Application Note

Oligonucleotide Analysis

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Molecular Confirmation of Oligonucleotides Using Agilent LC/MSD XT and OpenLab CDS

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Abstract

Mass spectrometry (MS) is an important tool for quality control during the development of synthetic oligonucleotides. This application note demonstrates molecular weight (MW) confirmation and simple characterization workflows for synthetic oligonucleotides using the Agilent LC/MSD XT single quadrupole LC/MS and MS spectral deconvolution feature in Agilent OpenLab CDS 2.8.

Introduction

Oligonucleotides are short nucleic acid sequences manufactured using phosphoramidite chemistry, a stepwise synthesis that incorporates nucleotides based upon a targeted complementary sequence. Depending on the application, synthetic oligonucleotides are significantly modified. These modifications can occur with nucleobases, the ribose or deoxyribose, and even the phosphate backbone if one considers the sulfurization with phosphorothioates. As such, confirmation of full-length product molecular weight is necessary, especially in medium- to high-throughput applications such as lead development.

Molecular weight determination for oligonucleotides is often performed with high-resolution accurate mass, especially if characterization and identification of impurities is necessary. However, unit mass detectors such as a single quadrupole mass spectrometer can be used to provide rapid results with sufficient accuracy to confirm the intended sequence.

In this application note, oligonucleotide standards, including a poly dT ladder and antisense oligonucleotide (ASO), were analyzed to demonstrate molecular confirmation workflows using LC/MS. Additionally, the ability to perform basic characterization using an LC/MSD XT single quadrupole LC/MS and spectral deconvolution is shown.

Experimental

Chemical and standards

Agilent DNA ladder standard (part number 5190-9029). Fully thiolated synthetic oligonucleotides were purchased from Integrated DNA Technologies (Coralville, IA, USA).

Sample preparation

The oligonucleotide ladder standards were dissolved with 1 mL of deionized (DI) water before use. The final concentration was 4 nmol/µL.

All synthetic oligonucleotide samples were also dissolved with 1 mL of DI water. Samples were then diluted to 50 µg/mL.

Instrumentation

An LC/MSD XT (G6135C) with an Agilent electrospray ionization (ESI) source (G1948B) was used to analyze the oligonucleotide samples. An Agilent 1290 Infinity II BioLC or a passivated Agilent 1290 Infinity II LC can be used for this analysis. LC method parameters applied for the analysis are shown in Table 1 and MS parameters are shown in Table 2.

Methods

Liquid chromatography

Table 1. Agilent 1290 Infinity II LC method.

Agilent 1290 Infinity II	
Parameter	**Value**
Column	Agilent AdvanceBio oligonucleotide columns, 2.1 × 50 mm, 2.7 µm (p/n 659750-702)
Sampler Temperature	4 °C
Mobile Phase A	100 mM HFIP and 15 mM TEA in water
Mobile Phase B	Methanol
Flow Rate	0.5 mL/min
Injection Volume	2 µL
Column Temperature	65 °C
Gradient Program	Time (min) %B 0.0 15 10.0 30 11 95

Mass spectrometry

Table 2. Agilent LC/MSD XT method.

Agilent LC/MSD XT (G6135C)	
Parameter	**Value**
Ion Source	AJS
Polarity	Negative
Drying Gas Temperature	275 °C
Gas Flow	12 L/min
Nebulizer Pressure	35 psi
Capillary Voltage Positive	4,500 V
Sheath Gas Temperature	350 °C
Sheath Gas Flow	12 L/min
Nozzle Voltage	2,000 V
Scan Range	1,000 to 3,000 *m/z* profile
Scan Time	1,500 ms
Fragmentor Voltage	175 V

Software

OpenLab CDS 2.8 with MS spectral deconvolution capabilities was used to operate the LC/MS instrument and perform the oligonucleotide analysis. The algorithm used in OpenLab CDS for spectral deconvolution is optimized for simplifying spectra of multiply charged molecules obtained from unit mass instrumentation. Provided the mass spectrum is high quality, only minimal adjustments for parameters are necessary. Table 3 summarizes the settings chosen for the target analytes. Note that advanced settings, including MW algorithm, MW algorithm threshold, and Envelope threshold, were optimized for oligos between 15mer to 40mer.

