UNIVERSITY of MARYLAND
SCHOOL OF PHARMACY

Jones Lab

Untangling the Complexity within Oligonucleotide Therapeutics

WCBP 2024

25-January-2024
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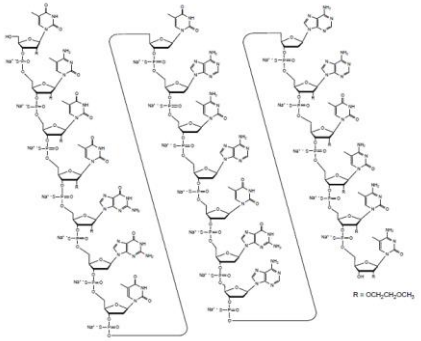
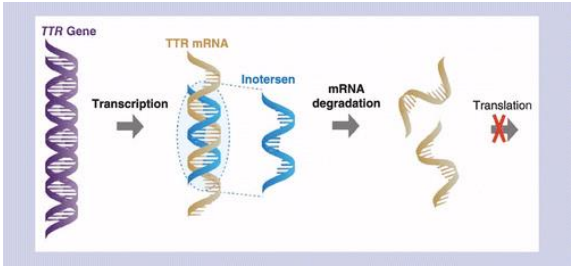
School of Pharmacy, University of Maryland

The use of mass spectrometry to determine oligonucleotide structure



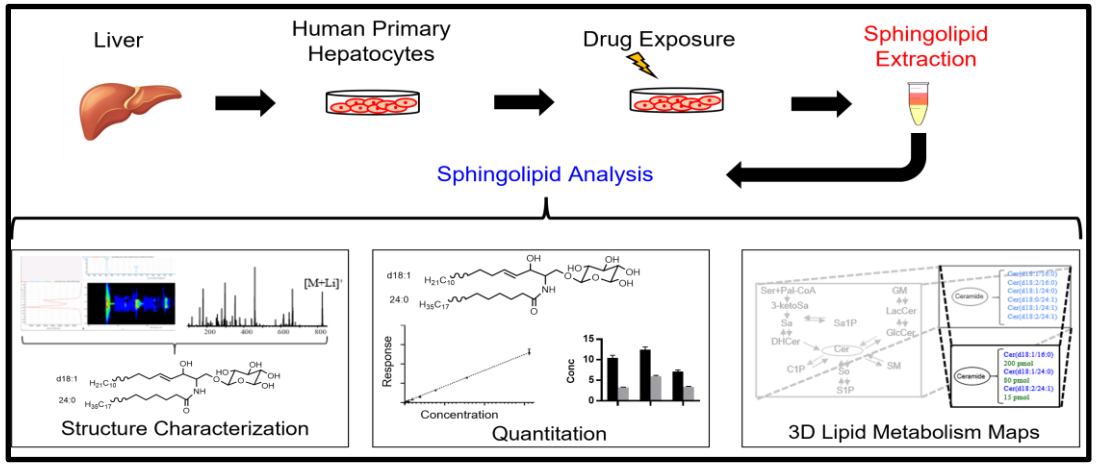
Frederick S. Brundick/cc-2.0/https://flic.kr/p/29umt8b



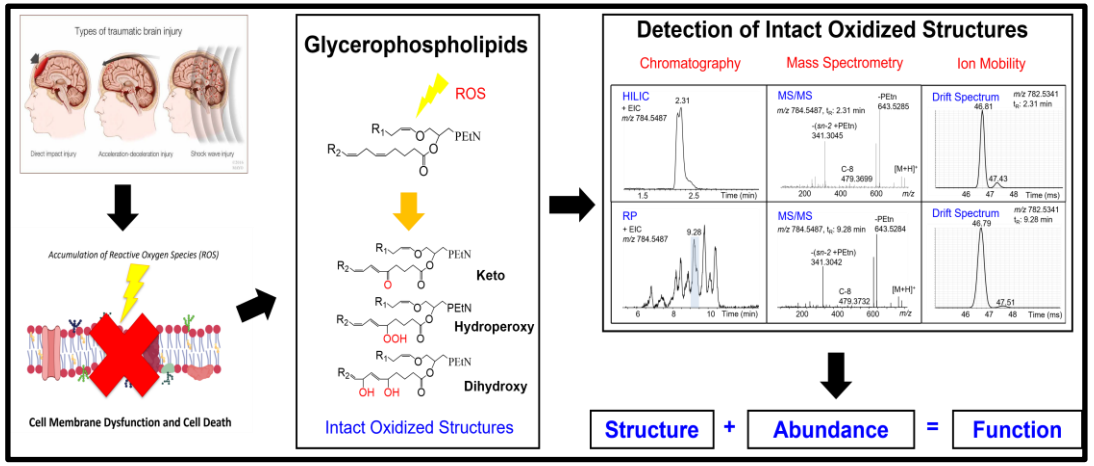
https://www.futuremedicine.com/doi/10.2217/nmt-2018-0037

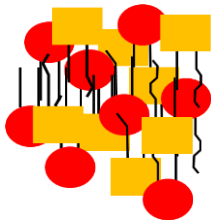
The use of mass spectrometry to determine lipid structure and abundance

Sphingolipid Metabolism as a Molecular Marker



Phospholipid Oxidation Susceptibility Following Lipid Peroxidation





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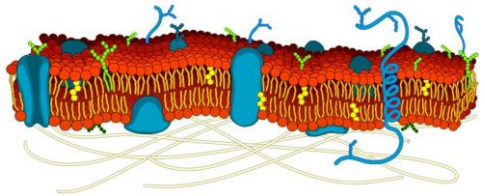
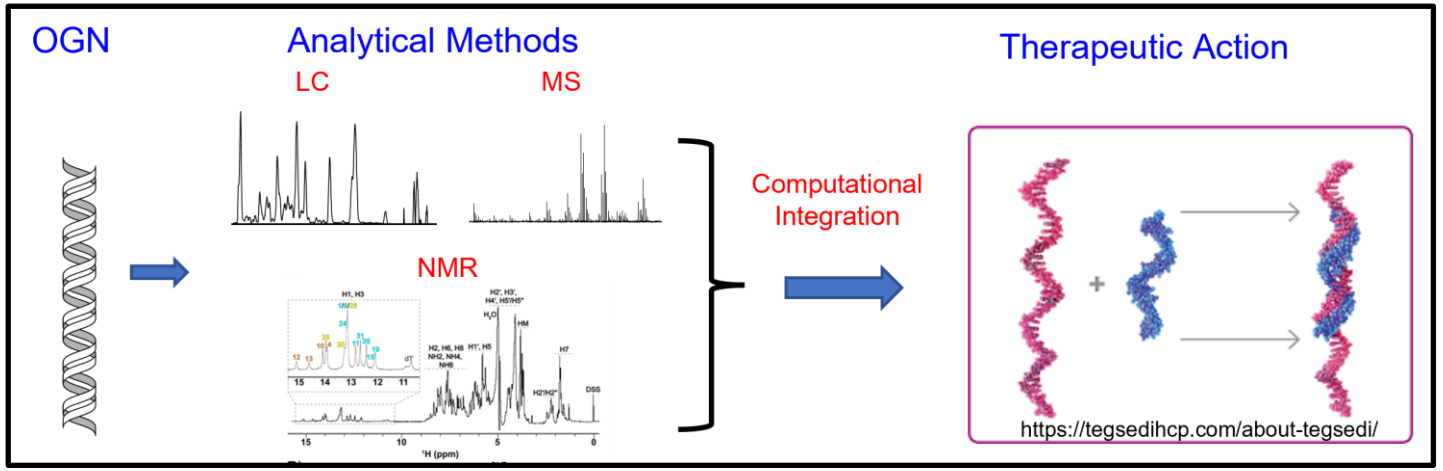
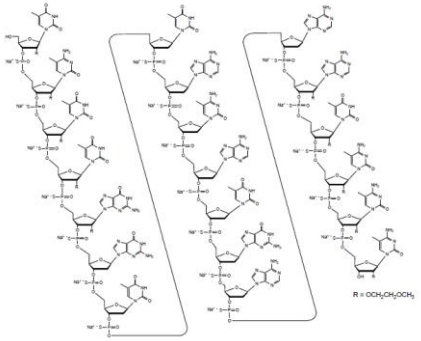


https://biologydictionary.net/lipid-bilayer/

The use of mass spectrometry to determine oligonucleotide structure

Oligonucleotide Therapeutics





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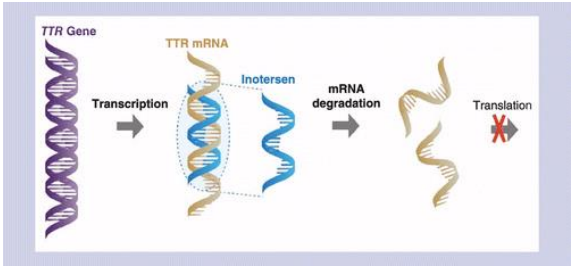

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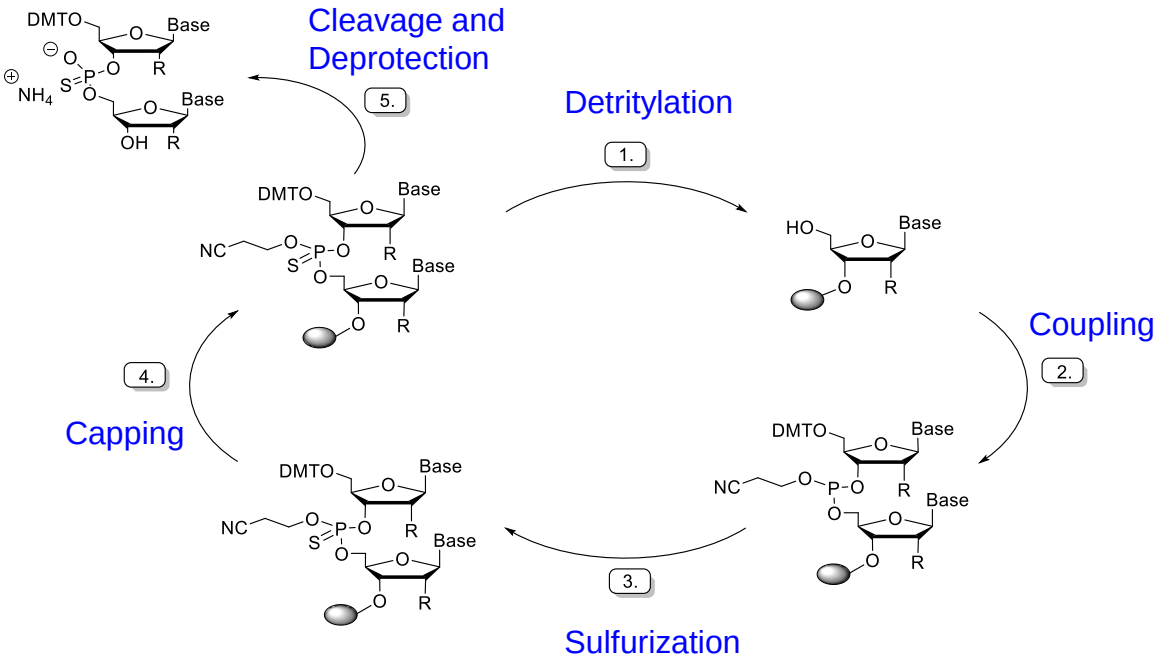
Chemical Synthesis of Oligonucleotides



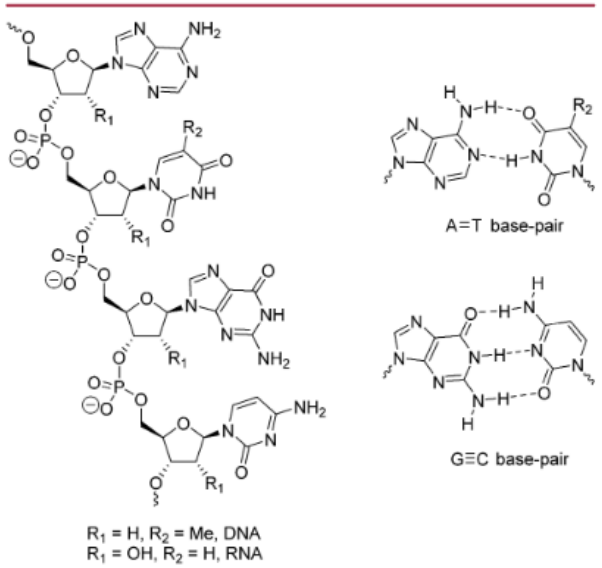


Figure 1. Structure of DNA and RNA and Watson–Crick base pairing.

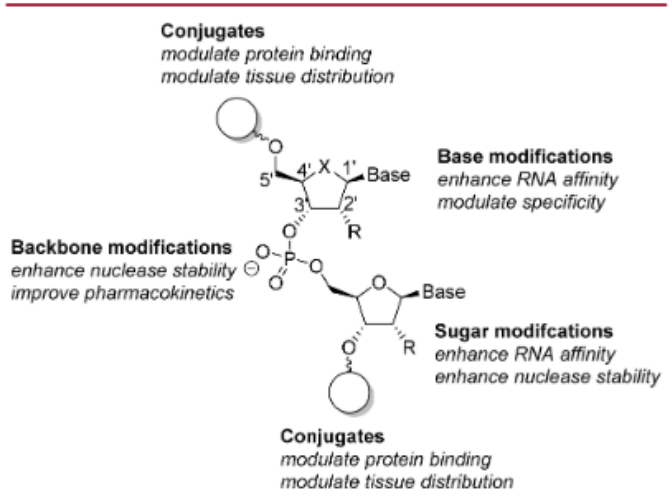


Figure 2. Sites for modification on a dinucleotide subunit.

Wan and Seth. J Med Chem. 2016 Nov 10;59(21):9645-9667.

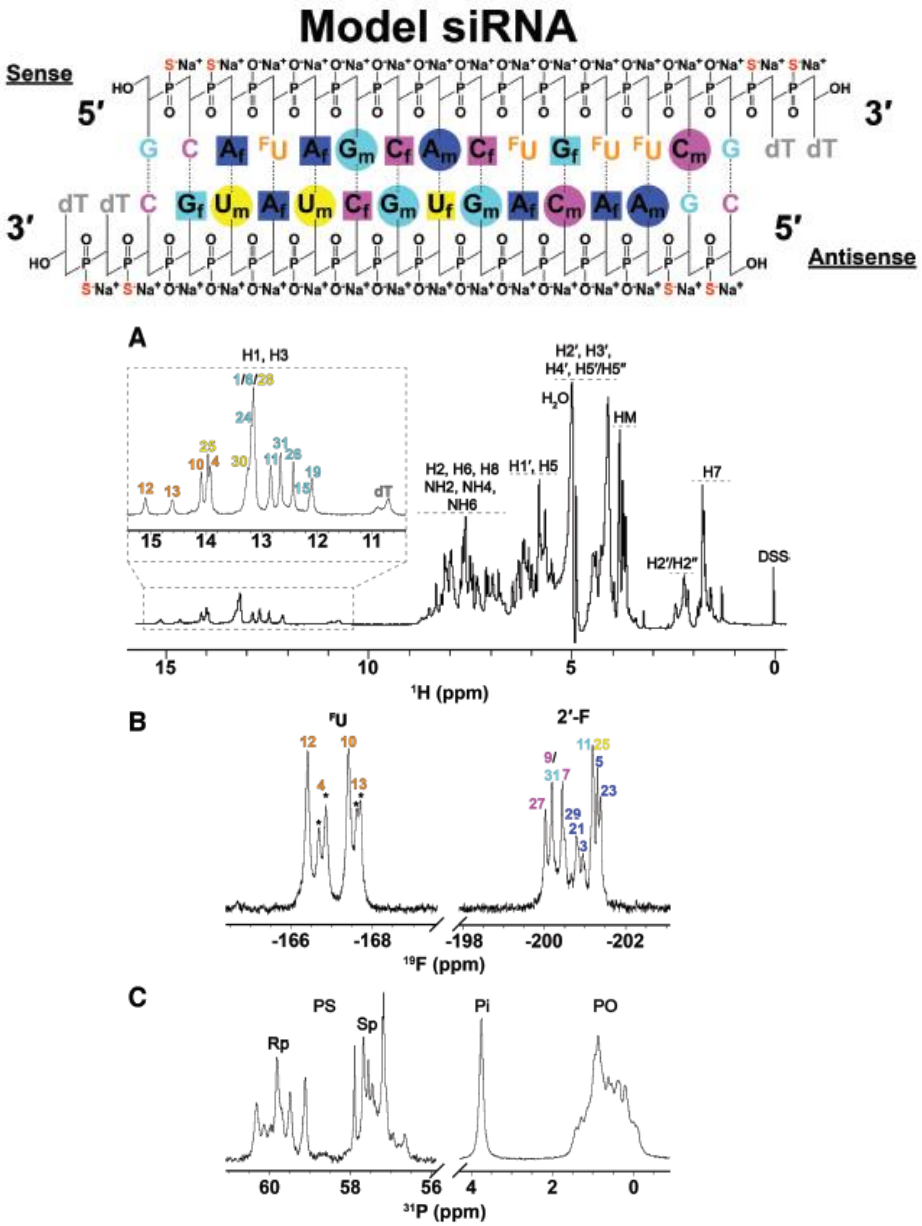
Chemical Modifications:

- Sugar modifications
  - O-Me, MOE, F
- Base modifications
  - 5-methylcytosine
- Conjugates (GalNAc)
- Backbone modifications
  - Phosphorthioate (PS)

Impurities:

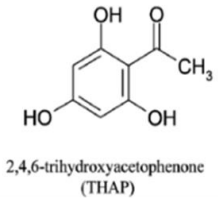
- Acetylation byproducts
- Truncations/Failed Sequences
- Incomplete deprotection
- Incomplete thiolation
- Deamination, Depurination

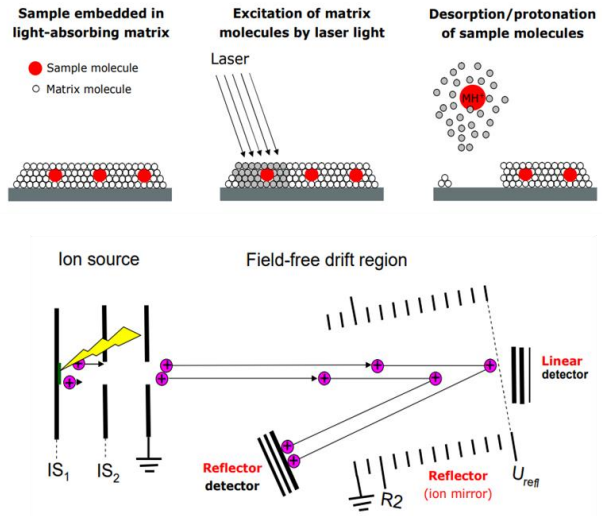
Use of Mass Spec is Complementary to NMR Structural Fingerprinting

