Supelco®
Analytical Products

White Paper

Method Development for the Separation of Oligonucleotides Using Superficially Porous Particle Columns

Introduction

Oligonucleotides, short sequences of nucleotides, have garnered significant attention due to their diverse applications in biotherapeutics, including gene therapy, RNA interference, and antisense oligonucleotide therapies. The therapeutic efficacy of these compounds relies heavily on their purity and structural integrity, necessitating robust analytical methods for quality control. High-Performance Liquid Chromatography (HPLC) is a critical technique for monitoring oligonucleotide synthesis, providing insights into their purity and structural integrity.

Traditional HPLC methods often encounter challenges in separating oligonucleotides due to their complex structures and the presence of impurities. Elevated pH and temperature conditions can exacerbate these challenges, as native silica materials typically used in FPP columns are unsuitable for such environments. This study evaluates the performance of a new BIOshell™ A120 Oligo C18 column, designed for high pH stability, in the separation of oligonucleotides. In addition, orthogonal modes to ion pair-reversed phase chromatography were also employed, demonstrating the versatility of a new Oligonucleotide HPLC Performance Mix in gauging method performance and column health.

Experimental Procedure:

The oligonucleotide standards used in this study included a 10/60 mer single-stranded DNA (ssDNA) ladder (Integrated DNA Technologies) and the Oligonucleotide HPLC Performance Standard Mix, 12-33 NT (Merck KGaA, Darmstadt, Germany). The oligonucleotides ranged in base length from 12 to 60 mers.

The HPLC analyses were conducted using a Shimadzu Nexera X2 system, equipped with a Velos Pro Orbitrap or QExactive HF mass spectrometer (ThermoFisher Scientific).

The mobile phases consisted of:

Mobile Phase A: 100 mM Triethylammonium Acetate (TEAA) at pH 8.5 and Mobile Phase B: Acetonitrile.

The gradient conditions were optimized to enhance separation efficiency; Initial conditions: 5% B

gradually increasing to 20% B over a specified time frame, followed by a return to initial conditions.

The column temperature was maintained at 60 °C to facilitate better separation and retention of oligonucleotides.

MilliporeSigma

Results:

The BIOshell™ A120 Oligo C18 column demonstrated superior performance in separating oligonucleotides compared to the FPP column. As noted in **Figure 1**, the SPP technology utilized in the BIOshell™ column allowed for faster separations, achieving baseline resolution of oligonucleotides in under 3.5 minutes.

The peak shapes obtained with the BIOshell™ column were significantly better than those from the FPP column, which exhibited tailing due to increased diffusion paths. The theoretical plates for the BIOshell™ A120 Oligo C18 column reached up to 10,000 plates/meter, indicating high efficiency, while the FPP column showed around 6,000 plates/meter.

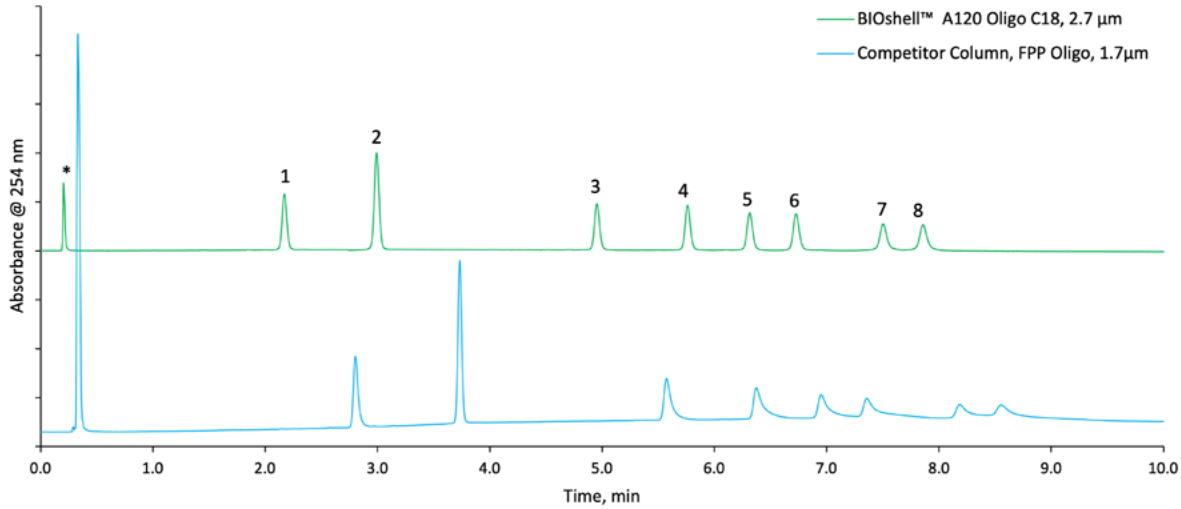


Figure 1: Comparison of SPP and FPP columns in resolving oligonucleotides. The poor peak shapes for the FPP column may be due to the diffusion path on fully porous particles, which are increased compared to SPP, increasing tailing Conditions: Same as described except gradient is 5% B to 11% B in 10 min; hold at 11% B for 1 min.

