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Comprehensive characterization of oligonucleotides and related impurities using advanced LC/Q-TOF analytical workflows

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Introduction

Therapeutic oligonucleotides are synthetic, short nucleic acid sequences designed to modulate gene expression by targeting RNA or DNA with high sequence specificity. However, their inherent structural heterogeneity makes impurity profiling essential, as during GMP manufacturing, impurities like phosphorothioate to phosphodiester, 5' and 3' truncations or extensions, depurination, and abasic species can directly impact both product safety and efficacy. Antisense oligonucleotides (ASO) like Nusinersen (7127.171 g/mol) are indicated for the treatment of spinal muscular atrophy (SMA). Their closely related variants coelute with the main peak, complicating their characterization in conventional one-dimensional LC/MS workflows. To address this challenge, a 2D LC/MS strategy was implemented using the Agilent 1290 Infinity III Bio LC system with multiple heart cutting, coupled to the 6545XT AdvanceBio LC/Q-TOF and MassHunter BioConfirm software. The first-dimension separation uses an ion pairing buffer-based method to achieve higher chromatographic peak resolution, while the second dimension provides MS compatible desalting, enabling enhanced mass spectrometric sensitivity and improved detection of low-level impurities.

Instrumentation

1290 Infinity III Bio 2D-LC & 6545XT Q-TOF includes:

- 1290 Infinity III Bio Flexible Pump (G7131A)
- 1290 Infinity III Bio High-Speed Pump (G7132A)
- 1290 Infinity III Bio Multisampler (G7137A)
- 1290 Infinity III Multicolumn Thermostat (G7116B)
- 1290 Infinity III Variable Wavelength Detector (G7114B)
- 1290 Infinity III Diode Array Detector (G7117B)
- 2D-LC System with MHC set up

Figure 2. Agilent 1290 Infinity III Bio 2D-LC coupled to an Agilent 6545XT AdvanceBio LC/Q-TOF.

