Technical Overview

Biopharma

Agilent OpenLab

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

Authors

Brian Rivera
Agilent Technologies, Inc.

Introduction

A challenge with therapeutic synthetic oligonucleotides is determining the sample purity, label claim or assay, and quantitation of impurities. Traditional high performance liquid chromatography (HPLC) methods relying on ultraviolet (UV) 260 nm detection may not have the sensitivity nor specificity for quality control testing. Using a more selective detector such as a mass spectrometer would be a viable approach. Although liquid chromatography/mass spectrometry (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 quality control (QC). Further, 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. Indeed, given its relatively high mass range and sensitivity in full scan mode, the Agilent InfinityLab LC/MSD XT single quadrupole detector can meet the demands for LC/MS in QC environments.

As demonstrated by Rentel et al (2022)[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, multi-step data analysis, which requires the extraction of several extracted ion chromatograms and manual integrations. Performing these operations using a compliance-ready chromatography data system (CDS) may be time consuming. Further, 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 be a deep dive into the Oligo Analysis Accelerator (OAA) for OpenLab CDS. This software add-on acts as the data analysis front end to OpenLab CDS, leveraging existing data integrity and compliance architecture. By performing overlays, integrations, and calculations in a fit-for-purpose user interface, data analysis is streamlined significantly, not only saving time-on-task but also improving 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 oligomer and consequently take on a multiply charge envelope during electrospray desorption prior to entering the mass spectrometry detector. Coeluted impurities lead to spectral overlap between target full-length product and other ions, thus 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 spectrometry resolution , the chemical similarity between parent oligo and impurities is extremely difficult, especially in a routine testing environment where methods must balance robustness to accuracy, repeatability, and specificity.

The LC/MS method developed by Rentel et al bypasses many of these challenges. First, the charge envelope is forced into primarily the 4-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, coeluted impurities are 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 coelute 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) that are integrated for quantitation.

Figure 1 gives a high-level, graphical representation of how the analytical workflow is performed. Each sample or standard injection has the UV signal integrated to determine the %UV purity by peak areas. 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 impurities.

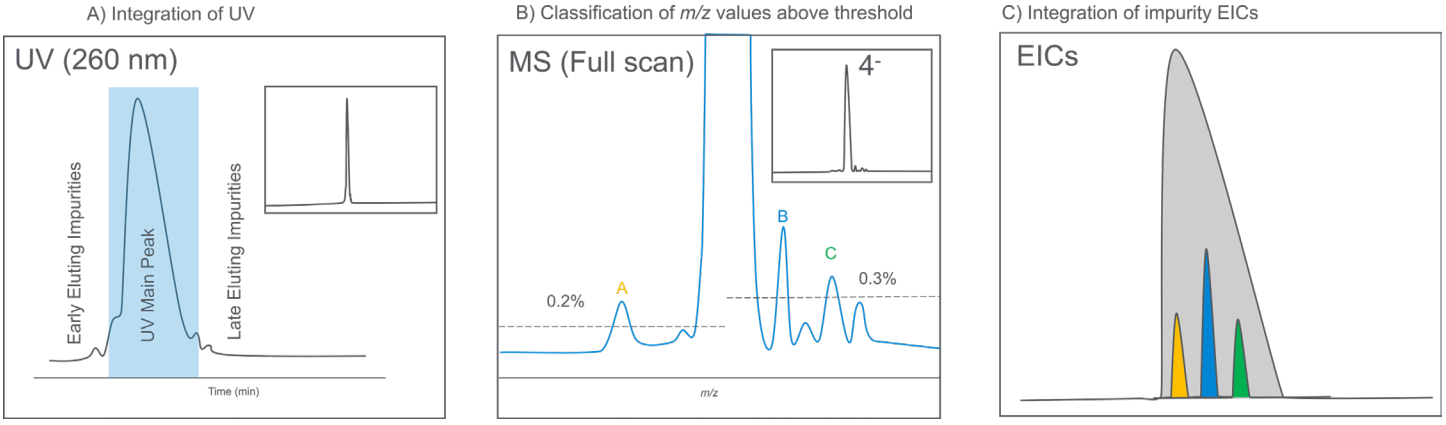

Figure 1. A) UV purity is calculated by integrating main peak from early-eluting and late eluting impurities. B) MS full scan spectrum of main peak is used to classify any impurities above threshold, which is 0.2% pre-FLP m/z and 0.3% post-FLP m/z. C) EICs for each impurity are then integrated to determine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

There are some other noteworthy aspects to the analytical workflow that make it unique from other QC methods. For example, a 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 peaks 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 near identical elution profiles, thus similar ionization efficiencies when compared to other partially separated impurities. With the phosphorothioate 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 4-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 an adduct. That is, for impurities that are near isobaric, if the harsh condition ion value is still above the threshold, the ion is classified as an impurity and EICs are performed for quantitation.

