Application Note

Agilent OpenLab

Automated LC/MS Quality Control of Anti-Sense Oligonucleotides

Using purpose-built software and high-sensitivity single quadrupole detection

Authors

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Abstract

Antisense oligonucleotides (ASOs) are a complex class of therapeutic nucleic acids that require precise and sensitive analytical methods for quality control (QC). This application note presents a streamlined LC/MS workflow for ASO purity assessment using the Agilent InfinityLab Pro iQ Plus LC/MS system and Oligo Analysis Accelerator (OAA) software for Agilent OpenLab CDS. The method builds on an established approach by using an extended mass range single quadrupole detection and purpose-built software to streamline traditionally manual steps such as extracted ion chromatogram (EIC) generation, impurity classification, and UV peak integration. By automating critical data analysis tasks and enabling direct comparison of spectra, the workflow improves accuracy in impurity profiling and facilitates method transfer to compliant QC environments.

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Introduction

Antisense oligonucleotides (ASOs) are an established therapeutic nucleic acid modality, typically manufactured by solid-phase synthesis. Due to their complexity, a common method to assess ASO purity is liquid chromatography mass spectrometry (LC/MS). As demonstrated by Rentel and colleagues[1] , single quadrupole LC/MS can be implemented for routine quality control (QC) lot release testing of single-stranded therapeutic oligos such as ASOs.

However, challenges remain in the analytical workflow, which requires several unique steps that most chromatography data systems (CDS) cannot perform without extensive manual intervention by the analyst. For example, commonly CDS nor MS data systems cannot perform the integration of a standard using extracted ion chromatogram (EIC) integration times to define UV peak integration.

This application note showcases how an extended mass range single quadrupole LC/MS, combined with purpose-built software, can be used to determine the purity of an ASO. The guided workflow simplifies method implementation and facilitates transfer for routine QC batch release.

Experimental

Instrument configuration

This experiment was conducted using the following instrument configuration:

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

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

Sample preparation

A 20-mer, non-HPLC purified antisense oligo ("ASO-1") was obtained from Integrated DNA Technologies (Coralville, IA, USA). The sample was reconstituted in de-ionized water to 1 mg/mL, then further diluted to 0.1 mg/mL in de-ionized water.

Software

Data acquisition was performed in Agilent OpenLab CDS, version 2.8, using the LC/MS parameters shown in Tables 1 and 2. Data analysis was performed in Oligo Analysis Accelerator (OAA) for OpenLab CDS, version 1.1.

LC/MS settings

MS parameters are shown in Table 1. Note the two distinct MS conditions required for each sample analysis. A comparison of full-scan spectra between

Table 1. Source parameters for Agilent InfinityLab Pro iQ Plus LC/MS system (G6170A).

Parameter	Value	
	Standard	**Harsh**
MS	G6170A	
Source	ESI	
Scan Mode	Negative Polarity	
Drying Gas Flow	12.0 L/min	13.0 L/min
Gas Temp	260 °C	350 °C
Nebulizer Pressure	25 psi	
Capillary Voltage	4,000 V	
Scan	m/z 1,450 to 2,175	
Scan Time	1,000 ms	
Fragmentor	100 V	
Gain Factor	1	

Table 2. LC parameters for Agilent Infinity II Bio LC.

Parameter	Value	
Column	Agilent AdvanceBio Oligonucleotide, 2.1 X 150 mm, 2.7 µm	
Sampler Temperature	8 °C	
UV Detection	260/4 nm (Ref 400/80 nm) Peak width > 0.05 min (5 Hz)	
Mobile Phase A	10% ACN, 5 mM tributylammonium acetate, 1 µm EDTA	
Mobile Phase B	80% ACN, 5 mM tributylammonium acetate, 1 µm EDTA	
Flow Rate	0.25 mL/min	
Multi Wash	20:80 water: methanol, flush port; 5 seconds 90:10 water: methanol, flush port; 3 seconds	
Column Temperature	50 °C	
Post Time	1.0 min	
Gradient Program	Time (min)	%B
	0	45
	22	80
	25	80
	26	45

so-called "standard" and "harsh" conditions is used to determine whether ions above the method threshold are adducts.

