Application Note

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MS/MS Oligonucleotide Sequencing Using LC/Q-TOF with HILIC Chromatography

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Introduction

Oligonucleotides are commonly analyzed by liquid chromatography/mass spectrometry (LC/MS) in negative ion polarity mode using ion-pairing reversed-phase (IP-RP) methods. Generally, this approach provides good chromatographic separation and MS response for a wide range of oligo samples. However, many ion-pairing reagents persist in the analytical system long after their use, present a strong MS response in positive ion polarity, and can be detrimental to subsequent analyses. Therefore, using ion-pairing methods on multipurpose systems can be challenging. In fact, normally these systems require substantial cleaning in between sample runs with IP-RP and non-ion-pairing conditions to provide optimal results. Therefore, LC/MS methods that provide high-quality data on oligos, but do not rely on ion-pairing conditions, are gaining attention.

LC/MS of oligos using hydrophilic liquid interaction chromatography (HILIC) can be an alternative to IP-RP conditions for a wide range of oligo targets. A recently published application note describes oligo characterization by HILIC resin on a quadrupole time-of-flight (LC/Q-TOF) in MS1 mode. This work used an Agilent InfinityLab Poroshell 120 HILIC-Z column and evaluated chromatographic separation, retention time stability, re-equilibration time, oligo size applicability, and performance on oligos of varying chemistries. Please see application note 5994-5631EN for further detail.[1] The use of the HILIC-Z resin in Agilent RapidFire analyses of oligos has also been described. In this case, oligos were characterized by MS1 data at a sustained rate of 12 seconds per sample (see application note 5994-4945EN).[2]

In this application note, the determination of oligo sequence confirmation using HILIC LC and high-resolution MS/MS data is described. As with the previous studies, an InfinityLab Poroshell 120 HILIC-Z column was used along with an Agilent 6545XT AdvanceBio LC/Q-TOF mass spectrometer.

Experimental

Instrumentation

- Agilent 1290 Infinity II LC including:
  - Agilent 1290 Infinity II High-Speed Pump (G7120A)
  - Agilent 1290 Infinity II Multisampler (G7167B) with Sample Thermostat (option #101)
  - Agilent 1290 Infinity II Multicolumn Thermostat (G7116B)
- Agilent 6545XT AdvanceBio LC/Q-TOF

Software

- Agilent MassHunter Data Acquisition software 11.0
- Agilent MassHunter BioConfirm software 12.0

Chemicals

LC/MS-grade acetonitrile and ammonium acetate were purchased from Sigma-Aldrich (St. Louis, Missouri). Water was obtained from a Milli-Q system (Millipore, Bedford, MA). All synthetic oligonucleotides (Table 1) were purchased from Integrated DNA Technologies, Inc. (Coralville, IA, USA).

Sample preparation

All synthetic oligonucleotide samples were dissolved in water without further purification. The final concentrations used were 100 µM for 20- and 30-mer DNA and 25 µM for ASO, aptamer, and 21-mer RNA. For each, 1 µL was injected, resulting in 100 or 25 pmol on column.

LC/MS analysis

LC/MS analyses were conducted on a 1290 Infinity II LC system coupled with a 6545XT AdvanceBio LC/Q-TOF, operated in 4 GHz high resolution mode, equipped with an Agilent dual spray Jet Stream ESI source. Agilent MassHunter data acquisition software 11.0 was used. LC separation was obtained with an InfinityLab Poroshell 120 HILIC-Z column, 2.1 × 50 mm, 2.7 µm (part number 699775-901). LC/MS method parameters are detailed in Table 2. These methods were based on application note 5994-5631EN and optimized with emphasis on method throughput.[1]

Table 1. Oligonucleotides used in this study and their associated code notations. All sequences are written in the 5' to 3' orientation.

