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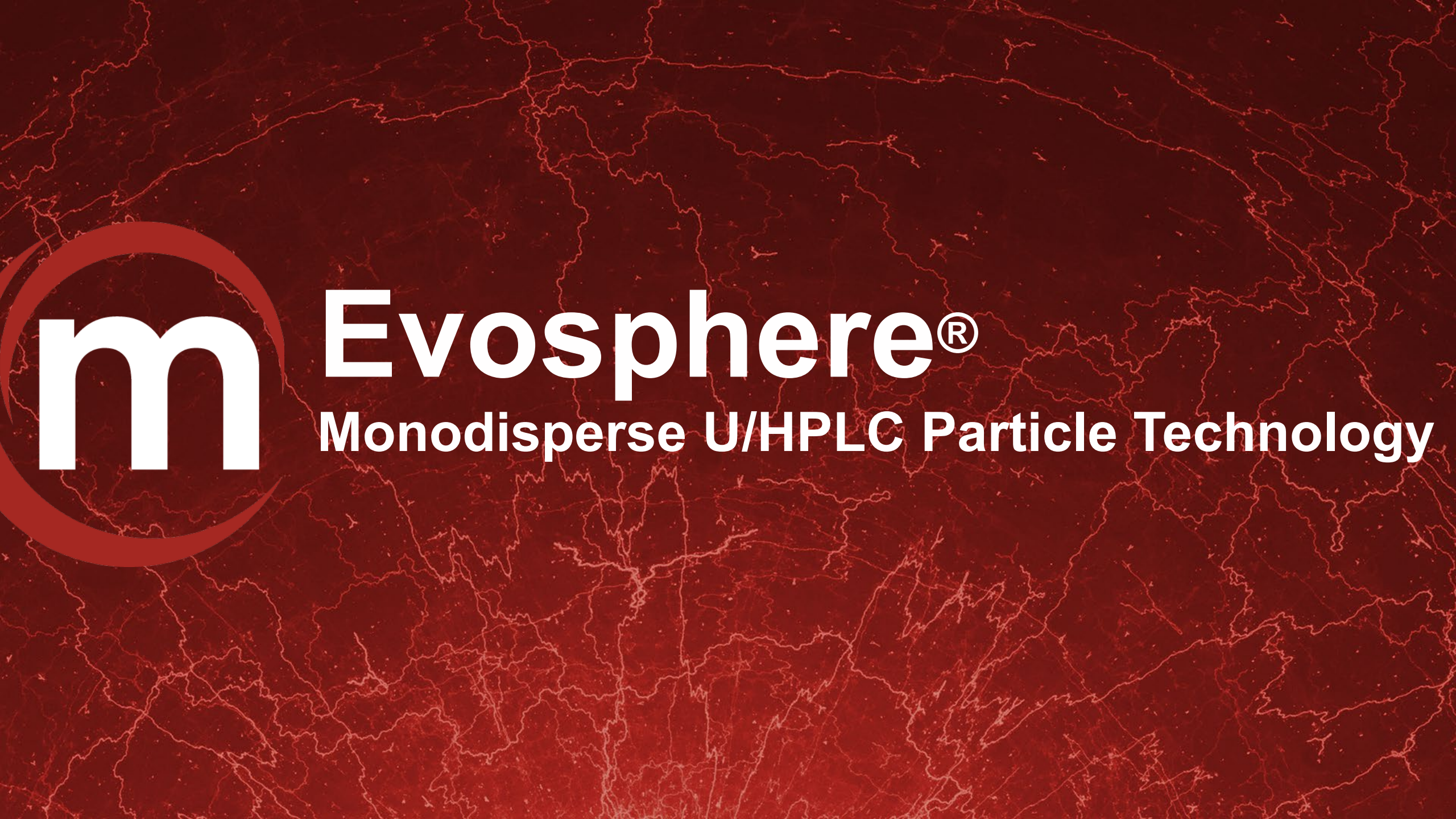
SMARTER CHROMATOGRAPHY

Oligonucleotide Analysis without Ion-Pairing Additives using Novel UHPLC Column Technology

(In Partnership with Sylwia Kowalska and Szymon Bocian at Nicolaus Copernicus University in Poland)

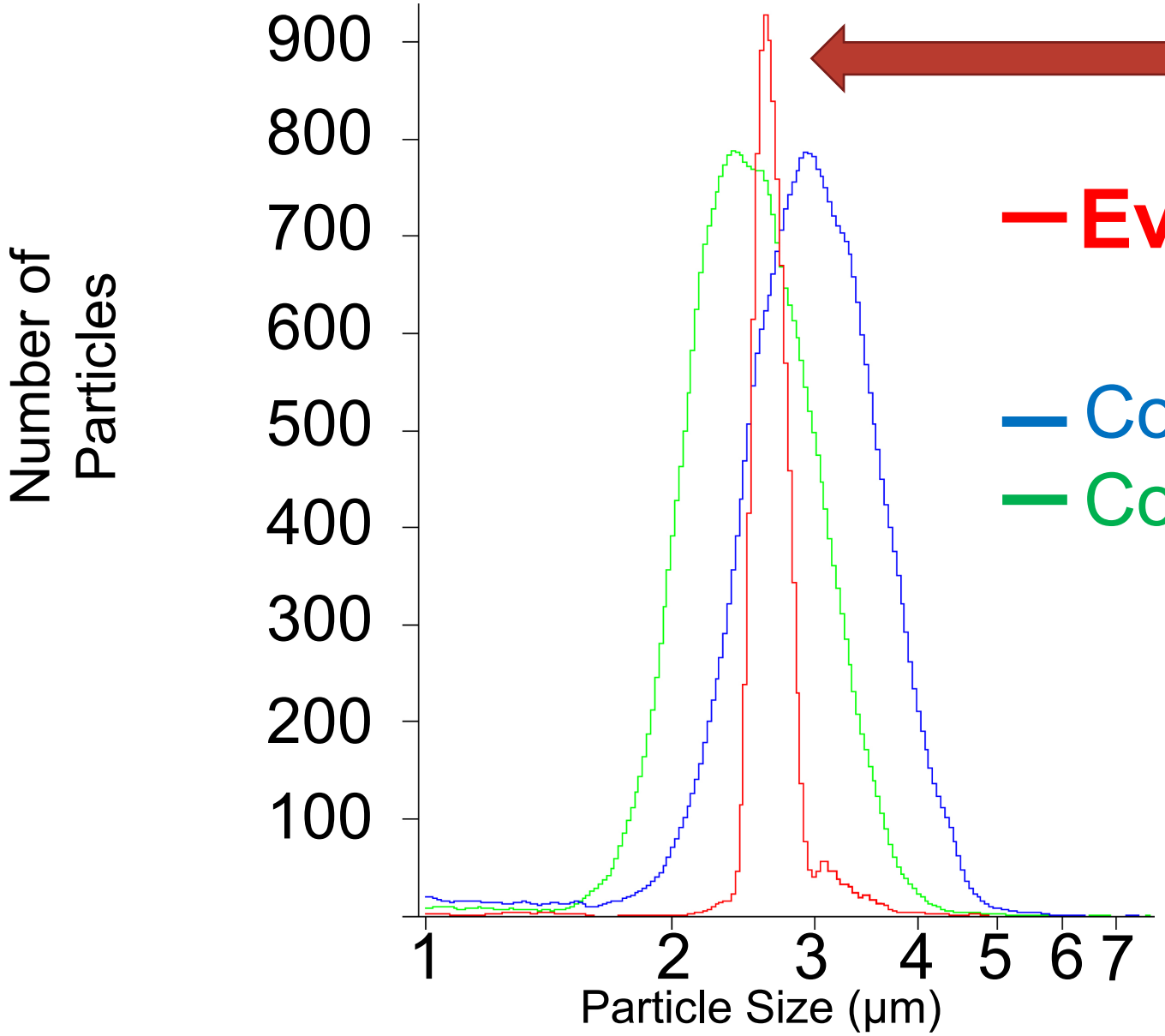

Evosphere®
Monodisperse U/HPLC Particle Technology

Evolution of U/HPLC Particles

- Morphology - Shape
- Size - Reduction
- Purity – Less Metals
- Size Distribution – Reduction in D90/D10

Ref: Historical Developments in HPLC and UHPLC Column Technology: The Past 25 Years, Dr. Ronald Majors

Particle Size Distribution Comparison



— Evosphere MFPP 3 µm

— Commercial FPP A 3 µm

— Commercial FPP B 3 µm

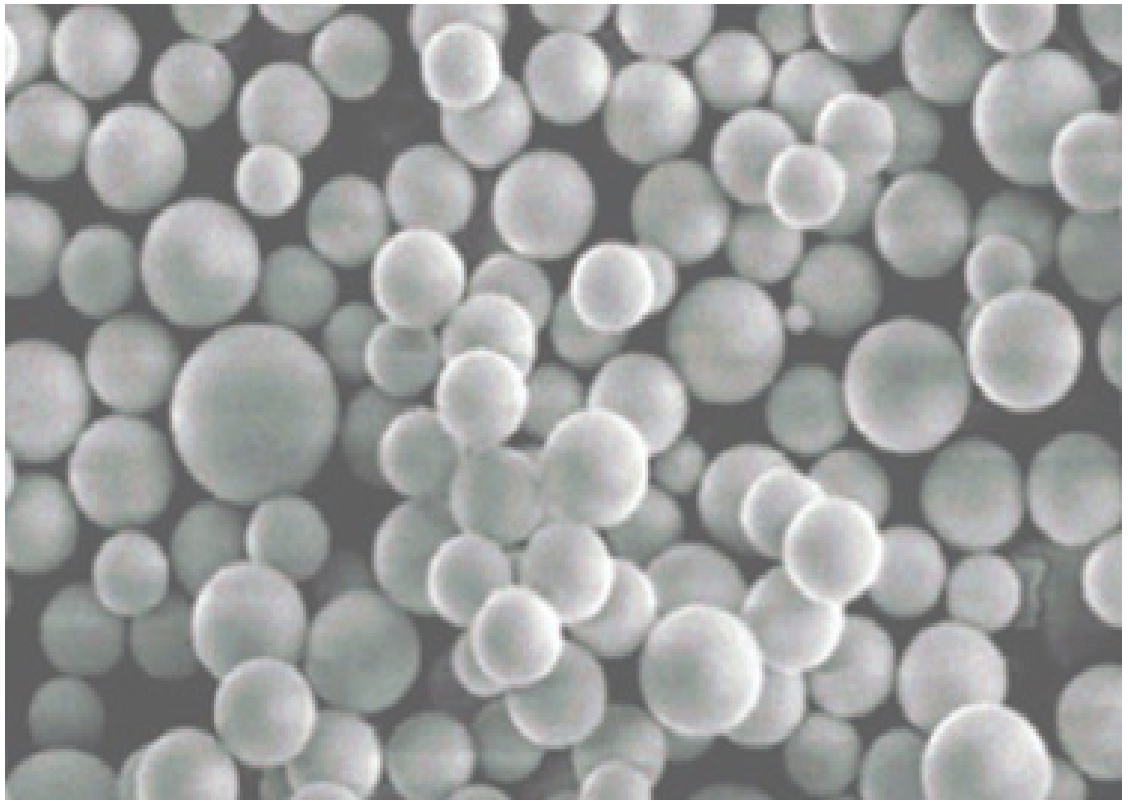
Particle Size Distribution Comparisons

	Monodisperse silica	Commercial 3u silica - A	Commercial 3u Silica-B
Mean particle size (d50) *	2.66µm*	2.48µm	2.97µm
SEM particle diameter	3.0µm	2.8µm	3.3µm
D90/10	1.12	1.58	1.61
Pore volume	0.89	0.88	0.89

