

Synthetic Oligonucleotide Impurity Analysis: Enhancing the Conventional Single Quad Method Using UPLC-ToF-MS

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Waters Corporation

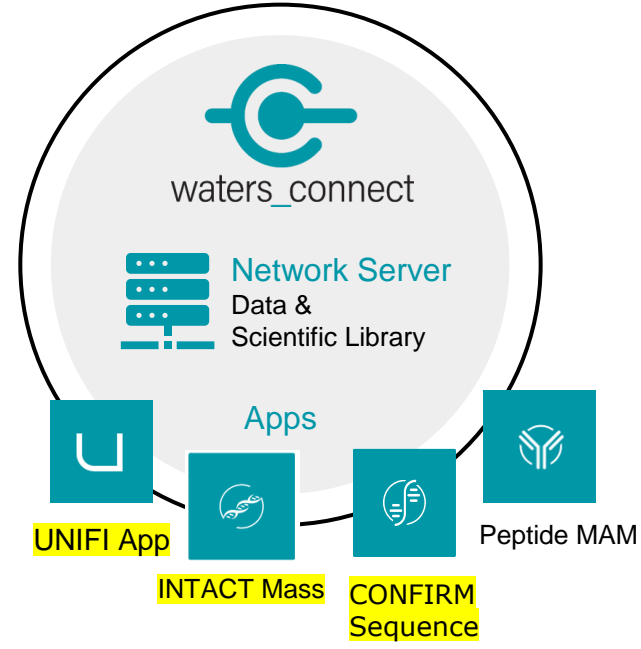
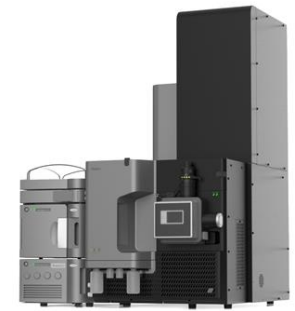
USP Workshop

04-09-2024

Overview of the Talk

- Benefit of MaxPeak™ Surface Technology
- Benefit of HRMS
- Informatics Tool (Three Apps)
 - Characterization (Intact Mass + CONFIRM Sequence)
 - Impurity Profiling using HRMS + Customized data processing (UNIFI)

Acquity PREMIER
COLUMNS AND SYSTEMS
with MAXPEAK™
HIGH PERFORMANCE SURFACES

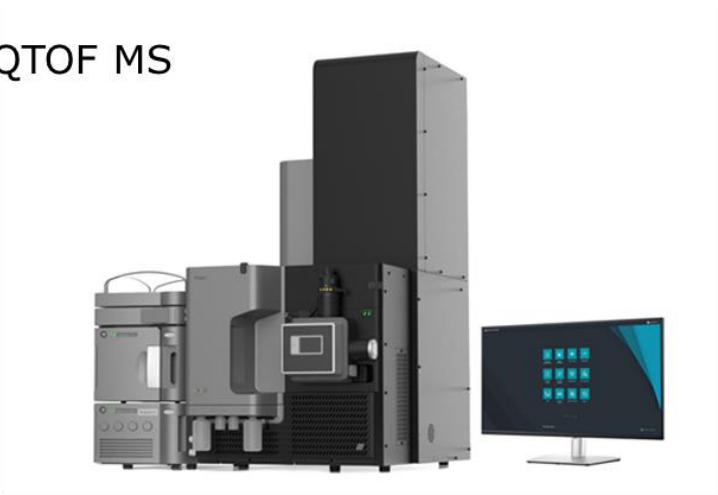


Tools for Oligonucleotide Analysis – Waters Corporation

IPRP-UPLC-UV-MS

Software Tools

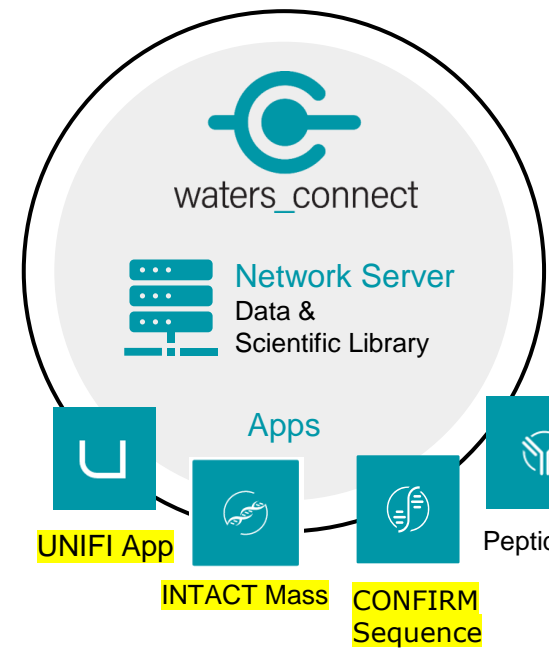
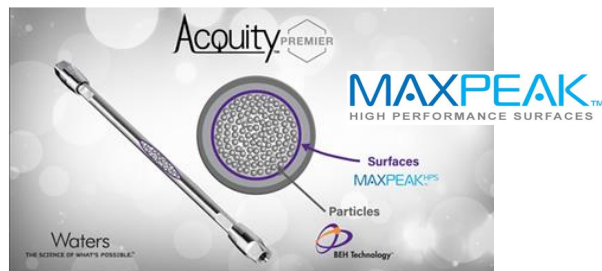
Xevo G3 QTOF MS



BioAccord (TOF MS)



OST Premier 2.1 x 50 mm, 130 Å, 300 Å column



Intact Mass (Classic Workflow)
Peptide Mapping
Released Glycans
Accurate Mass Screening

- ✓ Integrated compliance-ready data acquisition, processing and reporting
- ✓ A shared ecosystem that enables data traceability and transferability

UNIFI App: Impurity Screening

INTACT Mass App: Accurate Mass Measurement using MS 1

CONFIRM Sequence App: Oligo fragmentation data annotation

Some LC Separations Are More Challenging Than Others

Biomolecular analytes prone to metal interactions (NSA):

