Application Note

Biopharma

Analysis of Oligonucleotides Using Ion-Pairing Alternatives on the Agilent Pro iQ Plus

Authors

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Abstract

This application note presents a practical approach for medium- to high-throughput molecular confirmation of synthetic oligonucleotides using the Agilent InfinityLab Pro iQ Plus LC/MS system. Traditional ion-pair reversed-phase LC/MS methods often require dedicated instrumentation and toxic, costly reagents. In contrast, the method described here uses an ammonium bicarbonate-based method, eliminating the need for ion-pairing agents while maintaining sufficient chromatographic retention and MS sensitivity. Agilent OpenLab CDS MS spectral deconvolution simplifies data analysis workflows, enabling automated processing with minimal optimization. This approach provides robust LC/MS performance for various oligonucleotide types, including antisense oligonucleotides and siRNAs.

Introduction

Oligonucleotides are an emerging therapeutic modality that target and modulate gene expression through the silencing or degradation of mRNA. Once an mRNA target is identified, the antisense sequence must be optimized with strategically incorporated chemical modifications to improve pharmacokinetics[1], affinity, and minimize off-targeting or mismatching.[2]

Ion-pair reversed-phase LC/MS is commonly used to confirm the molecular weights of target oligo sequences to ensure proper synthesis. However, using alkylamines as ion-pairing agents often requires dedicated instrumentation. Furthermore, toxic and cost-prohibitive perfluorinated alcohols such as HFIP (hexafluoroisopropanol) are required for optimal chromatographic separation and MS sensitivity.

In this application, an alternative reversed-phase approach was used for molecular confirmation of oligonucleotides. The method used ammonium bicarbonate instead of ion pairing, while still providing sufficient chromatographic retention and MS sensitivity. Twenty replicate injections of three different antisense oligonucleotides and five replicates of a single siRNA were performed to ensure the applicability and reproducibility of the method.

Experimental

Instrument configuration

This experiment was conducted using the following instrument configuration:

- Agilent InfinityLab Pro iQ Plus LC/MS system (G6170A)
- Agilent Infinity II 1290 bio binary pump (G7120A)
- Agilent Infinity II 1290 bio multisampler (G7167B)
- Agilent Infinity II 1290 bio column compartment (G7116B)
- Agilent Infinity II 1260 diode array detector HS (G7117C)

Although this analysis used an Infinity II LC configuration, comparable results can be achieved on the Infinity III LC system with no changes to method parameters.

Sample preparation

All samples were resuspended in deionized (DI) water to a concentration of 50 µM and stored at –80 °C. Samples were transferred to polypropylene vials and stored in the temperature-controlled autosampler for up to two days prior to analysis. Oligonucleotide sample sequences are shown in Table 1.

Table 1. Oligonucleotide sample sequences.

Oligonucleotide Name	Length	Sequence
ASO-1	18	dU/MOErC//MOErA//MOErC/dUdUdU/MOErC// MOErA/dU/MOErA//MOErA/dU/MOErG/CdU/ MOErG/G
ASO-2	20	dU/MOErC/dUdU/MOErG/TT/MOErA//MOErC// MOErA//MOErT//MOErG//MOErA//MOErA// MOErA/dU/MOErC//MOErC//MOErC/C
Fomivirsen	21	G*C*G*T*T*T*G*C*T*C*T*T*C*T*T*C*T*T*G*C*G
Givosiran	22 S	mC*mA*mGmAmAmAfGmAfGmUfGmUfCmUfCmAm UmCmUmUmA/L96/
	23 AS	mU*mG*mGfUmCfUmUfUfCmUfCfAmCfAmGfAmGfU mAmGfA*fA*mU

Code	Description
/MOErA/	Methoxyethoxy A
/MOErC/	Methoxyethoxy C
/MOErT/	Methoxyethoxy T
/MOErG/	Methoxyethoxy G
dU	Deoxyuridine
fA	2-fluoroadenosine
fC	2-fluorocytidine
fG	2-fluoroguanadine
fU	2-fluorouridine
*	Phosphorothioate bond
A	2'-deoxyribose adenine
C	2'-deoxyribose cytosine
G	2'-deoxyribose guanine
T	2'-deoxyribose thymine
mA	2'-O-methyl A
mC	2'-O-methyl C
mG	2'-O-methyl G
mU	2'-O-methyl U
rA	Ribose adenine
rC	Ribose cytosine
rG	Ribose guanine
rU	Ribose uracil

LC/MS analysis

Source parameters for the Pro iQ Plus system are provided in Table 2, while high-performance liquid chromatography (HPLC) parameters are provided in Table 3.

