Application Note

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Oligonucleotide Analysis Using the Agilent InfinityLab Pro iQ and Altura Oligo HPH-C18 Column

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Abstract

The LC/MS analysis of oligonucleotides typically requires the use of alkylamine-based ion-pairing agents to achieve adequate retention and peak shape. However, selecting the optimal concentration of these reagents often involves a difficult trade-off: higher concentrations improve chromatographic resolution but lead to significant signal suppression and contamination in mass spectrometry.

This application note demonstrates a high-performance solution using the Agilent Altura Oligo HPH-C18 column in combination with the Agilent InfinityLab Pro iQ mass detector and the Agilent 1260 Infinity III Prime bio LC system. Featuring ultra-inert hardware technology, the Altura column minimizes non-specific secondary interactions between the oligonucleotides and metal surfaces. This allows for superior peak resolution—specifically for challenging n-1 mer impurities—even when using the reduced ion-pairing concentrations necessary for high-sensitivity MS detection.

By leveraging the *m/z* 2 to 1,600 scan range of the InfinityLab Pro iQ, we confirm that the system captures a sufficient ion set to support robust deconvolution and precise molecular weight confirmation of the target oligonucleotides. The results highlight how the synergy between ultra-inert column chemistry and sensitive mass detection provides a reliable workflow for the comprehensive impurity profiling and characterization of therapeutic oligonucleotides.

Introduction

The pharmaceutical industry is witnessing a rapid surge in oligonucleotide-based therapeutics, including antisense oligonucleotides (ASOs) and small interfering RNAs (siRNAs). These nucleic acid therapies represent a paradigm shift in drug development, offering the ability to directly modulate genetic targets that were previously considered "undruggable" by conventional small molecules or monoclonal antibodies. This makes them particularly innovative for treating rare genetic disorders and chronic diseases.[1]

In the manufacturing and quality control (QC) of oligonucleotides, purity is a critical parameter for ensuring drug safety and efficacy. While HPLC-UV has traditionally been the method of choice, the increasing complexity of therapeutic structures is exposing the limitations of relying solely on UV detection. Specifically, identifying and separating n-1 mer impurities—which share nearly identical physicochemical properties with the main component—is exceptionally challenging. Consequently, mass spectrometry (MS) has become essential for comprehensive purity analysis, impurity profiling, and molecular weight confirmation.[2]

Due to their negatively charged phosphate backbone, oligonucleotides are highly polar and exhibit poor retention on standard reversed-phase columns. This necessitates the use of ion-pairing agents such as triethylamine (TEA), dibutylamine (DBA), or hexylamine (HA).[3] While higher concentrations of these alkylamines generally improve peak resolution, they often lead to significant sensitivity suppression and instrument contamination in LC/MS analysis. Therefore, researchers are frequently forced to find a compromise between chromatographic resolution and MS sensitivity.

In this application note, we present an optimized oligonucleotide analysis using the Altura Oligo HPH-C18 column, the Pro iQ mass detector, and the 1260 Infinity III Prime bio LC system. The Agilent AdvanceBio Oligonucleotide column is already well-regarded for its superior resolution, particularly in separating n-1 mer species under UV-optimized conditions. The Altura Oligo HPH-C18 column utilizes the same high-performance packing material as the AdvanceBio line but features ultra-inert column hardware. This technology minimizes secondary interactions with the metal surfaces of the hardware, allowing for improved resolution even when ion-pairing reagent concentrations are reduced for MS compatibility.

Oligonucleotides typically form multiple charged ions in LC/MS, with major ASO ion envelopes typically appearing within the *m/z* 500 to 1,600 range. The Pro iQ, with its *m/z* 2 to 1,600 mass range, combined with the deconvolution capabilities of Agilent OpenLab CDS, enables precise molecular weight confirmation for both the main API and trace-level impurities.

The results of this study demonstrate that the Altura ultra-inert column ensures high resolution under low ion-pairing conditions, while the Pro iQ provides the sensitivity and sufficient mass range to effectively cover the oligonucleotide charge state envelopes. Together, this workflow offers a robust and standardized solution for the comprehensive characterization of therapeutic oligonucleotides.

