New Column Technology for Improved Oligonucleotide Separations

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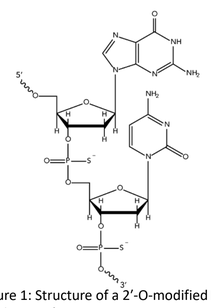
INTRODUCTION

Oligonucleotides continue to be of considerable interest due to their many applications in the bio therapeutic space. Treatments for neurodegenerative diseases, respiratory disorders, and cancers are examples of their promising therapies. Being able to monitor the synthesis of oligos via HPLC or LCMS is very important for producing safe therapeutics for potential patients. Analyzing nucleic acid oligomers requires LC method conditions that may include elevated pH and temperature. Due to the complex nature of polynucleotides and their potential for impurities and modifications the separation conditions can be challenging. These considerations demand the use of column technology that is high performance and robust. Comprehensive LC and LCMS characterization is required to provide analyses to support both research and therapeutic development of these new chemical entities.

AIM OF THE STUDY

In our study, we sought to evaluate the potential of a new SPP OLIGO C18 column for improved oligonucleotide separations through column comparisons and varying method conditions.



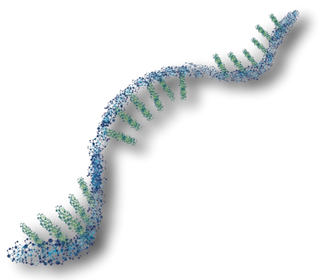


Figure 1: Structure of a 2'-O-modified RNA. (idtdna.com)

EXPERIMENTAL

Samples:

1. 10/60 ssDNA Ladder

Purchased from IDT as a lyophilized standard. The 10µg sample was first centrifuged to eliminate loss of sample. Next the sample was reconstituted in 1mL of 10mM Tris/1mM EDTA solution at pH 8.0 (Sigma-Aldrich).

2. Oligonucleotide Performance Standard Mix

Obtained from SigmaAldrich as a lyophilized standard. The sample, containing 6 oligonucleotides, ranging from 12 to 33 in base length was first centrifuged to eliminate loss of sample. Next sample was reconstituted in 100µL of de-ionized H_2O and diluted (1:10) in 10mM Tris/1mM EDTA solution at pH 8.0 (SigmaAldrich).

3. Mixed Primers

Multiple oligonucleotide standards obtained as lyophilized standards from IDT. Six different oligonucleotides of base length ranging from 15 to 21 were reconstituted in 10mM Tris/1mM EDTA. The six standards were then diluted to 1ng/mL in 10mM Tris/1mM EDTA.

Instrumentation:

LC System

All samples were analyzed on a Shimadzu Nexera X2 (Shimadzu Scientific Instruments, USA).

MS System

Velos Pro Orbitrap or QExactive HF (ThermoFisher Scientific, USA) operated in (-) ESI at 45000 Rs using the HESI-II Ion Max source (2.5kV, 350°C capillary).

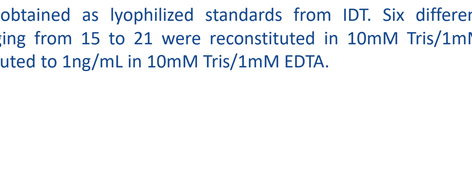
Gradient Conditions:

Mobile Phase A: 100mM TEAA, pH 8.5 (UV Detection, 1-2, 5)
100mM TEAA, pH 7.0 (UV Detection, 3)
50mM HFIP/5mM TEA, pH 8.4 (MS Detection, 6)

Mobile Phase B: Acetonitrile (UV Detection, 1-2, 5)
Methanol (UV Detection, 3)
Methanol (MS Detection, 6)

Flow Rate: 0.5mL/min

Temperature: 60°C

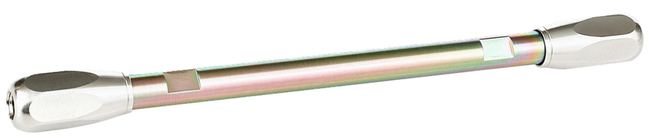


HALO 120Å 2.7µm OLIGO C18 Particle

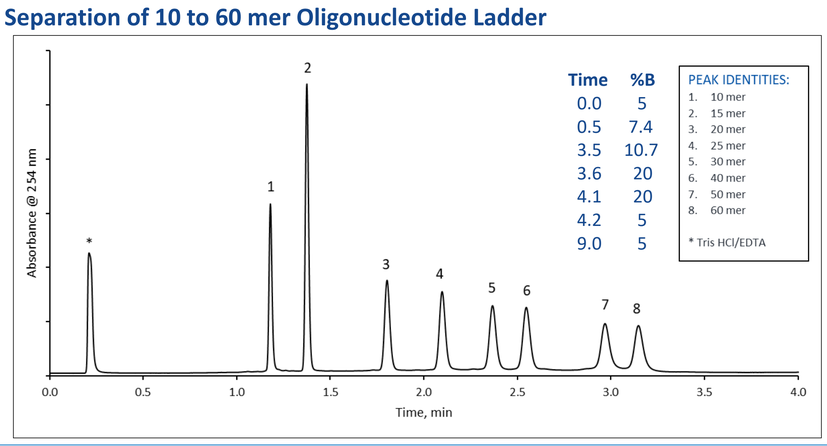
Columns in the Study:

A HALO 120 Å OLIGO C18, 2.7 µm, 2.1x50 mm
(Advanced Materials Technology, Wilmington, DE)

A 120 Å C18, FPP 1.9 µm, 2.1x50 mm



Separation of 10 to 60 mer Oligonucleotide Ladder



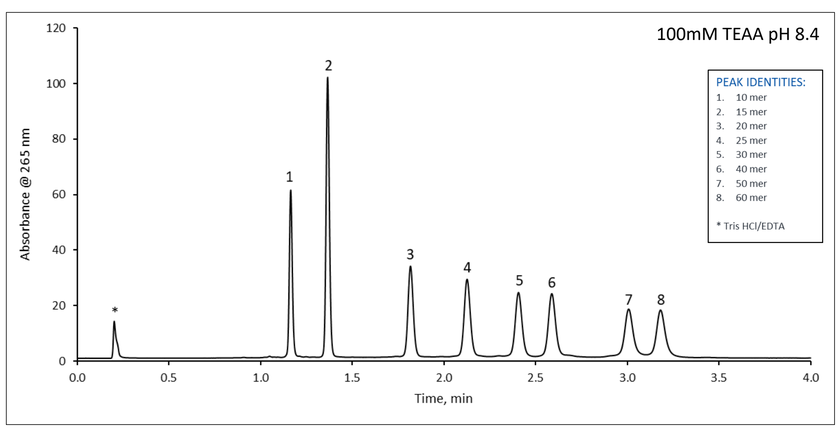
Peak #	Base Length	Sequence
1	10	ATC GCG GAT T
2	15	GCT GCG ACG AGG CTG
3	20	ATC GCG GAT TAG CAC TAC GT
4	25	ATC TCG GAT TAG CAC TAC GCA TCG G
5	30	ATC GCG GAT TAG CAC TAC GCA TCG GTT ACA
6	40	ATC GCG GAT TAG CAC TAC GCA TCG GTT ACA AAC GAG TAC C
7	50	ATC GCG GAT TAG CAC TAC GCA TCG GTT ACA AAC GAG GAC CTG ATG CAC TT
8	60	ATC GCG GAT TAG CAC TAC GCA TCG GTT ACA AAC GAG GAC CTG ATG CAC TTT GAC AGC ATG

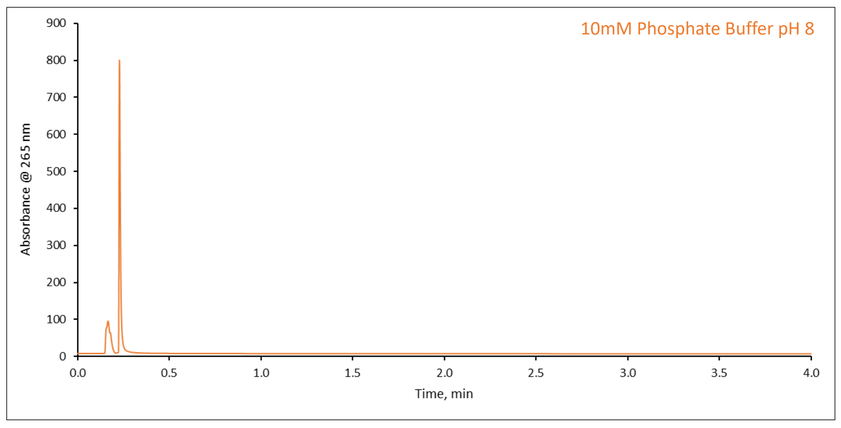
Table 1. Sequences in relation to oligonucleotide base length and elution order

- Native silica materials (SPP or FPP) are unsuitable for high temperature, high pH applications.
- The new HALO 120 Å OLIGO C18 column performs well under the UV conditions as shown above, as well as with different ion pairing conditions needed for MS analysis.
- Using SPP (superficially porous particle) technology the ladder of oligonucleotides can be completed in under 3.5 minutes while maintaining baseline resolution of each oligomer.

