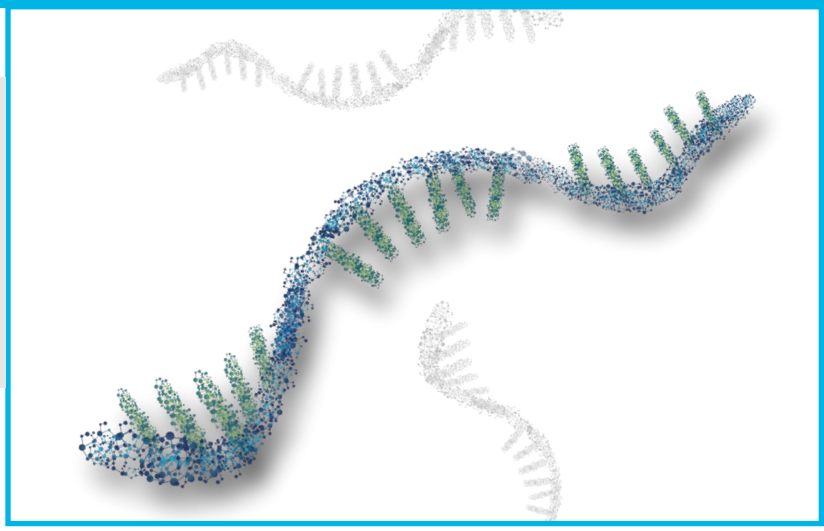
HALO®

TECHNICAL REPORT

TITLE: IMPROVED OLIGONUCLEOTIDE ANALYSIS USING HALO® OLIGO C18

MARKET SEGMENT: BIOPHARMACEUTICAL



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ABSTRACT

The analysis of polynucleotides, including DNA and RNA, is essential in various fields such as genetic research and pharmaceutical development. High-Performance Liquid Chromatography (HPLC) remains a vital tool for the separation and characterization of these complex biomolecules. This report explores the optimization of HPLC methods for polynucleotide analysis by examining the effects of key variables: column dimensions, particle size, mobile phase composition, and gradient conditions on separation performance.

INTRODUCTION

The analysis of polynucleotides, such as DNA and RNA sequences, is critical for a range of applications, from genetic research to pharmaceutical development. High Performance Liquid Chromatography (HPLC) has established itself as a powerful technique for the separation and characterization of these complex biomolecules due to its precision and versatility. However, optimizing HPLC methods for polynucleotide analysis involves careful consideration of several variables that can significantly impact separation efficiency and resolution.

Traditional HPLC methods for polynucleotide separations typically rely on specific column types, mobile phases, and gradient conditions. Recent advancements in HPLC technology and methodology provide analysts with the flexibility to modify several key parameters, including column dimensions, stationary phase, particle size, and mobile phase composition. These adjustments can enhance separation performance, reduce analysis time, and improve overall method robustness.

EXPERIMENTAL

A Shimadzu Nexera HPLC system (columbia, MD) was used for the separation of all data and chromatograms shown. Oligonucleotide standards were obtained via Integrated DNA Technologies (IDT) (Research Triangle park, NC), Zymo Research (Tustin, CA), and Sigma Aldrich (St. Louis, MO). Solvents and additives were obtained from Sigma Aldrich (St. Louis, MO). Columns used for the separations are indicated below.

Columns:

HALO 120 Å OLIGO C18, 2.7µm, 2.1 x 50mm, P2A62-402

HALO 120 Å OLIGO C18, 2.7µm, 2.1 x 100mm, P2A62-602 (Figure 6)

FPP 120 Å C18, 1.9µm, 2.1 x 50mm

Mobile Phase A: 100mM TEAA, pH 8.5 (Figure 1, 4, 9)

100mM TEAA, pH 7.0 (Figure 6, 8)

5mM TEA/50mM Hexafluoroisopropanol (HFIP), pH 8.3 (Figure 2)

5mM Ammonium Acetate, pH 7 (Figure 3)

Mobile Phase B: Acetonitrile (Figure 1, 4, 6, 9)

Methanol (Figure 8)

25/75 Acetonitrile/Methanol (Figure 2, 3)

Flow Rate: 0.4mL/min (Figure 2, 3, 6, 8, 9)

0.5mL/min (Figure 1, 4)

Temperature: 50 °C (Figure 8, 6)