Experimental

Instrumentation

The following instrumentation was used in this study:

- Agilent 1260 Infinity III bio flexible pump (product number G7131C)
- Agilent 1290 Infinity III bio multisampler (product number G7137C) with sample thermostat
- Agilent 1290 Infinity III multicolumn thermostat (product number G7116B) with Quick Connect bio heat exchanger standard flow (product number G7116-60071)
- Agilent 1260 Infinity III diode array detector (product number G7117C) with Max-Light cartridge cell LSS, 10 mm (product number G7117-60020)
- InfinityLab Pro iQ (G6160B)

Standards and reagents

The following standards were used:

- Oligonucleotide ladder standard: Agilent ssDNA (dT 15, 20, 25, 30, 35, 40 nt)(part number 5190-9029)
- Oligonucleotide resolution standard: Agilent ssRNA (part number 5190-9028)

Reagents

Hexylamine and 1,1,1,3,3,3,-hexafluoro-2-propanol were purchased from Sigma-Aldrich; acetic acid was purchased from Fisher scientific and acetonitrile was purchased from B&J.

Columns

- AdvanceBio Oligonucleotide column, 2.1 × 150 mm, 2.7 µm (part number 653750-702)
- Altura Oligo HPH-C18 column, 2.1 × 150 mm, 2.7 µm (part number 227215-702)

Software

The software used in this application note was OpenLab CDS software, version 2.8 with feature pack 2.

Methods

Table 1. Instrument parameters for the Agilent 1260 Infinity III Bio Prime LC under HAA condition.

Parameters	Details
Flow	0.6 mL/min
Mobile Phase	A) 100 mM HAA in water B) 100 mM HAA in acetonitrile
Injection Volume	20 µL
Sampler Temperature	4 °C
Column Temperature	60 °C
UV Detection	260 nm
Gradient	Time (min) %A %B 0 85 15 0.5 85 15 25 60 40 26 0 100 30 0 100 30.1 85 15 37 85 15
InfinityLab Pro iQ Parameters	
Ion Source	ESI (–)
Source Parameters	Gas Temperature: 325 °C Gas Flow: 11 L/min Nebulizer: 45 psi Capillary Voltage: 4,500V
Acquisition	Scan range: *m/z* 500–1,600 Fragmentor: Ramp Fragmentor (*m/z*) Voltage (V) 75 100 250 120 450 130 750 140 1,000 160 1,300 170 1,800 180 Scan time: 176 ms (5.59 Hz) Storage: Profile Diverter valve: 0 min – to waste 1 min – to MS 25 min – to waste

Table 2. Instrument parameters for the Agilent 1260 Infinity III Prime bio LC system and Agilent InfinityLab Pro iQ under HA-HFIP conditions.

Parameters	Details
Flow	0.6 mL/min
Mobile Phase	A) 15 mM HA + 25 mM HFIP B) Acetonitrile
Injection Volume	20 µL
Sampler Temperature	4 °C
Column Temperature	60 °C
UV Detection	260 nm
Gradient	Time (min) %A %B 0 85 15 0.5 85 15 25* 70 30 26 10 90 30 10 90 30.1 85 15 37 85 15

* For the oligonucleotide column, the mobile phase composition at the 25-minute mark was maintained at a 72:28 ratio of solvent A to solvent B.

Results and discussion

An oligonucleotide ladder standard (Oligo dT, 15 to 40 nt) and an oligonucleotide resolution standard (RNA, 14, 17, 20, and 21 nt) were analyzed under Method 1, as shown in Figure 1. In reversed-phase (RP) mode, ion-pair reagents are typically employed to facilitate the retention of highly polar oligonucleotides, where the reagent concentration directly impacts both retention time and resolution. Under 100 mM HAA conditions, the AdvanceBio Oligonucleotide and Altura Oligo HPH-C18 columns demonstrated comparable performance in the separation of the 39 and 40 nt species. This indicates that a high concentration of the ion-pair reagent can effectively offset inherent hardware differences.