Example injection sequence list is shown in Table 1, showing 4-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 acquisition method.

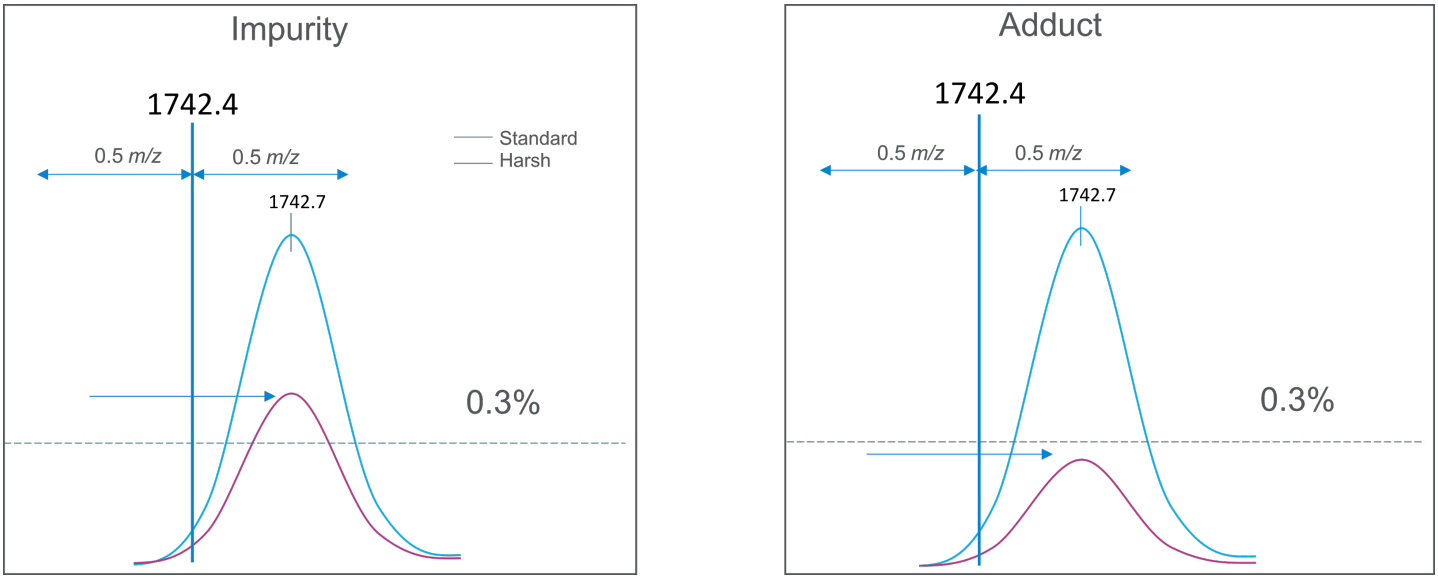

Figure 2. Because of the soft ionization required, two different MS acquisition methods are needed to determine if an ion above the threshold is an impurity or an adduct. If an ion still exceeds the threshold under harsh conditions, it is considered an impurity. Otherwise, the ion will be considered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

Table 1. Injection list for OAA acquisition method in OpenLab CDS. At least one blank is required, though three is 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. 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 charge state. Scan range is set to ±150 of the target FLP m/z for most oligo. 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 1260/1290 Infinity II LC system
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
Gradient	45-80% B in 22 minutes
Single Quadrupole Detector	G6135C LC/MSD or G6170A LC/MS
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, -150 to + 150 *m/z* e.g., for 1728.1, 1578.1-1878.1 *m/z*, 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. And 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 function 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 Agilent InfinityLab LC/MSD XT acquires data, and an optimized processing method ensures the integration settings, spectral extraction settings, and metadata are inputted into the correct fields. This original acquisition result set version is saved in a secure storage location on the server.