60 °C (Figure 1, 2, 3, 4, 9)

Detection: UV 254/265 nm

Pressure: 135 bar (HALO®); 302 bar (FPP 120 Å)

Sample(s):

- 10/60 ssDNA Ladder (IDT, P/N: 51-05-15-01)
- PolyT Mix (IDT, P/N's: 51-01-15-06, 51-01-15-07, 51-01-15-01)
- Oligonucleotide HPLC Performance Standard Mix (Sigma Aldrich, P/N: PHR8667-1EA)
- Small-RNA Ladder (Zymo Research, P/N: R1090)

Sample Solvent: 10mM Tris/1mM EDTA (pH 8)

Data Rate: 40 Hz

Response Time: 0.025 sec

Flow Cell: 1 µL

KEY WORDS:

polynucleotides, DNA, RNA, oligonucleotides, HALO 120 Å OLIGO C18 column, HPLC

RESULTS

Standard Conditions

A separation of eight different length oligonucleotides was performed on the HALO® OLIGO C18 column. This separation is completed in under four minutes, becoming our standard conditions to separate the 10/60 mer ladder from IDT (Figure 1). The ladder is well separated on the OLIGO C18 column, while maintaining a fast gradient to increase sample throughput. The peak order was later confirmed via LC-MS detection in conjunction with the sequence data acquired from IDT.

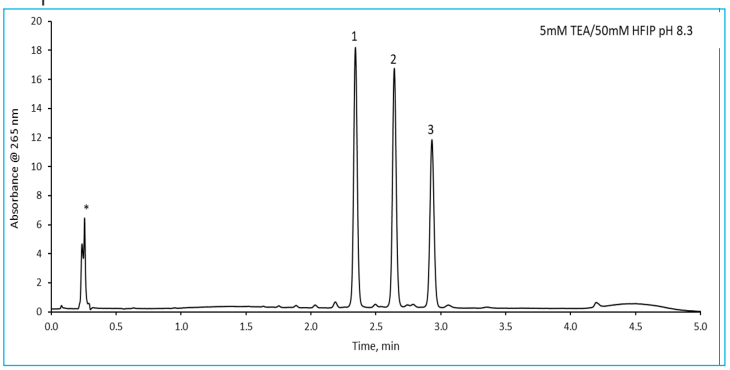

Figure 1. UV separation of the 10/60 mer ladder under ion-pairing conditions starting at 5% organic, increasing to 10.7% over 3.5 minutes. The peak identities are as follows, 10, 15, 20, 25, 30, 40, 50, 60 mer

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 of the oligonucleotides in the 10/60 ladder

Impact of Ion Pairing:

Ion pairing is essential for oligonucleotide analysis by HPLC because it enhances the separation of nucleotides by modifying their charge characteristics. Oligonucleotides are often highly polar and negatively charged due to their phosphate backbone, which can lead to poor retention and resolution during chromatographic separation. By introducing ion-pairing agents, such as triethylamine, the oligonucleotides form neutral or less charged complexes. This modification improves their interaction with the hydrophobic stationary phase of the HPLC column, allowing for more effective retention, better peak shapes, and enhanced resolution (Figure 2). Consequently, ion pairing facilitates accurate quantification and characterization of oligonucleotides in complex mixtures. When ion pairing is absent as in Figure 3, both retention and resolution are reduced.

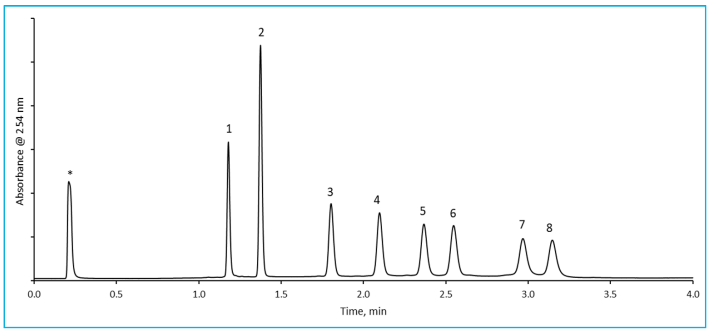

Figure 2. A separation of 3 polyT oligos (16, 18, 20 mer) under ion-pairing conditions