Conversely, when the HA concentration was reduced to 15 mM to ensure MS compatibility, a significant loss in resolution between the 39 and 40 nt oligo dT species was observed on the AdvanceBio Oligonucleotide column (Figure 2). However, since the separation of 20 and 21 nt RNA sequences was still successfully achieved, the AdvanceBio Oligonucleotide column remains a highly viable and effective solution for antisense oligonucleotide (ASO) analysis.

Notably, the Altura Oligo HPH-C18 column demonstrated superior performance under the 15 mM HA conditions, maintaining a level of resolution between the 39 and 40 nt species comparable to that achieved with the 100 mM HAA method (Figure 3). Furthermore, despite equivalent sample loading, the Altura Oligo HPH-C18 column exhibited significantly higher peak intensities relative to the AdvanceBio Oligonucleotide column. These findings underscore a critical factor in oligonucleotide analysis, the role of column hardware in mitigating non-specific secondary interactions. It is evident that utilizing specialized hardware materials—which either inherently prevent or effectively suppress these interactions—is paramount for ensuring optimal peak shape and recovery, particularly when ion-pair reagent concentrations are minimized for MS compatibility.

The six principal peaks of the oligonucleotide ladder standard, identified in Figure 3, exhibited distinct multiply charged ion envelopes within the *m/z* 500–1,600 range on the InfinityLab Pro iQ. Interestingly, most of these highly charged ions were concentrated between *m/z* 500 and 1,300. Furthermore, the Pro iQ performance enabled the acquisition of precise MS spectra for oligo dT sequences ranging from 4,500 to 12,000 Da. Subsequent deconvolution using OpenLab CDS allowed for the seamless and accurate verification of molecular weights for all six target peaks.

