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應用手冊

Developing a Novel, Integrated LC-MS Workflow for High-resolution Monitoring and Characterization of Oligonucleotides

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Abstract

This application note describes an integrated LC-MS workflow for identification, impurity profiling, and MS/MS characterization of oligonucleotides using high-resolution mass spectrometry.

The complete workflow consists of an ACQUITY UPLC H-Class Bio System coupled to an ACQUITY UPLC Tunable Ultraviolet (TUV) Detector and the Xevo G2-XS QTof Mass Spectrometer. System control and MS data acquisition is accomplished using Waters MassLynx Mass Spectrometry Software.

High-resolution oligonucleotide characterization was demonstrated by performing a full ladder sequencing of a siRNA sample using the MS/MS spectrum within MassLynx.

Benefits

- Optimized RP-UPLC mobile phase compositions for better oligonucleotide separation and lower salt adduct intensity in the MS spectra
- Newly enabled high-resolution, high-throughput data processing capability using ProMass HR integrated with MassLynx Software

- Enhanced MS/MS fragmentation with the Xevo G2-XS QTof MS System for full ladder sequencing of oligonucleotide samples

Introduction

Methods that enable structural characterization, molecular weight confirmation, and impurity analysis and profiling (e.g. failed sequences and other production-related impurities) are of great importance for therapeutic oligonucleotide development. These assays are often conducted using liquid chromatography coupled with both ultraviolet (UV) and mass spectrometry (MS) detection – enabling quantification via UV absorbance – and structural characterization, mass confirmation, and impurity monitoring via MS detection. The Waters Xevo G2-XS QTof Mass Spectrometer has proven to be an effective tool for characterizing biopharmaceutical drugs through analytical procedures such as intact mass analysis, subunit analysis, peptide mapping, and released glycan analysis.[1–4]

In this study, we describe an integrated LC-MS workflow for identification, impurity profiling, and MS/MS characterization of oligonucleotides using high-resolution mass spectrometry. The complete workflow consists of an ACQUITY UPLC H-Class Bio System coupled to an ACQUITY UPLC Tunable Ultraviolet (TUV) Detector and the Xevo G2-XS QTof Mass Spectrometer. System control and MS data acquisition is accomplished using Waters MassLynx Mass Spectrometry Software. Automated spectral deconvolution, data analysis, and reporting are enabled through an integrated version of ProMass HR (Novatia, Newtown, PA). High-resolution oligonucleotide characterization was demonstrated by performing a full ladder sequencing of a siRNA sample using the MS/MS spectrum within MassLynx.

Figure 1. From left to right: MassPREP Oligonucleotide Standard, Waters Oligonucleotide Separation Technology columns, the ACQUITY UPLC H-Class Bio System, and Xevo G2-XS QTof Mass Spectrometer.

Experimental

Reagents, solvents, and sample preparation

Triethylamine (99.5% purity) and 1,1,1,3,3,3-Hexafluoro-2-propanol (99.8% purity, LC-MS grade) were purchased from Sigma Aldrich. Mass spectrometry grade solvents (UHPLC grade) were purchased from Fisher Scientific. Mobile phase buffers were newly prepared prior to experiments. MassPREP Oligonucleotide Standard was purchased from Waters (P/N 186004135) and prepared at various concentrations (0.004~5 pmol/µL). siRNAs upper strand 5'-rUrCrGrUrCrArArGrCrGrArUrUrArCrArArGrGrTT-3' and the complementary lower strand 5'-TTrCrCrUrUrGrUrArArUrCrGrCrUrUrGrArCrGrA-3' were purchased from Integrated DNA Technologies (Coralville, Iowa) and prepared at a concentration of 5 pmol/µL in water. Injection volumes were varied depending on the experiments.

LC conditions

LC system:	ACQUITY UPLC H-Class Bio
Detectors:	ACQUITY UPLC TUV w/Ti flow cell absorption wavelength: 260 nm
Column:	Waters Oligonucleotide BEH C_{18} Column, 1.7 µm, 2.1 mm x 50 mm
Column temp.:	60 °C
Sample temp.:	6 °C
Mobile phase A:	15 mM TEA, 400 mM HFIP prepared in H_2O, pH 8.0
Mobile phase B:	15 mM TEA, 400 mM HFIP prepared in MeOH

Mobile phases were prepared gravimetrically.

