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A Novel and Highly Effective LC/MS Method for Phosphorodiamidate Morpholino Oligomer (PMO) Impurity and Sequencing Analysis

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Introduction

Phosphorodiamidate morpholino oligomers (PMOs) are a type of Antisense Oligonucleotides (ASOs), a rapidly growing class of RNA modalities. Currently, four ASOs based on PMO chemistry are approved by the FDA.[1,2] These PMOs possess a non-ionic backbone in which the ribose is replaced by a morpholine moiety and the phosphorodiester intersubunit bonds are replaced with phosphorodiamidate linkages (Figure 1). The PMO structure confers distinctive properties compared to other antisense strategies.[3,4] These structural differences also make PMO characterization by LC/MS unique to other oligonucleotides. In this work, we describe the characterization of PMOs by LC/MS using a unique method. Purity analysis, impurity identification and PMO sequence confirmation are described.

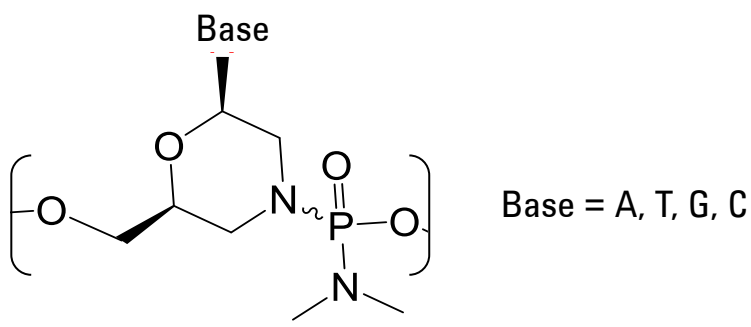


Figure 1. Chemical Structure of Phosphorodiamidate morpholino oligomers (PMOs)

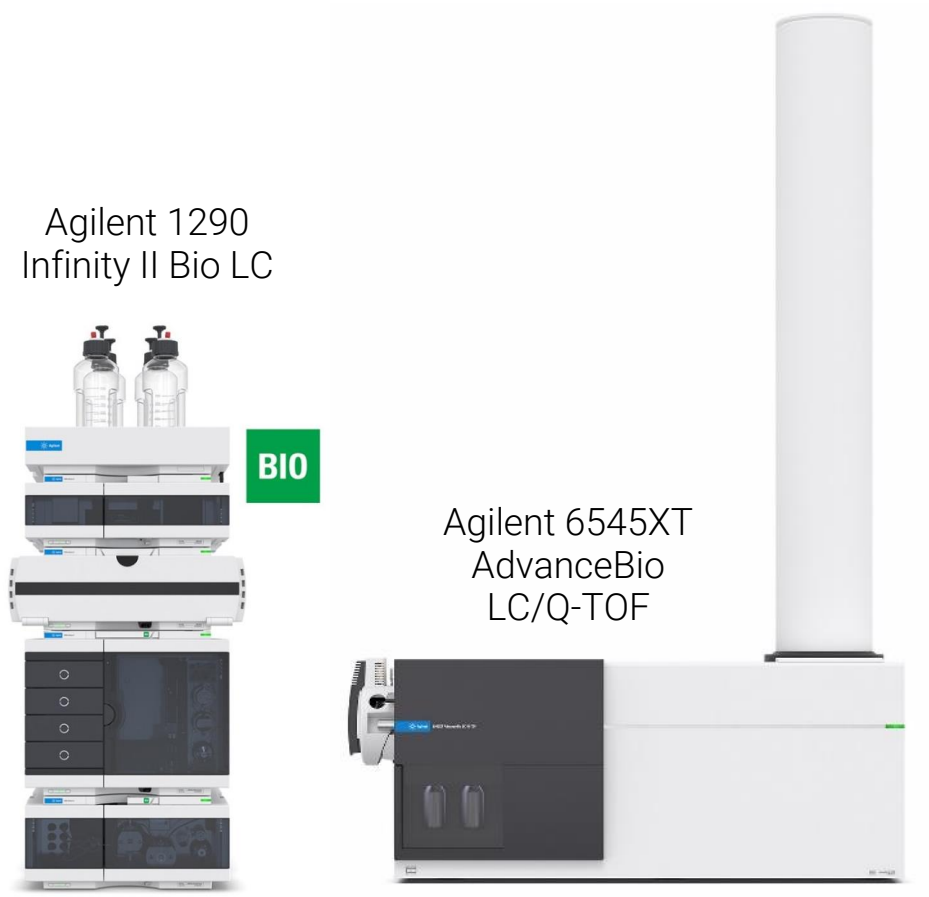


Figure 2. LC/MS Configuration

Experimental

Oligonucleotide Samples Analysis

LC/MS analyses were conducted on a 1290 Infinity II Bio LC system coupled with a 6545XT AdvanceBio LC/Q-TOF equipped with an Agilent Dual Jet Stream ESI source (Figure 2). LC separation was obtained with a ZORBAX RRHD 300Å StableBond C3 column, 1.8 µm, 2.1 x 50mm at 40 °C. For characterization and impurity analysis, data was acquired with a negative MS spectrum acquisition from 400 to 3200 *m/z*. For sequence confirmation analysis, data was acquired with targeted MS/MS mode on -3 charged ion. (Table 1). The resulting data were processed in Agilent MassHunter BioConfirm software 12.1 using both targeted and untargeted methods.