Results and discussion

OAA is a software using a web client in a client-server architecture that uses "RESTful APIs" to communicate with OpenLab CDS (Figure 1). Using OAA and API calls, users may perform complex data analysis with a more streamlined user interface. The guided workflow displays only the relevant data and information necessary to perform a particular task, effectively automating what otherwise would be highly manual steps within standard CDS software. Further, OAA acts as a front-end to the compliant environment within OpenLab to ensure user access control, change control/versioning, and traceability through audit trails and log files.

A system suitability requirement is that the reference standard purity must meet the method requirements. The Rentel LC/MS method takes a unique approach to assessing purity by generating EICs from the full-scan spectrum of the main peak in a reference standard or sample, because many impurities coelute with the full-length product. Therefore, a highly selective mass detector is required to identify closely related impurities, which are then quantified by integration of the EICs.

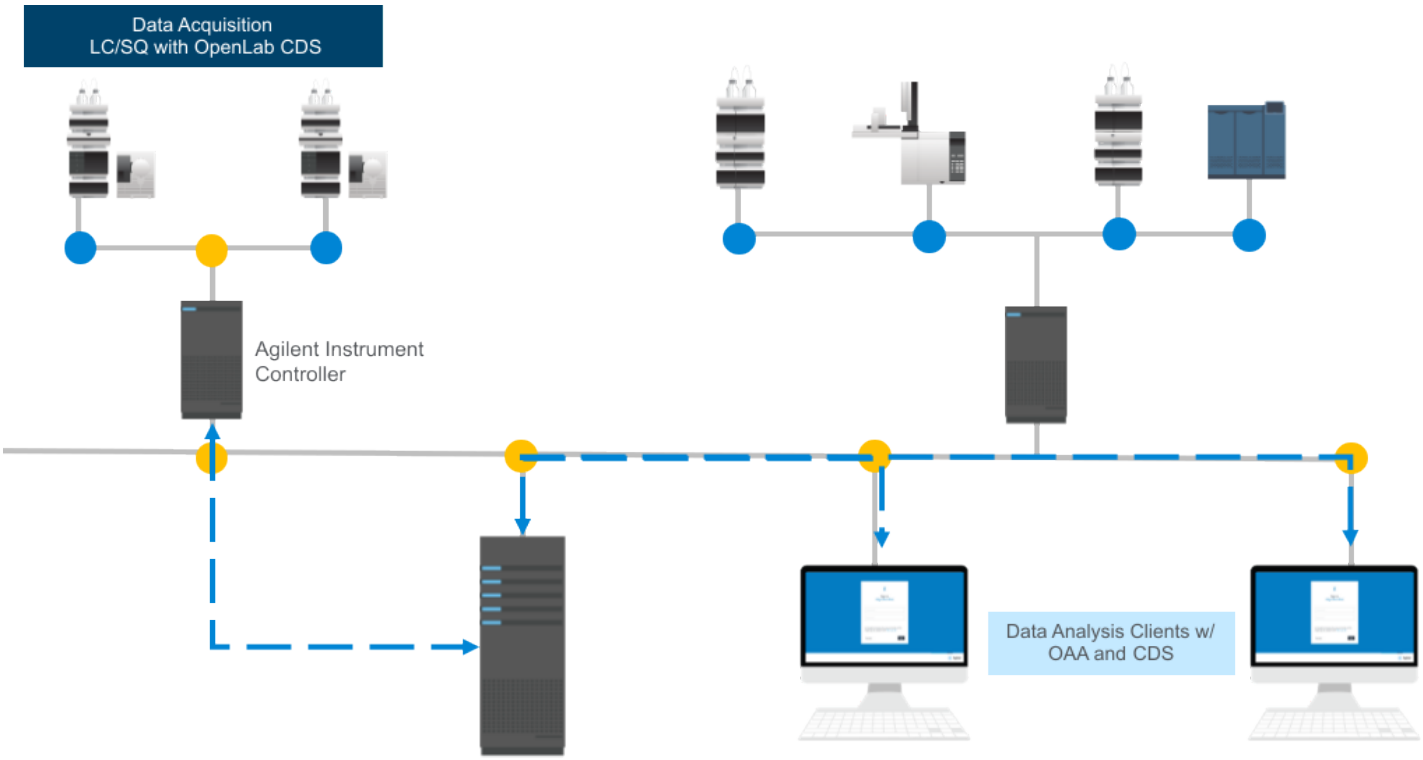

Figure 1. Client-server topology for Oligo Analysis Accelerator in Agilent OpenLab CDS. LC/MS instruments are connected to an Agilent Instrument Controller (AIC), which act as a central hub for instrument control and data acquisition. Data are then stored on the OpenLab Server.

A challenging step in the data analysis workflow is that the earliest eluting EIC defines the integration start of the UV peak, while the latest eluting EIC defines the end. To perform this step correctly, users must generate an EIC for all identified m/z values