Table 2. Source parameters for the Agilent Pro iQ Plus system.

Mass Spectrometry Parameters	
Parameter	**Value**
MS	Agilent Pro iQ Plus
Source	Agilent Jet Stream Electrospray Ionization (AJS-ESI) Source
Drying Gas Flow	13.0 L/min
Gas Temp	300 °C
Nebulizer Pressure	35 psi
Capillary Voltage	3,000 V
Sheath Gas Temp	250 °C
Sheath Gas Flow	11 mL/min
Nozzle Voltage	1,500 V
Mode	Positive
Scan	*m/z* 700–2,800
Scan Time	1,250 ms
Fragmentor	180 V
Gain Factor	5

Table 3. HPLC parameters used.

Parameter	Value
Column	Agilent AdvanceBio oligonucleotide column, 2.1 × 50 mm, 2.7 µm
Sampler Temperature	8 °C
UV Detection	260/4 nm (Ref 360/20 nm) Peak width > 0.1 min (2.5 Hz)
Mobile Phase A	20 mM ammonium bicarbonate in DI water
Mobile Phase B	Methanol
Flow Rate	0.7 mL/min
Injection Volume	2 µL
Multiwash	20:80 water:methanol; flush port; 5 seconds 90:10 water:methanol; flush port; 3 seconds
Column Temperature	75 °C
Post Time	1.0 min
Gradient Program	Time (min) %B 0 5 0.1 5 3.0 40 3.1 80 3.5 80 3.6 5

Results and discussion

Established in 1997, an alkylamine ion pair with perfluorinated alcohol as the acidic modifier is the preferred mobile phase for LC/MS analysis of oligonucleotides.[3] This is due to its chromatographic performance and electrospray efficiency, especially when compared to mobile phases using acetate as the counter ion. Extensive work has further demonstrated a wide experimental design space when using this powerful ion-pairing system.[4]

However, there are consequences when using alkylamine and HFIP for LC/MS methods. First, optimization of buffer components is necessary since oligo modifications and sequence affect electrospray desorption and chromatography.[5] Second, alkylamine may contaminate ionization sources and LC systems, leading to background peaks if polarity is switched back to positive mode. Consequently, this requires extensive cleaning/passivation of LC components and ion source surfaces. Even then, some labs may dedicate systems to negative mode due to the adsorption of alkylamines onto the LC/MS system. Finally, alkylamine-containing mobile phases have a short shelf life if not kept sealed under argon gas, and thus, mobile phases must be made fresh, sometimes daily, to ensure consistent method performance.

For labs performing molecular weight confirmation workflows where many oligos are analyzed, optimizing mobile phase conditions may not be feasible. Additionally, LC/MS downtime due to instrument maintenance and daily preparation of buffers may lead to sample backlogs, which for many labs can be a considerable challenge. A more practical approach would be to use a non-ion-pairing, reversed-phase methodology.

Recent work provides a more practical and cost-effective alternative to ion pairs, using an ammonium bicarbonate (NH_4HCO_3) buffer and methanol as the strong solvent.[6] This is an advantageous method for molecular weight confirmation, as ESI sensitivity and chromatographic performance are sufficient, even with minimal optimization of the mobile phase and gradient. Further, this referenced work postulates that carbon dioxide outgassing facilitates droplet formation, while ammonia evaporation contributes to proton adduction, thus allowing for positive mode analysis. This, therefore, eliminates the need to dedicate a system for negative mode analysis.

Because oligonucleotides are polar and negatively charged, there may be concerns that these analytes would not be retained or separate chromatographically without ion pairing. However, ammonium bicarbonate-based methods retain a wide variety of oligonucleotides and perform particularly well with modified oligos, such as antisense oligos (ASOs; Figure 1). The method demonstrated here provides a proof-of-concept for medium- to high-throughput analysis. Therefore, the gradient is 5% to 40% Mobile Phase B over three minutes. This relatively "ballistic" gradient provides excellent retention and peak shape, and the 0.7 mL/min flow rate does not negatively impact the spectral quality nor sensitivity.