Table 1. LC/MS method used in the study

Agilent 1290 Infinity II Bio LC Conditions		
Column	ZORBAX RRHD 300Å StableBond C3 column, 1.8 µm, 2.1 x 50 mm (pn: 857750-909)	
Injection volume	1 or 2 µL	
Mobile phase	A = Water + 5 mM Ammonium Acetate B = Acetonitrile	
Flow rate	0.4 mL/min	
Gradient program	Time (min) 0.00 4.00	B (%) 5 90
Stop time	4.00 min	
Post time	1.50 min	

6545XT AdvanceBio LC/Q-TOF Source Conditions	
Ion Polarity	Dual AJS Negative
Gas temperature	325 °C
Drying gas flow	12 L/min
Nebulizer gas	35 psi
Sheath gas temperature	350 °C
Sheath gas flow	12 L/min
Capillary voltage	3500
Nozzle voltage	2000V
Fragmentor	175 V

Experimental

Table 1. LC/MS method used in the study (continued)

6545XT AdvanceBio LC/Q-TOF Acquisition Conditions	
Characterization and Impurity Analysis	
Mass Range	400 – 3200 *m/z*
Acquisition Rate	1 spectra/sec
Sequence Confirmation	
MS Mass Range	400 – 3200 *m/z*
MS Acquisition Rate	4 spectra/sec
MS/MS Mass Range	100 – 3200 *m/z*
MS/MS Acquisition Rate	1 spectra/sec
Isotope Width	Medium (~ 4 *m/z*)
Collision Energies	55 V, 60 V, 65 V or 70 V
Targeted Mass	2068.7238 *m/z*
Retention Time	2.16 min
Delta Retention Time	1 min

Results and Discussion

Characterization and Impurity Analysis

Figure 3 shows the raw spectrum of PMO (200 ng on column), and the -3 charged ion dominated.