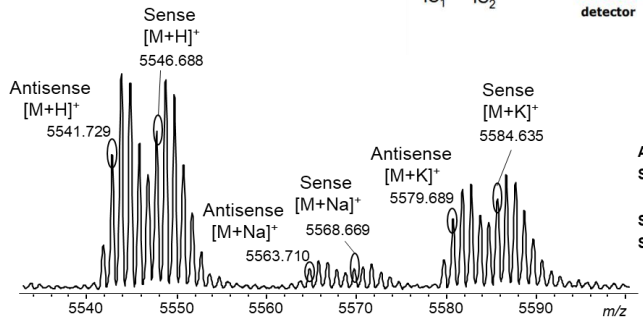


Robert Brinson, Ph.D.
NIST, IBBR

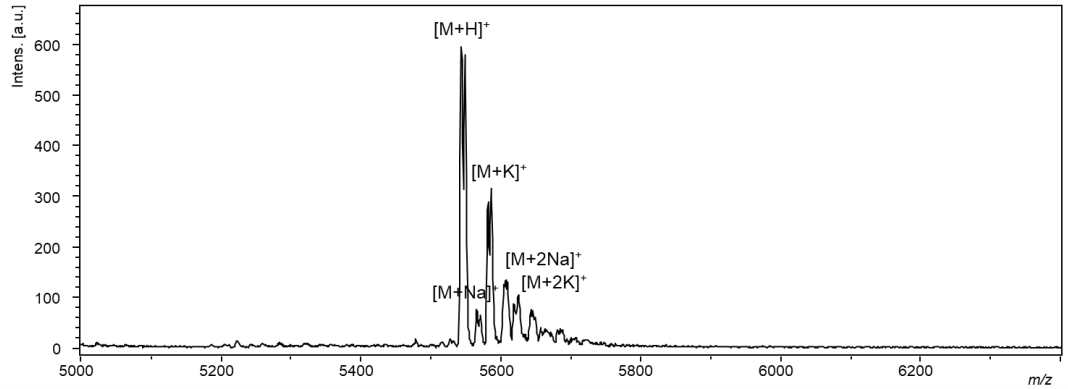
MALDI TOF Mass Spectrometry







	Adduct	Theoretical m/z	Experimental m/z	mass error (mmu)
Antisense Strand	[M+H]⁺	5541.761	5541.729	32.0
	[M+Na]⁺	5563.743	5563.710	32.9
	[M+K]⁺	5579.717	5579.689	27.8
Sense Strand	[M+H]⁺	5546.653	5546.688	34.6
	[M+Na]⁺	5568.635	5568.669	33.6
	[M+K]⁺	5584.609	5584.635	25.7



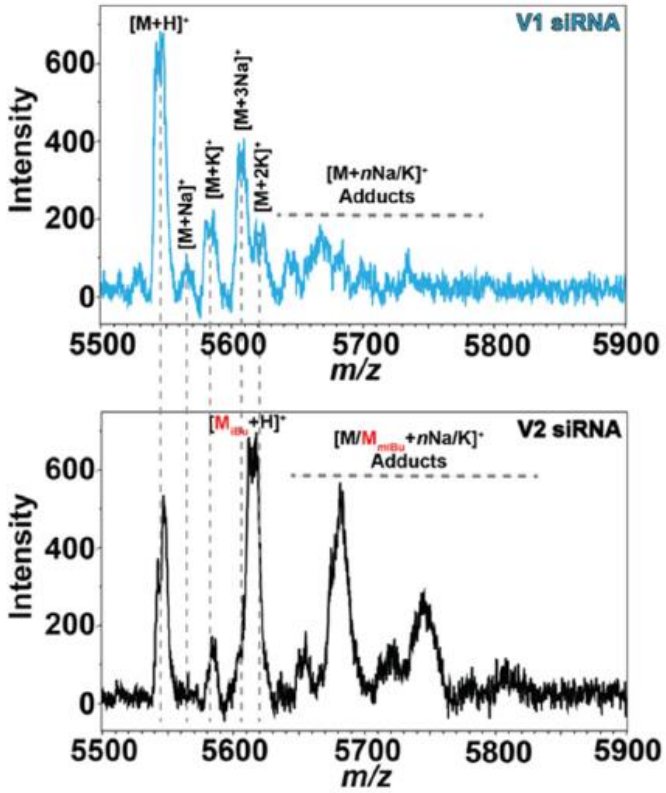
Use of Mass Spec to detect OGN Impurities

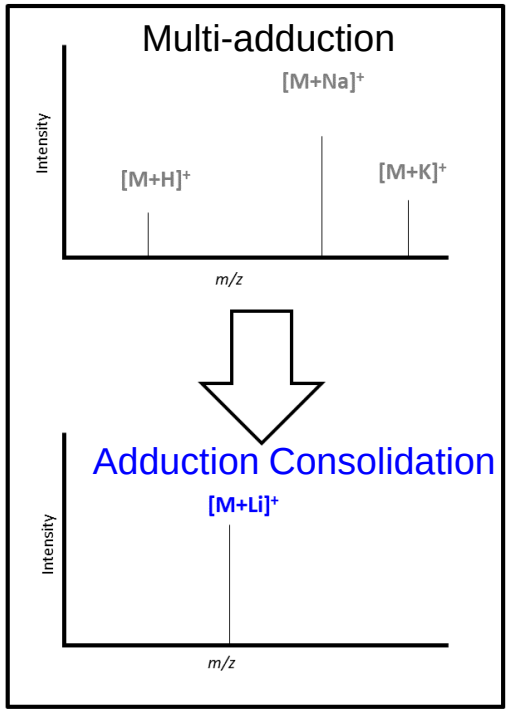
Sense 5′ G_s–C_s–A_f–^{F}U–A_f–G_m–C_f–A_m–C_f–^{F}U–G_f–^{F}U–^{F}U–C_m–G_s–dT_s–dT 3′

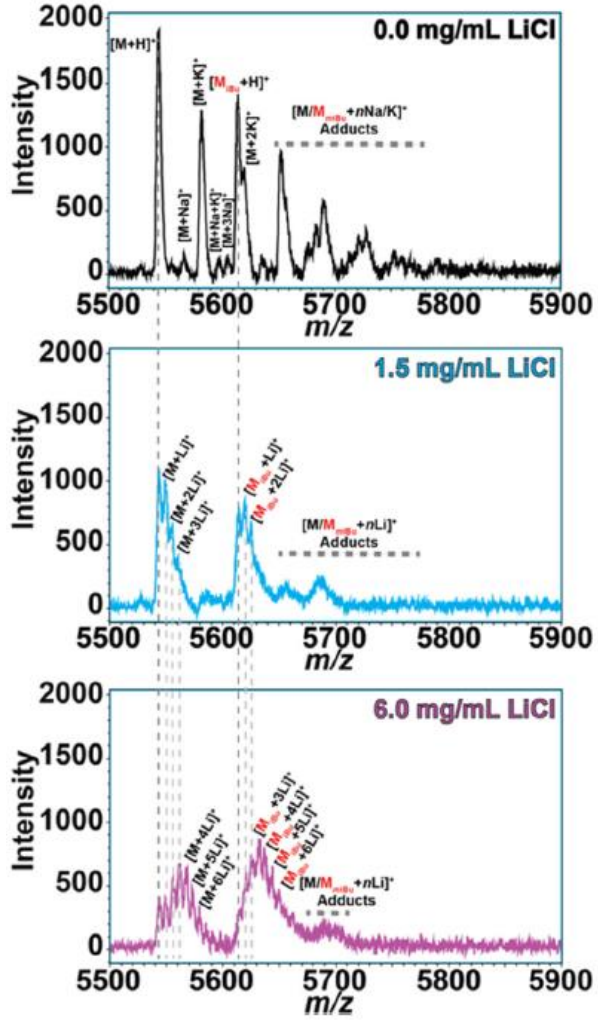
3′dT–dT_s–C_s–G_f–U_m–A_f–U_m–C_f–G_m–U_f–G_m–A_f–C_m–A_f–A_m–G_s–C_s 5′ **Anti-Sense**

A = adenosine C = cytidine G = guanosine U = uridine dT = deoxythymidine

s = phosphorothioate linkage f = 2′-fluoro m = 2′-*O*-methyl F = 5-fluoro







A Tran et al *JASMS*. 2021 Sep 1;32(9):2322-2333.
A Tran et al *JASMS*. 2021 Jan 6;32(1):289-300.

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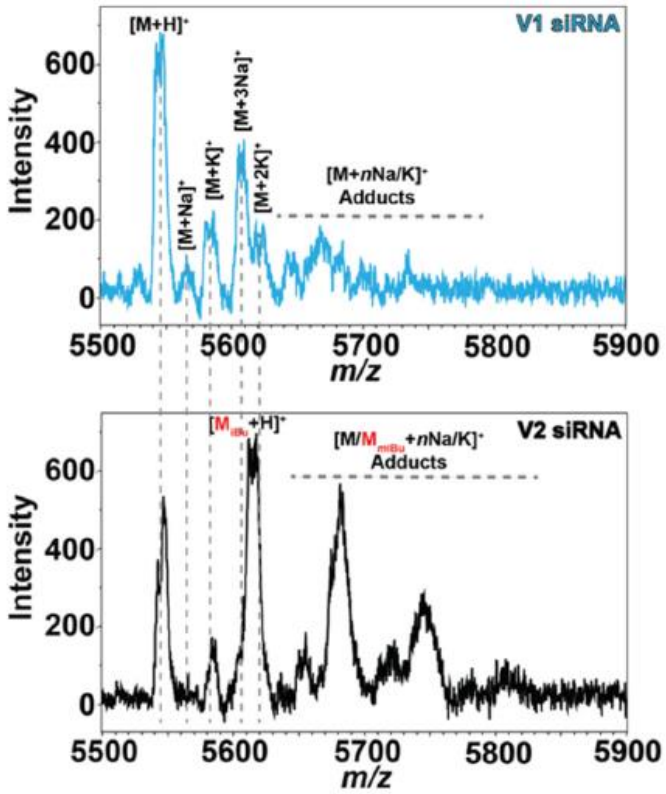
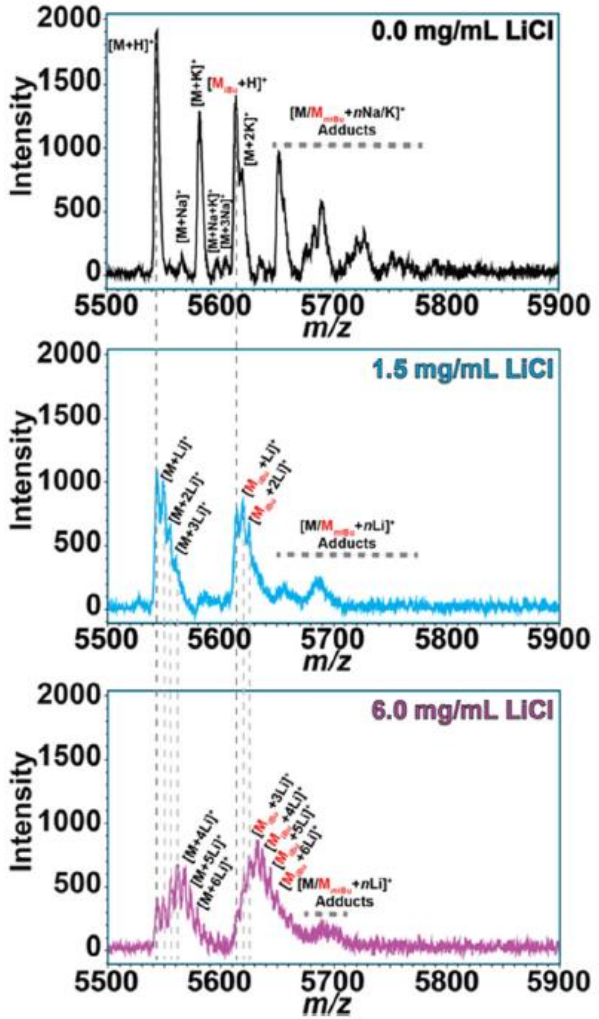
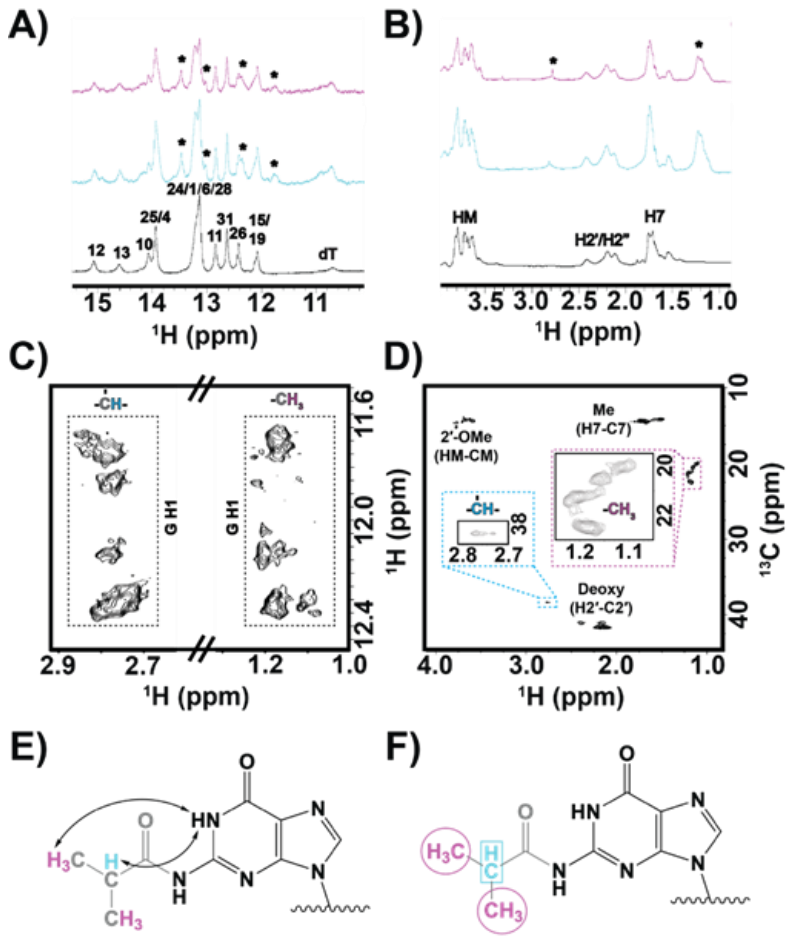
3′dT–dT_s–C_s–G_f–U_m–A_f–U_m–C_f–G_m–U_f–G_m–A_f–C_m–A_f–A_m–G_s–C_s 5′ **Anti-Sense**

A = adenosine C = cytidine G = guanosine U = uridine dT = deoxythymidine

s = phosphorothioate linkage f = 2′-fluoro m = 2′-*O*-methyl F = 5-fluoro

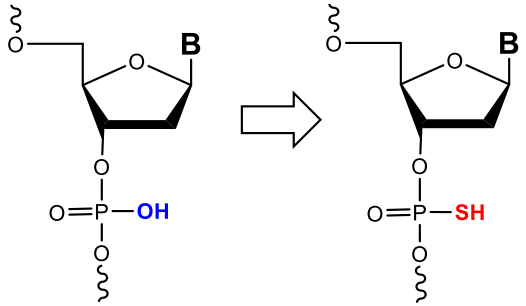


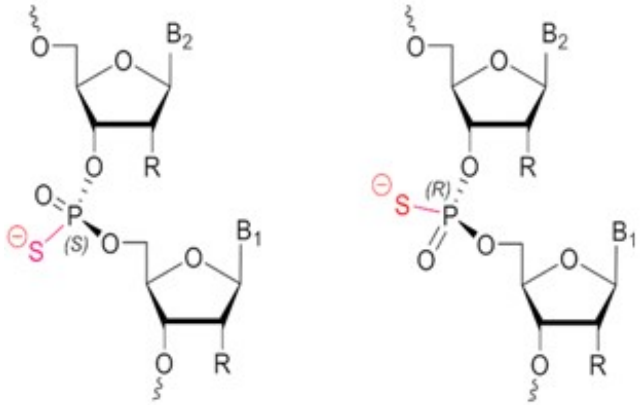




Multidimensional Approach to Characterize the Diastereomers of OGN

Phosphorothioate (PS) linkages





- PS linkage confers nuclease resistance and enhances protein binding
- PS linkage introduces chiral center
- Creates 2^n diastereomers (n is the number of PS linkages)
- A 20-mer potentially yields 2^{19} (524,288) possible stereoisomers
- PS stereoisomer mixtures potentially alter the pharmacological effects
- Adversely impact active ingredient sameness and bioequivalence

Reference Listed Drug (RLD)

- TEGSEDI (inotersen)
  - Used to treat polyneuropathy (nerve disease) of hereditary transthyretin-mediated amyloidosis
  - 20-mer Antisense Oligonucleotide (ASO)
  - FDA approved 2018 (first-in-class medication)

Key Objective:

- To develop a high-resolution analytical method for sensitive and robust determination of diastereomer composition in PS OGNs

Approach

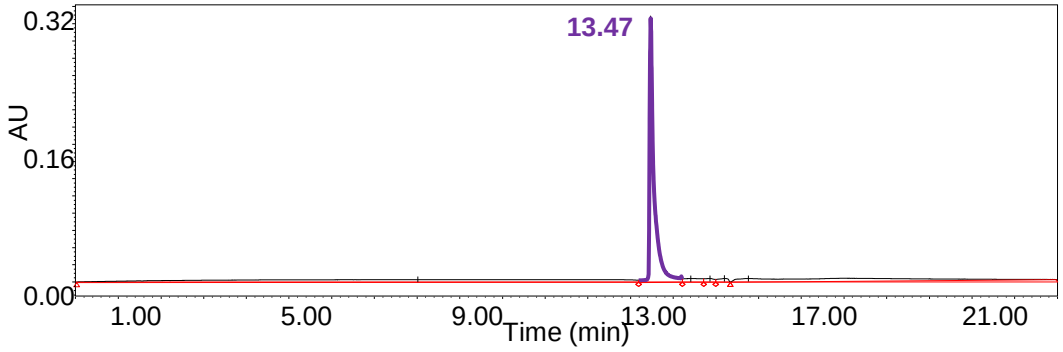
- Chemical synthesis of inotersen using different chemistries and computational integration of LC, MS, and NMR data
- Chemical synthesis (Fletcher Lab)
- Analytical Chemistry (LC, MS, NMR) (Jones and Brinson Labs)
- Computational Integration (Cummings Lab)

Outcome

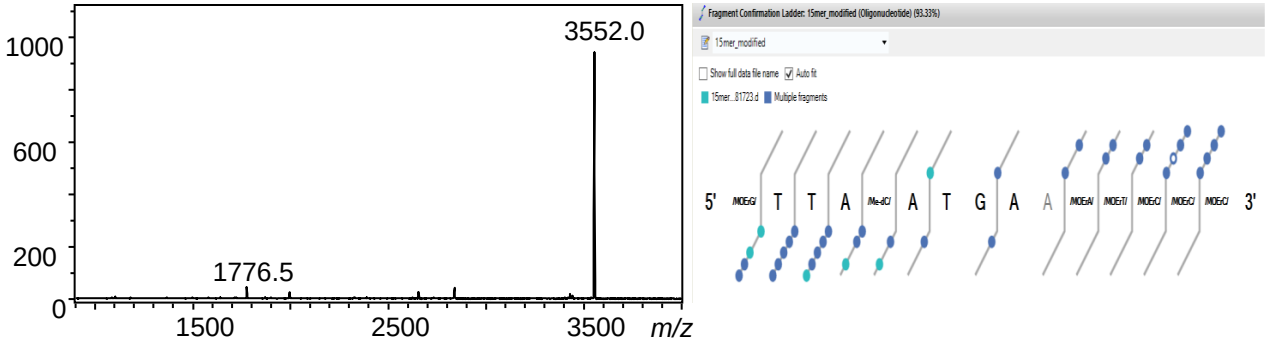
- Establish PS diastereomer composition as a well-defined chemical marker for ASO quality control