Oligonucleotide Name	Length	Sequence
20-mer DNA	20	CAGTCGATAGCAGTCGATAG
30-mer DNA	30	CAGTCGATAGCAGTCGATAGCAGTCGATAG
ASO	18	/52MOErT/*/i2MOErC/*/i2MOErA/*/i2MOErC/*/i2MOErT/*/i2MOErT/*/i2MOErT/*/i2MOErC/*/i2MOErA/*/i2MOErT/*/i2MOErA/*/i2MOErA/*/i2MOErT/*/i2MOErG/*/i2MOErC/*/i2MOErT/*/i2MOErG/*/32MOErG/
Aptamer	28	/52FC/mGmGrArA/i2FU//i2FC/mAmG/i2FU/mGmAmA/i2FU/mG/i2FC//i2FU//i2FU/mA/i2FU/mA/i2FC/mA/i2FU//i2FC//i2FC/mG/3InvdT/
21-mer RNA	21	rCrArGrUrCrGrArUrUrGrUrArCrUrGrUrArCrUrUrA

Code	Description
*	Phosphorothioate bond
A	2'-deoxyribose adenine
C	2'-deoxyribose cytosine
G	2'-deoxyribose guanine
T	2'-deoxyribose thymine
mA	2'-O-methyl A
mG	2'-O-methyl G
rA	Ribose adenine
rC	Ribose cytosine
rG	Ribose guanine
rU	Ribose uracil

Code	Description
/3InvdT/	3' inverted T
/32MOErG/	3' methoxyethoxy G
/52FC/	5' Fluoro C
/52MOErT/	5' 2-methoxyethoxy T
/i2FC/	Internal Fluoro C
/i2FU/	Internal Fluoro U
/i2MOErA/	Internal 2-methoxyethoxy A
/i2MOErC/	Internal 2-methoxyethoxy C
/i2MOErT/	Internal 2-methoxyethoxy T
/i2MOErG/	Internal 2-methoxyethoxy G

Table 2. LC/MS methods used in this study.

Agilent 1290 Infinity II LC Conditions	
Column	InfinityLab Poroshell 120 HILIC-Z, 2.1 × 50 mm, 2.7 µm (p/n 699775-901)
Column Temperature	30 °C
Injection Volume	1 µL
Autosampler Temperature	4 °C
Needle Wash	Methanol:water 50:50
Mobile Phase	A) 90% acetonitrile : 10% water + 15 mM ammonium acetate B) 10% acetonitrile : 90% water + 15 mM ammonium acetate
Flow Rate	0.4 mL/min
Gradient Program	**Time (min)** **B (%)** 0.50 25 5.00 75
Stop Time	5.00 min
Post Time	5.00 min

6545XT AdvanceBio LC/Q-TOF Conditions	
Ion Polarity	Dual AJS Negative
Data Storage	Both (Centroid and Profile)
Gas Temperature	350 °C
Drying Gas Flow	12 L/min
Nebulizer Gas	30 psi
Sheath Gas Temperature	400 °C
Sheath Gas Flow	12 L/min
Capillary Voltage	4,500 V
Nozzle Voltage	2,000 V
Fragmentor	180 V
Skimmer	65 V
Oct 1 RF Vpp	750 V
Acquisition Mode	Targeted MS/MS
MS Mass Range	400 to 3,200 *m/z*
MS Acquisition Rate	4 spectra/sec
MS/MS Mass Range	100 to 3,200 *m/z*
MS/MS Acquisition Rate	1 spectra/sec
Collision Energy	12 V, 15 V, 18 V, 20 V (*m/z* ≤1,500) 15 V, 20 V, 25 V, 30 V (*m/z* >1,500)
Targeted *m/z*	Calculated monoisotopic mass of the charged ion
Delta Retention Time	1 min
Isotope Width	Medium (~4 *m/z*)

Data processing

All data files of synthetic oligonucleotides samples were processed using Agilent MassHunter BioConfirm software 12.0. Method parameters are listed in Table 3.

Table 3. BioConfirm 12.0 data analysis methods.

Agilent MassHunter BioConfirm 12.0 Parameters	
Workflow	Oligonucleotides
Experiment	Sequence confirmation
Match Tolerance	Tolerance: 15 ppm Theoretical profile relative abundance ≥20%
Absolute Height Threshold	125
Matching Criteria	Warn if score is <90 Do not match if score is <85
Extraction MS/MS	Group by collision energy Two scans averaged

Results and discussion

Five synthetic oligonucleotides were analyzed using targeted MS/MS acquisition under HILIC conditions. The oligonucleotides were a 20-mer DNA (Figure 1), a 30-mer DNA (Figure 2), an 18-mer ASO (Figure 3), a 28-mer aptamer (Figure 4), and a 21-mer RNA (Figure 5) strand. All samples showed adequate retention on column with retention times between 1.5 and 3.5 minutes. Many of the corresponding *m/z* spectra showed bimodal charge state distributions, one of lesser charge (higher *m/z*) and one of higher charge (lower *m/z*), likely stemming from fractions of oligos in partially ordered and denatured states.

