Technical Overview

Biopharma

Agilent OpenLab

Purity, Assay, and Impurity Profiling of Single-Stranded Oligonucleotides Using Agilent Oligo Analysis Accelerator for OpenLab CDS

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Introduction

Challenges with therapeutic synthetic oligonucleotides include determining the sample purity, label claim or assay, and quantitation of impurities. Traditional HPLC methods that rely on 260 nm UV detection may not have the necessary sensitivity or specificity for quality control (QC) testing. Using a more selective detector such as a mass spectrometer is a viable approach for QC testing needs. Although LC/MS methods are a mainstay for characterization of oligos, many high-resolution accurate mass detectors may not have the robustness needed in a routine testing environment such as QC. Additionally, a high level of technical expertise is often required for MS operation, data analysis, and data interpretation.

A more practical approach uses a single quadrupole detector that is both sufficiently selective and sensitive enough for purity, assay, and impurity profiling of oligos. Given its relatively high mass range and sensitivity in full scan mode, the Agilent InfinityLab LC/MSD XT can meet the demands for LC/MS QC.

As demonstrated by Rentel et al.,[1] the LC/MSD XT can be used and validated for the QC lot release of oligos. However, one challenge that remains is the complex, multistep data analysis which requires the extraction of several extracted ion chromatograms and manual integrations. Performing these operations in a chromatography data system (CDS) while meeting compliance requirements can be time consuming. Manual transcriptions by the analyst may lead to a high margin of error, further delaying decision making.

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This technical overview will address the Oligo Analysis Accelerator (OAA) for Agilent OpenLab CDS. This software add-on acts as the data analysis front end to OpenLab CDS, leveraging built-in technical controls that help meet data integrity and compliance requirements. By performing overlays, integrations, and calculations in a fit-for-purpose user interface, data analysis is significantly streamlined. This not only saves time-on-task but also improves data quality.

Background on Ion-Pair Reversed Phase Liquid Chromatography Mass Spectrometry (IP-RPLC) for oligonucleotides

Although IP-RPLC for oligonucleotides is well-established, it does have some shortcomings. Oligos are short, negatively charged oligomers and consequently take on a multiply charged envelope during electrospray desorption prior to entering the mass spectrometry detector. Co-eluted impurities lead to spectral overlap between target full-length product and other ions, making for complex data analysis without the use of a high-resolution MS detector and a highly optimized LC method. Even with sufficient chromatographic and mass spectrometric resolution, the chemical similarity between parent oligo and impurities is extremely difficult, especially in a routine testing environment where methods must balance robustness with accuracy, repeatability, and specificity.

The LC/MS method developed by Rentel et. al. bypasses many of these challenges. Firstly, the charge envelope is forced into primarily the four-charge state using a combination of mobile phase/infusion solvent conditions (i.e. tributylammonium acetate in water and acetonitrile), in addition to relatively soft ionization conditions. Because one charge state is observed, determination and quantitation of chemically similar, co-eluted impurities is feasible. This has been demonstrated with good reproducibility to allow for a 0.2% impurity threshold.

Although optimal for minimizing charge states, tributylammonium acetate (TBAA) in the mobile phase provides less chromatographic separation for product impurities. However, TBAA is a strong ion pairing agent, thus requiring a high concentration of acetonitrile for elution. The resulting high organic electrospray ionization droplet ensures sufficient sensitivity. Any impurities that co-elute with the target can be detected above the threshold. Any m/z values exceeding this threshold- known or unknown impurities- are used for extracted ion chromatograms (EICs), which are integrated for quantitation.

Figure 1 gives a high-level summary of how the analytical workflow is performed. Each sample or standard injection has the UV signal integrated to determine the percent UV purity by peak areas. The MS spectrum in the UV main peak is extracted and evaluated for any m/z values which exceed a predefined threshold. EICs are then integrated to quantitate.

There are some other noteworthy aspects to the analytical workflow that make it unique from other QC methods. For example, four-point calibration curves with both UV and MS detection are used. The UV peak areas use a simple linear correlation for quantitation, which is necessary for determining the assay or concentration of drug substance and product. However, for the MS calibration, the peak areas for full-length product (FLP) and the FLP with a single oxidation (FLP, P=O) are summed together. This is because these two ions have nearly identical elution profiles, and as such, similar ionization efficiencies when compared to other partially separated impurities. With the phosphorothioate