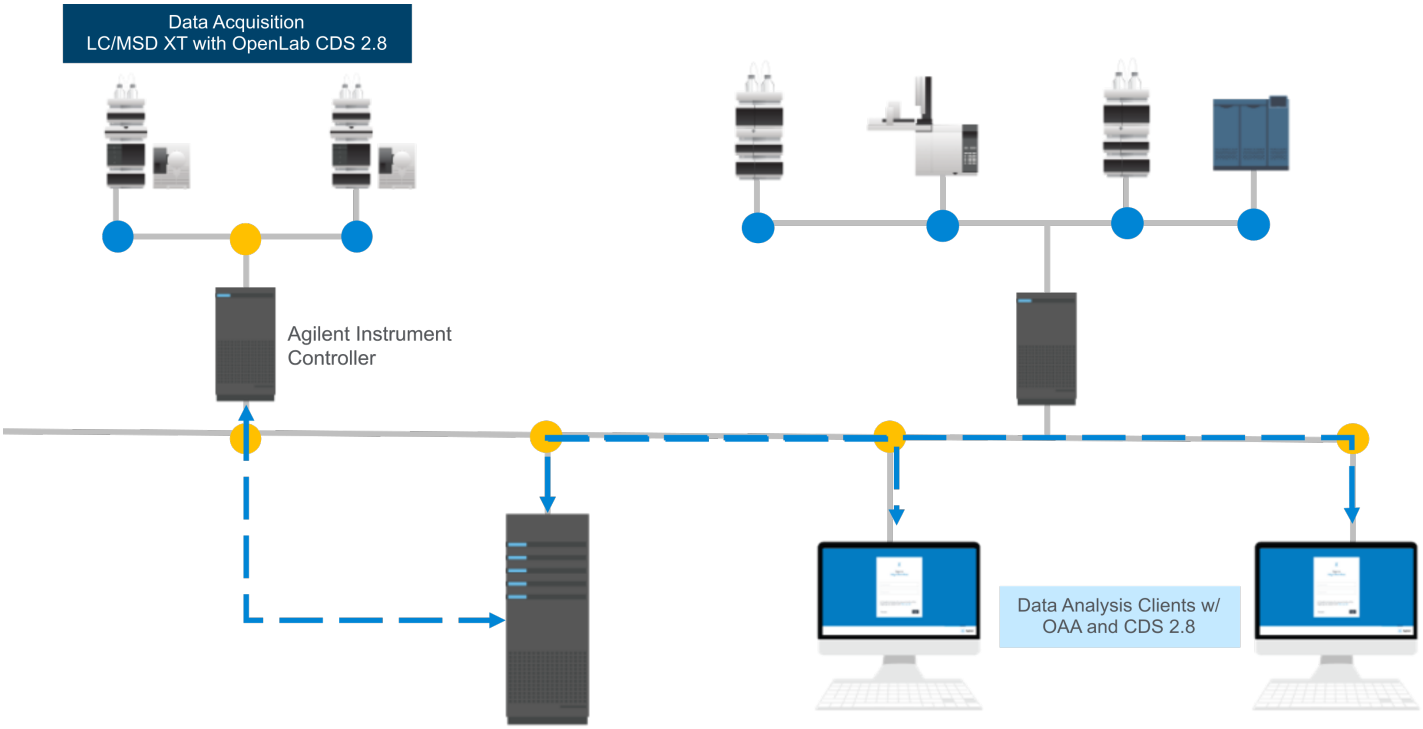

Figure 3. Client-server topology for Oligo Analysis Accelerator. Data is acquired with distributed InfinityLab LC/MSD XTs that are connected to an Agilent Instrument Controller (AIC). Data are then stored on the OpenLab Server. The OAA clients may then access the data via DA API, allowing for changes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

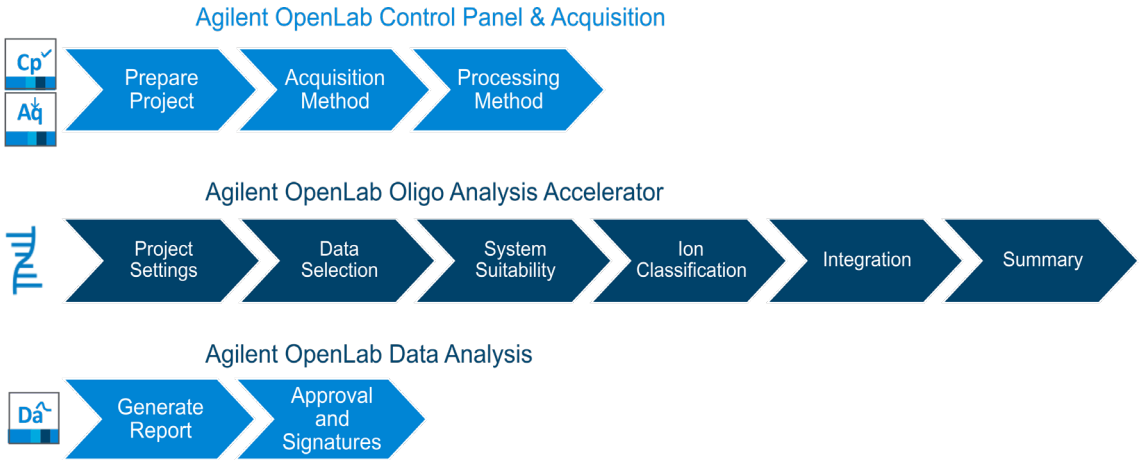

Figure 4. The complete analytical workflow for purity, assay, and impurity profiling of oligos using Oligo Analysis Accelerator. Project configuration and data acquisition are performed in OpenLab Control Panel and Acquisition, respectively. Data analysis occurs in Oligo Analysis Accelerator. Reports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

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

Importantly, as soon as data selection has begun, a new version of the OAA results set will be saved to ensure data integrity.

