Technical Overview

Agilent
Trusted Answers

Dynamic Binding Capacity and High-Resolution Oligonucleotide Analysis with Agilent Bio SAX Columns

Authors

Jordy Hsiao and Matt Turner
Agilent Technologies, Inc.

Abstract

This technical overview explores the use of nonporous anion exchange (AEX) chemistry for the separation of oligonucleotides from 25 to 100 bases in length. Demonstrated is how Agilent Bio SAX columns are uniquely suited for high-resolution LC-UV separations.

Introduction

Agilent Bio IEX columns contain nonporous polystyrene divinylbenzene (PS-DVB) particles grafted with a hydrophilic, polymeric layer that eliminates nonspecific binding, while increasing column efficiency. Having a nonporous particle, Bio SAX columns provide exceptional resolution for closely eluting oligonucleotide products and impurities making it uniquely suitable for LC-UV analysis.

Anion exchange chromatography is a versatile technique for both the purification and analytical characterization of biomolecules, such as oligonucleotides.[1] The negatively charged phosphate groups in the nucleic acid backbone are attracted to the positively charged functional groups on the particles. In general, the binding strength increases as oligonucleotide length increases. As such, the bound oligonucleotides are eluted in the order of increasing length as the gradient's salt concentration increases. A previous study examined the impact of mobile phase composition and column temperature on oligonucleotides separation using SAX.[2] This study investigates the dynamic binding capacity (DBC) and analytical separation of oligonucleotides using SAX chromatography.

Experimental

Reagent and chemicals

- All reagents were HPLC grade or higher.
- Agilent RNA resolution standard (part number 5190-9028) and crude DNA oligonucleotides from Integrated DNA Technologies were used.

Instrumentation

Agilent 1290 Infinity II Bio LC system comprising:

- Agilent 1290 Infinity II high-speed pump (G7132A)
- Agilent 1290 Infinity II Bio multisampler (G7137A) with Agilent InfinityLab sample thermostat (option 101)
- Agilent 1290 Infinity II multicolumn thermostat (G7116B) with biocompatible heat exchanger
- Agilent 1290 Infinity II diode array detector (G7115A) with a bio-inert flow cell (option 28)

Sample preparation

Oligonucleotide samples were dissolved in mobile phase A and final concentrations were measured using a NanoDrop spectrophotometer. Samples were placed on the instrument as eluent C.

HPLC method

Mobile phase preparation is outlined in Table 1, HPLC conditions for binding capacity are outlined in Table 2, and the oligo cleanup gradient profile is outlined in Table 3. HPLC conditions for the analytical runs are outlined in Tables 4 and 5.

Table 1. Mobile phase preparation for SAX chromatography.

	100x Stock Buffer	Mobile Phase A	Mobile Phase B
10 mM Tris, pH 8.0	1M Tris, pH 8.0, adjusted with HCl	10 mL stock buffer was mixed with 990 mL of water	10 mL of stock buffer was mixed with 116.88 g of NaCl (2 M), and water was added up to 1 L

Table 2. HPLC conditions for binding capacity.

Parameter	Value
Columns	– Agilent Bio SAX column, NP1.7, 4.6 × 50 mm (p/n 5190-2461) – Agilent Bio SAX column, NP5, 4.6 × 50 mm (p/n 5190-2468) – Agilent Bio SAX column, NP10, 4.6 × 50 mm (p/n 5190-2476)
Mobile Phase	Eluent A: 10 mM Tris, pH 8.0 in water Eluent B: 2 M NaCl in solvent A Eluent C: oligonucleotide solution
Flow Rate	0.5 mL/min
Column Temperature	25 °C
Detection (DAD)	270/4 nm (references 360/20 nm) Peak width >0.025 min (10 Hz)
Injection Volume	NA
Total Run Time	NA

Table 3. Oligo cleanup gradient profile.

Time (min)	%A	%B
0	100	0
2	0	100
10	0	100
10.1	100	0
20	100	0

Table 4. Analytical run gradient profiles for RNA standard, 25 and 50 mer oligos with column compartment temperature at 80 °C.