40% Reduction in D90/10

*Measured by Coulter Counter

SEM Images of Particles Technologies



Polydisperse

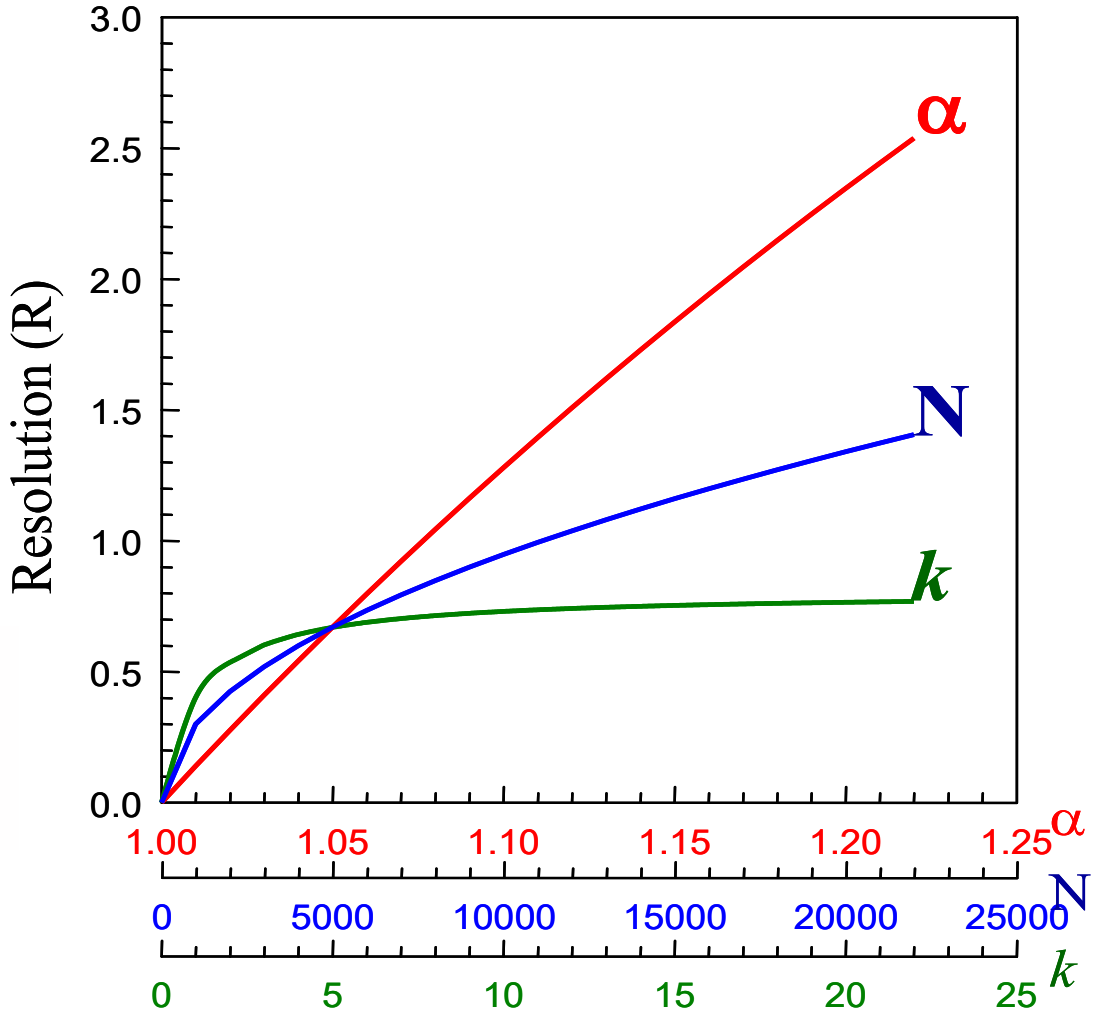
Monodisperse

Resolution Equation

Efficiency Retention Selectivity

$$R = \frac{\sqrt{N}}{4} \quad \frac{k'}{k'+1} \quad \frac{\alpha-1}{\alpha}$$

$$N = \frac{Length\ of\ Column}{HETP}$$



Simplified Van Deemter Equation

$$H = A + \frac{B}{u} + Cu$$

H: Height Equivalent to a Theoretical Plate

A Term: Eddy Diffusion (Multipath Effect)

B Term: Longitudinal Diffusion (Molecular Diffusion)

C Term: Resistance to Mass Transfer (Mobile Phase to Stationary Phase Transition)

Expanded Van Deemter Equation

$$H = 2\lambda d_p + \frac{2\gamma D_m}{u} + \left(\frac{\omega d_p^2 u}{D_m} + \frac{R d_f^2 u}{D_s} \right)$$

- H = Plate Height
- λ is packing factor
- d_p is particle diameter
- γ, ω, and R are constants
- d_f is the film thickness (approaches 0 for LC)

- D_m is the diffusion coefficient of the mobile phase
- d_c is the capillary diameter
- D_s is the diffusion coefficient of the stationary phase.
- u is the linear velocity

Expanded Van Deemter Equation

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- D_m is the diffusion coefficient of the mobile phase
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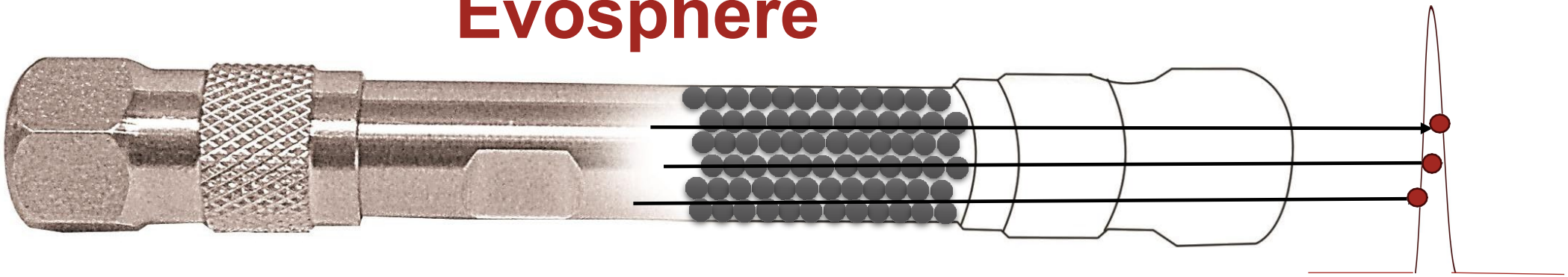
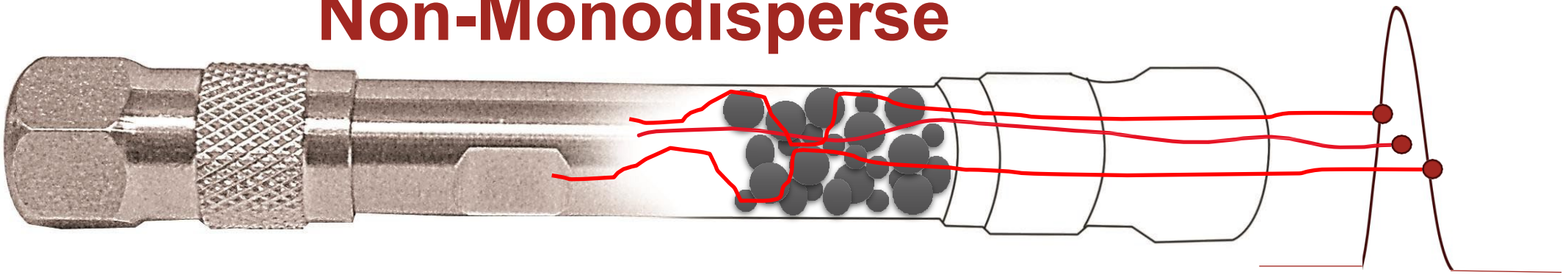
- D_s is the diffusion coefficient of the stationary phase.
- u is the linear velocity

Visual Representation of Eddy Diffusion (“A Term”)

Evosphere

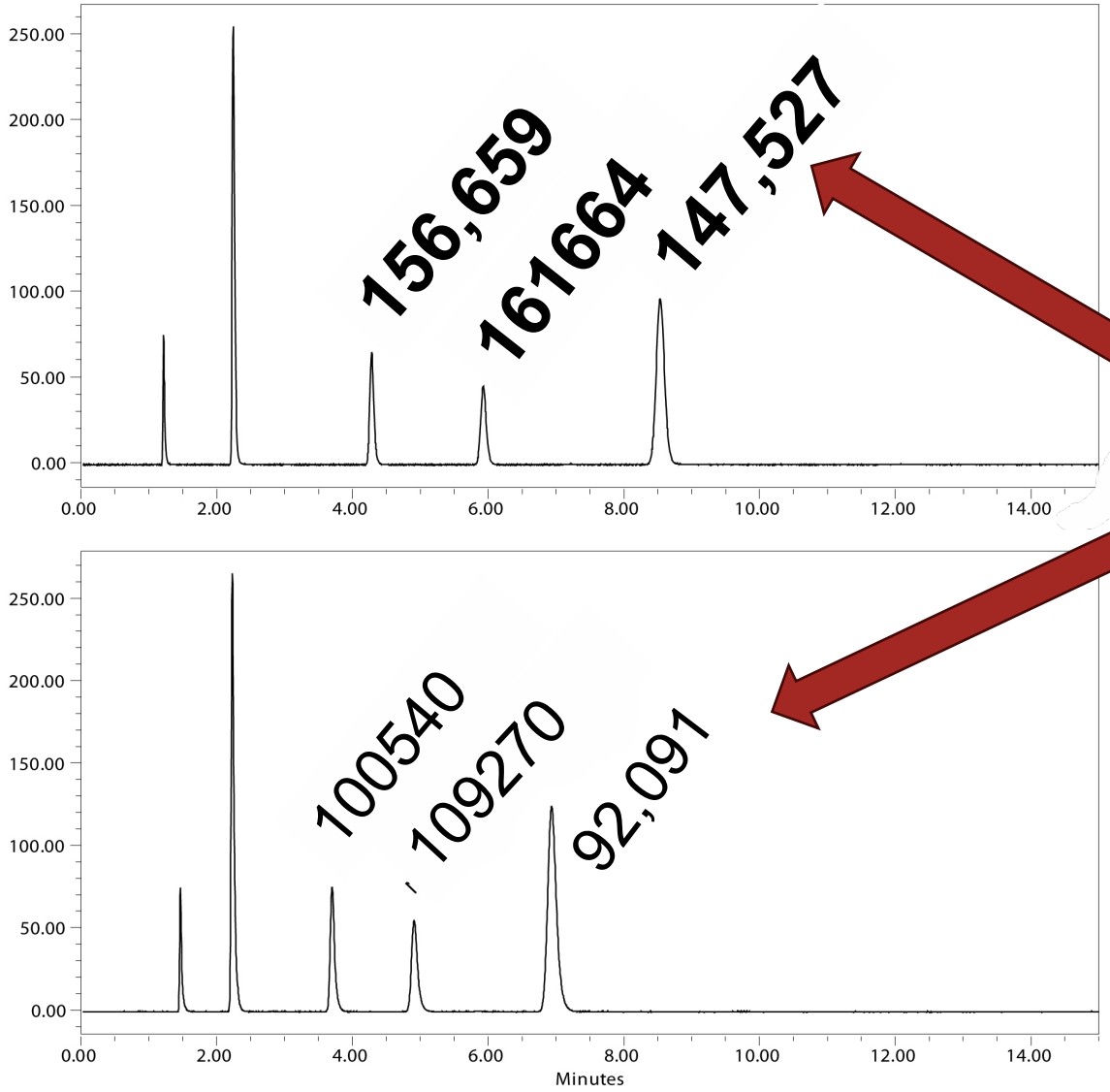


Non-Monodisperse



Flow through the column Evosphere vs. FPP

What does this look like chromatographically?



Evosphere C12

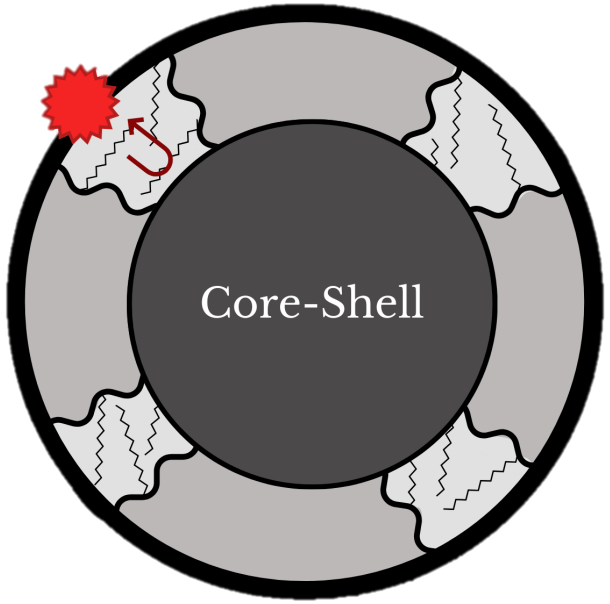
3µm, 4.6mm x 150 mm

60% Higher N

Popular Fully Porous C18
3µm, 4.6 x 150 mm

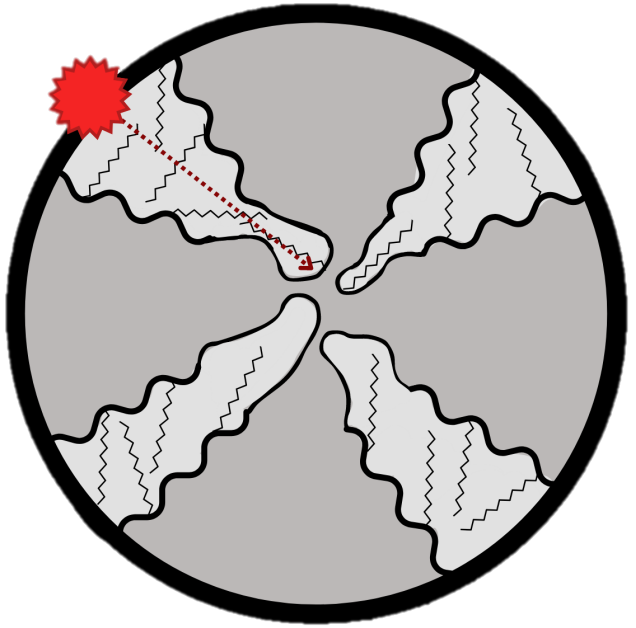
Core-Shell compared to Evosphere MFPP

Core-Shell



- Similar Efficiencies
- Greater Loading Capacity
- Scalability to Prep
- Increased Retention

