Oligonucleotide analysis

Detailed study into ASO impurity analysis, lessons learned, and myths dispelled while moving to compliant platform methods

Ulrik H. Mistarz[1], Marcus Hoffmann[2], Heiko Herrmann[2], Richard Law[3], Alexander Schwahn[4], Ken Cook[5]
[1]Thermo Fisher Scientific, Denmark, [2]Thermo Fisher Scientific, Germany, [3]Thermo Fisher Scientific, Austria, [4]Thermo Fisher Scientific, Switzerland, [5]Thermo Fisher Scientific, UK.

Abstract

Oligonucleotide analysis has gained considerable interest over the last few years with multiple synthetic short interfering RNA therapeutics now in clinical trials. More complicated new modalities have extended the need for novel analytics to determine impurities generated during manufacture. Despite this need, the chromatography and mass spectrometry methods for Ion Pair Reversed phase chromatography of oligonucleotides remains shrouded in myths and dated methodology. Amine ion pair and metal adducts create a quantitation problem due to multiple split signals with mass spectrometry and are still regarded as inevitable. Harsh source conditions to remove adducts, increases fragmentation in the source, generating false impurities. Here we will dispel some of the myths that surround oligonucleotide analysis that make the analysis seem daunting. Novel routines for the UHPLC systems allows for mixing applications on the same instrument and improves results for oligonucleotide analysis. Adhering to some simple but essential housekeeping rules for oligonucleotide analysis, yields much improved and more reproducible results.

Introduction

We will present data that shows the use of different charge states can improve reproducibility and accuracy for quantitation. The importance of the source and optimisation conditions to remove amine adducts without producing in-source fragmentation products. The generation of in-source impurities and adducts which are not present in the original sample is a problem which has now been solved. This knowledge has allowed a simple platform method to be developed for impurity analysis of ASO RNA with LC/HRMS. Quantitation without adducts is simpler and straightforward. A fully automated and GLP compliant workflow and report is also available for any ASO RNA drug product. The chromatography and quantitation method has been successfully applied to several different types of ASO RNA products. Deconvolution and automatically transferred the m/z values for XIC quantitation show both methods of analysis produced comparable results.

Materials and methods

Samples

Multiple Synthetic antisense oligonucleotide (ASO), see Figure 6

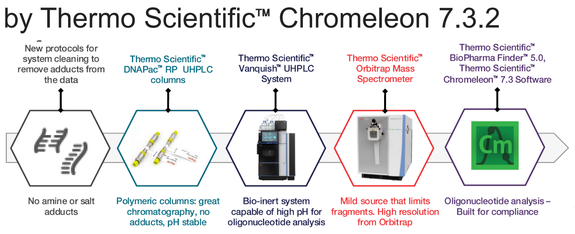
Equipment and workflow

LC-MS Measurement

UHPLC : RP separations were performed with a Thermo Scientific™ DNAPac™ RP column (4 µm, 2.1 ×100 mm) using a Thermo Scientific™ Vanquish™ Flex Binary UHPLC system. The system was flushed with a strong acid ion pair to remove adducts and achieve high sensitivity.

Thermo Scientific™ Chromacare™ UHPLC/MS solvents were used with borosilicate bottles and no glassware.

Mass Spectrometry: ASO purity analysis was performed at high resolution on Thermo Scientific™ Orbitrap Exploris ™ MS. Controlled by Thermo Scientific™ Chromeleon 7.3.2



Data Analysis: Chromeleon™ 7.3.2 software was used for identification and relative quantitation of the oligonucleotide full-length product (FLP) and their impurities. Characterization used deconvolution and XIC, with the impurities found by deconvolution transferred within the software to a component table for quantitation. A report was generated with flexible impurity annotation.

Workflow

- Ion pair reversed phase using Pentylamine and HFIP followed by deconvolution in CM
- Use of CM reporting engine 2.0 – faster, more functions, 64 bit, Thermo Scientific
- Automatic annotation of the full-length product (FLP) and filtered data to show true impurities
- Internal transfer within CM of identified components to an XIC component table. CM allows input of additional target XIC's
- Automatic reporting, including results tables, deconvoluted spectra, abundance values, XIC's, bar graphs
- Comparison of deconvoluted and targeted XIC results.