Time (min)	%A	%B
0	100	0
1	100	0
2	80	20
4	80	20
14	60	40
15	100	0
20	100	0

Table 5. Analytical run gradient profiles for 75 and 100 mer oligos with column compartment temperature at 80 °C.

Time (min)	%A	%B
0	100	0
1	100	0
2	70	30
4	70	30
14	50	50
15	100	0
20	100	0

Sequence

1. Oligo cleanup gradient (20 minutes)
2. Oligo binding (100% C until breakthrough)
3. Oligo cleanup gradient (20 minutes) × 2

Results and discussion

The binding capacity of Bio SAX columns packed with different particle sizes (1.7, 5, and 10 µm) were investigated. Four differently sized oligonucleotides were used to represent the binding capacity of a broad range of oligonucleotide classes based on length (Table 6).

A union was initially used in place of a column to determine the instrument's dead volume. The instrument was conditioned with 100% eluent A, then switched to 100% eluent C. The time taken before the oligonucleotide solution was detected at 270 nm was recorded as the dead volume of the system and then subtracted from each column run (Figures 1A to 1C). The measurement was taken at 25% full-scale deflection.

Before performing the DBC study, each column was subjected to a cleanup gradient lasting 20 minutes. Dynamic binding was measured when the oligonucleotide solution was detected to have saturated the column. Each column was then cleaned by repeating the cleanup gradient two times to remove any remaining bound oligonucleotides.

Since the Bio SAX columns use nonporous particles, the total stationary phase surface area decreases as the particle size increases in a packed column. Therefore, it is anticipated that the 1.7 µm Bio SAX particles would