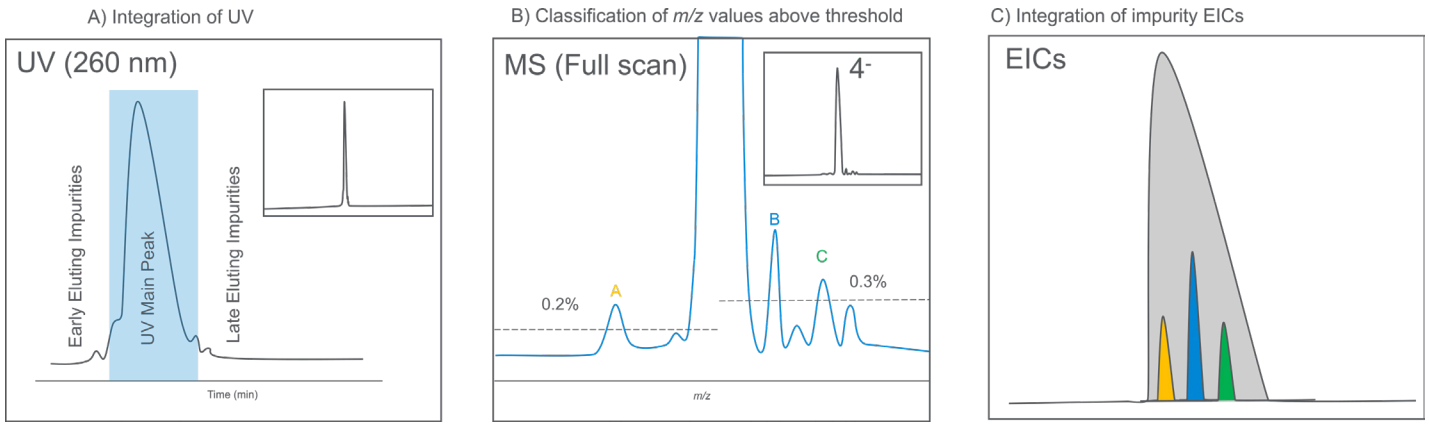

Figure 1. A) UV purity is calculated by integrating the main peak from early-eluting and late-eluting impurities. B) The MS full scan spectrum of the main peak is used to classify any impurities above threshold, which is 0.2% pre-FLP m/z and 0.3% post-FLP m/z. C) EICs for each impurity are then integrated to determine the MS purity of the standard or sample.

being a ubiquitous modification for therapeutic oligos, the single oxidation is a common impurity that should be quantitated accurately. The resulting summation of FLP and FLP (P=O) leads to a nonlinear response most notable at higher standard concentration levels. Consequently, a quadratic or second-order polynomial curve is used.

Additionally, each sample analyzed must be run under two different MS source conditions. Because the source must be run under soft conditions for the four-charge state, FLP adduct formation may occur, which may be problematic, especially with near-isobaric impurities present in the sample. As such, so-called "harsh" conditions are run to confirm the presence of adduct. That is, for impurities that are near isobaric, if the harsh condition ion value is still above threshold, the ion is classified as an impurity and EICs are performed for quantitation.

An example injection sequence list is shown in Table 1, showing four-point calibration using a "working standard solution" (WSS) and standard/harsh requirements for each sample. Table 2 shows recommendations for LC and MS parameters for the acquisition method.

One final consideration for this method is that because of the unique mobile phase, separation of duplex oligos (e.g. siRNA for denaturing conditions) is not practical since sense and antisense strands would co-elute. Additionally, the -4 charge state will limit the application to molecules less than oligos smaller than or equal to 40mer since the extended mass range of the Agilent InfinityLab LC/MSD XT has an upper limit of m/z 3000.

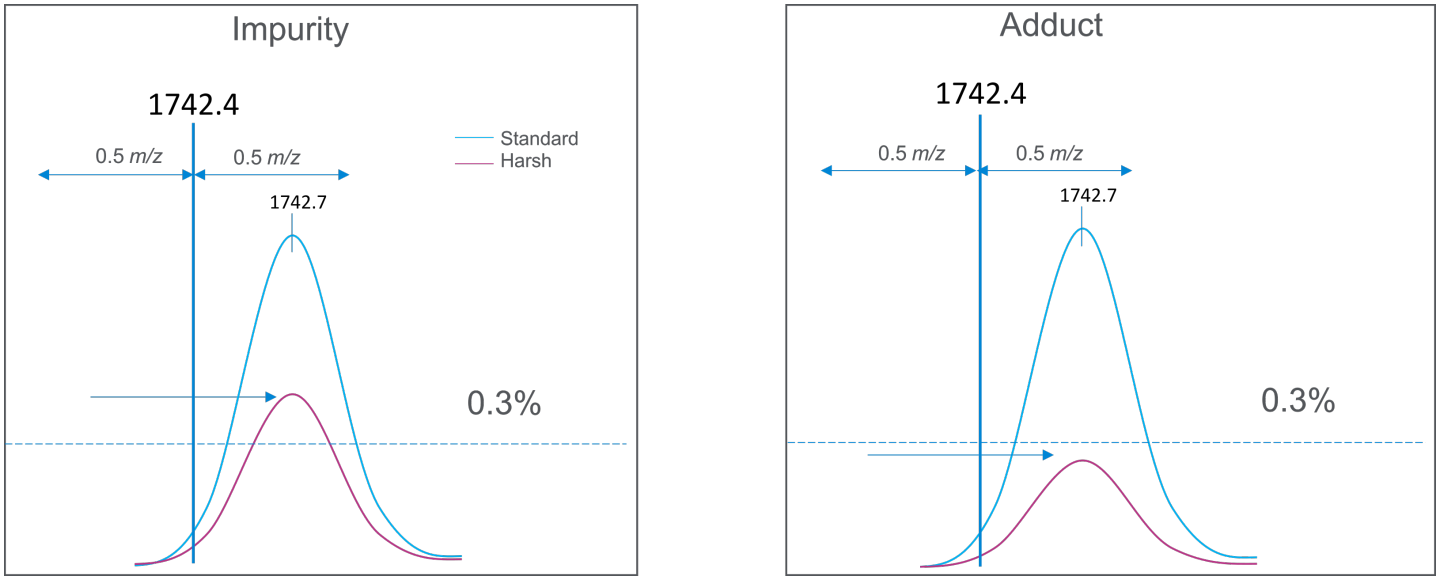

Figure 2. Due to the soft ionization required, two different MS acquisition methods are needed to determine if an ion above threshold is an impurity or an adduct. If an ion still exceeds the threshold under harsh conditions, it is considered an impurity. Otherwise, the ion will be considered an adduct and thus not used for quantitation.