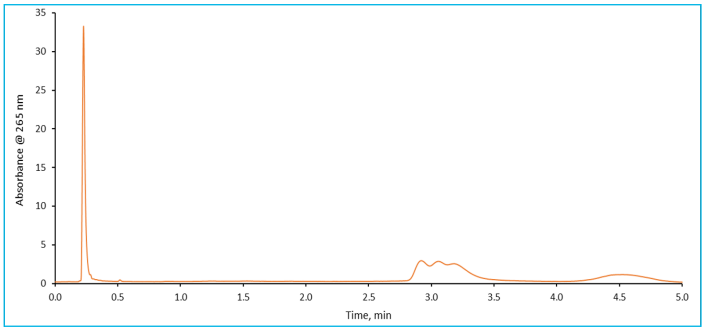

Figure 3. Failed separation of the polyT oligos (16, 18, 20 mer) under non ion-pairing conditions

Effect of pH on Oligonucleotides:

This ssDNA ladder is separated under the same gradient with changing pH in Figure 4. As pH is increased, the retention of the oligonucleotides is decreased. With the pH surpassing 9, the oligonucleotide ladder loses enough retention to cause a coelution and poor peak shape for the others in the ladder. This can be attributed to how oligos adopt a folded protonated or deprotonated state on either extreme of the pH scale (Figure 5). High pH compatible silica is highly recommended to increase column lifetime. HALO® OLIGO C18 has proven to show excellent stability while operating under the conditions below.

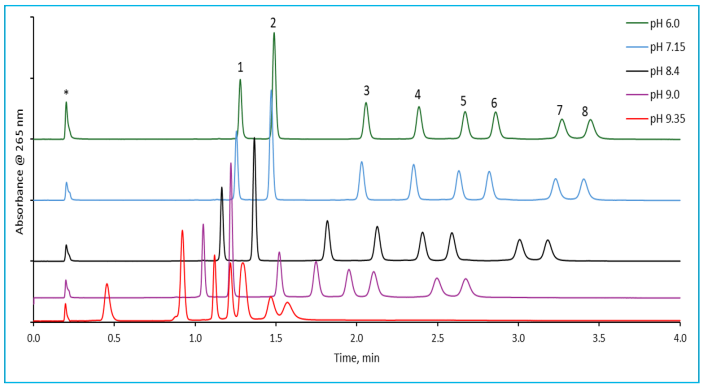

Figure 4. The 10/60 ladder separated under the same gradient conditions at 5 different pHs

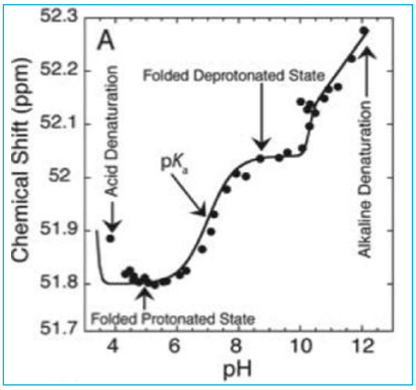

Figure 5. A graph of the different states that oligonucleotides take, spanning from 4 to 12 on the pH scale. (Source: Thaplyal, P., & Bevilacqua, P. C. (2014). Experimental approaches for measuring pKa's in RNA and DNA. Methods in enzymology, 549, 189–219. https://doi.org/10.1016/B978-0-12-801122-5.00009-X)

Modified Oligonucleotides:

Modifications to oligonucleotides can significantly impact their retention during chromatographic analysis due to alterations in their physicochemical properties. For instance, the introduction of chemical modifications such as phosphorothioate backbones, locked nucleic acids (LNAs), or 2'-O-methyl groups can enhance the hydrophobicity or steric bulk of the oligonucleotide. These changes can affect the degree of interaction with the stationary phase in HPLC, leading to variations in retention time. See Figure 6 for an example with 2 different 12 mers and how their retention is impacted. Moreover, modifications can influence the overall charge profile of the oligonucleotide, thereby impacting ion-pairing interactions in reverse-phase chromatography. As a result, careful consideration of these modifications is essential for optimizing separation conditions and achieving reliable analysis, particularly in complex biological samples.

