

## Workflow for Profiling Impurities in Synthetic Oligonucleotides Using the BioAccord™ LC-MS System with waters\_connect™ Informatics

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This is an Application Brief and does not contain a detailed Experimental section.

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### Abstract

As synthetic oligonucleotide therapeutics gain increasing interest as therapeutics, efficient methods to determine their impurity profiles are critical, especially as new modalities entering the pipeline increase in length and complexity. Currently established HPLC-MS (single quad) methods for analyzing impurities can be time consuming and require manual processing steps that introduce the risk of human error and create challenges with implementation in a regulated setting. Waters Corporation has responded to these challenges with the development of the Oligonucleotide Impurity Analysis Workflow Data Package. This workflow was developed on the BioAccord System, a UPLC-ToF MS that brings faster, more robust, and more resolving separations with higher mass resolution and sensitivity detection to this analysis. The workflow includes methods, with ready-made calculations and report templates, to streamline analysis by automating many of the data analysis steps and simplifying the few that still require user input. These tools and training data sets provide for a reduced user burden to facilitate the analysis of synthetic oligonucleotide impurity profiles.

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## Benefits

- Streamlined workflows reduce data analysis time from hours to minutes
- Automated, compliance-ready data processing within waters\_connect Informatics reduces manual analysis and risk of human error
- Easy-to-use and readily adaptable methods reduce training burden and required user expertise for routine method execution
- Improved chromatographic and mass spectrometric performance of the BioAccord System or Xevo QToF MS over the traditional single quad MS platforms<sup>1</sup>

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## Introduction

Nucleic acid therapeutics have gained substantial interest in recent years as new modalities are being explored as drug products. Among these classes of therapeutics are synthetic oligonucleotides, short nucleic acid chains synthesized through a cycle of protection, coupling, and deprotection reactions. While oligonucleotides are highly impactful due to selectively targeting specific genes, their synthesis can introduce impurities which affect their efficacy and safety. As the length and complexity of the synthetic oligonucleotide increases, the complexity of the impurity profile also increases, which can lead to challenges in assessing the product's impurity components.<sup>1</sup>

Methods have been established for measuring impurities in synthetic oligonucleotide products. However, these methods carry with them specific difficulties. Typically, impurity characterization has been carried out on a single quad mass spectrometer. While this type of instrument has proven difficult but effective for simple, short oligonucleotides, new modalities entering the development pipeline require more advanced LC-MS analytical systems that offer sufficient sensitivity and resolution to mass resolve near isobaric impurity components and quantify low level impurities. For this reason, switching to a time-of-flight (ToF) detector, such as with the BioAccord System or a Xevo QToF MS, is highly beneficial, as it improves analytical capability and can simplify the user experience.<sup>2</sup> There are also challenges with the data analysis, as existing workflows often require manual interventions during the processing of the data. This is not only time consuming, but carries risks associated with potential human error and makes it difficult to execute these methods in a regulated lab environment.

To address these challenges, this work details a newly released workflow specifically designed for impurity analysis of synthetic oligonucleotides. The workflow shown here used a BioAccord System with ACQUITY Premier LC for data collection with a short gradient that improved throughput and minimized solvent consumption and hazardous waste generation. Data analysis was performed in the UNIFI App of the waters\_connect Informatics Platform, which automates acquisition, processing, and reporting to streamline analysis of collected data. The data analysis is comprised of two workflows, as outlined in Figure 1, that account for expected and unexpected impurities in the oligonucleotide.