Table 1. Injection list for the Agilent Oligo Analysis Accelerator acquisition method in Agilent OpenLab CDS. At least one blank is required, though three are commonly performed to ensure system conditioning and equilibration. Three QC standards are used, one of which is also the third level of the calibration curve. The acquisition method for the samples requires two methods (standard and harsh), with a recommendation of a "harsh" condition blank injection. Labels are used in OpenLab CDS acquisition.

Sample Name	Sample Type	Label	Injection Volume (µL)	Acq. Method
Blank	Blank		25	Standard
Blank	Blank		25	Standard
Blank	Blank		25	Standard
WSS, 10 µL	Calibration Standard		10	Standard
WSS, 20 µL	Calibration Standard		20	Standard
WSS, 25 µL	Calibration Standard	QC	25	Standard
WSS, 30 µL	Calibration Standard		30	Standard
WSS	QC	QC	25	Standard
Sample (Standard)	Sample	S1	25	Standard
Blank	Blank		25	Harsh
Sample (Harsh)	Sample	H1	25	Harsh
Blank	Blank		25	Standard
WSS	QC	QC	25	Standard

Table 2. LC and MS recommended parameters. Tributylammonium acetate (TBAA) ensures lower charge states, with most single-stranded oligos resulting in a -4. Scan range is set to ± 150 of the target FLP m/z for most oligos. Standard and harsh MS conditions vary in both drying gas flow and gas temperature, with harsh conditions being higher to minimize adduct formation. However, this does lead to in-source fragmentation (primarily depurination) which is why standard conditions are still required, especially for quantitation.

HPLC	Agilent 1260 Infinity II LC/Agilent 1290 Infinity II LC
Column	Agilent AdvanceBio Oligonucleotide column, 2.1 x 150 mm
Mobile Phase	A: 10% ACN, 5 mM Tributylammonium Acetate, 1 µm EDTA B: 80% ACN, 5 mM Tributylammonium Acetate, 1 µm EDTA
Flow-rate	0.25 mL/min
Column Temp	50 °C
UV	Diode-Array Detector, 260 nm
Gradient	45-80% B in 22 minutes
MSD	G6135C
Source	ESI
Drying Gas Flow	12.0 L/min (standard) 13.0 L/min (harsh)
Gas Temp	260 °C (standard) 350 °C (harsh)
Nebulizer Pressure	25 psig
Capillary Voltage	4000 V
Mode	Negative
Scan	FLP, m/z -150 to + 150 e.g., for m/z 1728.1, 1578.1-1878.1, Profile
Scan Time	1149 ms (standard) 975 ms (harsh)
Fragmentor	100 V
Gain Factor	2

Oligo Analysis Accelerator software and architecture

Aside from the unique mobile phase and the requirement for two separate MS source conditions, the LC/MS acquisition workflow is relatively straightforward for oligo analysis. However, performing this analytical workflow in the confines of a standard chromatography data system can be time consuming, as it requires multiple manual signal overlays and manual transcription. Although there are software modules capable of performing the workflow, they may not have the data integrity and compliance necessary to meet 21 CFR Part 11 and other regulatory requirements.

As such, OAA was developed as a web client (Angular) that uses "RESTful APIs" to communicate with OpenLab CDS. This allows OAA to act as a front end to the compliant environment within OpenLab to ensure user access control, change control/versioning, and traceability via audit trails and log files.

Figure 3 illustrates how the web client is used in an OpenLab CDS client-server topology. In summary, an LC/MSD XT acquires data, and an optimized processing method ensures that the integration settings, spectral extraction settings, and metadata are input into the correct fields. This original acquisition result set version is saved in a secure storage location on the server.

Oligo Analysis Accelerator analytical workflow

This section highlights some of the key software features in the OAA application to guide the user through the purity, assay, and impurity profiling workflow. The workflow itself is divided into the following steps:

1. Project setup, processing method, data acquisition
2. Data selection
3. System suitability
4. Ion classification
5. Integration
6. Summary

Figure 4 highlights in which data system each workflow is performed. Importantly, as soon as data selection has begun, a new version of the OAA result set will be saved to ensure data integrity.