Project setup, acquisition, and processing method

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

Each molecule should have its own project folder configured in 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 then set up data acquisition. Sample and reference standard information are inputted in 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. Again, 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. First, the user logs into the web portal with their OpenLab log in credentials, as inputting any parameters in data selection are managed with user roles and privileges as assigned in Control Panel. Project level parameters can then be set. This includes importing of 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 inputted, the user will move into initial set up. 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 this using Excel worksheets or even 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.

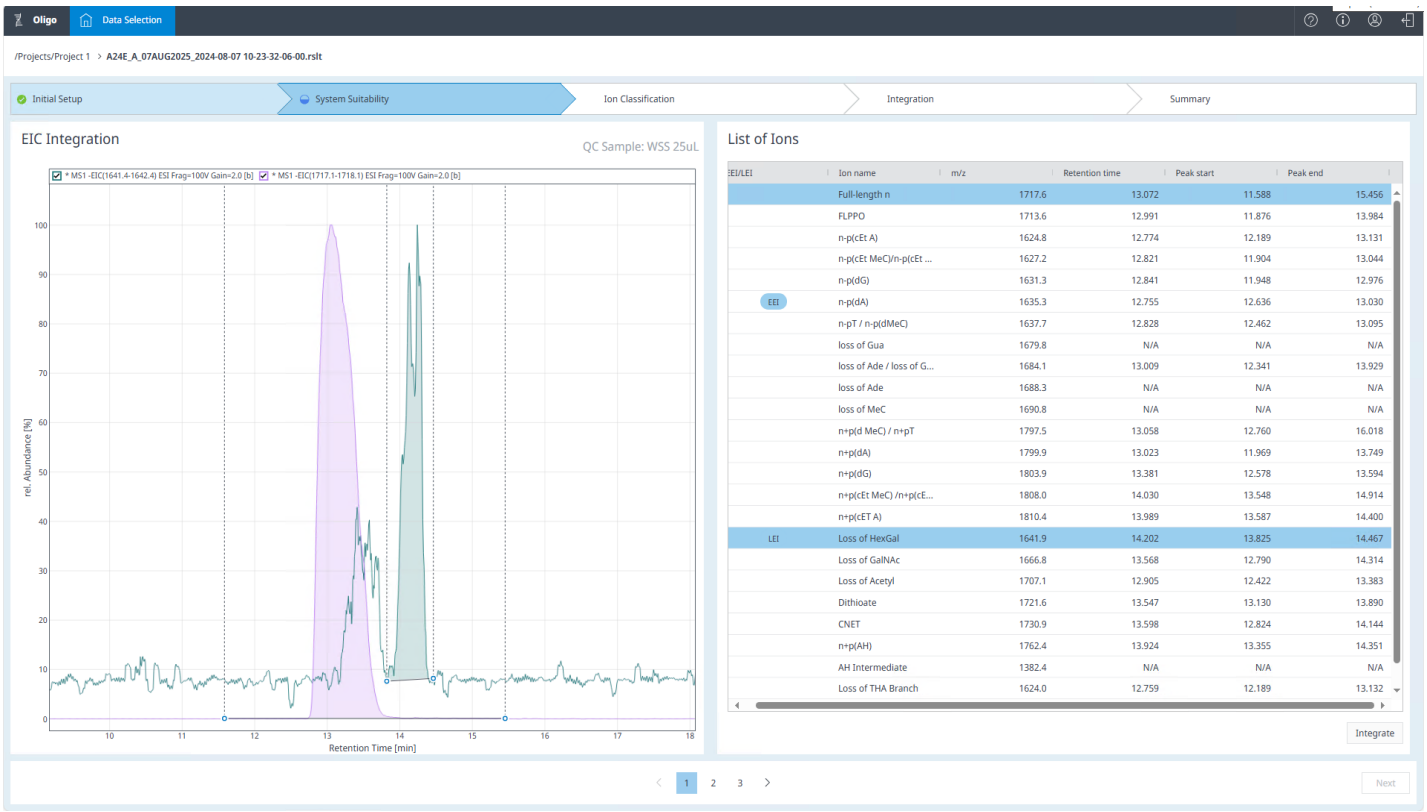

Figure 5. The software automatically extracts any ions in the reference standard exceeding the threshold. Integration of each extracted ion chromatogram is performed based upon processing method parameters. The user may then review the integration for any of these ions, taking care to ensure both EEI and LEI are integrated properly, as they will determine the integration boundaries for the UV main peak of the reference standard.