Synthesis and Product Characterization

Liquid Chromatography

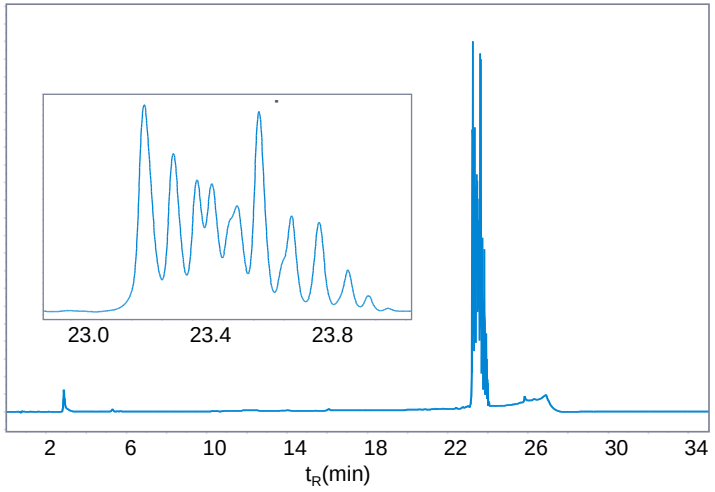


Mass Spectrometry

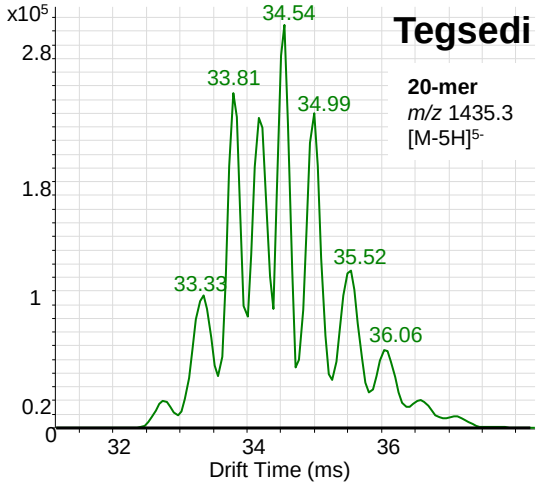


Diastereomer Composition Characterization

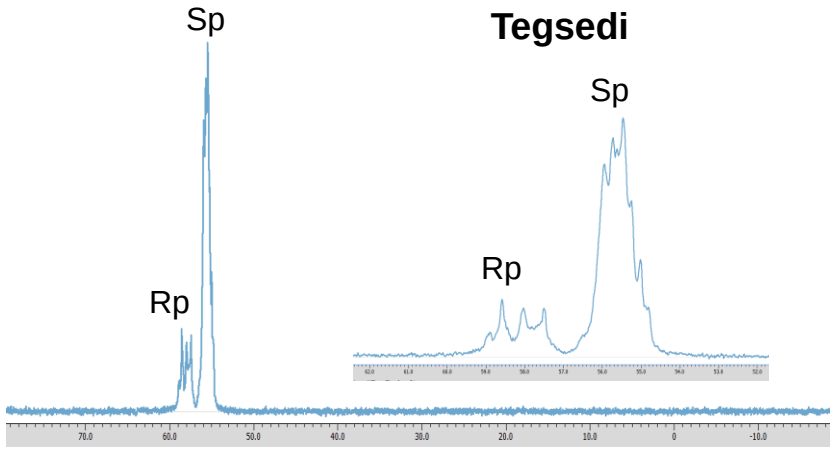
Liquid Chromatography



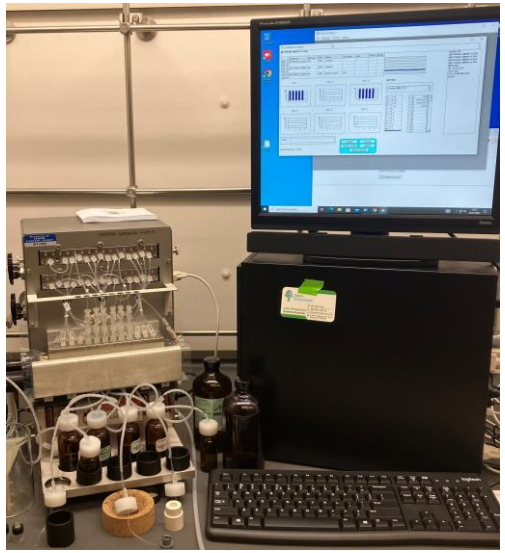
Ion Mobility



^{31}P NMR



Synthesis and Product Characterization



Steven Fletcher, Ph.D.
UMB SOP

Inotersen:

- Synthetic 20-mer ASO, all PS linkages (19)
- 2’-MOE modifications at positions 1–5 and 15–20
- All C modified with 5-methyl group
- T*^{M}C*T*T*G*G*T*T*A*^{M}C*A*T*G*A*A*A*T*^{M}C*^{M}C*^{M}C
- Synthesize in-house to control chemistry (choice of activator)
- n=3 per sequence

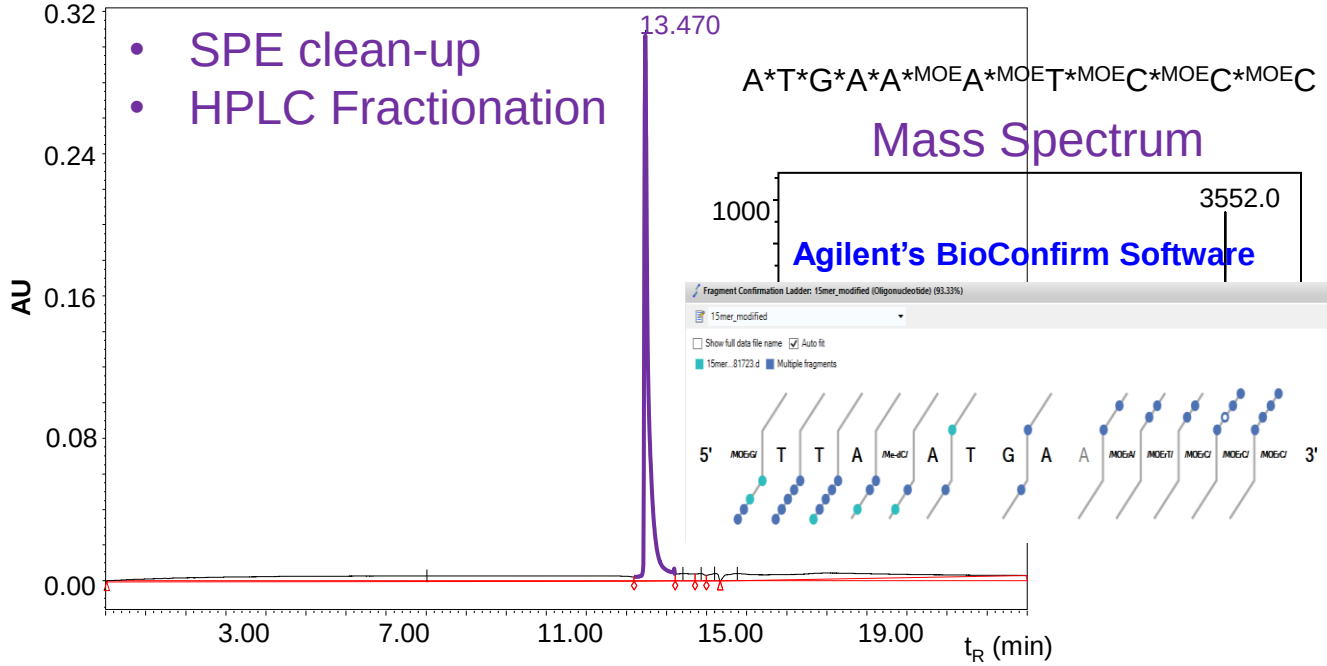
Sequence #	Oligo length	Activator	# of PS linkages	# of potential Diastereomers
Sequence 1	2-mer	DCI	1	2
	5-mer		4	16
	10-mer		9	512
	15-mer		14	16384
	20-mer		19	524288
Sequence 2	2-mer	ETT	1	2
	5-mer		4	16
	10-mer		9	512
	15-mer		14	16384
	20-mer		19	524288

Note, additional sequences including RLD and other sequences were purchased from vendors.

DCI: 4,5-dicyanoimidazole
ETT: 5-(ethylthio)-1H-tetrazole

In-house Synthesis

- SPE clean-up
- HPLC Fractionation



Liquid Chromatography: Analytical Separations

Collaboration with Agilent (Graham Robinett)

- 1290 Infinity Bio LC System

CC)

graphy (HILIC)

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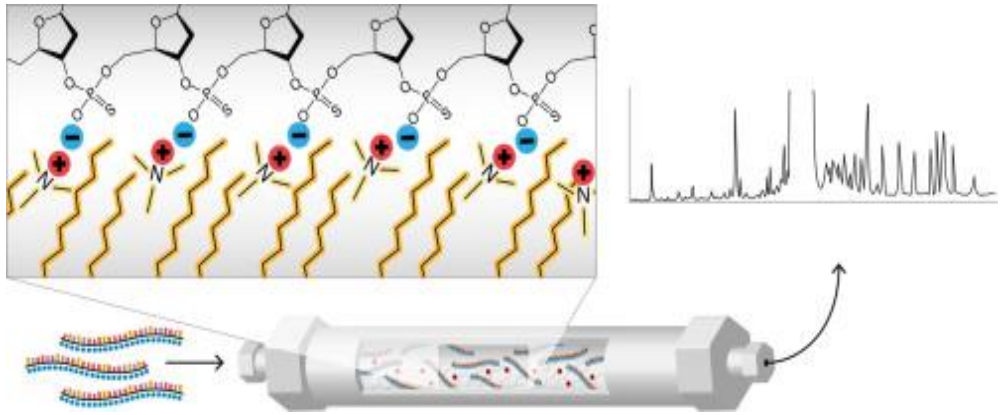
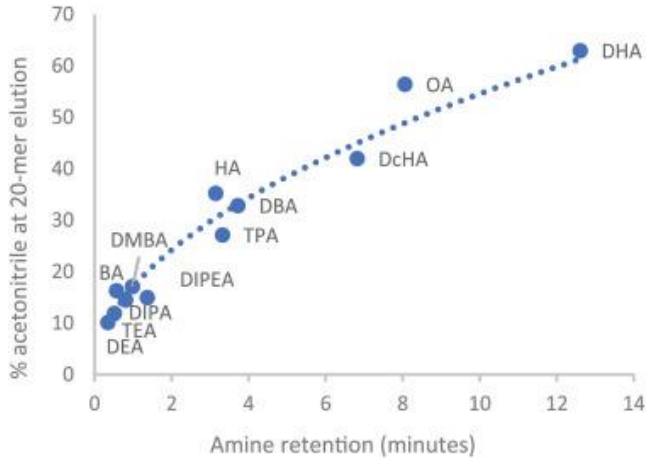
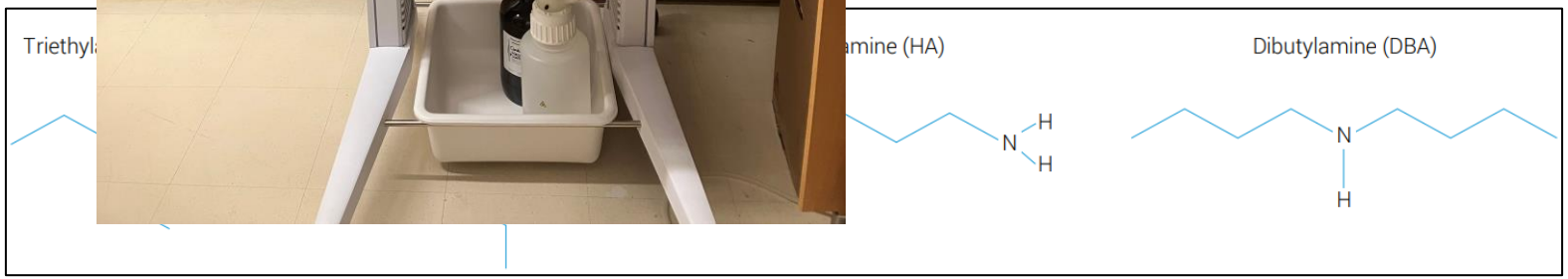


OGNs interact with the stationary phase on which ion-pairing reagents are adsorbed and result is chromatographic separation

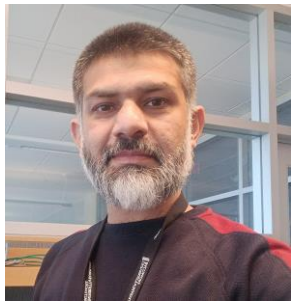


Correlation between alkyl amine retention time and the concentration of acetonitrile (20-mer)



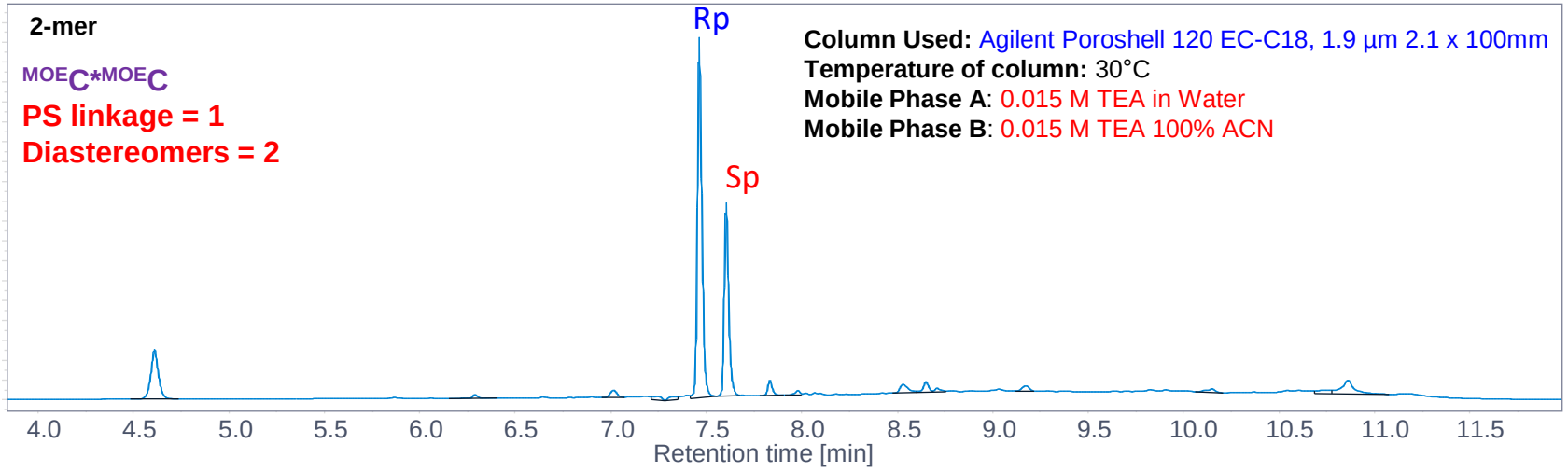
Journal of Chromatography Open 3 (2023) 100079

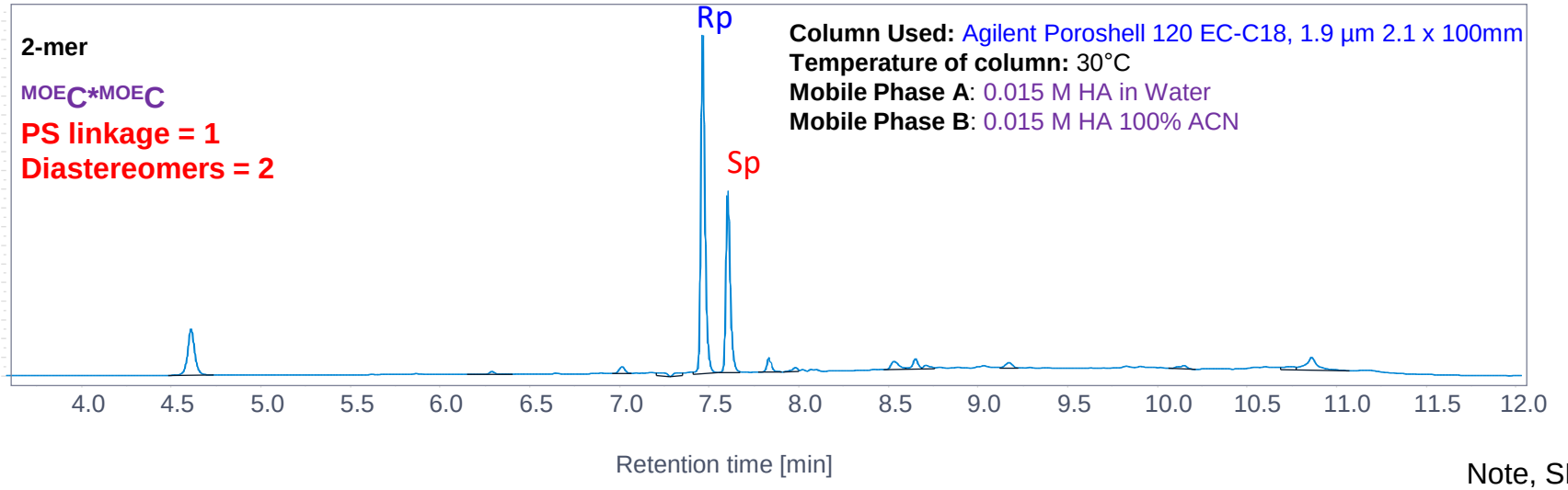
In-house 2-mer IP-RP chromatography



Mohsin Ali, Ph.D.