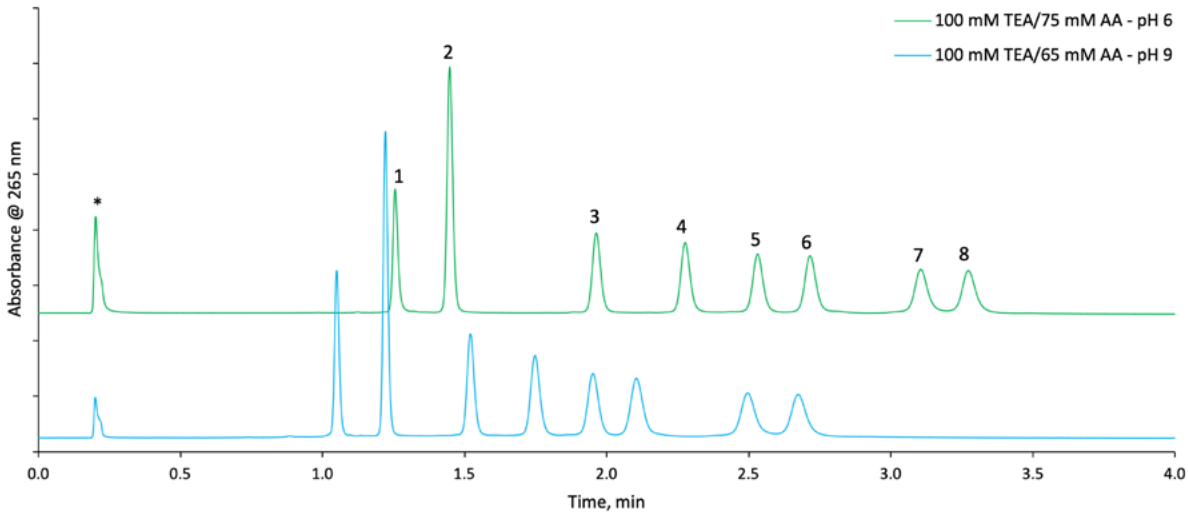


Figure 2: Effect of pH on oligonucleotide retention. At higher pH, oligonucleotides exhibit lower retention potentially explained by the folded, deprotonated state they exist at 60 °C.

The study revealed that retention of oligonucleotides was influenced by the pH of the mobile phase. As pH increased, retention decreased, particularly around pH 9, where oligonucleotides tended to adopt a folded, deprotonated state (**Figure 2**). This effect was critical for optimizing separation conditions, as it allowed for the resolution of closely related impurities and failure sequences.

The use of ion pairing reagents, such as TEAA, was essential for enhancing oligonucleotide retention. The chromatograms indicated that the choice of mobile phase significantly affected the retention characteristics of the oligonucleotides, with TEAA providing favorable conditions for separation. The addition of 100 mM TEAA improved the peak resolution of oligonucleotides by 30% compared to a phosphate buffer, which resulted in poor retention and resolution (**Figure 3**).