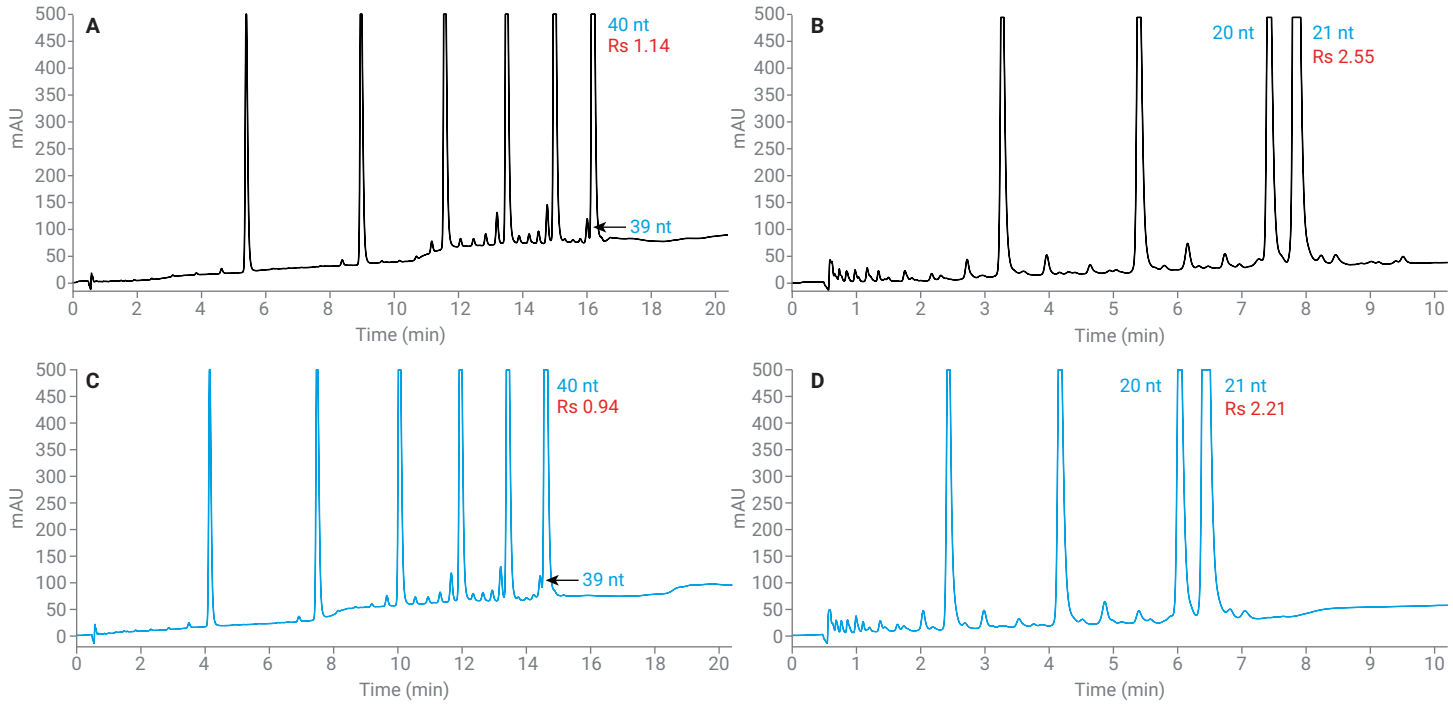

Figure 1. UV chromatograms of oligonucleotide ladder standard (A, C) and oligonucleotide resolution standard (B, D) analyzed in 100 mM HAA using Agilent Altura Oligo HPH-C18 (A, B) and Agilent AdvanceBio Oligonucleotide (C, D) columns.