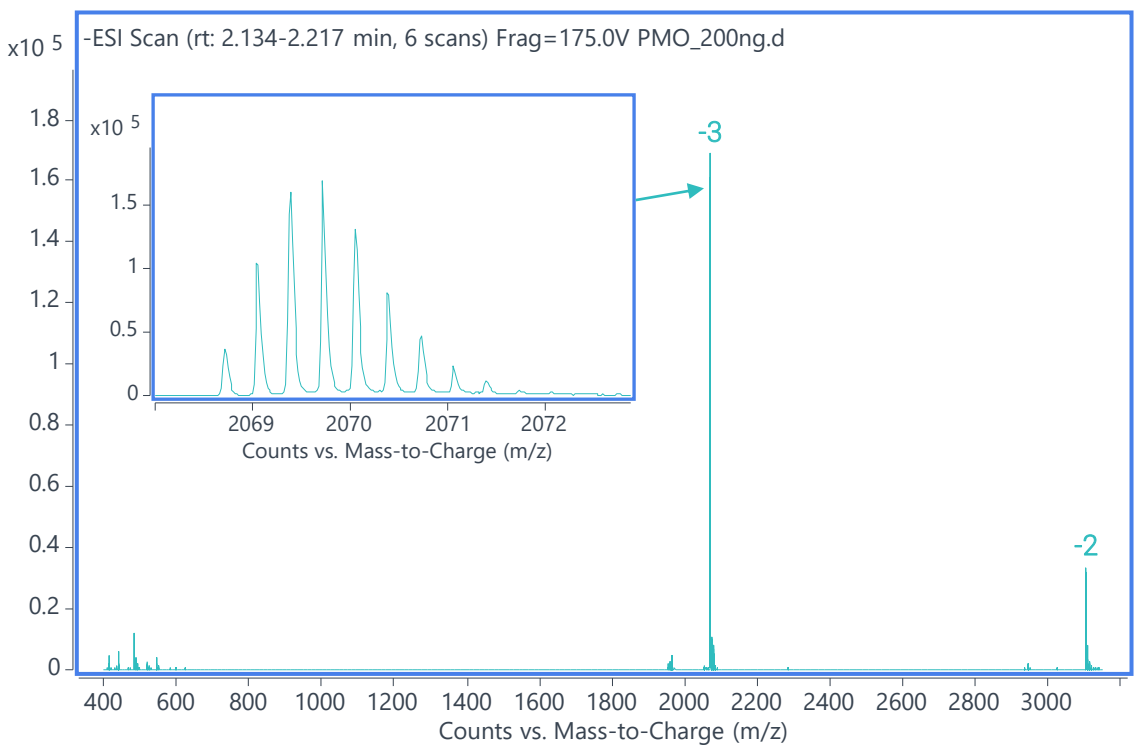
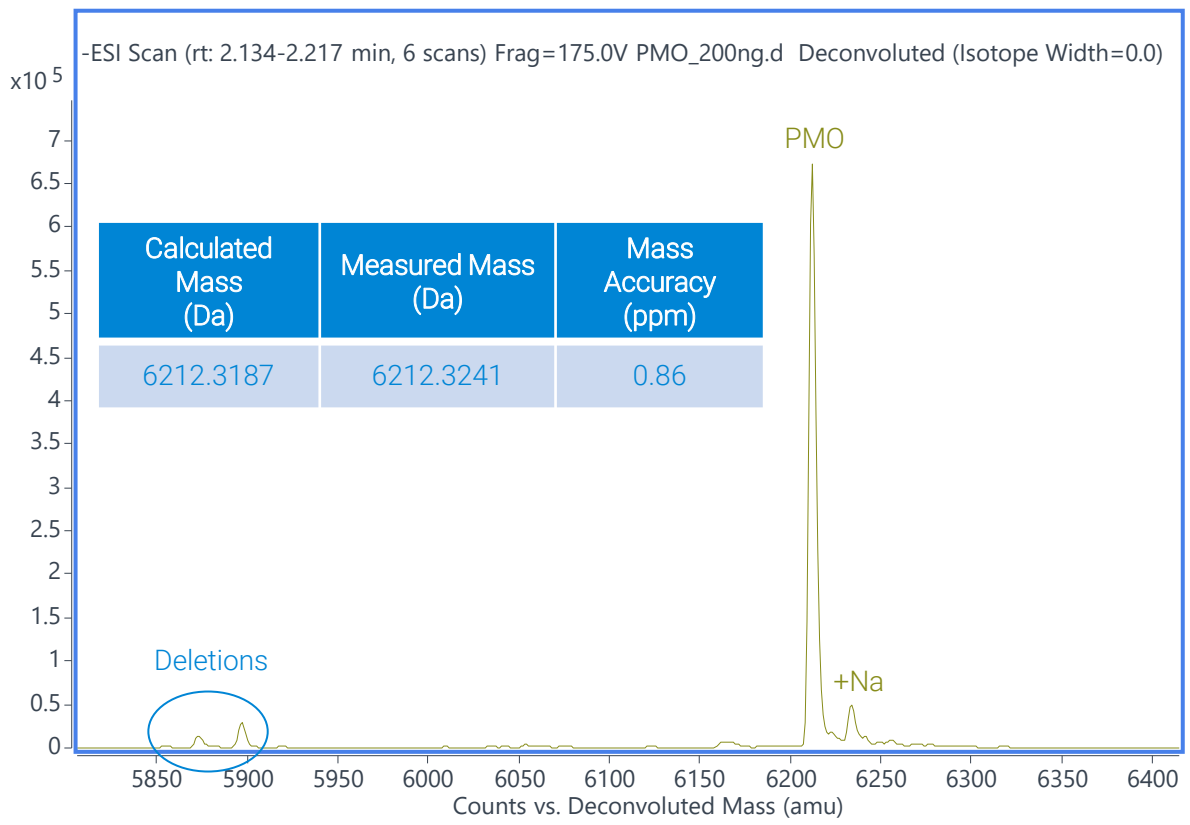


Figure 3. Raw spectrum for PMO.

Results and Discussion

Characterization and Impurity Analysis

Figure 4 shows the deconvolution result of PMO, and mass accuracy of PMO was 0.86 ppm. Multiple low-abundance impurities were identified: A deletion, C deletion and T deletion.



Calculated Mass (Da)	Measured Mass (Da)	Mass Accuracy (ppm)
6212.3187	6212.3241	0.86

Figure 4. The deconvoluted spectrum for PMO.

Sequence Confirmation Analysis

For sequence confirmation analysis, targeted MS/MS was conducted on -3 charge state precursor (Figure 3) using 4 different collision energies.