Like most large molecules with multiple ionizable functional groups, oligos appear as multiply charged ions when analyzed by ESI-MS. Importantly, depending on the alkylamine ion pair and source parameters, charge state distribution of the oligo may vary.[7] This can be problematic with heated ionization sources, which tend to yield higher charge states. Consequently, deconvolution of the spectrum may lead to artifacts and potential misidentifications. On the other hand, ammonium bicarbonate (ABC) tends to yield oligos with lower charge states (Figure 2). The higher *m/z* values will have less spectral overlap with matrix interferences, impurities, or other components, thus improving confidence in the deconvoluted data.

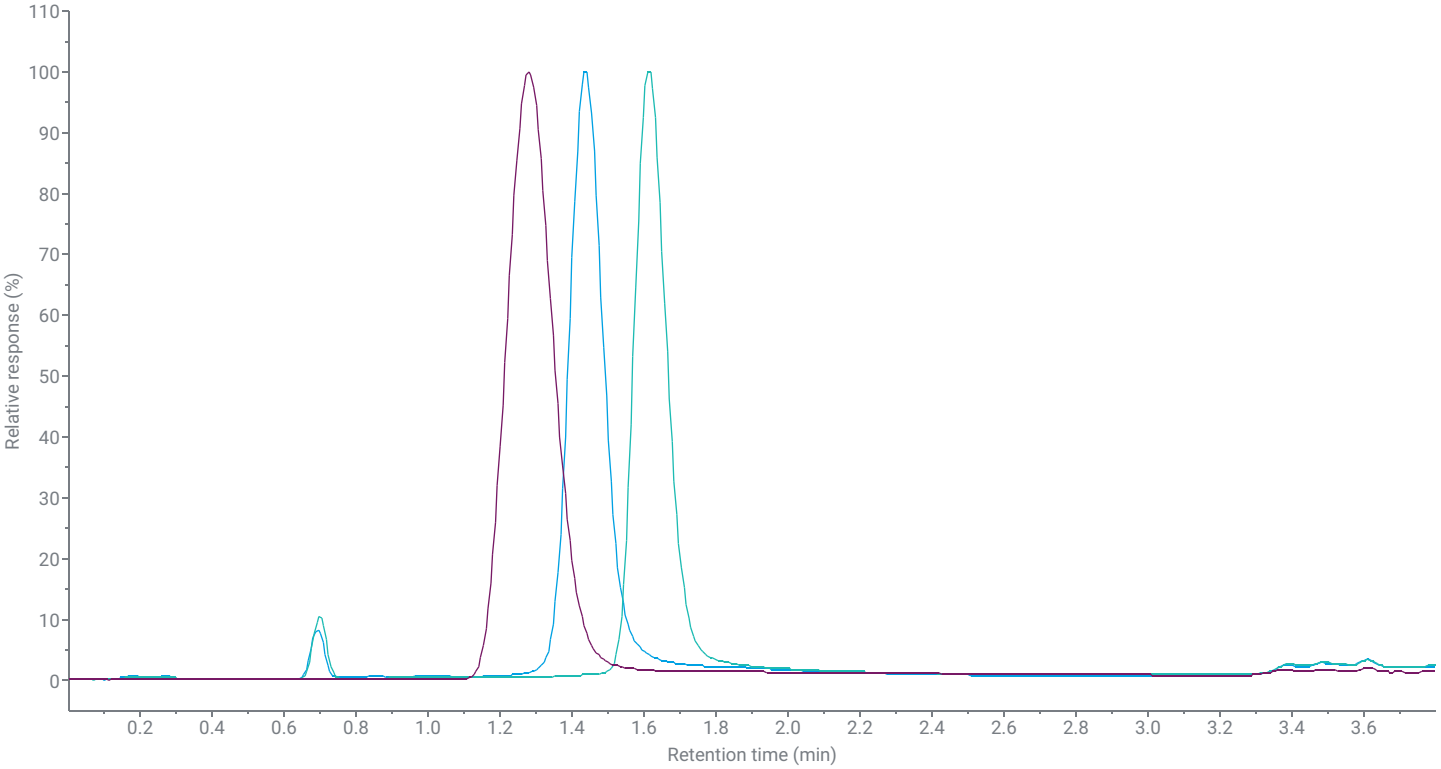

Figure 1. UV chromatograms for three antisense oligos (ASOs). Fomivirsen is a 21mer ASO with no 2'-modifications. Thus, it elutes earlier than the two shorter, fully thioated, 2'-modified ASOs.

