Application Note

Biopharma

Oligonucleotide Mass Confirmation and Impurity Identification

Using Agilent InfinityLab LC/MSD XT and Agilent OpenLab CDS

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Abstract

This application note highlights the use of the Agilent InfinityLab LC/MSD XT system on molecular weight (MW) confirmation and impurity identification for a diverse range of synthetic oligonucleotides (oligos). The MS spectral deconvolution feature in Agilent OpenLab CDS version 2.8 streamlines data analysis. The results show high mass accuracy and excellent reproducibility in oligo MW determination and impurity identification.

Introduction

Synthetic oligonucleotides are chemically synthesized, and usually modified to increase molecule stability or enhance biological efficacy. They are present in various sizes and forms and are usually classified into categories such as small interfering RNA (siRNA), antisense oligonucleotides (ASO), microRNA (miRNA), aptamers, and single-guide RNA (sgRNA).

The primary product quality attributes of synthetic oligonucleotides are their mass, purity, product-related impurities, and sequence composition. The characterization is usually conducted using high-resolution mass spectrometry (HRMS).[1] Unit mass spectrometry with sufficient mass accuracy such as single quadrupole MS (SQ) is also capable of determining the MW of full-length product (FLP) and related impurities. Compared to HRMS, SQ is more cost-effective and has greater ease-of-use with sufficient mass accuracy for MW determination.

In this application note, a diverse range of oligos were analyzed to confirm their identity by MW determination of their respective FLPs using the InfinityLab LC/MSD XT system. Impurities in one of the antisense oligonucleotide (ASO) samples were also identified under similar conditions. The user-friendly deconvolution feature of OpenLab CDS Data Analysis version 2.8 streamlines oligo data processing and enables data automation.

Experimental

Chemical and standards

Triethylamine (TEA) and 1,1,1,3,3,3-hexafluoro-2-propanol (HFIP) were purchased from Sigma-Aldrich (St. Louis, MO, U.S.). RNase-free water was purchased from Integrated DNA Technologies, Inc. (Coralville, IA, U.S.). Methanol (InfinityLab Ultrapure LC/MS grade, part number 5191-4497) was obtained from Agilent Technologies.

Sample and sample preparation

Oligonucleotide RNA Standard (part number 5190-9028) and a 21-mer ASO sample were obtained from Agilent. Other samples were purchased from Integrated DNA Technologies, Inc. (Coralville, IA, U.S.) with standard desalting purification. Sample details are listed in Tables 1 and 2.

Table 1. Oligonucleotide sample details.

Name	Length	Sequence
ASO-18mer	18-mer	/52MOErT/*/i2MOErC/*/i2MOErA/*/i2MOErC/*/ i2MOErT/*/i2MOErT/*/i2MOErT/*/i2MOErC/*/ i2MOErA/*/i2MOErT/*/i2MOErA/*/i2MOErA/*/ i2MOErT/*/i2MOErG/*/i2MOErC/*/i2MOErT/*/ i2MOErG/*/32MOErG/
ASO-21mer	21-mer	rArCrA rUrArU rUrCrC rCrUrG rArUrG rArGrG rUdTdT
16S rRNA Rev	21-mer	ACG GCT ACC TTG TTA CGA CTT
Aptamer	28-mer	/52FC/mGmGrArA/i2FU//i2FC/mAmG/ i2FU/mGmAmA/i2FU/mG/i2FC//i2FU//i2FU/mA/ i2FU/mA/i2FC/mA/i2FU//i2FC//i2FC/mG/3InvdT/
cDNA Cloning Primer	38-mer	GGC CAC GCG TCG ACT AGT ACT TTT TTT TTT TTT TTT TV

Table 2. Oligonucleotide codes and descriptions.

Code	Description
*	Phosphorothioate bond
A	2'-Deoxyribose adenine
C	2'-Deoxyribose cytosine
G	2'-Deoxyribose guanine
mA	2'-O-Methyl A
mG	2'-O-Methyl G
rA	Ribose adenine
rG	Ribose guanosine
/52MOErT/	5' 2-Methoxyethoxy T
/i2MOErA/	Internal 2-methoxyethoxy A
/i2MOErC/	Internal 2-methoxyethoxy C
/i2MOErT/	Internal 2-methoxyethoxy T
/i2MOErG/	Internal 2-methoxyethoxy G
/32MOErG/	3' 2-Methoxyethoxy G
/52FC/	5' Fluoro C
/i2FU/	Internal Fluoro U
/i2FC/	Internal Fluoro C
/3InvdT/	3' Inverted T
V	Any of the three bases (A, G, C)

The RNA standard was dissolved with 1 mL of RNase-free water before use. The final concentration was 2 pmol/µL. Other oligo samples were dissolved and diluted with RNase-free water to 50 ng/µL.