Results and discussion

Oligos often take high charge states during electrospray ionization, which may complicate LC/MS analysis and molecular weight confirmation workflows. For example, a 21mer (Figure 1) may demonstrate a wide charge distribution, with the 8 or 9 charge state being the most dominant. The resulting mass spectrum may also be non-Gaussian, with a wide distribution that may overlap with interferences at lower *m/z* values. Further, mobile phase, oligonucleotide length, and sequence may affect the charge state distribution, making it critical to empirically determine molecule behavior prior to implementing an LC/MS method for routine testing.[1,2]

For this reason, the acquisition method should be optimized using a suitable standard, such as the Agilent DNA ladder. Because the test mixture contains oligonucleotides of varying lengths (15, 20, 25, 30, and 35mer), assessing method performance with this standard ensures the LC/MS method can accommodate for different types of oligos.

Software

Table 3. Agilent OpenLab CDS spectral deconvolution processing method for DNA ladder standard.

Parameter	Value
Spectrum Extraction Type	Peak apex spectrum
Background Mode	Spectrum at peak start and end
Use *m/z* Range	Disabled
Low Molecular Weight	4,000
High Molecular Weight	13,000
Maximum Charge	40
Minimum Peaks in Set	4
MW Agreement (0.01%)	5
Absolute Noise Threshold	1,000
Relative Abundance Threshold (%)	15
MW Algorithm	Curve Fit
MW Algorithm Threshold	40
Envelope Threshold	10

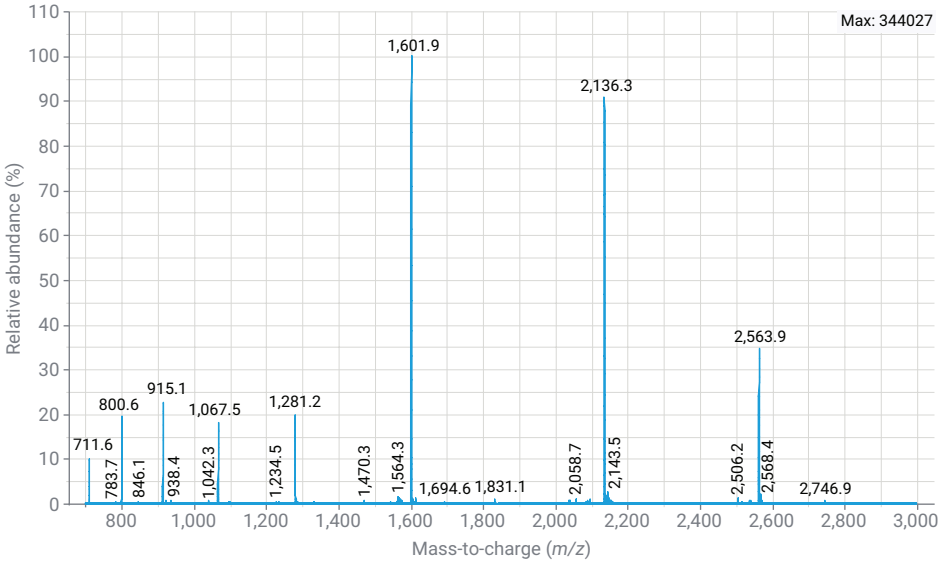

Figure 1. Mass spectrum for a 21mer DNA oligonucleotide.

Although this is highly source- and mobile phase-dependent, in general, longer oligonucleotides take higher charge states with wider distributions. Figure 2 shows an example of the poly dT ladder and differences in charge state distribution observed for each oligonucleotide. The 15mer poly dT oligonucleotide has primarily one dominant charge state (z = 3 at 1,499.3 *m/z*), while the 40mer has significantly more charge states, ranging from 5 to 13, from 750 to 2,500 *m/z*. The LC/MSD XT has an upper mass range of 3,000 *m/z* for the detection of larger molecule species, making it ideal for a wide range of oligonucleotides.

Additionally, the data analysis processing method should also be optimized to accommodate the different charge envelopes for oligos of varying lengths and sequences. The deconvolution results shown in Table 4 demonstrate how the same data analysis parameters might be used for different length oligonucleotides. Minimum peaks in set and Envelope threshold (%) were both optimized to accommodate for variance in charge states and distribution, respectively. Deconvoluted masses for oligonucleotides were within at least ±1 Dalton, which is to be expected for the unit mass detection of oligonucleotides varying in molecular weight.

Although the settings used for the DNA ladder standard may be suitable when analyzing many types of samples, optimization of the processing method may be necessary. This is useful when deviating from routine testing, such as performing ad hoc preliminary characterization if unexpected chromatographic peaks or abnormalities in the spectrum are observed.