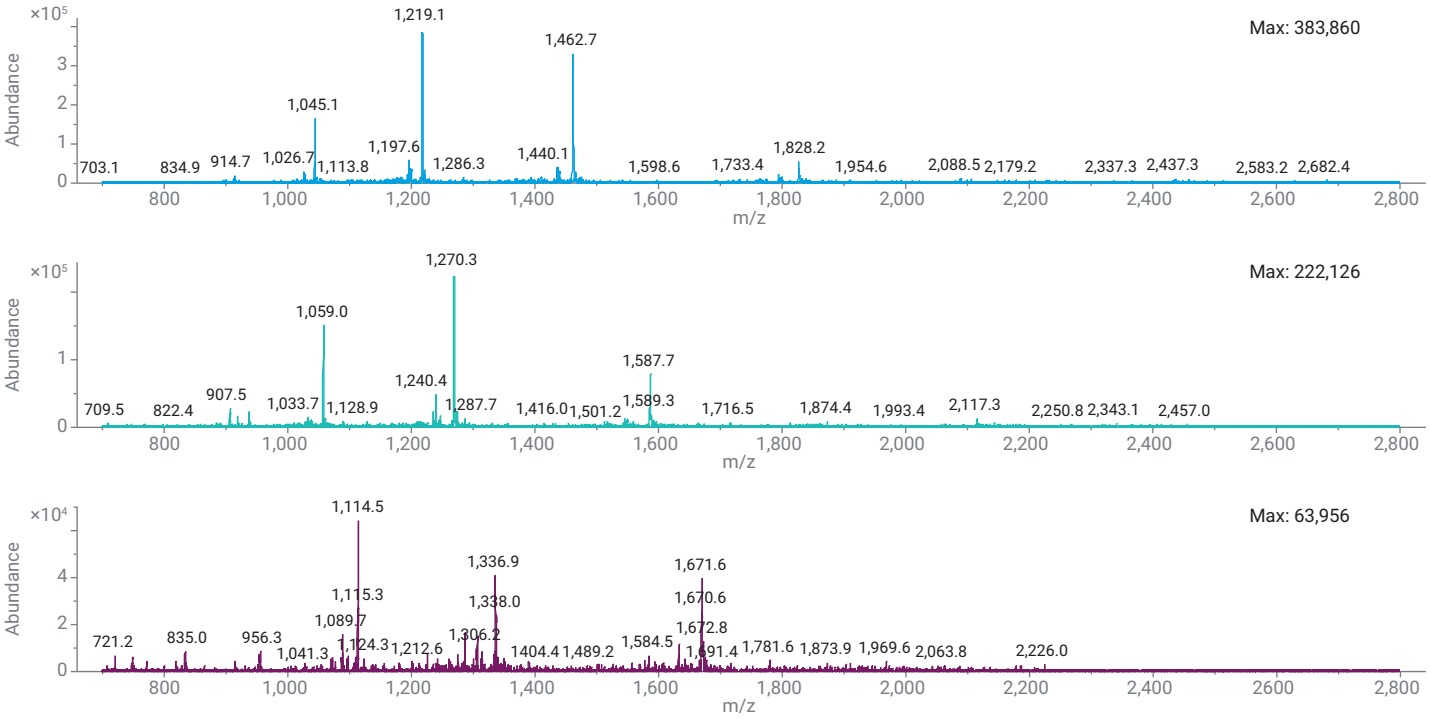

Figure 2. Full-scan spectra for samples. Charge states of 4 to 6 are predominant for each ASO.