Results

Optimisation of source conditions

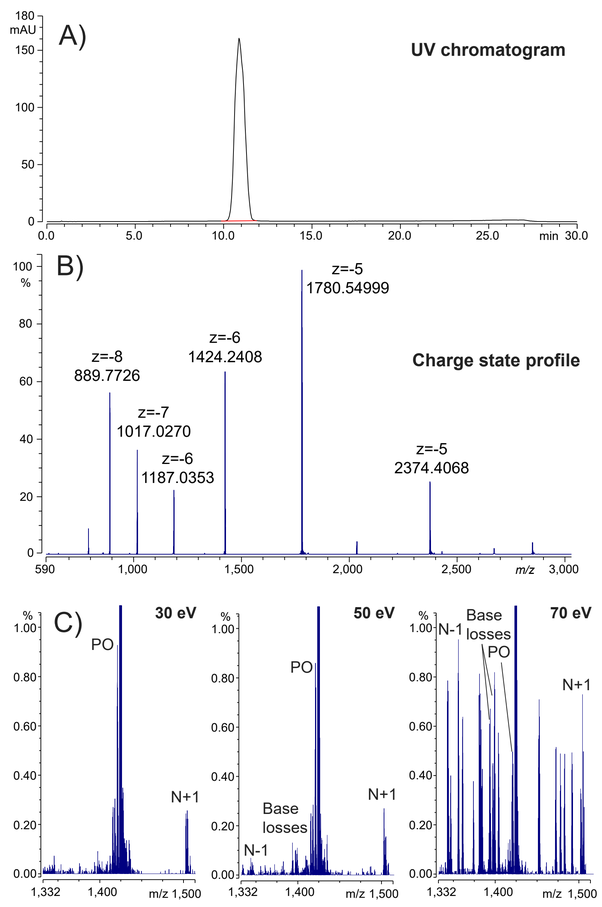


Figure 1. A) UV chromatogram, B) charge state profile, and C) zoomed in profile of a single charge state at the indicated in-source collision energies.

Optimisation of the source conditions is critical to remove adducts without producing in-source fragments that can be mistaken for real impurities. **Figure 1C** shows the effect on in-source impurity generation with increasingly harsher source conditions. Nusinersen is quite stable and shows little indication of breaking down in the source until 70 eV is applied. At this voltage base loss appears as well as N-1 and other impurities that are being generated in source.

Figure 1B shows there are multiple charge states present in the HRMS profile. This can be used to indicate which impurities are real and require monitoring by XIC. Figure 2 shows a zoom of the charge states -6, -5, and -4. The PO impurity remains constant in all charge states where the impurity shown with a red star appears much higher in charge state -4.

This shows that choosing the charge state for quantitation is very important as the lower charge state has an overlapping adduct interfering with quantitation.

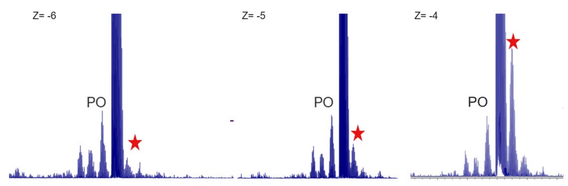


Figure 2. Zoom of different charge states showing differences in the impurities present.

Figure 3 shows how sliding windows moves a narrow window of deconvolution across the chromatography (A) to allow an XIC to be generated from the windows in which the component was found. **Figure 3 B** shows the full TIC with an XIC of a N-2 impurity found before the FLP

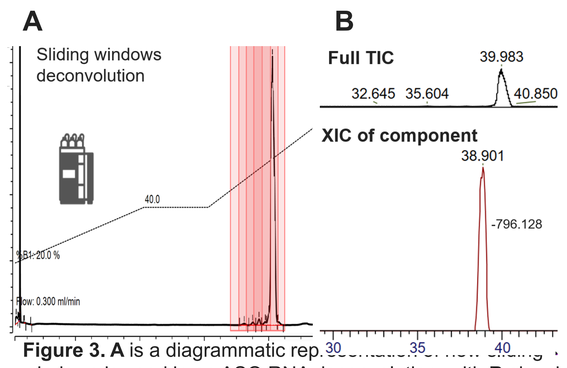


Figure 3. A is a diagrammatic rep[illegible] windows is used in an ASO RNA deconvolution, with **B** showing the TIC and an XIC of an N-2 impurity with delta mass -796.128.

Figure 4 shows how the XIC values for the different impurities found are transferred to a component table in CM for quantitation. The values from all charge states are transferred. Additional components can still be added to the component table manually if required. The charges states to be used in the calculations can be selected, the mode of quantitation and the integration parameters can then be optimised.