Padmapriya Sridhar





Time [min]	A [%]	B [%]	Flow [mL/min]	Max. Pressure Limit [bar]
0.00	95.00	5.00	0.400	1300.00
2.00	95.00	5.00	0.400	---
7.00	75.00	25.00	0.400	---
10.00	60.00	40.00	0.400	---
11.00	95.00	5.00	0.400	---
13.00	95.00	5.00	0.400	---

Note, SPE clean-up includes TFA to cleave DMT group

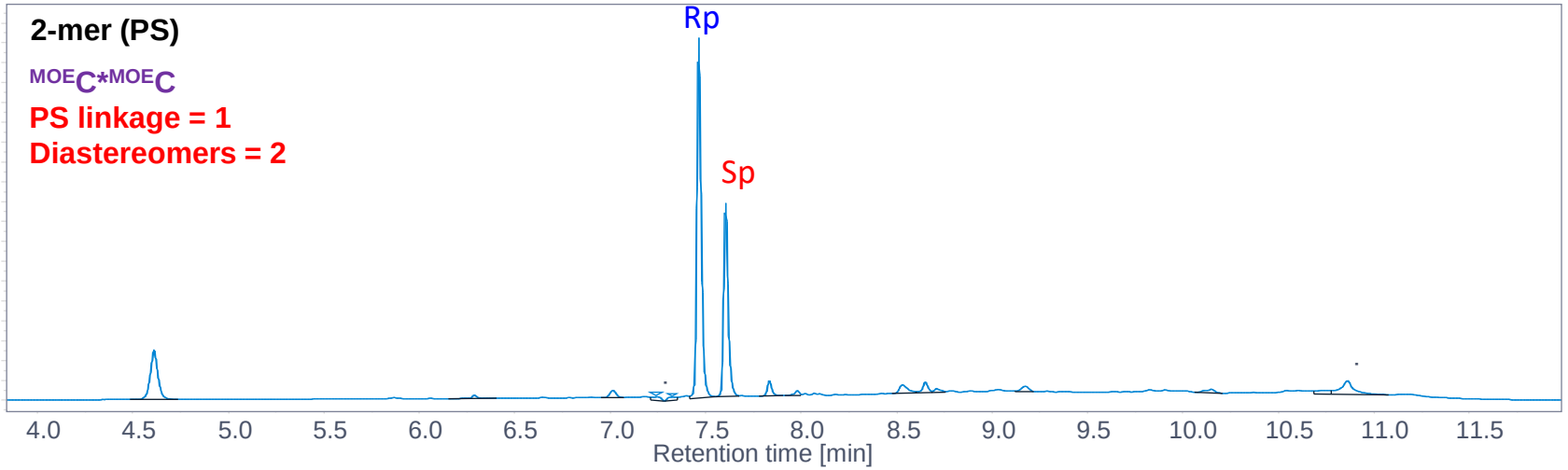
In-house 2-mer IP-RP chromatography: Comparison of PO and PS linkages

Column Used: Agilent Poroshell 120 EC-C18, 1.9 µm 2.1 x 100mm
Temperature of column: 30°C
Mobile Phase A: 0.015 M TEA in Water
Mobile Phase B: 0.015 M TEA 100% ACN

2-mer (PS)

$^{MOE}C^{*MOE}C$

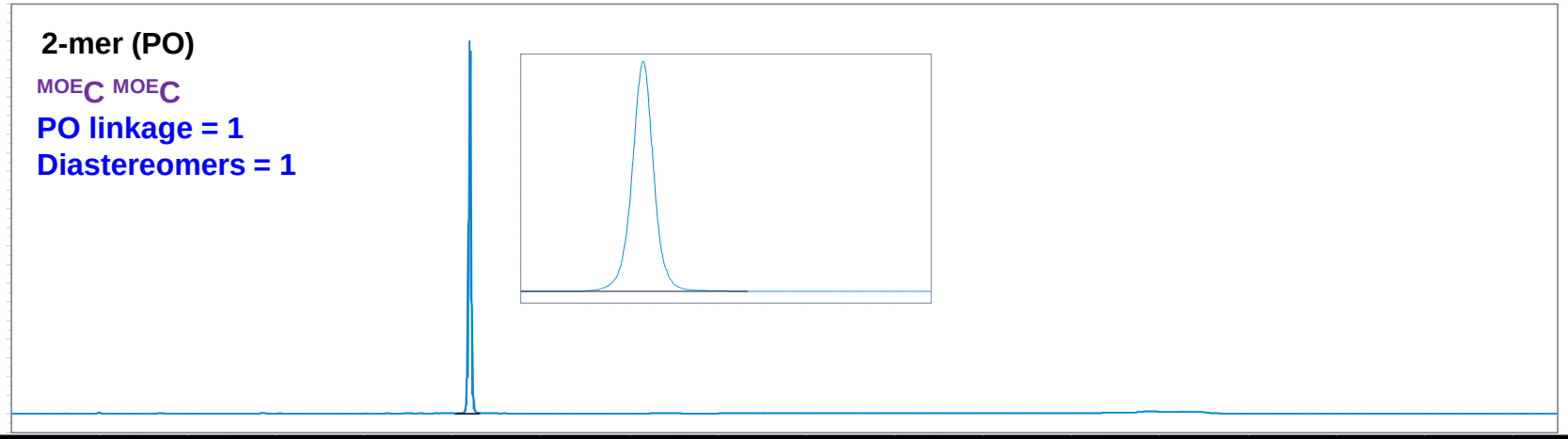
PS linkage = 1
Diastereomers = 2



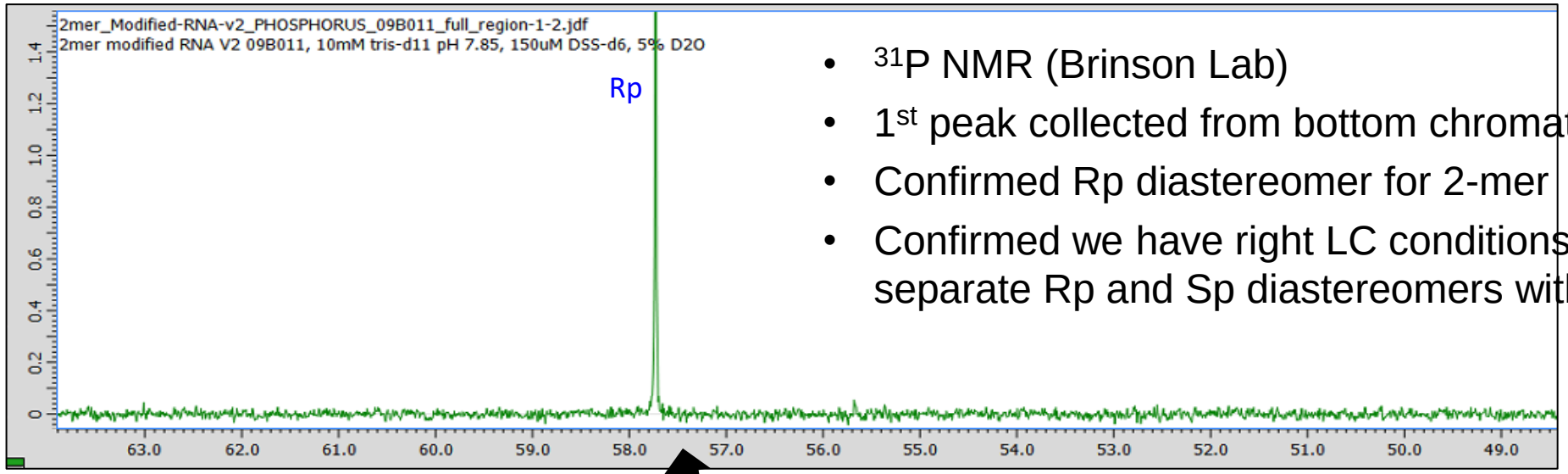
2-mer (PO)

$^{MOE}C\ ^{MOE}C$

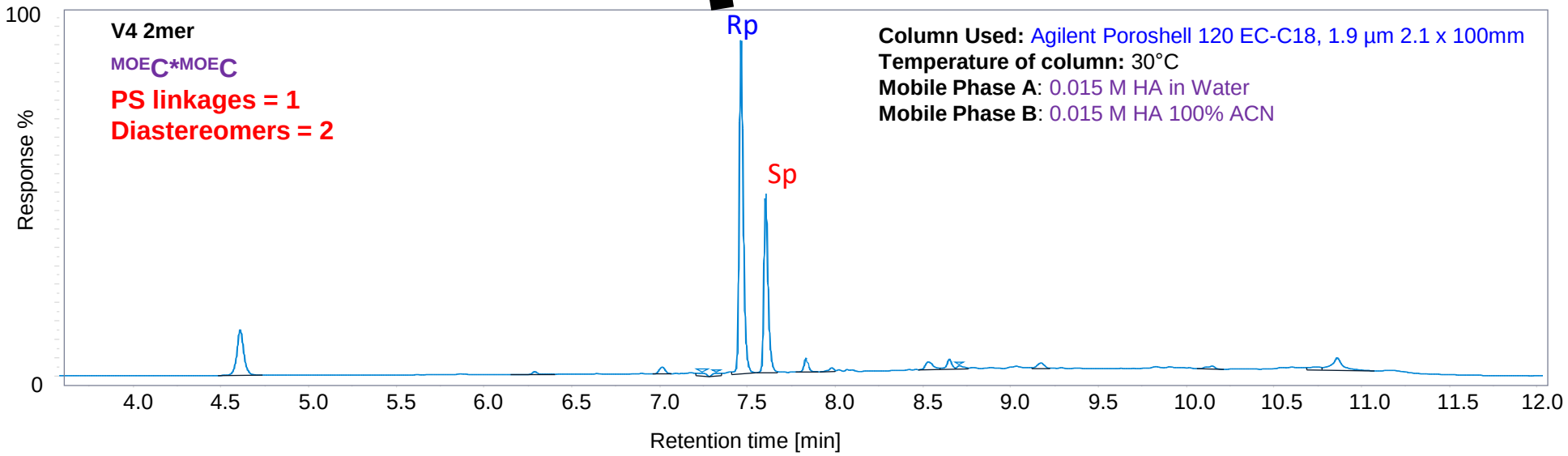
PO linkage = 1
Diastereomers = 1



IP-RP Diastereomer Separation and NMR Confirmation

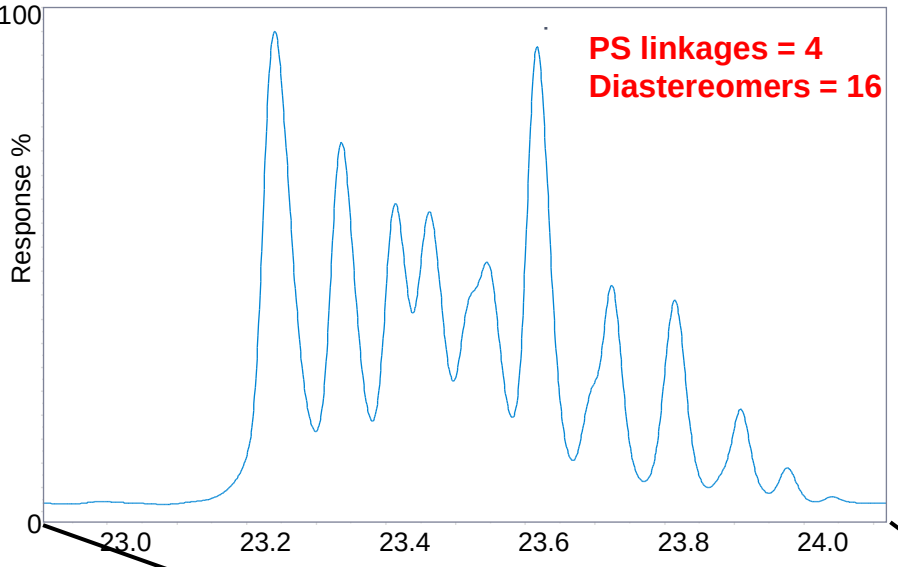


- ^{31}P NMR (Brinson Lab)
- 1st peak collected from bottom chromatogram
- Confirmed Rp diastereomer for 2-mer
- Confirmed we have right LC conditions to separate Rp and Sp diastereomers without DMT

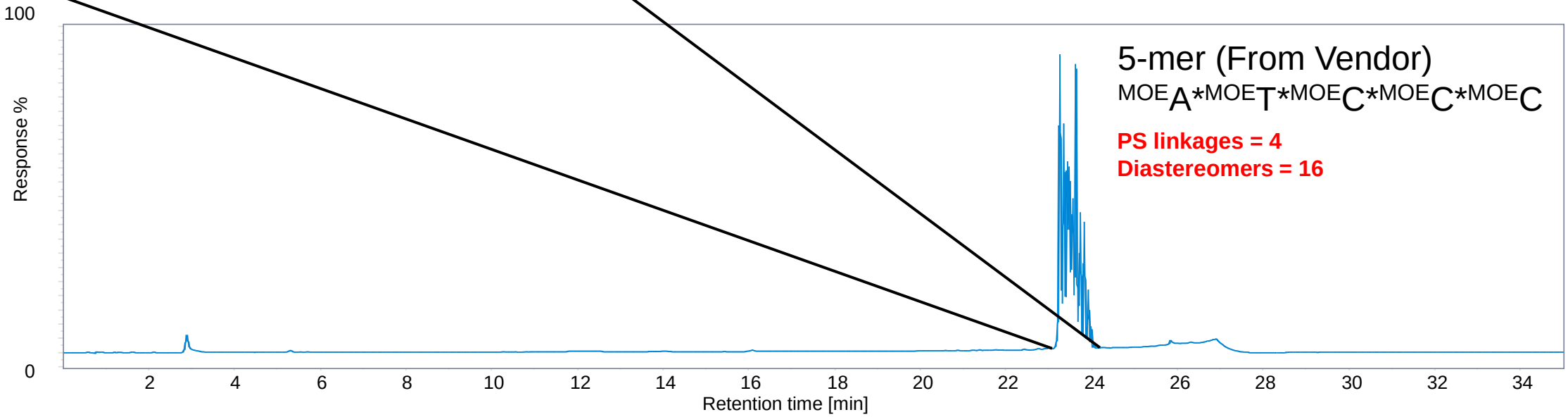


IP-RP separation of 5-mer

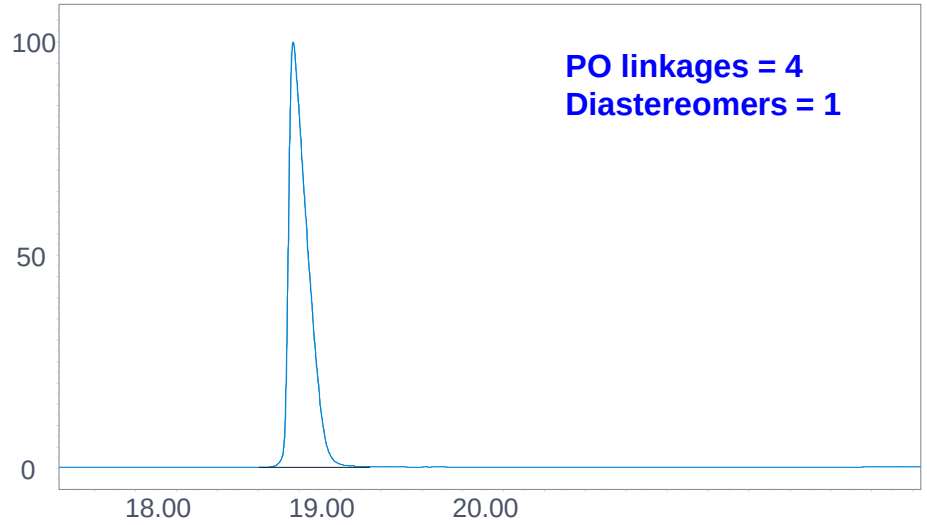
Time [min]	A [%]	B [%]	Flow [mL/min]	Max. Pressure Limit [bar]
0.00	95.00	5.00	0.400	1300.00
2.00	95.00	5.00	0.400	---
22.00	75.00	25.00	0.400	---
26.00	60.00	40.00	0.400	---
27.00	95.00	5.00	0.400	---
35.00	95.00	5.00	0.400	---

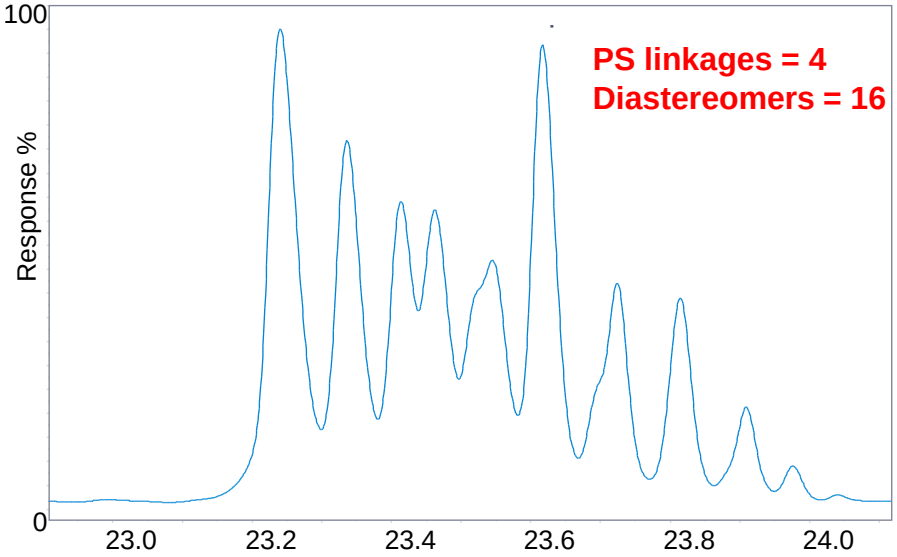


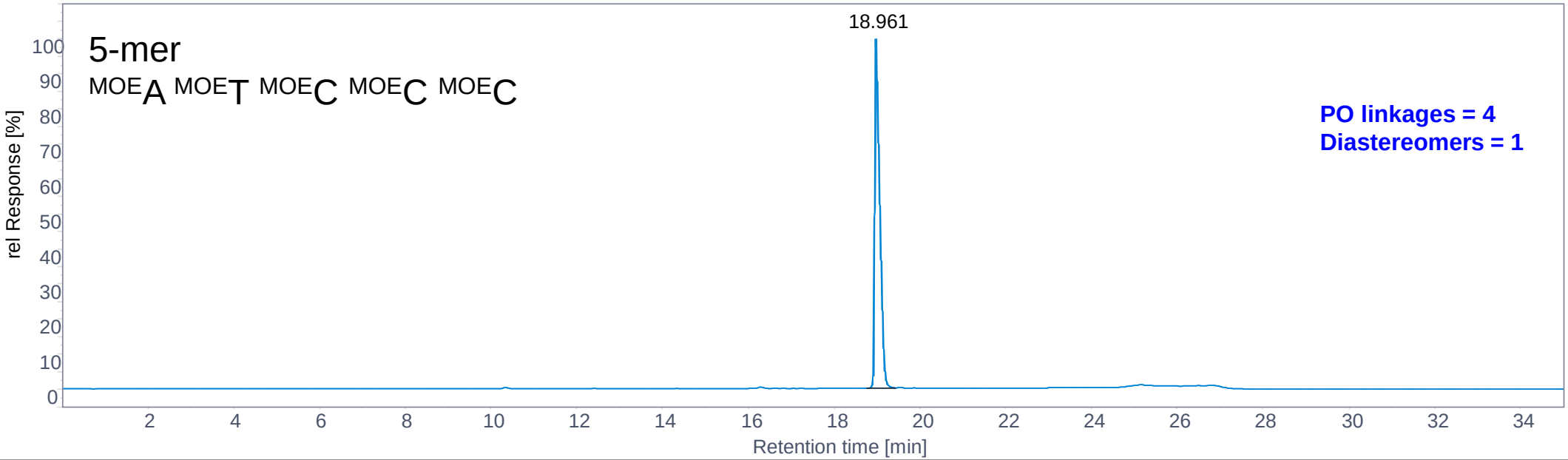
Column: Agilent Poroshell 120 EC-C18, 1.9 µm 2.1 x 100mm
Temperature = 30°C
Mobile Phase A = 0.015 M HA in Water
Mobile Phase B = 0.015 M HA in ACN