Agilent OpenLab CDS uses a unique spectral deconvolution that is optimized for unit mass detectors. Maximum entropy calculates the most probable mass from a spectrum by increasing dominant features while minimizing noise. In contrast, OpenLab CDS spectral deconvolution simply identifies ions in the spectrum and matches them to target masses based on user inputs. These values are then fit to a linear regression or centroided to calculate a molecular weight average. This simple approach allows for easy molecular weight confirmation using generic processing methods, which can be fully automated with minimal optimization. Triplicate injections of each sample yield nearly identical results, with relative standard deviations (%RSDs) < 0.1%. The automated deconvolution settings used in the sample sequence for acquisition are shown in Table 4, and a summary of deconvolution results is shown in Table 5.

Table 4. MS spectral deconvolution settings.

Parameter	Value
Automatic Deconvolution Settings	
Run Automatic Deconvolution	Enabled
RT Window	1–2.7 min
TIC Peak Type	All peaks
TIC Top (n) Peaks	3
Basic Settings	
Use *m/z* Range	Disabled
Low/High Molecular Weight	3,000–10,000
Maximum Charge	10
Minimum Peaks in Set	3
Advanced Settings	
MW Agreement (0.01%)	10
Absolute Noise Threshold	100
Relative Abundance Threshold	10
MW Algorithm	Curve fit
MW Algorithm Threshold (%)	40
Envelope Threshold (%)	50

Figure 3 shows the deconvoluted spectrum of ASO-1, an 18mer phosphorothioate with 2'-MOE modifications. Component A is a full-length product, showing good agreement with the calculated mass. Component B is likely depurination (loss of guanine). Component C is likely a sodium adduct. As shown, the InfinityLab Pro iQ Plus, which combines unit mass accuracy with simple deconvolution, can monitor oligonucleotides and identify unexpected impurities or components. Further characterization can be performed by high-resolution MS.

Table 5. Deconvoluted results of twenty replicates of each ASO.

Oligonucleotide Name	Calculated Mass (Da)	Average Measured Mass (Da)	Delta Mass (Da)
ASO-1	6,348.3	6,347.9	−0.4
ASO-2	7,309.2	7,308.8	−0.4
Fomivirsen	6,682.4	6,681.8	−0.6

A duplex, tri-antennary GalNAc-conjugated siRNA (Givosiran) was also analyzed to demonstrate broad applicability of the method. Slight modifications to the LC/MS parameters were required with the duplex conjugate. Specifically, the gradient program was adjusted slightly (5% to 45% in 3 minutes) to ensure baseline separation of the sense and antisense strands. Additionally, the fragmentor voltage was decreased to 120 V to minimize in-source fragmentation of the fragile oligosaccharide conjugate. Figure 4 shows an overlay of UV chromatograms for five injections of Givosiran.

Although no ion pair is used, the ABC mobile phase still provides baseline separation of sense and antisense strands (Figure 5). This separation is required because any spectral overlap may result in misidentifications in deconvolution. Spectra are shown in Figure 5, with similar charge state distributions to the previously mentioned ASOs. Analysis of the deconvoluted spectrum for each peak show the antisense strand eluting earlier than the sense strand. Interestingly, the sense strand may indicate GalNAc losses (Figure 6). A summary of Givosiran results is shown in Table 6.

Table 6. Deconvoluted results for each injection of Givosiran.

Oligonucleotide Name	Calculated Mass (Da)	Average Measured Mass (Da)	Delta Mass (Da)
Givosiran, Antisense	7,563.8	7,563.1	0.7
Givosiran, Sense	8,736.5	8,735.6	0.9