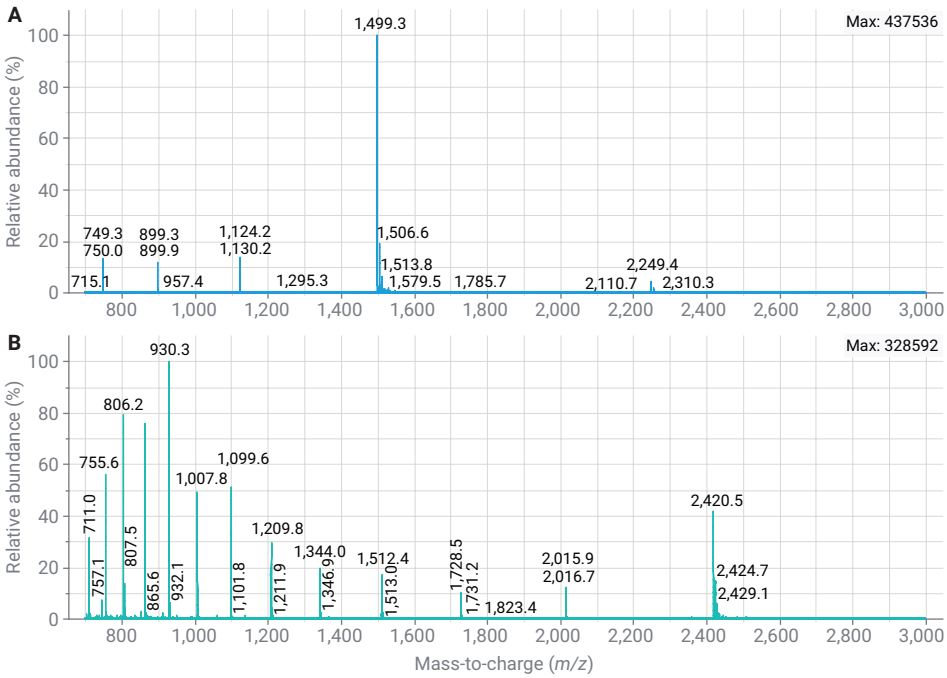

Figure 2. Comparison of mass spectrum of 15mer DNA oligo (A) and 40mer DNA oligo (B). Longer oligonucleotides generally have higher charge states, with broader charge state distributions. This is both ion source- and mobile phase/infusion solvent-dependent.

Table 4. DNA ladder deconvolution results, standard settings.

Sample Name	Spectrum RT (min)	Calculated Mass	Experimental Mass (Da)	Δ Mass (Da)	Mass Accuracy (ppm)
15mer	2.38	4,501.0	4,501.0	0.0	9
20mer	3.594	6,022.0	6,022.0	0.1	11
25mer	4.387	7,543.0	7,543.6	0.6	84
30mer	4.924	9,063.9	9,064.5	0.5	59
35mer	5.274	10,584.9	10,585.5	0.5	51
40mer	5.554	12,105.9	12,106.8	0.9	76

For deconvolution of the 18mer and 20mer ASOs, the target mass range was narrowed down to 6,000 to 8,000 MW; all other parameters remain as they were in Table 3. This allowed for proper identification of full-length product as well as shortmer and longmer impurities. Deconvoluted masses are reported in Table 5; delta masses between calculated and measured are within expected performance for unit mass instrumentation.

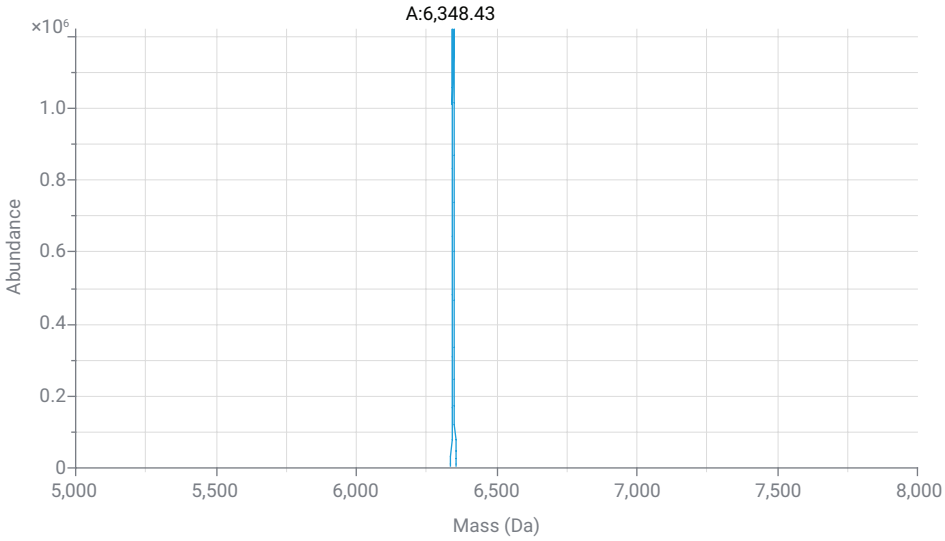

Figure 3. Deconvoluted spectrum for 18mer ASO using optimized deconvolution parameters.