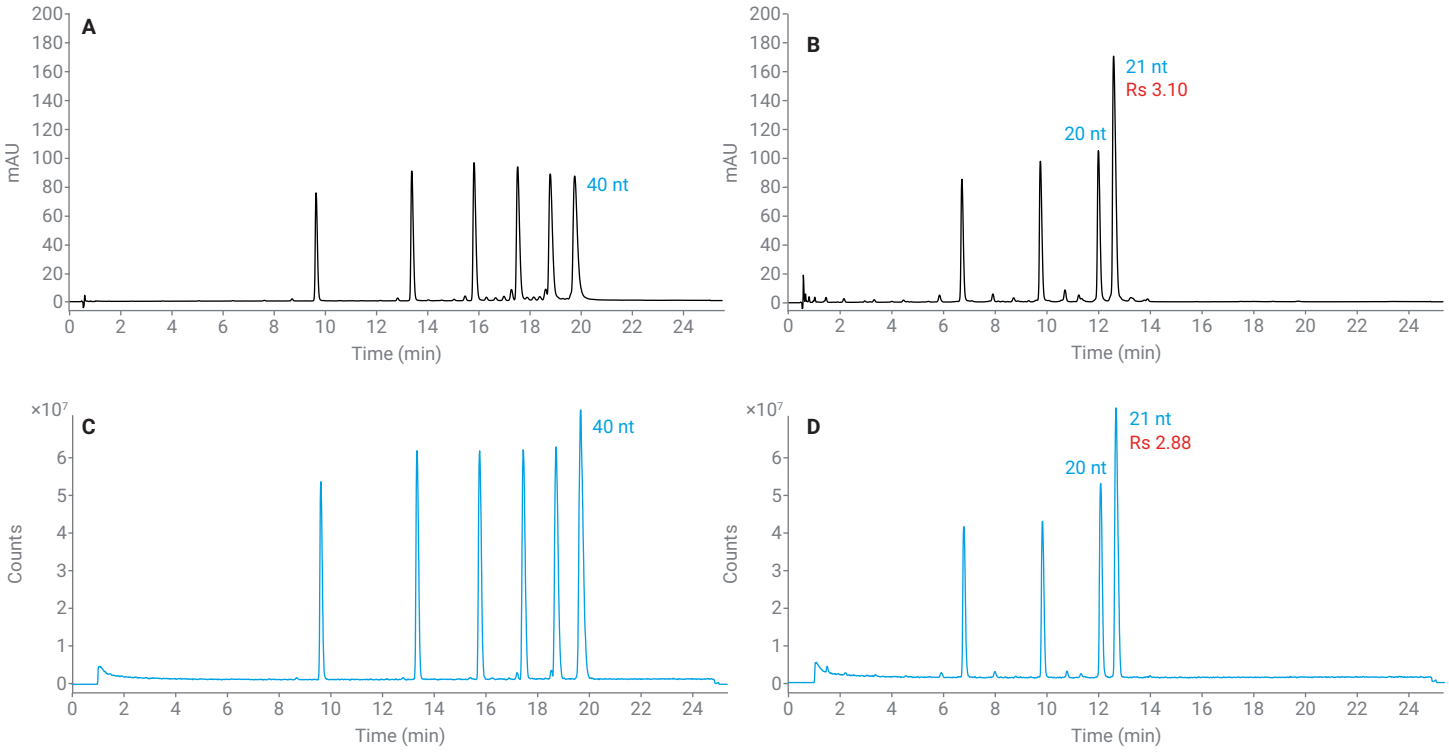

Figure 2. UV chromatogram and Agilent InfinityLab Pro iQ total ion chromatograms (TIC) of oligonucleotide ladder standard (A, C) and oligonucleotide resolution standard (B, D) analyzed in 15 mM HA/25 mM HFIP using an Agilent AdvanceBio Oligonucleotide column (black: UV chromatogram, blue: TIC).