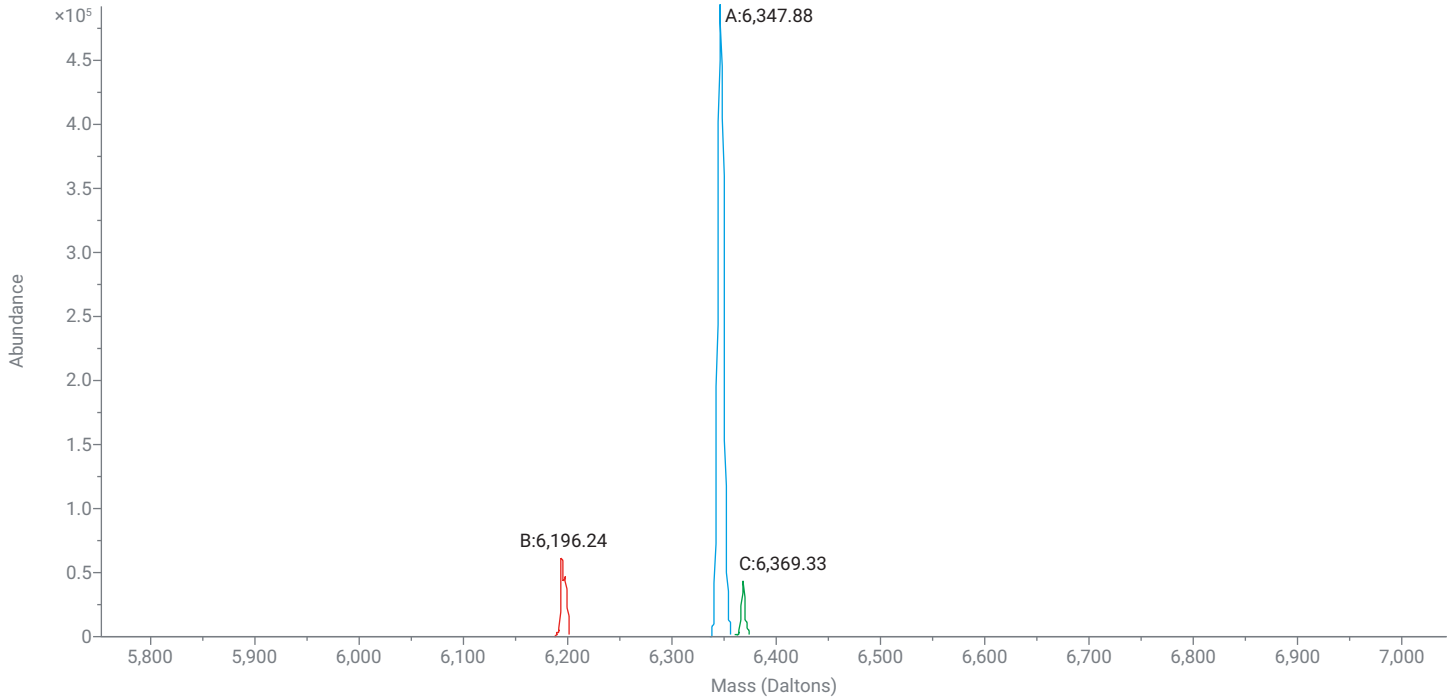

Figure 3. Deconvoluted spectrum for ASO-1.

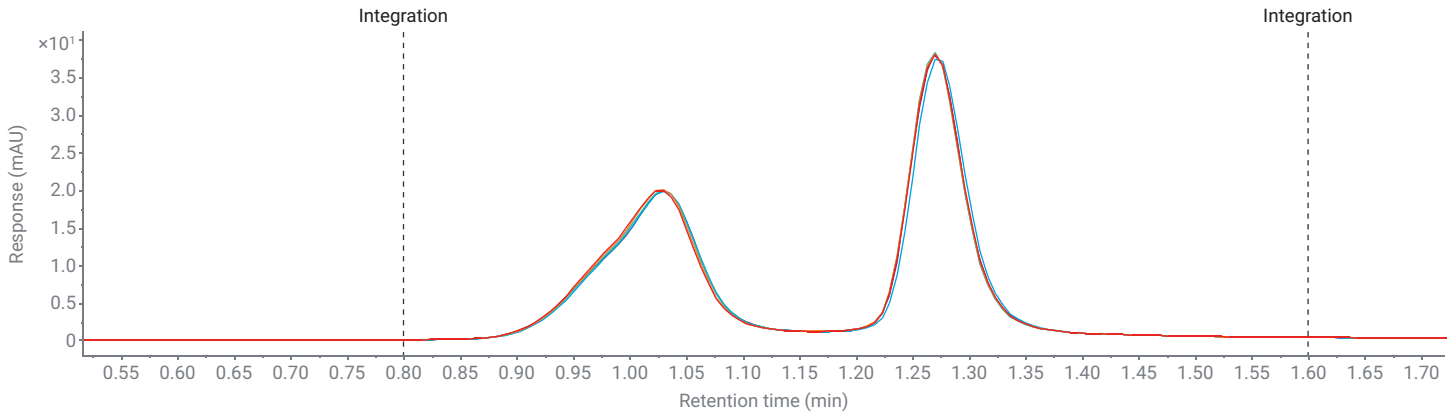

Figure 4. Overlay of UV chromatograms for five injections of Givosiran.