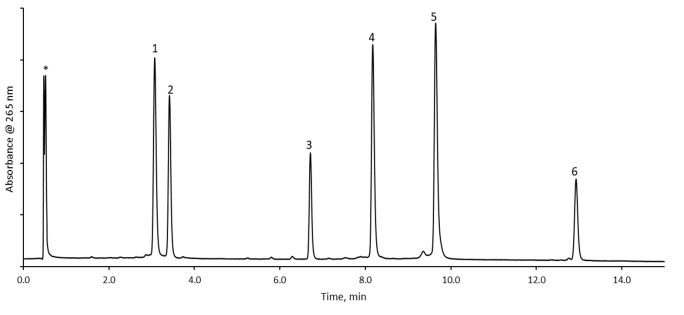

Figure 6. A separation of the "Oligonucleotide HPLC Performance Standard Mix" from Sigma Aldrich under ion-pairing conditions, starting at 7.5% and increasing to 15% over 20 minutes. The mixture contains 6 oligonucleotides, with two oligonucleotides being 12 base lengths, but the later eluting 12 mer has a biotin modification. The elution order is as follows, 20, 15, 12, 25, 33, and 12(Biotin) mer.

Sequence	Mer	Chemical Formula	Molecular Weight	Content nmol/vial
TTT TTT TTT TTT	12	$C_{120}H_{157}N_{24}O_{82}P_{11}$	3588.40	0.8
TTT TTT TTT TTT 3'mod {BtnTg} [1]	12	$C_{142}H_{197}N_{27}O_{92}P_{12}S_1$	4157.98	1.0
AGC TGT ACT TTT TTT TTT TTT TTT T	25	$C_{248}H_{320}N_{64}O_{165}P_{24}$	7580.90	1.0
AGC TGT ACT TTT TTT TTT TTT TTT TTT TTT TTT	33	$C_{328}H_{424}N_{80}O_{221}P_{32}$	10014.40	1.0
TGT GAC CAC GTA GAC TGA CT	20	$C_{195}H_{246}N_{75}O_{118}P_{19}$	6117.04	1.0
TCT CTC TCT CTC TCT	15	$C_{143}H_{189}N_{37}O_{96}P_{14}$	4395.90	1.0

[1] TTT TTT TTT TTT 3'mod {BtnTg} structure below.

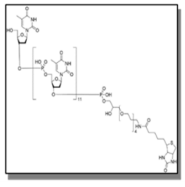

Figure 7. Sequences of the Oligonucleotide HPLC Performance Standard Mix from Sigma Aldrich

Effect of Particle Morphology on Oligonucleotide Retention:

The SPP HALO® OLIGO C18 2.7 µm column outperformed the FPP C18 in oligonucleotide separations, providing a faster separation while achieving efficiencies similar to the 1.9 µm FPP. It also exhibited higher resolution between critical peak pairs (peaks 1-2 and peaks 3-4) along with a significant reduction in back pressure. Additionally, the HALO® OLIGO C18 demonstrated similar peak widths compared to the FPP column even with a larger particle size (Figure 8).

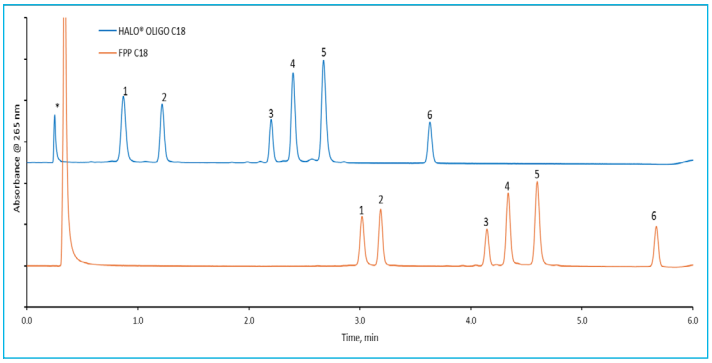

Figure 8. A comparison of the "Oligonucleotide HPLC Performance Standard Mix" on the SPP HALO® column and a FPP C18 column. A gradient starting at 17% and increasing to 30% in 5 minutes.

ssRNA Under Ion-Pairing Conditions:

Single-stranded RNA (ssRNA) may exhibit lower retention characteristics under ion-pairing conditions due to its inherent structural and charge properties. ssRNA is highly negatively charged due to its phosphate backbone, which can lead to strong electrostatic repulsion when interacting with the ion-pairing agents. Additionally, ssRNA has a relatively flexible structure that can reduce its hydrophobic interactions with the stationary phase in HPLC. As a result, ssRNA may not form stable complexes with the ion-pairing agents, leading to decreased retention times and less effective separation compared to other oligonucleotides or modified forms. Even with the reduced retention of the ssRNA ladder, the OLIGO C18 column separates all 4 oligonucleotides in under 4 minutes (Figure 9).