IP-RP chromatography of 5-mer: comparison of PO and PS



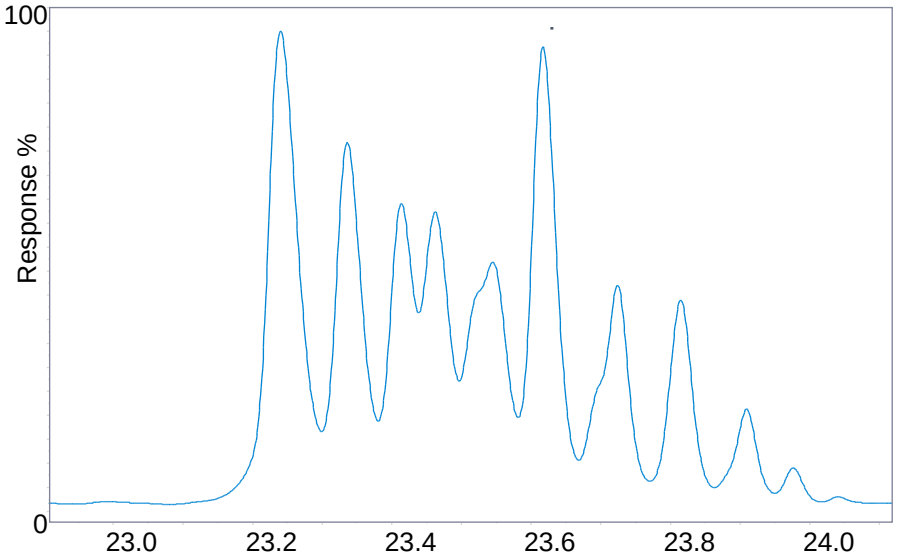




5-mer: Diastereomer Evaluation via IP-RP and NMR

Robert Brinson, Ph.D.
NIST, IBBR

IP-RP Chromatogram



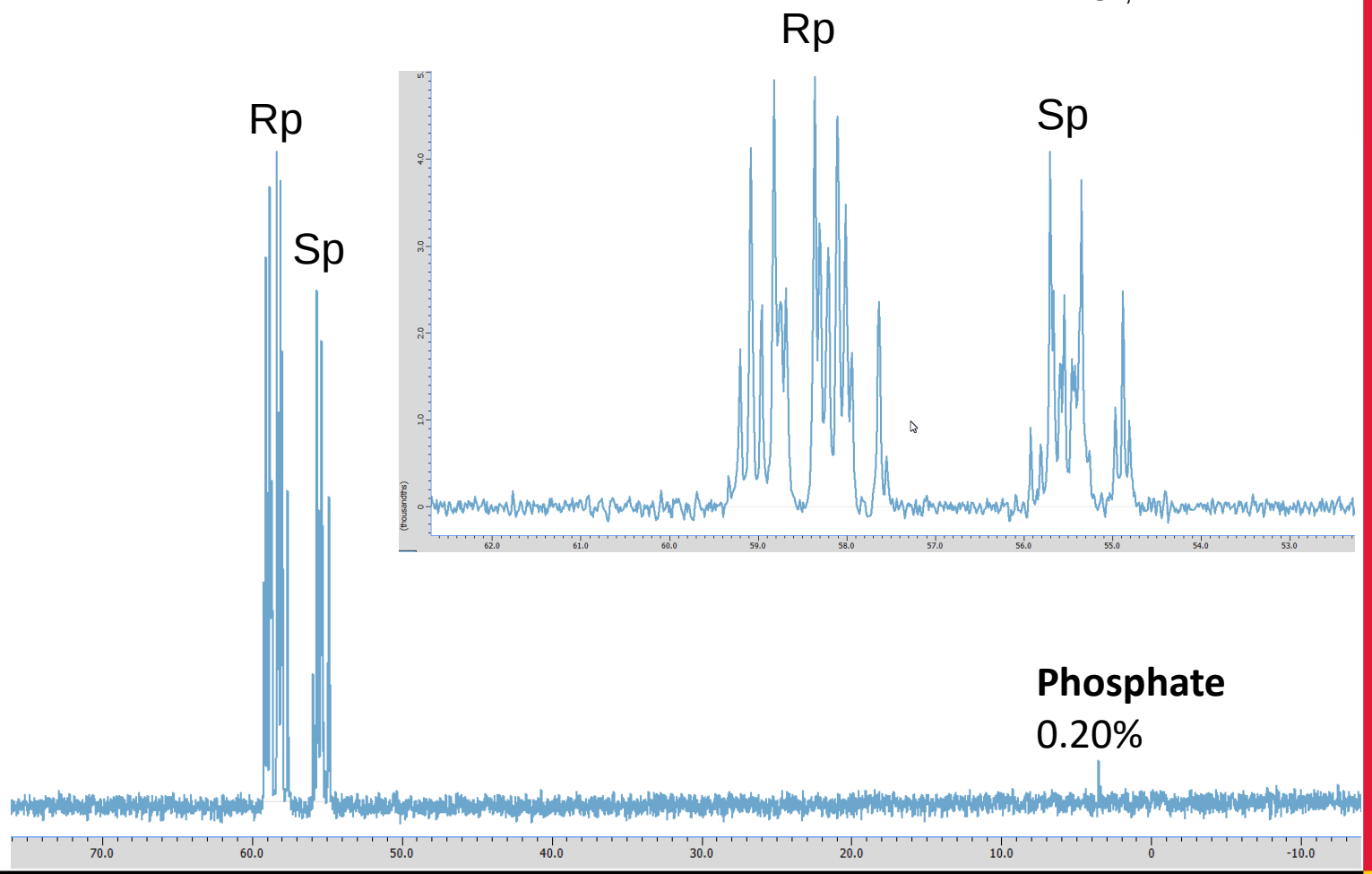
5-mer (From Vendor)

$^{MOE}A*^{MOE}T*^{MOE}C*^{MOE}C*^{MOE}C$

PS linkages = 4

Diastereomers = 16

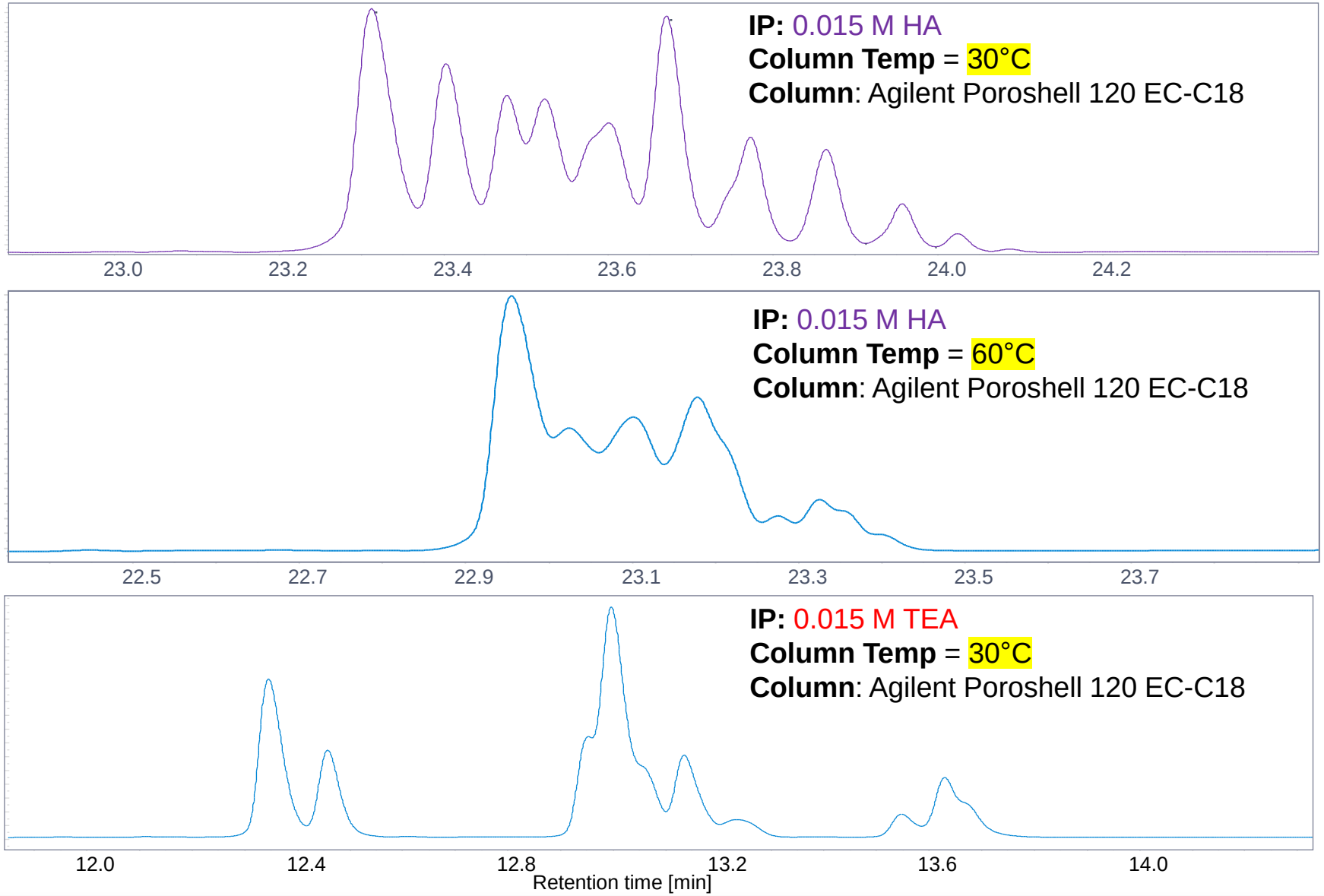
^{31}P NMR



Phosphate

0.20%

Ion Pair Reagent and Temperature Effect Chromatographic Separation



5-mer (Vendor)

$^{MOE}A*^{MOE}T*^{MOE}C*^{MOE}C*^{MOE}C$

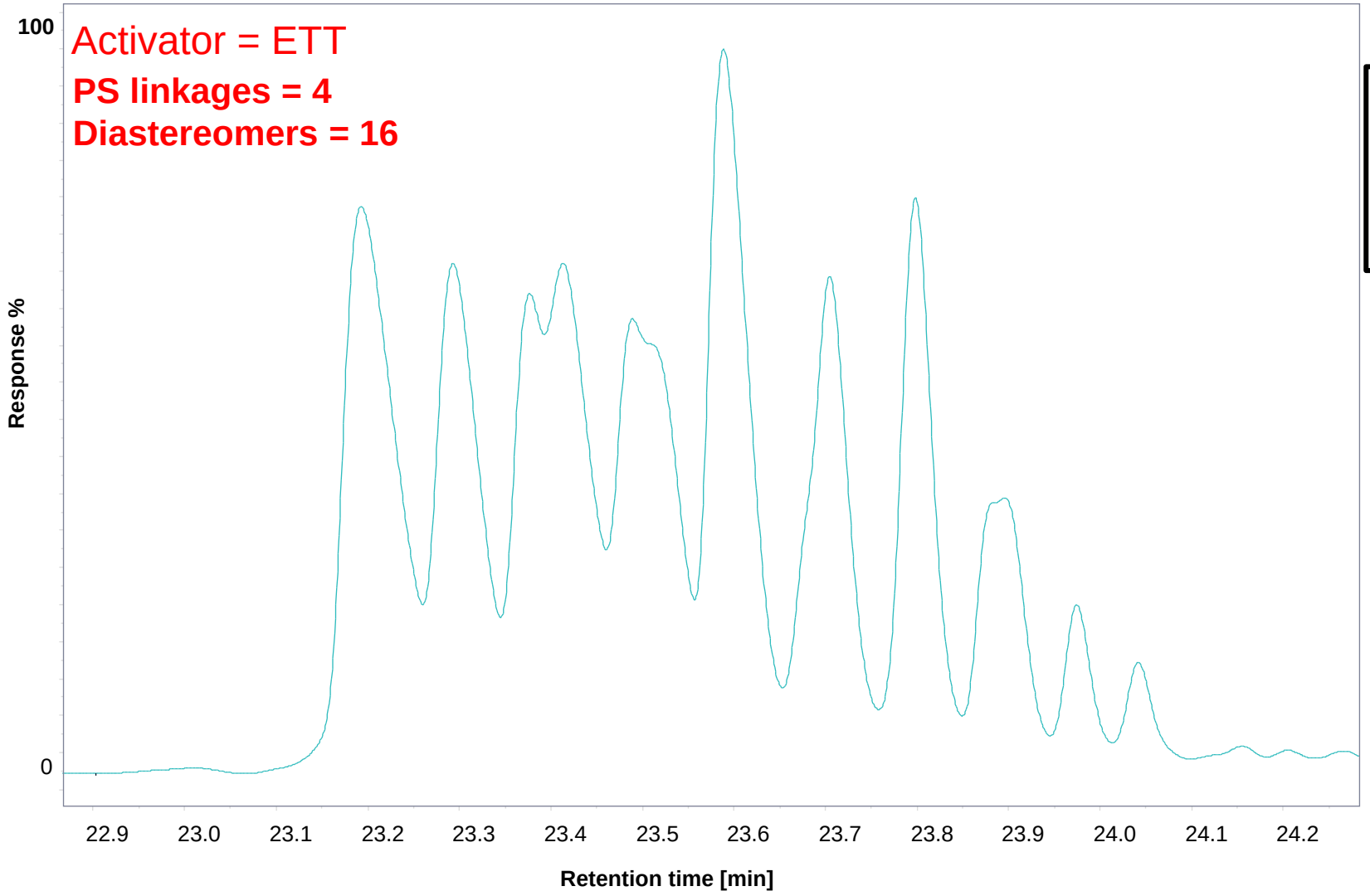
PS linkages = 4

Diastereomers = 16

IP RP of 5-mer (In-house synthesis, ETT)

5-mer (in-house ETT)

$^{MOE}A*^{MOE}T*^{MOE}C*^{MOE}C*^{MOE}C$



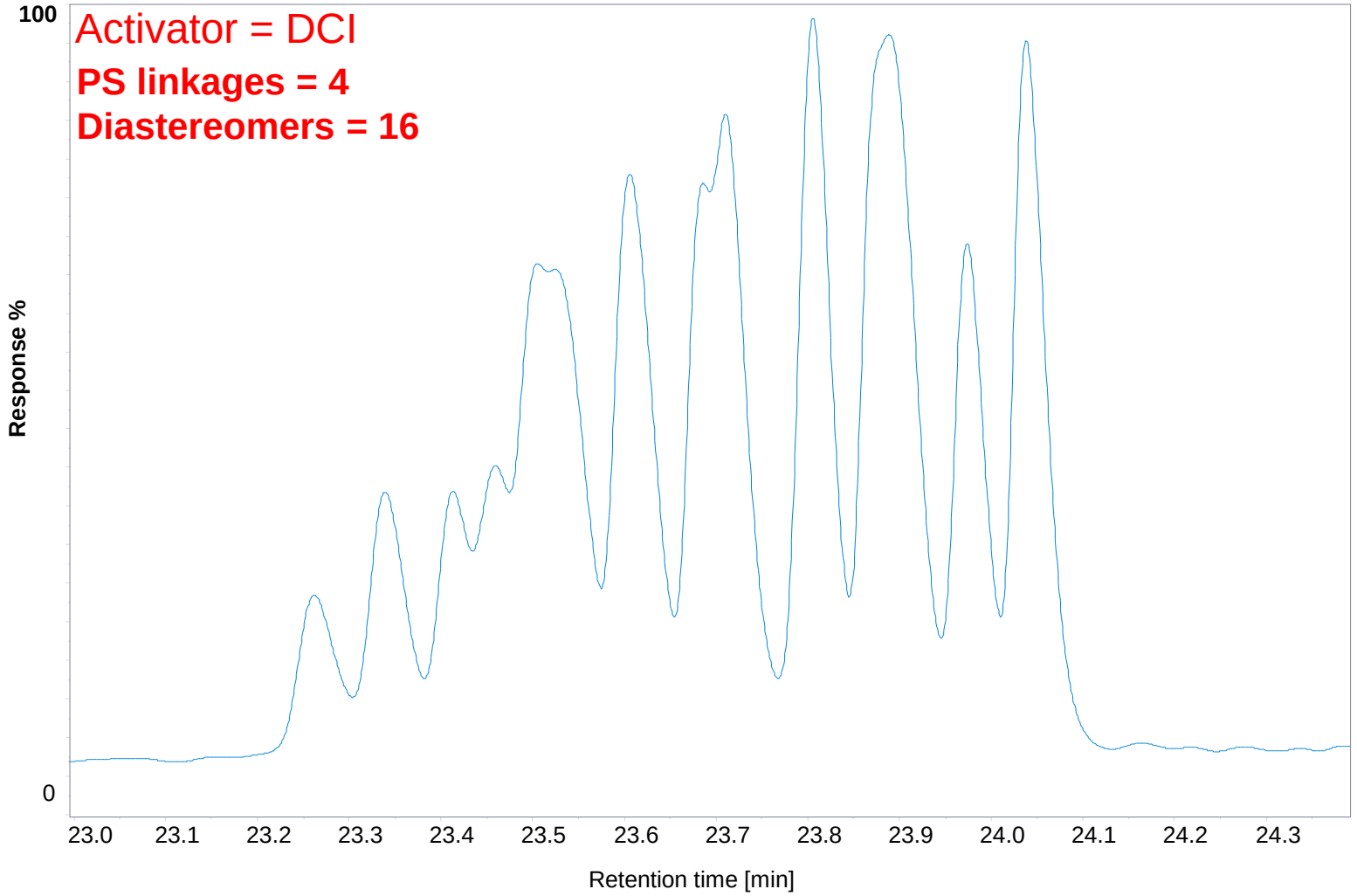
Column: Agilent Poroshell 120 EC-C18 1.9 µm 2.1 x 100mm
Temperature = 30°C
Mobile Phase A = 0.015 M HA in Water
Mobile Phase B = 0.015 M HA in ACN

Time [min]	A [%]	B [%]	Flow [mL/min]	Max. Pressure Limit [bar]
0.00	95.00	5.00	0.400	1300.00
2.00	95.00	5.00	0.400	---
22.00	75.00	25.00	0.400	---
26.00	60.00	40.00	0.400	---
27.00	95.00	5.00	0.400	---
35.00	95.00	5.00	0.400	---

IP RP of 5-mer (In-house synthesis, DCI)

5-mer (in-house DCI)

$^{MOE}A*^{MOE}T*^{MOE}C*^{MOE}C*^{MOE}C$



Column: Agilent Poroshell 120 EC-C18 1.9 µm 2.1 x 100mm
Temperature = 30°C
Mobile Phase A = 0.015 M HA in Water
Mobile Phase B = 0.015 M HA in ACN

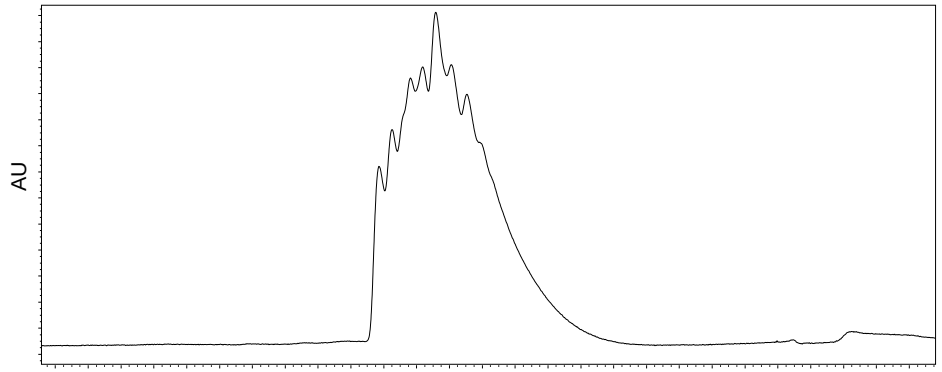
Time [min]	A [%]	B [%]	Flow [mL/min]	Max. Pressure Limit [bar]
0.00	95.00	5.00	0.400	1300.00
2.00	95.00	5.00	0.400	---
22.00	75.00	25.00	0.400	---
26.00	60.00	40.00	0.400	---
27.00	95.00	5.00	0.400	---
35.00	95.00	5.00	0.400	---