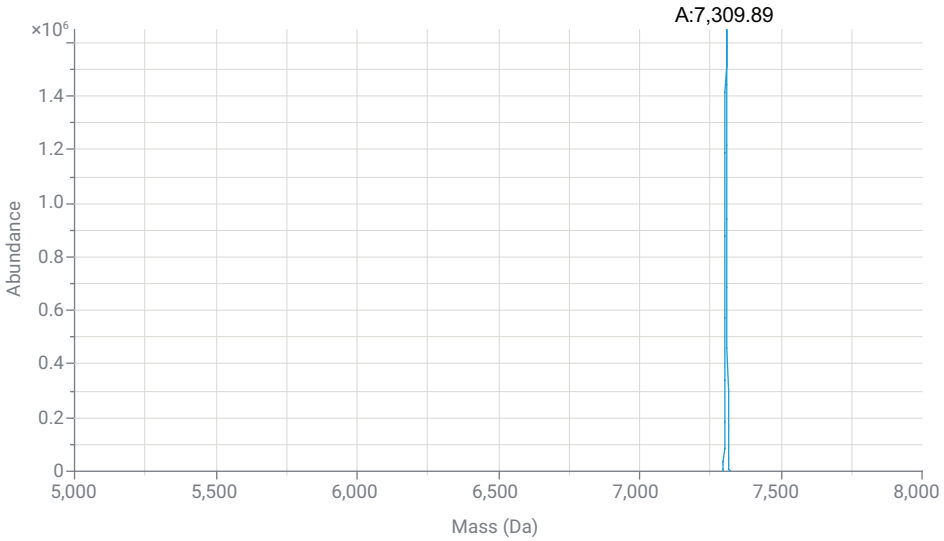

Figure 4. Deconvoluted spectrum for 20mer ASO.

Table 5. Deconvolution results for 18mer and 20mer antisense oligonucleotides.

Sample	Sequence	Spectrum RT (min)	Calculated Mass	Experimental Mass (Da)	Δ Mass (Da)	Mass Accuracy (ppm)
18mer	5'- U*/i2MOErC/*/i2MOErA/* /i2MOErC/*U*U* U*/i2MOErC/*/i2MOErA/* U*/i2MOErA/*/i2MOErA/* U*/i2MOErG/*C* U*/i2MOErG/*G -3'	5.764	6,348.2	6,348.4	0.2	36
20mer	5'- U*/i2MOErC/*U* U*/i2MOErG/*T* T*/i2MOErA/*/i2MOErC/* /i2MOErA/*/i2MOErT/*/i2MOErG/* /i2MOErA/*/i2MOErA/*/i2MOErA/* U*/i2MOErC/*/i2MOErC/* /i2MOErC/*C -3'	7.280	7,309.2	7,309.9	0.7	94
20mer, n-1	NA	7.012	NA	6,913.49	0.7	NA

When the data analysis processing method is optimized, with sufficient separation and signal intensity, low level impurities can still be detected. Figure 5 shows the total ion current (TIC) of an 18mer ASO, with baseline resolution of putative low molecular weight impurity. Spectrum extraction and deconvolution was performed, with the result (6,913.49) strongly indicating that this was indeed an n-1 impurity and was 0.12% of the main peak by absolute abundance of deconvoluted spectrum.