MS conditions

MS system:	Xevo G2-XS Qtof

Data mass range:	400–3000 Da
Mode:	ESI negative resolution
Cone voltage:	80 V for MS and 120 for MS/MS
Capillary voltage:	2.0 kV
Source offset:	80 V
Source temp.:	125 °C
Desolvation temp.:	500 °C
Desolvation gas flow:	800 L/Hr
Lockmass:	Glu Fibrinopeptide B at 100 fmol/µL in 50–50 H2O-ACN, 0.1% FA
Informatics for data collection and processing	MassLynx v4.1 SCN 9.25 with MaxEnt1 and MaxEnt3 ProMass HR ProMass Bridge 1.1

For Waters OST Standard (contains a mix of 5 polyT oligonucleotides, 15–35 nt)					
Time (min)	Flow (mL/min)	%A	%B	%C	%D
Initial	0.20	80.50	19.5	0.0	0.0
15.00	0.20	72.00	28.0	0.0	0.0
16.00	0.20	50.00	50.0	0.0	0.0
17.00	0.20	80.50	19.5	0.0	0.0
21.00	0.20	80.50	19.5	0.0	0.0

Gradient Table 1.

For ssRNA Samples					
Time (min)	Flow (mL/min)	%A	%B	%C	%D
Initial	0.20	87.0	13.0	0.0	0.0
10.00	0.20	77.0	23.0	0.0	0.0
10.10	0.20	50.0	50.0	0.0	0.0
11.10	0.20	50.0	50.0	0.0	0.0
11.20	0.20	87.0	13.0	0.0	0.0
16.00	0.20	87.0	13.0	0.0	0.0

Gradient Table 2.

Results and Discussion

In this section we review the experimental results generated using the aforementioned high-resolution LC-MS workflow for oligonucleotide analysis; demonstrate the basic performance of the system in terms of mass

accuracy, salt adduct reduction, and MS detection sensitivity; and discuss the fit-for-purpose capabilities for high-resolution MS screening and impurity profiling of oligonucleotides, as well as high-resolution characterization via MS/MS ladder sequencing.

Figure 2 shows excellent separation of the polyT oligonucleotides and their truncation sequences (achieved using Waters OST column with BEH C_{18} chemistry). As described in recent studies, LC-MS grade reagents, such as TEA and HFIP, and mobile phase solvents, such as acetonitrile and water, were used to ensure the best LC peak separations.[5,6] The LC-MS grade reagents and solvents also play an important role in helping generate high-quality MS spectra. An MS spectrum example is shown in Figure 3 that displays the multiple charged species distribution/charge envelope of the 25 nt polyT, which demonstrates minimal sodium adduct formation (<5%) and excellent isotopic peak resolution in the zoom-in region of the M^{3-} peak.