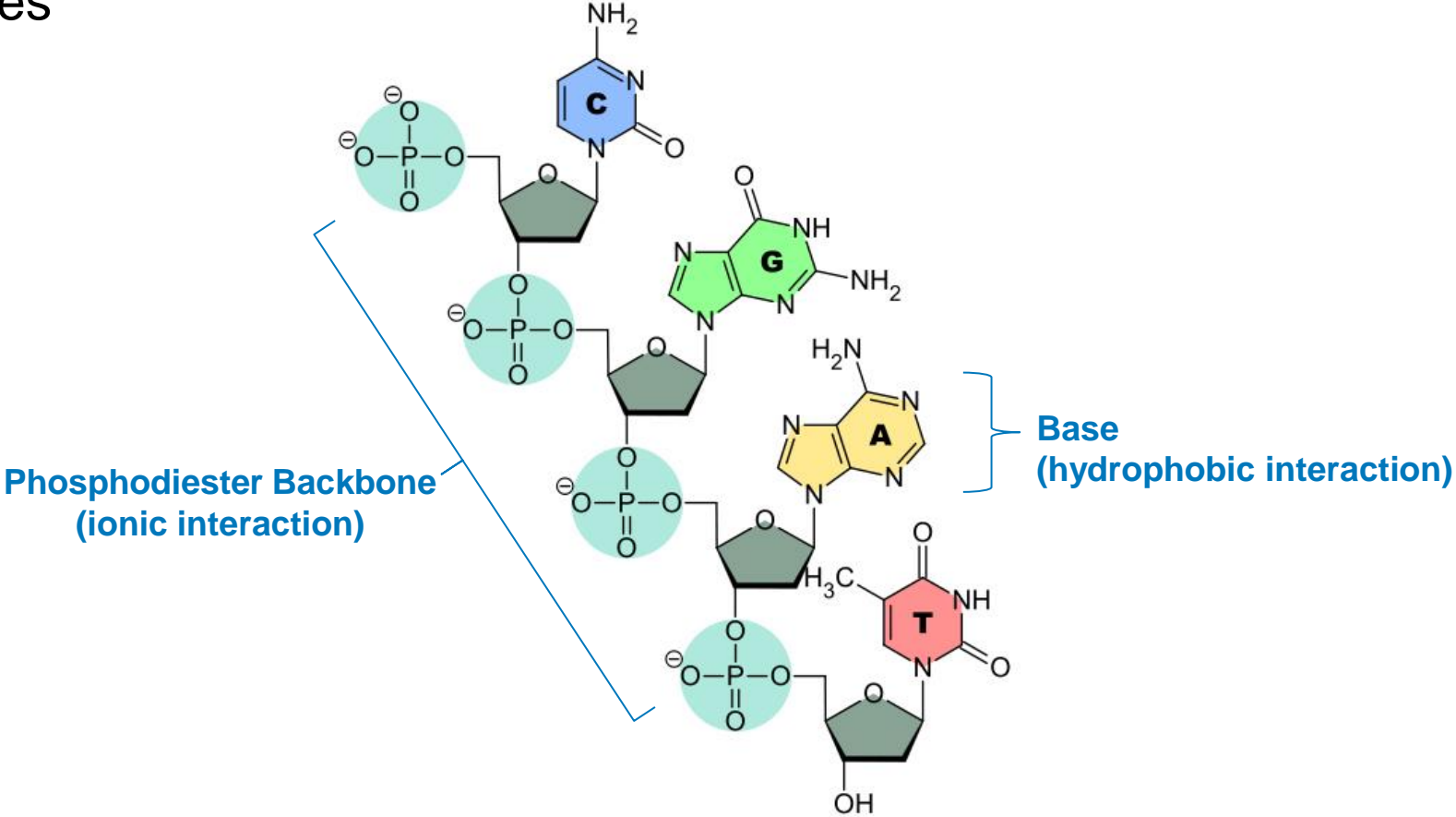
Acidic & Phosphorylated Peptides

Nucleotides

Oligonucleotides

Sialylated glycans

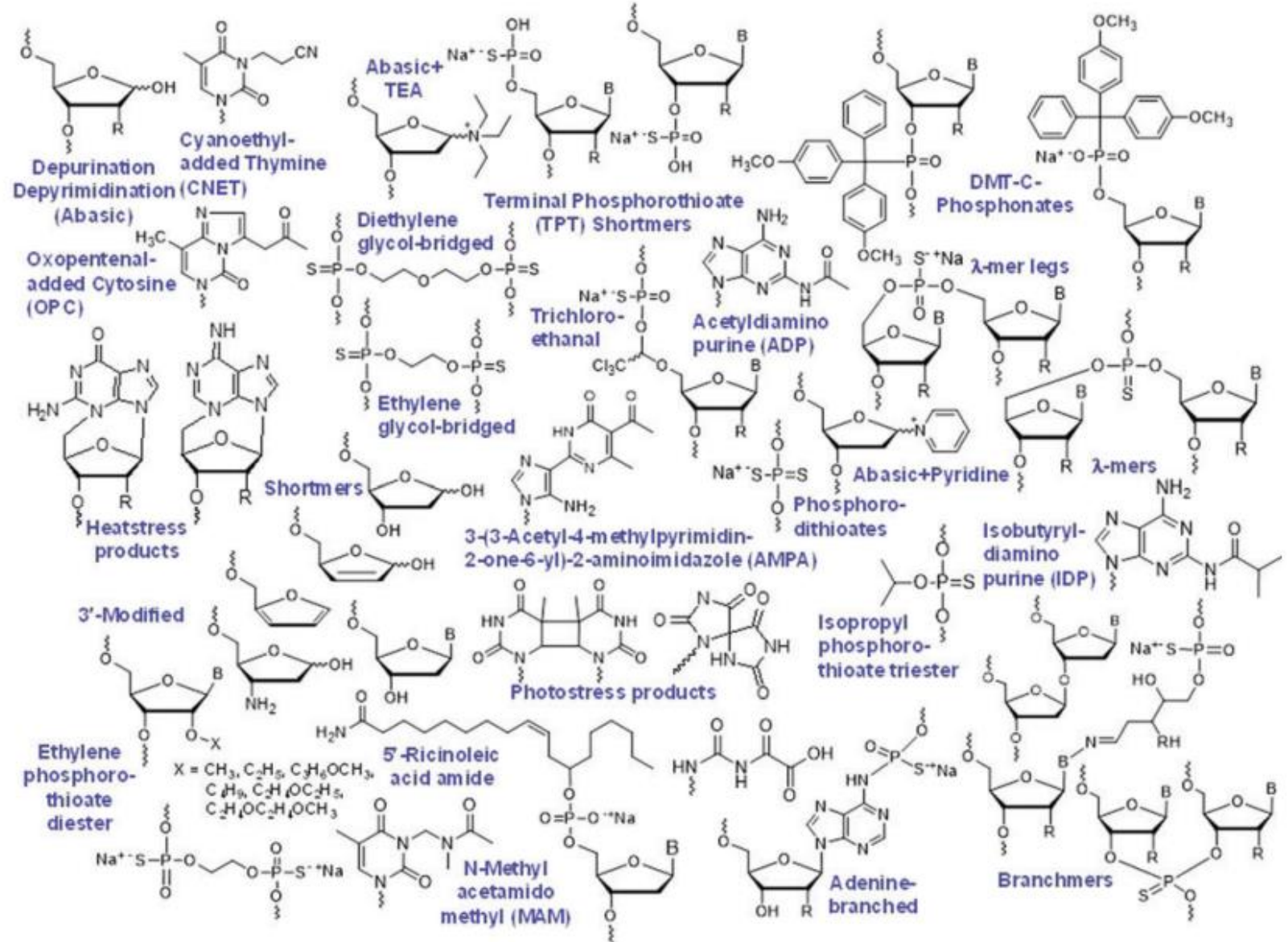
Glycoproteins, et.al.



(Photo Credit : Spunk (talk)/Wikimedia Commons)

Too Many Impurities

- Deletion/Extension
- Truncation
- Modifications on FLP



Benefit of the MaxPeak™ Surface Technology

High Performance Surfaces that mitigate unwanted surface interactions and adsorption