Evosphere



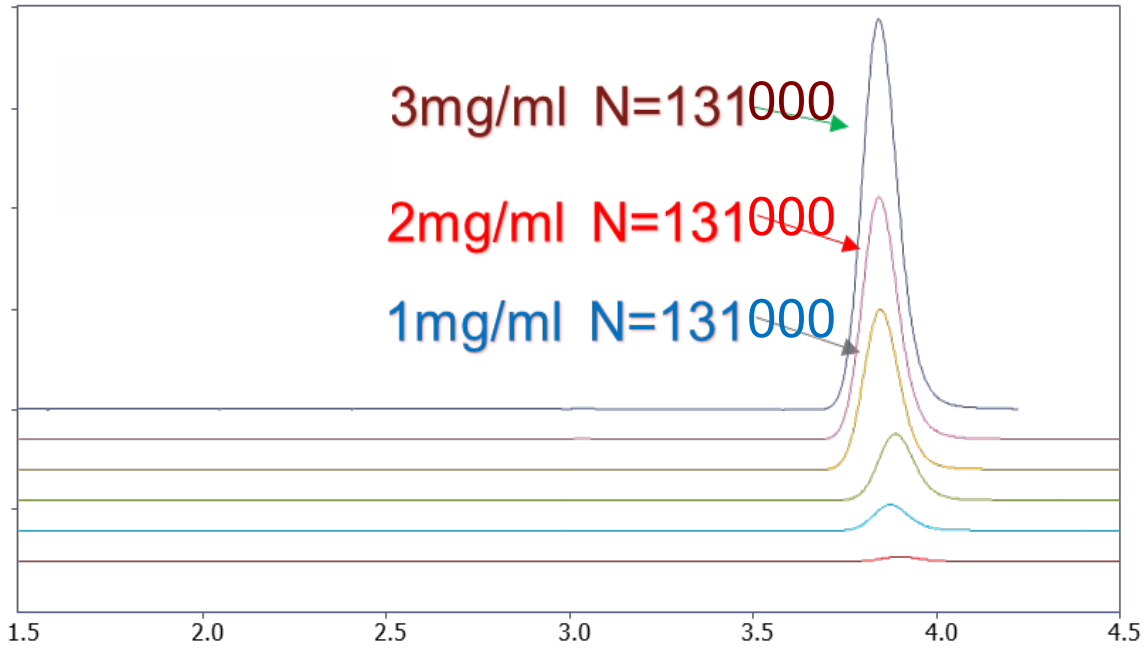
SA = ~130 m^2/g

~3X Surface Area

SA = 350 m^2/g

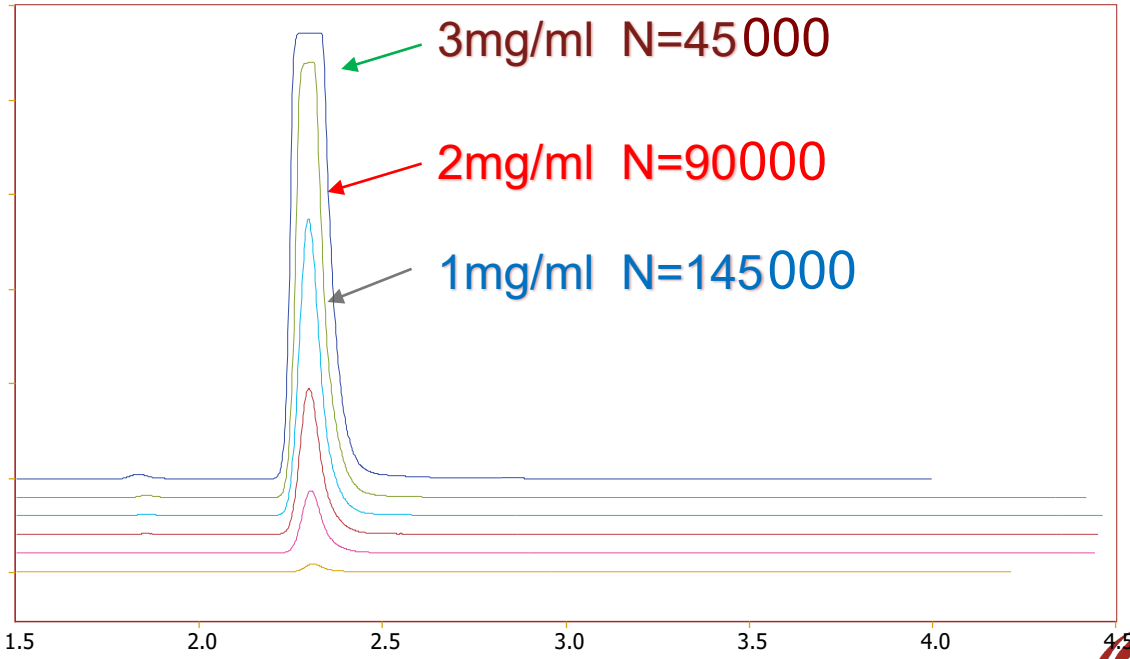
Fortis® Evosphere®
Improves Loading and Increases Retention

Evosphere L1 Surface Area = 350 m²/g



Rt = 3.8 min

Core Shell L1 Surface Area = 130 m²/g



Rt = 2.25 min

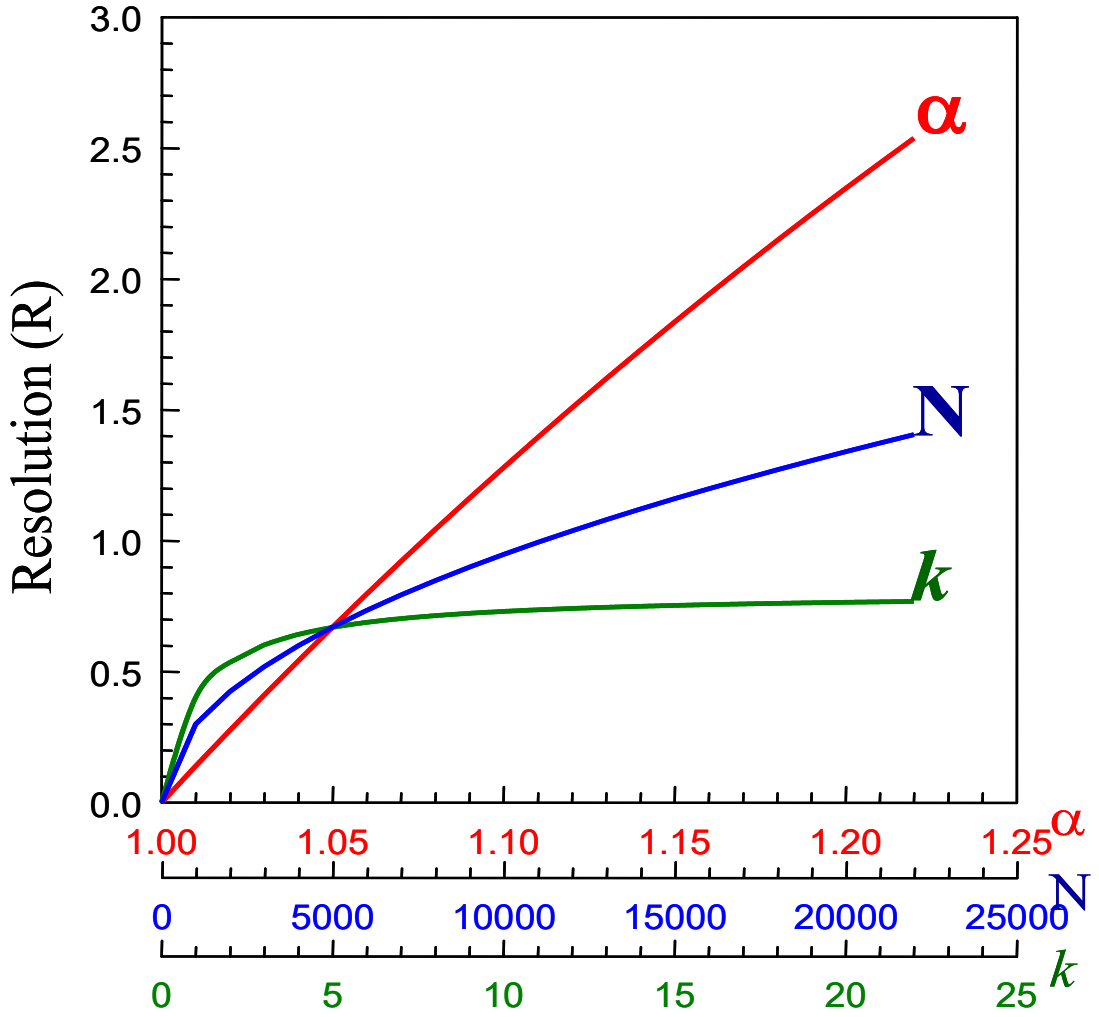
Resolution Equation

Efficiency Retention Selectivity

$$R = \frac{\sqrt{N}}{4} \quad \frac{k'}{k'+1} \quad \frac{\alpha-1}{\alpha}$$

$$\alpha = \frac{k_2}{k_1}$$

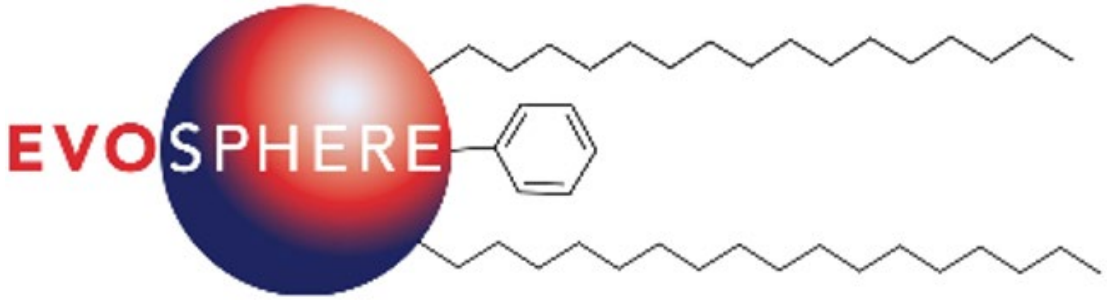
- Selectivity (α) has the greatest impact on improving resolution.



Goals of Experiment

- Develop a unique approach for Modified and Unmodified Oligonucleotide Analysis by UHPLC/UV/MS which achieves excellent separations without the use of Ion-Pairing Reagents (TEA/HFIP or DIPEA/HFIP).
- Study the impacts of salt concentration, type, and pH on separation capability.

Experimental Conditions



Column: Evosphere Max C18/AR 1.7 µm (2.1 mm x 100 mm)
Mobile Phase - (Unmodified Oligonucleotides) - 10-30% v/v MeOH in 10 minutes
Mobile Phase - (Modified Oligonucleotides) - 15-50% v/v MeOH in 10 minutes
Temperature - 30°C or 60°C
Flow Rate - 0.3 mL/min
Injection Volume - 0.5 µL
Instrument - Thermo Scientific™ Vanquish™ Horizon UHPLC system with DAD
Autosampler Temperature - 4°C
UV Wavelength – 260 nm

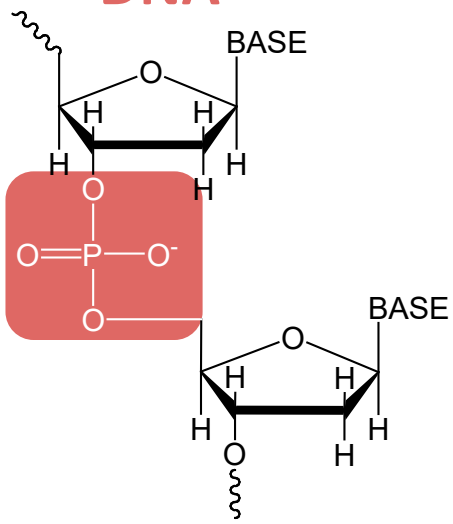
Unmodified Oligonucleotide Standards

Shortcut	Sequence 5'→3'	Modification type
UNMODIFIED OLIGOS		
20A	ATCGATCGATCGATCGATCA	-
20G	ATCGATCGATCGATCGATCG	-
20C	ATCGATCGATCGATCGATCC	-
18hA	AAAAAAAAAAAAAAAAAA	-

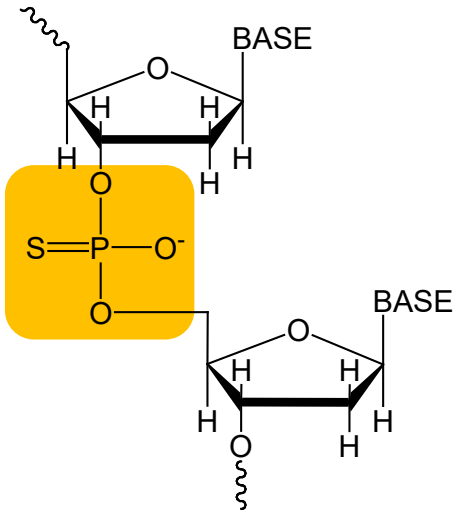
Modified Oligonucleotide Standards

Shortcut	Sequence 5'→3'	Modification type
MODIFIED OLIGOS		
DNA20	GCCCAAGCTGGCATCCGTCA	-
PS20	GCCCAAGCTGGCATCCGTCA	phosphorothioate
ME20	GCCCAAGCTGGCATCCGTCA	2'-O-methyl
MOE20	GCCCAAGCTGGCATCCGTCA	2'O-methoxyethyl

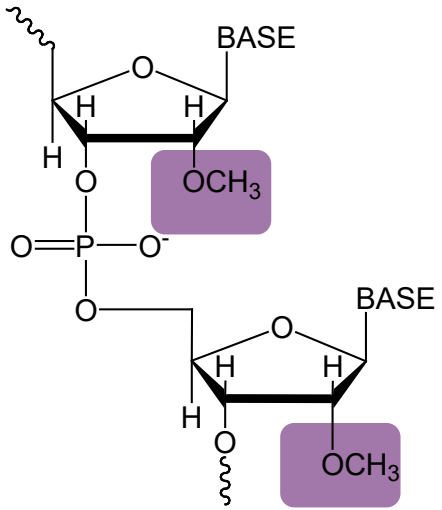
DNA



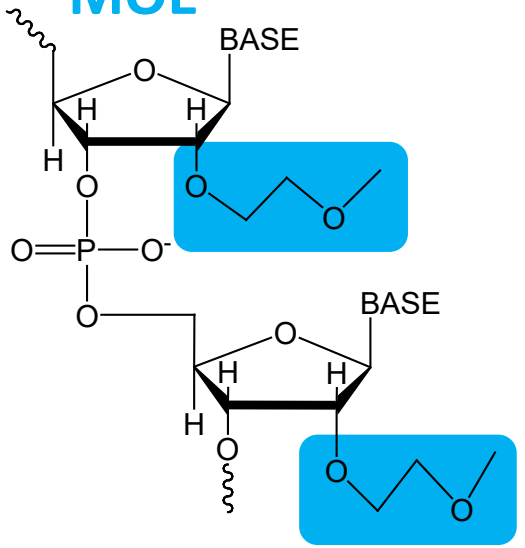
PS



ME



MOE



Unmodified and phosphorothioate oligonucleotide standards were purchased from Sigma Aldrich (Dorset, UK). 2'-O-(2-methoxyethyl) and 2'-O-methyl OGNs were obtained from Eurogentec (Seraing, Belgium).