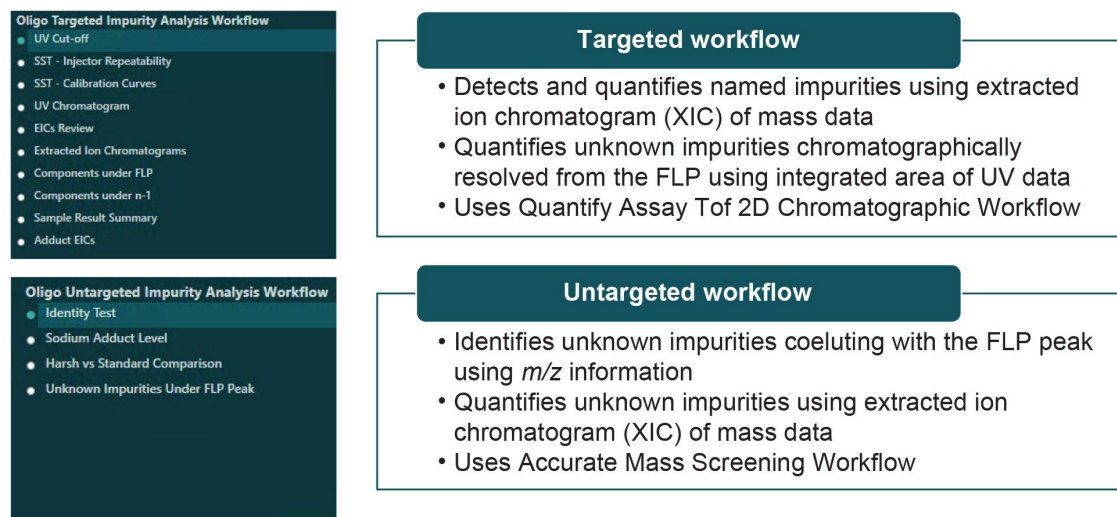


Figure 1. Summary of the two workflows used in the Oligonucleotide Impurity Analysis Workflow Package. The guided data review window steps for each workflow are shown on the left.

## Experimental

### Sample Description

Nusinersen was diluted in pure water and varying volumes were injected on column to generate a calibration curve ranging from 0.8  $\mu\text{g}$  to 2.4  $\mu\text{g}$ . Two replicates of the sample were acquired, one under softer ionization conditions (400  $^{\circ}\text{C}$ ) and one under harsher ionization conditions (550  $^{\circ}\text{C}$ ). An example sample set is shown in

Figure 2.

The sequence of Nusinersen is U-\*C-A-\*C-\*U-\*U-\*U-\*C-A-\*U-A-A-\*U-G-\*C-\*U-G-G (methylation on "C" and "U"). The exact mass is at 7122.2763 Dalton.

Item name	Sample type	Level	Sample position	Processing options	Desolvation temperature (°C)
Blank H2O	Blank		1:A,1		400
Nusinersen 60V 400oC 0pt8uL	Standard	Level 1	1:C,6	Quantitation standard	400
Nusinersen 60V 400oC 1pt6uL	Standard	Level 2	1:C,6	Quantitation standard	400
Nusinersen 60V 400oC 2uL	Standard	Level 3	1:C,6	Quantitation standard	400
Nusinersen 60V 400oC 2pt4uL	Standard	Level 4	1:C,6	Quantitation standard	400
Nusinersen 60V 400oC 2uL	QC	Level 3	1:C,6		400
Nusinersen standard 50V 550oC 0.5uL	Reference		1:A,8		550
Nusinersen standard 50V 400oC 0.5uL	Unknown		1:A,8		400

Figure 2. Example of a sample set including Working Standard Solution injections at varying concentration levels and two injections of a sample at different ionization conditions.

Method Conditions

## LC Conditions

LC system:	ACQUITY™ Premier (Binary)
Detection:	ACQUITY Premier TUV; $\lambda = 260$ nm
Vials:	QuanRecovery with MaxPeak HPS vials (p/n: 186009186)
Columns:	ACQUITY Premier Oligonucleotide C <sub>18</sub> Column (130 Å, 1.7 $\mu$ m, 2.1 x 50 mm) (p/n: 186009484)

Column temperature:	50 °C
Sample temperature:	6 °C
Injection volume:	0.5–2.4 µL
Flow rate:	0.250 mL/min
Mobile phase A:	5 mM TBuAA; 1 µM EDTA; Water:ACN 90:10
Mobile phase B:	5 mM TBuAA; 1 µM EDTA; Water:ACN 20:80