Instrumentation and software

Agilent 1290 Infinity II bio LC, including:

- Agilent 1290 Infinity II bio high-speed pump (G7132A)
- Agilent 1290 Infinity II bio multisampler (G7137A) with Agilent Infinity II sample cooler (option #100)
- Agilent 1290 Infinity II multicolumn thermostat with Quick Connect bio heat exchanger (G7116B)
- Agilent 1290 Infinity II Diode Array Detector (G7117B) with Max-Light cartridge cell LSS, 10 mm

Agilent InfinityLab LC/MSD XT system (G6135C)

Agilent OpenLab CDS, version 2.8, with MS spectral deconvolution feature

LC/MS analysis

LC/MS method parameters are listed in Tables 3 and 4.

Table 3. Liquid chromatography parameters.

Agilent 1290 Infinity II Bio LC System	
Parameter	**Value**
Column	Agilent AdvanceBio Oligonucleotide, 2.1 × 50 mm, 2.7 µm (p/n 659750-702)
Thermostat	4 °C
Solvent A	15 mM TEA and 100 mM HFIP in water
Solvent B	Methanol
Flow Rate	0.5 mL/min
Gradient	Time (min) %B 0.0 5 10.0 30 10.5 95
Post Time	9 min
Injection Volume	1 µL, with needle wash flush port 10 seconds, 50% methanol
Column Temperature	65 °C
Detection (DAD)	260 nm/4 nm, Ref: 355 nm/20 nm Peak width > 0.013 min (20 Hz)

Table 4. MS data acquisition parameters.

Agilent InfinityLab LC/MSD XT System	
Parameter	**Value**
Source	AJS
Polarity	Negative
Gas Temperature	275 °C
Gas Flow	12 L/min
Nebulizer	35 psi
Sheath Gas Temperature	350 °C
Sheath Gas Flow	12 L/min
Capillary Voltage	4,500 V
Nozzle Voltage	2,000 V
Fragmentor	175 V
Scan Mode	Scan only
Scan Range	*m/z* 1,000 to 3,000 in profile mode
Scan Time	1,500 ms

Data processing

Optimization of spectral deconvolution parameters is discussed in a separate publication.[2] Table 5 summarizes the primary and advanced deconvolution settings for the samples analyzed in this application note.

Table 5. Agilent OpenLab CDS spectral deconvolution parameters.

Parameter	Value
Spectrum Extraction Mode	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	3
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

Results and discussion

Separation of RNA standard

Agilent supplies oligonucleotide standards for both DNA and RNA samples. It is a good practice to run the standards prior to analyzing the actual samples as a system suitability test of system performance, such as column resolution, detector sensitivity, and deconvolution settings. In this application note, Agilent RNA standard was used to check the method performance. Excellent chromatographic resolution down to a single nucleotide was achieved among four different lengths of standards (14-mer, 17-mer, 20-mer, and 21-mer), as shown in Figure 1A. The peak apex mass spectrum was extracted from each peak for deconvolution. Figure 1B displays the peak apex spectrum of the 21-mer standard. The measured masses for each standard are all within ± 0.5 Da of their respective theoretical masses (Table 6). The study's findings highlight the capability of using unit mass resolution instruments to deliver precise mass accuracy measurements, which is essential in QA/QC settings.