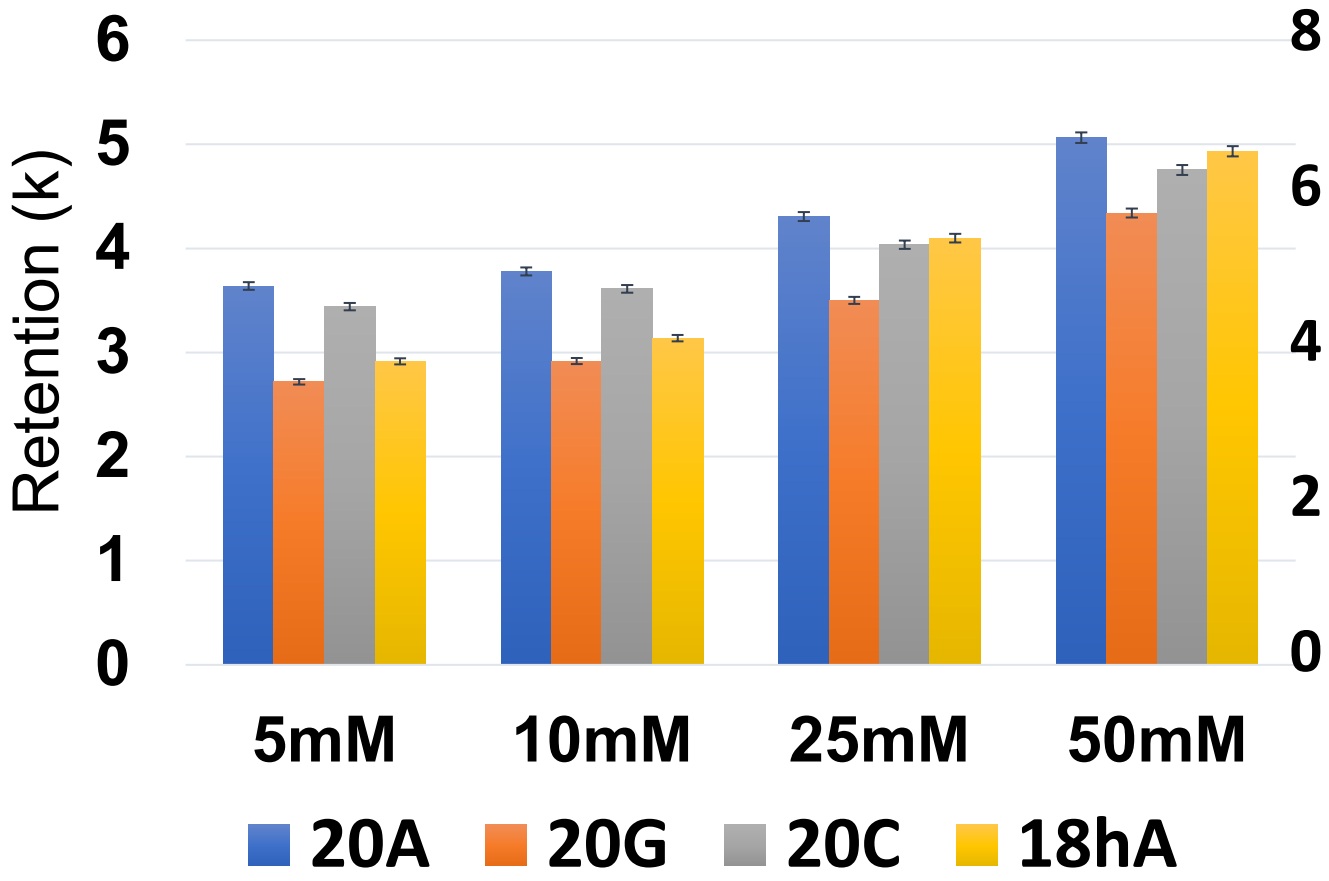
Method Conditions to Optimize

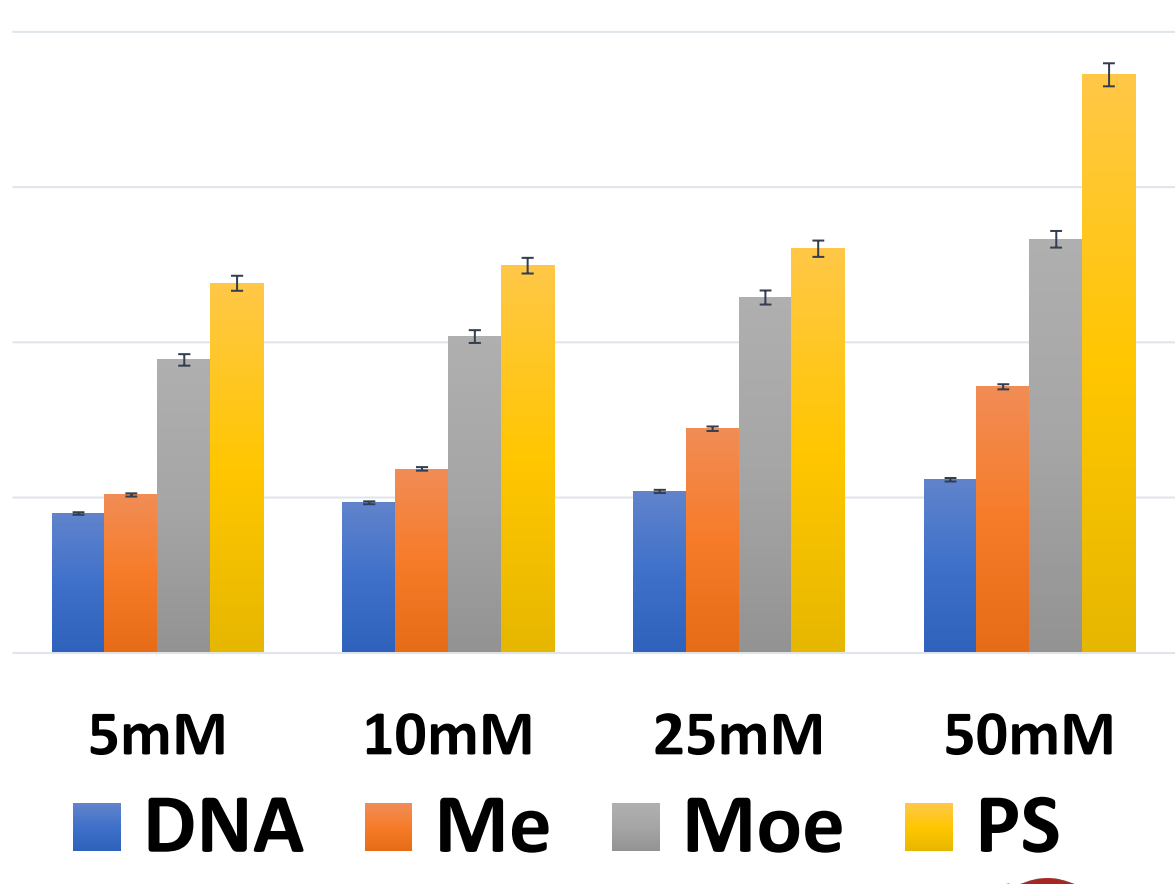
IMPACT OF SALT CONCENTRATION
AMMONIUM ACETATE
5, 10, 25, 50 mM

IMPACT OF SALT TYPE
AMMONIUM ACETATE (AA)
AMMONIUM FORMATE (AF)
AMMONIUM BICARBONATE (AB)

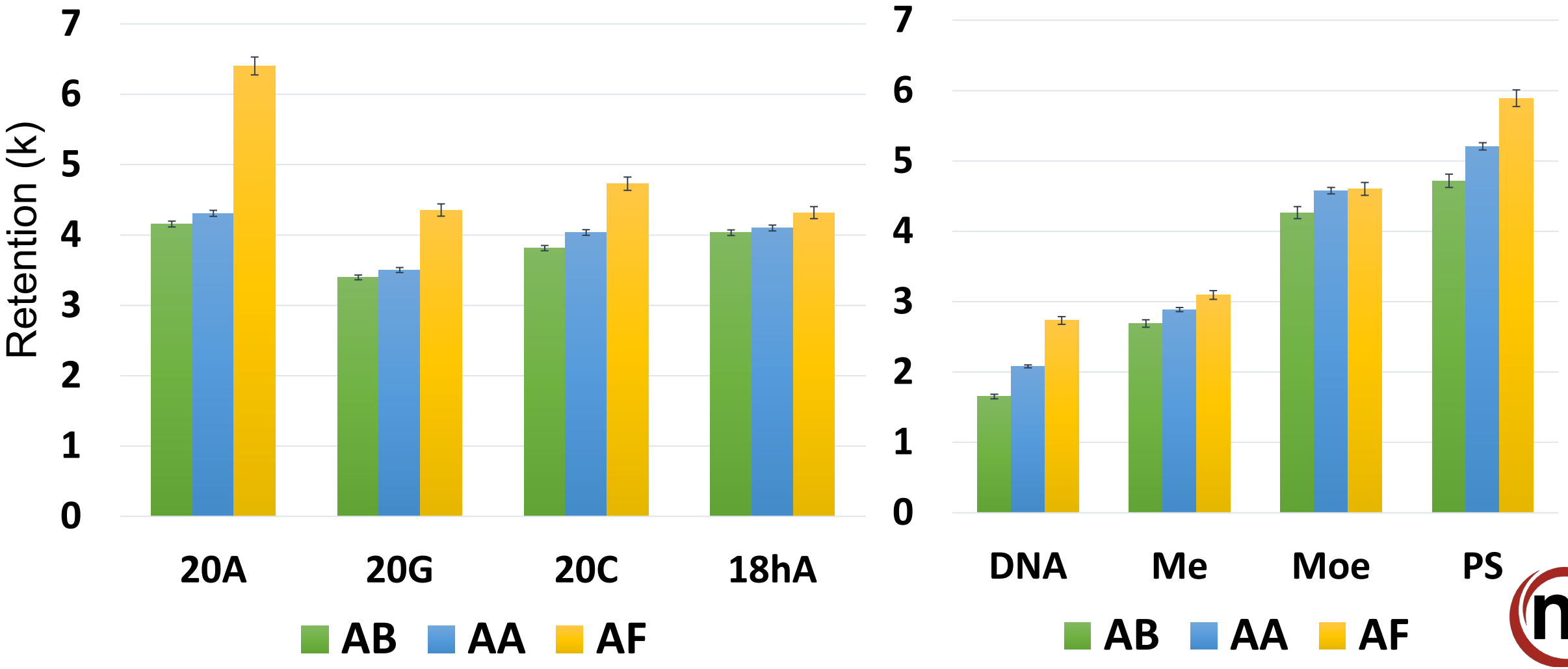
IMPACT OF SALT pH
25mM AA
pH 3.0, 4.5, 6.0, 7.5

Impact of Salt Concentration (Amm Ac.)