## Gradient Table

Time (min)	Flow (mL/min)	%A	%B	Curve
0.00	0.250	55.0	45.0	Initial
11.00	0.250	20.0	80.0	6
12.40	0.250	20.0	80.0	6
12.50	0.250	55.0	45.0	6
15.50	0.250	55.0	45.0	6

## MS Conditions

MS system:	ACQUITY RDa™ Detector
Ionization mode:	ESI, negative
Acquisition range:	High (400–5000 <i>m/z</i> )
Capillary voltage:	0.80 kV

Cone voltage:	50 V
Desolvation temperature:	Soft conditions: 400 °C Harsh conditions: 550 °C
Intelligent data capture:	On

## Data Management

Data were acquired and processed with the waters\_connect informatics platform (version 3.2.0) using the 2D Quan and Accurate Mass Screening workflows of the integrated UNIFI App (version 3.6.0.21).

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## Results and Discussion

A cumbersome bottleneck in impurity profiling of oligonucleotides has been the amount of time it takes to perform the required data analysis, especially when manual steps are required to supplement those automatically performed by the data analysis software. Currently, the most widely adopted method for analyzing impurities in synthetic oligonucleotides is an HPLC Ion Pair RP-UV-single quad method originally developed by Ionis Pharmaceuticals.<sup>3</sup> While this method has proven fit-for-purpose for simple oligonucleotides, it requires manual analysis steps, and lacks the analytical capability of assessing next generation oligonucleotides of greater length and complexity.

To address the needs for supporting growing and evolving oligonucleotide pipelines, a UPLC method with time-of-flight based mass detection has been developed. A shorter UPLC separation offered by the ACQUITY Premier UPLC System decreases solvent consumption and consequential generation of toxic waste while increasing separation quality and analytical throughput. Utilizing high-resolution mass detection offered by the BioAccord System reduces mass measurement errors for impurity assignments and improves the detection of near-isobaric impurities. For example, the sequence impurities n-U and n-C only differ by approximately 1 Da in mass. At the -4 charge state, their  $m/z$  values (1681.0468 and 1681.2928, respectively) are too similar to be distinguishable with a single quad MS but are easily resolved by the ToF detection on the BioAccord System.

The traditional Ionis method also exhibits throughput limitations due to its reliance on numerous steps that

require manual input from the user. This reliance on manual intervention makes the method not only time-consuming, but also difficult to train, and challenging to execute in regulated environments. Substantial training (often weeks to months) is required for new users to confidently carry out the traditional method, and reliance on manual data processes increases risks associated with human error.

The new Oligonucleotide Impurity Analysis Workflow streamlines data analysis by automating many of the data analysis steps and simplifying the few that still require user input to reduce time, risk of errors, and required user training. With this automated data processing approach, datasets that would have taken a day to process can now be reviewed in less than an hour. The default analysis method provided with the data package can be readily adapted to different full-length products and their associated impurities.

The methods for data processing consist of two key workflows, labeled as Targeted and Untargeted. The Targeted Workflow includes steps for system suitability, checks of repeatability, as well as calibration curve generation, UV integration of impurities chromatographically resolved from the Full Length Product (FLP), and facilitated review of extracted ion chromatogram (XIC) integrations of named impurities coeluting with the FLP. The Untargeted Workflow includes system suitability tests for confirming FLP identity (based on mass and sodium adduct level) and review of XIC integration of all unknown impurities coeluting with the FLP.

Collectively, these two workflows, summarized in Figure 1, work seamlessly with a single acquired data set on the BioAccord LC-MS instrument, taking advantage of chromatographic performance of the ACQUITY Premier UPLC System and ACQUITY Premier Oligonucleotide Column to enable a faster separation (roughly half of the traditional HPLC-SQ method length) with improved impurity separations using correspondingly less solvent and generating less hazardous waste – a key challenge with the solvents and toxic fluorinated ion pairing agents used for this analysis.