For each oligo, targeted MS/MS was conducted on a variety of charge states using several different collision energies to evaluate their effect on sequence coverage. Results indicated that fragmenting higher charged precursors generally provided more sequence coverage, and this trend was especially true for longer oligonucleotides. However, fragmentation of the most abundant charge states was required for complete sequence coverage for several oligo samples studied. Please refer to the figure titles for the precursors targeted, as well as the number of replicates, used to achieve 100% sequence coverage for each oligonucleotide in this study.

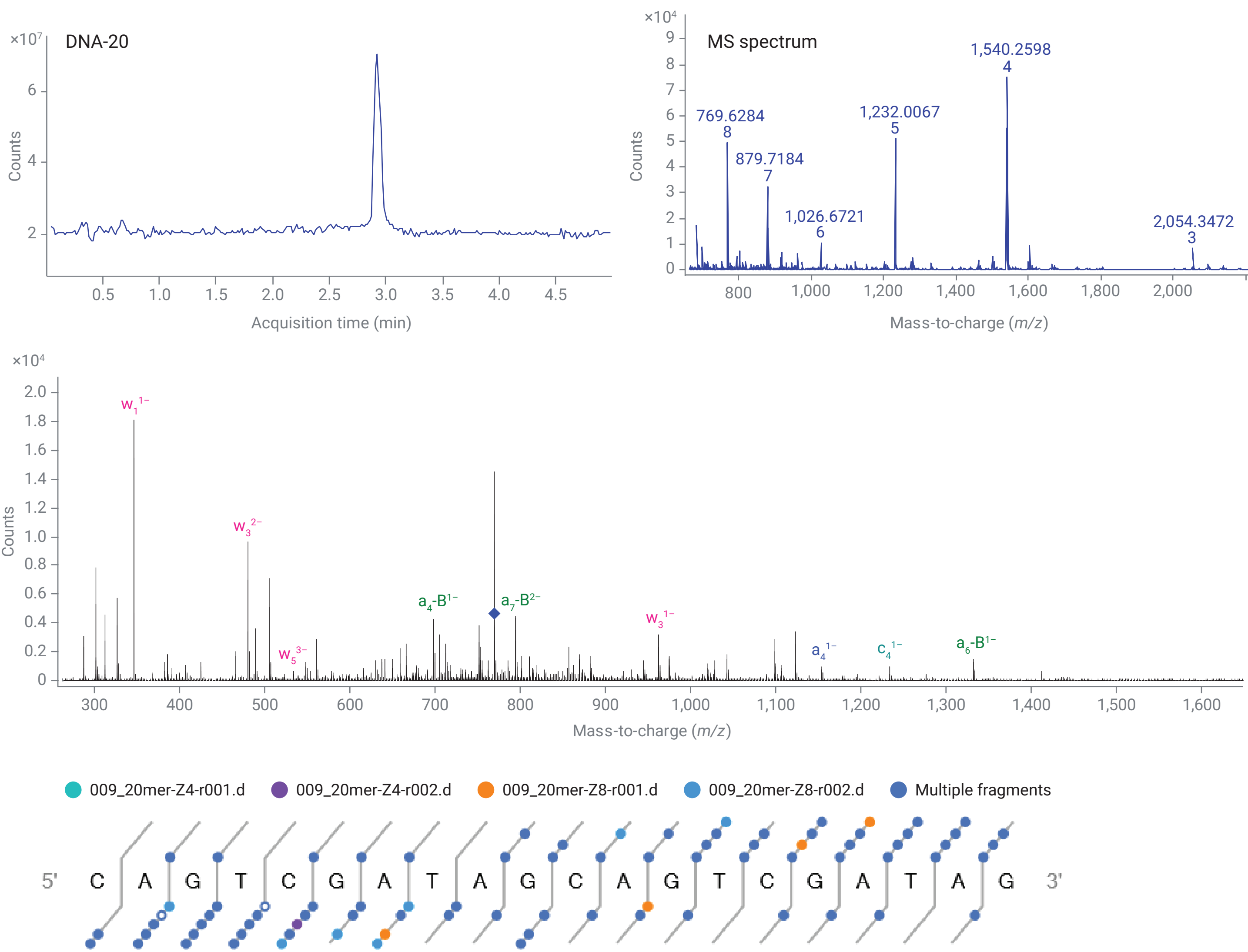

Figure 1. 20-mer DNA data. Sequence coverage was achieved by targeting the –4 and –8 charge states in duplicate.

