Method Development Approach to Separating Oligonucleotides Under UV and MS Detection

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Introduction to Oligos

- Why are oligonucleotides being analyzed?
  - Oligonucleotides are being developed as drugs
    - These drugs can be used to modulate gene expression
  - Impurity analysis of said drugs is required for effective products
  - The development of mRNA vaccines (Covid-19)

- What are the challenges of separating oligonucleotides?
  - Complicated structures and sizes
  - A phosphate backbone that reduces retention under RPLC
  - Modifications can range broadly: phosphorothioates, lipids, sugars or nucleobases
  - Sample stability requires suitable handling (nucleases and metals)
  - Hybrid structure formation can be either intended or unwanted

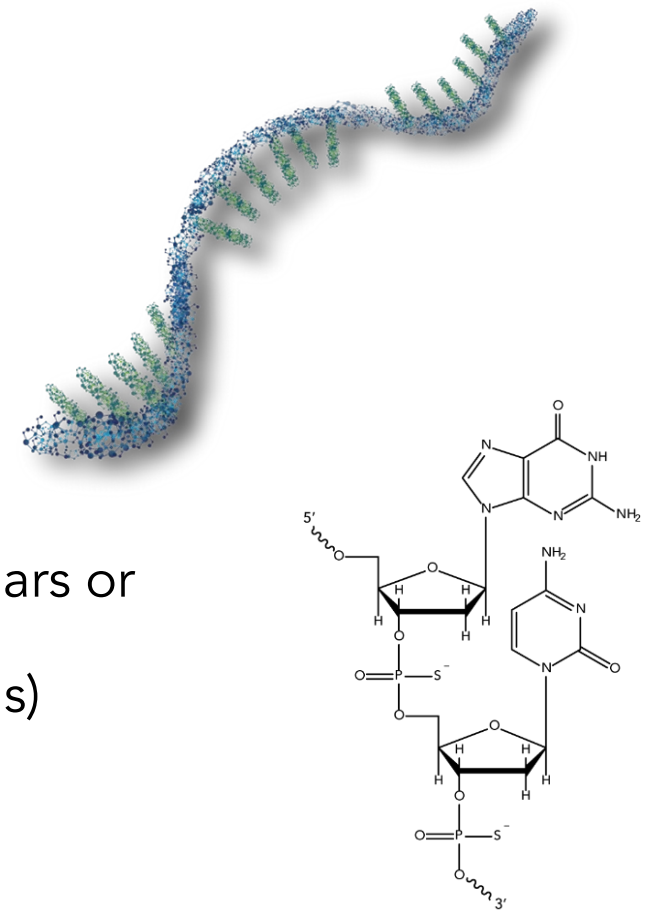


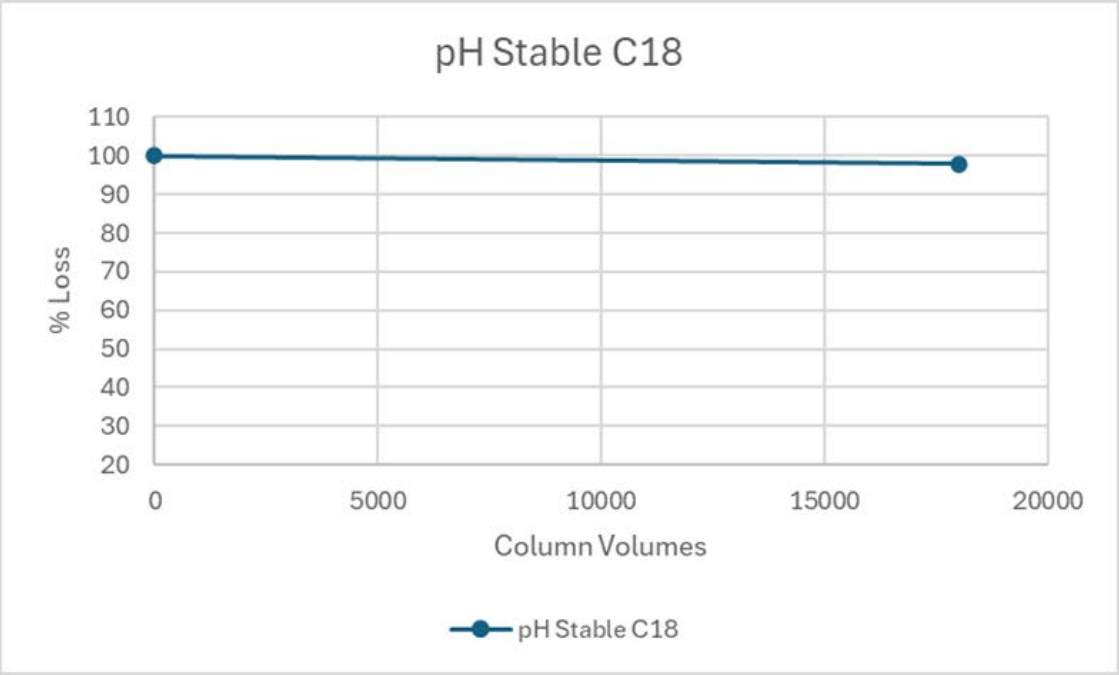
Figure 1: Structure of a 2'-O-modified RNA. (idtdna.com)

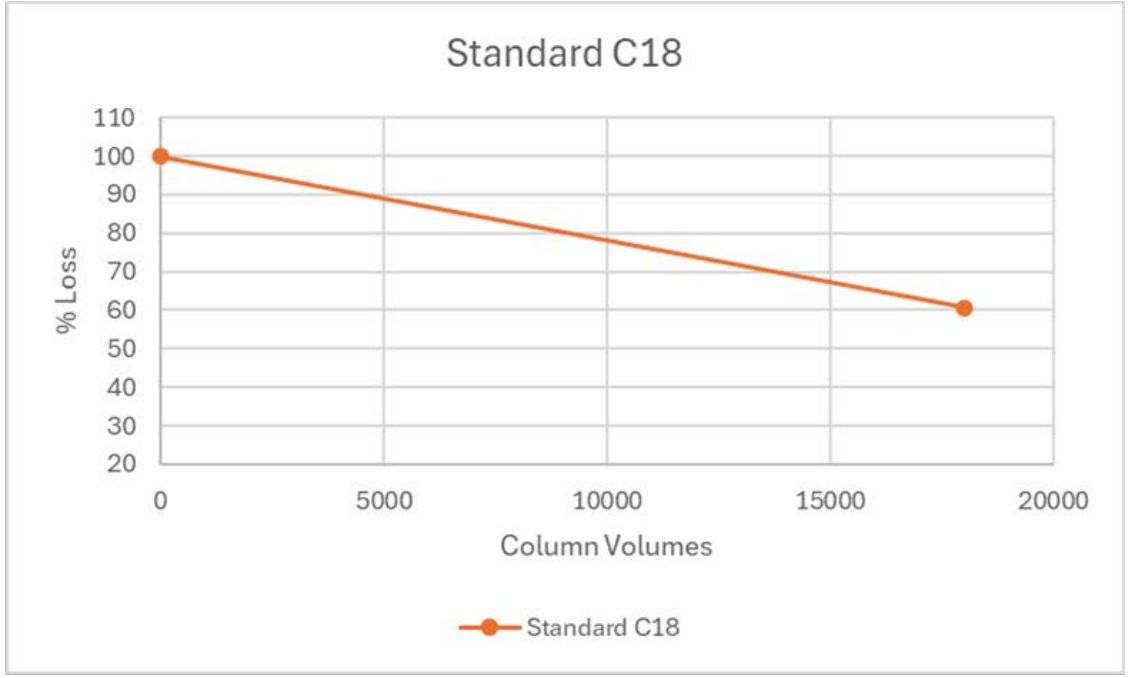
Technical Considerations for Oligonucleotide Separations

- Reversed-phase or ion-pairing RP HPLC methods have largely replaced gel electrophoretic methods for small (<20 nts) and medium size (<60 nts) oligo/poly nucleotides.
- IP-RP methods can be rapid, and with suitable choices can be used with online MS detection for identity and sequence analysis.
- Most IP-RP approaches are at pH 6-11, and often at elevated temperature, especially when complementary hybrids can be formed (internal or intermolecular hybrids).
- Hybrid formation is temperature, ionic strength and solvent dependent, and defined by sequence (GC vs AT or AU), as well as backbone (RNA vs DNA).