## Targeted Impurity Analysis Workflow

The Targeted Workflow contains system suitability checks for data quality analysis and integration of impurity peaks by either UV or MS data using the Quantify Assay ToF 2D Chromatographic Workflow within the UNIFI App. The first step of the workflow enables the user an option to perform a manual adjustment of the UV integration. Oligonucleotides often exhibit notable peak-tailing, reduced significantly with the Premier UPLC Systems and Oligonucleotide Chemistries, which can make it difficult to determine where the appropriate integration dropline is. The established standard methodology specifies that the cut-off point for FLP integration should be determined based on the retention time of the latest eluting impurity co-eluting with the FLP.<sup>3</sup> This workflow step calculates where the cut-off point should be based on those criteria and reports this value. The

user simply manually adjusts the UV dropline to be at this reported cut-off point.

The next step of the workflow is the evaluation of injection repeatability, which is based on repeated injections of a Working Standard Solution (WSS) at a single concentration. This guided data review step shows relevant information for the FLP UV peak in each injection in a tabular format, such as observed retention time, UV response, and calculated concentration. The bottom of the table shows the mean and % relative standard deviation (RSD) for each of these values. This information is used to assess instrument performance and data quality.

Similarly, the next guided data review step shows calibration curves generated from the WSS injections at varying concentrations to ensure appropriate quantitation of the results. Calibration curves are generated for both the UV and MS data, and the trendline equation and  $R^2$  value are reported for each curve. Figure 3 shows a screenshot of what the calibration curve review step looks like within the workflow. Limit checks are included in the method to flag any results with values outside of acceptance criteria.

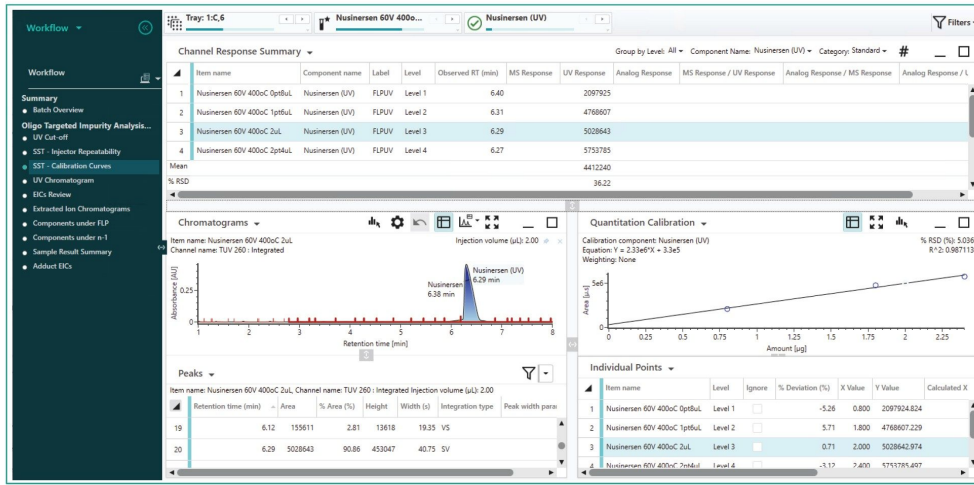


Figure 3. Workflow guided data review step showing the calibration curves of the Full Length Product (FLP) in the Working Solution Standard (WSS) using the Targeted Workflow for known impurity quantification. The table (TOP) shows information for each injection included in the calibration curve. The calibration curve (BOTTOM RIGHT) includes the equation for the trendline and the  $R^2$  value indicating the quality of the fit.



The next three guided data review steps show peak integration using the UV channel and MS channel, respectively, as shown in Figure 4. Peaks that are chromatographically resolved from the FLP are quantified with UV integration. These peaks are shown in yellow in the chromatogram (Figure 4, left), and their retention time, area, and relative area (%) are reported in the table below the chromatogram. Also included in the table is the % area sum for early eluting (before the FLP) and late eluting (after the FLP) peaks, which can be used for purity calculations.