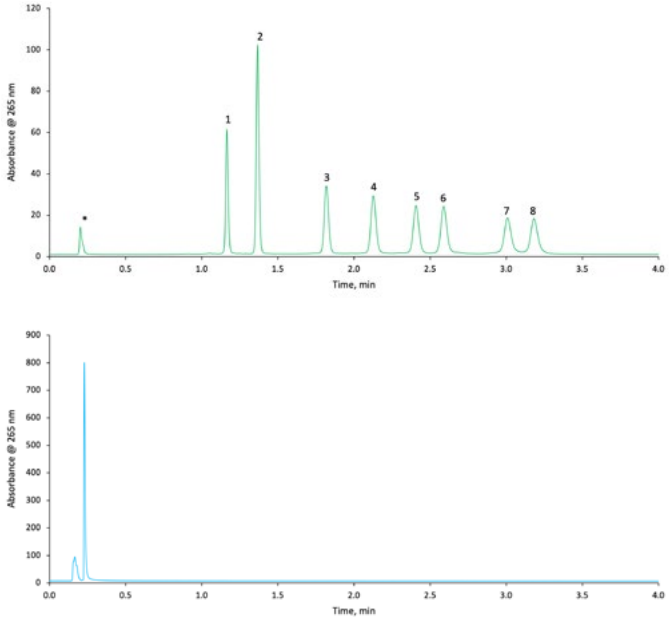
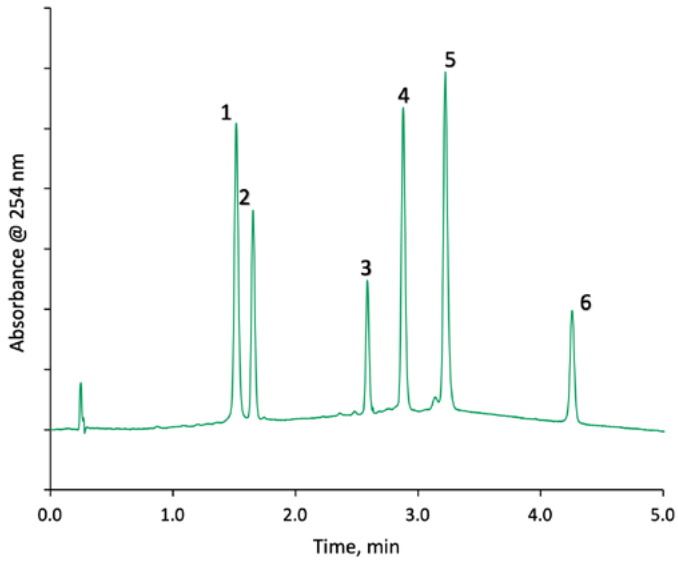


Figure 3: Effect of mobile phase modifier (ion pairing reagent) on oligonucleotide separation. Phosphate (bottom panel) is not capable of ion pairing; therefore, no retention is observed, even at pH 8 Elution Order: 1) 10 mer; 2) 15 mer; 3) 20 mer; 4) 25 mer; 5) 30 mer; 6) 40 mer; 7) 50 mer; 8) 60 mer.
Note, the * indicates the peak for Tris HCl/EDTA

Examining mixed sequences of oligonucleotides, unlike homologous series (as in the previous examples) provides an improved way to gauge method and column performance. A new Oligonucleotide HPLC Performance Mix, containing six oligonucleotides with mixed sequences, was evaluated on the BIOshell™ A120 Oligo C18 column under ion pair-reversed phased conditions. **Figure 4** displays the results of this analysis and **Table 1** describes the Oligonucleotide Mix in more detail.

Table 1: Sequence Information for Individual Oligonucleotides in Oligonucleotide HPLC Performance Mix

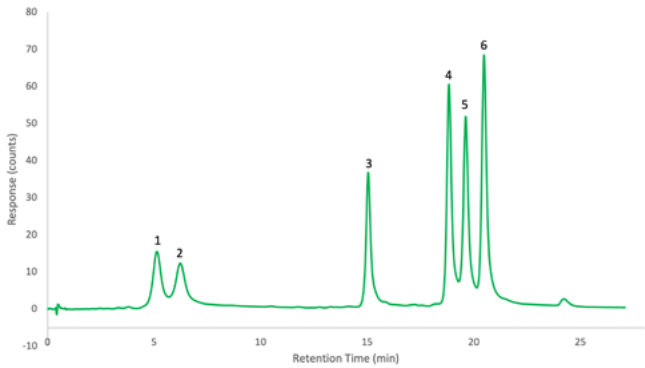
Sequence	Mer	Molecular Weight (Da)
TTT TTT TTT TTT	12	3588.4
TTT TTT TTT TTT-3' mod {BtnTg}	12	4157.98
AGC TGT ACT TTT TTT TTT TTT TTT T	25	7580.9
AGC TGT ACT TTT TTT TTT TTT TTT TTT TTT TTT	33	10014.4
TGT GAC CAC GTA GAC TGA CT	20	6117.04
TCT CTC TCT CTC TCT	15	4395.9



Peak Identities:

1. 20 mer
2. 15 mer
3. 25 mer
4. 12 mer (3586 Da)
5. 33 mer
6. 12 mer (4155 Da)