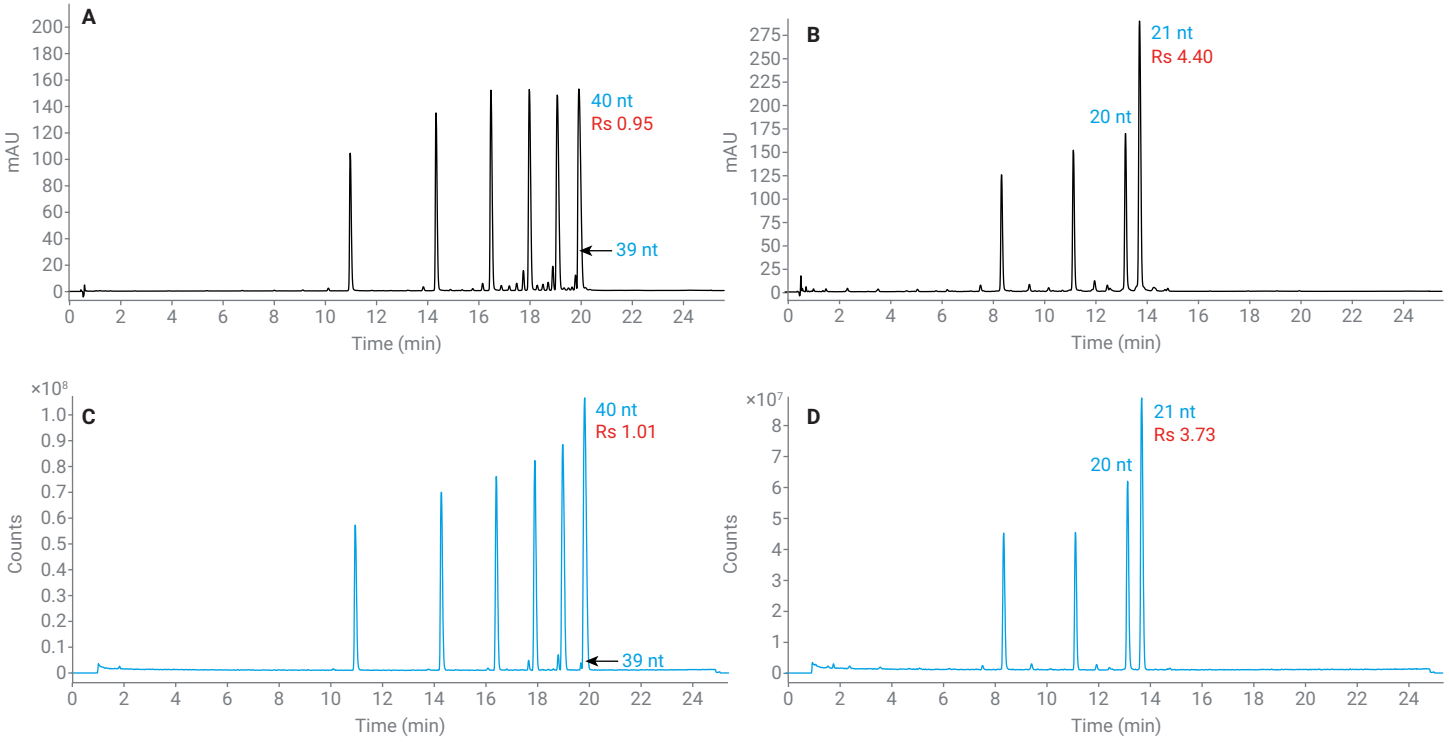

Figure 3. UV chromatogram and Agilent InfinityLab Pro iQ total ion chromatograms (TIC) of oligonucleotide ladder standard (A, C) and oligonucleotide resolution standard (B, D) analyzed in 15 mM HA/25 mM HFIP using an Agilent Altura Oligo HPH-C18 column (black: UV chromatogram; blue: TIC).

Characterizing the 39 nt oligonucleotide impurity is inherently challenging due to its close elution with the 40 nt main peak, necessitating an MS system with a sufficiently high scan speed to accurately sample narrow chromatographic windows. As illustrated in Figure 5, the 39 nt species was successfully resolved from the 40 nt peak. Subsequent deconvolution revealed a mass reduction of 304 Da relative to the 40 nt sequence, facilitating the precise identification of this truncated impurity.