The following guided review step provides an opportunity to review the XIC integration of impurity peaks coeluting with the FLP that have been included in the targeted component list. This step provides an opportunity to compare these peaks in the injections acquired under softer ionization and harsher ionization conditions. Adducts are often generated during the ionization process. If the adducts are unknown, they can be mistaken as impurities. To distinguish adduct peaks from impurity peaks, the sample is often ionized under harsher conditions (*e.g.*, using a higher desolvation temperature). Adducts are liberated under these harsher conditions, therefore comparing peaks at softer and harsher conditions reveals any contribution of adducts to impurities with the same *m/z*.

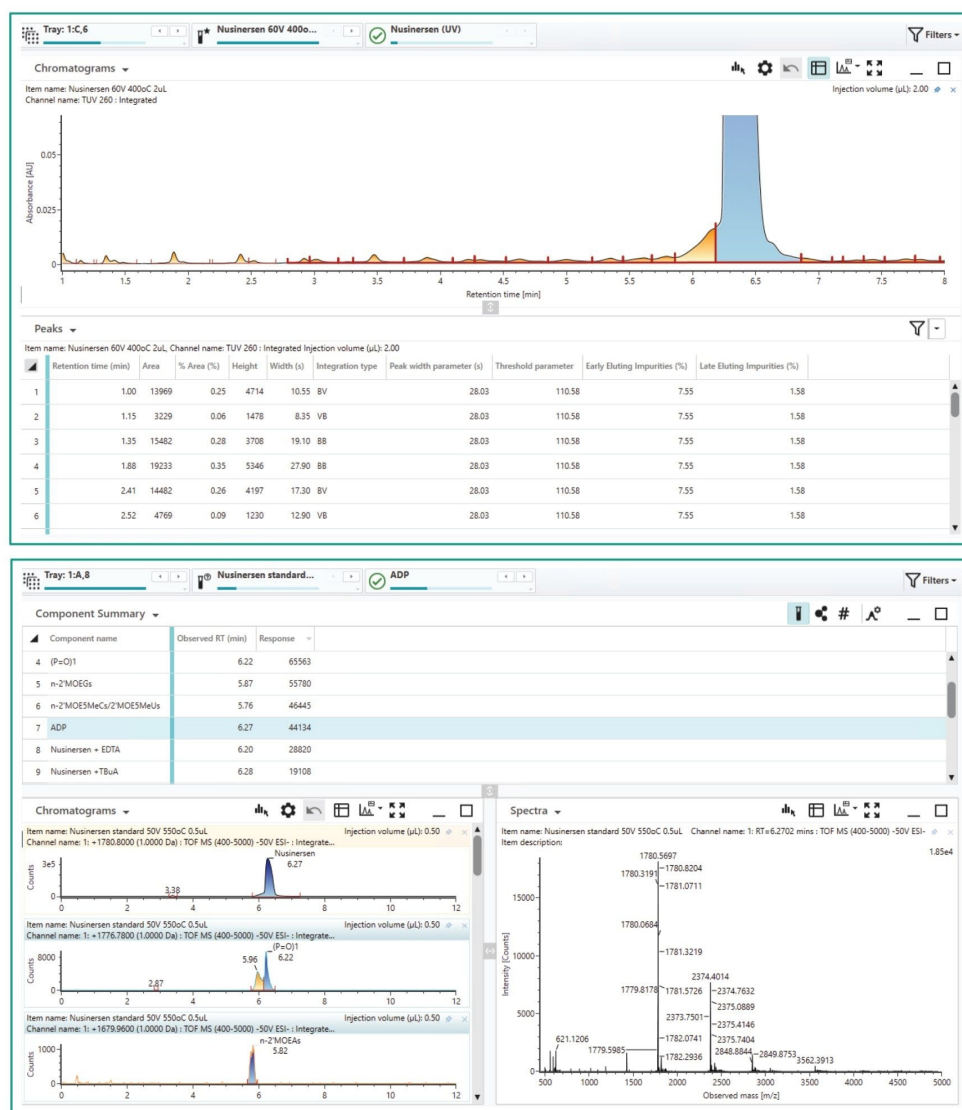


Figure 4. Summary of integration results using (top) UV data and (bottom) extracted ion chromatograms (XIC) from the MS data to quantify chromatographically resolved impurities and known, coeluting impurities, respectively.