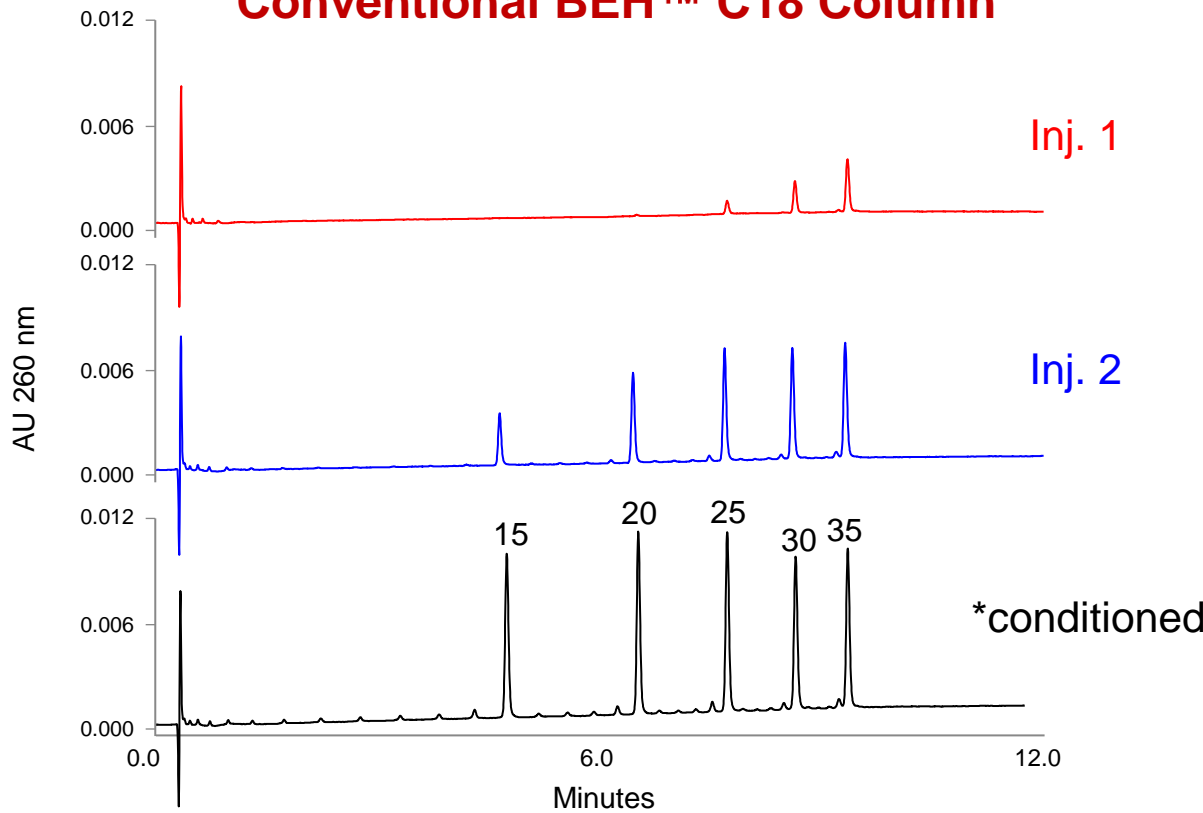
The diagram illustrates the adsorption of metal-sensitive analytes on a metal surface. At the top, two chemical structures are shown: a carboxylate group (R-COO⁻) and a phosphate group (R-O-PO₃H⁻). Below these, a grey arrow points to a blue bar representing a 'Metal' surface. Above the metal surface, several '+' signs indicate the presence of metal ions. Below the metal surface, two boxes represent the periodic table entries for Iron (Fe, atomic number 26, atomic weight 55.845) and Titanium (Ti, atomic number 22, atomic weight 47.868). Below the diagram, the text reads: 'On conventional LC systems, metal sensitive analytes are adsorbed on to metal surfaces'.

The diagram shows the HPS surface structure, which is a hybrid organic-inorganic silica. It consists of a blue 'Metal' layer topped with a thin grey layer, and a thick purple 'HPS surface' layer. Above the HPS surface, the same carboxylate and phosphate chemical structures are shown. A large red 'X' is placed over a grey arrow pointing from the structures down to the HPS surface, indicating that adsorption is minimized. Below the diagram, the text reads: 'MAXPEAK™ HIGH PERFORMANCE SURFACES' and 'Waters™ MaxPeak High Performance Surface is designed to minimize metal-analyte interactions'. At the bottom, it states: 'HPS is hybrid organic-inorganic silica'.