IP-RP separation of 5-mer on different UHPLC under same conditions

MOE A*MOE T*MOE C*MOE C*MOE C

PS linkages = 4

Diastereomers = 16



Column: Agilent Poroshell 120 EC-C18 1.9 µm 2.1 x 100mm

Temperature = 30°C

Mobile Phase A = 0.015 M HA in Water

Mobile Phase B = 0.015 M HA in ACN

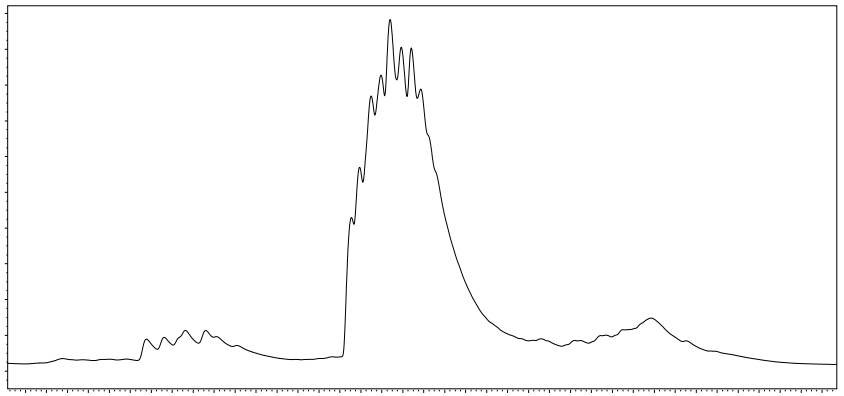
Gradient

	Time	Flow (mL/min)	%A	%B	%C	%D	Curve
1	Initial	0.400	95.0	5.0	0.0	0.0	Initial
2	2.00	0.400	95.0	5.0	0.0	0.0	6
3	22.00	0.400	75.0	25.0	0.0	0.0	6
4	26.00	0.400	60.0	40.0	0.0	0.0	6
5	27.00	0.400	95.0	5.0	0.0	0.0	6
6	35.00	0.400	95.0	5.0	0.0	0.0	6

IP-RP separation of 5-mer (ETT) on different UHPLC under same conditions

MOE A*MOE T*MOE C*MOE C*MOE C

PS linkages = 4
Diastereomers = 16



Gradient

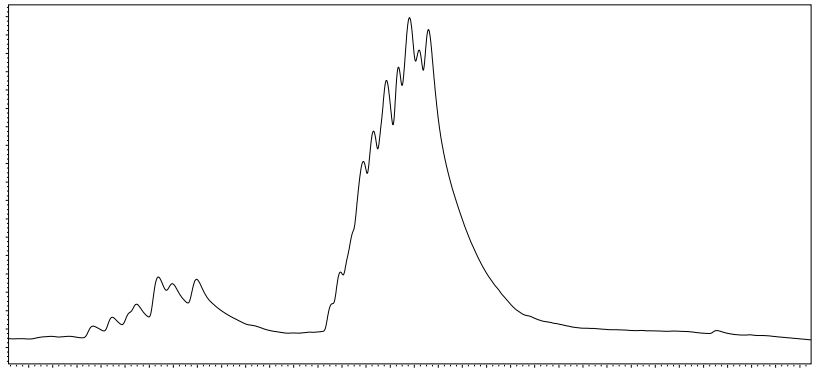
	Time	Flow (mL/min)	%A	%B	%C	%D	Curve
1	Initial	0.400	95.0	5.0	0.0	0.0	Initial
2	2.00	0.400	95.0	5.0	0.0	0.0	6
3	22.00	0.400	75.0	25.0	0.0	0.0	6
4	26.00	0.400	60.0	40.0	0.0	0.0	6
5	27.00	0.400	95.0	5.0	0.0	0.0	6
6	35.00	0.400	95.0	5.0	0.0	0.0	6

Column: Agilent Poroshell 120 EC-C18
1.9 µm 2.1 x 100mm
Temperature = 30°C
Mobile Phase A = 0.015 M HA in Water
Mobile Phase B = 0.015 M HA in ACN

IP-RP separation of 5-mer (DCI) on different UHPLC under same conditions

$^{MOE}A^{*MOE}T^{*MOE}C^{*MOE}C^{*MOE}C$

PS linkages = 4
Diastereomers = 16



Column: Agilent Poroshell 120 EC-C18 1.9 µm 2.1 x 100mm
Temperature = 30°C
Mobile Phase A = 0.015 M HA in Water
Mobile Phase B = 0.015 M HA in ACN

Gradient

	Time	Flow (mL/min)	%A	%B	%C	%D	Curve
1	Initial	0.400	95.0	5.0	0.0	0.0	Initial
2	2.00	0.400	95.0	5.0	0.0	0.0	6
3	22.00	0.400	75.0	25.0	0.0	0.0	6
4	26.00	0.400	60.0	40.0	0.0	0.0	6
5	27.00	0.400	95.0	5.0	0.0	0.0	6
6	35.00	0.400	95.0	5.0	0.0	0.0	6

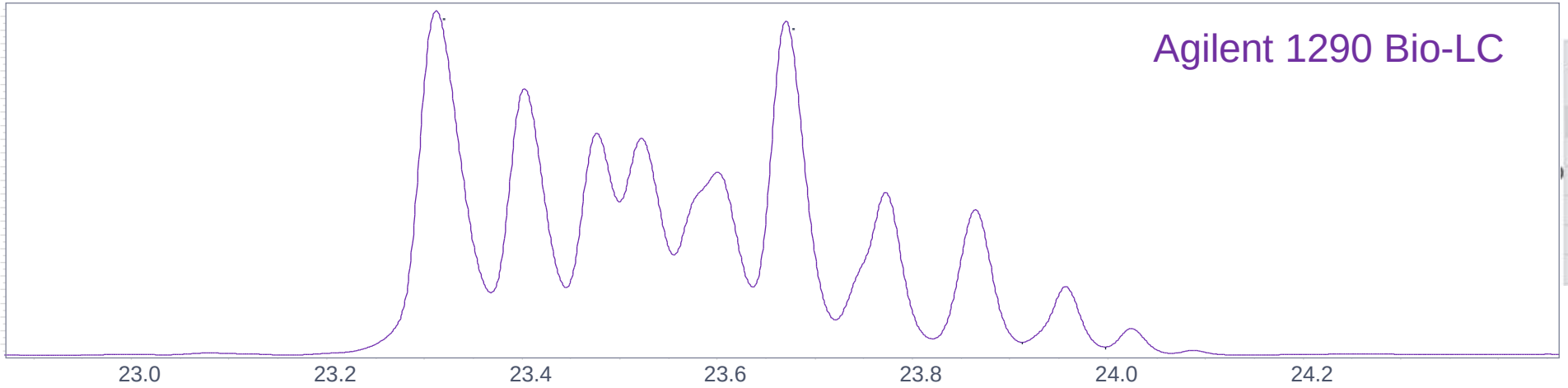
AU
0.32
0.24
0.16
0.08
0.00
0.00 4.00 8.00 12.00 16.00 20.00 24.00 28.00 32.00
Minutes

5-mer comparison using Agilent 1290 Bio-LC vs Another LC

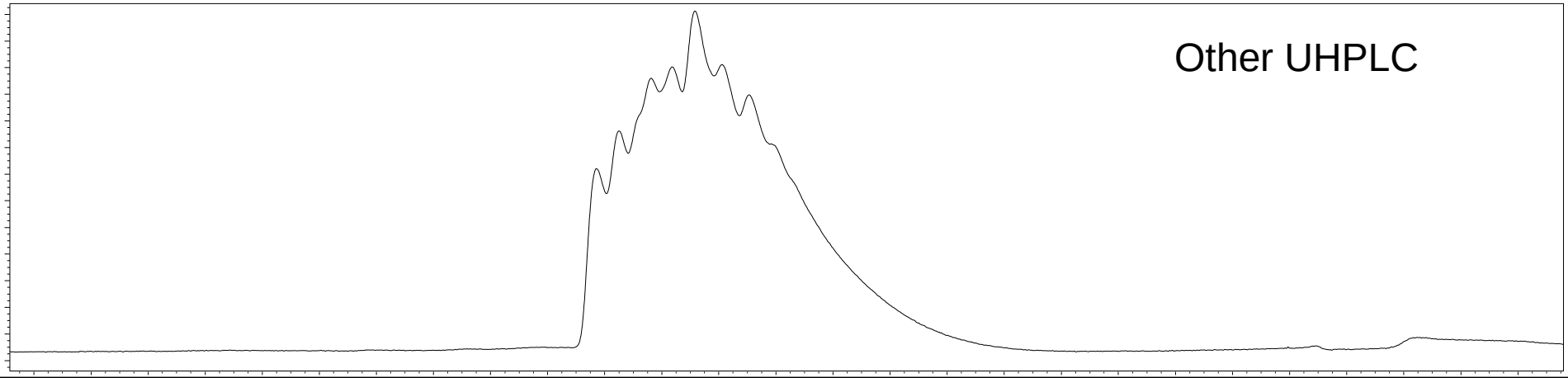
$^{MOE}A*^{MOE}T*^{MOE}C*^{MOE}C*^{MOE}C$

PS linkages = 4
Diastereomers = 16

Column: Agilent Poroshell 120 EC-C18
Temperature = 30°C
Mobile Phase A = 0.015 M HA in Water
Mobile Phase B = 0.015 M HA in ACN



Time [min]	A [%]	B [%]	Flow [mL/min]	Max. Pressure Limit [bar]
0.00	95.00	5.00	0.400	1300.00
2.00	95.00	5.00	0.400	---
22.00	75.00	25.00	0.400	---
26.00	60.00	40.00	0.400	---
27.00	95.00	5.00	0.400	---
35.00	95.00	5.00	0.400	---



Gradient

	Time	Flow (mL/min)	%A	%B	%C	%D	Curve
1	Initial	0.400	95.0	5.0	0.0	0.0	Initial
2	2.00	0.400	95.0	5.0	0.0	0.0	6
3	22.00	0.400	75.0	25.0	0.0	0.0	6
4	26.00	0.400	60.0	40.0	0.0	0.0	6
5	27.00	0.400	95.0	5.0	0.0	0.0	6
6	35.00	0.400	95.0	5.0	0.0	0.0	6

IP RP Had Unique Chromatographic Profiles for Different Activators

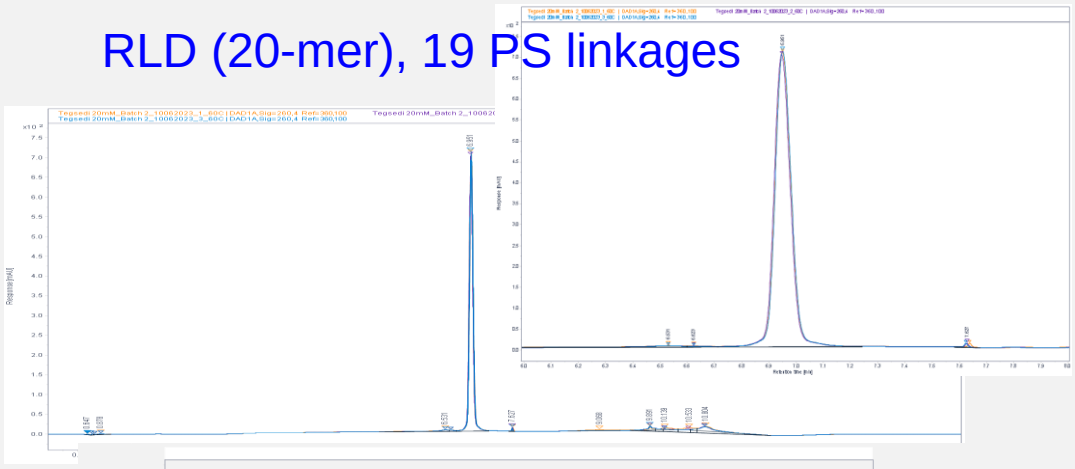
In-house synthesized 5-mer (ETT)
Activator = ETT

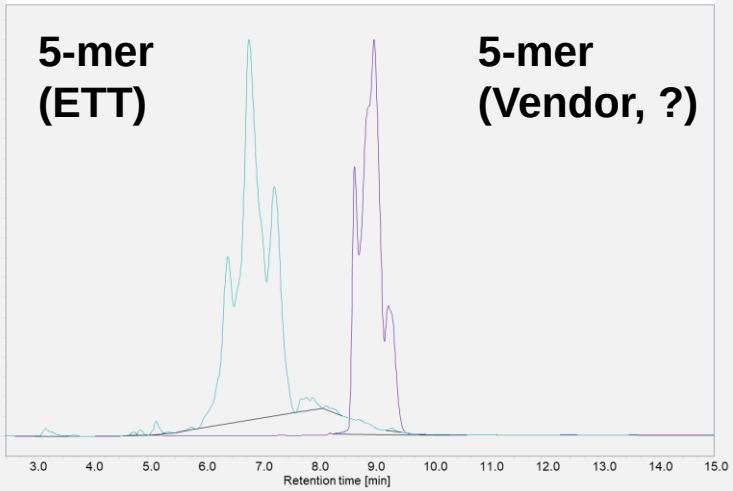
Next steps:

- Continued IP-RP separation of other length oligos and RLD
- Use of other LC separations (MICC, SAX, HILIC)
- Work with the Cummings Lab to develop statistical/computational models to comprehensively evaluate and visualize diastereomer composition

Michael P. Cummings, Ph.D.
University of Maryland

RLD (20-mer), 19 PS linkages

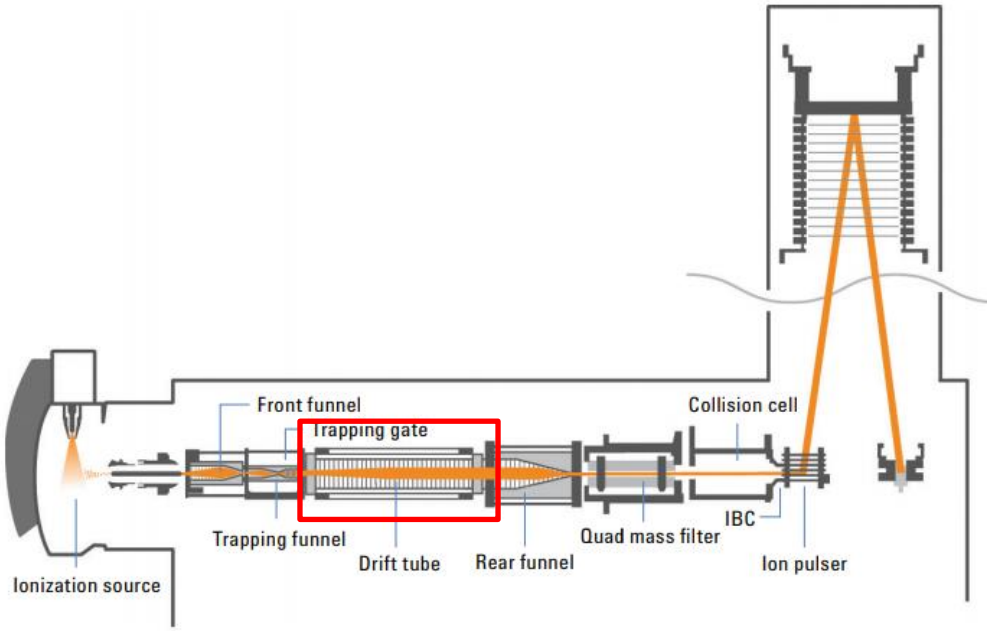




- **MICC**: Agilent ZORBAX SB-C18 (150 x 2.1mm, 3.5µm)
- Temperature: 35°C
- Mobile Phase A: 10 mM NH_4PO_4 + 20 ppm Potassium Acetate, pH 6.8 – 6.9
- Mobile Phase B: 100% ACN

Use of Ion Mobility to characterize OGN structure

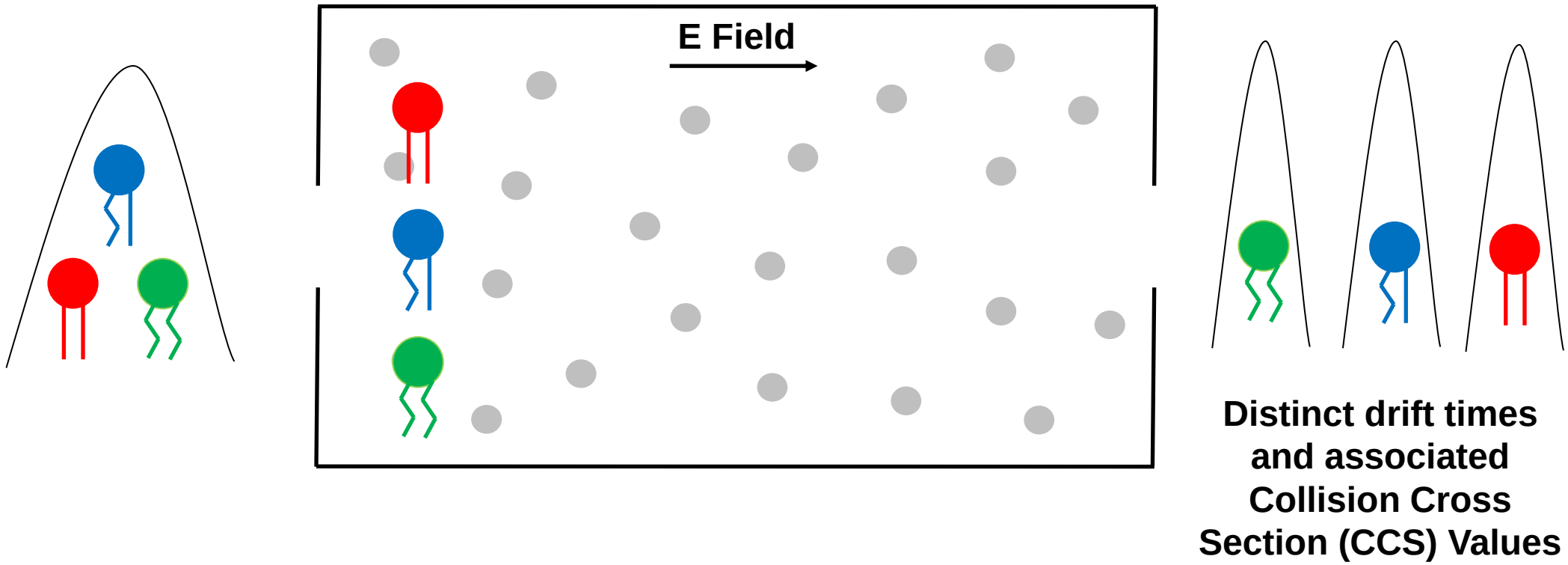
- Gas phase separation
- Based on shape, size and charge
- Allows direct collisional cross section (CCS) calculation



Agilent DT-IM-QTOF 6560

Drift Tube Ion Mobility

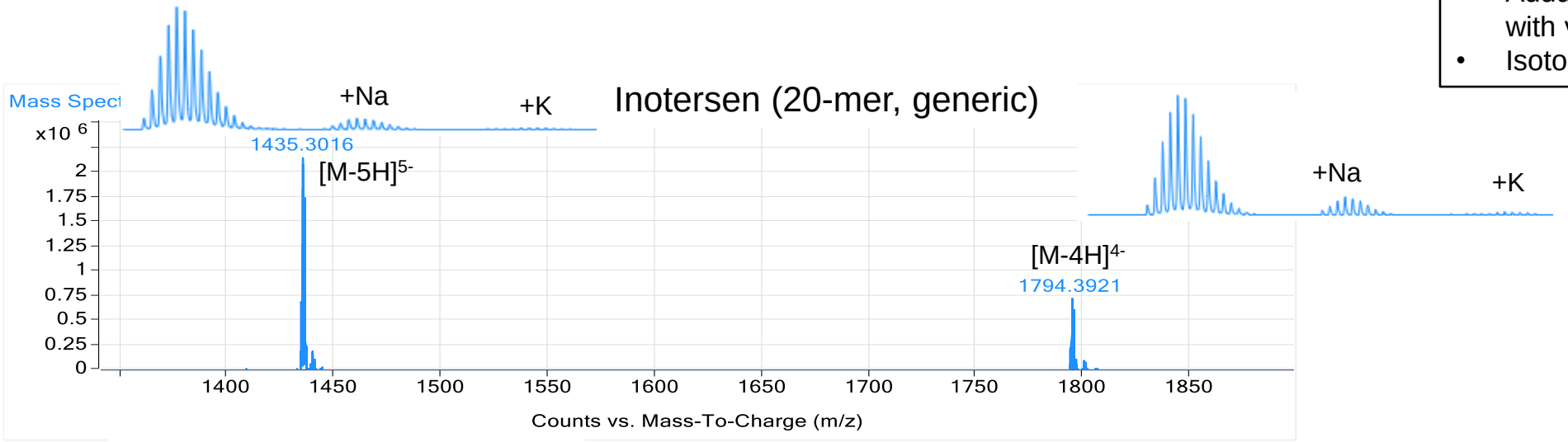
Another dimension of separation to effectively isolate isobaric and isomeric structures that may not be resolved through LC-MS.