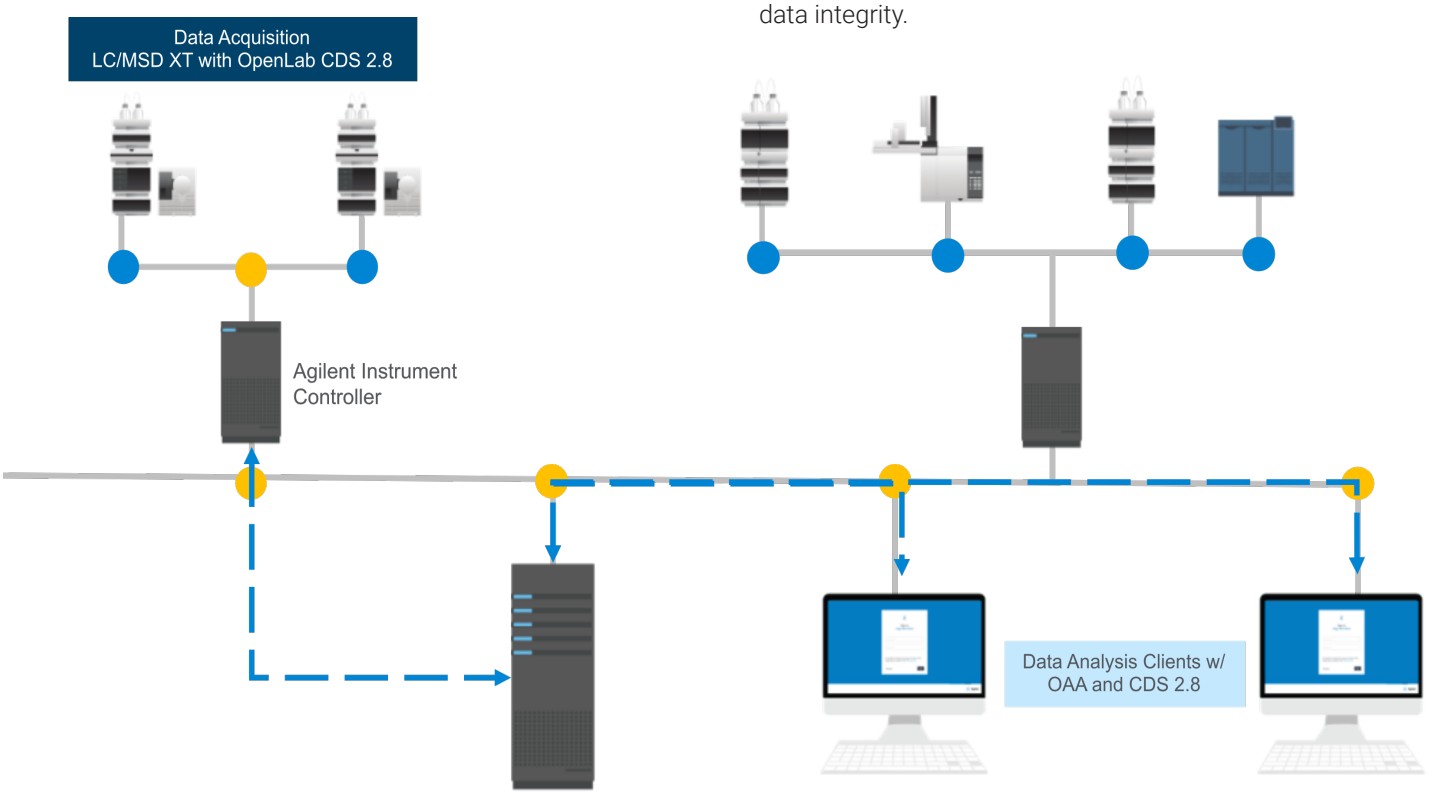

Figure 3. Client-server topology for Agilent Oligo Analysis Accelerator for OpenLab CDS. Data is acquired with distributed Agilent InfinityLab LC/MSD XTs, which are connected to an Agilent Instrument Controller (AIC). Data are then stored on the Agilent OpenLab Server. The OAA clients may then access the data via DA API, allowing for changes to the processing method. Any changes to the processing method are maintained in respective log files and audit trails within OpenLab.

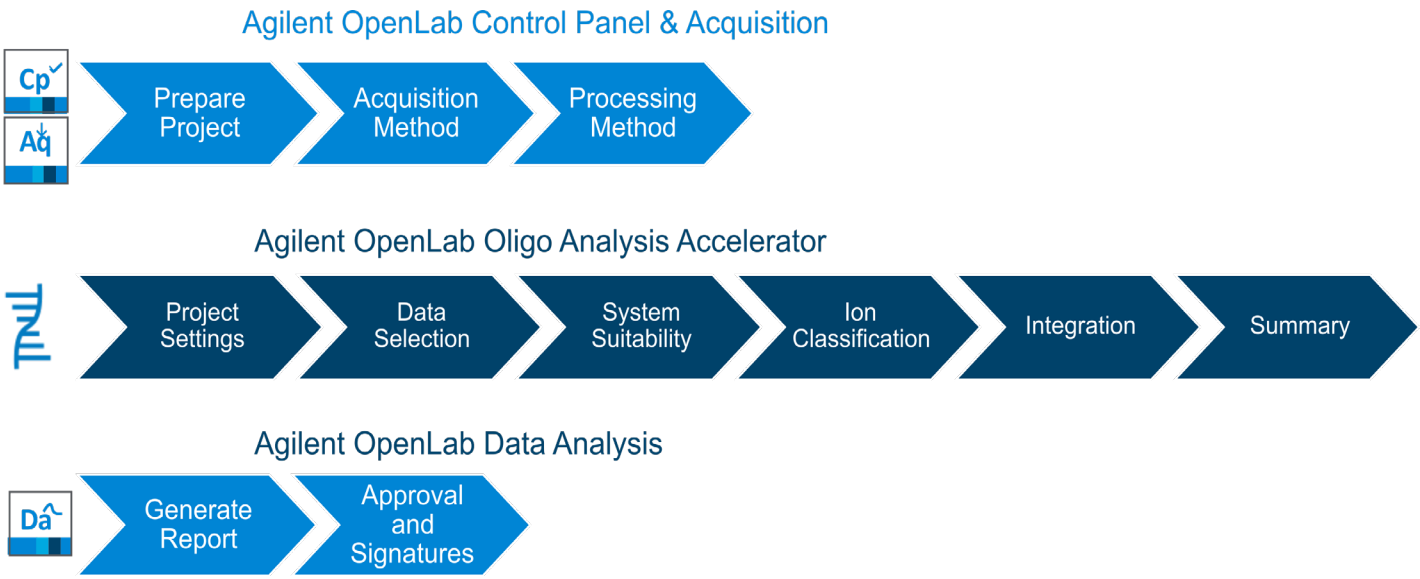

Figure 4. The complete analytical workflow for purity, assay, and impurity profiling of oligos using Agilent Oligo Analysis Accelerator for OpenLab CDS. Project configuration and data acquisition is performed in Agilent OpenLab Control Panel and Acquisition, respectively. Data analysis occurs in Agilent Oligo Analysis Accelerator. Reports and approvals occur in Agilent OpenLab Data Analysis.