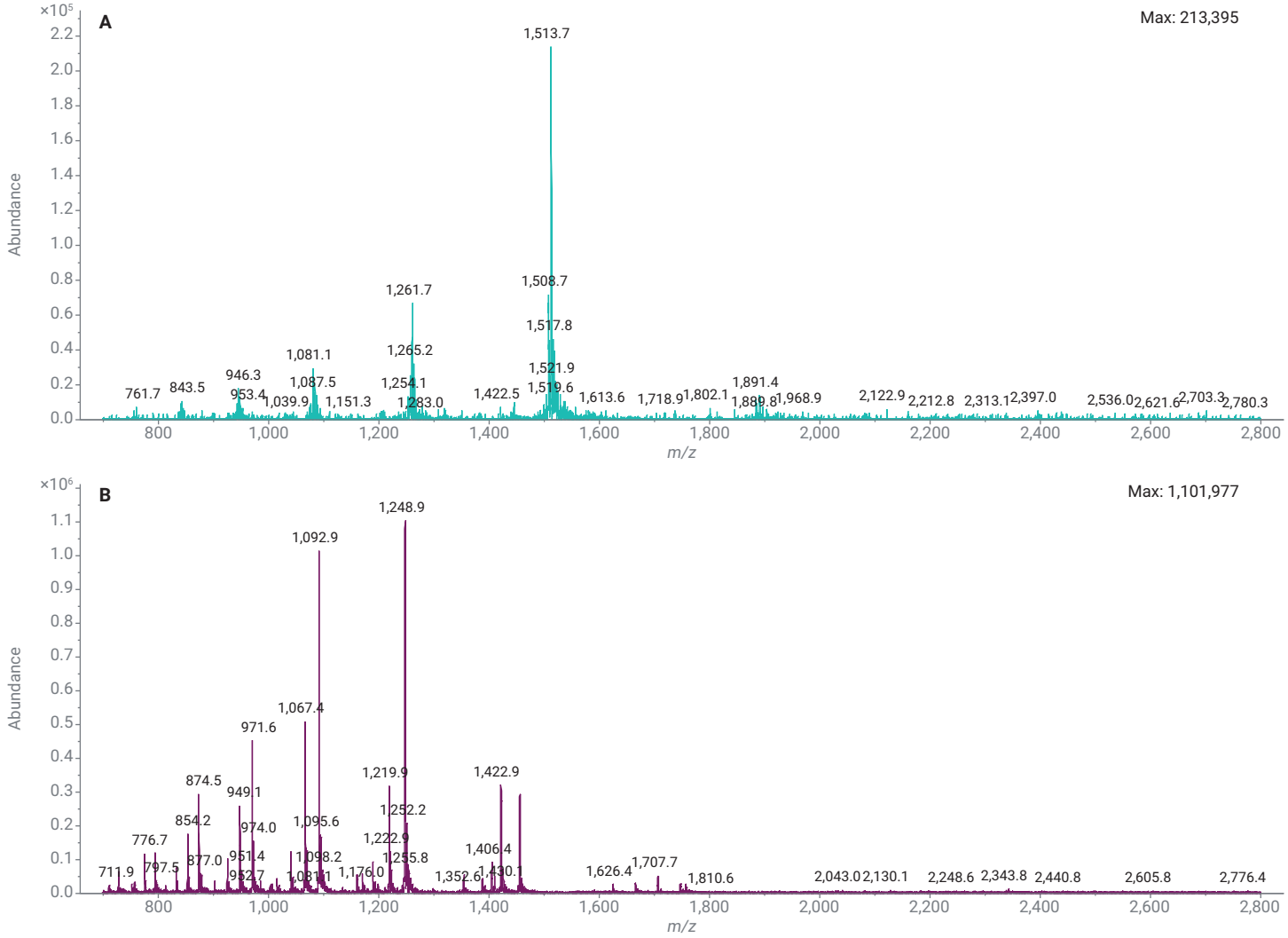

Figure 5. Spectra for antisense (A) and sense (B) RNA strands. Charge state distribution is similar to that of antisense oligos analyzed using a similar method. The 5– charge state is the most abundant for antisense, and the 6– charge state is the most abundant for the GalNAc-conjugated sense strand.