Table 2. MassHunter BioConfirm method parameters used for PMO sequence confirmation analysis

Agilent MassHunter BioConfirm 12.1 Parameters	
Workflow	Oligonucleotides
Experiment	Sequence confirmation
Match Tolerance	Tolerance: 5 ppm Theoretical profile relative abundance ≥ 12%
Absolute Height Threshold	350
Matching Criteria	Warn if score is < 90 Do not match if score is <85
Extraction MS/MS	Group by collision energy 2 scans averaged

Results and Discussion

Sequence Confirmation Analysis

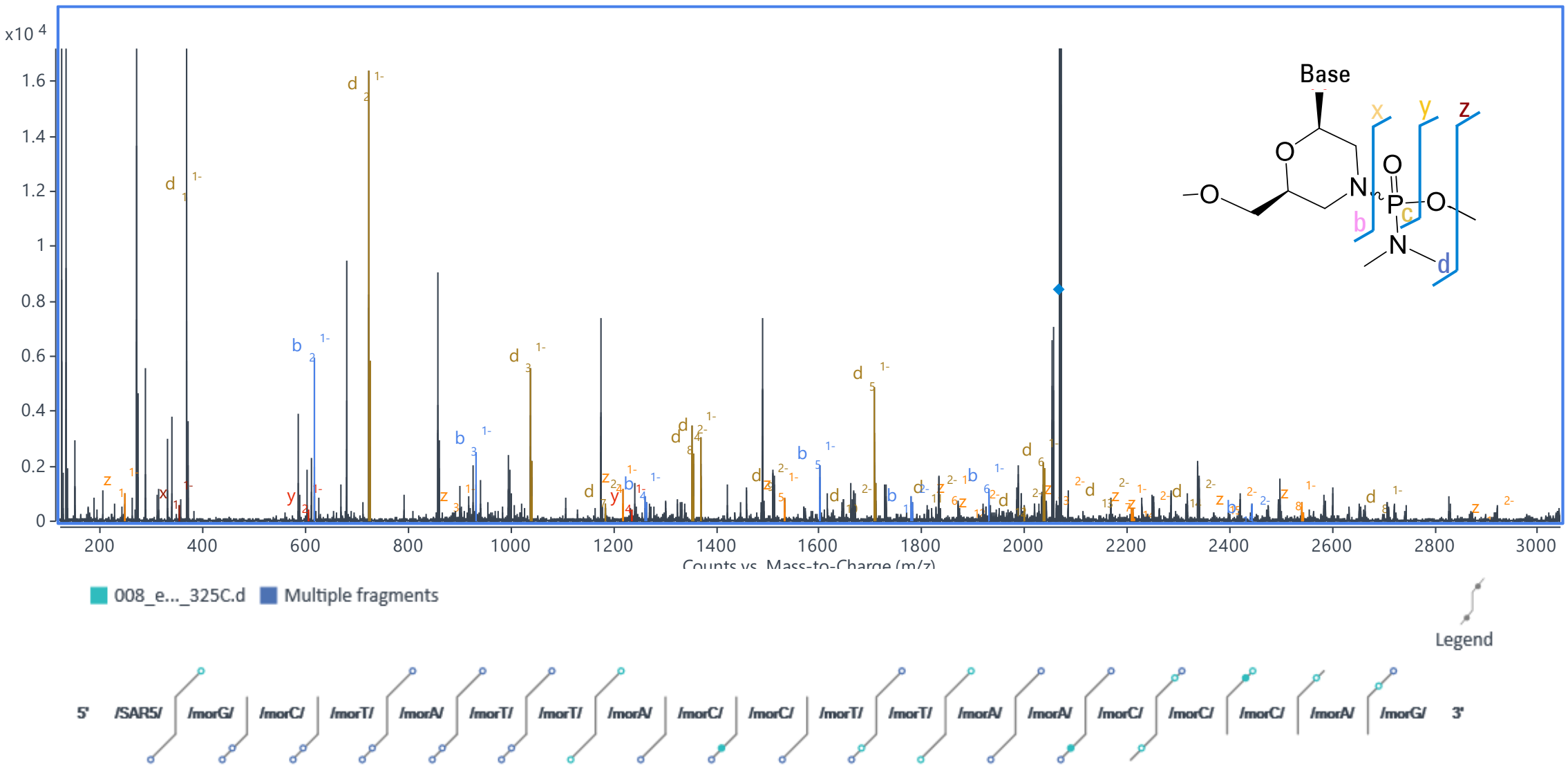


Figure 6. PMO sequence confirmation data. 100% sequence coverage achieved.

Complete sequence coverage was achieved in Agilent MassHunter BioConfirm software (Table 2) using the data from 2 µL injection of 0.2 mg/mL PMO sample (Figure 6). From the fragment confirmation ladder in Figure 6, b, d and z ions dominated. Based on PMO structure, a and w ions should not be seen.

Conclusions

- This work describes an original LC/MS method for PMO impurity and sequencing analysis.
- Excellent mass accuracy for PMO was achieved.
- Multiple low-abundance impurities were identified.
- 100% sequence coverage was obtained with a single injection.

References

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