First, the user must confirm the EIC integration of the early eluting impurity (EEI) and late eluting impurity (LEI), with impurity names based upon the order by which they elute. The software algorithm calculates which impurities exceed the threshold, and then subsequently will automatically extract the EICs. Proper peak integration is critically important to calculate system suitability, as EEI and LEI define the UV main peak integration start and end point, respectively. Should the user have to manually adjust the integration of either EEI or LEI, this can be performed directly in the web application. It is also important to note that both EEI and LEI integration points and retention times used to define UV integration automatically includes the retention time delay of the MS signal.

However, there is some variation with the integration methodology for determination of the 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 may simply drag the dashed line into 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 and 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.

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, which is 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 that exceed a specific threshold. For m/z values less than the -4 charge state of the FLP, the threshold is commonly set at ≤ 0.2

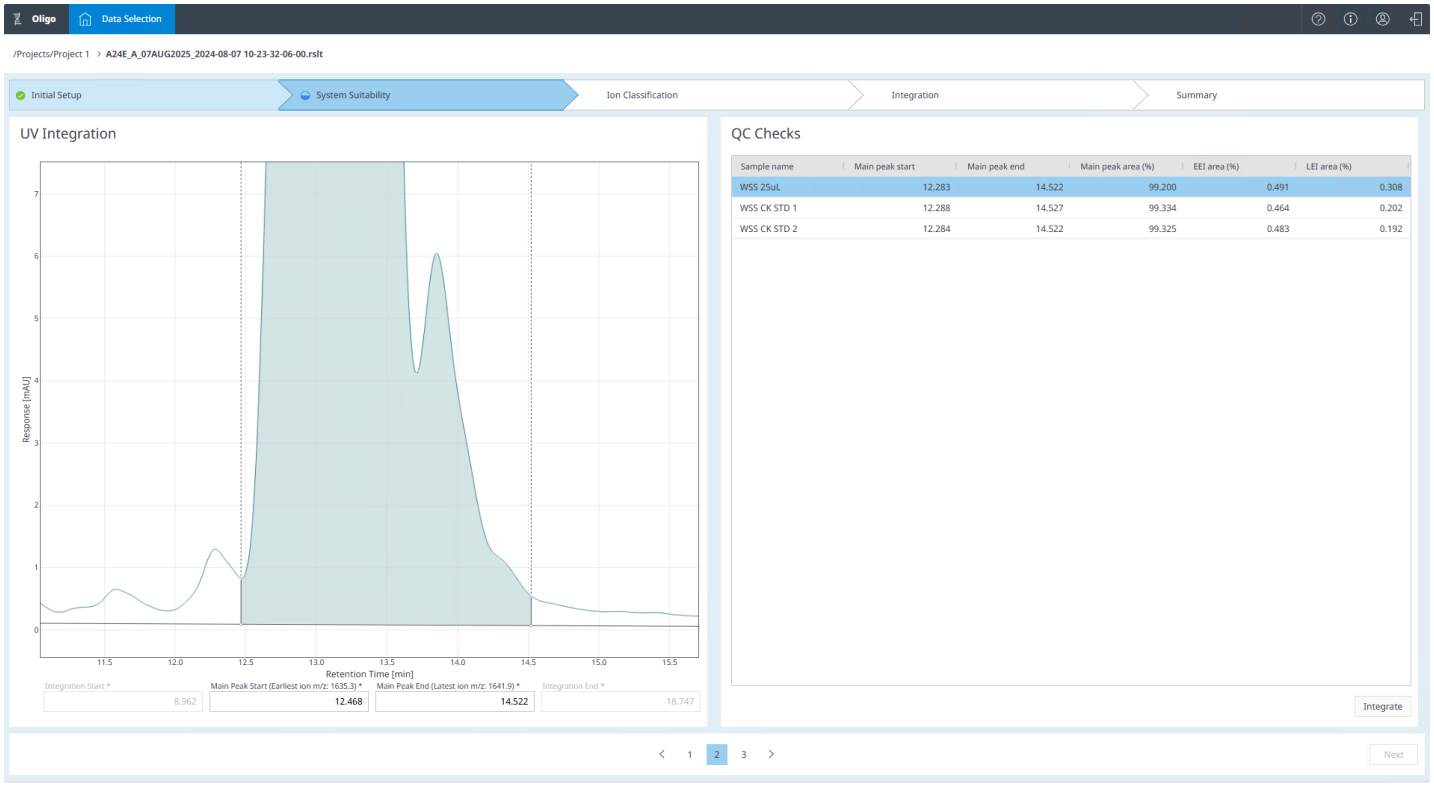

Figure 6. The software will automatically determine the UV main peak integration by using the extracted ion chromatograms (EICs) of earliest and latest eluting impurities. However, the user may also manually integrate the UV signal directly into the software simply by clicking and dragging.

% relative abundance. For m/z values more than the -4 charge state ion of the FLP, the threshold is ≤ 0.3% relative abundance.