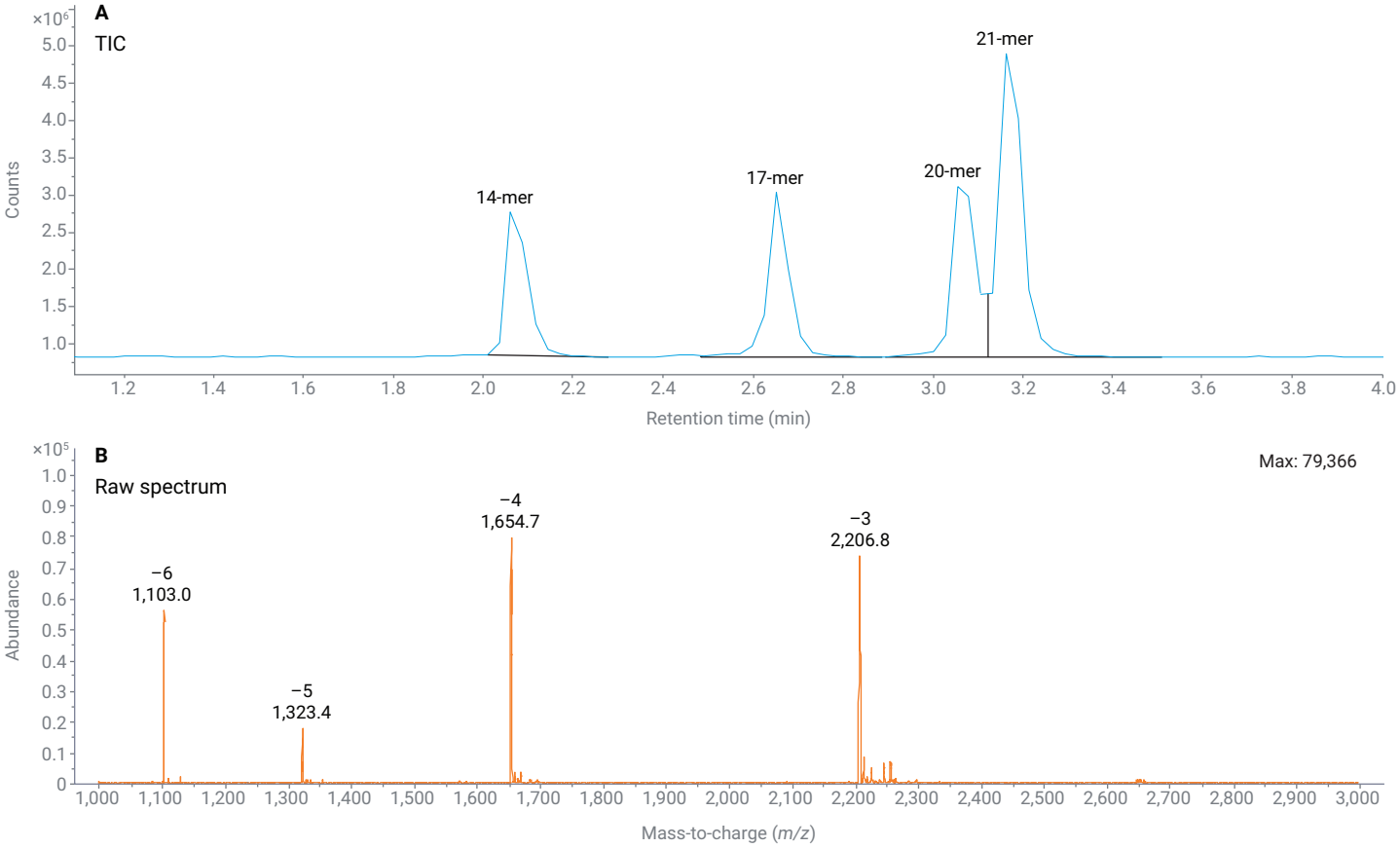

Figure 1. LC/MS chromatogram and mass spectrum of Agilent RNA standard. (A) Total ion chromatogram (TIC) of RNA standard. (B) MS spectrum of 21-mer RNA standard with charge state and *m/z* value labeled above each charge isoform.

Table 6. RNA standard deconvolution results.

Sample	Peak Spectrum RT (min)	Theoretical Mass (Da)	Measured Mass (Da)	Δ Mass (Da)
14-mer	2.07	4,397.7	4,397.8	0.1
17-mer	2.65	5,338.3	5,338.2	−0.1
20-mer	3.08	6,278.8	6,278.5	−0.3
21-mer	3.17	6,624.0	6,623.8	−0.3

MW determination of oligo samples

The LC/MSD XT enables the detection of multiple charge states in oligos and enhances confidence in MW determination. In this study, the five oligo samples ranging from 4 to 12 kDa in MW were analyzed. Two samples, ASO-18mer and aptamer, are highly modified. Regardless of the length and composition variations, the optimized method successfully separated and determined the MW of each oligo.

Figure 2 shows the overlaid TIC of the samples. Under the reversed-phase LC condition, the elution order of oligos is associated with the chain length, linker type, and the degree of chemical modification. The phosphorothioation of ASO-18mer, together with full methoxyethoxylation at the 2'-position leads to more hydrophobic retention on RPLC.