Figure 2. 30-mer DNA data. Sequence coverage was achieved by targeting the –6, –11, and –12 charge states in triplicate.

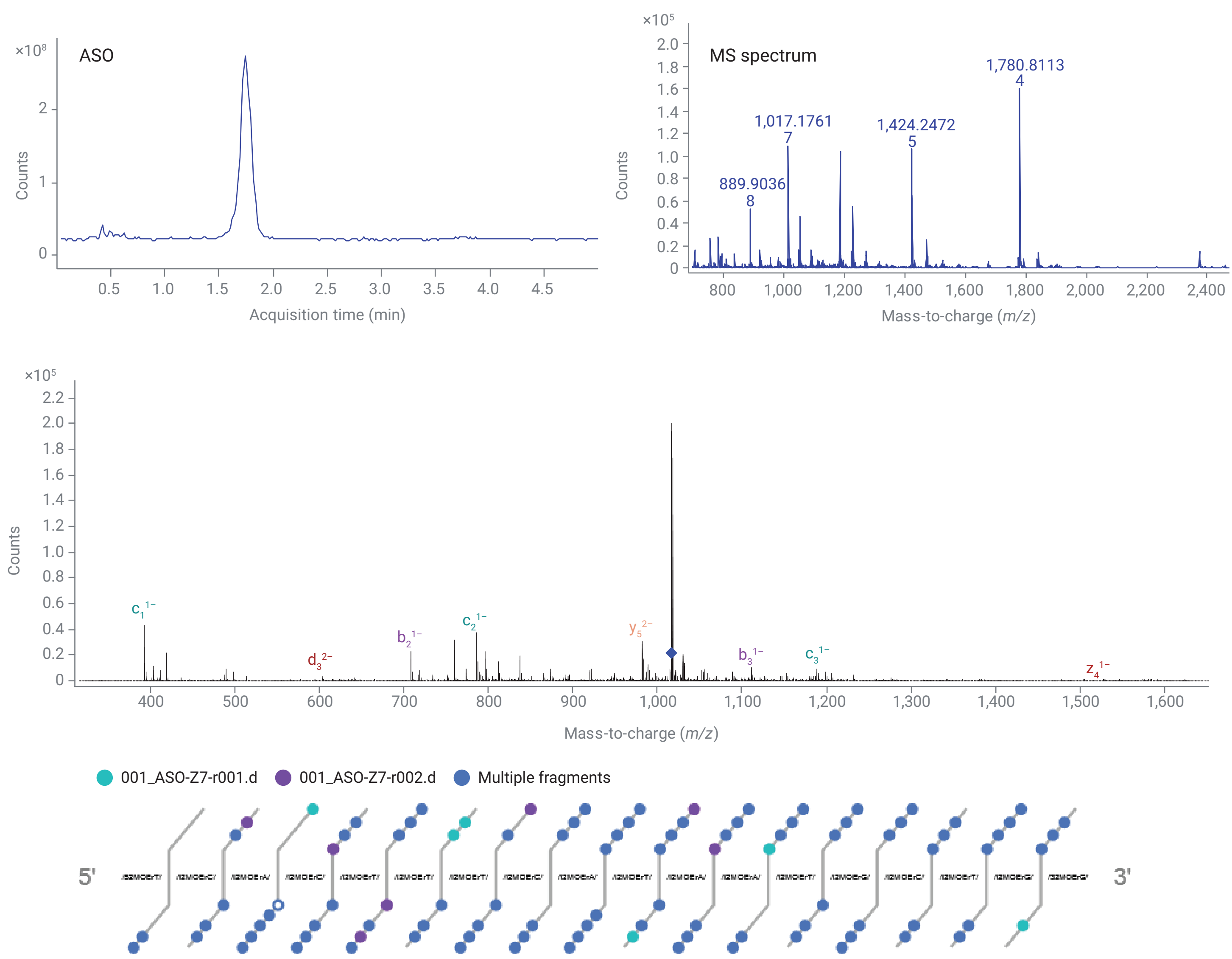

Figure 3. ASO data. Sequence coverage was achieved by targeting the –7 charge state in duplicate.