#	Result Component	Matching Component	Monoisotopic Mass	Relative Abundance	Fractional Abundance	Delta Mass
1	Result Component 1	(No match)	7122.28270	100.00	98.43	0.00000
2	Resu	Copy	7106.28191	0.58	0.57	-16.00079
3	Resu	Add to Component Table	7175.30886	0.48	0.47	53.02616
4	Result Component 4	(No match)	7078.25113	0.19	0.19	-44.03157

m/z values for each charge state transferred to the component table

Charge State	Intensity	MZ Centroid	Calculated Mass
4	1.78E+10	1779.56193	7122.27233
5	2.17E+10	1423.44804	7122.27375
6	1.62E+10	1186.03881	7122.27277
7	1.43E+10	1016.46079	7122.27077
8	6.38E+09	889.27729	7122.27237

Figure 3. Transfer of the XIC values of selected deconvoluted components to the quantitation component table in Chromeleon software.

Quantitation in MS is most often done using the XIC signals of the targeted components, Oligonucleotides have multiple charges states that could potentially be used. In this workflow, the quantitation by deconvolution is presented alongside the results using the targeted XIC signals. This gives an extra layer of confidence in the results and aids in choosing the best charge states to use, as shown in **Figure 2**. It also allows new peak detection of any new impurity that was not originally targeted with XIC.

Chromeleon can use the UV as well as the XIC MS signals for quantitation in a GLP environment. This is a market need that has not been previously fulfilled. The quantitative results shown in Table 1 reveal comparable results obtained by XIC and deconvolution.

Component	Deconvolution	XIC component table
FLP	97.88	97.47
PO	1.22	1.30
CNET	0.30	0.35
-44	0.22	0.25
N+1	0.15	0.12

Table 1. Component quantitation: Comparable results between deconvolution and XIC

Figure 4 shows the annotated impurities found at low level in a Nusinersen sample. The data is clean and easy to quantitate due to the removal of adducts and source induced impurities. Similar results (not shown) have been obtained using a triple quadrupole MS with the same source and LC conditions.

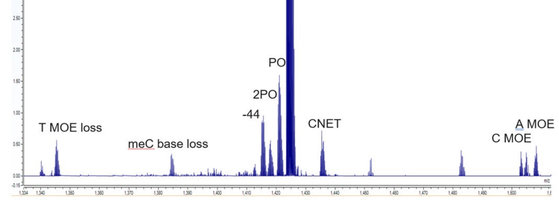


Figure 4. Zoom of the impurity isotopic profiles around the full-length product (FLP) with identifications.

The Quantitative data can be used in a custom report as shown in Figure 5. Several different samples were run in the same sequence. The target ASO RNA is identified from the chemical formula input into a column in the run sequence. Impurities are identified and annotated from a customizable delta mass impurity table in the report. The relative abundance by MS and UV is also shown in the report.

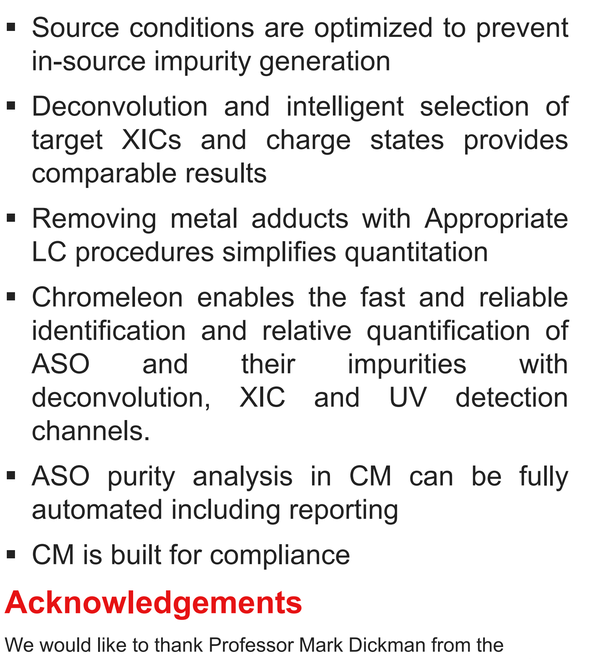


Figure 6. Example report in Chromeleon software for identification by expected mass and a deeper view on each sample with impurity analysis, MS spectra, TIC and identification

Conclusions

- IPRP-LC HRAM MS is a powerful technology to analyze the purity of ASOs
- Source conditions are optimized to prevent in-source impurity generation
- Deconvolution and intelligent selection of target XICs and charge states provides comparable results
- Removing metal adducts with Appropriate LC procedures simplifies quantitation
- Chromeleon enables the fast and reliable identification and relative quantification of ASO and their impurities with deconvolution, XIC and UV detection channels.
- ASO purity analysis in CM can be fully automated including reporting
- CM is built for compliance

Acknowledgements

We would like to thank Professor Mark Dickman from the University of Sheffield and Dr Jonathan Bones from NIBRT for advice and supplying additional purified samples to further authenticate these results.

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