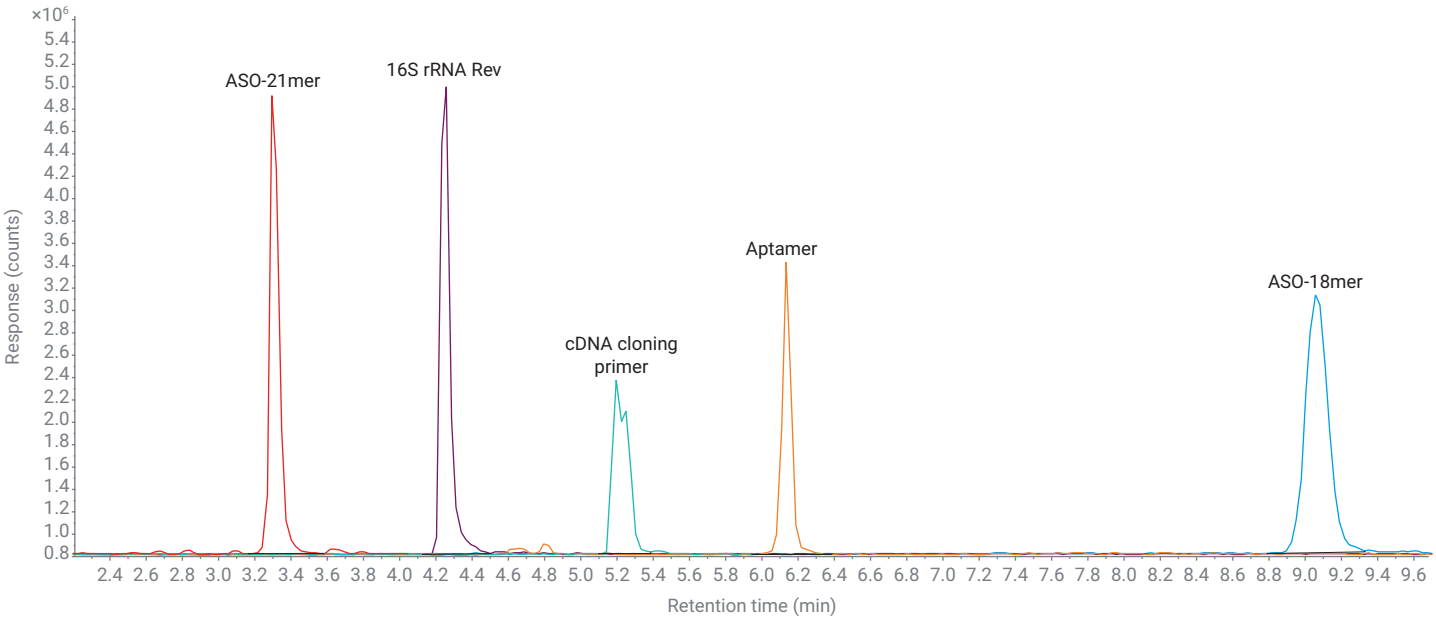

Figure 2. Overlaid TICs of the five oligo samples.

The longest oligo sample in this study was the cDNA cloning primer, with 38 nucleotides in the sequence. The sample is heterogenous, containing three sequence variants which differ at the last nucleobase. The three variants chromatographically coeluted, causing the shouldering of the peak. The mass spectrum of the cDNA cloning primer exhibits a broad charge state distribution (Figure 3). The wide mass range of the LC/MSD XT enabled the detection of up to seven charge states. The sufficient ion sets enabled accuracy in mass determination. At each charge state in the mass spectrum, the three variants were clearly resolved from each other, as shown in the inset of Figure 3. As a result, the masses of the three variants were determined and confirmed even though they were coeluting.

Optimization of the MS deconvolution data processing method is necessary to manage the diverse samples with varying lengths and sequence compositions. Previously published results[2] have addressed the impact of each parameter and provided guidance on method optimization. Using the optimal settings, all five oligo samples were processed. The deconvolution results are summarized in Table 7. The results indicated a high degree of mass accuracy and confirmation of the FLPs.

Table 7. Deconvolution results of the samples.

