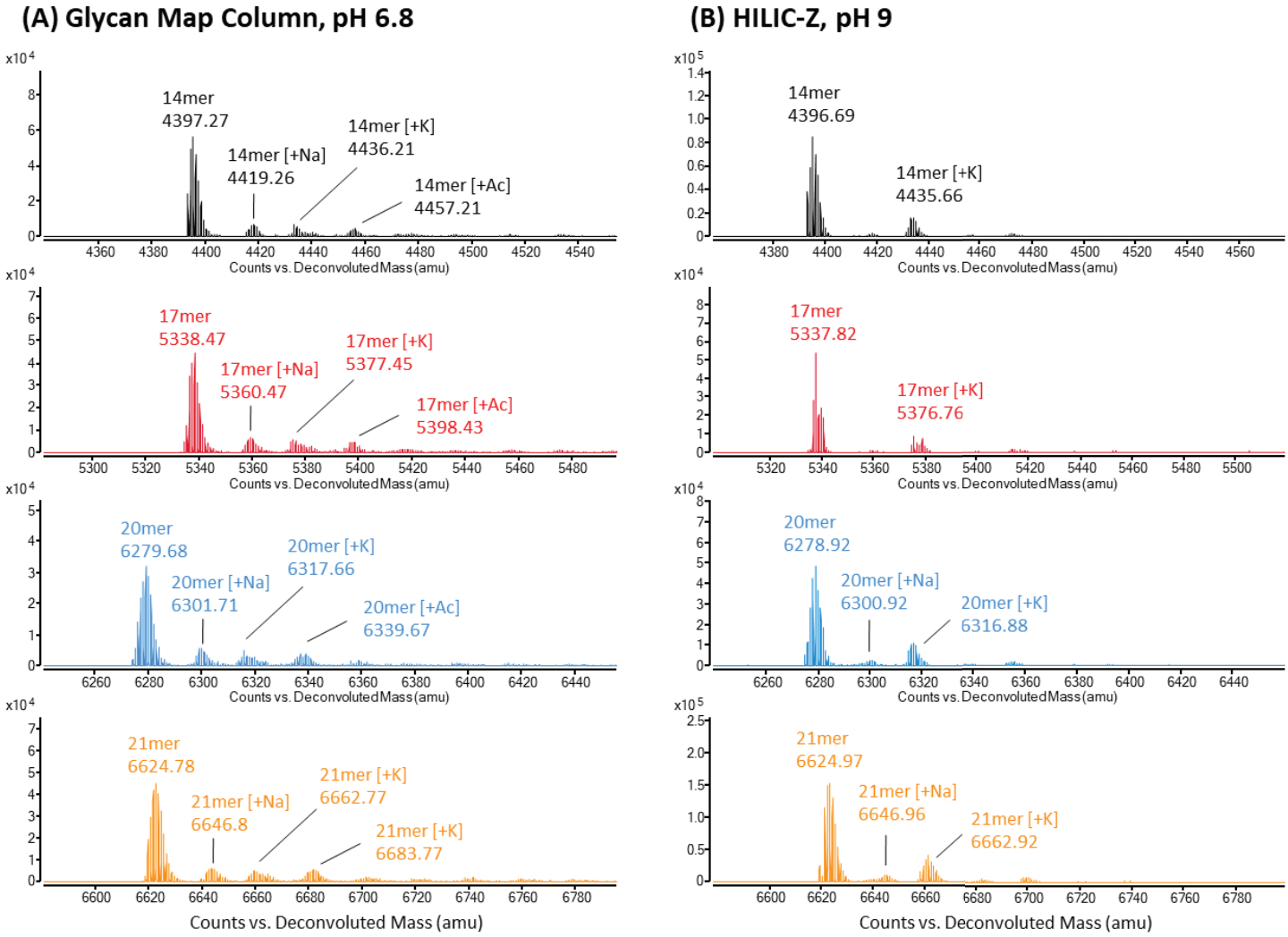


Figure 5. Spectral deconvolution of 14-, 17-, 20-, 21-mer RNA, showing the applicability of the LC/MS method for intact mass determination.

To evaluate the optimized HILIC-MS methods with a heavily modified oligonucleotide, an ASO with multiple 2-methoxyethoxy (2MOE) modifications were analyzed using the Glycan Mapping and HILIC-Z column (Figure 6). The results showed that both columns yielded a sharp peak for the ASO, with the Glycan Mapping column having better sensitivity relative to the HILIC-Z column (Figure 6A). As expected, the Glycan Mapping also showed a broader charge state distribution with the 8- charge state being the highest charge state detected.

In contrast, the HILIC-Z column highest charge state detected was 6- for the ASO (Figure 6B). Lastly, the ASO identity was confirmed through the deconvoluted mass spectra from both experiments (Figure 6C).

The stability of the Glycan Mapping column was verified by running and storing it with 10 mM ammonium acetate at pH 6.8 and 30 °C over 17 days (Figure 7). The column underwent repeated RNA sample injections for 3 days, paused for 2 days, run for 5 days, paused for 3 days, then run for an additional 5 days to complete 1,000 injections. This stop/start pattern simulates a more realistic usage scenario for oligonucleotide analysis. The chromatographic results demonstrated that the column maintained optimal separation between the n–1 oligonucleotide pair (i.e., change in retention time (ΔRT) between 20- and 21-mer) throughout the 1,000 injections. Additionally, the method achieved excellent retention stability, with a %RSD of less than 3% for all monitored oligonucleotides.