Importance of High pH Stability

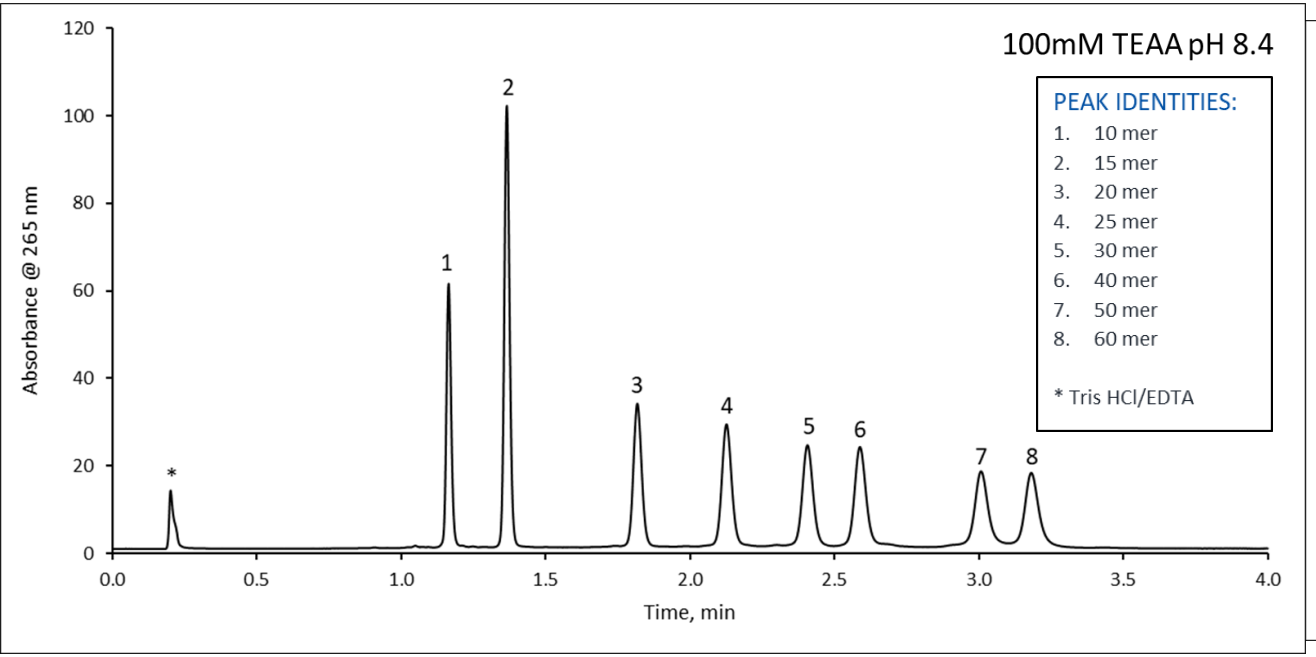
- At high pHs, native silica will be solubilized unless treated for high pH stability
- The graph measures the result of efficiency (plates) of both a standard silica and the new surface modified silica from AMT after stability testing.
- The pH stable phase maintained good efficiency after the stability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

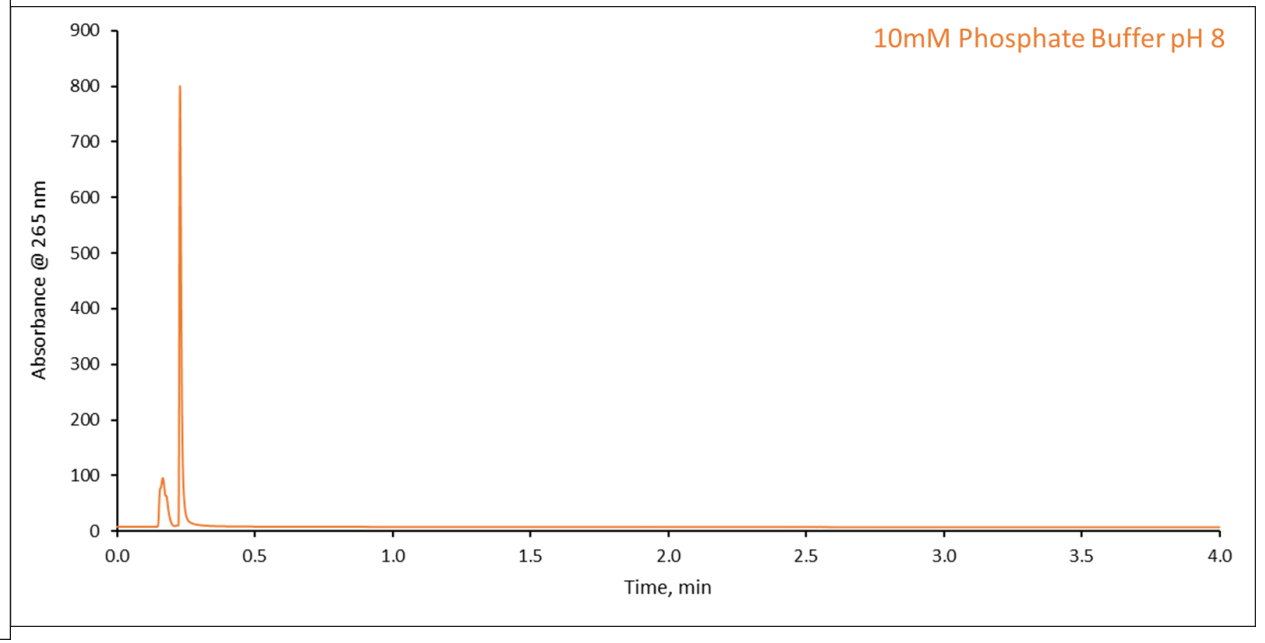




Why Ion Pairing?

- 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.