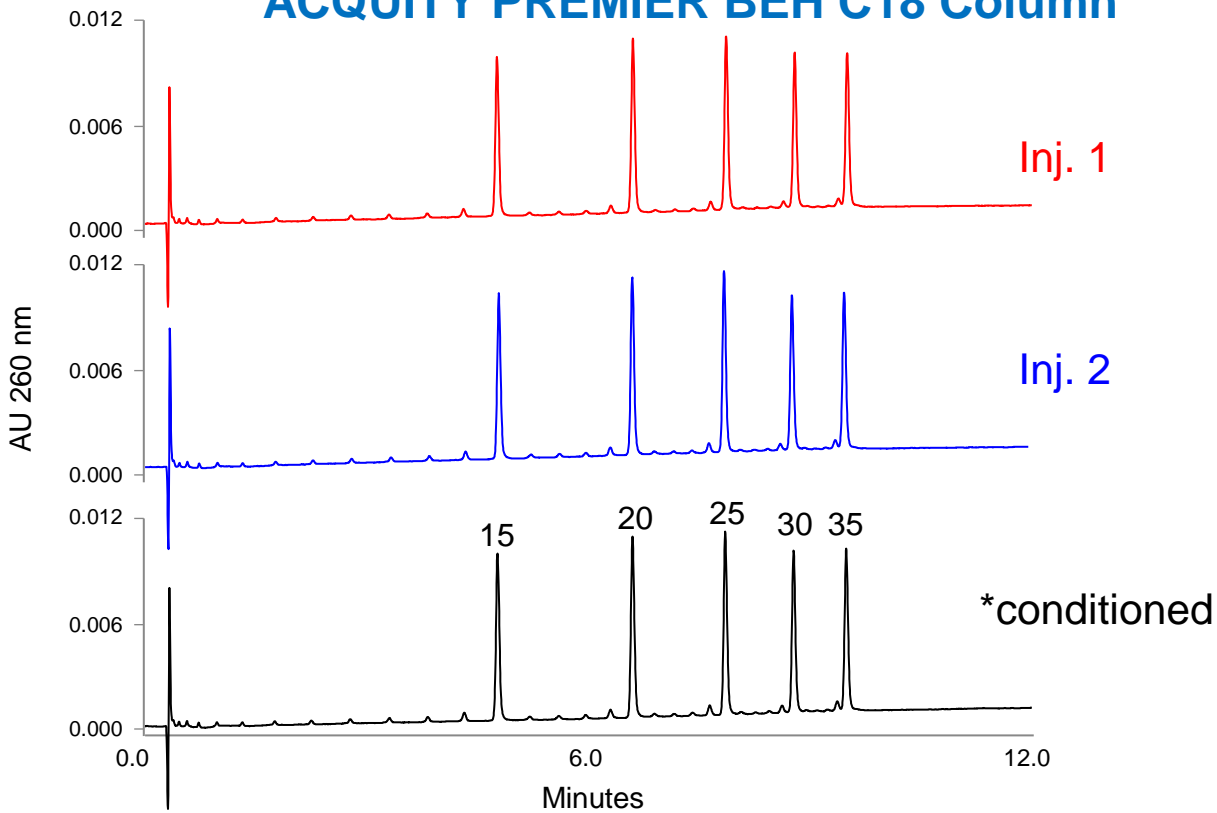
Anal. Chem. 2021, 93, 14, 5773–5781

Sample: MassPREP™ Oligonucleotide Standard

Conventional BEH™ C18 Column

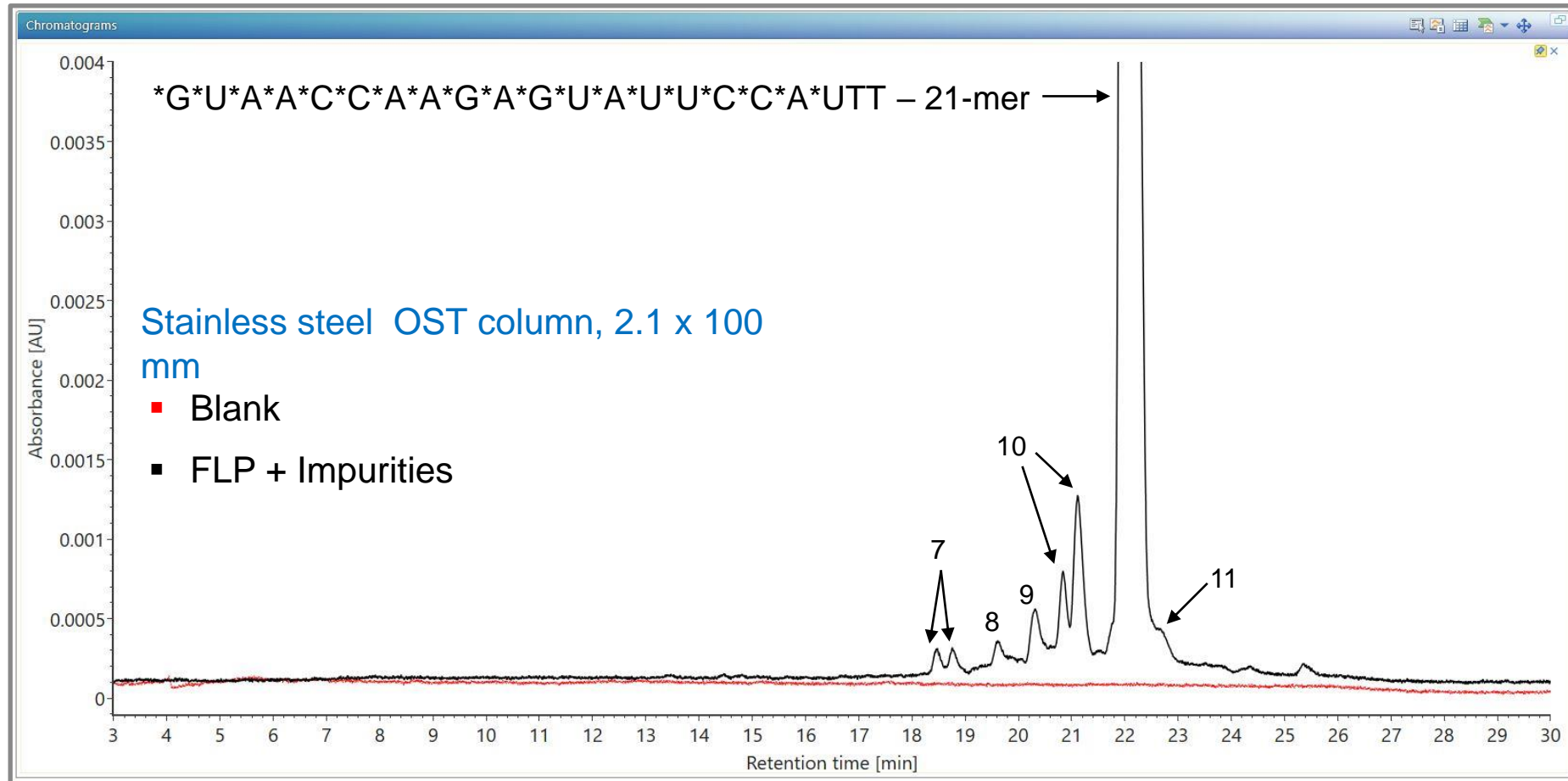


ACQUITY PREMIER BEH C18 Column

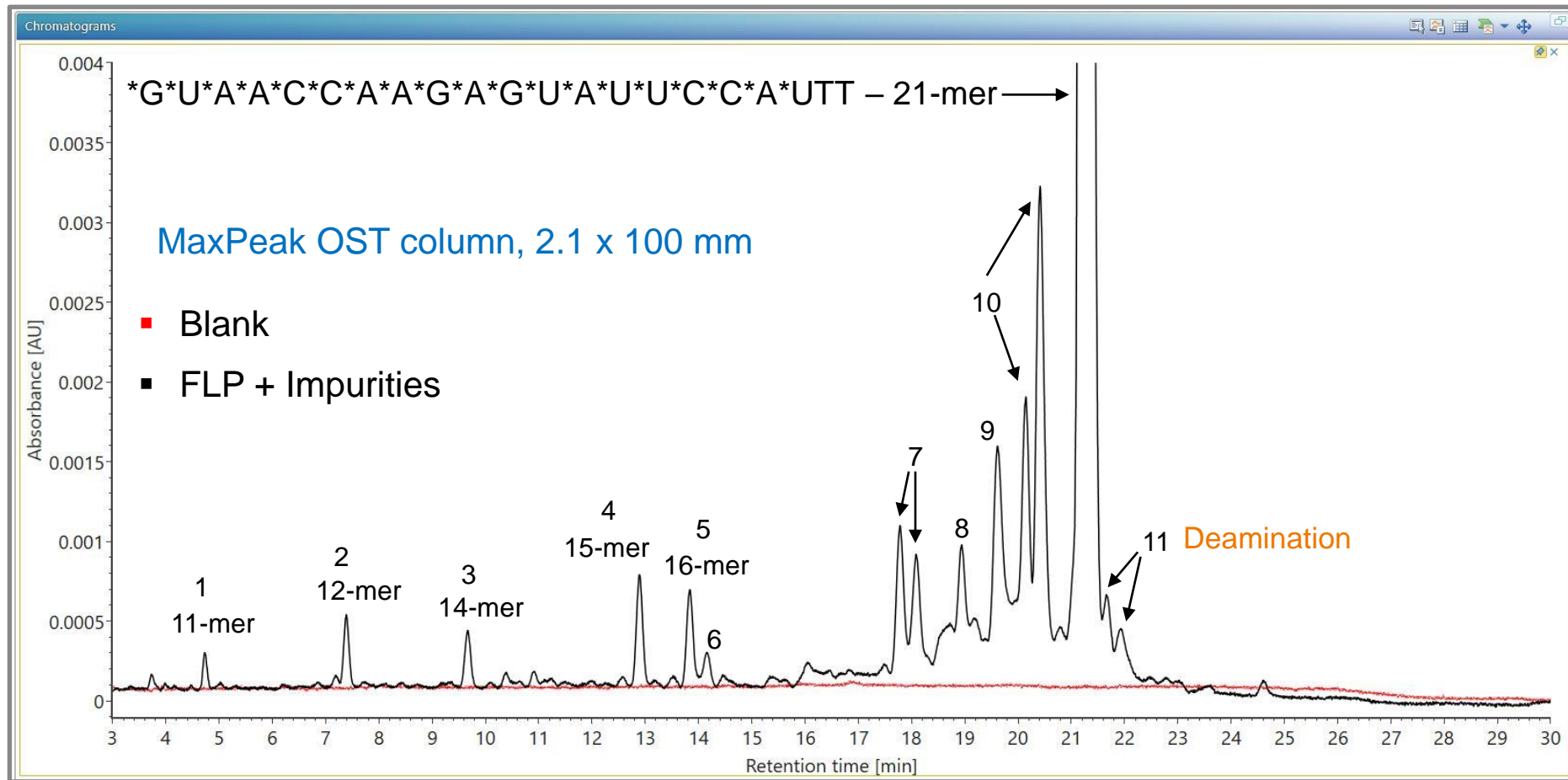


Injection of 2 μ L of OST standard, 10 pmol of each oligonucleotide on column, m.p. 25 mM HAA, pH 6
*Conditioning: 500 pmol injection of 35 mer followed by "post conditioning" injection of 10 pmol of standard

Impurities Observed from Regular S.S. Column



MaxPeak OST Column Recovers More Impurities

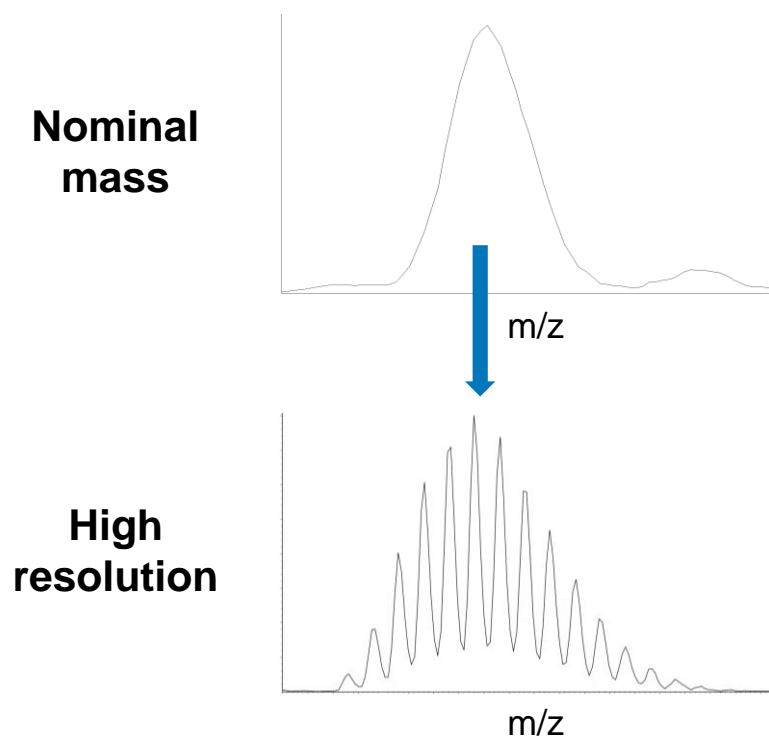


Benefit of HRMS



Low Resolution MS	High Resolution MS
Nominal mass resolution	Isotope resolved
Difficulty measuring charges (z)	Can measure multiple charges (z)
Good for some targeted analysis	Targeted and untargeted (determines unknowns)
Requires good chromatographic separation	Fragmentation for structure elucidation

HRMS for Increasing Structural Complexity?