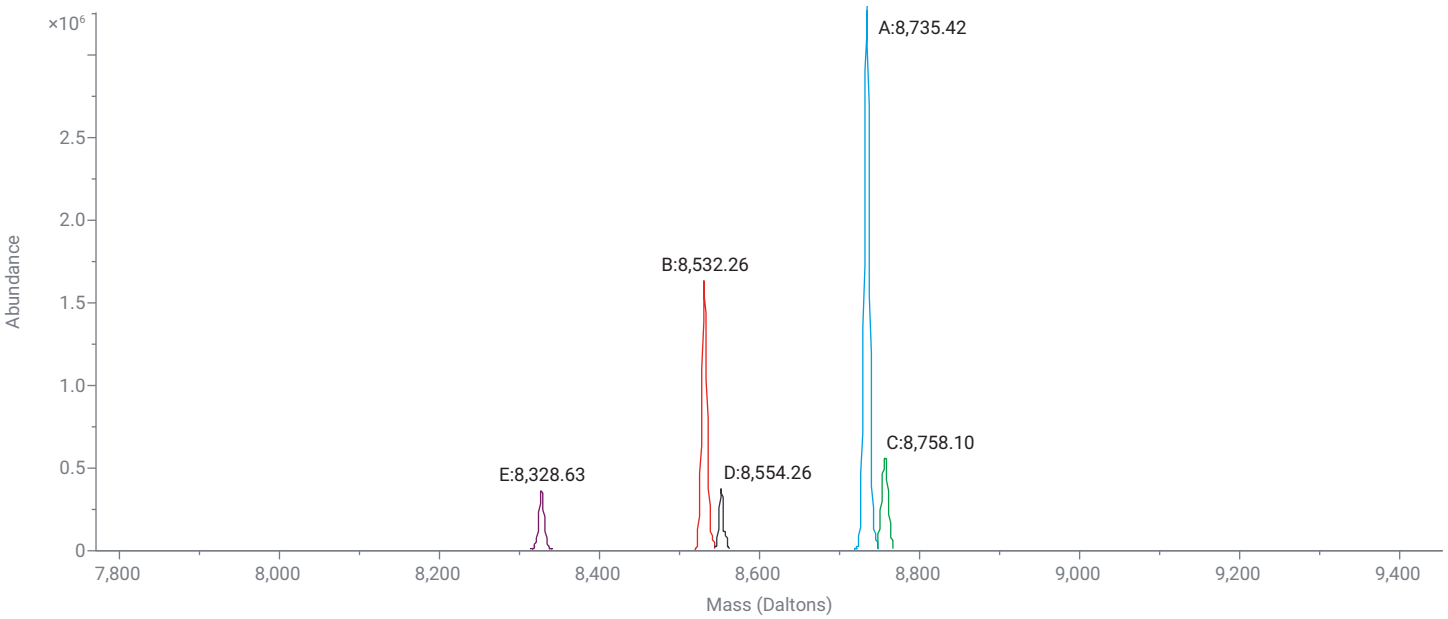

Figure 6. Deconvoluted spectrum for the Givosiran sense strand. The approximately 200 Da shift observed could be loss of GalNAc.

Conclusion

The method described in this application note provides a practical approach for medium- to high-throughput molecular confirmation of synthetic oligonucleotides. The Agilent Pro iQ Plus demonstrates excellent resolution and sensitivity for a unit mass detector, even at high *m/z* ranges that exceed the capabilities of many single quadrupole detectors. The method uses a novel ammonium bicarbonate mobile phase, providing sufficient LC/MS performance for labs analyzing many types of oligos, including antisense oligonucleotides and siRNAs. LC and MS results described in this application note are reproducible. Data analysis workflows are simplified using Agilent OpenLab CDS MS spectral deconvolution, which is automated by unattended processing methods that require minimal optimization.

References

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