Project setup, acquisition, and processing method

Prior to importing the result set into OAA for data processing, the user must configure the project. This includes project folder setup, optimizing processing method, and data acquisition. These three steps are all performed within OpenLab Control Panel and CDS.

Each molecule should have its own project folder configured in Agilent OpenLab Control Panel. Sample Custom Parameters are used to enter relevant metadata needed for reporting of drug substance and drug assay. This includes sample preparation information, Karl Fischer measurements for moisture, sample lot numbers, and more. Additionally, the processing method configuration is used to minimize the amount of manual integration and inputs in the web application. This includes peak integration optimization based upon expected chromatographic performance for UV signal and EICs.

With both the project folder and process method configured, the analyst can set then up data acquisition. Sample and reference standard information are input into the sequence table, and the processing method can be assigned to each sample. Of critical importance are the two separate acquisition methods for sample standard and harsh conditions. These source settings are required for correct determination of adducts and impurities.

Data selection

The data selection step is the first step in OAA that is performed in the web application. The user logs into the web portal with their OpenLab login credentials, as the input of parameters in data selection is managed with user roles and privileges, as assigned in the control panel. Project-level parameters can then be set. This includes importing the ion list, which is a .CSV of the all the known ions or m/z values. Additionally, system suitability requirements and sample parameters are entered, along with parameters for drug substance and drug product.

Once project-level parameters have been input, the user will move into initial setup. If needed, the user can change anything at the result set level. Otherwise, metadata and inputs from the result set can be reviewed prior to system suitability.

System suitability

The System Suitability Test (SST) has several different calculations that must be performed for both UV and MS signals. Having to perform the SST using Microsoft Excel worksheets or custom reporting in OpenLab CDS can be very time consuming. As such, the OAA web application will automatically calculate SST using the data already processed in the result set.

However, the one step that may need to be performed in the web application is the integration of the UV main peak. This determines the UV purity of the working standard solution, and is used to generate the UV calibration curve.

The OAA algorithm determines the integration of the UV main peak in the following way:

The earliest eluting impurity (EEI) is determined based upon the retention time of all known impurities in the ion list. The EIC integration start is then used as the UV main peak integration start point. The latest eluting impurity (LEI) is determined based upon the latest retention time of all known impurities in the ion list, and the EIC integration end time is used to determine the UV main peak integration end point (Figure 5). Both EEI and LEI integration points and retention times are adjusted by a tool to accommodate for UV and MS offset- the user does not have to perform any calculations, as this occurs automatically.

However, there is some variation with the methodology for determination of retention time start point for the main peak. Namely, some methods simply implement a dropline representing the n-2 inflection point. In this case, the user simply may drag the dashed line in the OAA interface to adjust as needed.

After UV main peak integration has been finalized, system suitability results can be reviewed prior to the next step in the workflow. Results include calibration curves and equations. Any parameters not meeting the criteria will be marked appropriately- the user may still proceed with the test, go back to integration of UV to ensure proper settings, or back into OpenLab CDS Data Analysis to review the data further.