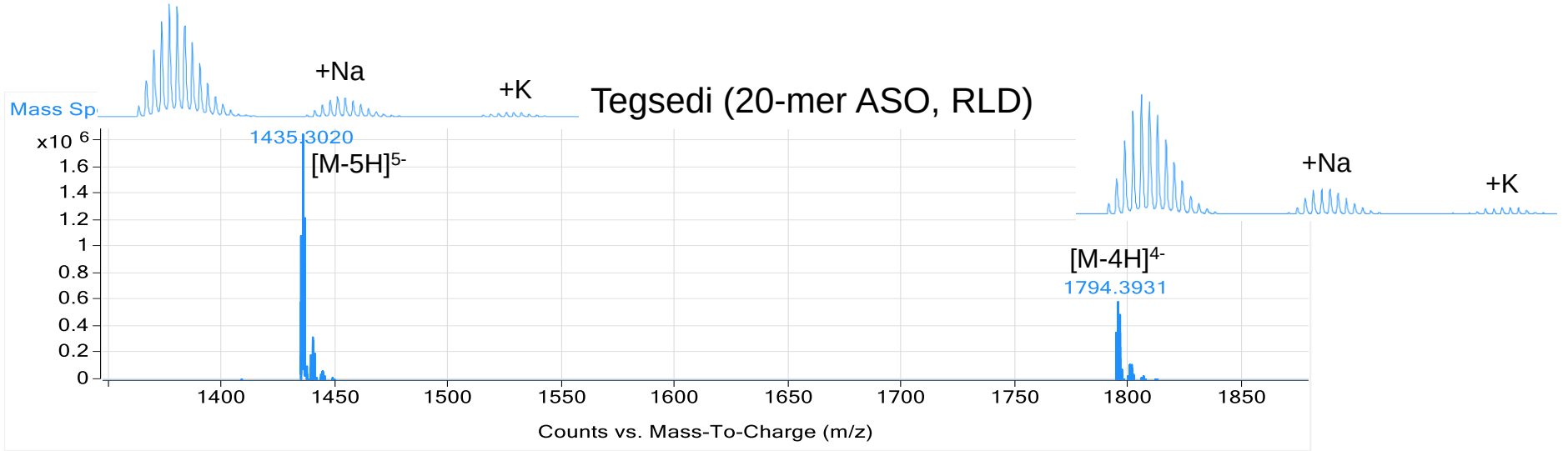
High Resolution Mass Spectrometry

- Accurate Mass
- Charge State Distribution
- Adduct distribution (deprotonation with various Na and/or K)
- Isotopic Envelope

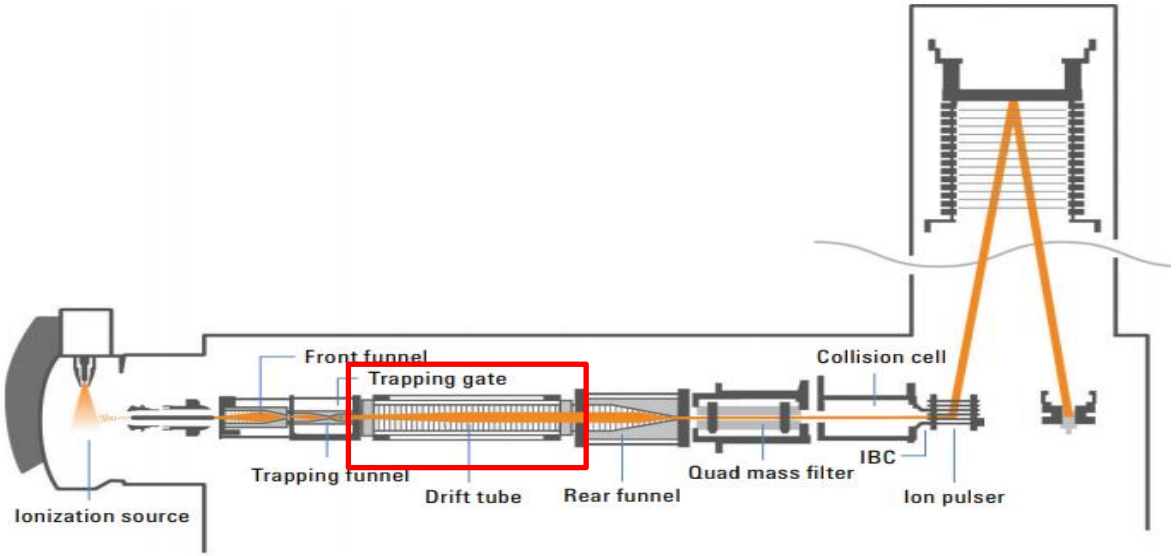
Inotersen (20-mer, generic)



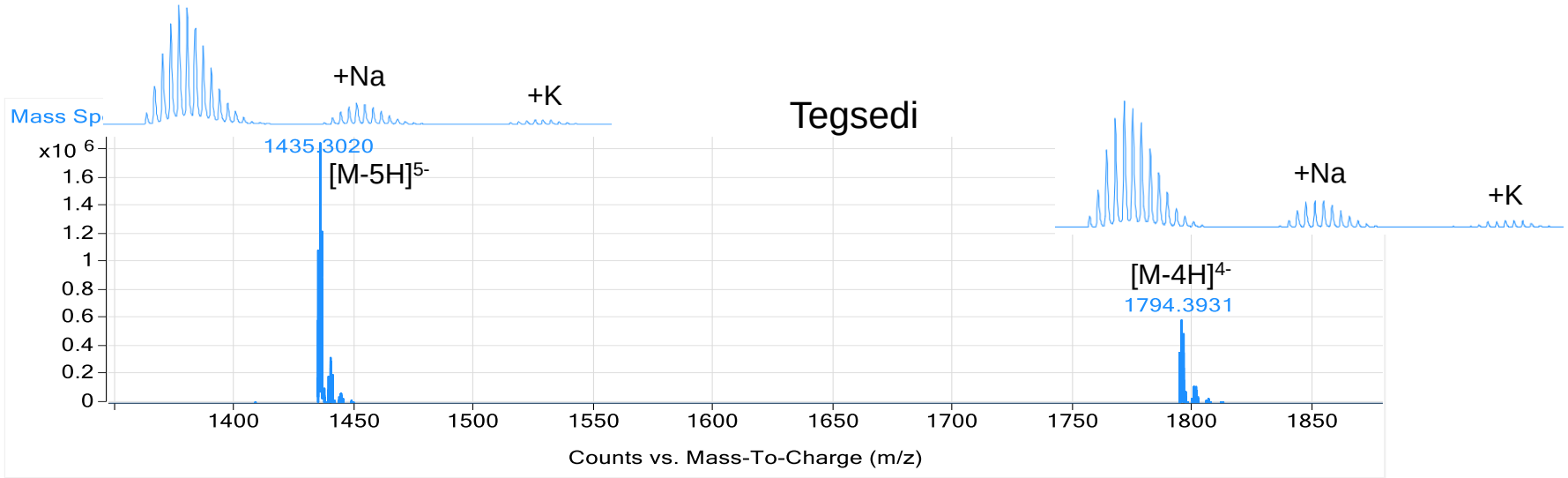
Tegsedi (20-mer ASO, RLD)



Drift Tube Ion Mobility (DT-IM)



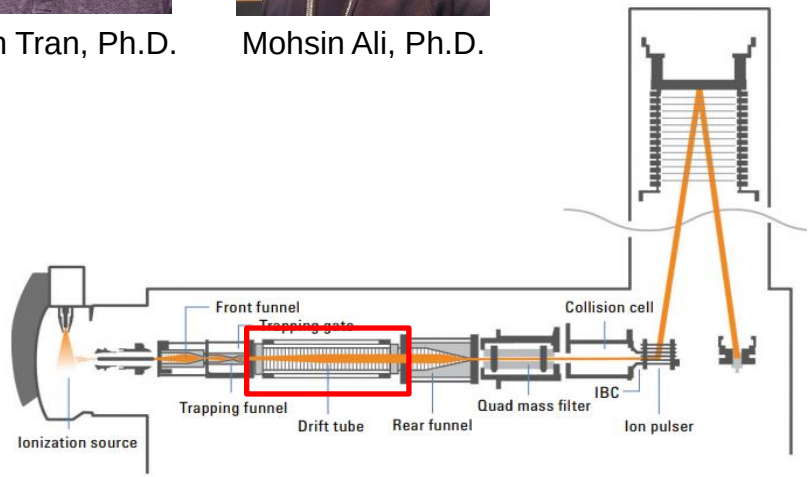
DT-IM
- Charge State
- Adduct
- Isotopic Envelope or individual peak

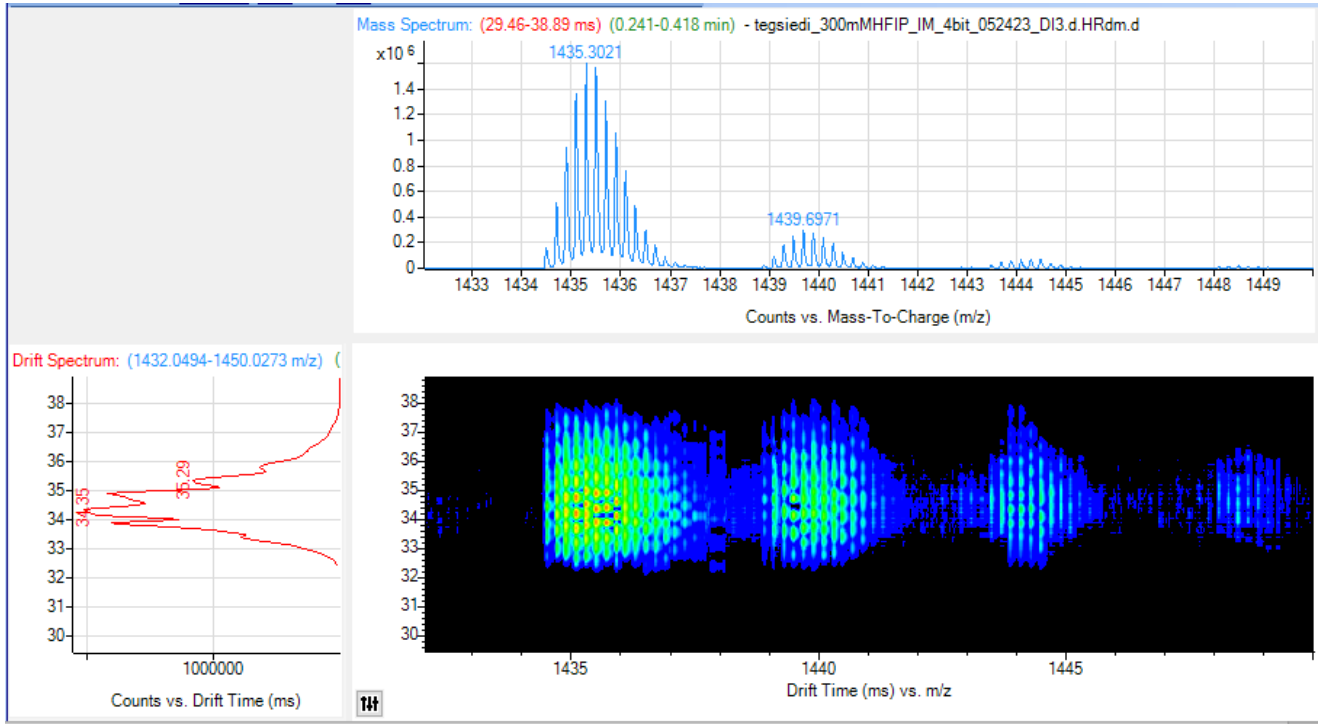


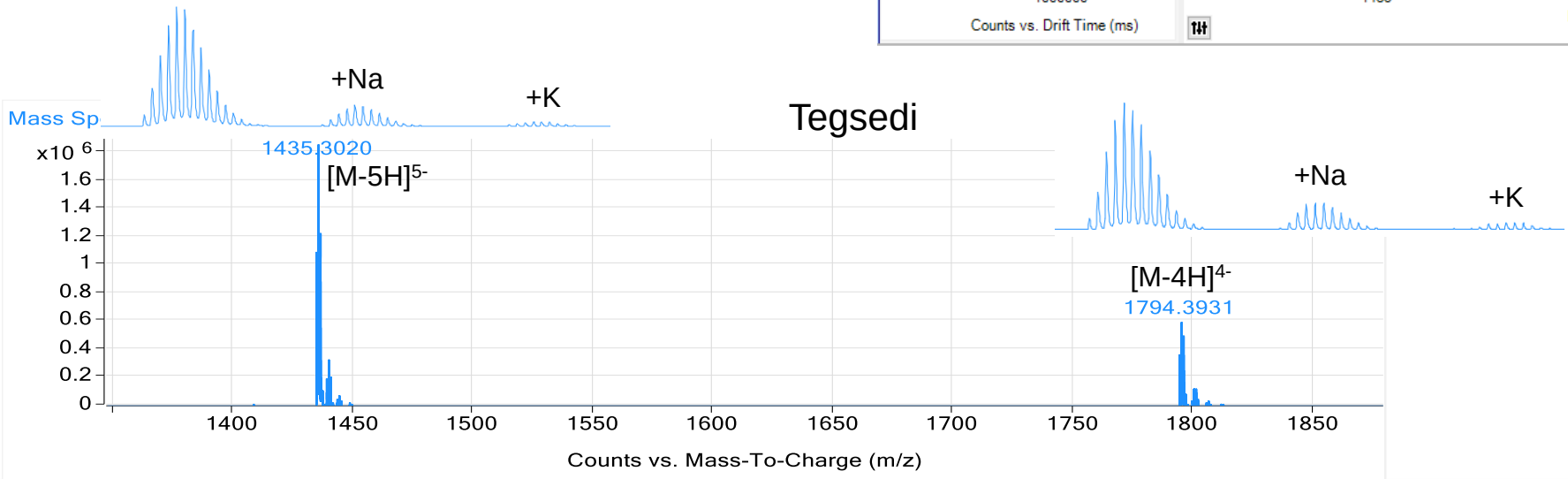
Drift Tube Ion Mobility (DT-IM)

Anh Tran, Ph.D.

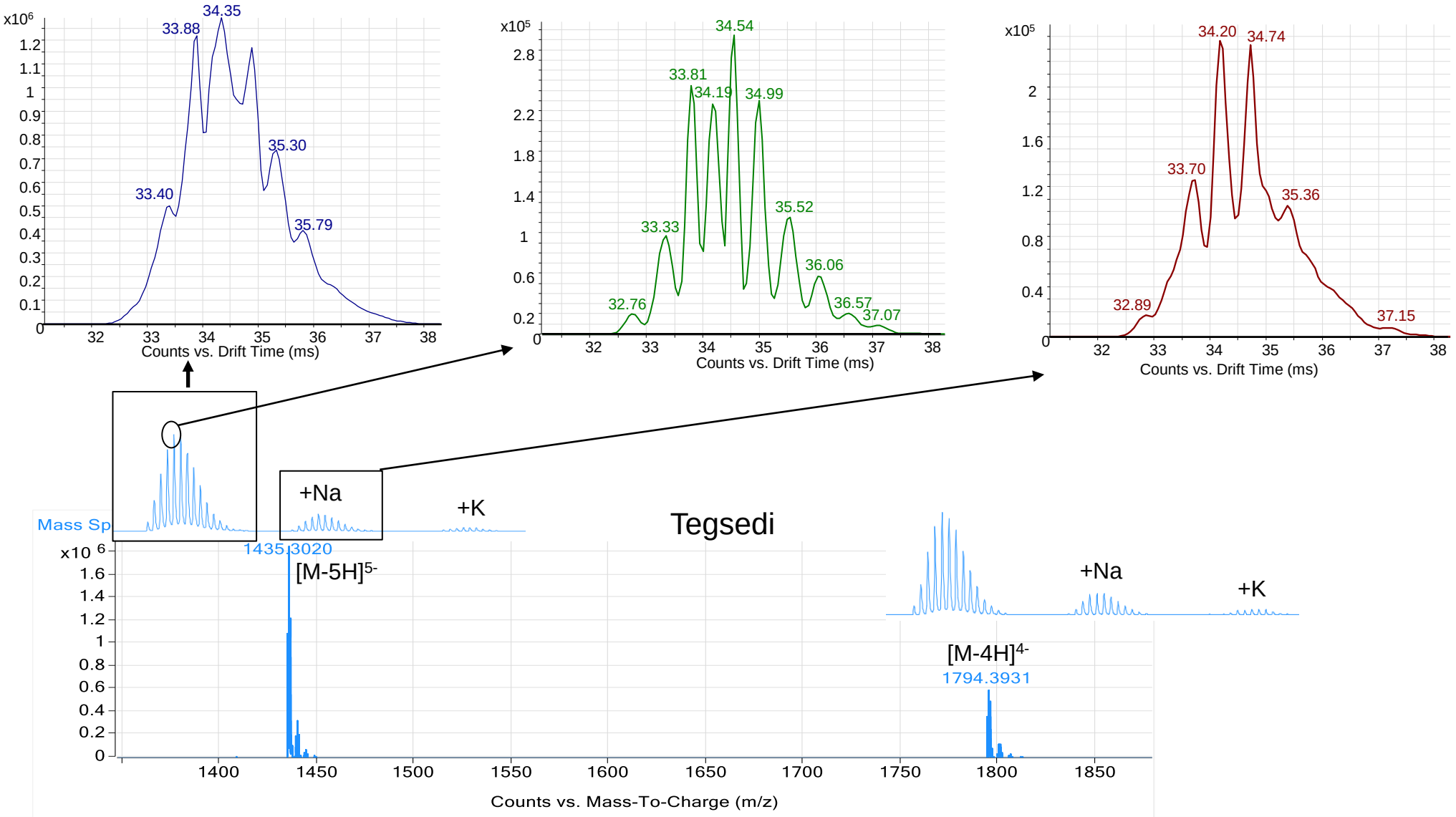
Mohsin Ali, Ph.D.