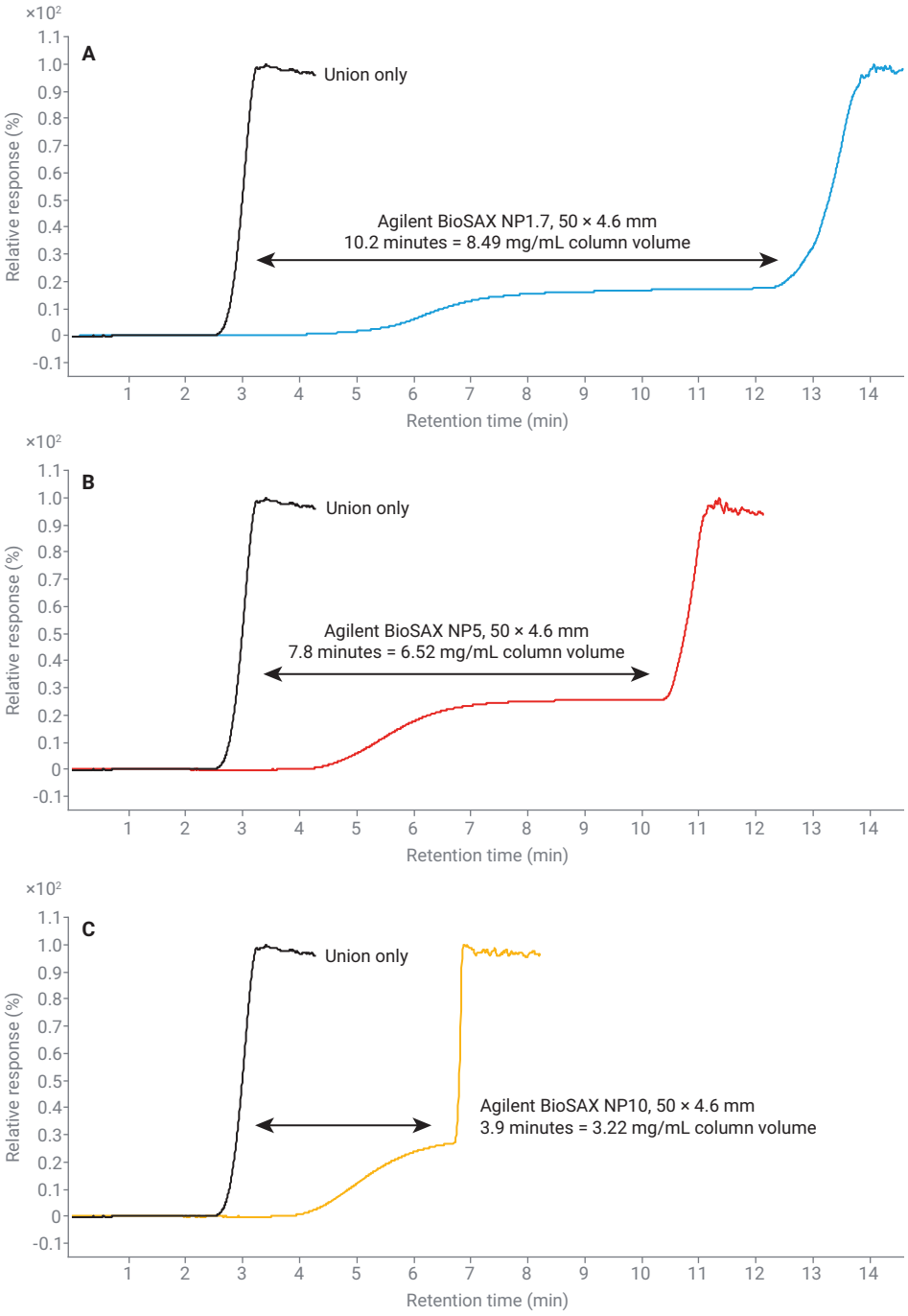

Figure 1. 50 mer oligonucleotide breakthroughs using Agilent Bio SAX columns of different sizes: (A) 1.7 µm, (B) 5 µm, and (C) 10 µm.

Table 6. Oligonucleotides used in this investigation.

25 mer	CATATAAGTTGCGTTACTTCGGCCT
50 mer	CCTAACCGCACCCTTAGCACGAAGA CAGATTCGTTCTTACCCATACTCCA
75 mer	CCGTTGGCAGGGGGATCGCATGTCC CACGTGAAACATTGCTAAACCCTCA GGTCTCTGAGCGACAAAAGCTTTAA
100 mer	AGGGAAATTCGCGCCCATAACTTGGT CCGAATACGGGTTCTTGCATCGTTC GACTGAGTTTGTTTTATATAAAACGGGCGCAATGTCTGCTTTGATCAAC

have higher DBC when compared to the 5 and 10 µm Bio SAX particles. As expected, the 1.7 µm particles yielded the highest binding capacity, while the 10 µm particles yielded the lowest binding capacity (Figure 2). Interestingly, the binding capacity for each particle size was independent of oligonucleotide lengths that were tested (Figure 2).

The analytical separation of the oligonucleotides is shown in Figure 3, together with a zoomed region to demonstrate the column's ability to resolve many of the impurities that are present within the crude samples.

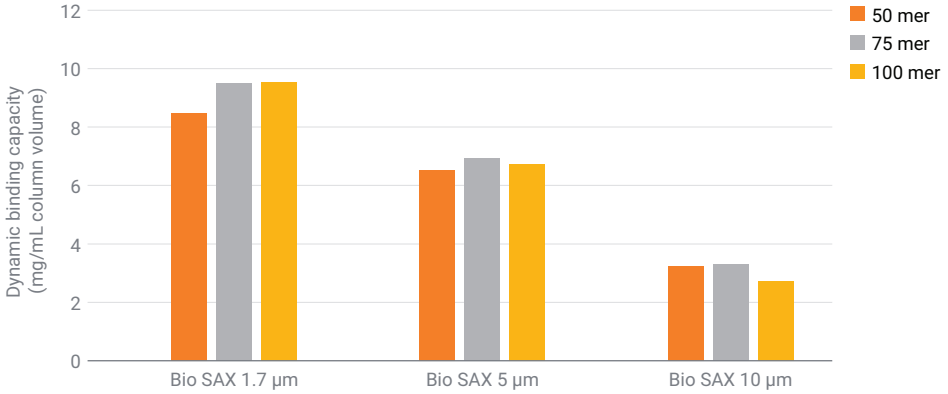

Figure 2. Comparison of binding capacity of different size oligonucleotides.