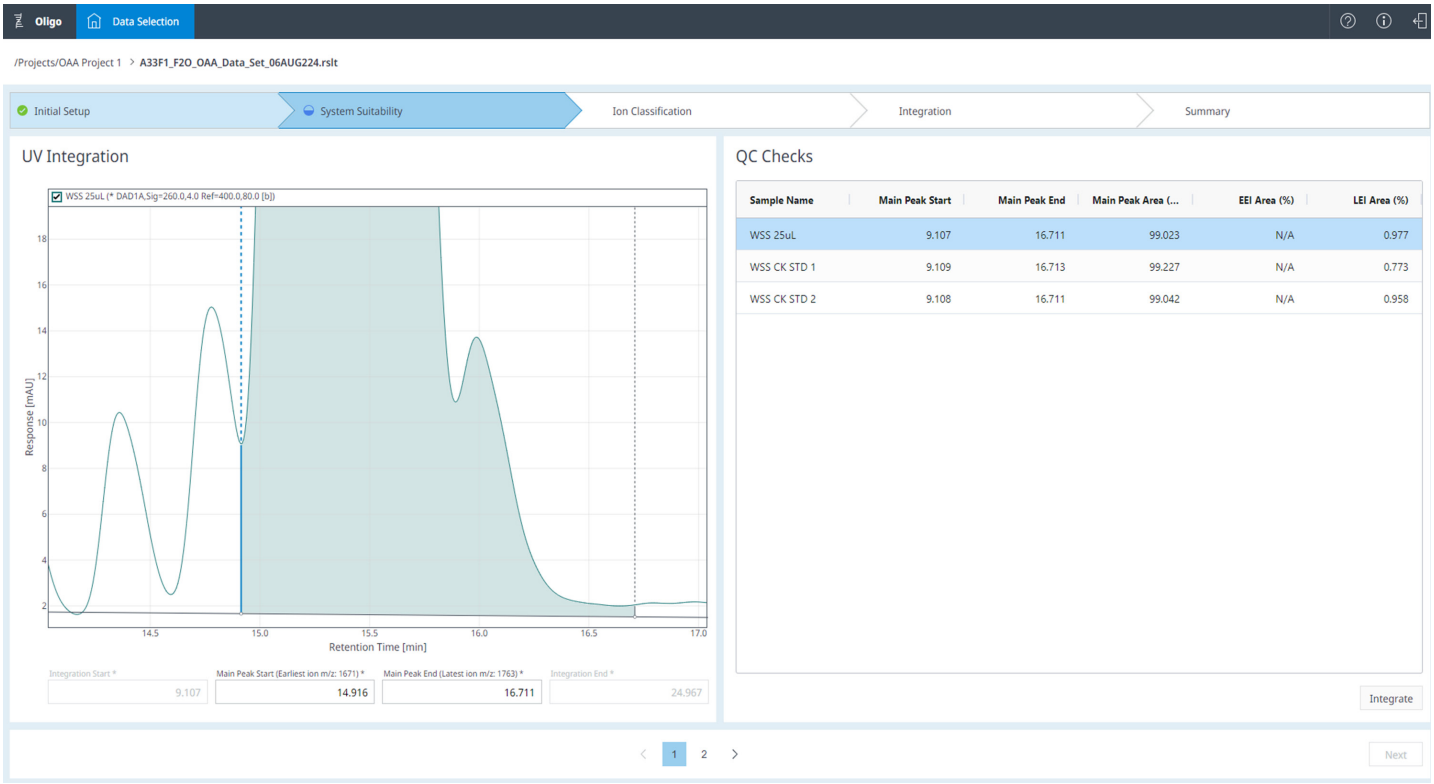

Figure 5. The software will automatically determine the UV main peak integration by using the extracted ion chromatograms (EICs) of earliest and latest eluting impurities. However, the user may also manually integrate the UV signal directly in the software user interface by simply clicking and dragging.

Ion classification

Prior to quantitation of the peak areas of impurities above the threshold that coelute with the FLP in the UV main peak, m/z values must be classified. That is, any ions above the spectral threshold are identified and matched to the ion list. Any matches are then used for extraction to determine EIC peak areas for purity calculations. This classification must be done separately for each sample analyzed.

The first step in the workflow simply shows the integrated total ion current (TIC) with each respective average MS spectrum extracted. The user can overlay each sample injection standard and harsh spectra, a useful feature to ensure each injection was properly labeled in the acquisition and processing method.

The next step is the ion classification itself. Each ion has already been classified by the OAA algorithm, identifying any m/z values which exceed a particular threshold. For m/z values less than the -4 charge state of the FLP, the threshold is ≤ 0.2% relative abundance. For m/z values more than the -4 charge state ion of the FLP, The threshold is ≤ 0.3% relative abundance. Definitions of each ion are shown in Table 3.

Ion classification is typically a manual process. This is because CDS platforms do not typically have built-in features for assessing spectra values above a threshold, and the user would need to manually identify m/z values above two different thresholds. Any values above the threshold would then be matched to the ion list, and each ion extraction would be extracted appropriately. For classification of each known impurity/possible adduct and unknown impurity, an overlay of spectra from both injections of standard and harsh MS conditions is necessary, making visual inspection of ions that exceed the threshold increasingly difficult.

Conversely, OAA classifies these items automatically and reports theoretical values against measured values. Additionally, the user interface allows for further inspection if needed, and the user may select another classification than what the software algorithm determines (Figure 6). Once all ions are classified as either known or unknown, EICs are performed for each m/z value.

Integration

The integration step evaluates each extracted EIC to ensure proper quantitation. Resulting areas that are above the threshold of 0.2% relative quantitation are then used to determine the purity of the UV main peak, and thus overall purity of the sample. Although manual integration is typically not ideal for a routine testing application due to sample complexity and insufficient chromatographic resolution, EICs generated can become quite complex. An m/z ± 0.5 window for unit mass detection can result in challenging peak integration.

To facilitate this workflow, three overlays are generated in the user interface: FLP, EIC standard, and EIC harsh, as shown in Figure 7. The FLP EIC is used as a useful reference to guide proper integration for co-eluted peak areas. This is especially pertinent for any near-isobaric known impurity/possible adduct ions, where the standard EIC should be compared to the harsh EIC. The peak areas under the FLP are presumed to be related to adduct, and as such, only relevant peak areas are integrated for the standard EIC.

Table 3. Definitions for ion classification.

Ion	Description
Insufficiently Resolved	MS spectra peaks within m/z ± 1.0 resolution between known and unknown impurities
Adduct	Known adduct with no overlap (i.e. m/z ≥ 0.5 resolution between unknown impurities)
Known Impurity/ Possible Adduct	Known adduct with insufficient resolution between adduct and impurity (i.e. m/z ≤ 0.5 resolution between adduct and known impurities); requires overlay of standard and harsh spectrum for confirmation
Unknown Impurity	Ion which exceeds threshold but does not match any of the values in the ion list; requires overlay of standard and harsh spectrum for confirmation
Not Detected	Known ion is below the threshold