Standard Separation with Ion Pairing

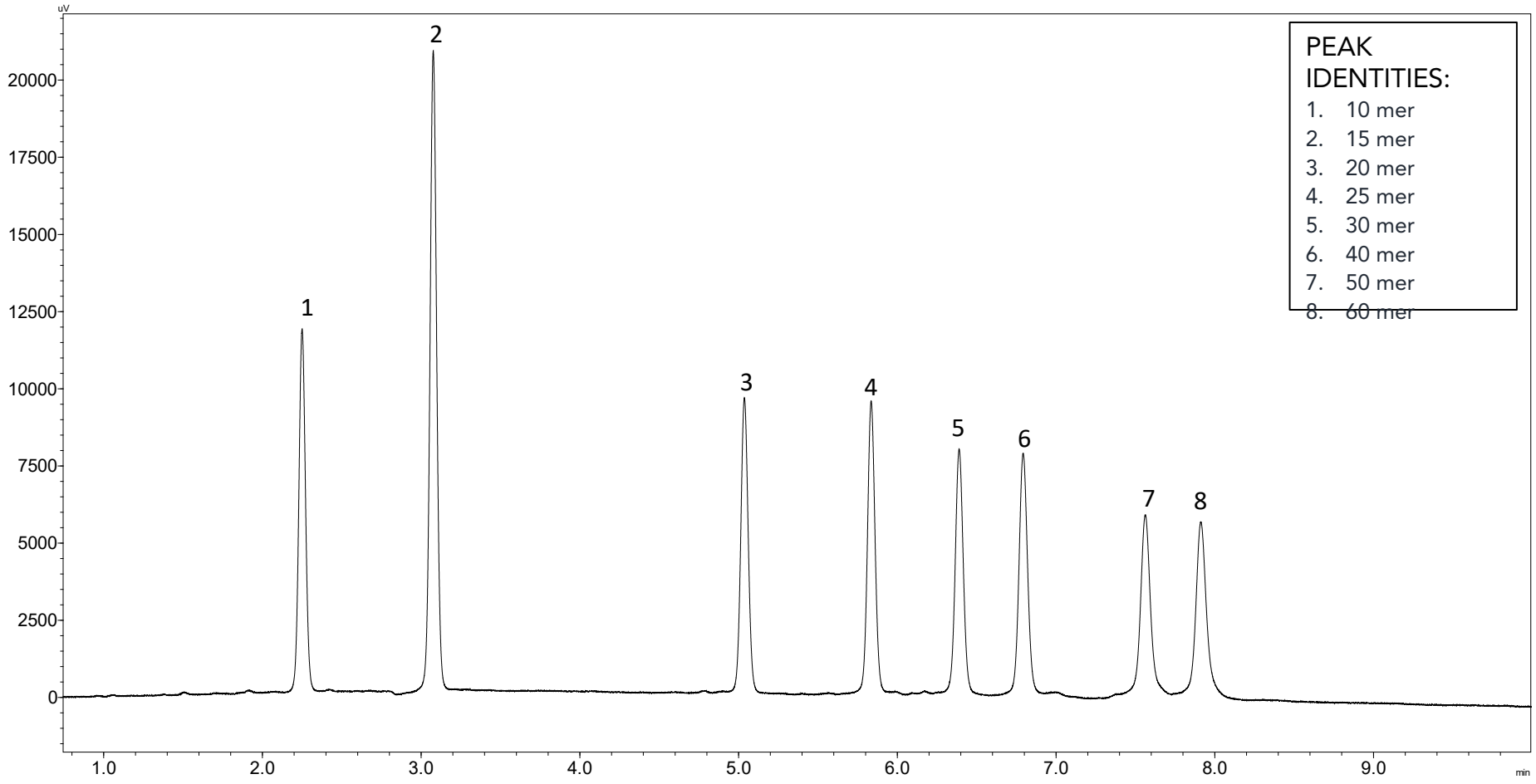
HALO®

Testing Conditions:

Column: HALO 120 Å OLIGO, 2.7 µm, 2.1 x 50 mm
Mobile Phase: A: 100mM TEAA
Adjusted to pH = 8.5
B: ACN
Gradient:

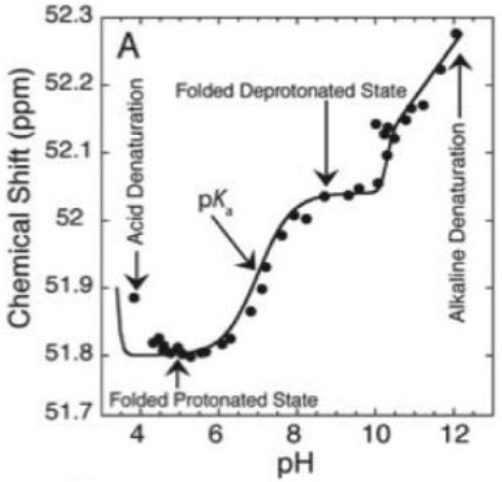
Time	%B
0.0	5
10.0	11
11.0	11
11.5	0
16.5	0

Flow Rate: 0.5 mL/min
Back Pressure: 140 bar
Temperature: 60 °C
Injection: 1.0 µL, 10µg on Column
Sample Solvent: 10mM Tris HCl/1mM EDTA pH=8.0
Wavelength: PDA, 254 nm
Flow Cell: 1 µL
Data Rate: 100 Hz
Response Time: 0.025 sec.
LC System: Shimadzu Nexera X2



Effects of pH on Oligonucleotides

- Five different pHs were used to evaluate how the retention of oligonucleotides differs at the pHs 6 and 9.5
- 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



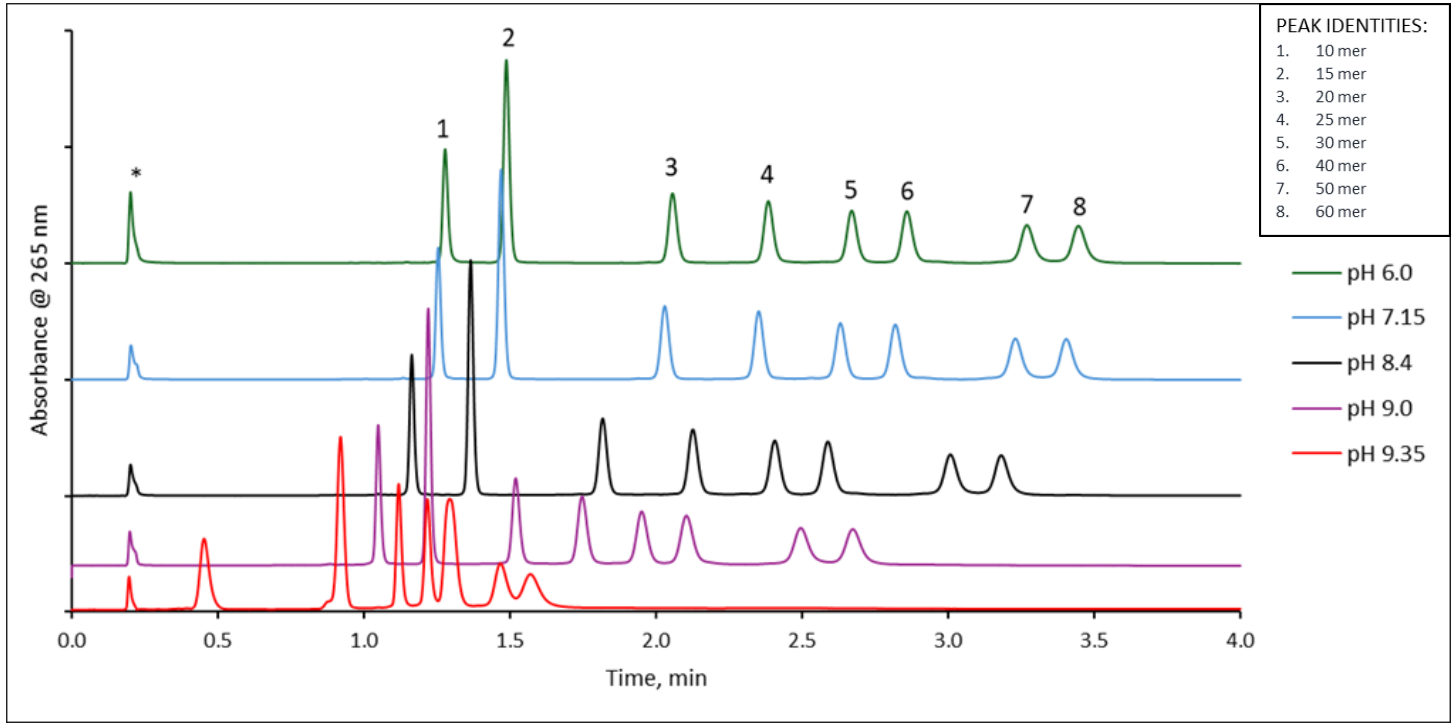
Testing Conditions:

Column: HALO 120 Å OLIGO, 2.7 µm, 2.1 x 50 mm
Mobile Phase: A: Refer to Chromatogram
B: ACN

Gradient:

Time	%B
0.0	5
0.5	7.4
3.5	10.7
3.6	20
4.1	20
4.2	5
9.0	5

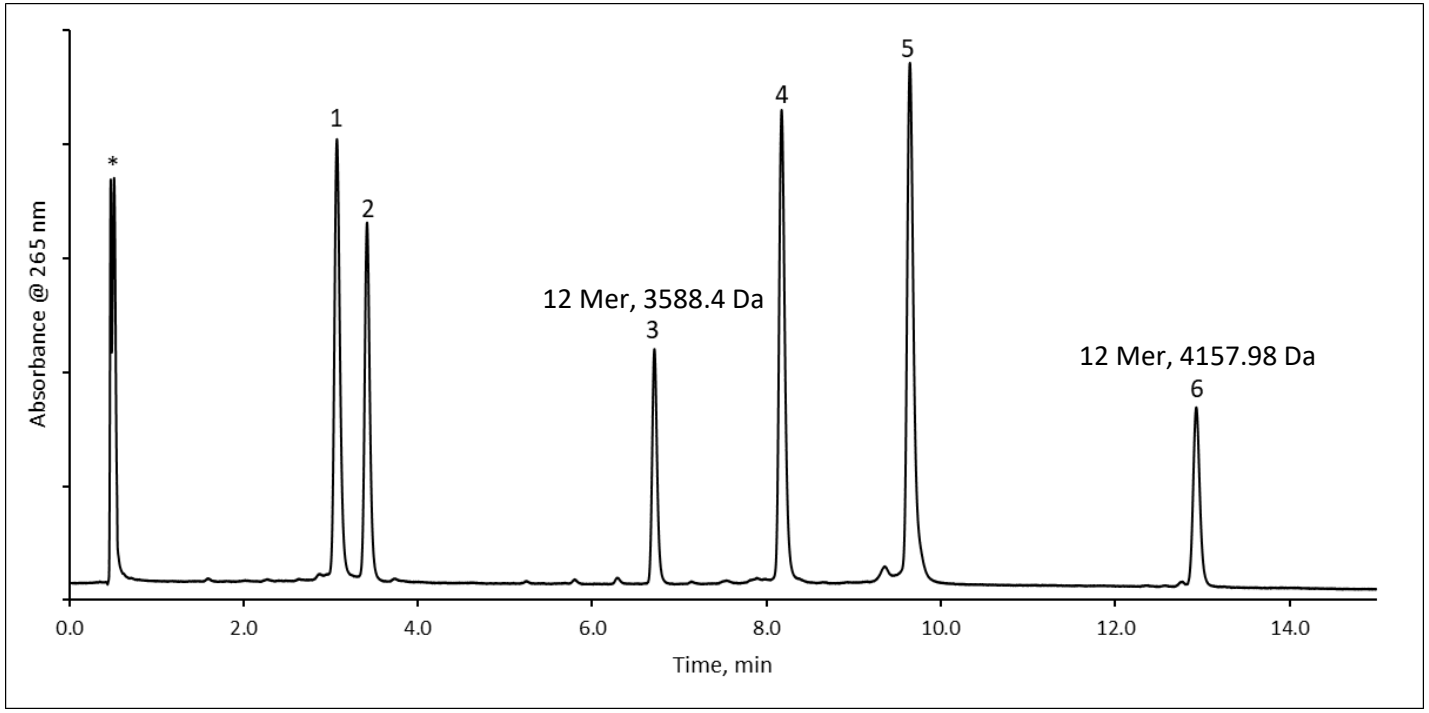
Flow Rate: 0.5 mL/min
Back Pressure: 140 bar
Temperature: 60 °C
Injection: 1.0 µL, 10µg on Column
Sample Solvent: 10mM Tris HCl/1mM EDTA pH=8.0
Wavelength: PDA, 265 nm
Flow Cell: 1 µL
Data Rate: 40 Hz
Response Time: 0.05 sec.
LC System: Shimadzu Nexera X2



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

Oligomer Composition Matters!

- 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 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 3588 Da compared to the later eluting oligomer which has a mass of 4157 Da.



Testing Conditions:

Column: HALO 120 Å OLIGO C18, 2.7 µm, 2.1 x 100 mm
Part Number: P2A62-602
Mobile Phase: A: 100mM TEAA @ pH 7.0
B: Acetonitrile
Gradient:

Time	%B
0.0	7.5
20.0	15
20.3	60
20.6	60
20.8	7.5

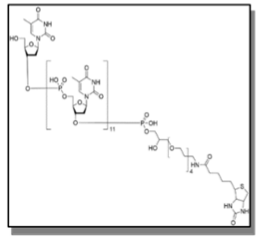
Flow Rate: 0.4 mL/min
Back Pressure: 142 bar
Temperature: 50 °C
Injection: 4 µL of Oligonucleotide Performance Standard Mix, 12-33 NT
P/N: PHR8667-1EA
Sample Solvent: 10mM Tris HCl/ 1mM EDTA
Wavelength: PDA, 265 nm
Flow Cell: 1 µL
Data Rate: 40 Hz
Response Time: 0.05 sec.
LC System: Shimadzu Nexera X2

PEAK IDENTITIES:

1. 20 mer
2. 15 mer
3. 12 mer
4. 25 mer
5. 33 mer
6. 12 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.



LCMS Oligonucleotide N+1/2 Separations

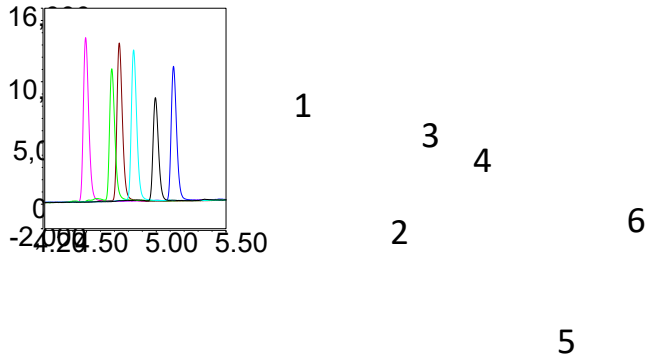
- Six different oligonucleotides are individually separated on the HALO® OLIGO C18 column
- Using overlays of each injection it can be seen that the column has very little trouble separating each oligonucleotide under MS friendly conditions
- Oligonucleotide base length can help predict retention behavior
- Base type also plays a major role in oligomer separations and by using the HALO® OLIGO C18 column even minor changes in base type or length can be separated

Testing Conditions:
Column: HALO 120 Å OLIGO C18, 2.7 µm, 2.1 x 50 mm
Part Number: P2A62-402
Mobile Phase: A: 5mM TEA/50mM HFIP, pH 8.4
B: Methanol
Gradient:

Time	%B
0.0	5
7.0	18

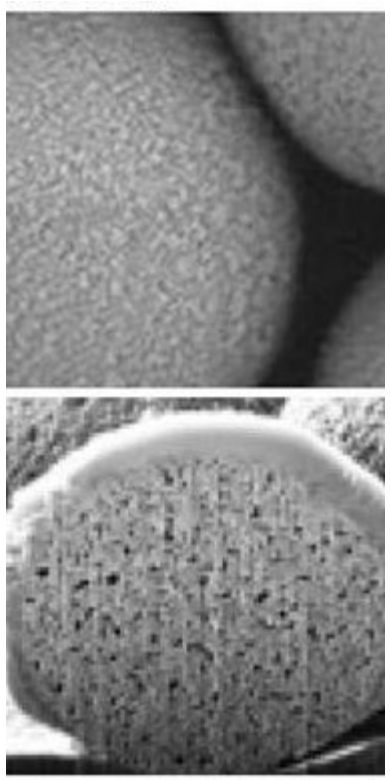
Flow Rate: 0.4 mL/min
Back Pressure: 106 bar
Temperature: 50 °C
Injection: 1.0 µL, (10µg)
Sample Solvent: 10mM Tris HCl/1mM EDTA pH=8.0
Wavelength: PDA, 260 nm
Flow Cell: 1 µL
Data Rate: 100 Hz
Response Time: 0.025 sec.
LC System: Shimadzu Nexera X2
MS System: Thermo Velos Pro Orbitrap

MS CONDITIONS:
Detection: (-) HESI
Spray Voltage: 2.5 kV
Sheath gas: 35
Aux gas: 10
Capillary temp: 350 °C
Source Heater temp: 300 °C
S lens: 60
microscan: 1
max ion time: 200 ms



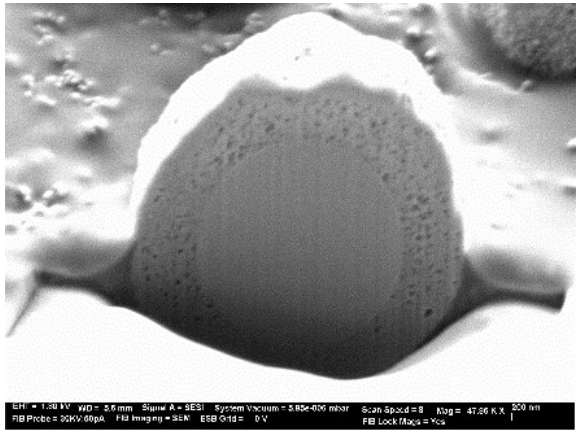
PEAK IDENTITIES:
1. 19 mer
2. 20 mer
3. 20 mer
4. 21 mer
5. 15 mer
6. 16 mer

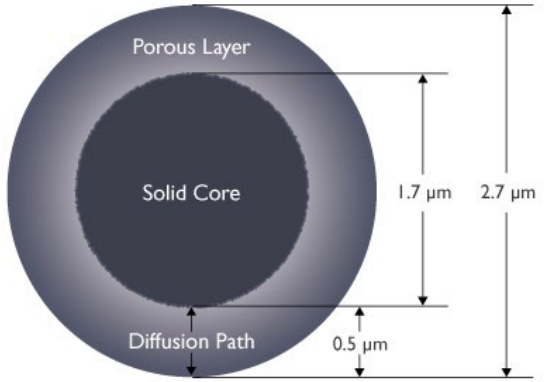
SPP Technology



Fully Porous Particle (FPP)