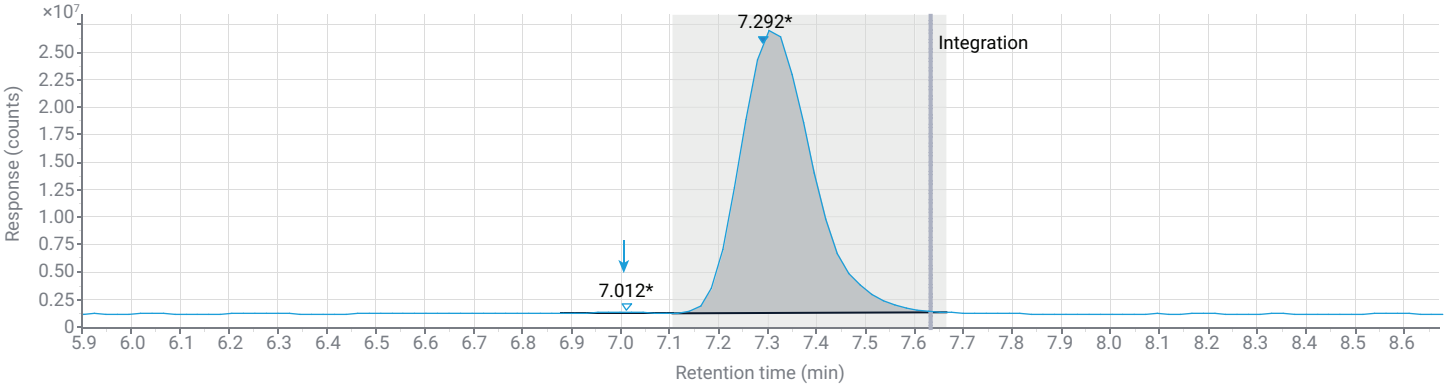

Figure 5. Total ion current (TIC) for the 20mer oligo. Early eluting impurity was manually integrated and spectrum extracted for deconvolution.

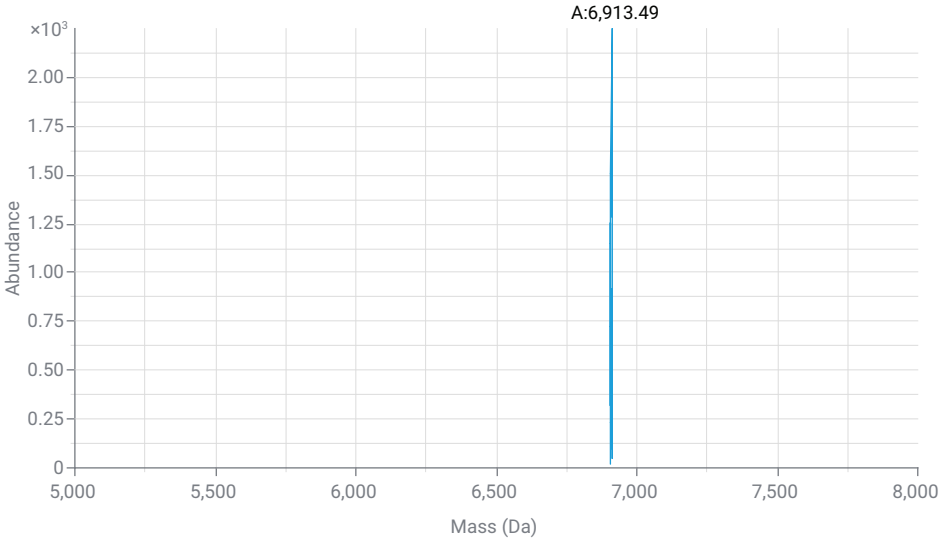

Figure 6. Deconvoluted spectrum for an early-eluting impurity. Deconvoluted mass of 6,913.49 Da strongly indicates this as n-1; further confirmation and characterization would need to be performed using accurate mass detection.

Conclusion

The combination of the Agilent 1290 Infinity II LC/MSD XT and OpenLab CDS 2.8 with MS spectral deconvolution is a useful tool for rapid molecular weight confirmation and purity assessment of oligonucleotides. This combination of analytical tools provides user-friendly software and robust hardware to enable oligonucleotide analysis workflows where speed-to-answer is prioritized. Spectral deconvolution in OpenLab CDS can be used for routine molecular weight confirmation for oligonucleotides of varying lengths, as demonstrated by analysis of a DNA ladder.

References

1. Chen, B.; Mason, S. F.; Bartlett, M. G. The Effect of Organic Modifiers on Electrospray Ionization Charge-State Distribution and Desorption Efficiency for Oligonucleotides. *Journal of the American Society for Mass Spectrometry* **2013**, *24(2)*, 257–264. https://doi.org/10.1007/s13361-012-0509-5
2. Basiri, B.; Murph, M. M.; Bartlett, M. G. Assessing the Interplay Between the Physicochemical Parameters of Ion-pairing Reagents and the Analyte Sequence on the Electrospray Desorption Process for Oligonucleotides. *Journal of the American Society for Mass Spectrometry* **2017**, *28(8)*, 1647–1656. https://doi.org/10.1007/s13361-017-1671-6

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