RESULTS

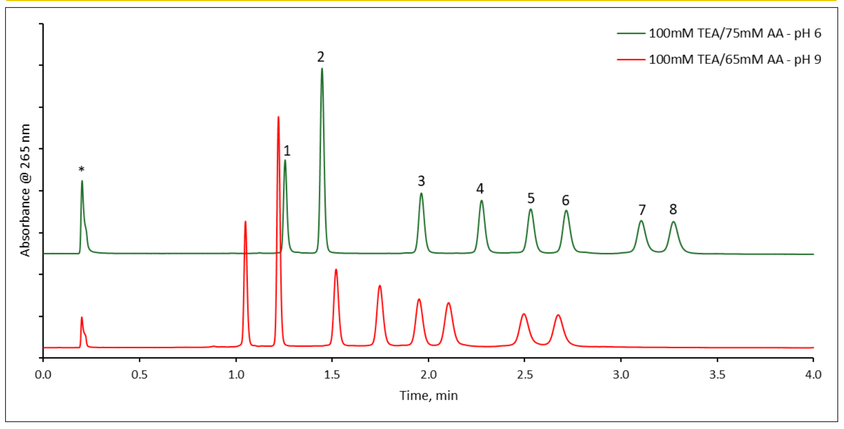
1) Impact of Ion Pairing on Oligonucleotide Retention





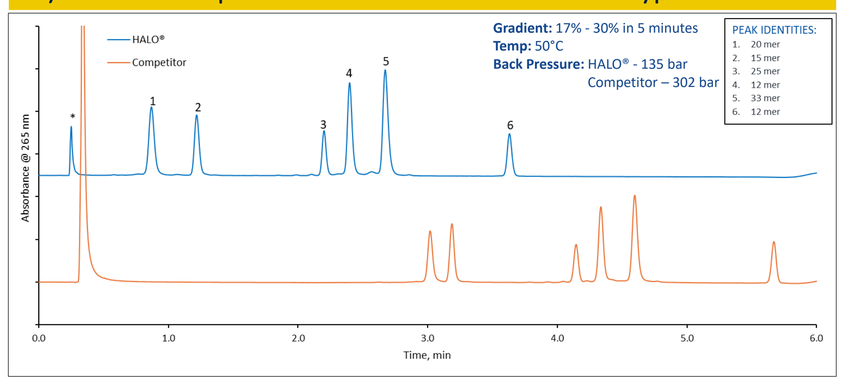
- The above chromatograms give an example of why pH is not the only important mobile phase modifier for oligonucleotide separations.
- Contemporary oligonucleotide analysis is frequently conducted using ion pair reversed phase chromatography (IPRP).
- TEA (triethylamine) or TEA-Acetate (TEAA) buffer is a common IPRP modifier, creating favorable IPRP separations.
- Phosphate buffer does not support IPRP, meaning little to no retention.

2) Effects of pH on Oligonucleotide Retention



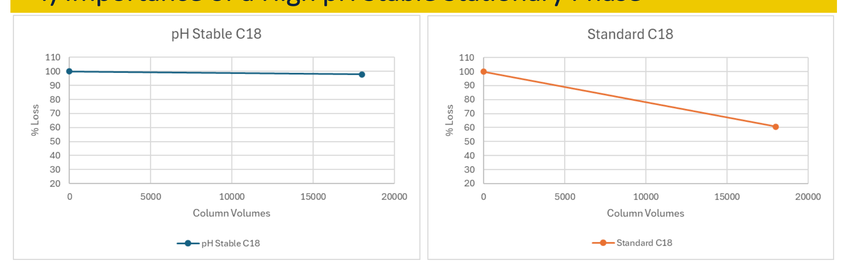
- Two different pHs were used to evaluate how the retention of oligonucleotides differs at the pHs 6 and 9.
- TEAA was used as the ion pairing reagent for each mobile phase and acetic acid was used to adjust the pH as specified.
- The concentration of TEA was maintained at 100mM to ascertain the role that pH has on oligonucleotide retention.
- By adjusting pH with acetic acid, we can see, retention modestly decreases as pH increases.
- Around a pH of 9, it is suggested that at 60°C oligonucleotides will adopt a folded deprotonated state, reducing retention.