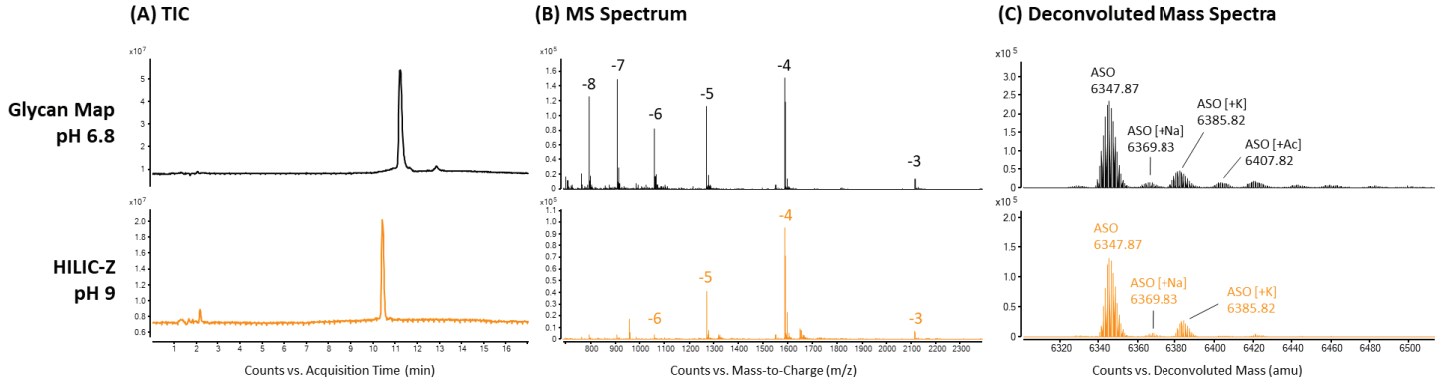
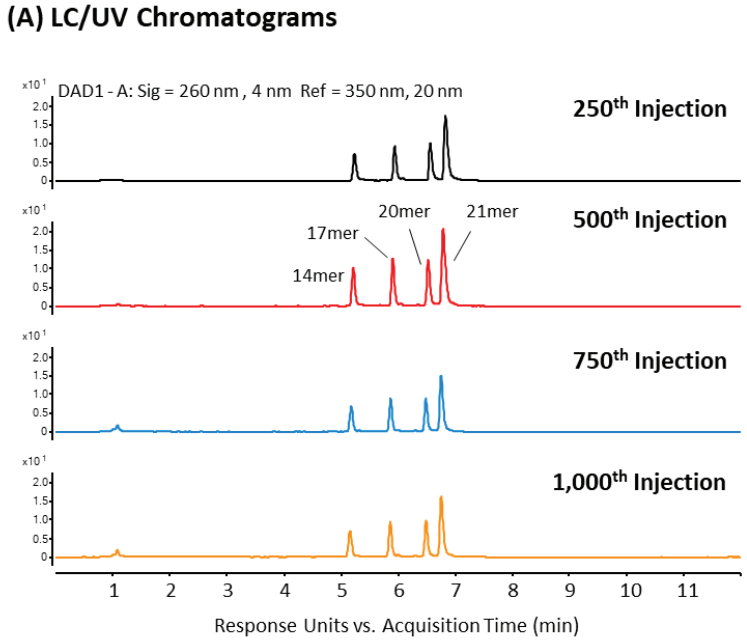
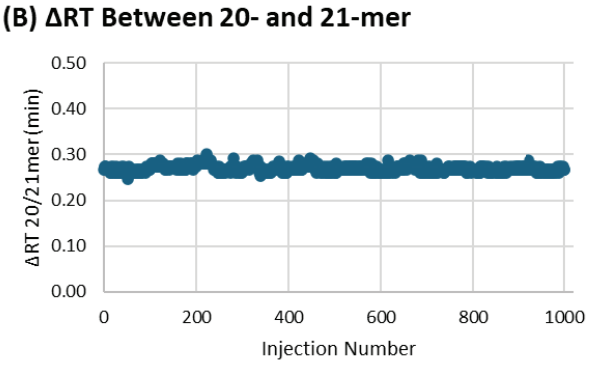


Figure 6. Comparison of the Glycan Mapping and HILIC-Z columns for characterization of an 18-mer ASO.





(C) Retention Time %RSD Over 1,000 Injections

	14mer	17mer	20mer	21mer
Average	5.29	5.98	6.61	6.88
STD	0.14	0.17	0.14	0.14
%RSD	2.69	2.83	2.13	2.05

Figure 7. Reproducibility study with the Glycan Mapping column using an Agilent 1290 Infinity diode array detector over 1,000 injections.

Conclusion

This study systematically evaluated five different HILIC stationary phases with varying mobile phase pH levels to determine the optimal conditions for chromatographic peak shape, resolution, and MS signal for oligonucleotides. It was found that different mobile phase pH conditions can impact the charge state distribution of the analyte, which is important to consider when working with longer oligonucleotides. The developed methods ultimately facilitated the LC/MS analysis of a heavily modified ASO, with the potential for sequence confirmation analysis through tandem MS. This work confirms that HILIC chromatography can be an attractive alternative to IP-RP for the analytical characterization of oligonucleotides. These methods could be applied to other emerging oligonucleotide modalities, including siRNA, aptamers, single-stranded guide RNA (sgRNA), and mRNA sequencing in the future.

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