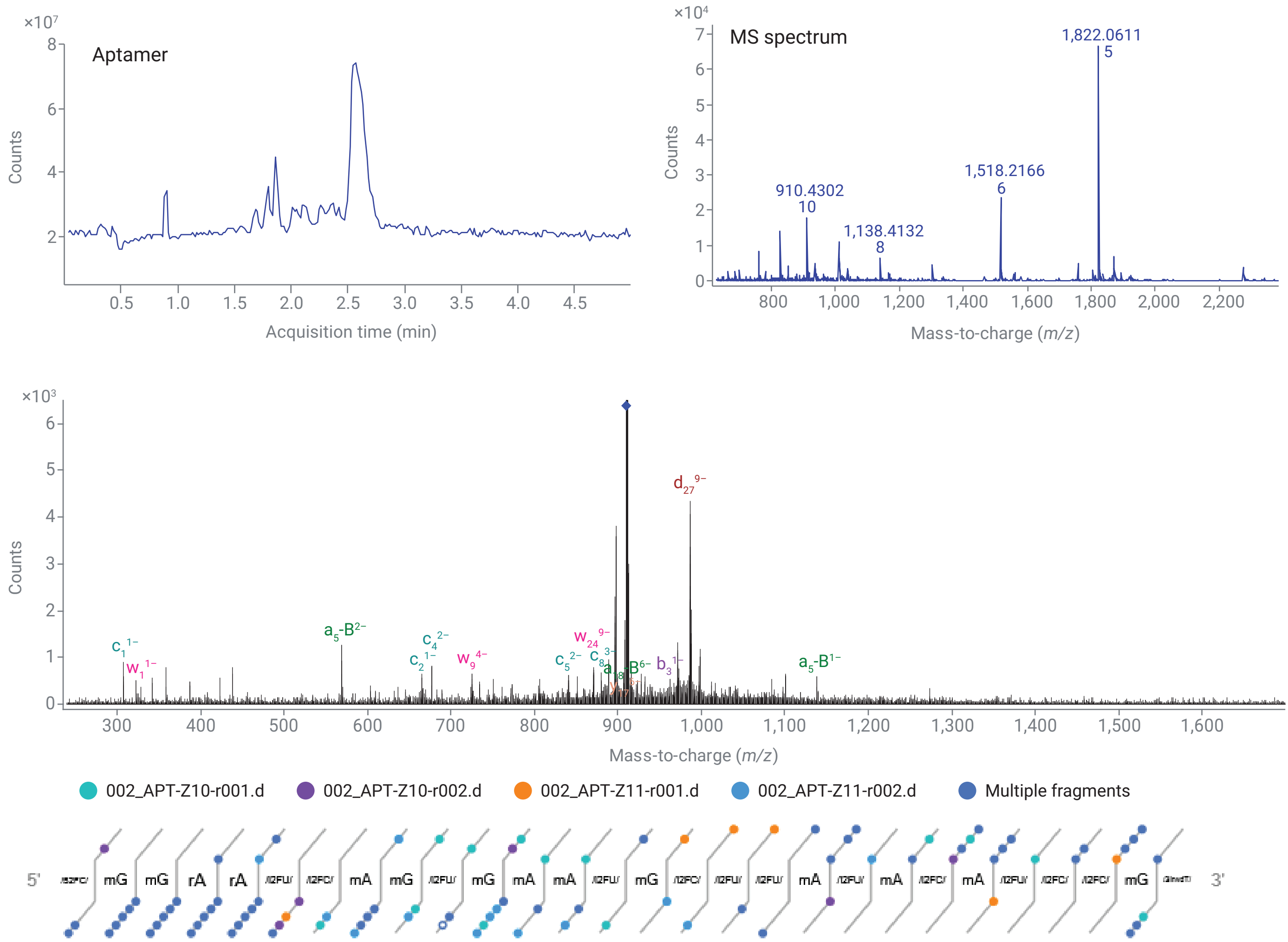

Figure 4. Aptamer data. Sequence coverage was achieved by targeting the –10 and –11 charge states in duplicate.