corresponding to impurities. Each impurity EIC must then be properly integrated, and their elution order determined or confirmed. The integration start and end times of respective early- and late-eluting impurity EICs are then used to integrate the UV peak. This process would be tedious and manual in many chromatography or MS data systems.

To streamline the workflow, the OAA software automatically generates the EICs based on user-uploaded inputs ("ion list") and evaluates whether any of these ions exceed the method-specific threshold. EICs are automatically integrated using predefined processing method integration parameters. Early- and late-eluting impurities are identified using the software algorithm. Conversely, users may predefine a late-eluting impurity, if that impurity is already known. Manual integration of EICs is also supported, if necessary, allowing for overlay with any other EICs to facilitate the process (Figure 2). Any manual integration is recorded in log files and respective audit trails to ensure data integrity. Once all EICs have been integrated according to method specifications, the integration parameters are then automatically applied to the UV signal of the standard.

Another laborious step in the data analysis workflow is the "ion classification" step. Similar to the process used to assess the purity of a reference standard, determining ASO sample purity involves extracting a full-scan spectrum from the main peak. Any m/z values in the average spectrum that exceed an established threshold can then be used to generate an EIC. Note that the full scan will be performed at m/z ±150 of the 4– charge state of the full-length product (FLP). For example, an oligo with a molecular