Nusinersen FLP: 2'-O-MOE, PS modified RNA 18-mer

U-C-A-C-U-U-U-C-A-U-A-A-U-G-C-U-G-G (C and U are methylated)

Exact mass = 7122.2763



Nominal mass MS can not difference them

Impurities	Exact Mass (Da)	m/z (-4)
n - u	6728.2163	1681.0468
n - c	6729.2003	1681.2928

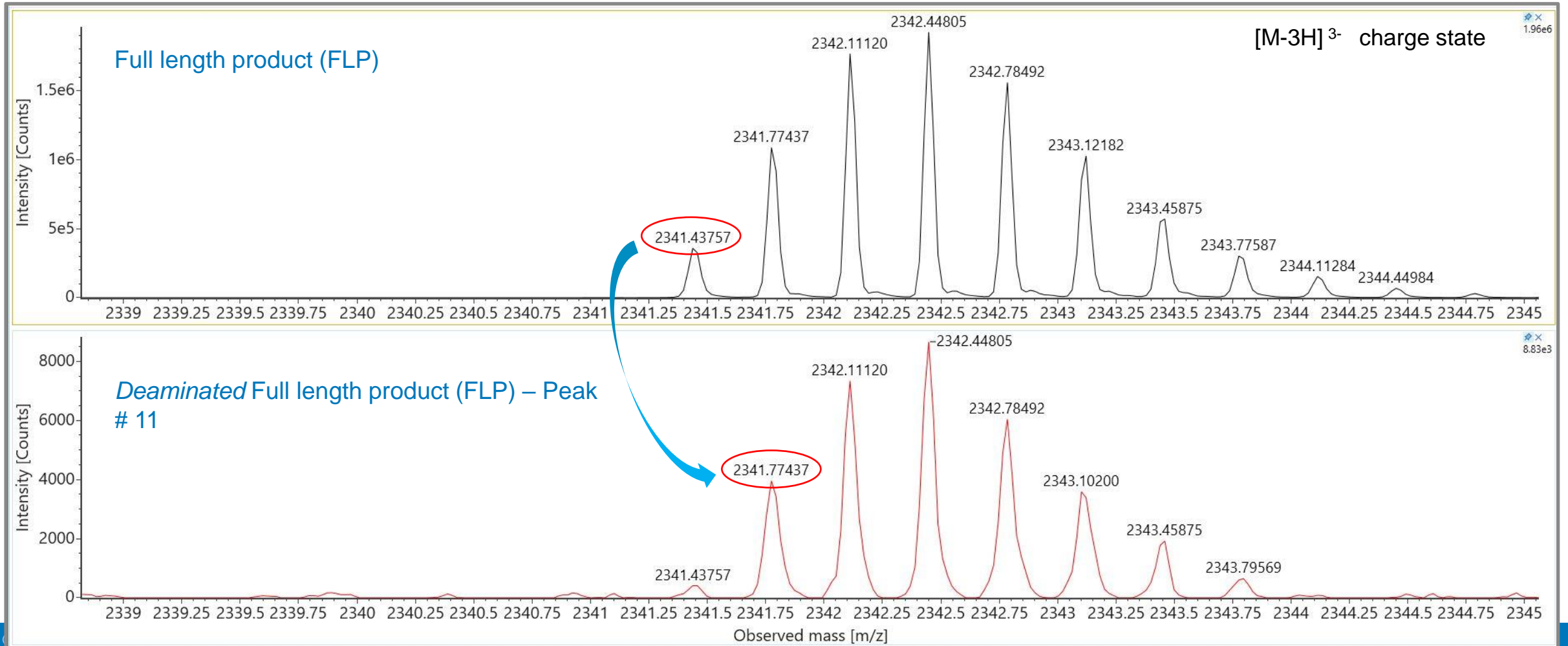
[Ref: In-Depth Impurity Assessment of Synthetic Oligonucleotides Enabled by HRMS \(fda.gov\)](#)

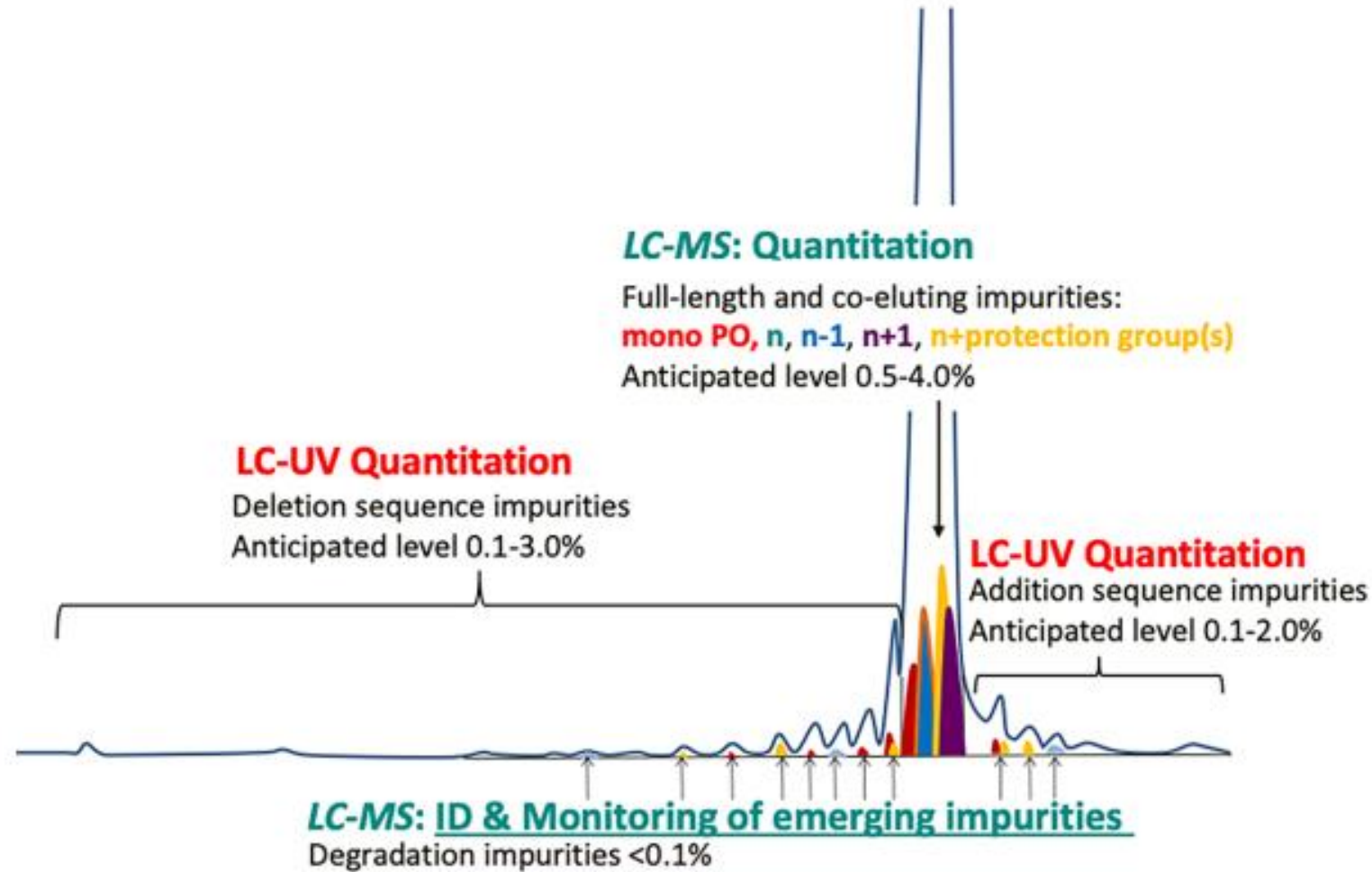
HRMS Can Measure Impurities with Small Mass Change from FLP