HALO 90 Å, 2.7 µm





Superficially Porous Particle (SPP)

Brief terminology lesson:

FPP – Fully Porous Particle

TPP- Totally Porous Particle

SPP – Superficially Porous Particle

Terms associated with SPP – Fused-Core®, solid-core, core shell

Effects of SPP Technology

HALO®

Testing Conditions:

Column: HALO 120 Å OLIGO C18, 2.7 µm, 2.1 x 50 mm
Part Number: P2A62-402
Competitor: FPP 120 Å C18, 1.9 µm, 2.1 x 50 mm
Mobile Phase: A: 100mM TEAA, pH 7
B: MeOH
Gradient:

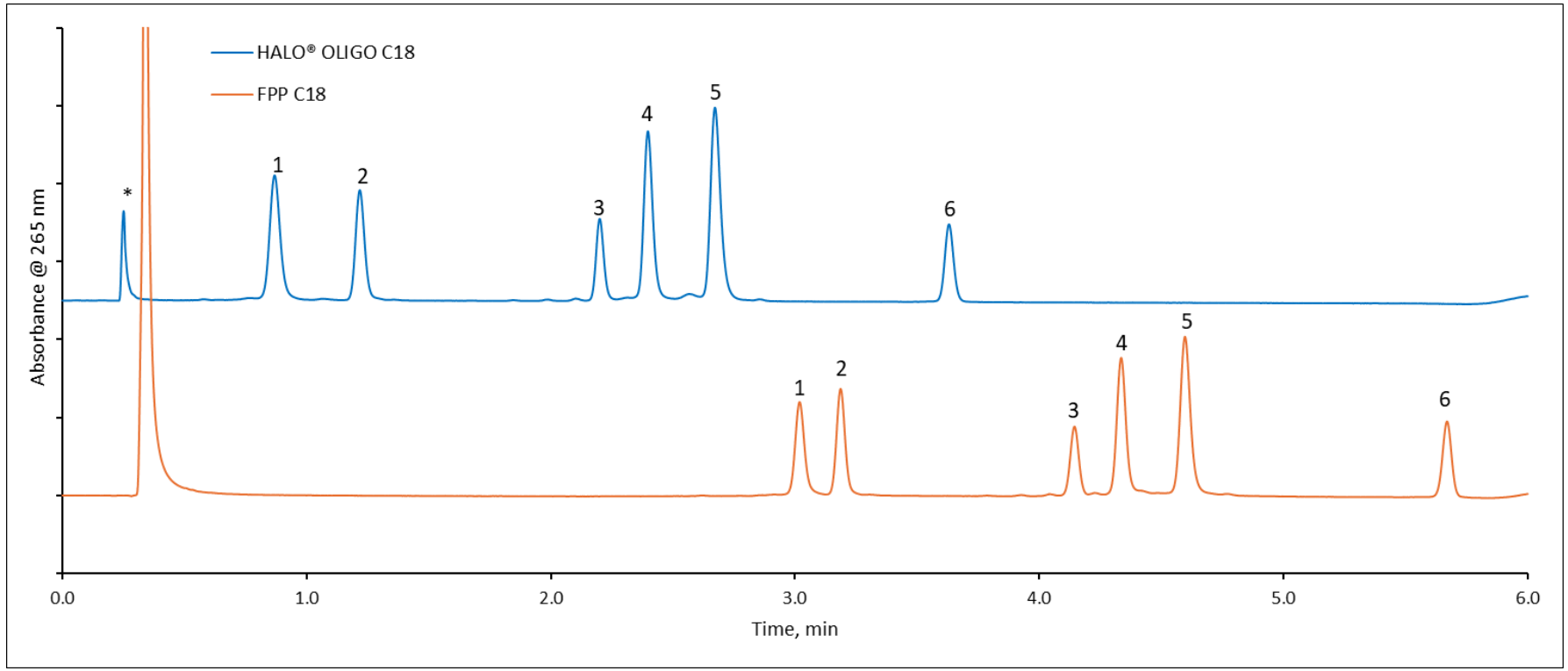
Time	%B
0.0	17
5.0	30
5.3	60
5.6	60
5.8	17

Flow Rate: 0.4 mL/min
Back Pressure: HALO® - 135 bar
FPP - 302 bar
Temperature: 50 °C
Injection: 1.0 µL
Sample Solvent: 10mM Tris HCl/1mM EDTA pH=8.0
Wavelength: PDA, 265 nm
Flow Cell: 1 µL
Data Rate: 40 Hz
Response Time: 0.05 sec.
LC System: Shimadzu Nexera X2

PEAK IDENTITIES:

1. 20 mer
2. 15 mer
3. 12 mer
4. 25 mer
5. 33 mer
6. 12 mer

* Tris/EDTA



Pore Size Effects on ssDNA

- Baseline separation on all peaks but 6 and 7
- Reaching the limits of a 120Å pore size
- Does a larger pore size increase separation between peaks 6 and 7?

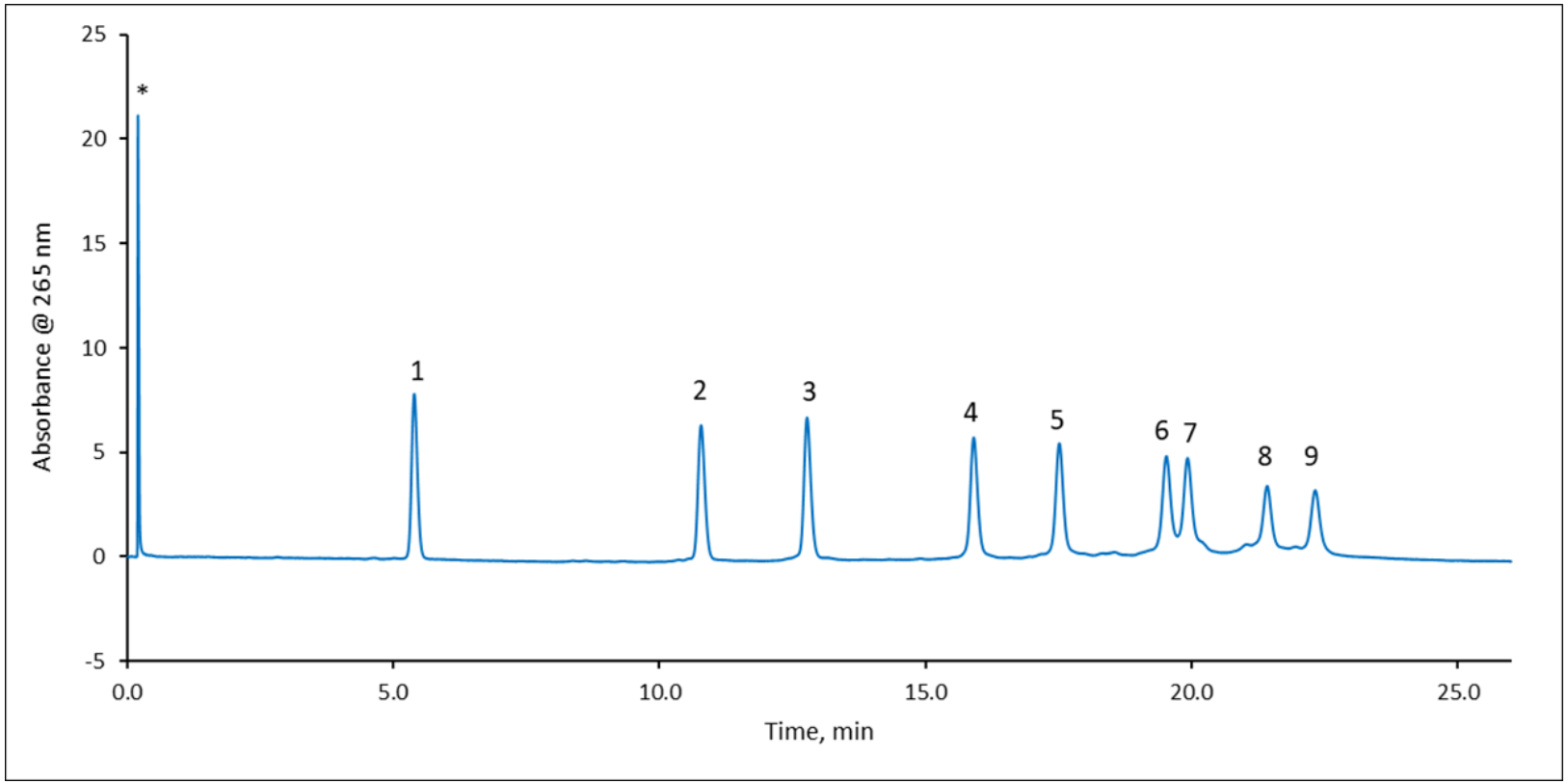
Testing Conditions:
Column: HALO 120 Å OLIGO C18, 2.7 µm, 2.1 x 50 mm
Part Number: P2A62-402
Mobile Phase: A: 100mM TEAA, pH 7
B: Acetonitrile
Gradient:

Time	%B
0.0	6.5
30	11
31	11
31.1	6.5
35	6.5

Flow Rate: 0.5 mL/min
Back Pressure: 144 bar
Temperature: 60 °C
Injection: 2.0 µL, (10µg)
Sample Solvent: 10mM Tris HCl/1mM EDTA pH=8.0
Wavelength: PDA, 265 nm
Flow Cell: 1 µL
Data Rate: 40 Hz
Response Time: 0.05 sec.
LC System: Shimadzu Nexera X2

PEAK IDENTITIES:
1. 20 mer
2. 30 mer
3. 40 mer
4. 50 mer
5. 60 mer
6. 70 mer
7. 80 mer
8. 90 mer
9. 100 mer

* Tris HCl/EDTA



Greater Peak Capacity for Larger Pore SPP: TEA/HFIP Acetonitrile

(0.25% AcN/min)

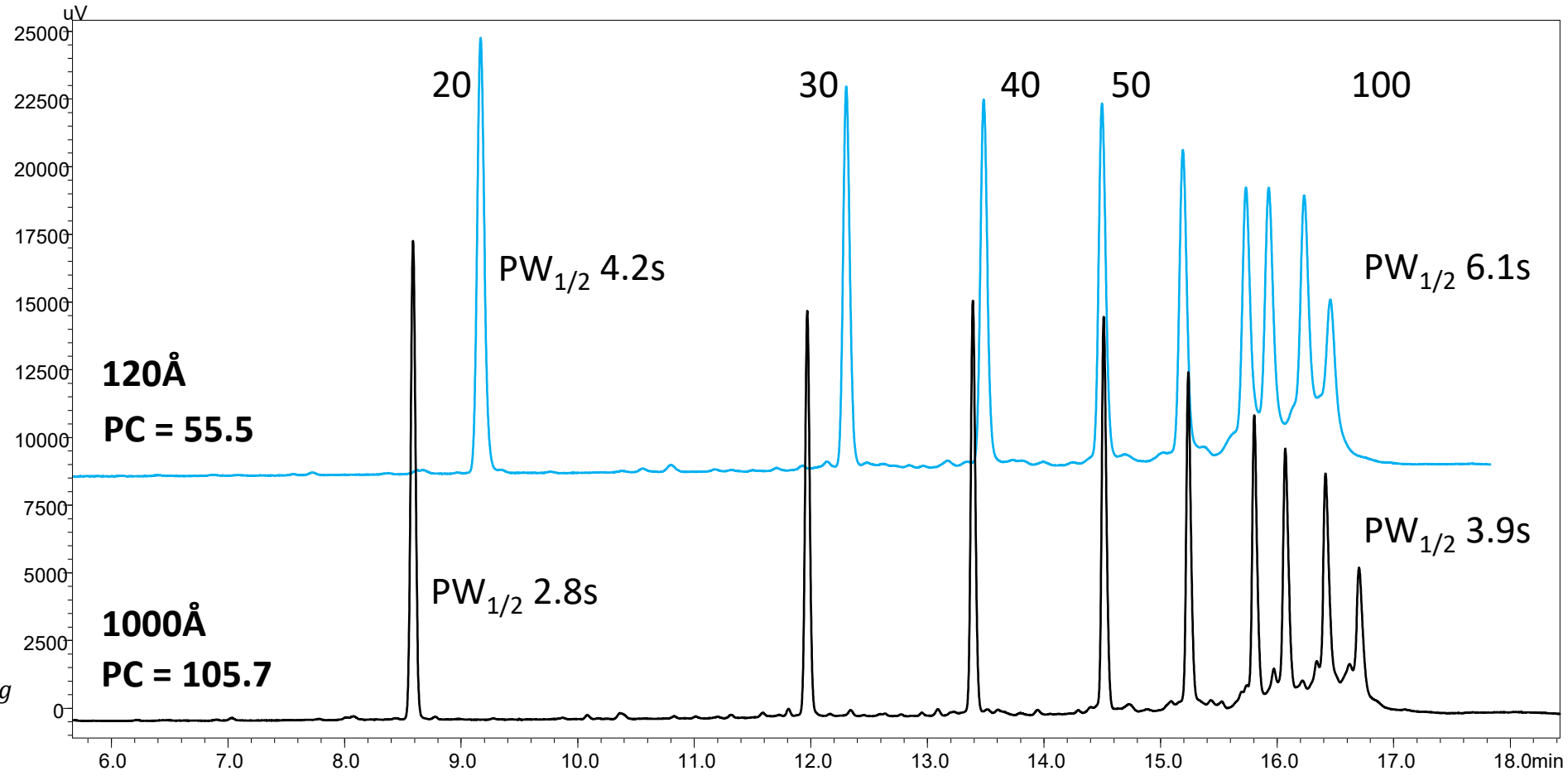
Conditions:

Columns: 2.1 x 100 mm, Inert HW
Flow Rate: 0.5 mL/min
Temp: 60°C
A- 15 mM TEA/50 mM HFIP, pH 8.9
B- Acetonitrile

Gradient:	Time	%
	0.0	1.5
	20	6.5
	21	15
	22	15
	22.5	1.5
	30	Stop

Detection: 260 nm, 10 nm
Sample: 1 µL, 20/100 IDT @ 10ng

$$PC = \Delta(Rt_{100} - Rt_{20})/4\sigma_{avg}$$



Resolution of Longer Oligonucleotides

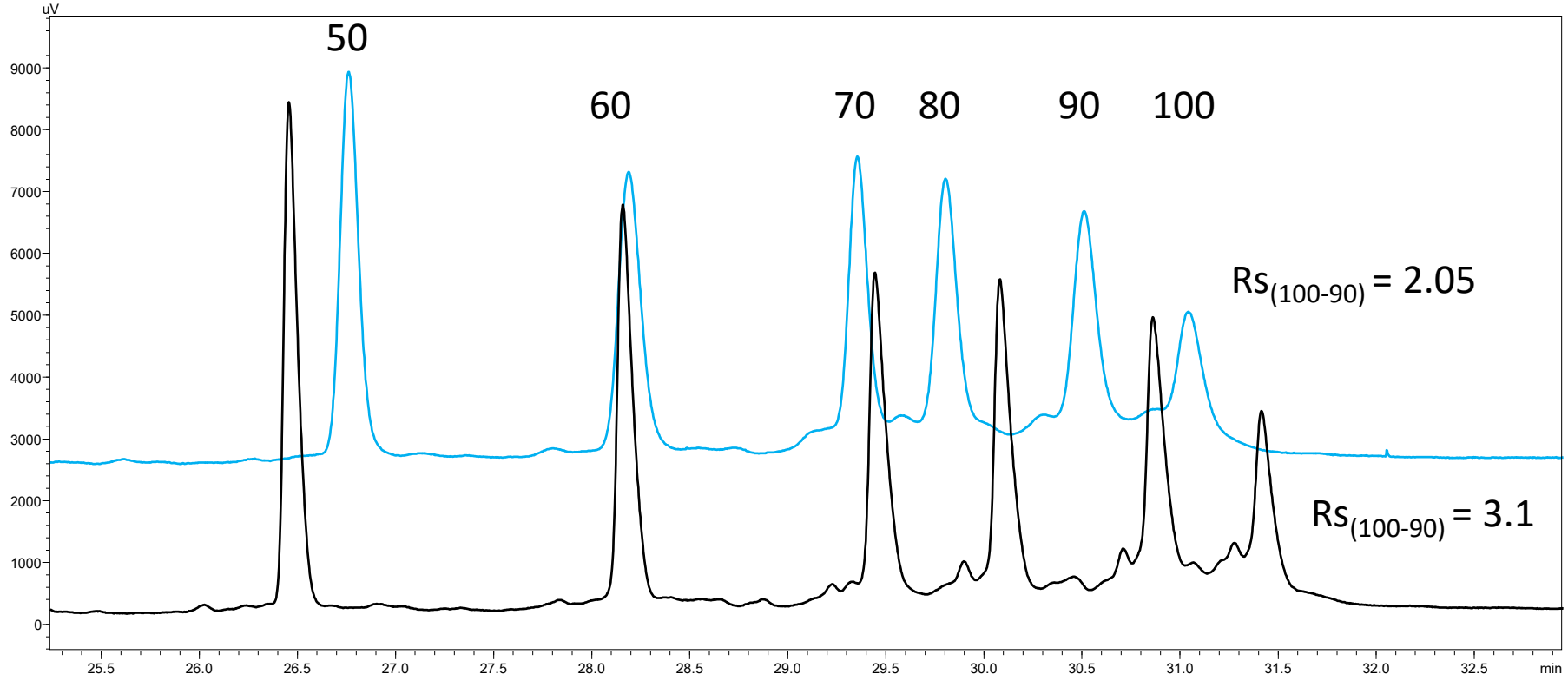
Conditions:

Columns: 2.1 x 100 mm, Inert HW
Flow Rate: 0.5 mL/min
Temp: 60°C
A- 15 mM TEA/50 mM HFIP, pH 8.9
B- Acetonitrile

Gradient:	Time	%
	0.0	1.5
	40	6.5
	41	15
	42	15
	43	1.5
	50	Stop

Detection: 260 nm, 10 nm
Sample: 1 µL, 20/100 IDT @ 10ng

(0.125% AcN/min)



- Adjusting gradient for closer matching of Rt across this sample yields little effect on Rs or PC

ssRNA Under Ion Pairing

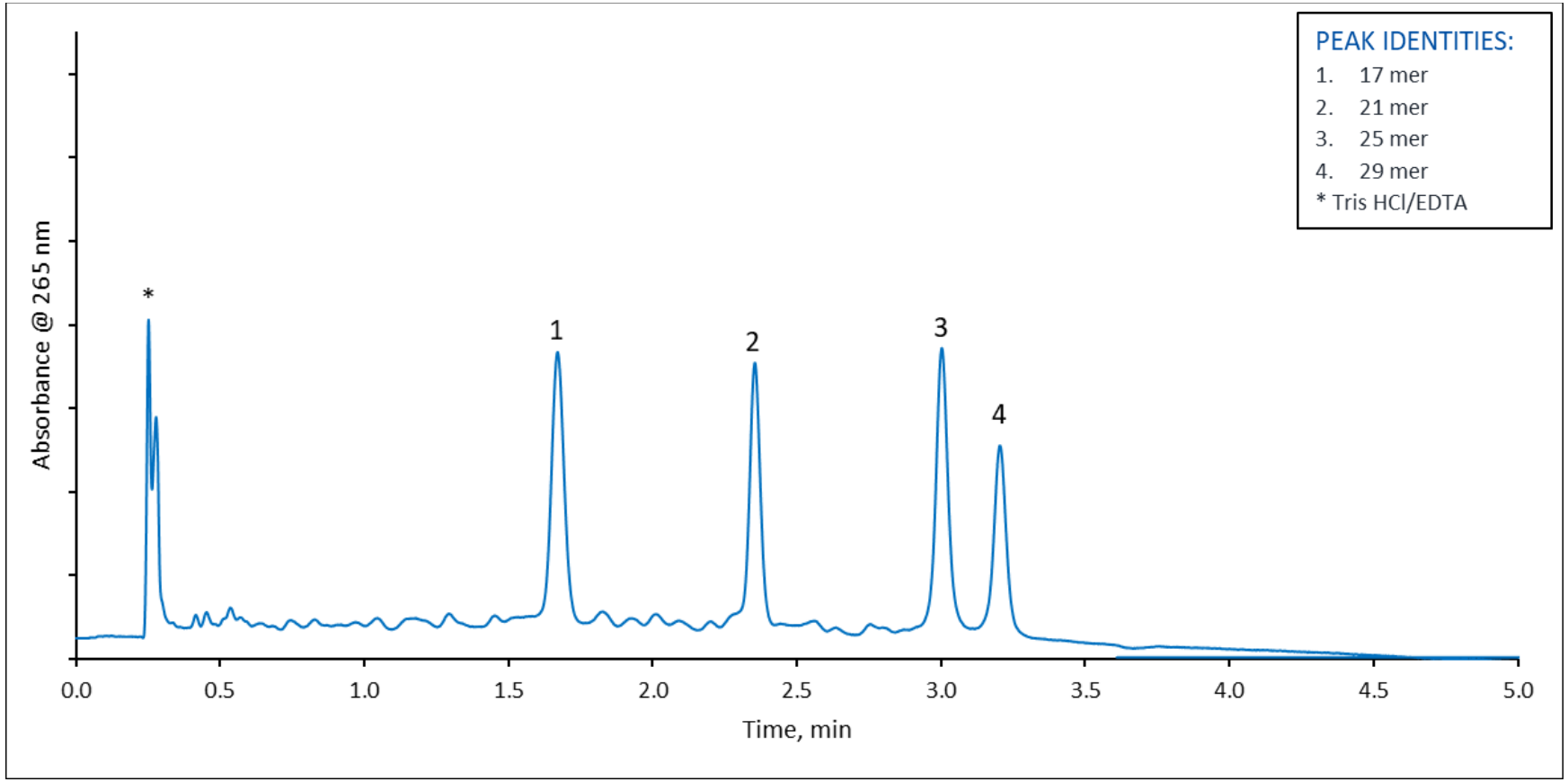
- ssRNA has less retention compared to ssDNA under the same conditions
- ssRNA has a relatively flexible structure that can reduce its hydrophobic interactions
- As a result, ssRNA may not form stable complexes with the ion-pairing agents

Testing Conditions:

Column: HALO 120 Å OLIGO C18, 2.7 µm, 2.1 x 50 mm
Part Number: P2A62-402
Mobile Phase: A: 100mM TEAA, Adjusted to pH = 8.56
B: ACN
Gradient:

Time	%B
0.0	5
5.0	10
5.3	60
5.6	60
5.7	5
9.0	5

Flow Rate: 0.4 mL/min
Back Pressure: 116 bar
Temperature: 60 °C
Injection: 3.0 µL, 30µg on Column
Sample Solvent: 10mM Tris HCl/1mM EDTA pH=8.0
Wavelength: PDA, 265 nm
Flow Cell: 1 µL
Data Rate: 40 Hz
Response Time: 0.05 sec.
LC System: Shimadzu Nexera X2