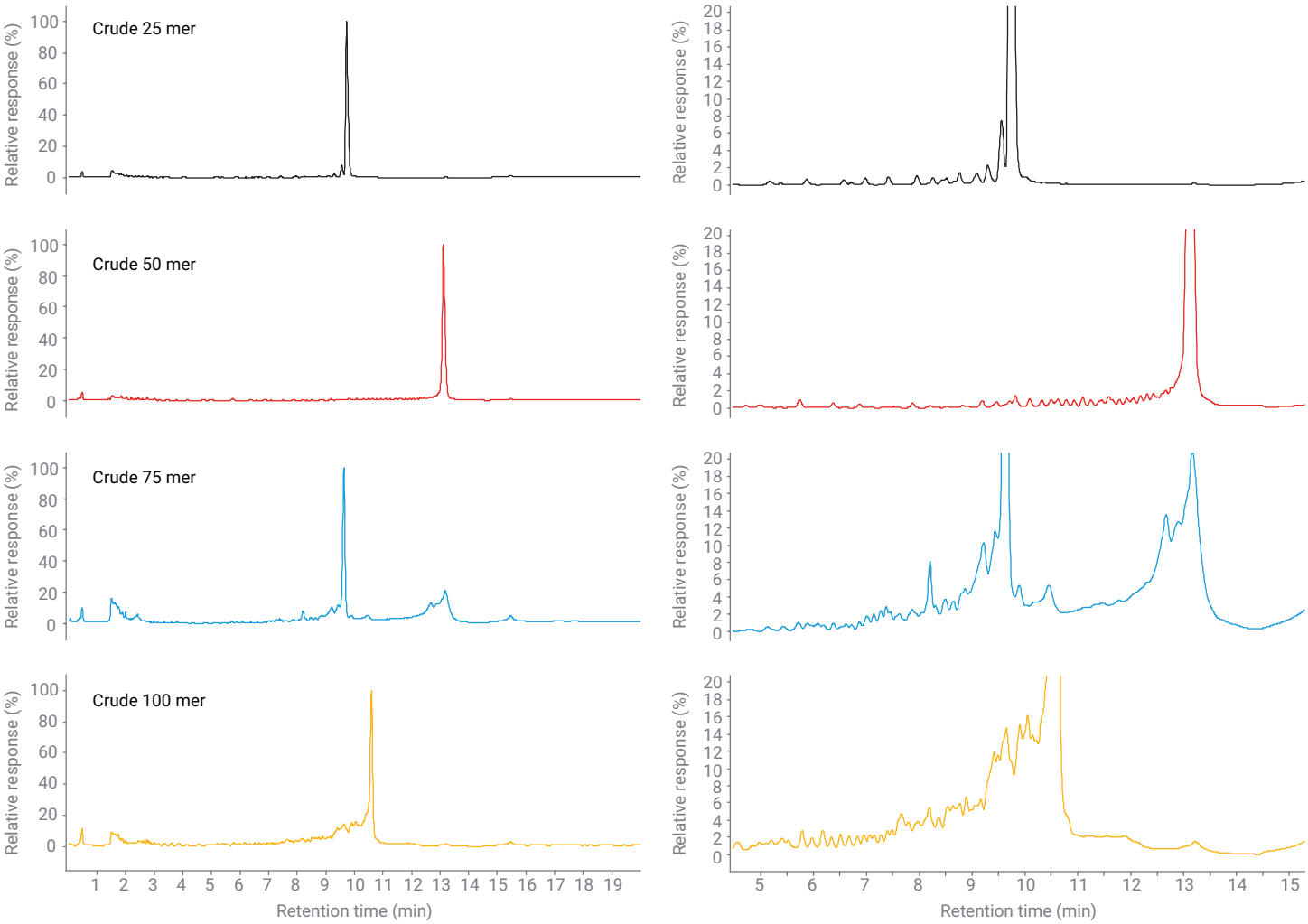

Figure 3. Analytical separation of crude 25, 50, 75, and 100 mer using an Agilent Bio SAX 5 µm column (left) with zoomed regions (right).