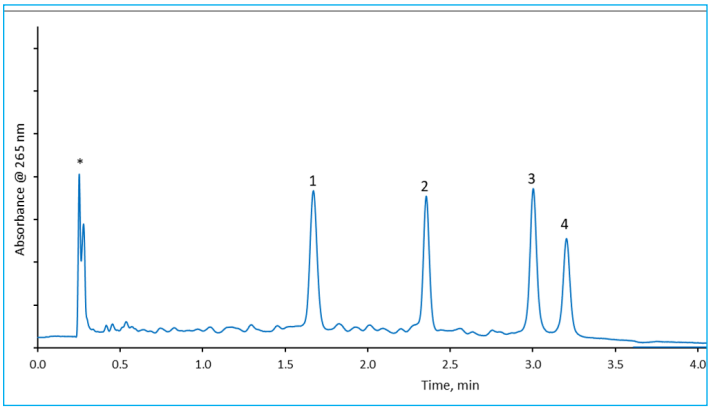

Figure 9. Separation of 4 ssRNA nucleotides under ion-pairing conditions. The elution order is as follows, 17, 21, 25, 29 mer. The gradient starting at 5% and increasing to 10% over 5 minutes.

Peak #	Base Length	Sequence
1	17	5´CAGUGGCUGGUUGAGAU 3'
2	21	5´AGCAGUGGCUGGUUGAGAUUU 3´
3	25	5´AGAGCAGUGGCUGGUUGAGAUUUAA 3´
4	29	5'AGAGAGCAGUGGCUGGUUGAGAUUUAAUU 3'

Table 2. Sequences of the oligonucleotides in the small ssRNA ladder

CONCLUSION:

Oligonucleotides are analyzed primarily for their roles as research and diagnostic reagents, therapeutic agents, and in drug development to modulate gene expression. Accurate impurity analysis is essential for ensuring the safety and efficacy of these products. However, challenges in separating oligonucleotides arise due to their complex structures and sizes, as well as their negatively charged phosphate backbone. Some of the factors discussed play a large role in the separation of polynucleotides. Ion pairing plays a crucial role in enhancing the analysis of oligonucleotides by reversed phase chromatography, significantly improving their retention and resolution through the formation of neutral or less charged complexes with ion-pairing agents.

Elevated pH levels can adversely affect retention, leading to coelution and poor peak shapes due to the altered states of oligonucleotides. Modifications to oligonucleotide structures, such as phosphorothioates, lipids, sugars, or nucleobases, further complicate their analysis, necessitating careful optimization for effective separations. While single-stranded RNA (ssRNA) exhibits lower retention under ion-pairing conditions due to strong electrostatic repulsion and structural flexibility, the HALO® OLIGO C18 column successfully resolved the mixture. Overall, the integration of ion pairing, careful consideration of pH, and particle morphology are critical for optimizing oligonucleotide analysis in complex biological samples.

REFERENCES:

1. 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

2. Sakharam P. Waghmare, Petros Pousinis, David P. Hornby, Mark J. Dickman,

Studying the mechanism of RNA separations using RNA chromatography and its application in the analysis of ribosomal RNA and RNA:RNA interactions, Journal of Chromatography A, Volume 1216, Issue 9,2009,Pages 1377-1382,ISSN 0021-9673, https://doi.org/10.1016/j.chroma.2008.12.077

3. John K. Frederiksen, Joseph A. Piccirilli, Chapter 14 - Separation of RNA Phosphorothioate Oligonucleotides by HPLC, Methods in Enzymology, Academic Press, Volume 468, 2009, Pages 289-309, ISSN 0076-6879, ISBN 9780123743992, https://doi.org/10.1016/S0076-6879(09)68014-9

4. Mirlinda Biba, Joe P. Foley, Christopher J. Welch, Chapter 6 - Liquid chromatographic separation of oligonucleotides, Editor(s): Salvatore Fanali, Paul R. Haddad, Colin F. Poole, Marja-Liisa Riekkola, Liquid Chromatography (Second Edition), Elsevier, 2017, Pages 159-182, ISBN 9780128053928, https://doi.org/10.1016/B978-0-12-805392-8.00006-2