Experimental

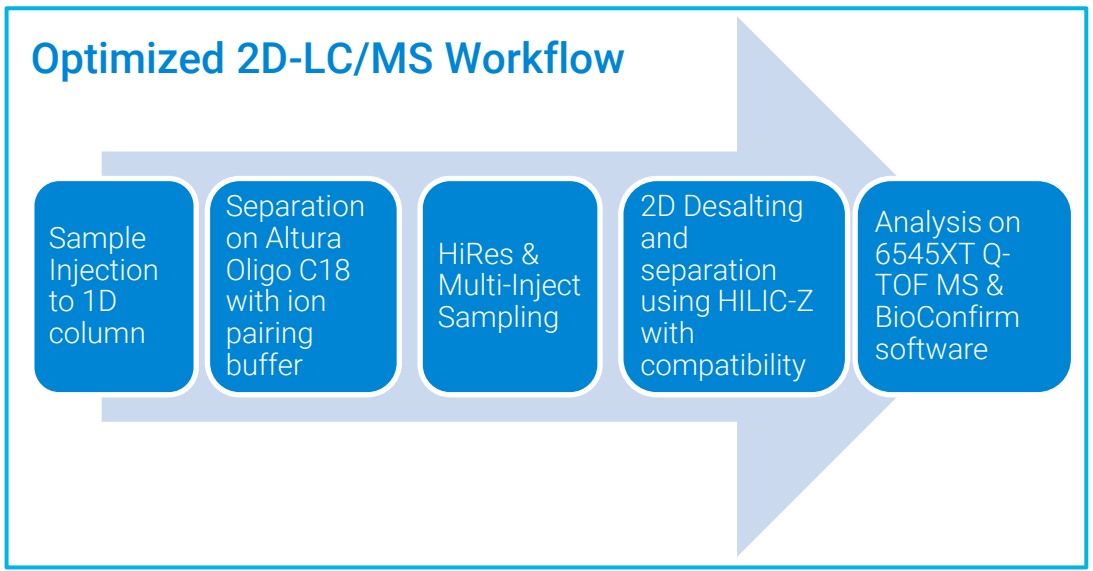
Table 1: 1D and 2D Method Parameters

Parameters	Details								
First-dimension									
Mobile Phase	Buffer: 0.2% Dibutyl amine and 0.2% HFIPA into 1L water; Mobile phase A: Buffer: methanol (80:20; v/v) Mobile phase B: Methanol: buffer (80:20; v/v)								
Flowrate	0.4 ml/min								
Gradient	Time (min)	0	2	5	10	20	27	31	32	35
	B (%)	05	05	35	70	70	95	95	05	05
Injection Vol	10 µL								
Column	Altura Oligo HPH-C18, 4.6 × 150 mm, 2.7 µm								
Column Temp	30 °C								
Detector	UV 260 nm								
Second- dimension									
Mobile Phase	Buffer: 15 mM Ammonium acetate in water Mobile phase A: Acetonitrile: buffer (70:30; v/v) Mobile phase B: Buffer: Acetonitrile (70:30; v/v)								
Flowrate	0.4 ml/min								
Gradient	Time (min)	0	0.5	1.2	2.4	3.0	3.5	4.0	
	B (%)	15	15	90	90	15	15	15	
Column	Altura Poroshell HILIC-Z, 2.1*100mm, 2.7 µm								
Column Temp	30 °C								
Flowrate	0.3 ml/min								
2D Cycle Time	46mins								
ASM Setting	Factor: 3; Flush factor: 5								

Table 2: MS Parameters

System	6545XT AdvanceBio LC/Q-TOF
Gas Temp	350 °C
Drying Gas	13 L/min
Nebulizer	35 psi
Sheath Gas Temp	250°C
Sheath Gas Flow	12 L/min
VCap	4500 V
Nozzle Voltage	2000 V
Fragmentor	180 V
Mass Range	*m/z* 400 to 3,200
Auto MS/MS Range	*m/z* 50 to 3,200
Polarity	Negative
Sort Precursors	By abundance only

Optimized 2D-LC/MS Workflow



Results and Discussion

Nusinersen Characterization Using BioConfirm

Step 1: Acquisition Setup 6545XT LC/Q-TOF

- MS & MS/MS data collected
- Wide mass range & optimized Collision energy used

Step 2: Define Sequence and Modifications

- Input nusinersen full length product (FLP) sequence into BioConfirm with backbone as phosphorothioate (PS)
- Define 2' O methoxyethyl (2' MOE) ribose modifications to enable accurate identification of targeted main peak and impurities

Step 3: BioConfirm Target Plus Impurities Workflow

- Identify the FLP at MS level alongside isoforms, truncated variants

Step 4: BioConfirm Sequence Confirmation Workflow

- Tolerance set to MS: ±10 ppm & MS/MS: ±20 ppm
- Identify and confirm presence of FLP, truncated, variants, isoforms, degradants etc.