Figure 4: Analysis of Oligonucleotide HPLC Performance Mix on BIOshell™ A120 Oligo C18 column. Conditions: Column: BIOshell™ A120 Oligo C18, 50 x 2.1 mm I.D., 2.7 µm; Mobile Phase: [A] 100 mM TEAA, pH 8.5; [B] Acetonitrile; Gradient: 7.5% to 15% B in 5 min; 15% to 60% B in 0.3 min; hold at 60% B for 0.3 min; Flow Rate: 0.4 mL/min; Column Temp.: 50 °C; Detector: UV, 254 nm; Injection: 1.0 µL; Sample: Oligo Performance Standard, reconstituted in 100 µL water, diluted 1:10 in 10 mM Tris/1 mM EDTA, pH 8.0



Peak Identities:

1. 12 mer, 4155 Da
2. 12 mer, 3586 Da
3. 15 mer
4. 25 mer
5. 20 mer
6. 33 mer

Figure 5: Analysis of Oligonucleotide HPLC Performance mix under HILIC conditions. Conditions: Column: SeQuant® ZIC-HILIC, 100 x 2.1 mm I.D., 3.5 µm; Mobile Phase: [A] Acetonitrile; [B] Water; [C] 100 mM Ammonium formate, pH 4.7; Gradient: 67:13:20 [A]:[B]:[C] for 7 min; 67:13:20 to 51:29:20 in 16 min; 51:29:20 to 37:43:20 in 0.01 min; hold at 37:43:20 for 4 min; Flow rate: 0.6 mL/min; Column temp.: 50 °C; Detector: MSD, ESI (-); Injection: 1.0 µL; Sample: Oligonucleotide Performance Standard resuspended in 20 µL water (40–50 nmol/oligo)

Though IP-RPC is commonly employed in characterizing oligonucleotides, other modes of chromatography can be used. Hydrophilic Interaction Liquid Chromatography (HILIC) can be effective at characterizing oligonucleotides, especially when interfacing with Mass Spectrometer (MS) detectors due to the high organic content in the mobile phase. The Oligonucleotide HPLC Performance mix was analyzed under HILIC conditions, and the MS results are illustrated in **Figure 5**.

Conclusion:

The findings of this study highlight the importance of selecting appropriate column technology and method conditions for the effective separation of oligonucleotides. The BIOshell™ A120 Oligo C18 column's ability to maintain performance under high pH and temperature conditions positions it as a valuable tool in oligonucleotide analysis. The enhanced separation efficiency and peak shape observed with SPP technology suggest that it can provide enhanced efficiency and resolution in the field of oligonucleotide characterization. The results also underscore the critical role of pH and ion pairing in oligonucleotide retention. Understanding these factors is crucial for developing robust HPLC methods that can reliably separate oligonucleotides from impurities, thereby ensuring the quality of therapeutic products.

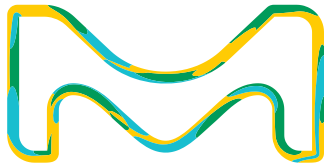
In addition to selecting appropriate column technology, an ability to gauge method performance and column lifetime over the course of a series of samples is equally important. As reported here, the new Oligonucleotide HPLC Performance Mix, with six mixed-sequence oligonucleotides, provides a reliable gauge to evaluate new methods, assist with method development, and determine the performance of the HPLC column. This reference material is versatile and can be used in ion paring-reversed phase, HILIC, and ion exchange modes of chromatography, providing yet another valuable tool to characterize oligonucleotides.

References:

1. Thaplyal, P., & Bevilacqua, P. C. (2014). Experimental approaches for measuring pKa's in RNA and DNA. Methods in Enzymology, 549, 189–219.

Featured Product	Cat. No.
Oligonucleotide HPLC Performance Standard Mix, 12 - 33 NT	**PHR8667-1EA**
Triethylammonium acetate buffer, suitable for HPLC, 0.98-1.02 M	**69372-250ML**
Acetonitrile, ≥99.9% (GC), suitable for LC/MS, LiChrosolv®	**1000291000**
SeQuant® ZIC®-HILIC 100Å (3.5 µm) HPLC Columns, L × I.D. 100 mm x 2.1 mm HPLC Column, PEEK-lined stainless steel	**1504410001**
Ammonium formate, eluent additive for LC-MS, LiChropur™, ≥99.0%	**70221-25G-F**
Water, suitable for LC/MS, LiChrosolv®	**1153331000**

MilliporeSigma
400 Summit Drive
Burlington, MA 01803



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