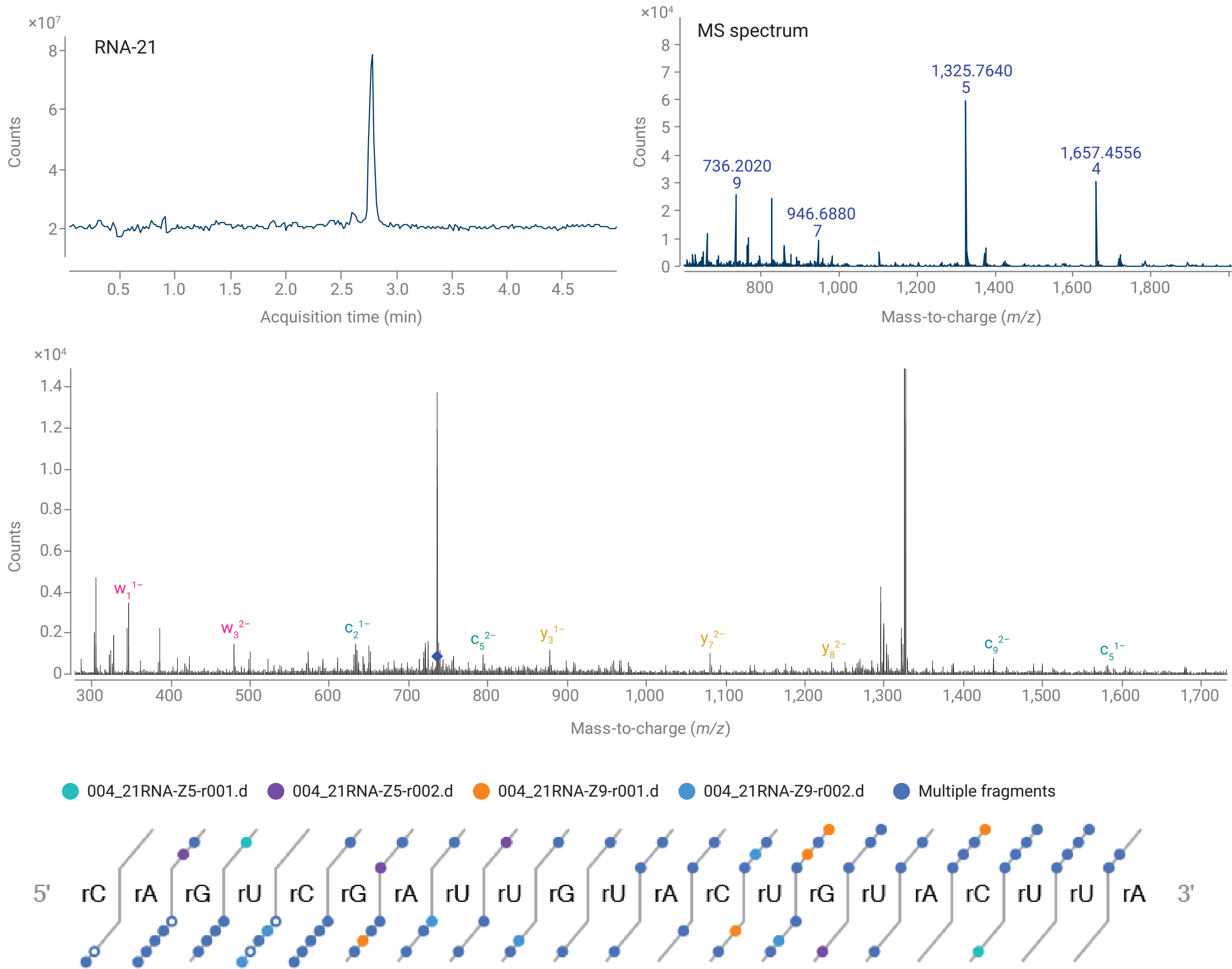

Figure 5. 21-mer RNA data. Sequence coverage was achieved by targeting the –5 and –9 charge states in duplicate.

Based on these experiments, the suggested approach for similar studies is to run individual injections targeting the most abundant charge state and the charge state one higher than the most abundant, for both charge state distributions. Under typical circumstances, individually targeting these four charge states for fragmentation is expected to provide informative scouting data.

In addition to the selection of charge states for fragmentation, multiple collision energies were evaluated for their effect on sequence coverage. These efforts illustrated that high collision energies do not generally provide good sequence coverage, likely because of the formation of internal fragments, which are not considered during analysis. However, in some cases, slightly elevated collision energies were required to obtain sequence coverage on the end of the sequences. In contrast, lower collision energies provided substantial coverage in the middle of the sequences studied (data not shown).