Ion classification is typically performed manually. CDS platforms do not typically have built-in features to assess spectra values above a threshold. To perform the ion classification workflow, 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. Further, for classification of each known impurity/possible adduct and unknown impurity, an overlay of spectrum 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 automatically and reports theoretical against measured values (Figure 7). The user interface allows for further inspection if needed, and the user may select another classification than what the software algorithm determines. Additionally, the threshold is user-configurable; if a particular method has a post-peak threshold of 0.5%, for example, this can be changed in the project or

Table 3. Definitions for ion classification.

Ion	Description
Known Impurity	Known product/process related impurity in the ion list; sufficient resolution between adducts (within ± 0.5 m/z of known ion)
Insufficiently Resolved	MS spectra peaks within ± 1.0 m/z resolution between known and unknown impurities
Known Impurity/ Possible Adduct	Known adduct with insufficient resolution between adduct and impurity (i.e., ≤ 0.5 m/z 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

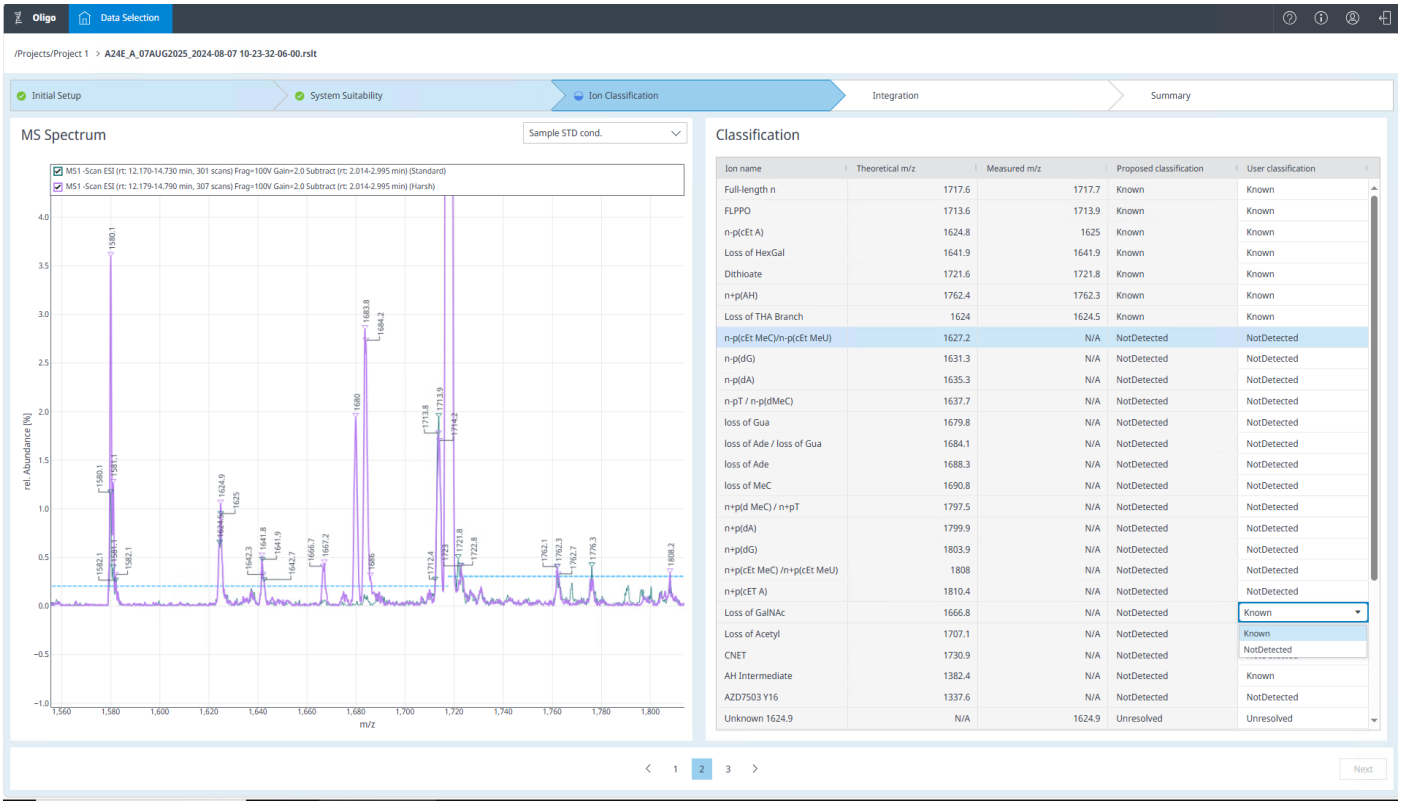

Figure 7. During the Ion Classification workflow, the user reviews software proposed classification. Spectral overlays of MS conditions are provided to facilitate in confirmation of adducts. The user can also adjust classification if needed. This may be useful for impurities which may be classified as not detected, but are not resolved due to overlapping spectra or the presence of adducts. Any changes in classification by the user are recorded in the log file and audit trail for traceability and data integrity.

result set parameters. The change in threshold is also reflected in the UI. Once all ions are classified as either known or unknown, EICs are performed for each m/z value. Finally, before proceeding to the next step, the user can select whether any unknown impurities are degradation products.