While manually comparing the peaks between the two conditions can be time consuming, the workflow facilitates this step by allowing an overlaid comparison of the two injections, as shown in Figure 5. Here, the difference between harsh and soft ionization can be observed in the XIC corresponding to the  $m/z$  of a known impurity. When the two injections are overlaid, it can be seen that part of the peak is absent from the harsh

ionization injection, while the rest of the peak persists. The part of the peak that is absent in the harsh injection can be attributed to an unknown adduct at the same  $m/z$  as the impurity, and so it can be removed from the integration. As a result, the % area of the impurity is lower and more representative of the actual impurity amount in the sample. After reviewing each of the named impurities in the list and manually adjusting as necessary, the resulting XIC integrations for each peak are shown in the following guided data review workflow step (Figure 4 right).

The final four guided data review workflow steps summarize the impurities and adducts both by grouping them based on impurity type (as indicated by their labels in the target component list) and by summarizing their mean amount across multiple injections.

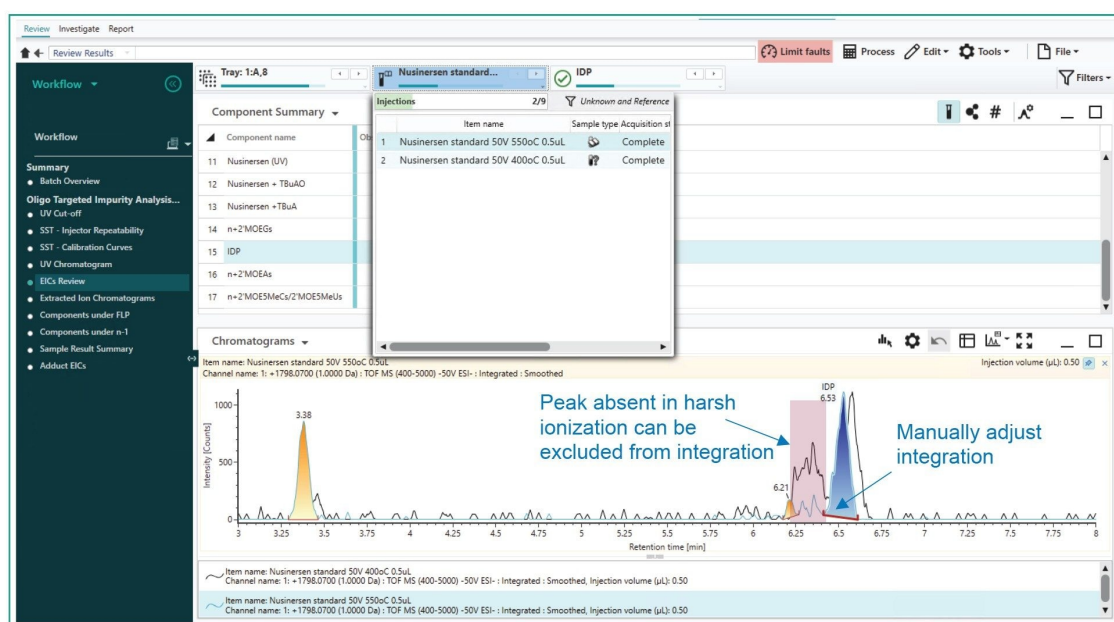


Figure 5. Guided data review workflow step for comparing harsh versus soft ionization in the Targeted Workflow to distinguish adduct peaks from known impurities of the same  $m/z$ .