Moreover, the baseline separation of n – 1 oligonucleotides with the Bio SAX column is shown using an RNA standard containing 20 and 21 mer oligonucleotides (Figure 4).

Conclusion

Agilent Bio SAX nonporous materials are ideal for oligonucleotide separations, especially when there is a need to avoid volatile buffers, such as those used in ion-pair reversed-phase (IP-RP) chromatography. This can be attractive for performing LC-UV analysis and impurity profiling of the final oligonucleotide product. Ultimately, it is important for users to conduct more extensive testing and validation for each individual target biomolecule to ensure that chromatographic separation and methodology meets the user's purity criteria.

References

1. Cook, K.; Thayer, J. Advantages of Ion-Exchange Chromatography for Oligonucleotide Analysis. *Bioanalysis* **2011**, *3(10)*, 1109–1120.
2. Hsiao, J.; Apffel, A.; Turner, M. Optimizing Separation of Oligonucleotides with Anion-Exchange Chromatography. *Agilent Technologies application note*, publication number 5994-4753EN, **2022**.

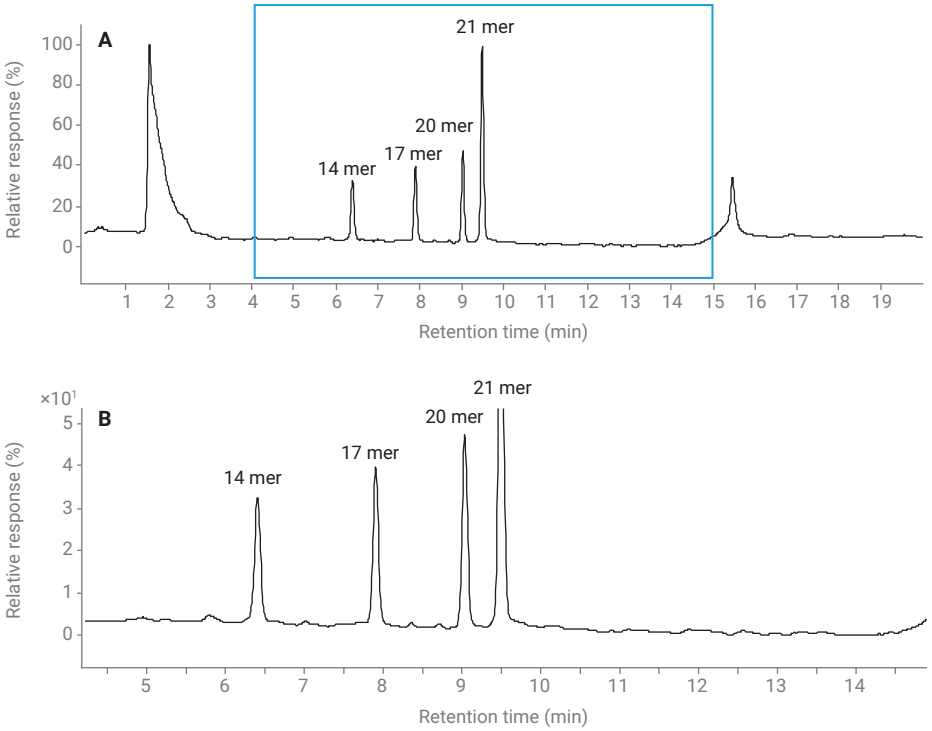

Figure 4. Analytical separation of RNA standards using an Agilent Bio SAX 5 µm column (A) with zoomed region (B).

www.agilent.com

DE50136108

Printed in the USA, July 22, 2022
5994-5109EN

Agilent
Trusted Answers