Integration

The integration step evaluates each extracted EIC to ensure proper peak integration and thus 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, because of sample complexity and insufficient chromatographic resolution, EICs generated can get quite complex. Indeed, a ± 0.5 m/z window for unit mass detection can result in a challenging peak integration.

To facilitate peak integration, 3 overlays are generated in the user interface: FLP, EIC standard, and EIC harsh. The FLP EIC is as useful reference to guide proper integration for coeluted 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.

Importantly, an m/z value in the spectrum may not necessarily result in an EIC that can be integrated. That is, the abundance of the m/z ion and the extraction parameters use result in an EIC which is no more than noise. As such, should the integration processing method parameters inadvertently integrate baseline noise as a chromatographic peak, the user has the option of removing the peak. Again, this is captured in both log files and audit trail, maintaining data integrity (Figure 8).

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. Otherwise, the user can still manually integrate the UV main peak if needed.

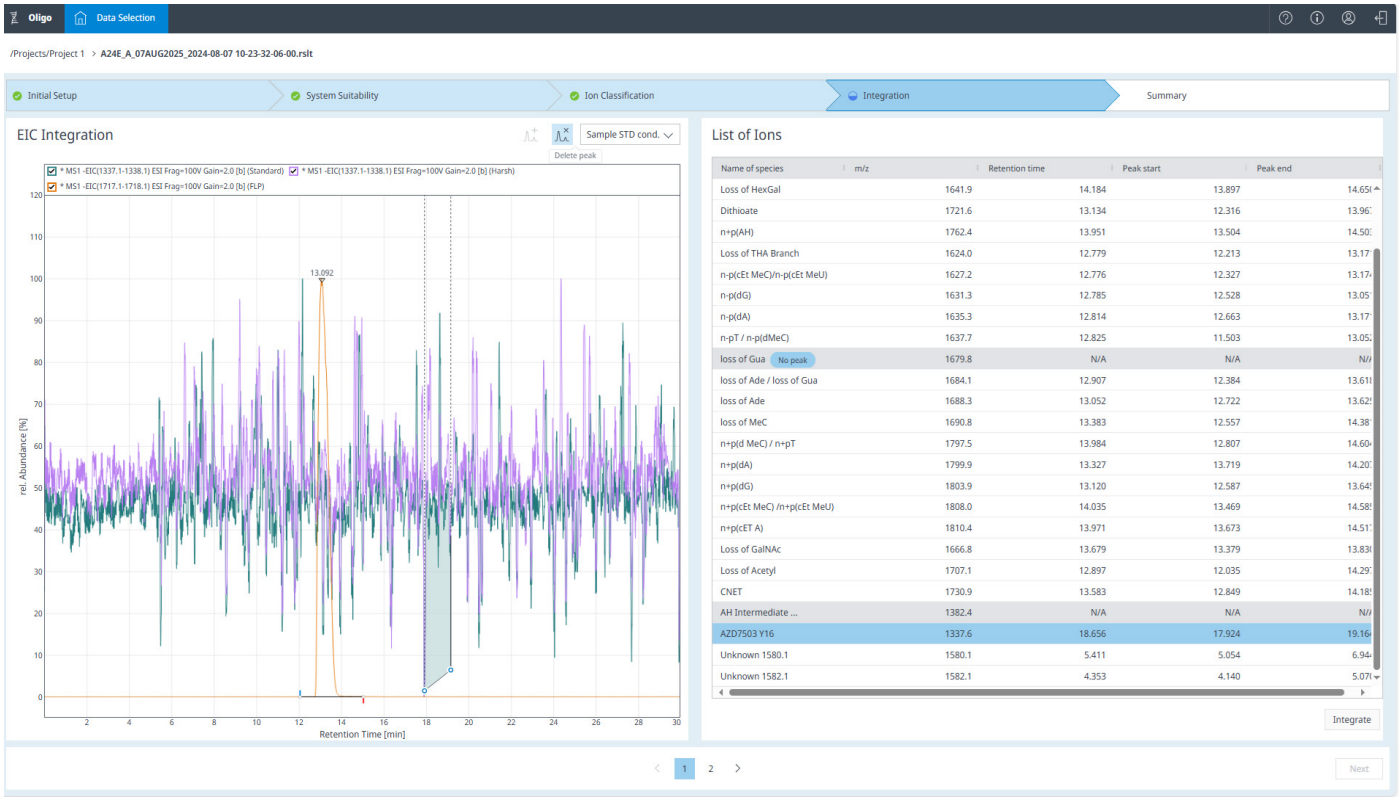

Figure 8. Depending on the processing method integration parameters, baseline noise may be misidentified as a chromatographic peak. The user has the option to remove the peak to ensure accuracy of purity calculations. Note that this manual action is captured in the audit trail and log file for full traceability.