## Untargeted (Coeluting Unknown) Impurity Analysis Workflow

The Untargeted Workflow uses the Accurate Mass Screening Workflow within the UNIFI App to perform additional system suitability tests and quantify unknown impurities coeluting with the FLP, based on their summed XIC. The first guided data review workflow step is an Identity Test, which confirms the identity of the

FLP by comparing the measured  $m/z$  in the sample to either the  $m/z$  of the same FLP in the WSS injection or the theoretical value notated in the untargeted component list. If the  $m/z$  difference is less than the specified threshold (e.g., 0.2  $m/z$  here), the test passes. This step also shows the FLP XIC and the MS spectrum with the isotopic envelope highlighted in green (Figure 6).

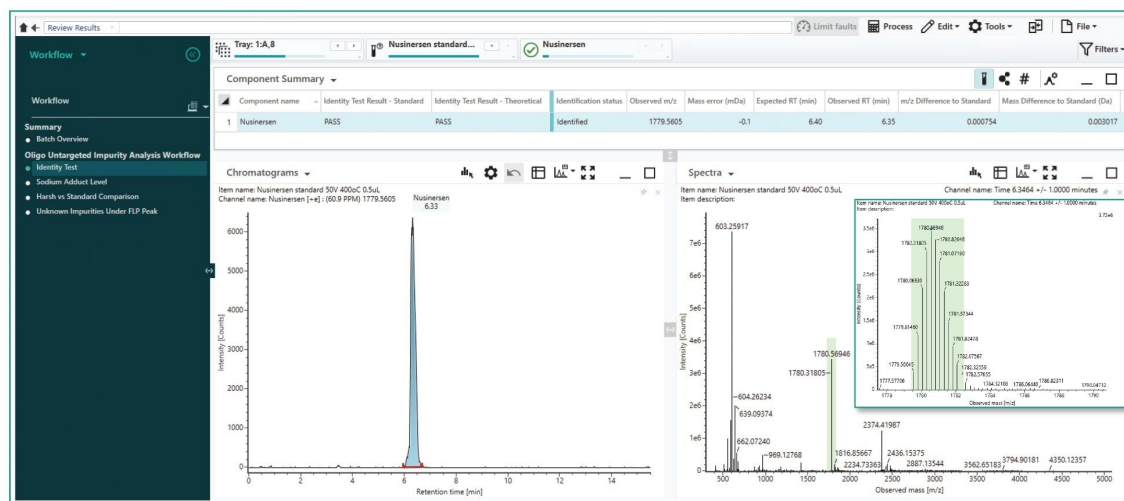


Figure 6. Guided data review workflow step for visualizing the results of the Full Length Product (FLP) identity test using the Untargeted Workflow.

The sodium adduct test functions similarly and passes if the relative intensity of the FLP sodium adduct peak is less than a specified threshold (e.g., 3% here) compared to the FLP peak. The threshold values for both of these tests can readily be edited in the method.

Similarly to the Targeted Workflow, the Untargeted Workflow also includes an opportunity for the user to distinguish impurity peaks from adduct peaks by comparing soft and harsh ionization. The display (Figure 7) contains mirror plots showing the comparison of soft and harsh XIC chromatograms and spectra. The green trace in the chromatogram indicates the difference, enabling the user to easily see which part of the peak, if any, is due to the presence of an adduct. Manual changes can then be made to the integration, accordingly.

The final step of the guided data review workflow lists all detected unknown impurities that are coeluting with the FLP and shows their spectra and XIC integration that is used for quantification. Impurities are only included in this list if they are above a specified detection threshold (e.g., 0.2% relative intensity for  $m/z$  lower than the

FLP and 0.3% relative intensity for  $m/z$  above the FLP in this case). To avoid double-counting, this list excludes anything chromatographically resolved from the FLP or present in the component list as a named impurity.



Figure 7. Guided data review workflow step comparing harsh versus soft ionization for distinguishing adducts from impurity peaks of the same  $m/z$  using mirror plots in the Untargeted Workflow.