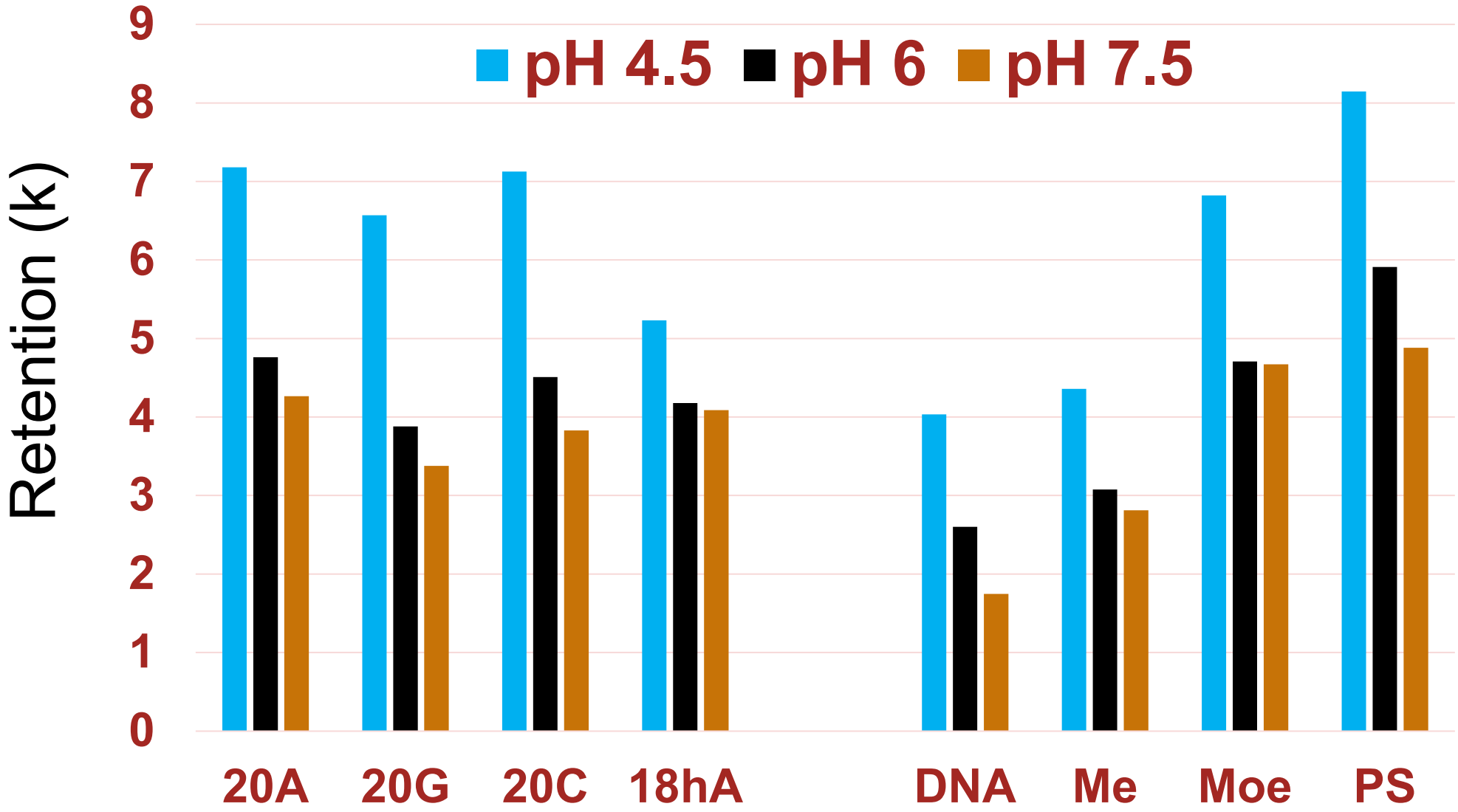




Impact of Salt Type

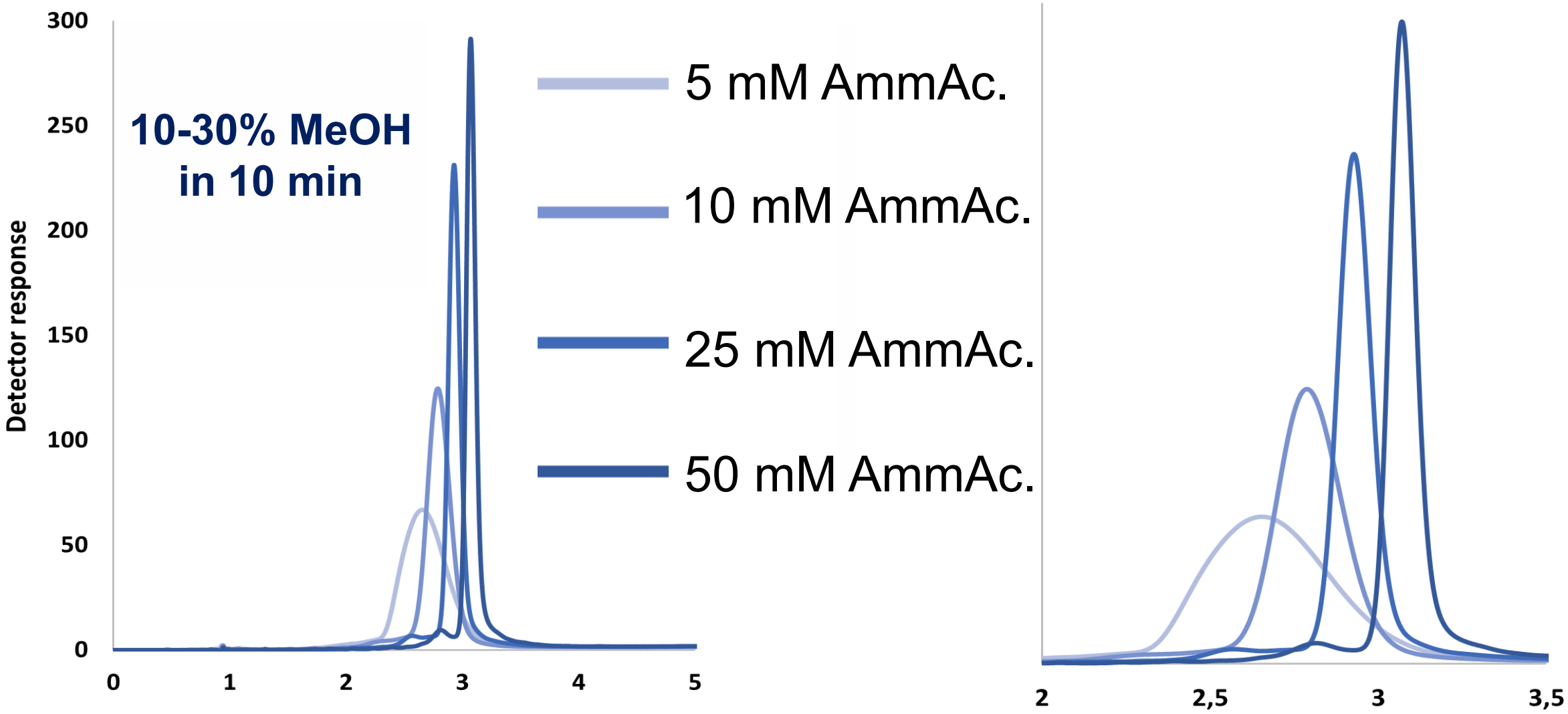


Impact of pH on Retention

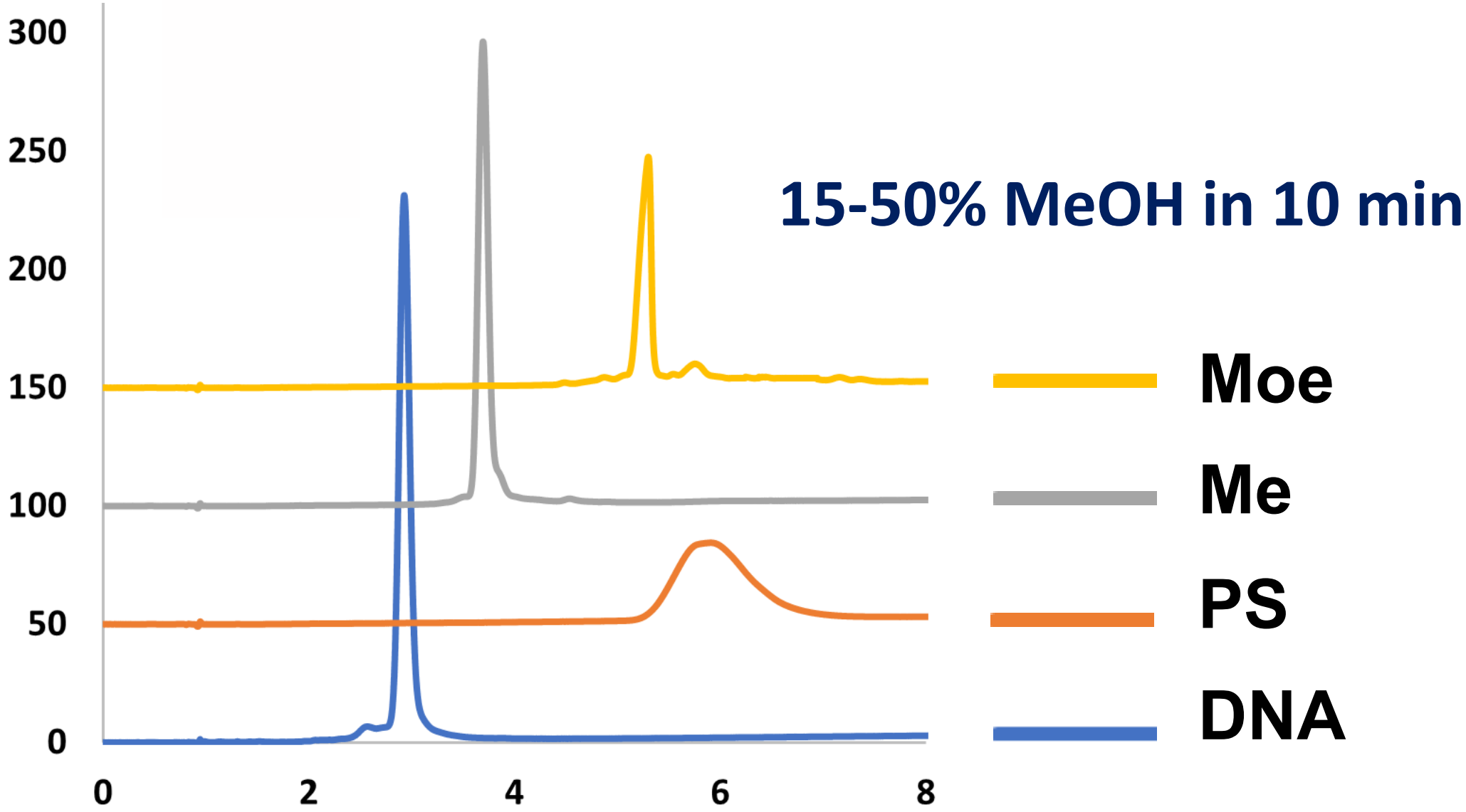


Peak Shape for DNA20 Oligos

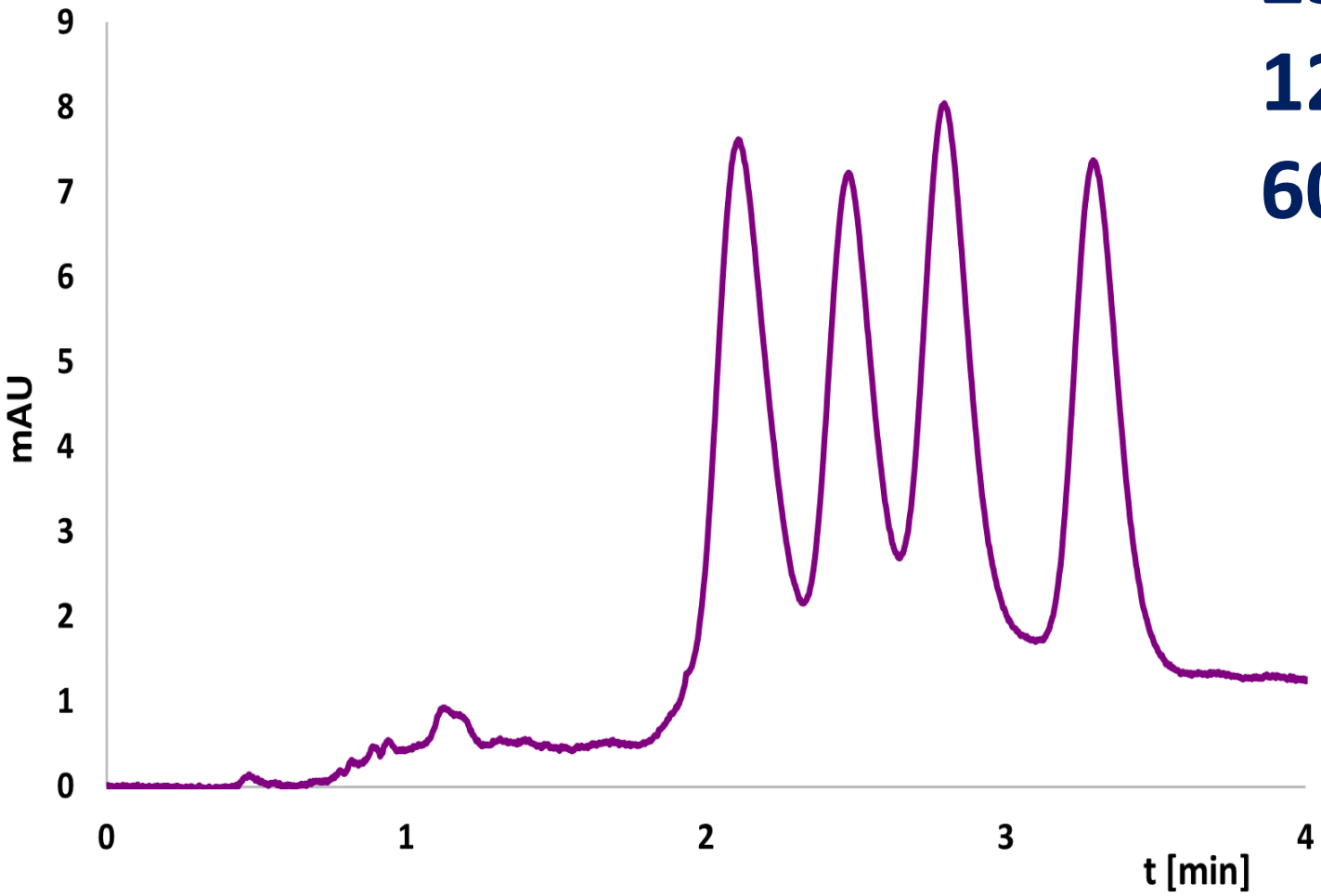
Oligonucleotide Sample - GCCCAAGCTGGCATCCGTCA