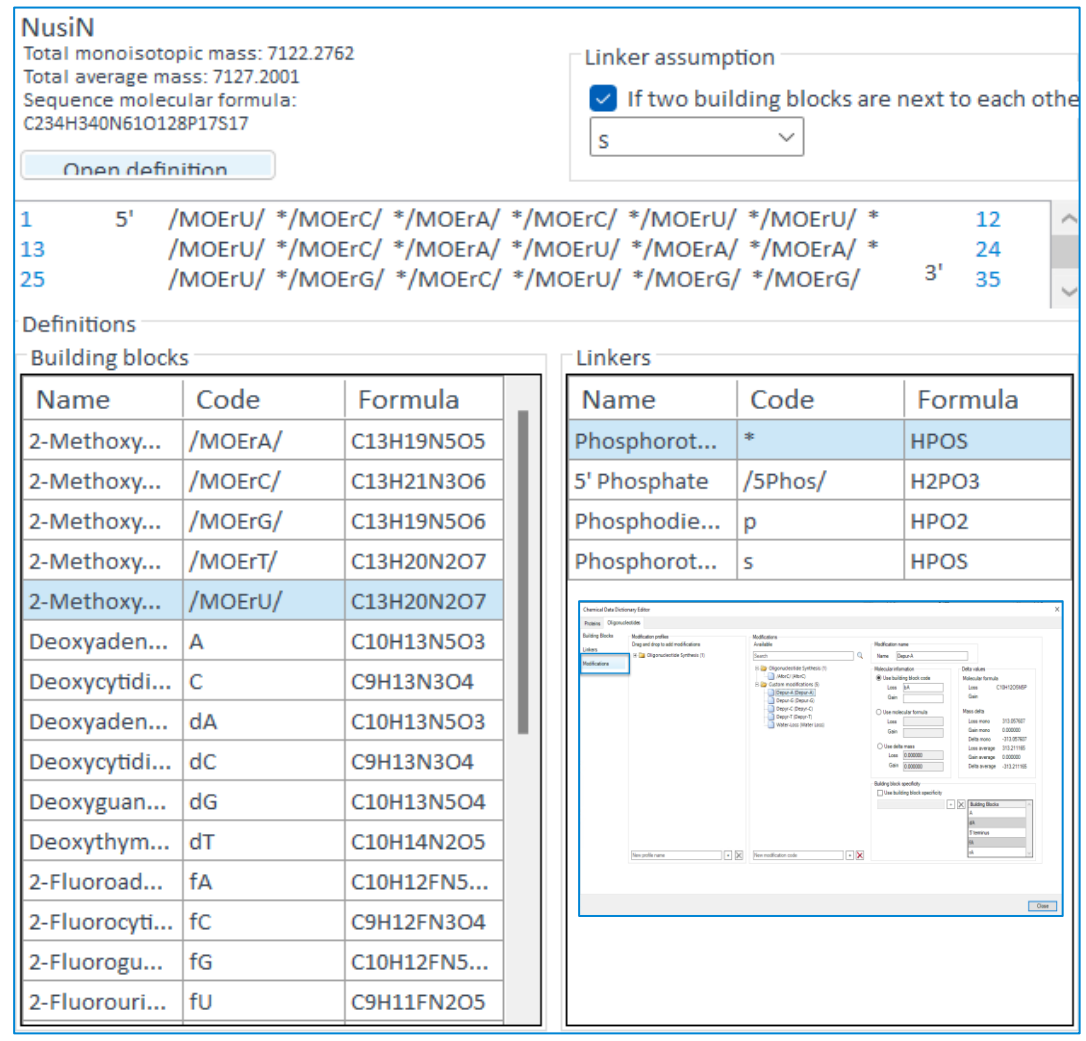
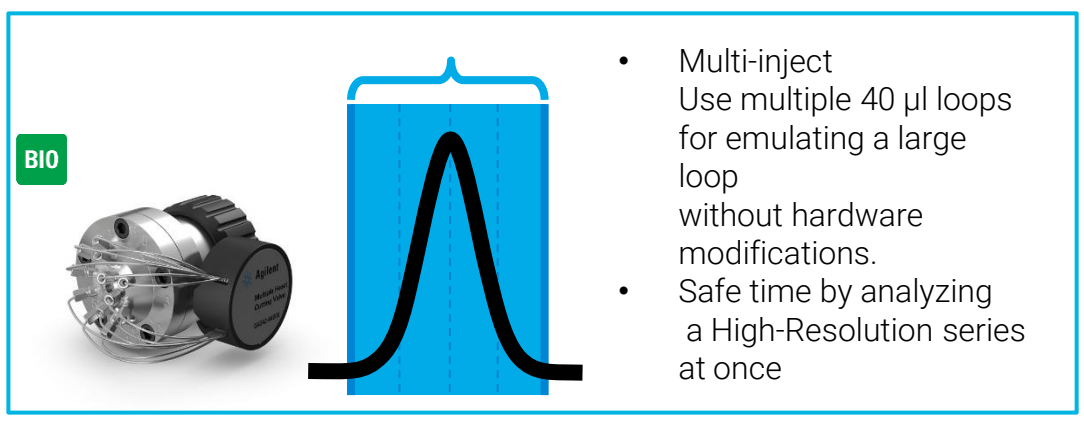


Figure 3. Nusinersen backbone nucleotide sequence and chemical modifications 2' O methoxyethyl (2' MOE) ribose in sequence using Chemical data dictionary set up in BioConfirm.

2D-LC Separation and HiRes Sampling Strategy

- Orthogonal 2D-LC using Altura oligo HPH (1D) and Altura HILIC-Z (2D) enabled high-resolution separation and MS-compatible desalting.
- HiRes sampling with multi-inject allowed combined transfer of heart-cuts, improving throughput and efficiency and identification of trace level peaks.



- Multi-inject
  Use multiple 40 µl loops for emulating a large loop
  without hardware modifications.
- Safe time by analyzing
  a High-Resolution series at once

Figure 5. Multi-Inject with Bio MHC Valve & Biocompatible Loops

Two targeted experiments:

- Experiment 1:
  - Focused on main peak at 16.0 min with the coeluting impurities peaks.
  - Targeted HiRes cuts captured main peak impurities
  - Enabled detailed profiling of FLP and coeluting impurities

- Experiment 2:
  - Focused on all the other peaks before 16.0 min
  - Multi-inject features transferred 10 heart-cuts per run, ensuring high-resolution separation and full MS data acquisition across all peaks.