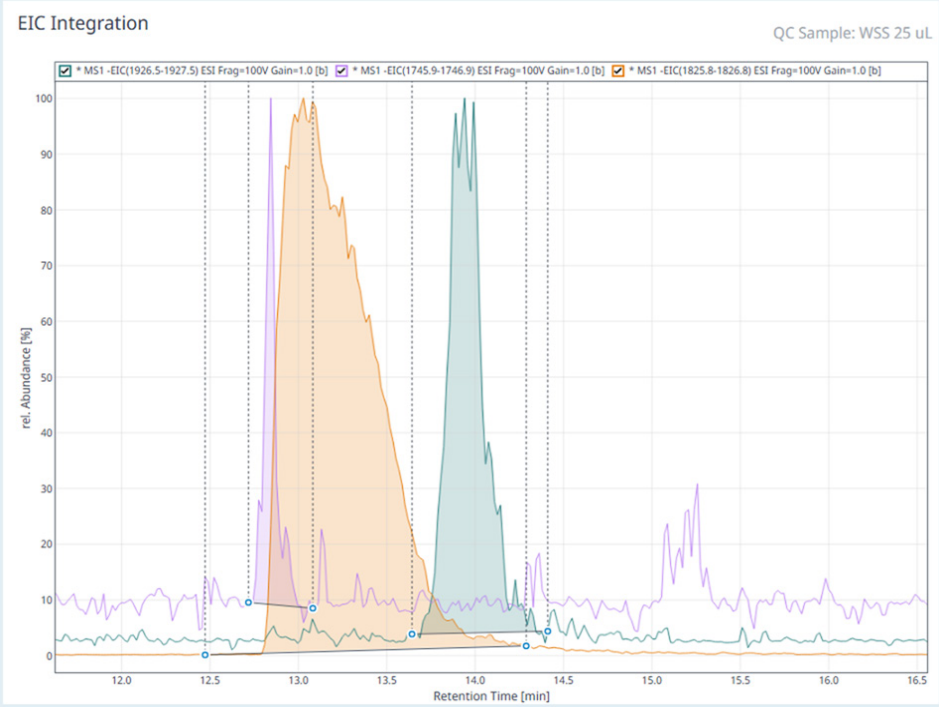

Figure 2. Overlay of extracted ion chromatograms (EICs) for the early- and late-eluting impurities, and full-length product. Users can manually integrate any of the EICs.

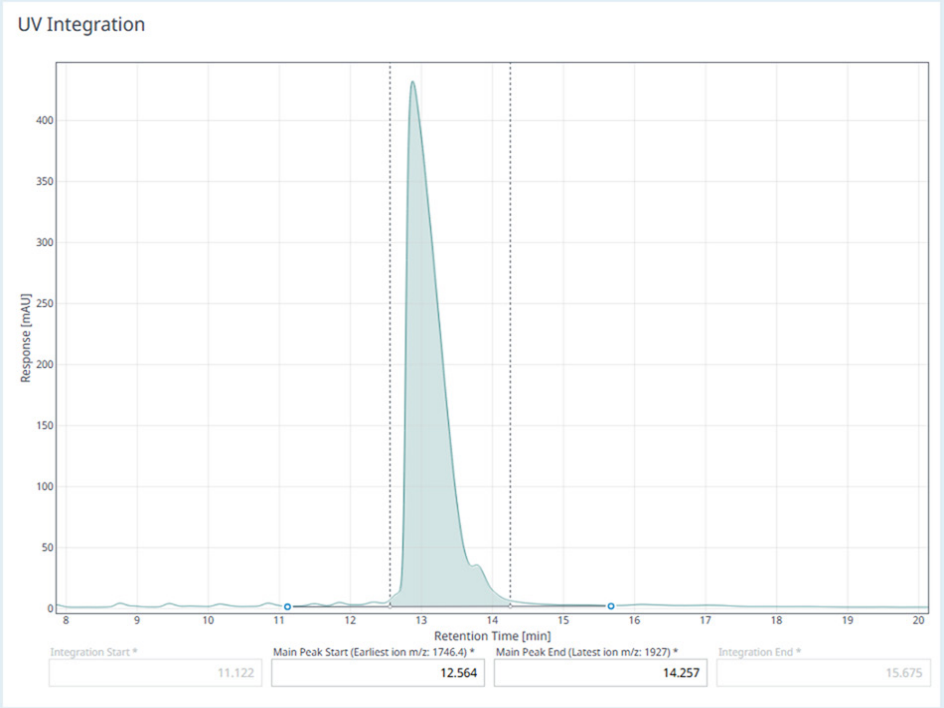

Figure 3. Integration of the UV signal, resulting from parameters defined by impurity extracted ion chromatogram integration. Impurities used to define the integration are shown in Table 3. Users may also manually adjust both UV integration start and end times.

weight of 8,000 Da would have a scan from m/z 1,750 to 2,150. As such, an extended mass range single quadrupole detector is commonly required. In this experiment, the Pro iQ Plus, with a mass range of m/z 2 to 3,000, was used.

The complexity in the method arises not only from determining whether an ion exceeds the threshold, but also from comparing the sample spectra when analyzed under different MS conditions. Figure 1 shows the overlay of so-called "standard" and "harsh" spectra from the ASO-1 sample. This overlay of spectra on relative scale is used to determine if any ions exceeding the threshold are adducts. Specifically, ions that exceed the threshold under standard conditions but fall below the threshold under harsh conditions are considered "adducts," as the higher temperature used in harsh conditions minimizes adduct formation during electrospray desorption of the ASO. The spectra obtained under both conditions demonstrate excellent selectivity and sensitivity for the ions in the 4– charge state.