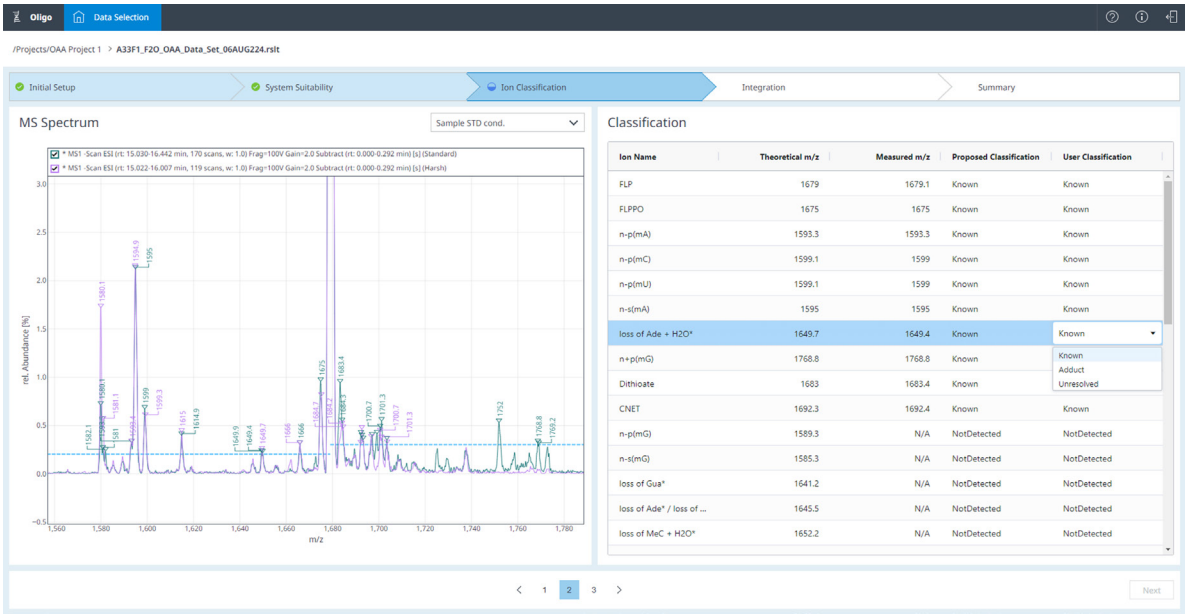

Figure 6. During the Ion Classification workflow, the user reviews software-proposed classification. Spectral overlays of MS conditions are provided to facilitate confirmation of adducts. The user can also adjust classification if needed- any changes are recorded in the log file for traceability and data integrity.

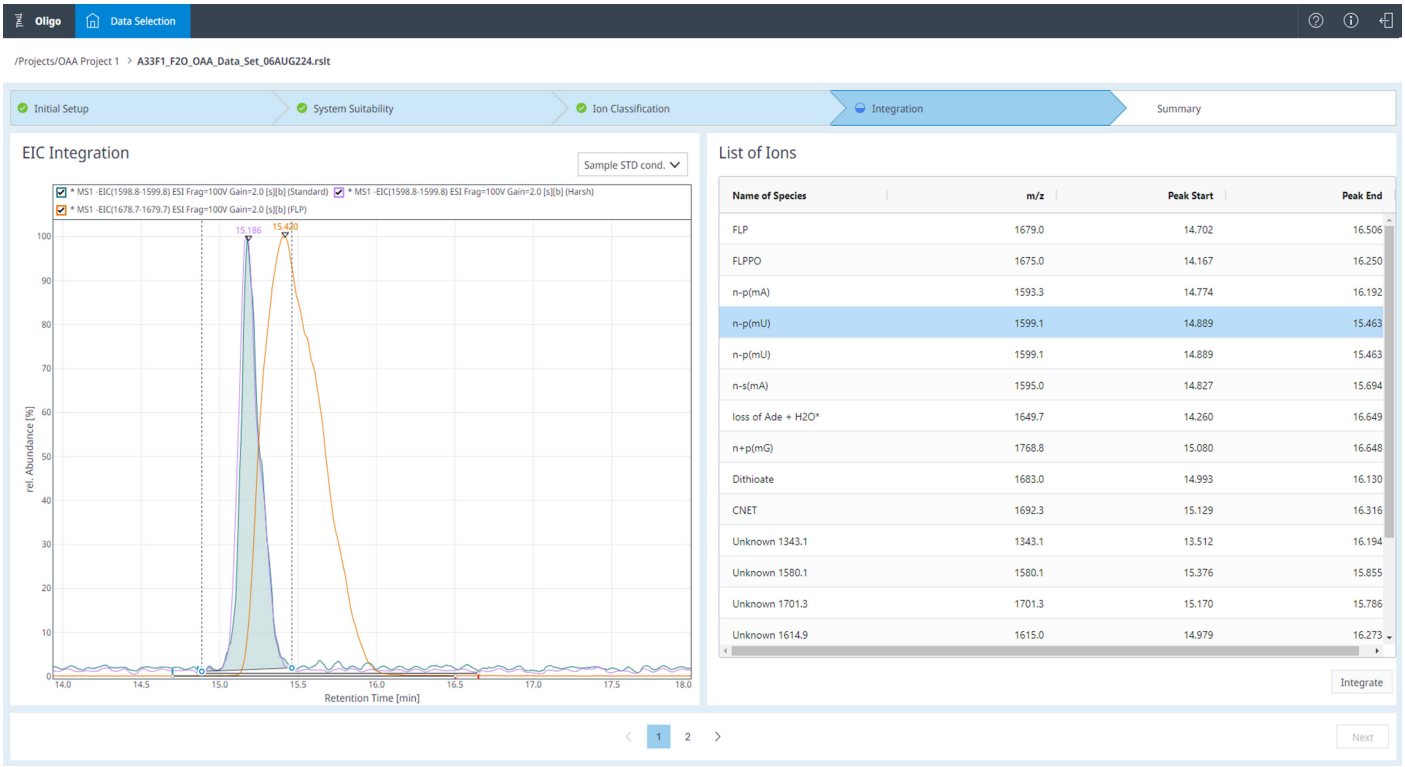

Figure 7. The overlay of EICs for full-length product and standard/harsh conditions is automatically generated in the user interface to facilitate integration. The user may select and/or deselect FLP and harsh EIC signals, as only the standard EIC integration is necessary.