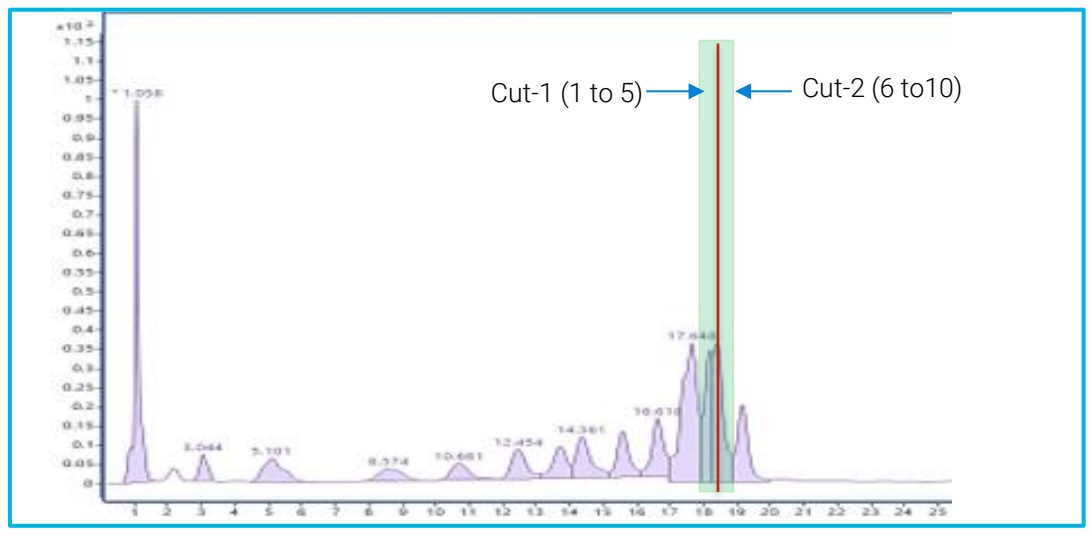
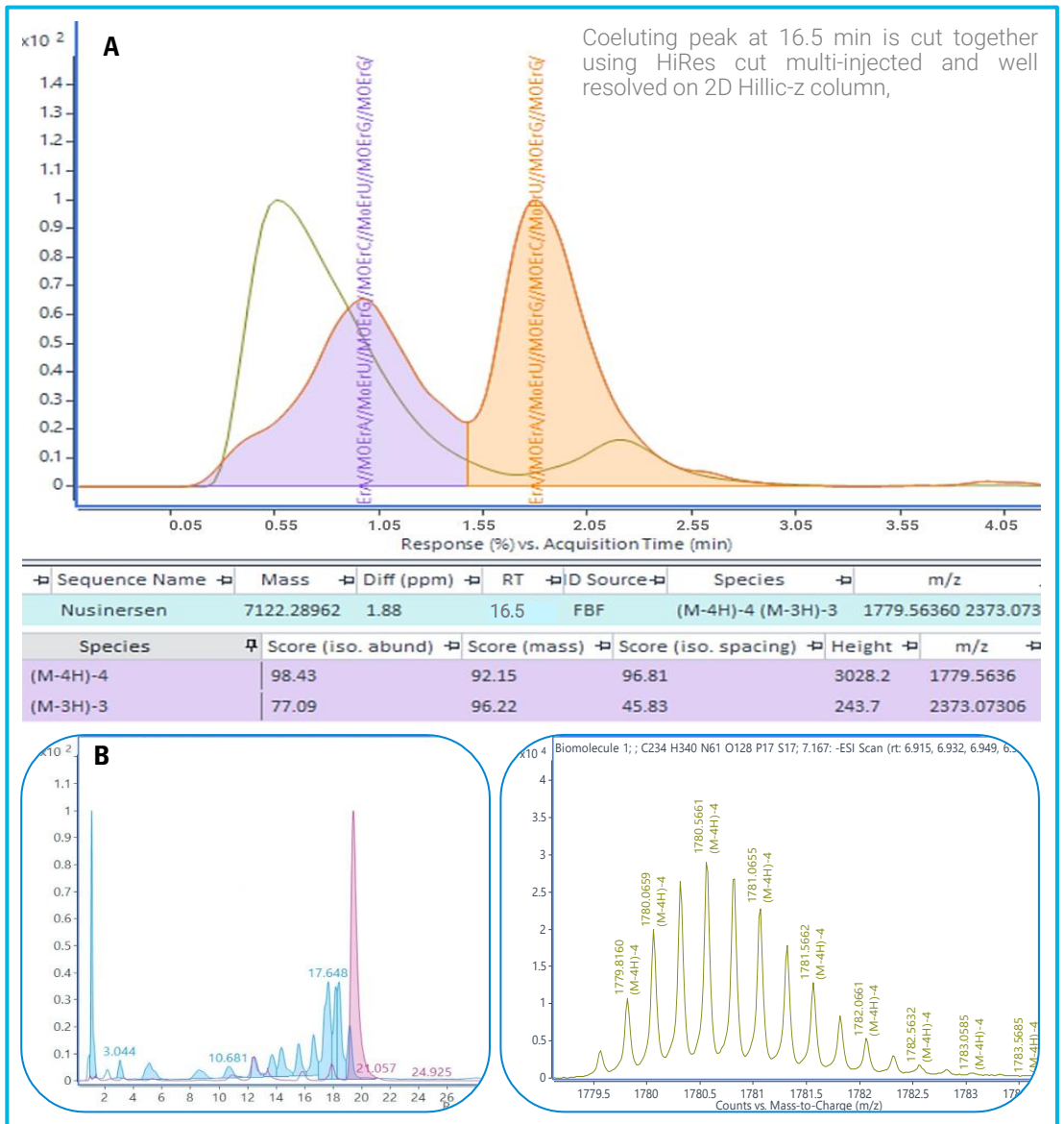