*G*U*A*A*C*C*A*A*G*A*G*U*A*U*U*C*C*A*UTT – 21-mer

Example: Deamination: + 0.98 Da

5-methyl-cytosine → Thymidine





Oligo Characterizations

INTACT Mass App



CONFIRM Sequence App

- Automated charge deconvolution for accurate mass measurement
- Targeted or untargeted data process
- LC-UV and LC-MS quantitation



- Build sequence in scientific library
- Structure elucidation using MS fragment ions
- Sequence coverage viewer

SYNTHETIC Library is Used to Construct Oligo Sequence

SYNTHETIC Library

[Hub](#)
[Help](#)
[Feedback](#)
[waters_connect A.](#)

View Sequence
*G*U*A*A*C*C*A*A*G*A*G*U*A*U*U*C*C*A*UTT – 21-mer

Sequence

1 OMEG 2 2'-OMe-5Me Uridine 3 OMEA 4 OMEA 5 2'-OMe-5-Me Cytidine 6 2'-OMe-5-Me Cytidine 7 OMEA 8 OMEA 9 OMEG 10 OMEA 11 OMEG 12 2'-OMe-5Me Uridine 13 OMEA 14 2'-OMe-5Me Uridine

15 2'-OMe-5Me Uridine 16 2'-OMe-5-Me Cytidine 17 2'-OMe-5-Me Cytidine 18 OMEA 19 2'-OMe-5Me Uridine 20 dT 21 dT

#	ID	Base	Sugar	Linker	Elemental composition	Monoisotopic mass (Da)
1	OMEG	Guanine	2'-OMe	Phosphodiester	C11H14N5O7P(OH)	376.0658
2	2'-OMe-5Me Uridine	5'-Methyluracil	2'-OMe	Phosphodiester	C11H15N2O8P	334.0566
3	OMEA	Adenine	2'-OMe	Phosphodiester	C11H14N5O6P	343.0682

Sequence details

ID
Oligo D423

Sequence
OMEG 2'-OMe-5Me Uridine OMEA OMEA 2'-OMe-5-Me Cytidine 2'-OMe-5-Me Cytidine OMEA OMEA OMEG OMEA OMEG 2'-OMe-5Me Uridine OMEA 2'-OMe-5Me Uridine 2'-OMe-5Me Uridine 2'-OMe-5-Me Cytidine 2'-OMe-5-Me Cytidine OMEA 2'-OMe-5Me Uridine dT dT

5' terminus
OH (R group)

3' terminus
OH (R group)

3' linker
Removed

Monoisotopic mass (Da)
7,027.3761

Elemental composition
C229H306N76O143P20

Date created
3/31/2022

Predicted fragment ion monoisotopic mass (Da)

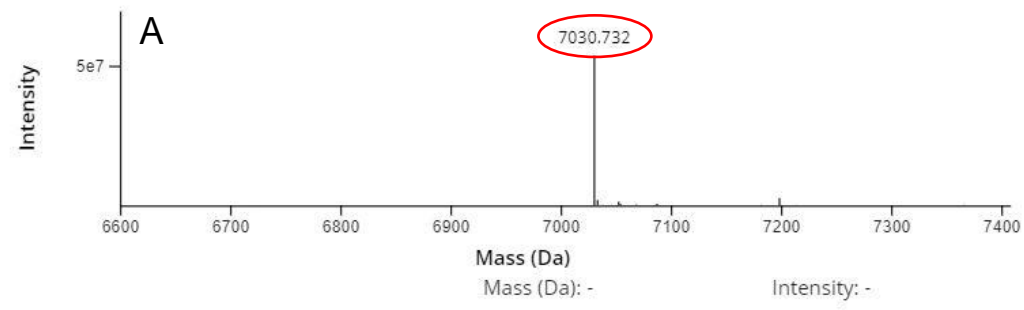
Ion series	Previous monomer	Next monomer	Ion	Elemental composition	Expected mass (Da)
1/20	OMEG	2'-OMe-5Me Uridine	a1	C11H13N5O4	279.0968
1/20	OMEG	2'-OMe-5Me Uridine	b1	C11H15N5O5	297.1073
1/20	OMEG	2'-OMe-5Me Uridine	c1	C11H14N5O7P	359.0631

INTACT Mass App Data Processing Results



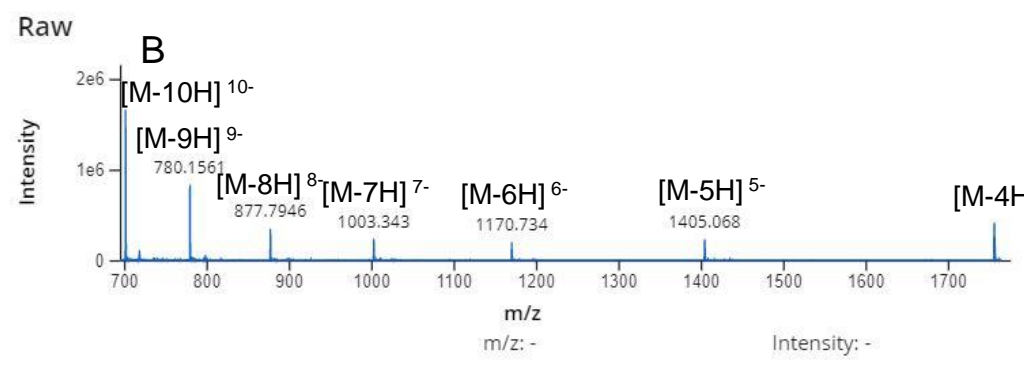
*G*U*A*A*C*C*A*A*G*A*G*U*A*U*U*C*C*A*UTT – 21-mer

Deconvolved

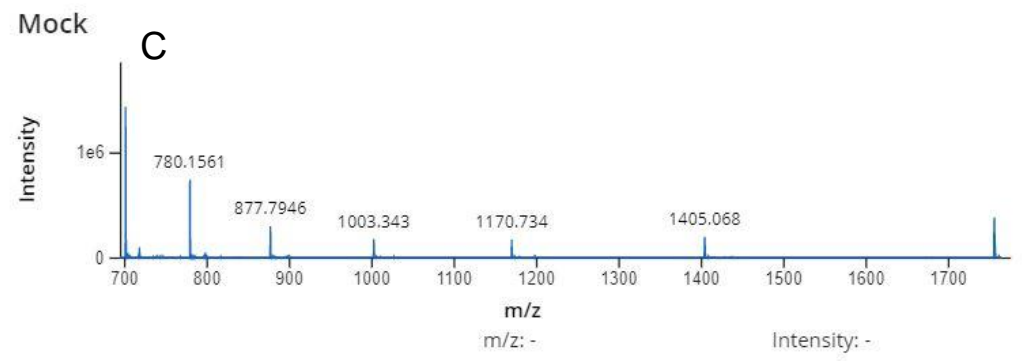


Peak no:

	Component	Observed mass (Da)	Expected mass (Da)	Mass error (ppm)	Identity result	Observed TIC RT (mins)	Observed UV RT (mins)	LC area	LC amount (%)
1	D1423 n-OMeA[5] & n-OMe5MeC[2] & n-OMeG[2] & n-OMe5MeU	3,593.702	3,593.707	-1.5	Pass	4.74	4.69	1,222	0.2
2	D1423 n-OMeA[4] & n-OMe5MeC[2] & n-OMeG[2] & n-OMe5MeU	3,936.762	3,936.775	-3.4	Pass	7.37	7.34	2,737	0.5
3	D1423 n-OMeA[3] & n-OMe5MeC[2] & n-OMeG & n-OMe5MeU	4,638.916	4,638.907	1.9	Pass	9.66	9.61	2,625	0.5
4	D1423 n-OMeA[2] & n-OMe5MeC[2] & n-OMeG & n-OMe5MeU	4,981.988	4,981.975	2.7	Pass	12.88	12.83	5,276	0.9
5	D1423 n-OMeA[2] & n-OMe5MeC & n-OMeG & n-OMe5MeU	5,315.046	5,315.048	-0.3	Pass	13.80	13.77	4,704	0.8
6	D1423 n-OMeA & n-OMeG & n-OMe5MeU	5,991.181	5,991.188	-1.2	Pass	17.76	17.72	7,942	1.4
7	D1423 n-OMeA	6,684.310	6,684.308	0.3	Pass	19.59	19.55	13,473	2.4
9	D1423 n-OMe5MeU	6,693.332	6,693.320	1.9	Pass	20.13	20.08	13,283	2.3
10	D1423 n-OMe5MeC	6,694.315	6,694.304	1.8	Pass	20.39	20.34	26,001	4.5
8	D1423 unknown[-NH5]	7,008.354	7,008.334	2.9	Pass	20.13	20.08	13,283	2.3
	D1423	7,027.390	7,027.376	2	Pass	21.22	21.19	469,430	82.0
11	D1423 Deamination	7,028.315	7,028.360	-6.5	Pass	20.39	20.34	26,001	4.5



MAIN PEAK



Customized Workflow for Impurity Profiling

-Method Transfer from Single Q to TOF MS



Oligo Targeted Impurity Analysis Workflow

- UV Cut-off
- SST - Injector Repeatability
- SST - Calibration Curves
- UV Chromatogram
- EICs Review
- Extracted Ion Chromatograms
- Components under FLP
- Components under n-1
- Sample Result Summary
- Adduct EICs

Oligo Untargeted Impurity Analysis Workflow

- Identity Test
- Sodium Adduct Level
- Harsh vs Standard Comparison
- Unknown Impurities Under FLP Peak

Targeted Workflow

- Detects and quantifies named impurities using extracted ion chromatogram (XIC) of mass data
- Quantifies unknown impurities chromatographically resolved from the FLP using integrated area of UV data
- Uses Quantify Assay ToF 2D Chromatographic Workflow

Untargeted Workflow

- Identifies unknown impurities coeluting with the FLP peak using m/z information
- Quantifies unknown impurities using extracted ion chromatogram (XIC) of mass data
- Uses Accurate Mass Screening Workflow

Targeted Workflow Automated Data Processing Steps

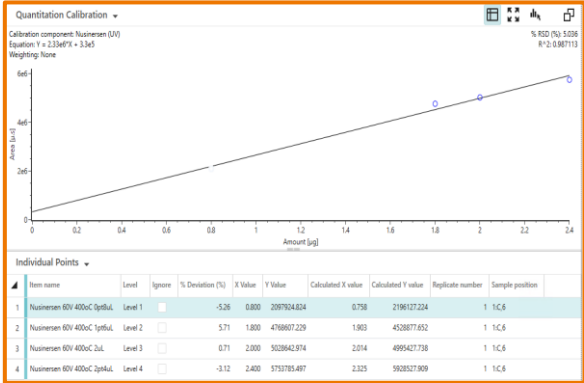
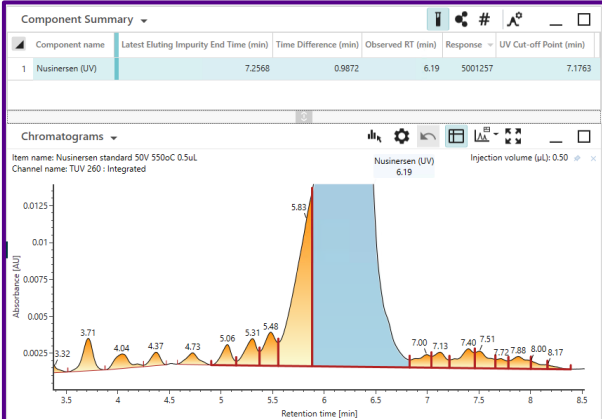
Steps for analysis of known impurities and impurities chromatographically resolved from FLP

1
Manual Adjustment

2
SST and Calibration Curves

3
Integration of UV data

4
Integration of XICs



Item name	Level	Ignore	% Deviation	X Value	Y Value	Calculated X value	Calculated Y value	Replicate number	Sample position
Nusinersen 60V 400c 0p5uL	Level 1	<input checked="" type="checkbox"/>	-5.26	0.000	2057034.824	0.758	2196127.224	1	1 C6
Nusinersen 60V 400c 1p5uL	Level 2	<input type="checkbox"/>	5.71	1.800	4768607.229	1.903	4528877.652	1	1 C6
Nusinersen 60V 400c 3uL	Level 3	<input type="checkbox"/>	0.71	2.000	5028642.914	2.014	4995427.738	1	1 C6
Nusinersen 60V 400c 5p5uL	Level 4	<input type="checkbox"/>	-3.12	2.400	5753795.497	2.325	5928527.909	1	1 C6

Region events (Full Range)

Type	Start	Stop	Value
1 Detect shoulder peaks	1	8	
2 Inhibit integration	8	12	

Region parameters (Full Range)

Chromatographic peak width
 Automatic peak width
Peak width: 60.0 seconds

Peak detection threshold
 Automatic detection
Detection threshold: []

Peak integration
Liftoff: 0.0 %
Touchdown: 0.0 %

Peak rejection
Minimum height: 0.0
Minimum area: 300.0

Peak detection limit
Maximum allowed number of peaks per chromatogram: 10

Untargeted Workflow Automated Data Processing Steps

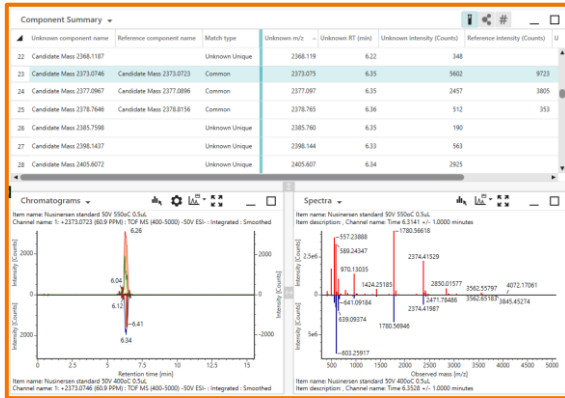
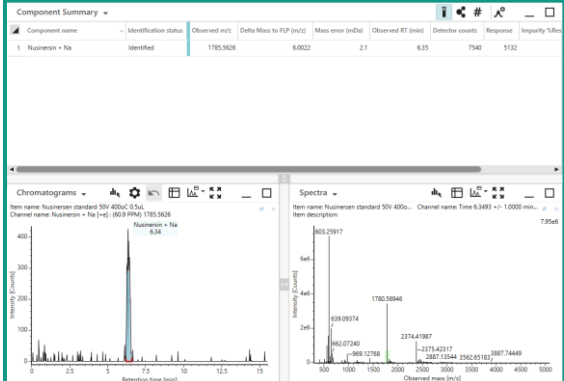
Steps for analysis of unknown impurities coeluting with FLP

1
Identity Test

2
Sodium Adduct Test

3
Harsh vs Soft

4
XIC of Unknown Impurities



Report Generation

Built-In Report Templates Enable Rapid Interrogation of Data

The collage displays several report pages from the UNIFI software interface, illustrating the built-in report templates for data analysis. The reports are for 'Nusinersen standard 50V 4000c 0.5ul' and 'Nusinersen standard 50V 4000c 0.5ul'.