Peak Shape for Modified Oligos



Separation of Unmodified Sequence Isomers



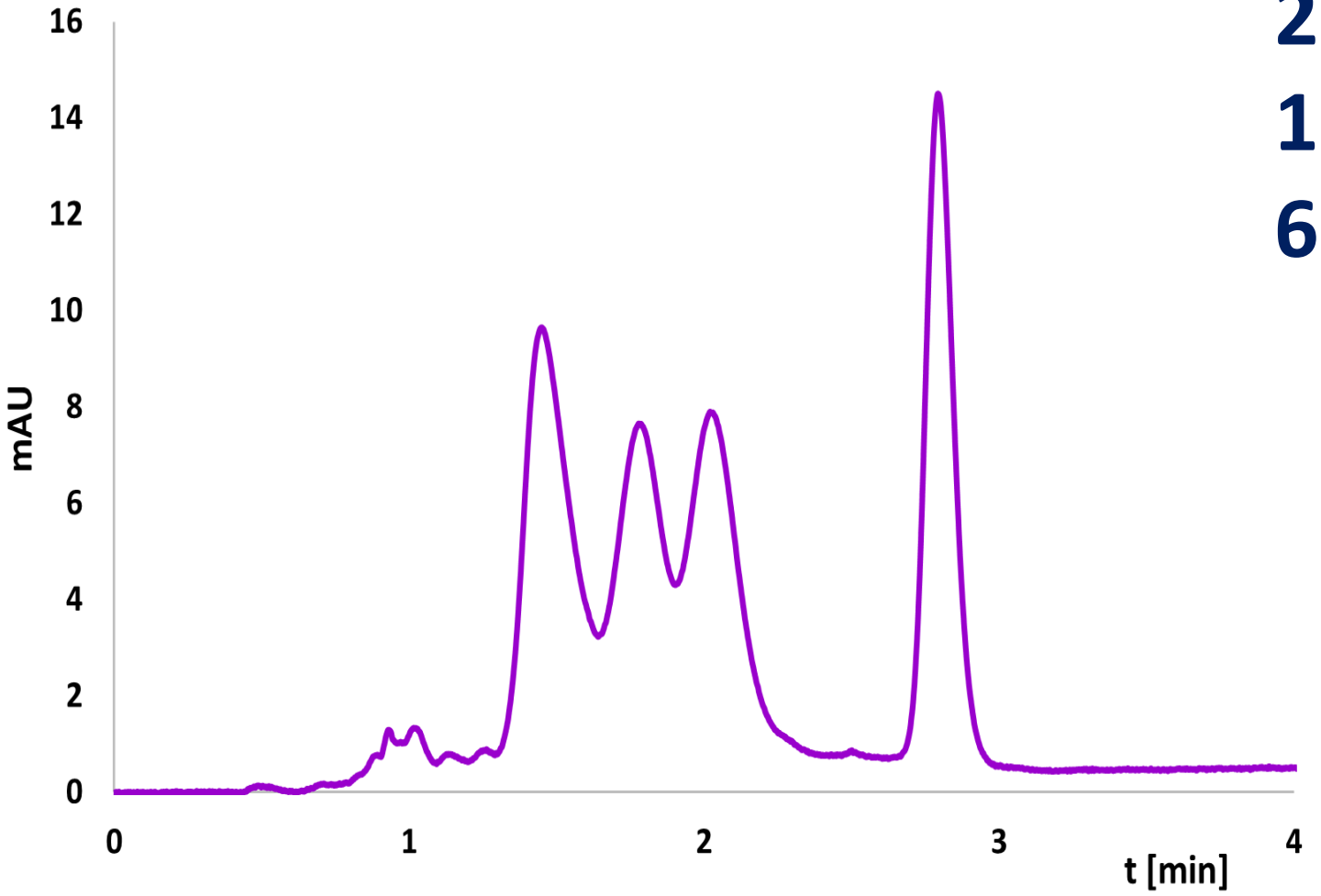
25mM AA pH=6

12-20 % MeOH in 10min

60 °C

1 - ATCGATCGATCGATCGATCG
2 - ATCGATCGATCGATCGATCT
3 - ATCGATCGATCGATCGATCC
4 - ATCGATCGATCGATCGATCA

Separation of Sequence Isomers



25mM AA pH=6

12-20 % MeOH in 10min

60 °C

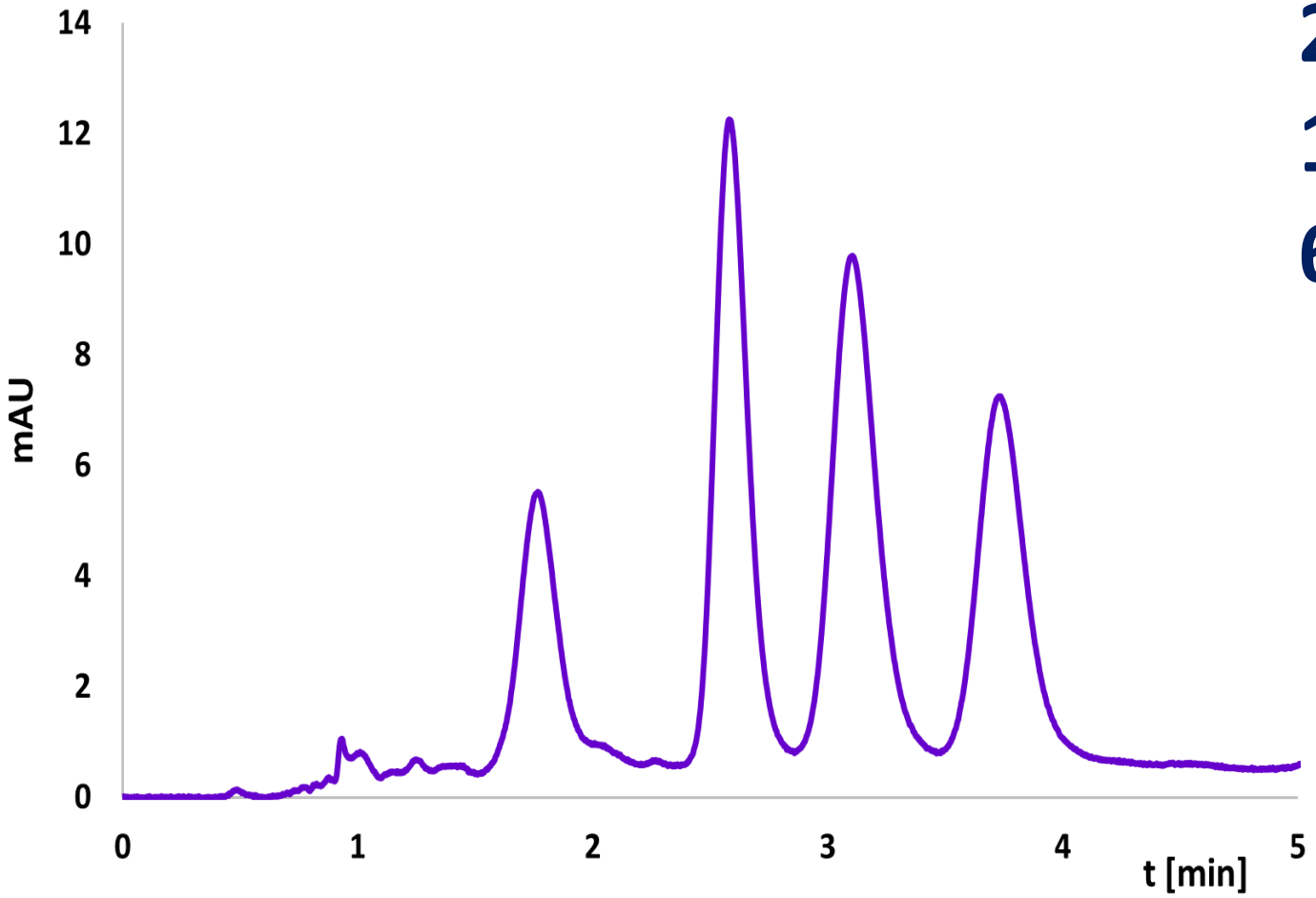
1 - ATCGATCGAGCGATCGATCG

2 - ATCGATCGAACGATCGATCG

3 - ATCGATCGATCGATCGATCG

4 - ATCGATCGACCGATCGATCG

Separation of Sequence Isomers



25mM AA pH=6

12-20 % MeOH in 10min

60 °C

1 - ATCGATCGAACGATCGATCG

2 - ATCGATCGATAGATCGATCG

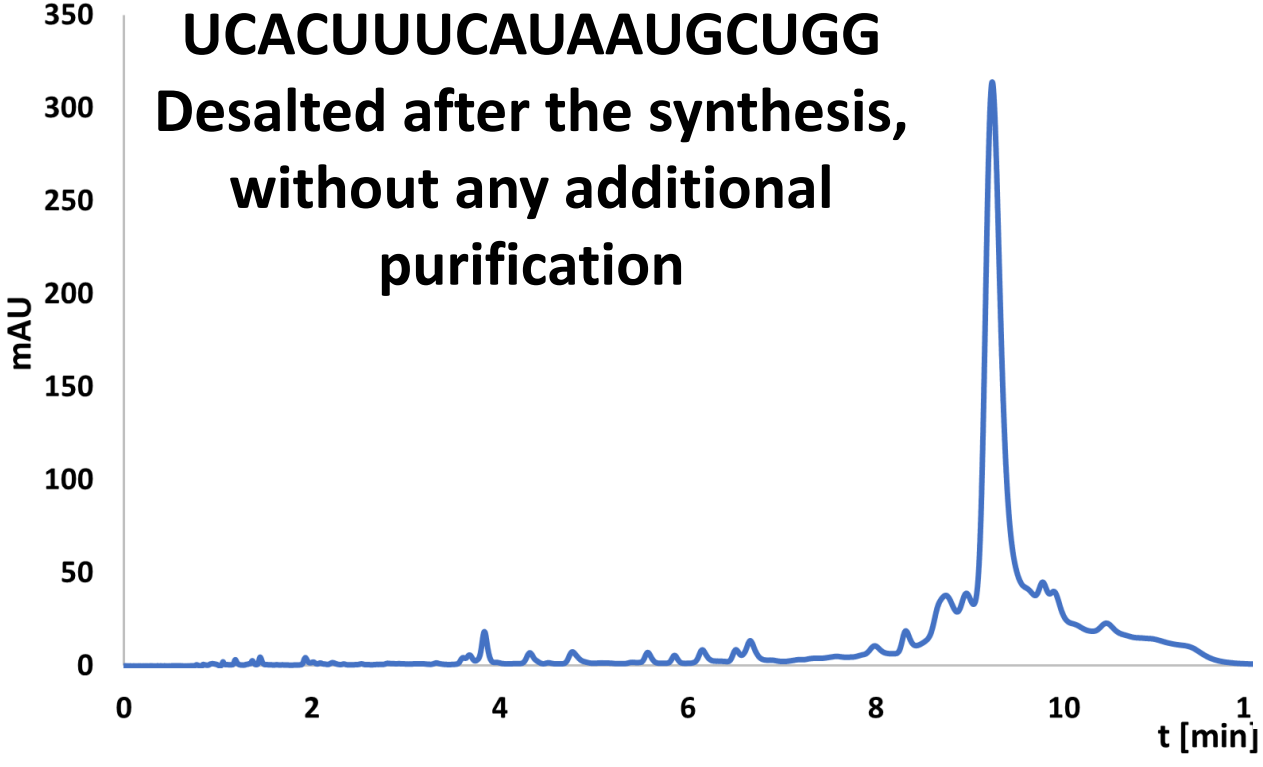
3 - ATCGATCGATCGATCGAACG

4 - ATCGATCGATCGATCGATCA

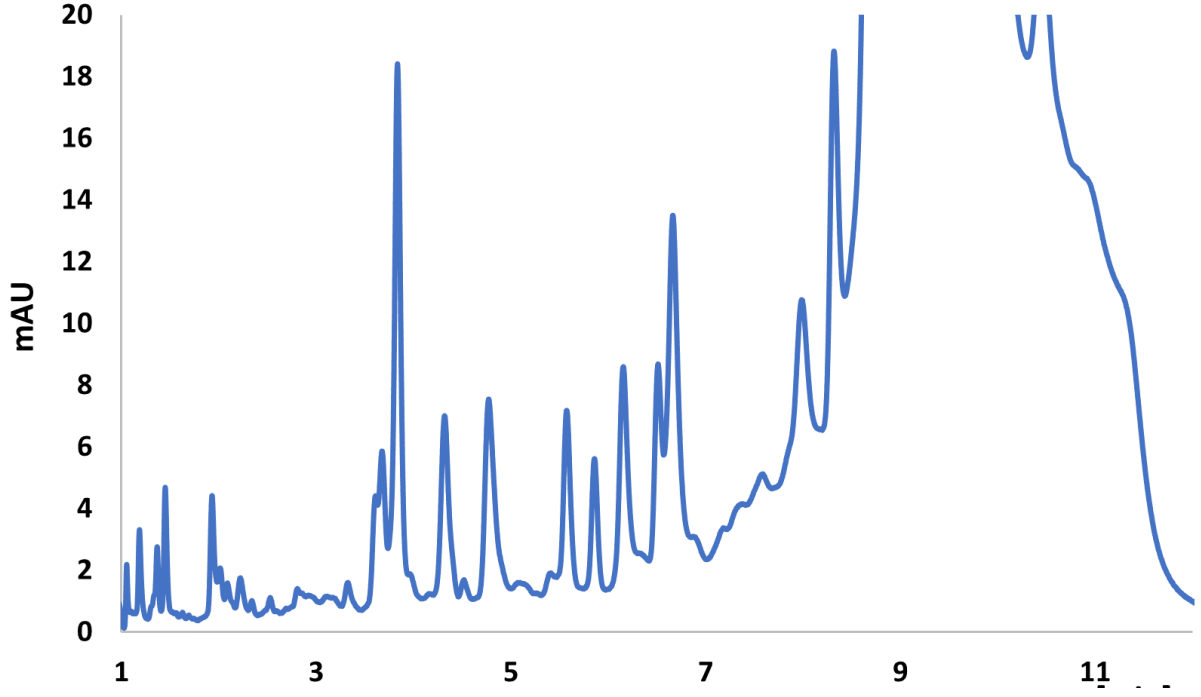
Unpurified RNA Oligonucleotide Separation