Sample	Theoretical Mass (Da)	Measured Mass (Da)	Δ Mass (Da)
ASO-18mer	7,127.2	7,127.2	0.0
ASO-21mer	6,631.0	6,630.9	−0.1
16S rRNA Rev	6,372.2	6,372.1	−0.1
Aptamer	9,116.6	9,116.5	−0.1
cDNA Cloning Primer	G: 11,603.5 C: 11,563.4 A: 11,587.5	11,603.7 11,563.7 11,587.3	0.2 0.3 −0.2

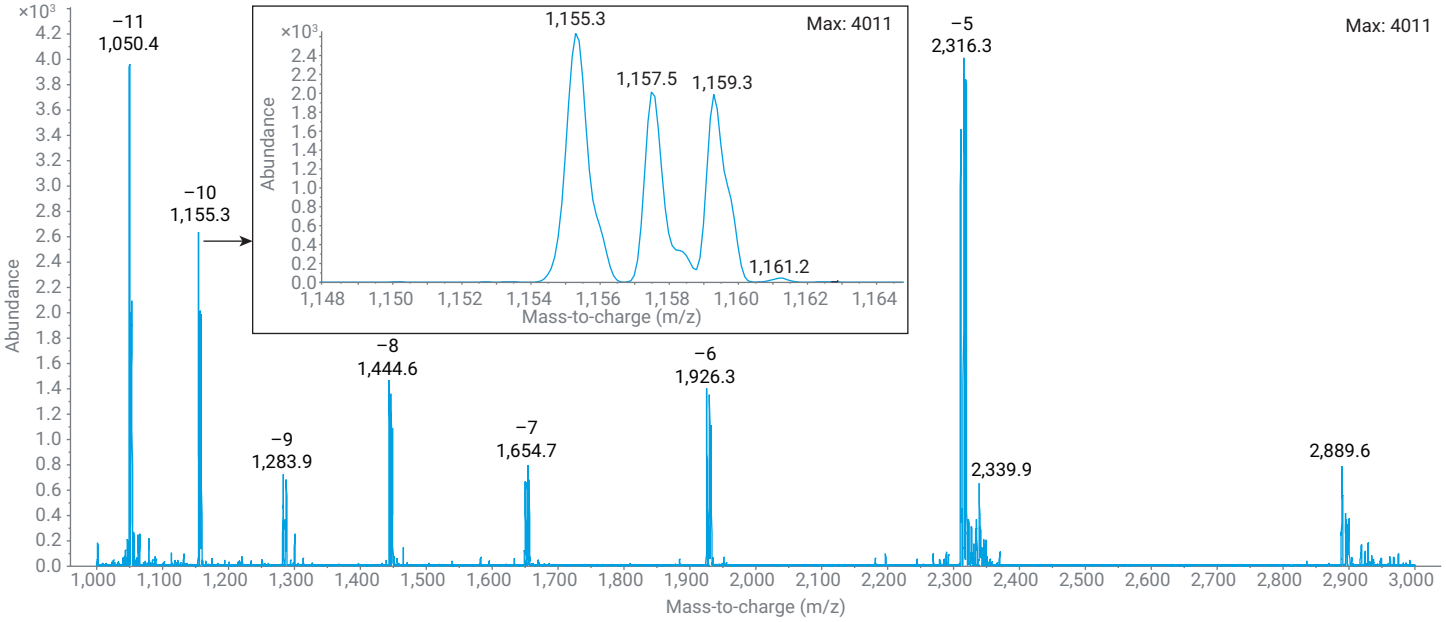

Figure 3. Mass spectrum of the cDNA cloning primer.

Impurity identification

Although solid-phase synthesis has progressed significantly over the years, synthetic oligonucleotides still contain some low-level impurities, which may impact their efficacy and safety. Therefore, impurity analysis is an indispensable part in the characterization of synthetic oligonucleotides.

The impurities can exist in multiple forms, such as truncations, additions, and adducts[3], among others. In this application note, a phosphorothioate ASO-21mer sample was analyzed for its impurities using the LC/SQ system. The LC gradient was shallowed from 5 to 15% B within 10 minutes to improve chromatographic separation. The impurities eluted both before and after the FLP peak, as shown in the UV chromatogram of the sample (Figure 4).

When the automatic deconvolution function was activated in the data processing method, the deconvoluted masses of all identified peaks were populated in the deconvolution results list. The peaks that eluted before FLP were primarily shortmers, whereas those that eluted after FLP were the various adducts of the ASO. The shortmers were identified as 5'-truncation series, ranging from n-1 to n-9. High mass accuracy was achieved within ± 0.5 Da for each shortmer. Relative quantification of impurities was determined on peak area percent in the UV chromatogram. The relative UV response factor is more consistent among different lengths of oligo impurities, unlike the differing ionization efficiency in MS.[4]

Replication of five injections was performed to verify the reproducibility of the measurement. The method also demonstrated excellent RT and percent area reproducibility, at < 0.3 and < 5% RSD, respectively. This indicated the robustness of the method performance. The overall results are summarized in Table 8.