Figure 6. Overlay of 1D chromatograms with HiRes cut markers: Experiment 1 (Coeluting peak at 16 min) & Experiment 2 (Peaks prior to 16 min).

Results and Discussion

Experiment 1: Coeluting peak at 16.0 min



Sequence Name	Mass	Diff (ppm)	RT	D Source	Species	m/z
Nusinersen	7122.28962	1.88	16.5	FBF	(M-4H)-4 (M-3H)-3	1779.56360 2373.073

Species	Score (iso. abund)	Score (mass)	Score (iso. spacing)	Height	m/z
(M-4H)-4	98.43	92.15	96.81	3028.2	1779.5636
(M-3H)-3	77.09	96.22	45.83	243.7	2373.07306

Figure 7. (A) Separation between the two peaks (7122.29202 m/z with isomeric impurities) coeluting at 16 min on 2D column; (B) Identified FLP biomolecule overlaid with 1D UV chromatogram; (C) Charge envelope for identified mass 7122.29202 m/z

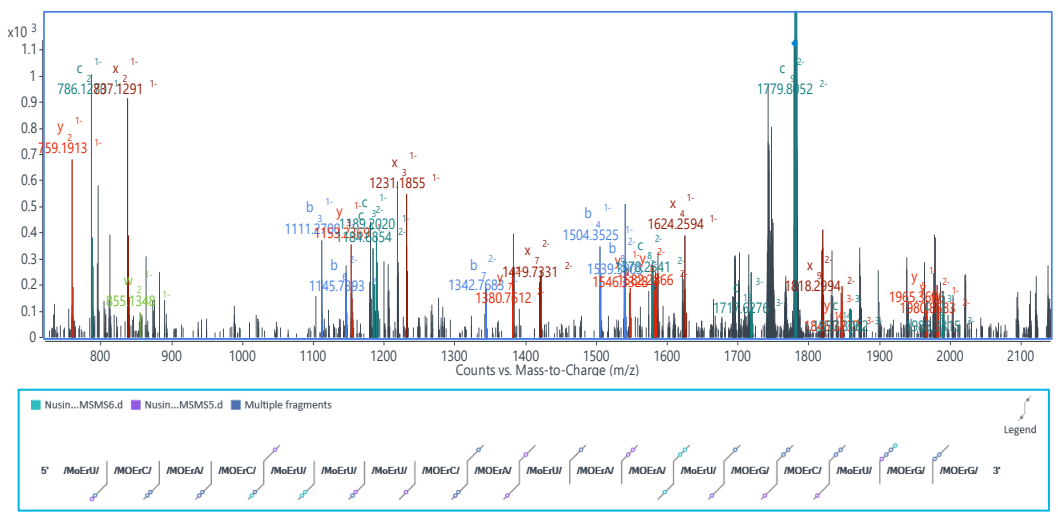


Figure 8. MS/MS fragmentation ladder of nusinersen (z = 4) showing y, b and w ions.