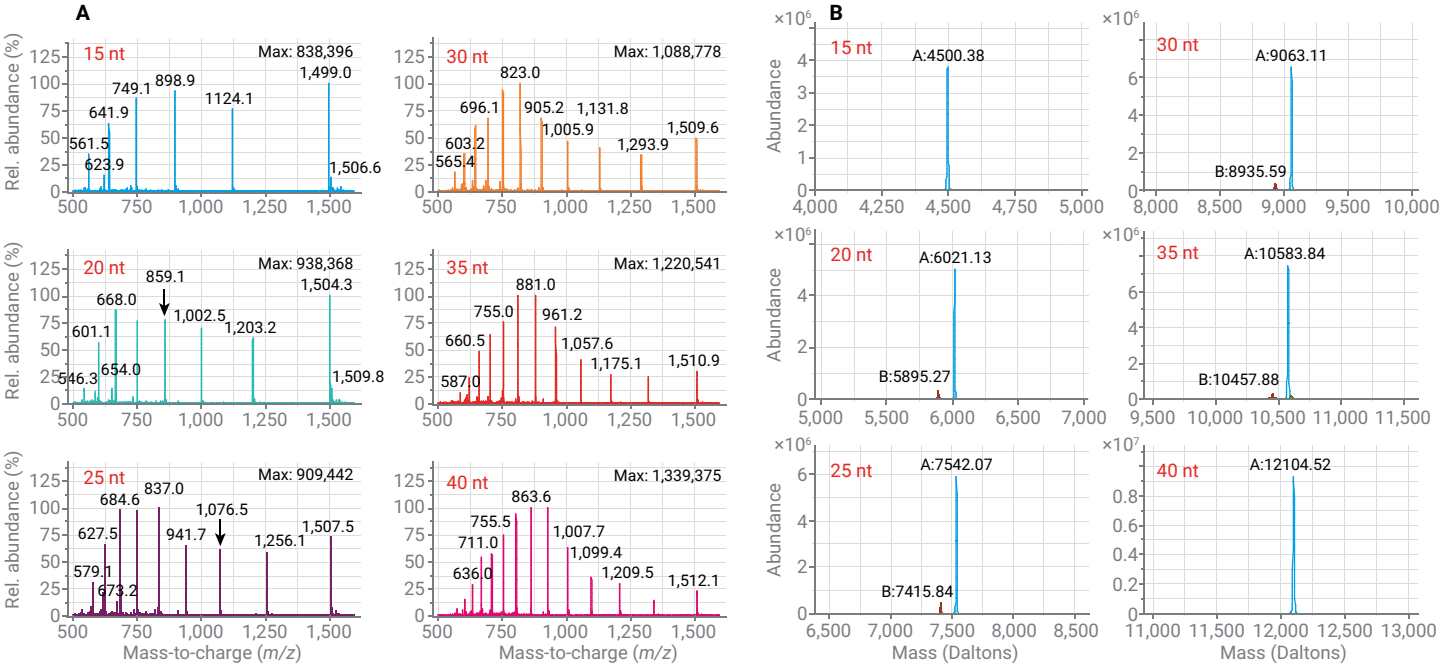

Figure 4. MS raw spectra (A) and deconvoluted spectra (B) of 15, 20, 25, 30, 35, and 40 nt oligonucleotide ladder peaks obtained using an Agilent InfinityLab Pro iQ and Agilent OpenLab CDS.

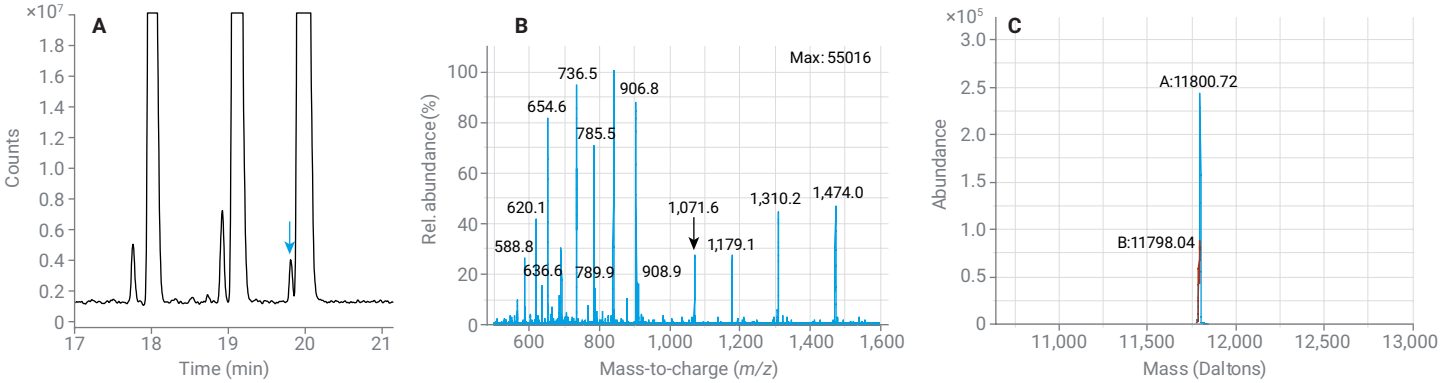

Figure 5. MS raw and deconvoluted spectra of the 39 nt oligonucleotide identified in Figure 3; (A) zoomed TIC; (B) MS raw spectra; (C) deconvoluted spectra.

Conclusion

This application demonstrates that high-resolution separation and molecular weight verification of oligonucleotide impurities are achievable even at a reduced ion-pair concentration of 15 mM HA, leveraging the combined power of the Agilent InfinityLab Pro iQ and Agilent OpenLab CDS deconvolution.

While mitigating column hardware-induced peak broadening typically necessitates high concentrations of ion-pair reagents, the implementation of ultra-inert coating technology significantly enhances chromatographic resolution under MS-friendly conditions. This advancement offers several critical advantages: it ensures more accurate sample purity assessments, reduces operational costs through lower reagent consumption, minimizes chemical noise, and streamlines LC/MS method development.

Furthermore, the rapid scan speed of the InfinityLab Pro iQ preserves the integrity of the improved peak resolution, enabling the precise characterization of larger species such as the 39 nt oligonucleotide impurity. This capability is particularly significant, as such high-molecular-weight impurities have historically been challenging to resolve and analyze under standard LC/MS conditions.

References

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