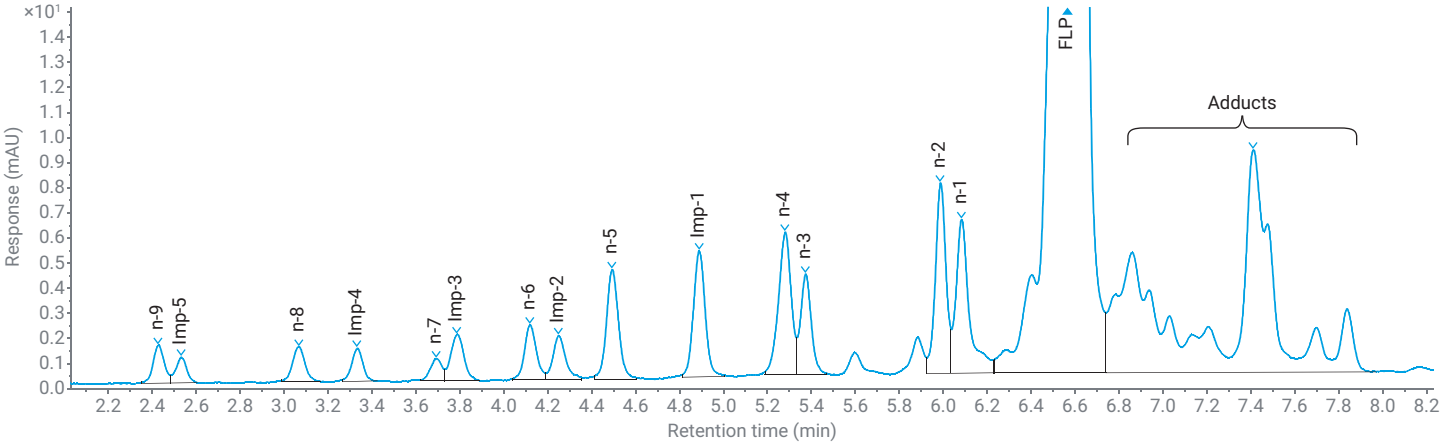

Figure 4. UV chromatogram of ASO-21mer FLP, shortmers, adducts, and other impurities.

Table 8. Summary of the ASO-21mer impurity identification results (n = 5).

Peak	Name	Calculated Mass (Da)	Measured Mass (Da)	Δ Mass (Da)	RT Average	RT %RSD	Area% Average	%RSD
1	FLP	6,631.00	6,631.04	0.04	6.56	0.15	86.57	0.07
2	n-1	6,301.80	6,301.39	−0.41	6.07	0.10	1.09	1.55
3	n-2	5,996.61	5,996.33	−0.29	5.98	0.09	1.07	0.73
4	n-3	5,667.41	5,667.18	−0.23	5.37	0.12	0.57	0.79
5	n-4	5,361.24	5,361.09	−0.15	5.28	0.12	0.96	1.05
6	Imp-1	NA	5,087.92	NA	4.88	0.21	0.78	0.45
7	n-5	5,032.04	5,031.68	−0.35	4.49	0.22	0.69	0.45
8	Imp-2	NA	4,758.30	NA	4.24	0.21	0.29	1.03
9	n-6	4,725.87	4,725.73	−0.14	4.11	0.19	0.35	1.43
10	Imp-3	NA	4,413.07	NA	3.78	0.21	0.32	1.40
11	n-7	4,419.70	4,420.19	0.49	3.68	0.19	0.14	4.61
12	Imp-4	NA	4,107.18	NA	3.32	0.17	0.19	0.67
13	n-8	4,114.52	4,114.39	−0.13	3.06	0.26	0.23	1.50
14	Imp-5	NA	3,778.08	NA	2.53	0.22	0.15	0.97
15	n-9	3,809.34	3,809.21	−0.13	2.42	0.20	0.23	1.06
16	Adducts	NA	NA	NA	7.42	0.14	6.38	0.82

Conclusion

In this application note, the Agilent 1290 Infinity II bio LC system, in combination with the MS spectral deconvolution feature in Agilent OpenLab CDS, version 2.8 has been proven to be a reliable and user-friendly tool for oligonucleotide mass determination and impurity identification. The results demonstrated high mass accuracy and excellent reproducibility. The method can serve as a good starting point for the analysis of diverse types of oligos.

References

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