Table 3 lists ions identified using the OAA algorithm. These ions meet two criteria: they exceed the threshold under both standard and harsh conditions, and any "known" ions are within m/z ±0.5 of values in the imported ion list. Interestingly, several unknown ions with m/z values greater than the full-length product are observed. These ions may be longmers (for example, n+1) or other process-related impurities. As these species are not chromatographically resolved, their detection by the MS detector is critically important.

The ions listed in Table 3 are subsequently used to generate EICs, which are then integrated in the next step of the workflow. This information serves two purposes: first, the relative peak areas are used to calculate the MS purity of the peak, and second, the earliest and latest eluting impurities are used to determine the UV integration of the main peak. This

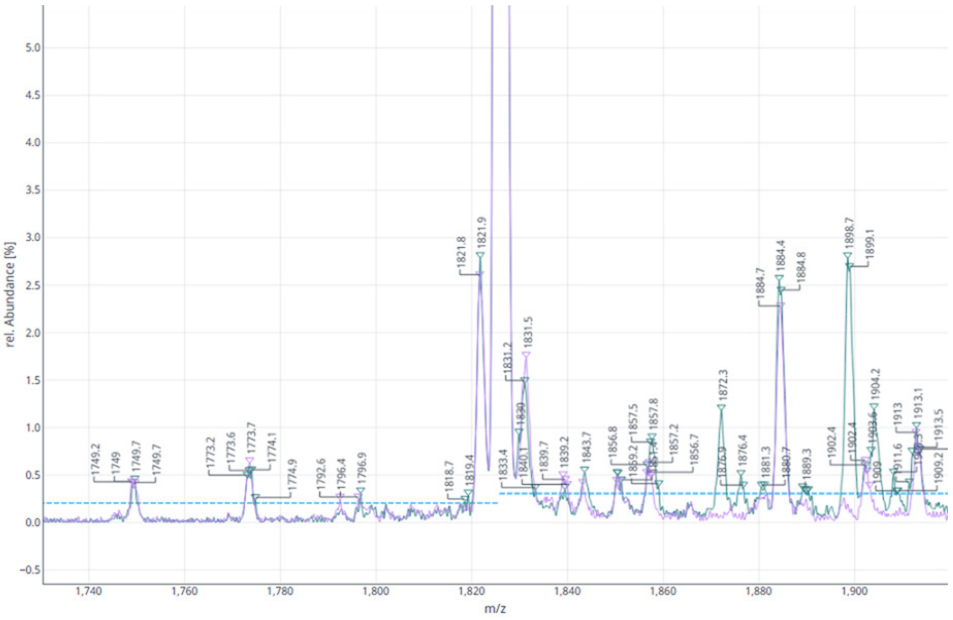

Figure 4. Overlay of ASO-1 full-scan spectra under standard (teal) and harsh (violet) conditions. The Oligo Analysis Accelerator software user interface allows for direct inspection of the overlaid spectra. Dashed lines indicate the user-configurable thresholds: 0.2% "pre-peak", or m/z values less than the 4– charge state of the full-length product (FLP), and 0.3% "post-peak," or m/z values greater than the 4– charge state of FLP. The software automatically classifies ions based on whether they remain above the threshold in harsh conditions.

Table 3. Classified ions from ASO-1 exceeding method thresholds.