Best	Sequence Name	Mass	Diff (ppm)	ID Source	Species	m/z	Score (Bio)	Sequence	Variable Mods
	Nusinersen	7106.31028	1.57	FBF	(M-9H)-9 (M-7H	789.02784 1	94.29	/MoErU//MOErC//MOErA//	{/PS to PO/}

Score (iso. abund)	Score (mass)	Score (iso. spacing)	Height	Species	m/z
0	96.58	0	56.9	(M-9H)-9	789.02784
42.91	96.46	79.4	358.8	(M-7H)-7	1014.17684
82.93	92.87	98.12	413.8	(M-5H)-5	1420.25464
89.96	94.18	99.72	1129.3	(M-4H)-4	1775.56961
29.14	99.77	70.74	139.5	(M-3H)-3	2367.75518

Figure 9. Another coeluting peak identified as a PS to PO modified sequence with mass 7106.31028 m/z. The identity is further confirmed by MS/MS results which produced a set of ions containing 5' terminus (a-B, b & d), loss of H2O, and d & w ions.

Experiment 2: Impurity prior to the peak at 16.0 min

m/z	Mass	Variable Mods	Diff (Bio, ppm)	Score (Bio)	Sequence	Flags (Bio)
2374.42194	7122.28367		1.04	99.05	/MoErU//MOErC//MOErA//MOErC//MoErU//MoErU//MoErU//MOErC//MOErA//MoErU//MOErA//MoErU//MOErG//MOErC//MoErU//MOErG/	
2243.06823	6728.22278	{5-Trunc-L}	0.97	98.96	/MoErU//MOErC//MOErA//MOErC//MoErU//MoErU//MoErU//MOErC//MOErA//MoErU//MOErA//MoErU//MOErG//MOErC//MoErU//MOErG/{5-Trunc-L}	
1583.78019	6335.14643	{5-Trunc-L(x2)}	0.97	99.13	/MoErU//MOErC//MOErA//MOErC//MoErU//MoErU//MoErU//MOErC//MOErA//MoErU//MOErA//MoErU//MOErG//MOErC//MoErU//MOErG/{5-Trunc-L(x2)}	
1482.76224	5932.07508	{5-Trunc-L(x3)}	1.06	98.66	/MoErU//MOErC//MOErA//MOErC//MoErU//MoErU//MoErU//MOErC//MOErA//MoErU//MOErA//MoErU//MOErG//MOErC//MoErU//MOErG/{5-Trunc-L(x3)}	
1384.49378	5538.99933	{5-Trunc-L(x4)}	1.18	98.68	/MoErU//MOErC//MOErA//MOErC//MoErU//MoErU//MoErU//MOErC//MOErA//MoErU//MOErA//MoErU//MOErG//MOErC//MoErU//MOErG/{5-Trunc-L(x4)}	
1714.97361	5144.93755	{5-Trunc-L(x5)}	0.91	98.86	/MoErU//MOErC//MOErA//MOErC//MoErU//MoErU//MoErU//MOErC//MOErA//MoErU//MOErA//MoErU//MOErG//MOErC//MoErU//MOErG/{5-Trunc-L(x5)}	
1583.28617	4750.87596	{5-Trunc-L(x6)}	0.65	99.2	/MoErU//MOErC//MOErA//MOErC//MoErU//MoErU//MoErU//MOErC//MOErA//MoErU//MOErA//MoErU//MOErG//MOErC//MoErU//MOErG/{5-Trunc-L(x6)}	
1451.93276	4356.81551	{5-Trunc-L(x7)}	0.6	99.27	/MoErU//MOErC//MOErA//MOErC//MoErU//MoErU//MoErU//MOErC//MOErA//MoErU//MOErA//MoErU//MOErG//MOErC//MoErU//MOErG/{5-Trunc-L(x7)}	
1320.90702	3963.73897	{5-Trunc-L(x8)}	0.51	99.44	/MoErU//MOErC//MOErA//MOErC//MoErU//MoErU//MoErU//MOErC//MOErA//MoErU//MOErA//MoErU//MOErG//MOErC//MoErU//MOErG/{5-Trunc-L(x8)}	
1981.85787	3963.73784	{5-Trunc-L(x8)}	0.23	85.97	/MoErU//MOErC//MOErA//MOErC//MoErU//MoErU//MoErU//MOErC//MOErA//MoErU//MOErA//MoErU//MOErG//MOErC//MoErU//MOErG/{5-Trunc-L(x8)}	