## Report Templates

The Oligonucleotide Impurity Analysis Workflow data package also includes report templates that systematically summarize the data in a clear, succinct way for rapid communication of the data and results. Individual reports are generated for the Targeted and Untargeted Workflows. Example report pages for the Untargeted Workflow (Figure 8) contain a header page that shows basic information about the analysis, followed by a summary of system suitability information generated by that workflow. Additional pages show information related to quantification of the impurities, such as tables listing all detected impurities and their relative response. The final report sections contain information for data quality verification, such as the individual XICs for each quantified impurity.

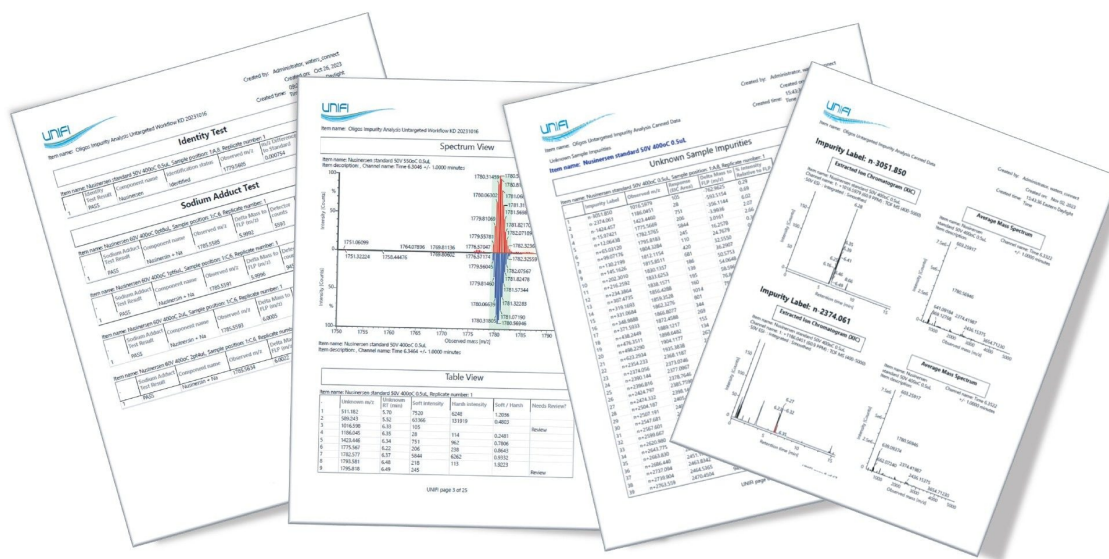


Figure 8. Example pages from a report generated from the customized report template designed for the impurity analysis untargeted workflow.

## Conclusion

As synthetic oligonucleotides become more prominent as therapeutics, new analysis methods are necessary to rapidly and confidently analyze complex impurity profiles. Reducing manual data analysis is key to increase throughput and reduce the risk of human error. In our approach, shortcomings from a well-established HPLC-single quad MS method were addressed using an updated workflow with automated data processing. This data package was developed on a UPLC-ToF MS system operated using the compliance-ready UNIFI App within waters\_connect. The overall data package automates a large portion of the required data analysis and guides the user to easily execute steps that still require manual intervention. These integrated guided data review workflow steps walk the user through each part of the data analysis, and built-in report templates distill the large volume of data into clear, concise tables and visuals for rapid communication. The workflow package has been designed to be easily adapted to different synthetic oligonucleotide products and impurities, reducing the challenge of prolonged method development for each new molecule. Ultimately, the next generation of oligonucleotides will require the tools and methodology discussed in this note.

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## References

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2. Doneanu CE, Knowles C, Fox J, Harry E, Yu YQ, Fredette J, Chen W. Analysis of Oligonucleotide Impurities on the BioAccord System with ACQUITY Premier. Waters Application Note. 2021 July; [720007301](#).
3. Rentel C, Gaus H, Bradley K, Luu N, Kolkey K, Mai B, Madsen M, Pearce M, Bock B, Capaldi D. Assay, Purity, and Impurity Profile of Phosphorothioate Oligonucleotide Therapeutics by Ion Pair-HPLC-MS. *Nucleic Acid Therapeutics.* 2022 March, 32(3), 206–220.

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