25mM Amm. Ac. pH 6, 30 °C
5-15% MeOH 10min

UCACUUUCAUAAUGCUGG
Desalted after the synthesis, without any additional purification

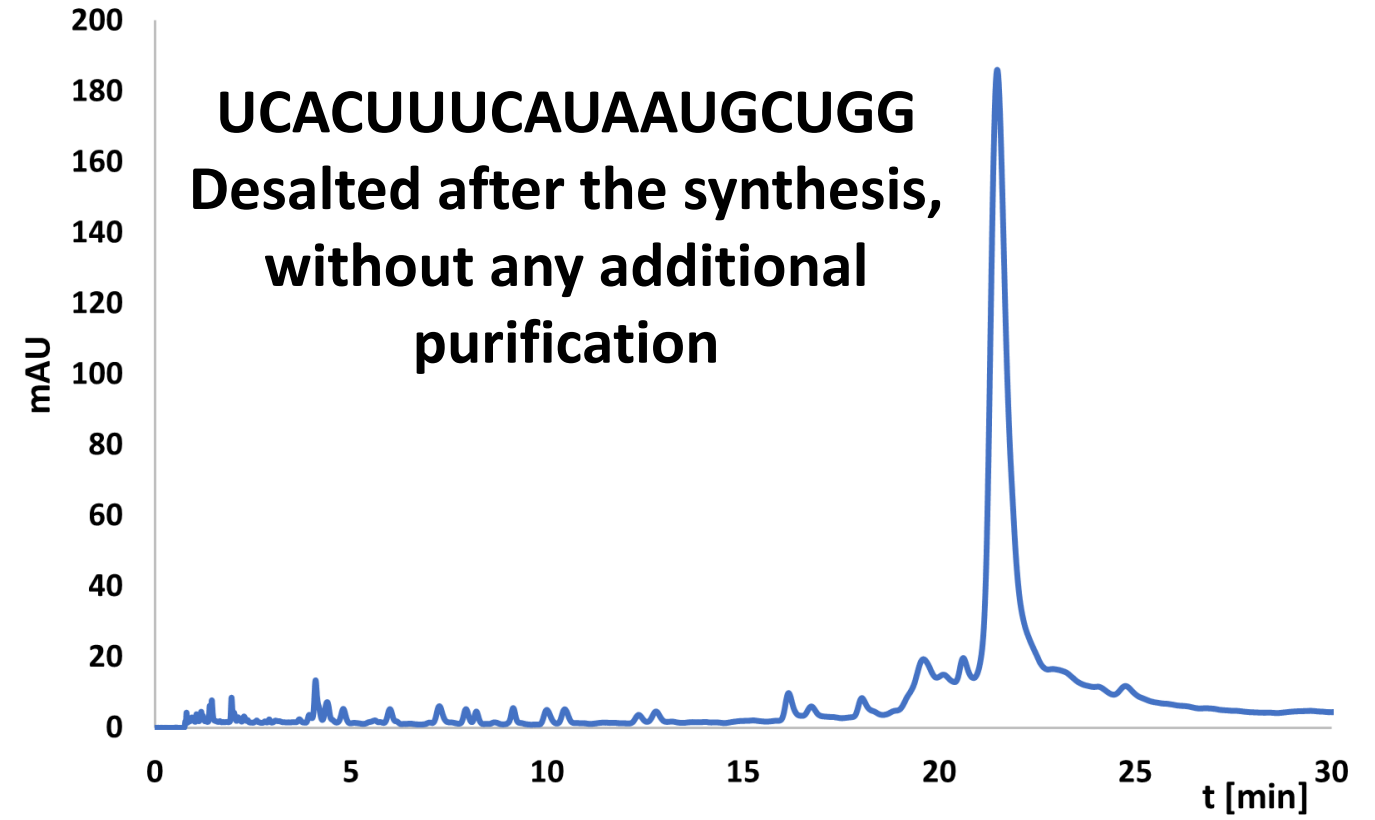


Magnified Chromatogram

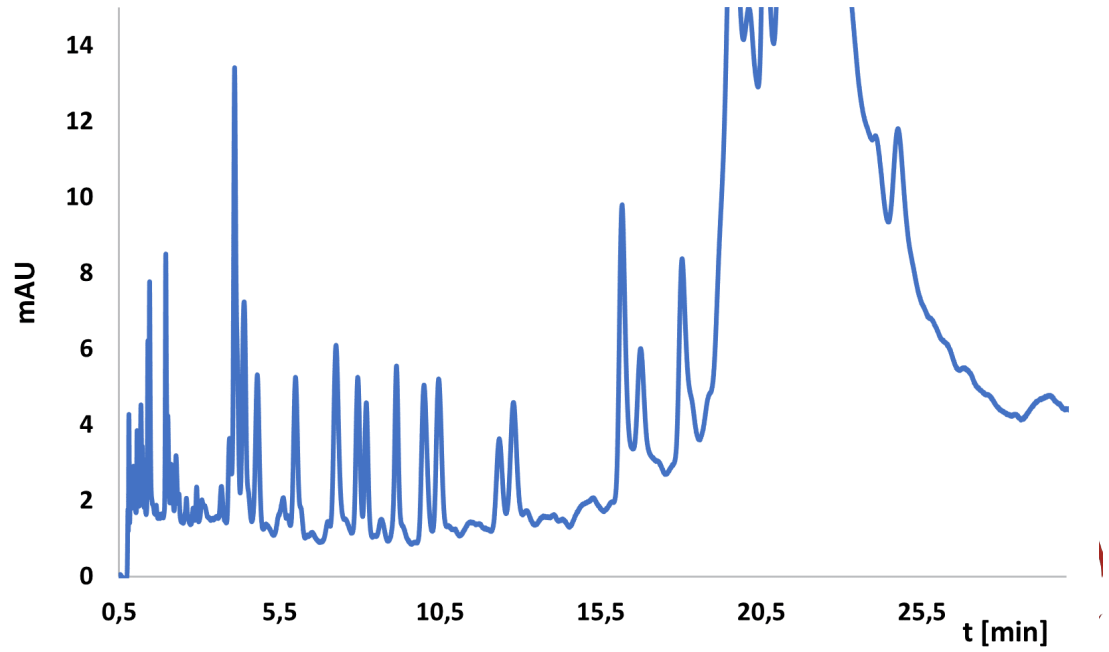


Unpurified RNA Oligonucleotide Separation

25mM Amm. Ac. pH 6 30 °C
5-15% MeOH 30min



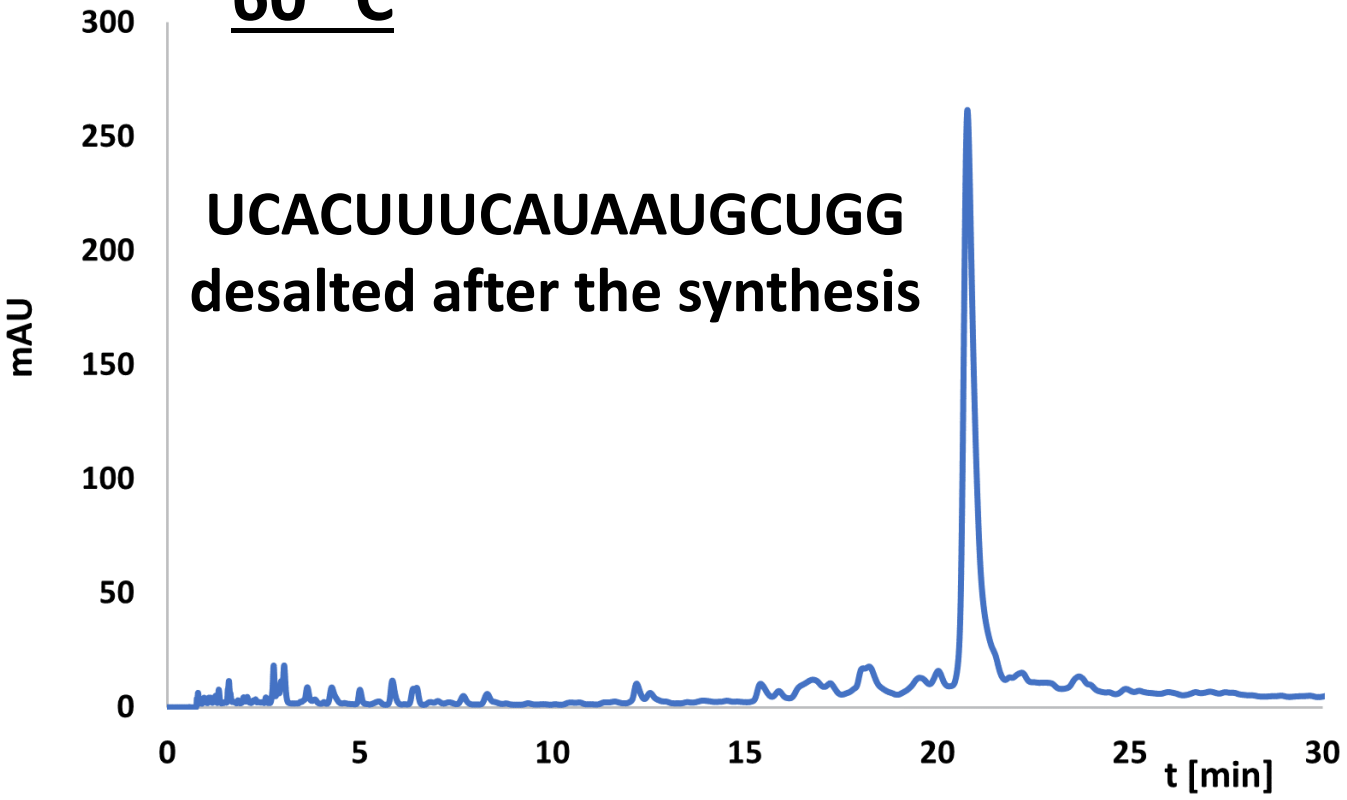
Magnified Chromatogram



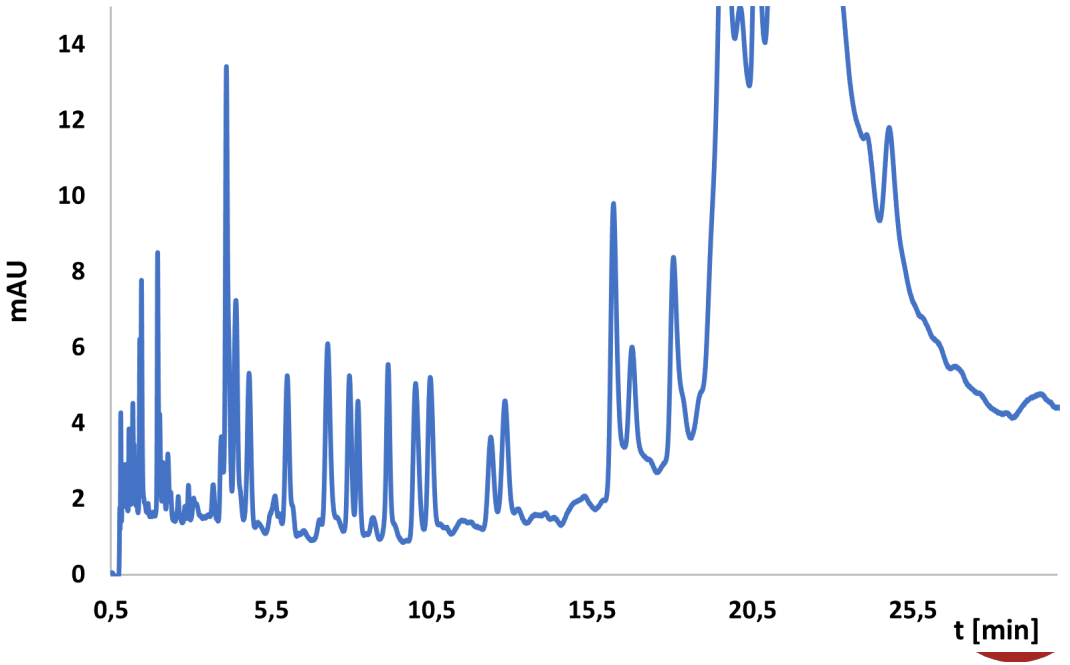
Unpurified RNA Oligonucleotide Separation