1780.32845	3560.66831	{5-Trunc-L(x9)}	0.82	99.19	/MoErU//MOErC//MOErA//MOErC//MoErU//MoErU//MoErU//MOErC//MOErA//MoErU//MOErA//MoErU//MOErG//MOErC//MoErU//MOErG/{5-Trunc-L(x9)}	
1582.7973	3166.60616	{5-Trunc-L(x10)}	0.23	99.42	/MoErU//MOErC//MOErA//MOErC//MoErU//MoErU//MoErU//MOErC//MOErA//MoErU//MOErA//MoErU//MOErG//MOErC//MoErU//MOErG/{5-Trunc-L(x10)}	
1381.26219	2763.53595	{5-Trunc-L(x11)}	0.75	99.41	/MoErU//MOErC//MOErA//MOErC//MoErU//MoErU//MoErU//MOErC//MOErA//MoErU//MOErA//MoErU//MOErG//MOErC//MoErU//MOErG/{5-Trunc-L(x11)}	
1179.7257	2360.46243	{5-Trunc-L(x12)}	0.03	99.25	/MoErU//MOErC//MOErA//MOErC//MoErU//MoErU//MoErU//MOErC//MOErA//MoErU//MOErA//MoErU//MOErG//MOErC//MoErU//MOErG/{5-Trunc-L(x12)}	
982.19537	1966.40452	{5-Trunc-L(x13)}	1.09	96.42	/MoErU//MOErC//MOErA//MOErC//MoErU//MoErU//MoErU//MOErC//MOErA//MoErU//MOErA//MoErU//MOErG//MOErC//MoErU//MOErG/{5-Trunc-L(x13)}	
772.66215	1547.33757	{5-Trunc-L(x14)}	1.07	97.86	/MoErU//MOErC//MOErA//MOErC//MoErU//MoErU//MoErU//MOErC//MOErA//MoErU//MOErA//MoErU//MOErG//MOErC//MoErU//MOErG/{5-Trunc-L(x14)}	
576.12383	1154.26159	{5-Trunc-L(x15)}	1.41	95.21	/MoErU//MOErC//MOErA//MOErC//MoErU//MoErU//MoErU//MOErC//MOErA//MoErU//MOErA//MoErU//MOErG//MOErC//MoErU//MOErG/{5-Trunc-L(x15)}	
759.19324	760.20043	{5-Trunc-L(x16)}	0.59	99.61	/MoErU//MOErC//MOErA//MOErC//MoErU//MoErU//MoErU//MOErC//MOErA//MoErU//MOErA//MoErU//MOErG//MOErC//MoErU//MOErG/{5-Trunc-L(x16)}	
759.19297	760.20022	{5-Trunc-L(x16)}	0.3	99.13	/MoErU//MOErC//MOErA//MOErC//MoErU//MoErU//MoErU//MOErC//MOErA//MoErU//MOErA//MoErU//MOErG//MOErC//MoErU//MOErG/{5-Trunc-L(x16)}	

Figure 10: (A) Multiple heart cut across the impurity at Rt_12.45 min; (B) MS/MS fragmentation of the impurity points to a truncation (N-1); (C) Table shows sequences for all the peaks at different Rt were identified as truncated impurity

Conclusions

- A 2D-LC/MS workflow enabled effective separation and identification of coeluting impurities.
- HiRes sampling with multi-injection improved resolution and throughput while preserving 1D separation for MS-compatible desalting.
- 6545XT AdvanceBio LC/Q-TOF delivered accurate mass and MS/MS data for low-abundance impurity detection.
- MassHunter BioConfirm software enabled automated sequence confirmation and impurity identification.
- This integrated platform offers a robust solution for oligonucleotide characterization in therapeutic development.

References

Analysis of GLP-1 Agonists Using the Agilent 1290 Infinity II Bio LC System and Altura Ultra Inert HPLC Column. Agilent publication number5994-8837EN, 2025.

https://www.agilent.com/en/promotions/asms

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