Identity Test

Item name	Component name	Identified
Nusinersen standard 50V 4000c 0.5ul	Nusinersen	Identified

Sodium Adduct Test

Item name	Component name	Observed m/z	Delta Mass to FLP (m/z)	Detector counts
Nusinersen 60V 4000c 0.5ul	Nusinersen + Na	1785.5585	5.9992	5593
Nusinersen 60V 4000c 1pt6ul	Nusinersen + Na	1785.5591	5.9996	9451
Nusinersen 60V 4000c 2ul	Nusinersen + Na	1785.5593	6.0005	9358
Nusinersen 60V 4000c 3pt6ul	Nusinersen + Na	1785.5634	6.0022	

Spectrum View

Intensity [Counts] vs Observed mass [m/z]. Peaks labeled with m/z values: 1780.31459, 1780.81759, 1780.06302, 1781.06662, 1781.31957, 1781.56981, 1779.81069, 1781.82170, 1782.07189, 1779.55781, 1782.32367, 1790.0522, 1751.06099, 1764.07896, 1769.81136, 1776.57047, 1782.32559, 1790.04712, 1779.56045, 1782.07567, 1781.82478, 1779.81460, 1781.57344, 1780.06639, 1781.32283, 1780.31805, 1781.07190, 1780.56946.

Unknown Sample Impurities

Item name	Impurity Label	Observed m/z	Response (IIC Area)	(Delta Mass to FLP (m/z))	% Intensity Relative to FLP
Nusinersen standard 50V 4000c 0.5ul	n-3051.850	1016.5979	28	-762.9625	0.29
	n-2374.061	1186.0451	28	-356.1144	0.69
	n-1424.457	1423.4460	206	-1.9936	2.66
	n-15.97421	1775.5669	5844	3.0161	0.36
	n+12.06438	1782.5765	245	24.7679	0.37
	n+65.03120	1795.8183	110	32.5550	1.0
	n+99.07176	1804.3284	420	36.2907	
	n+130.2199	1812.1154	681	50.5753	
	n+145.1626	1815.8511	196	64.0648	
	n+202.3010	1830.1357	196	76.8684	
	n+216.2592	1838.6253	195	79.79	
	n+234.3864	1838.1571	160	82.2	
	n+307.4735	1856.4288	1014	82.2	
	n+319.1693	1862.3276	344		
	n+331.0684	1866.8077	269		
	n+348.9888	1872.4588	155		
	n+371.5933	1889.1217	134		
	n+438.2449	1898.6482	267		
	n+476.3511	1904.1177	227		
	n+498.2294	1935.3838	348		
	n+623.2934	2368.1187			
	n+2374.056	2373.0746			
	n+2390.144	2377.0967			
	n+2396.816	2378.7646			
	n+2424.797	2385.7598			
	n+2474.332	2398.1437			
	n+2504.187	2405.607			
	n+2507.191	2406.3			
	n+2547.681	2411			
	n+2567.601	2411			
	n+2599.667	2411			
	n+2620.980	2440.004			
	n+2643.775	2445.5181			
	n+2663.830	2451.1707	362		
	n+2686.440	2463.8342	578		
	n+2737.094	2464.5365	949		
	n+2739.904	2470.4504			
	n+2763.559				

Impurity Label: n-3051.850

Extracted Ion Chromatogram (EIC) showing Intensity [Counts] vs Retention time [min]. Peak at 6.35 min.

Impurity Label: n-2374.061

Extracted Ion Chromatogram (EIC) showing Intensity [Counts] vs Retention time [min]. Peak at 6.32 min.

Average Mass Spectrum

Intensity [Counts] vs Observed mass [m/z]. Peaks labeled with m/z values: 603.25917, 1780.56946, 641.09184, 2374.41987, 2436.15375, 3654.71230.

Table View

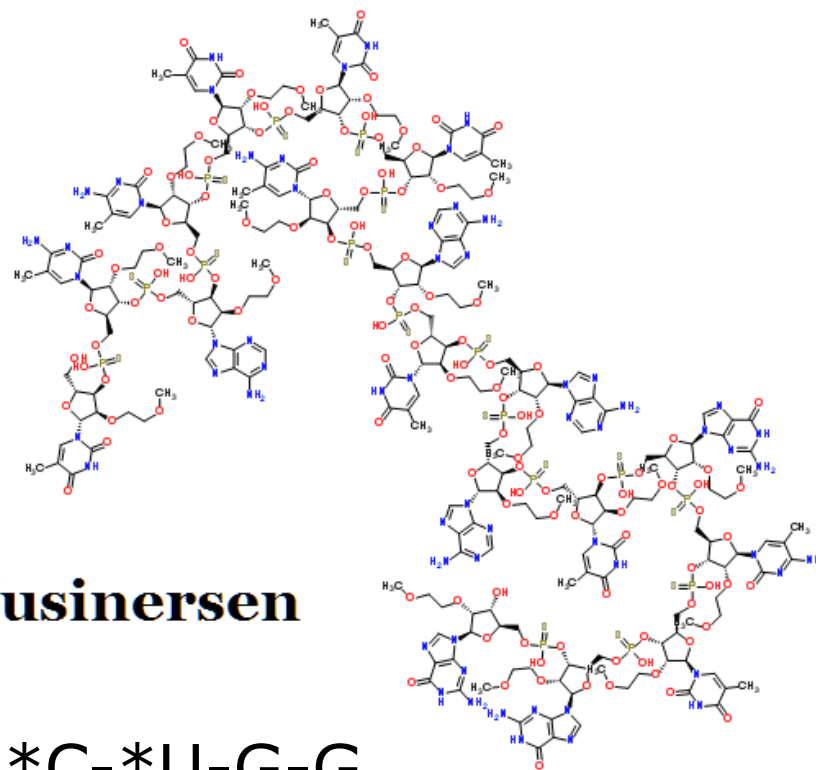
Unknown m/z	Unknown RT (min)	Soft intensity	Harsh intensity	Soft / Harsh	Needs Review?
511.182	5.70	7520	6248	1.2036	
589.243	5.52	63366	131919	0.4803	
1016.598	6.33	105	105		
1186.045	6.35	28	114	0.2481	Review
1423.446	6.34	751	962	0.7806	
1775.567	6.22	206	238	0.8643	
1782.577	6.37	5844	6262	0.9332	
1793.581	6.48	218	113	1.9223	Review
1795.818	6.49	245			

Data Package Available: Nusinersen

Build your own method based on this data package

- Nusinersen is an 18-mer antisense oligonucleotide used to treat spinal muscular atrophy
- The exact mass is 7122.2763 Dalton
- Sequence:

U-*C-A-*C-*U-*U-*U-*C-A-*U-A-A-*U-G-*C-*U-G-G
(methylation on "C" and "U")



MaxPeak HPS Technology

Greater consistency and repeatability, enhanced sensitivity and dynamic range, less passivation/conditioning time and cost

HRMS and Informatics Solutions

Improved sensitivity and mass resolution to address challenges analyzing increasingly complex oligonucleotides

Oligonucleotide Impurity Analysis Workflow Package

Semi-automated data analysis workflow package to streamline data analysis, reducing time, required training, and risk of error

Questions?

Waters™

Solving problems that matter

Ying_Qing_Yu@waters.com