3) Column Comparisons: Particle Size and Particle Type



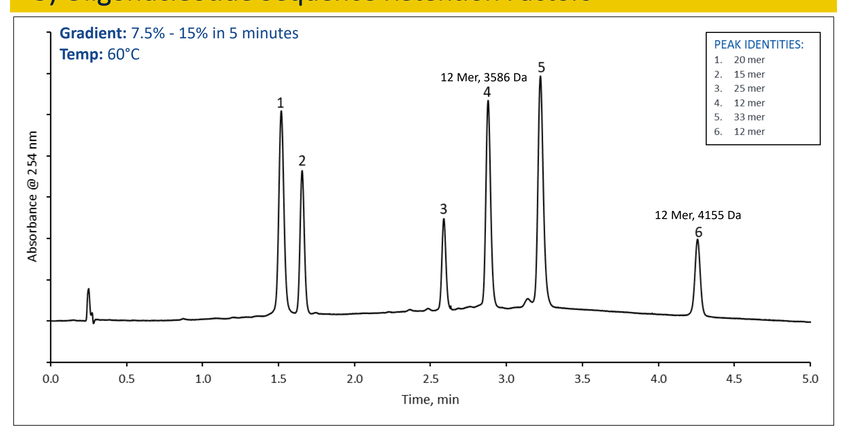
- The HALO® OLIGO C18 2.7 µm column performed favorably compared to an FPP C18 demonstrated for oligonucleotide separations, yielding a faster separation while maintaining efficiencies similar to the 1.9 µm FPP.
- The resolution between the critical pairs, peaks 1-2 and peaks 3-4, is higher on the SPP HALO® column.
- By recording the peak width values of each peak, the efficiency of each column can be measured by calculating peak capacity. The HALO® OLIGO C18 has a capacity of 68 compared to the competitor with a capacity of 66.

4) Importance of a High pH Stable Stationary Phase



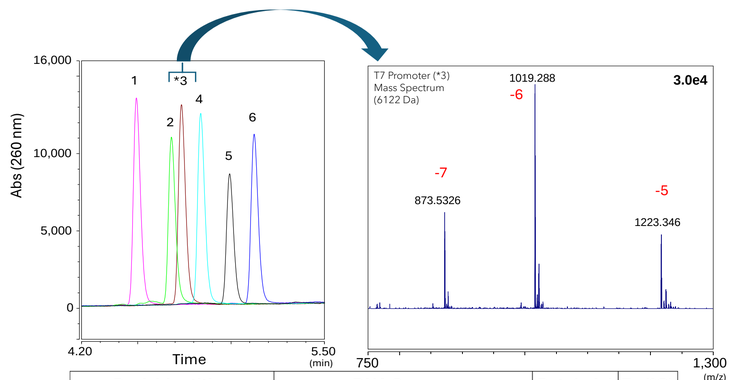
- At high pHs, native silica will be solubilized unless treated for high pH stability
- The graph measures the result of efficiency (plates) of both a standard silica and the new surface modified silica from AMT after stability testing.
- The pH stable phase maintained good efficiency after the stability while a standard, silica particle bonded phase material, lost efficiency.

5) Oligonucleotide Sequence Retention Factors



- Oligonucleotide sequences can change retention characteristics if composition differs, but base length stays the same.
- Two oligomers in this mix are the same base length, 12 mer, but are well resolved on the HALO® OLIGO C18 column
- The difference in retention can be attributed to a difference in sequence which also affects mass.
- The earlier eluting 12 mer oligonucleotide has a mass of 3586 Da compared to the later eluting oligomer which has a mass of 4155 Da.

6) LCMS Analysis of Mixed Primers



Peak Identities		ssDNA Sequence	Ret. (min)	T_m (°C)
1	T7 Terminator$_{19mer}$	GCT AGT TAT TGC TCA GCG G	4.49	60.3
2	16s rRNA$_{20mer}$	AGA GTT TGA TCC TGG CTC AG	4.68	61.3
3	T7 Promoter$_{20mer}$	TAA TAC GAC TCA CTA TAG GG	4.74	55.2
4	16s rRNA$_{21mer}$	ACG GCT ACC TTG TTA CGA CTT	4.84	63.5
5	dT$_{15mer}$	TTT TTT TTT TTT TTT	4.99	38.6
6	dT$_{16mer}$	TTT TTT TTT TTT TTT T	5.12	40.9

- This example shows synthetic oligonucleotides of 15-21 bases resolved using 5 mM TEA as the ion pairing reagent, with 50 mM HFIP buffer additive. (left side, Abs 260 nm)
- In series, online ESI-MS analysis was obtained (right side), showing the charge states for the 6122 amu MW T7 promoter synthetic oligonucleotide primer.
- Retention of this ssDNA is determined by length and sequence (composition), which permits closely related impurities and failure sequences (n-1) to be well resolved using the HALO® OLIGO C18 column

CONCLUSIONS

- The obtained results demonstrate a method development approach to improve oligonucleotide separations using a new SPP column (HALO® OLIGO C18).
- The ability to run at higher temperatures coupled with a high pH ion pairing mobile phase is critical in oligonucleotide retention.
- It was demonstrated that oligonucleotide sequence or composition is an important variable to consider when creating methods focused on oligonucleotide separation.

REFERENCES

Thaplyal, P., & Bevilacqua, P. C. (2014). Experimental approaches for measuring pKa's in RNA and DNA. Methods in enzymology, 549, 189–219. https://doi.org/10.1016/B978-0-12-801122-5.00009-X