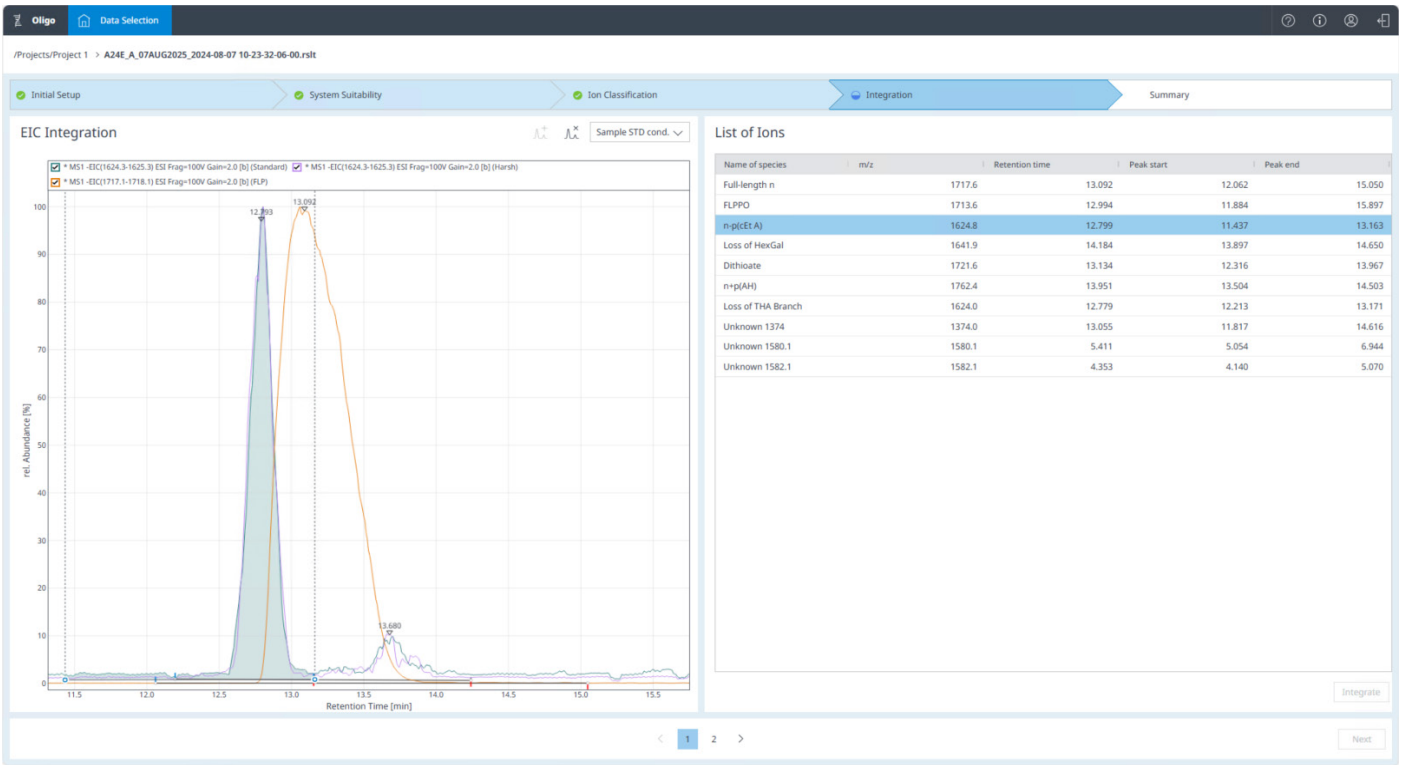

Figure 9. Overlay of EICs for full-length product and standard/harsh conditions are automatically generated in the UI to facilitate integration. User may select and deselect FLP and harsh EIC signals, as only the standard EIC integration is necessary.

Summary

The final step in the workflow is to review the results. This includes the SST results which were 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 template must still be used to report the results for review. Further, any electronic signatures and approvals occur within OpenLab CDS, not in OAA. This is because OAA leverages the 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 spectrum 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.

Oligo Analysis Accelerator is a paradigm shift in the way software is being developed. Instead of the user having to select signals to perform overlays, while needing to manually perform both integration and spectrum extraction, OAA acts as an abstraction layer to the OpenLab CDS Data Analysis subsystem. The UI only shows what is necessary for the analyst to complete the workflow, so the focus is on the specific task. Further, since OAA is still working with OpenLab, audit trails, log files, and versioning are still enabled, which is a primary concern for meeting regulatory requirements.

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

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