Primer Mix on OLIGO C18

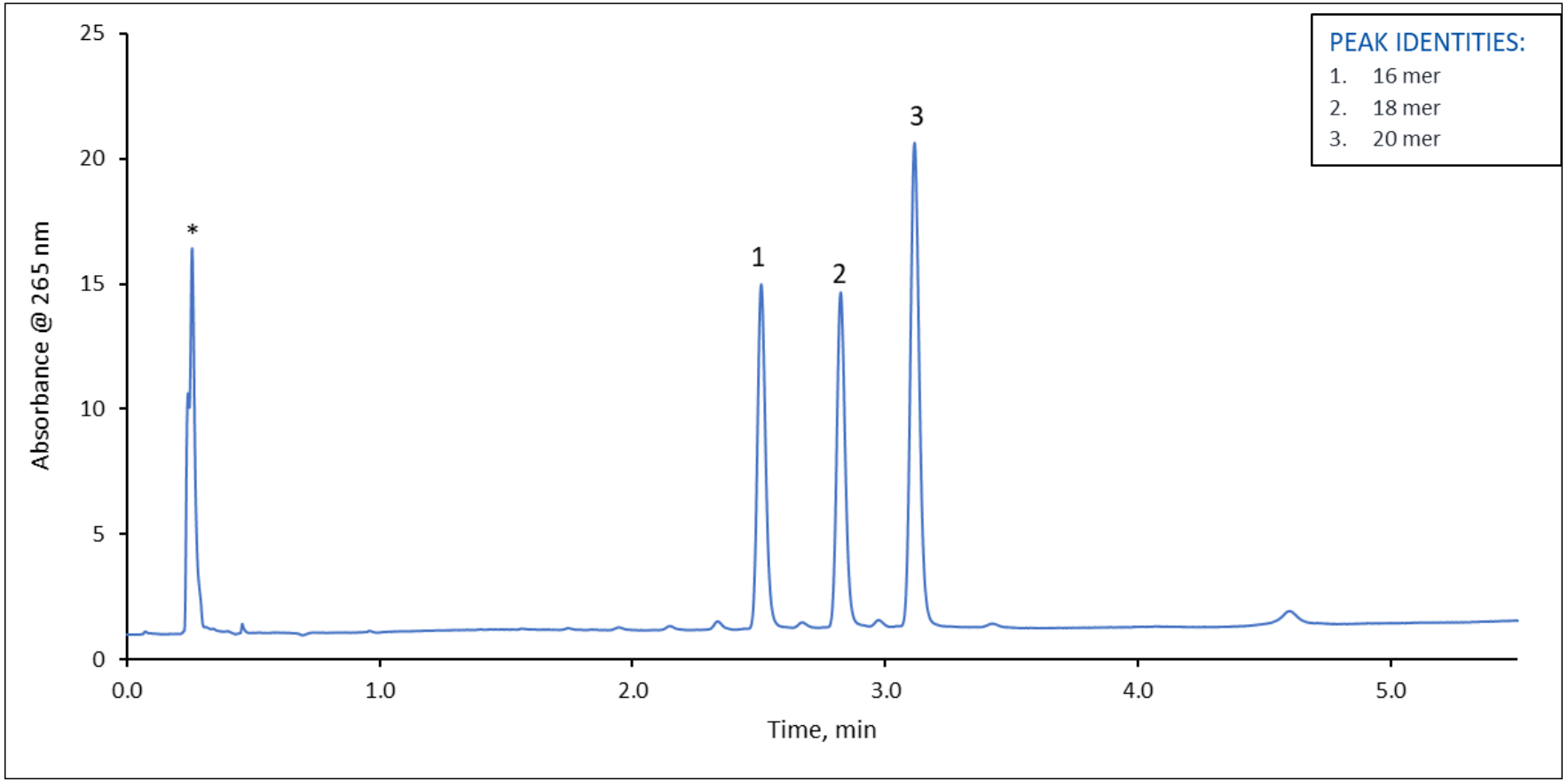
HALO®

Testing Conditions:

Column: HALO 120 Å OLIGO C18, 2.7 µm, 2.1 x 50 mm
Part Number: P2A62-402
Mobile Phase: A: 5mM TEA/50mM HFIP @ pH 8.3
B: 25/75 ACN/MeOH
Gradient:

Time	%B
0.0	5
0.5	8
3.5	13
5.0	20
5.4	20
5.5	5
9.0	5

Flow Rate: 0.4 mL/min
Back Pressure: 219 bar
Temperature: 60 °C
Injection: 3µL Primer Mix
Sample Solvent: 10mM Tris HCl/ 1mM EDTA
Wavelength: PDA, 265 nm
Flow Cell: 1 µL
Data Rate: 40 Hz
Response Time: 0.05 sec.
LC System: Shimadzu Nexera X2



Primer Separated on OLIGO C18

HALO®

Testing Conditions:

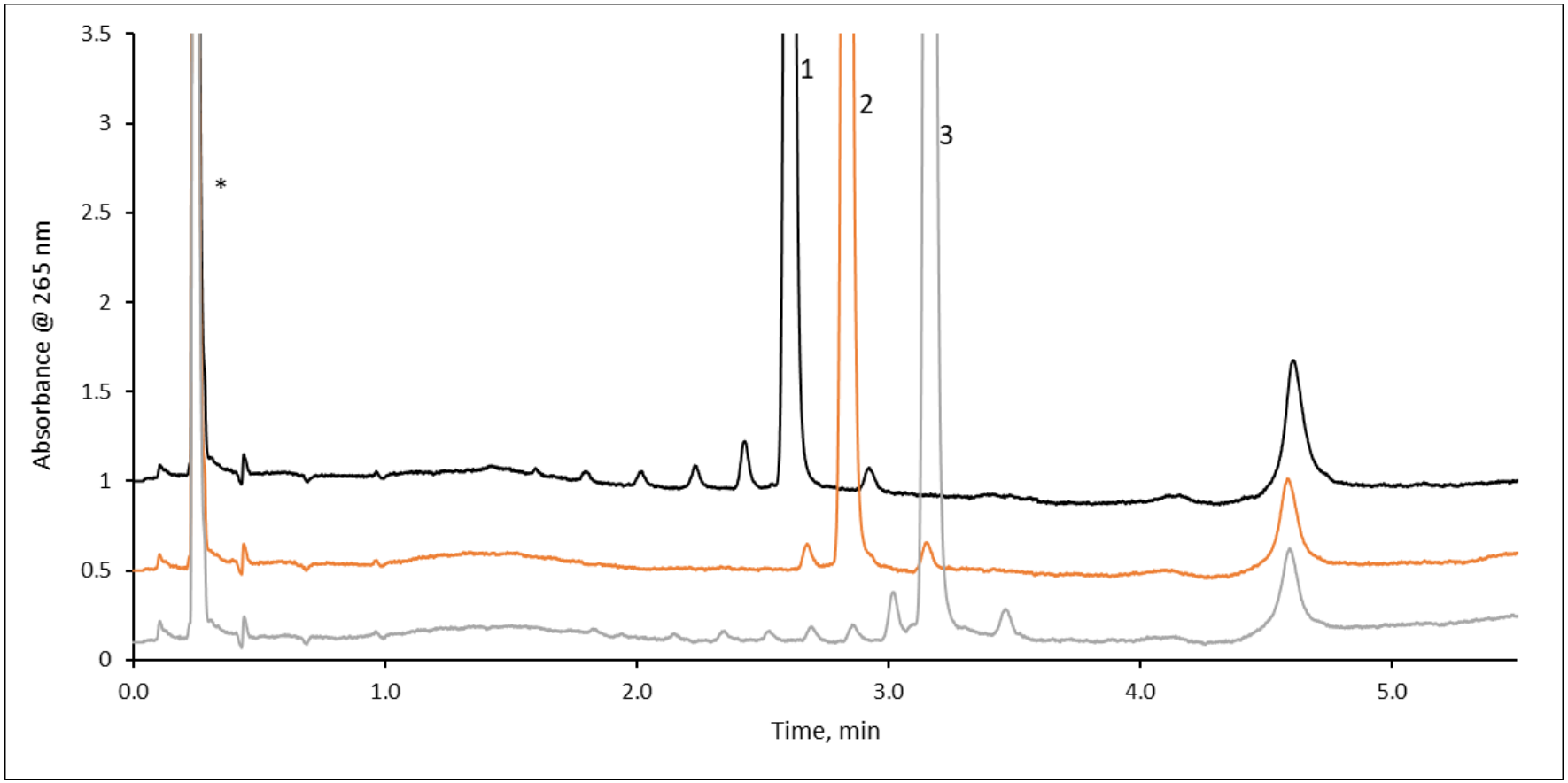
Column: HALO 120 Å OLIGO C18, 2.7 µm, 2.1 x 50 mm
Part Number: P2A62-402
Mobile Phase: A: 5mM TEA/50mM HFIP @ pH 8.3
B: 25/75 ACN/MeOH
Gradient:

Time	%B
0.0	5
0.5	8
3.5	13
5.0	20
5.4	20
5.5	5
9.0	5

Flow Rate: 0.4 mL/min
Back Pressure: 219 bar
Temperature: 60 °C
Injection: 1µL Primer
Sample Solvent: 10mM Tris HCl/ 1mM EDTA
Wavelength: PDA, 265 nm
Flow Cell: 1 µL
Data Rate: 40 Hz
Response Time: 0.05 sec.
LC System: Shimadzu Nexera X2

PEAK IDENTITIES:

1. 16 mer
2. 18 mer
3. 20 mer



Questions?

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