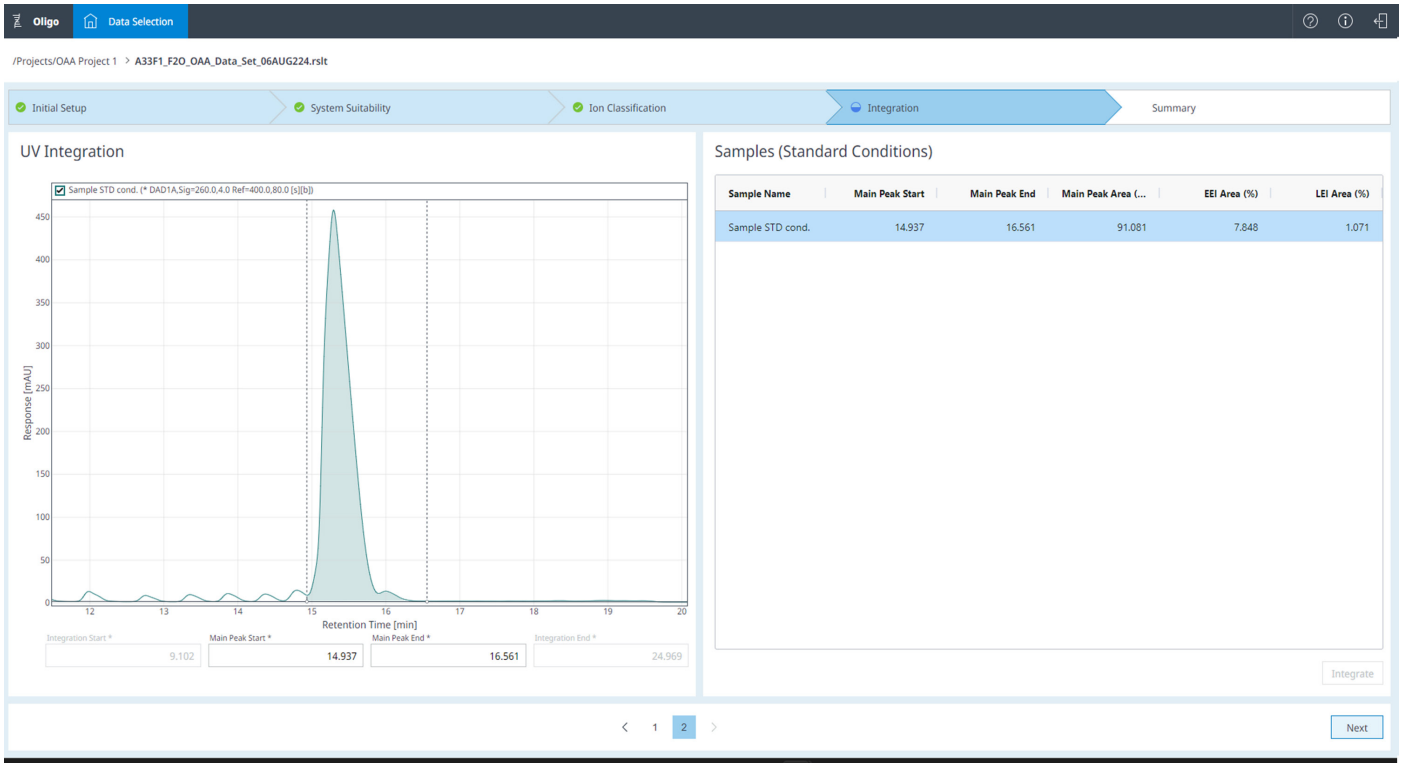

Figure 8. Sample UV integration is based upon early- and late-eluting impurities, similar to the system suitability step. The user may also adjust the integration start and end points, if necessary.

Once EIC peaks have been integrated for all samples, OAA will then apply the EEI and LEI settings for the UV main peak integration, similar to the SST main peak integration. That is, EEI peak integration start is used for the start of the main peak integration, and LEI peak integration end is used for the end of main peak integration; UV and MS signal offsets occur automatically. Figure 8 shows main peak integration is performed automatically based upon EEI and LEI integration. Otherwise, the user can still manually integrate the UV main peak if needed.

Summary

The final step in the workflow is to review the results. This includes the SST results shown in previous steps, as well as purity and identity results. Assay results may vary depending on which formulation was selected for the sample.

It is important to note that the summary itself is not the report. An OpenLab CDS report template must still be used to report the results for review. Further, any electronic signatures and approvals occur within OpenLab CDS, not in OAA. Again, this is because OAA leverages the already existing data compliance engine within the OpenLab CDS platform.

Future considerations

LC/MS data analytical workflows have been confined to data systems either used to interpret spectra or chromatograms. However, the need for more user-friendly, fit-for-purpose data analysis is becoming increasingly important for the development of complex therapeutics such as oligonucleotides.

Agilent Oligo Analysis Accelerator for OpenLab CDS is a paradigm shift in the way software is being developed.

References

1. Rentel, C.; Gaus, H.; Bradley, K.; Luu, N.; Kolkey, K.; Mai, B.; Madsen, M.; Pearce, M.; Bock, B.; and Capaldi, D. Assay, purity, and impurity profile of phosphorothioate oligonucleotide therapeutics by ion pair–HPLC–MS. Nucleic Acid Ther. **2022**, 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

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