25mM Amm. Ac. pH 6
2-8% MeOH 30min
60 °C

UCACUUUCAUAAUGCUGG
desalted after the synthesis



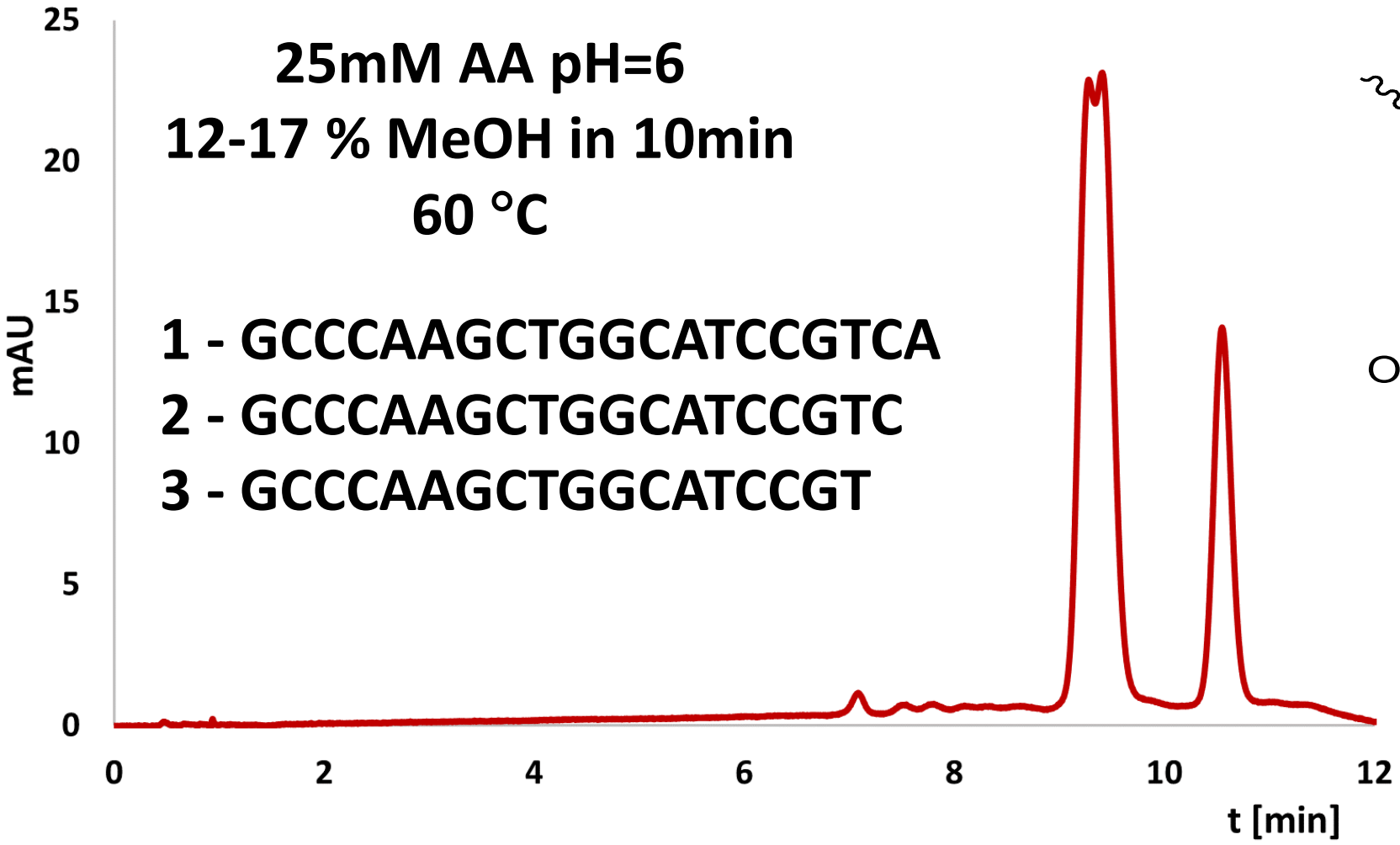
Magnified Chromatogram



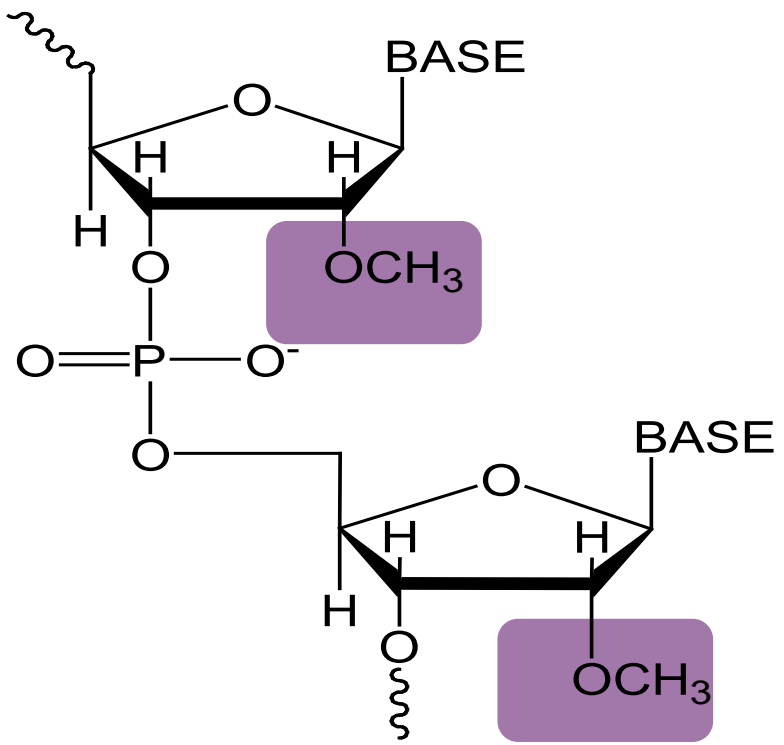
Modified Oligonucleotide Separation

25mM AA pH=6
12-17 % MeOH in 10min
60 °C

1 - GCCCAAGCTGGCATCCGTCA
2 - GCCCAAGCTGGCATCCGTC
3 - GCCCAAGCTGGCATCCGT

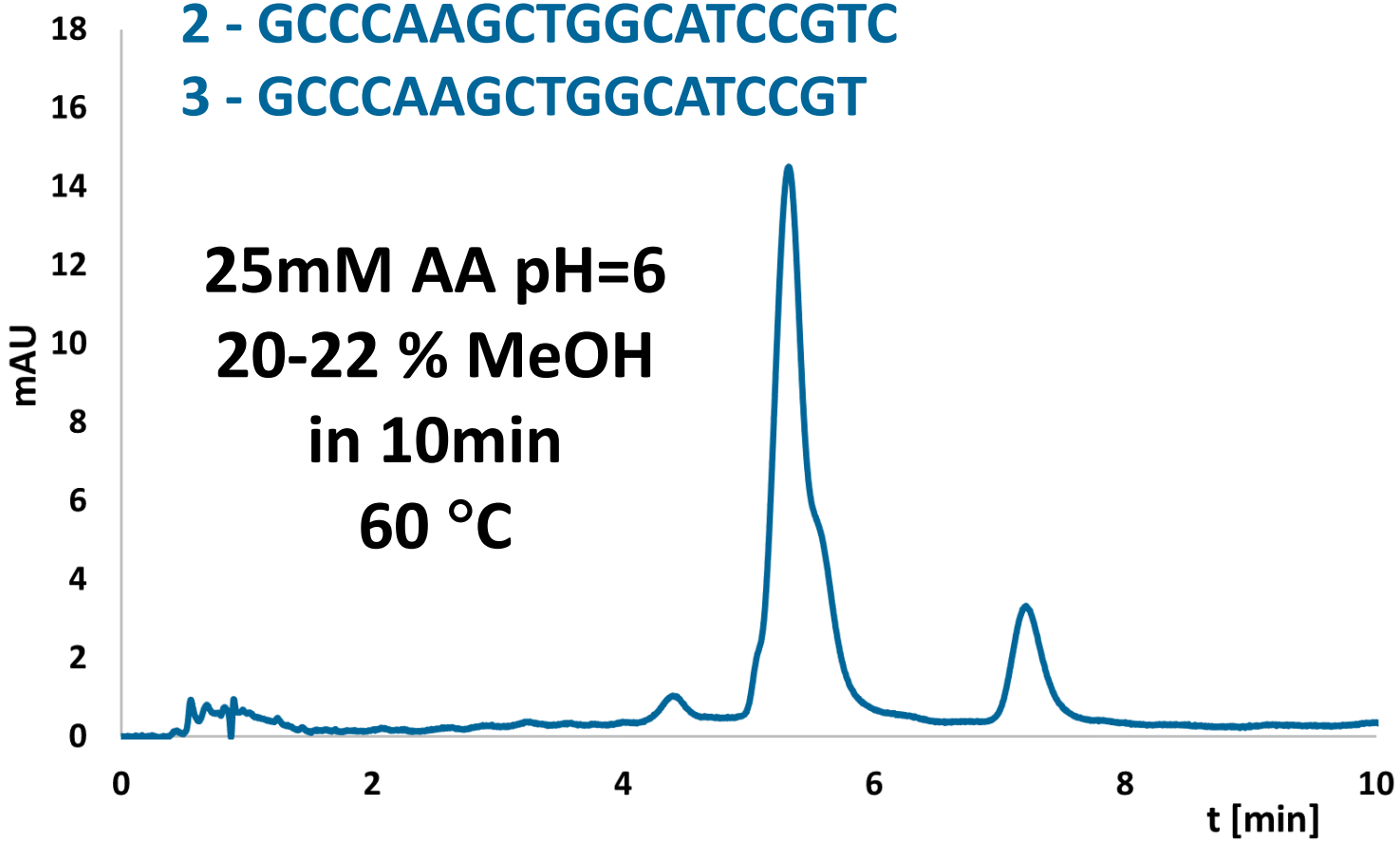


ME

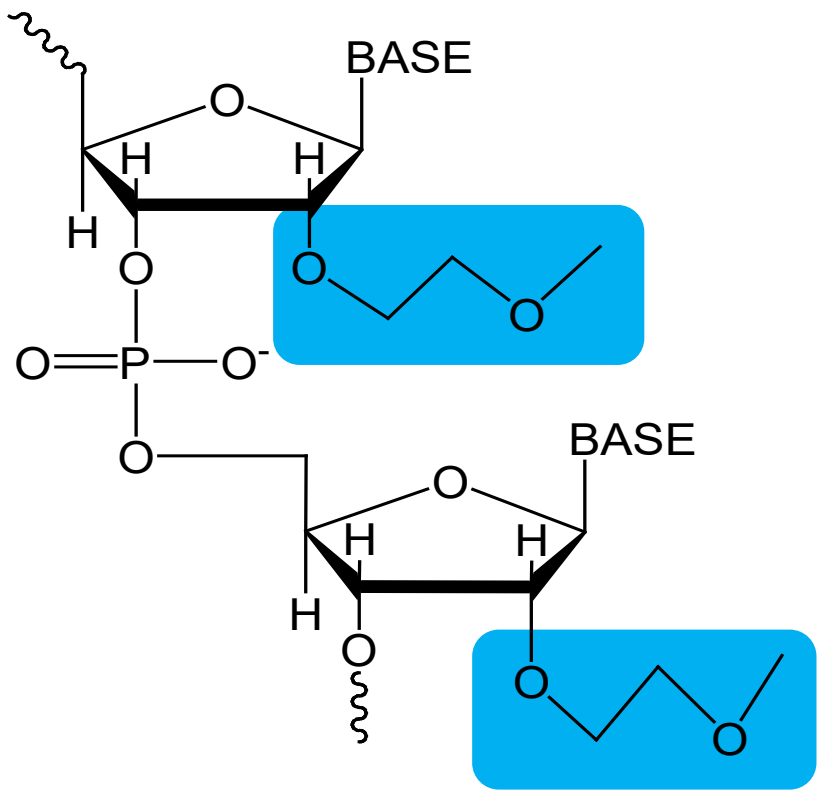


Modified Oligonucleotide Separation

1 - GCCCAAGCTGGCATCCGTCA
2 - GCCCAAGCTGGCATCCGTC
3 - GCCCAAGCTGGCATCCGT



MOE



Conclusions and Future Work

- Conclusions:
  - Evosphere MAX C18/AR is a viable candidate to evaluate potential non-ion-pair mobile phase systems for modified and unmodified Single-Stranded Oligonucleotides
- Future Work:
  - Evaluate Evosphere 300 Å C18/AR material for longer Single Stranded Oligonucleotides
  - HILIC for Double Stranded Oligonucleotides
  - Improve Sample Prep Workups
  - Evaluate Evosphere MAX C12 for traditional TEA/HFIP or DIPEA/HFIP ion-pairing mobile phases

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SMARTER CHROMATOGRAPHY

Thank you for your time
Questions?