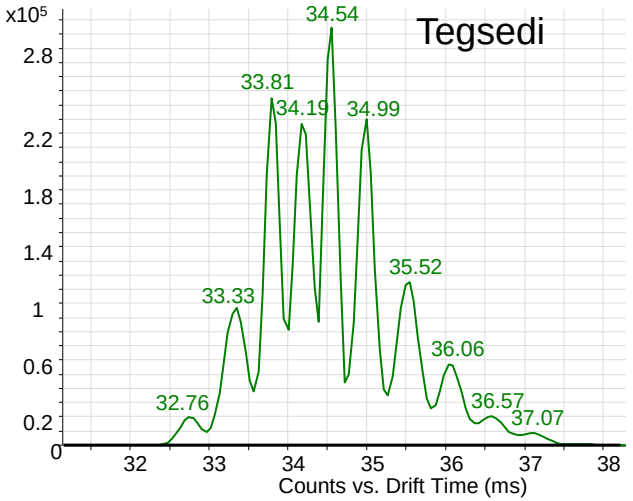


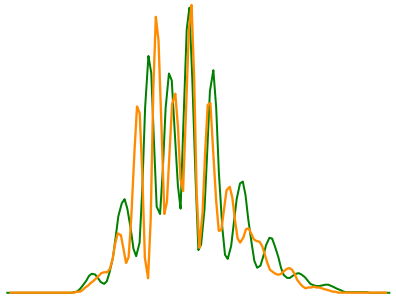
Generation of Drift Spectra to Profile OGN Structure

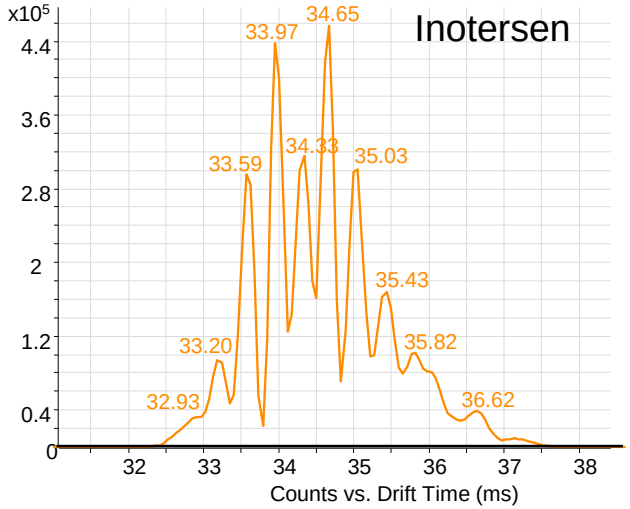


Drift Time Mobiliograms: OGN fingerprints

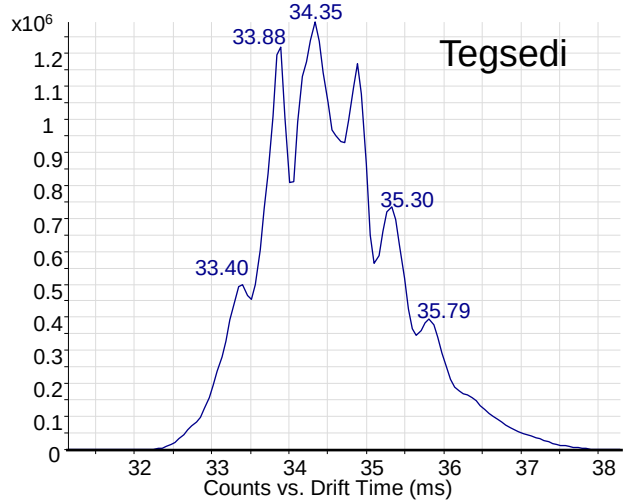
$[M-5H]^{5-}$, *m/z* 1435.3; monoisotopic peak

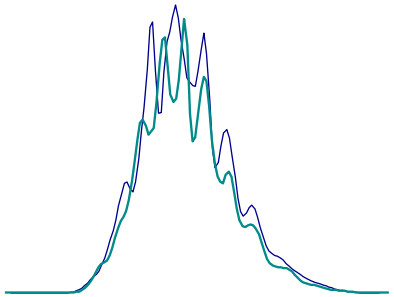


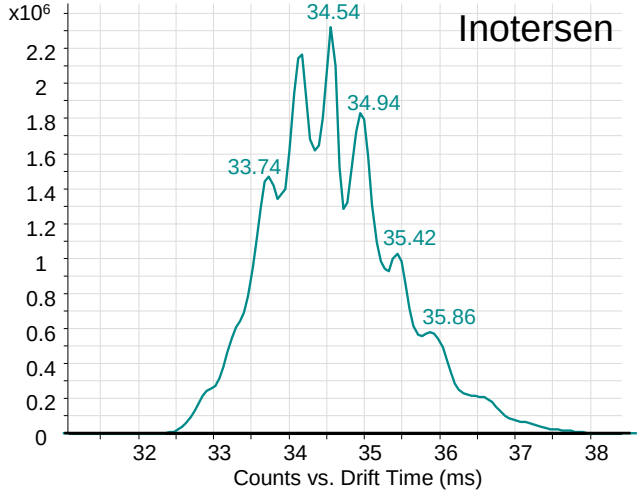




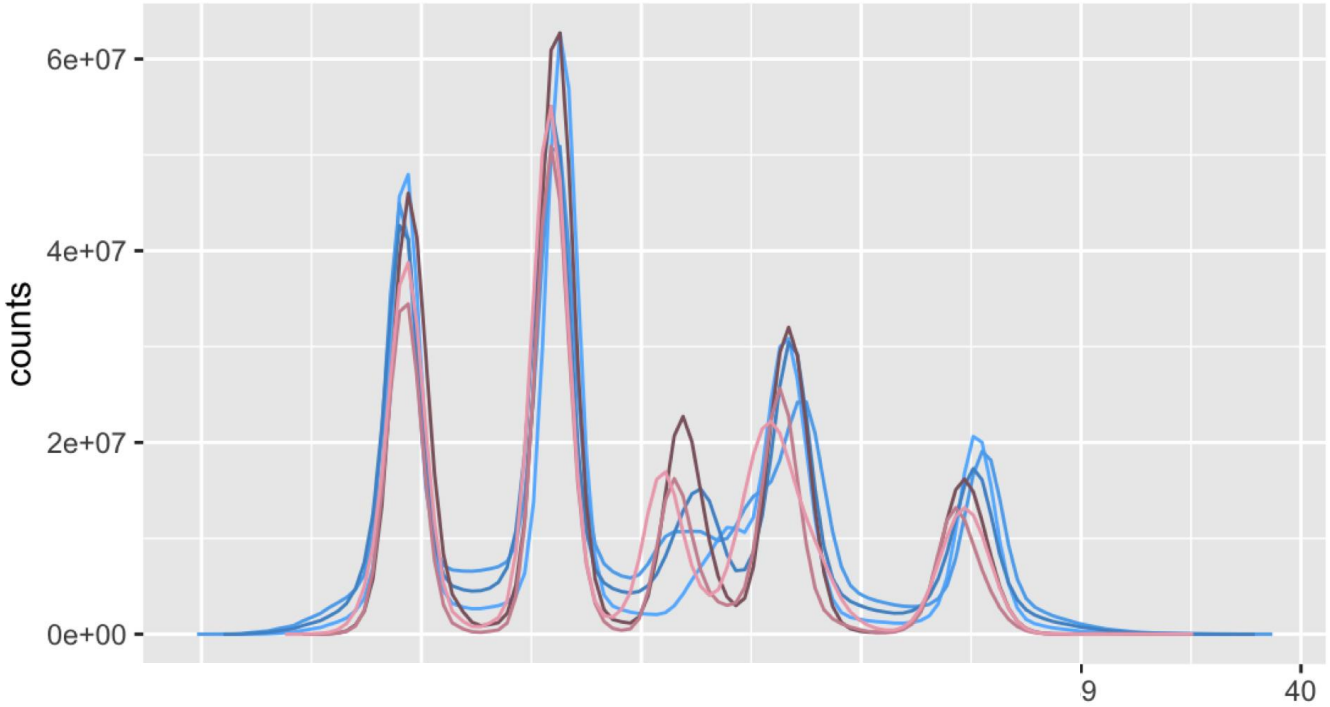
$[M-5H]^{5-}$, *m/z* 1434-1438; isotopic envelope



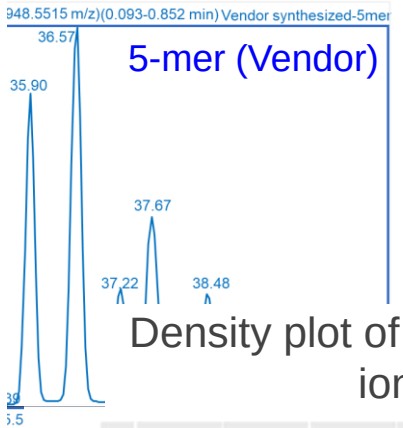




Drift Time Mobiliograms: OGN fingerprints



ectrum (Monoisotopic Peak)



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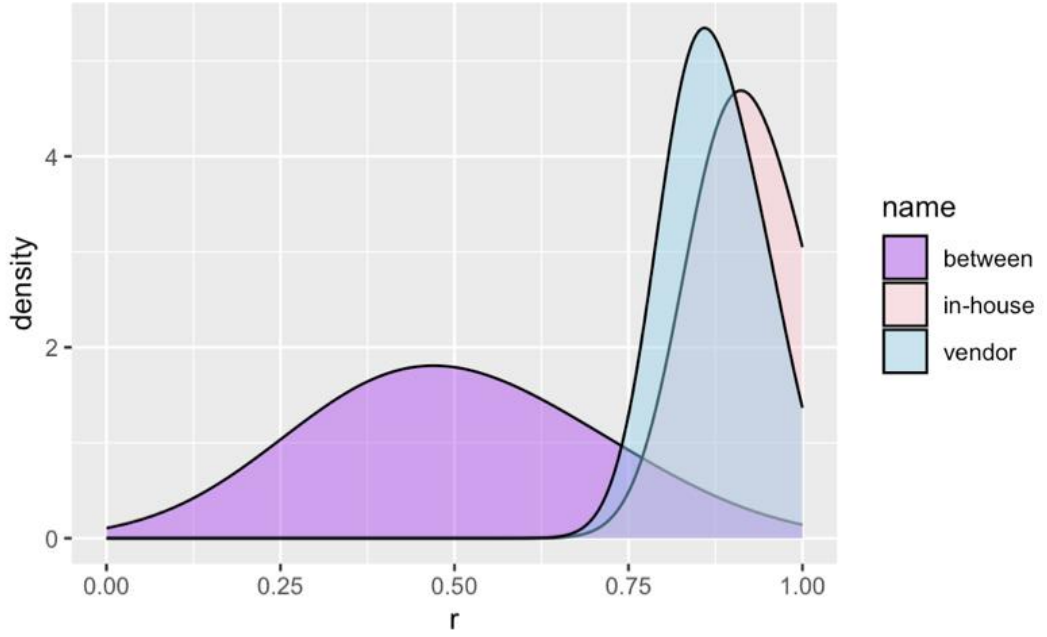
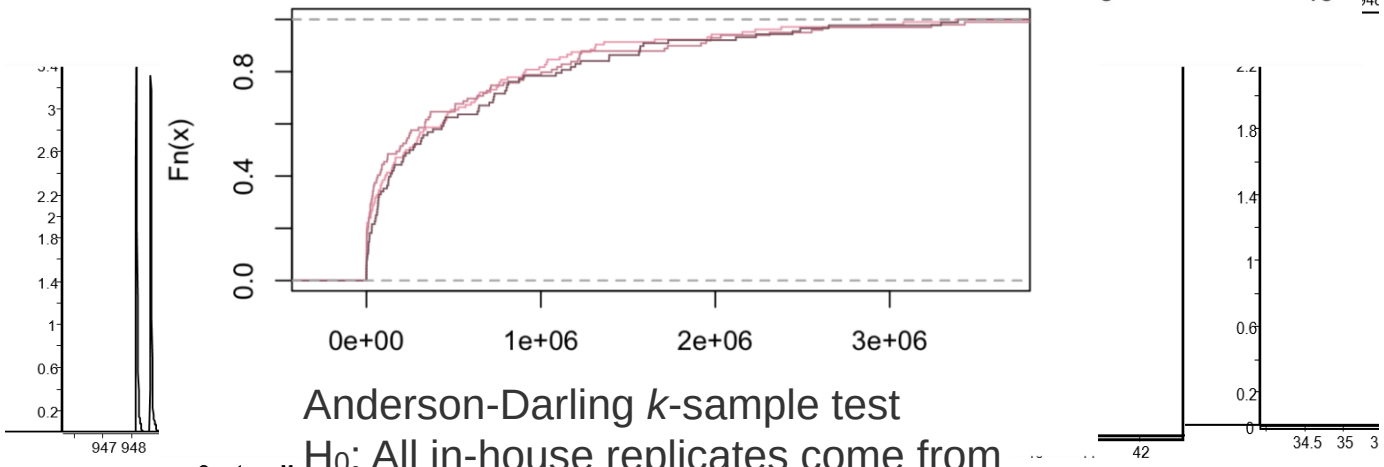
University of Maryland

Density plot of correlation coefficients for ion-mobility data

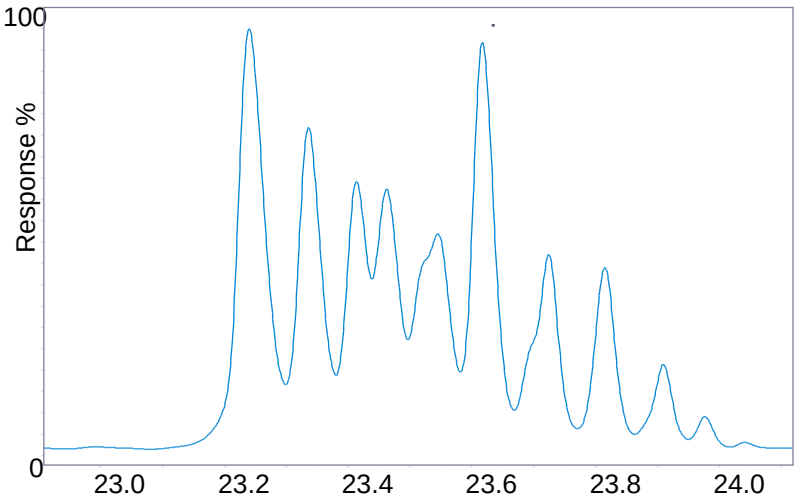




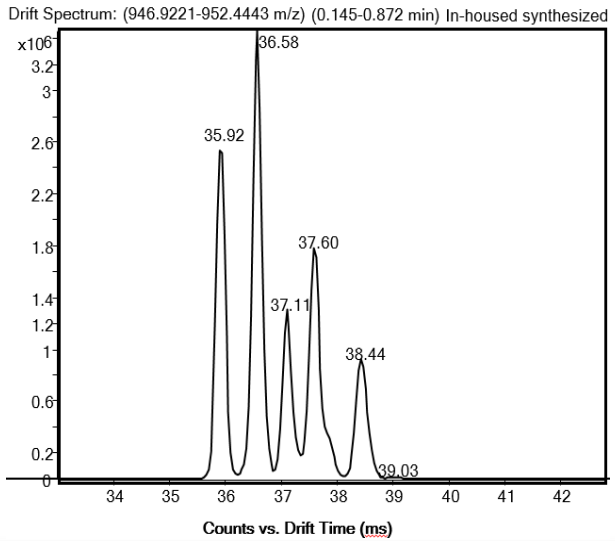
Anderson-Darling *k*-sample test
H_0: All in-house replicates come from a common population

Diastereomer Characterization via IP-RP, IM, and NMR

IP-RP Chromatography



Ion Mobility

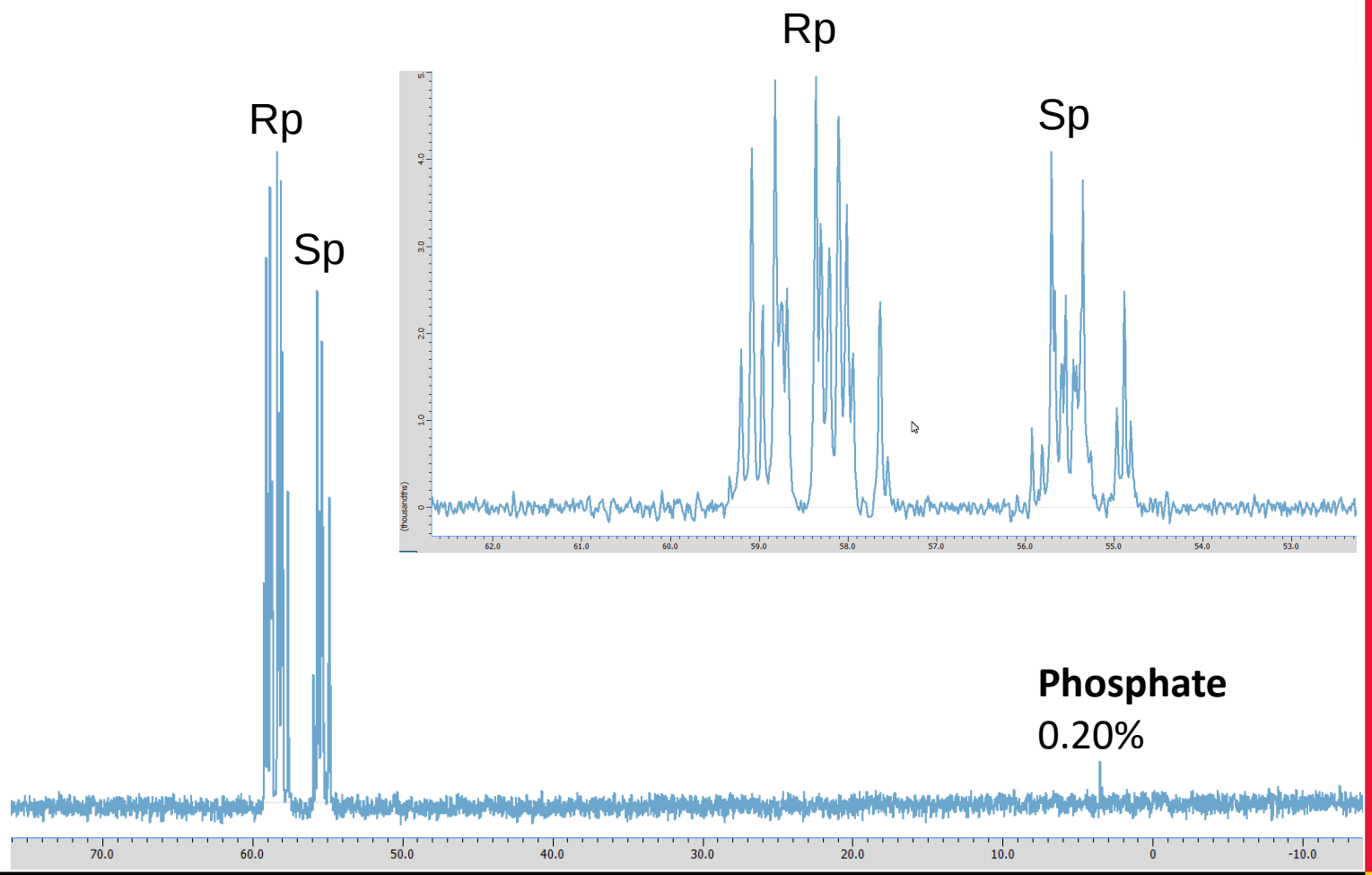


5-mer (From Vendor)

$^{MOE}A*^{MOE}T*^{MOE}C*^{MOE}C*^{MOE}C$

PS linkages = 4
Diastereomers = 16

^{31}P NMR



Phosphate
0.20%

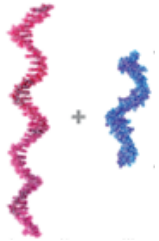
Mass Spec/Ion Mobility

Chromatography

NMR

Chemical Synthesis

Stats/Computation



OGN Structure

Frederick S. Brundick/cc-2.0/https://flic.kr/p/29umt8b

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