Mass Spectrometry Parameters	m/z
Full-length n	1826.3
Full-length n (P=O)1*/Loss of methylene	1822.3
n – p(MOE A)	1725.5
n – p(MOE MeU)/n-p(MOE MeC)	1727.9
n – p(dA)	1744
Abasic depurination species (loss of Ade + H20)	1797
n + p(dA)	1908.5
n + p(dG)	1912.5
Dithioate / thioate	1830.2
N3-(2-cyanoethyl)thymine (CNET)	1839.5
Unknown	1884.4
Unknown	2087.1
Unknown	1857.8
Unknown	1651.1
Unknown	1902.4
Unknown	1774.1
Unknown	1843.7
Unknown	1850.7

approach mirrors the methodology used for the reference standard and system suitability, with the only difference being the comparison of "standard" and "harsh" spectra required for determining if any unknown peaks are adducts. Once relative quantitation by peak areas is complete, both MS and UV purities can be used to determine the overall purity of the ASO sample.

The relative peak areas for each EIC are shown in Table 4. The resulting MS purity of 86.9% contrasts with the calculated UV purity of 98.0% (Table 5). Although this method was intentionally designed to allow coelution of closely related impurities, chromatographic method development may help ensure more accurate quantitation. This is especially important given that many of the unknown impurities are likely n – 1 or alkylated impurities, which elute later than the FLP. In either case, a sensitive and selective MS detector is crucial for determining the purity of an oligonucleotide.

Table 4. Relative peak area results for all ions.

m/z	Name	Category	EIC % Peak Area
1826.3	Full-length n	Full-length n	85.126
1822.3	Full-length n (P=O)1*/loss of methylene	Full-length (P=O)	2.184
1725.5	n – p(MOE A)	n – 1	0.197
1727.9	n – p(MOE MeU)/n – p(MOE MeC)	n – 1	0.489
1797	Abasic depurination species (loss of Ade + H20)	Abasic	0.271
1908.5	n+p(dA)	n + 1	0.271
1912.5	n + p(dG)	n + 1	0.578
1924.6	n + p(MOE MeU)/n+p(MOE MeC)	n + 1	0.338
1927	n + p(MOE A)	n + 1	0.663
1931	n + p(MOE+G)	n + 1	0.624
1830.2	Dithioate / thioate	Others	0.688
1839.5	N3-(2-cyanoethyl)thymine (CNET)	Others	0.21
1884.4	Unknown	-	1.831
2087.1	Unknown	-	0.59
1857.8	Unknown	-	0.545
1913.8	Unknown	-	0.614
1651.1	Unknown	-	0.334
1902.4	Unknown	-	0.47
1774.1	Unknown	-	0.453
1843.7	Unknown	-	0.356
1850.7	Unknown	-	0.25
1749.7	Unknown	-	0.256
1833.4	Unknown	-	0.216
1478.4	Unknown	-	0.242
1457	Unknown	-	0.162
1507.4	Unknown	-	0.113

Table 5. Experimental results

Criteria	Result	Expected Result
UV Purity	97.9518%	98.0%
MS Purity	86.9059	-
Most Abundant Mass (Da)	7307.6	7307.4

Conclusion

The method proposed by Rentel and colleagues has become the gold standard in LC/MS purity, assay, and impurity profiling for therapeutic, single-stranded oligonucleotides. However, the associated data analysis workflow can be time-consuming, as the multi-step process requires several manual tasks such as peak integration and spectral investigation. Oligo Analysis Accelerator for Agilent OpenLab CDS offers a guided workflow that significantly reduces manual processing. This streamlining enables a smoother method transfer and implementation of the LC/MS method in compliant, routine testing QC labs.

References

1. Rentel, C., Gaus, H., Bradley, K., Luu, N., Kolkey, K., Mai, B., Madsen, M., Pearce, M., Bock, B., & Capaldi, D. (2022). Assay, purity, and impurity profile of phosphorothioate oligonucleotide therapeutics by Ion Pair–HPLC–MS. Nucleic Acid Therapeutics, 32(3), 206–220. https://doi.org/10.1089/nat.2021.0056

To learn more about Oligo Analysis Accelerator for OpenLab CDS, visit:
www.agilent.com/biopharma/oligo-analysis-accelerator

To learn more about InfinityLab Pro iQ Plus, visit:
www.agilent.com/lcms/pro-iq-series

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