From these experiments, it was determined that the optimal collision energy correlated better with the *m/z* value of the charge state fragmented than the length of the oligonucleotide being analyzed. These observations allowed a simplified collision energy (CE) schema to be derived for all the oligonucleotides in this application note. When the precursor *m/z* was less than 1,500, 12, 15, 18, and 20 V were set for CEs. If the precursor *m/z* was more than 1,500, 15, 20, 25, and 30 V were used for CEs. These settings were entered in the collision energy tab, in the Targeted MS/MS Mode, as shown in Figure 6.

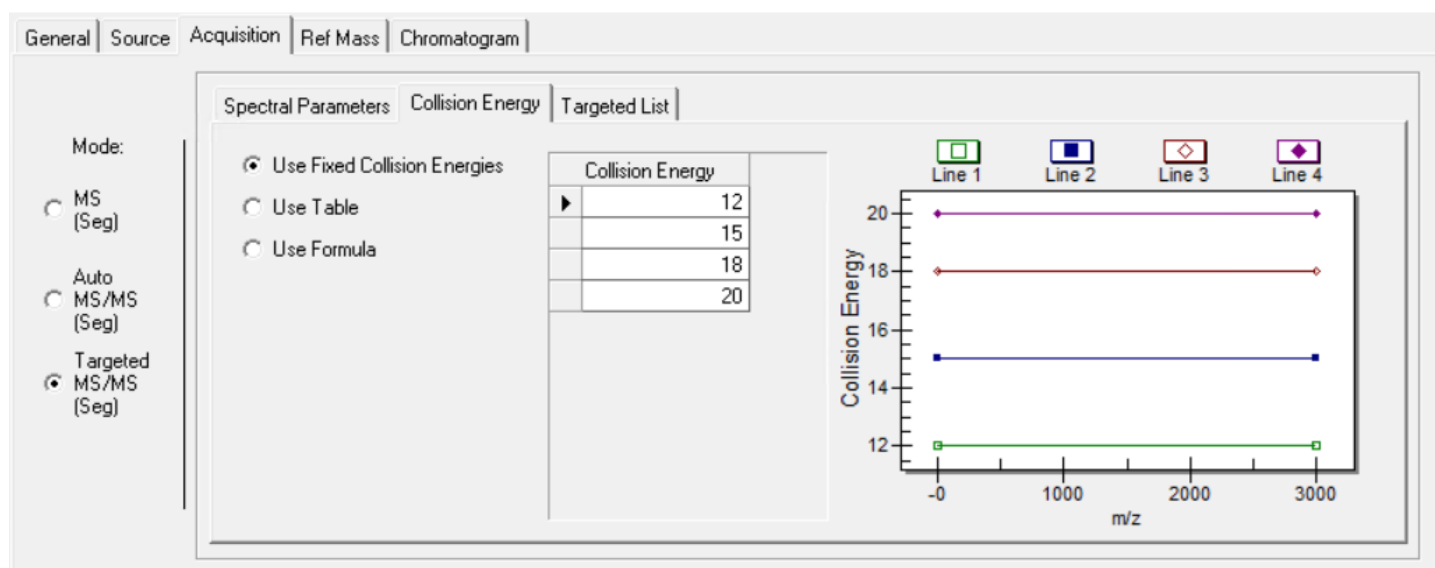

Figure 6. Suggested collision energy settings for fragmenting precursors less than *m/z* 1,500. For precursors bigger than *m/z* 1,500, replace 12, 15, 18, and 20 V with 15, 20, 25, and 30 V, respectively.

Conclusion

- A HILIC (non-ion-pairing) MS/MS method for oligo sequencing was described.
- The method used an InfinityLab Poroshell 120 HILIC-Z column, a 6545XT AdvanceBio LC/Q-TOF mass spectrometer, and the BioConfirm 12.0 software.
- The method provided 100% sequence coverage of all five oligos studied – including 20- and 30-mer DNA, a 21-mer RNA, an aptamer, and an ASO sample.
- Simplified criteria for target selection and CE setting were determined to minimize optimization requirements with the described methods.

References

1. Rye, P.; Schwarzer, C. MS1 Oligonucleotide Characterization Using LC/Q-TOF with HILIC, *Agilent Technologies application note*, Chromatography publication number 5994-5631EN, **2023**.
2. Rye, P. High-Throughput, Ion-Pairing-Free, HILIC Analysis of Oligonucleotides Using Agilent RapidFire Coupled to Quadrupole Time-of-Flight Mass Spectrometry, *Agilent Technologies application note*, publication number 5994-4945EN, **2022**.

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