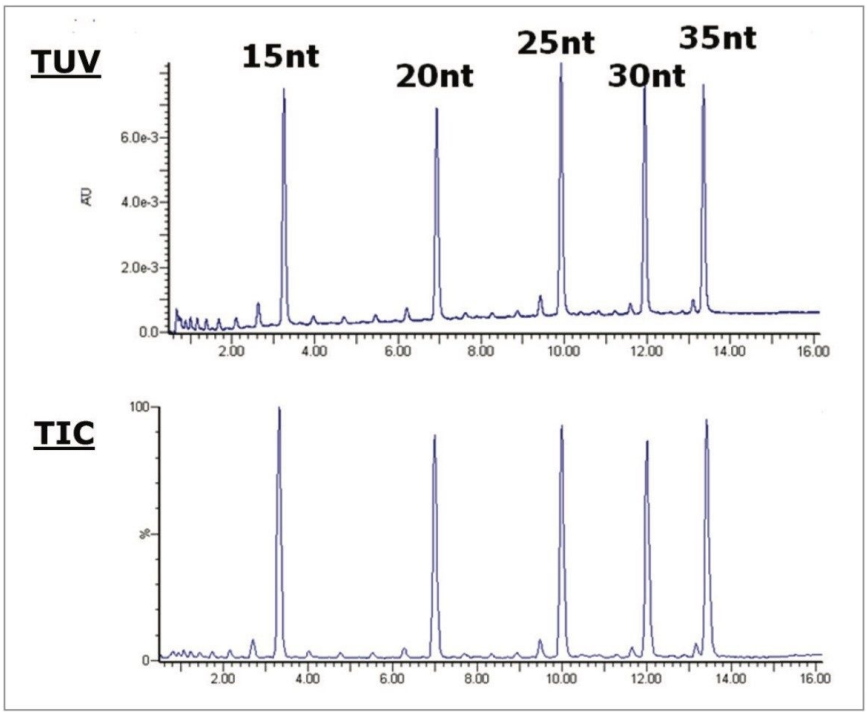

Figure 2. In-line orthogonal TUV and TIC chromatograms of the Waters MassPREP Oligonucleotide Separation Technology Standard, containing a mixture of five polyT oligonucleotides from 15 to 35 nt, with expected synthesis-related impurities.

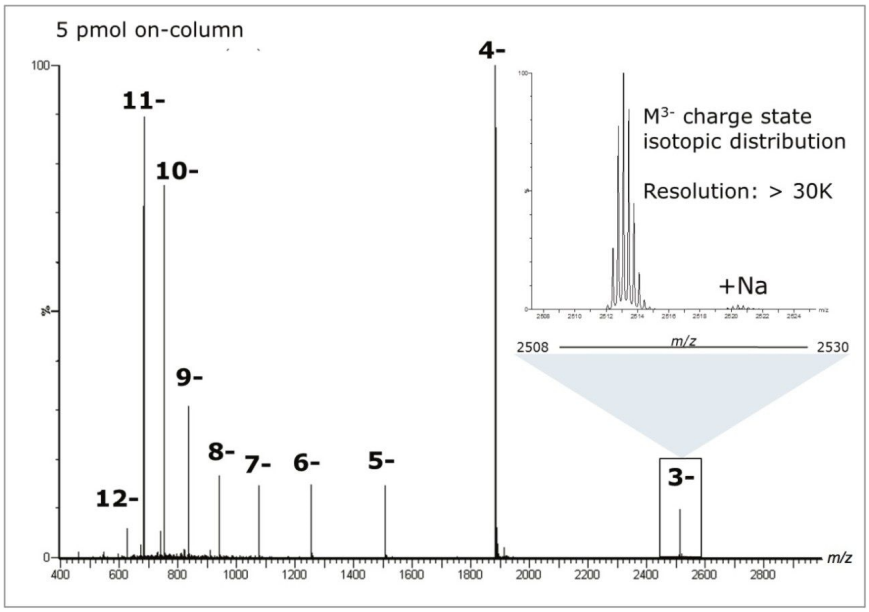

Figure 3. Combined raw ESI spectrum for the 25 nt polyT oligonucleotide from the MassPREP OST Standard.

Using ProMass HR*, data acquired by MassLynx can be automatically processed to obtain the deconvoluted exact monoisotopic masses contained within each sample. Table 1 is a summary from the ProMass HR processed report that displays the monoisotopic mass accuracy for the five polyT oligonucleotides contained within the MassPREP OST Standard. The average mass accuracy across all five polyT oligonucleotides was about 1.25 ppm.

The limit of detection (LOD) of the LC-MS system using the MassPrep OST Standard was determined to be 20 fmol on-column as shown in Figure 4. The comparison between the TUV and TIC chromatograms in the figure suggests the LOD is limited by the optical/TUV signal, and not by the Xevo G2-XS QTof MS instrument.

Using MassLynx in combination with ProMass HR, high-resolution screening and impurity profiling of synthetic oligonucleotides can be performed with automated data acquisition, processing, and analysis for greater productivity. The MassLynx sample list in Figure 5 shows the fields that should be included for this automated process.

**ProMass HR can be used to process high-resolution MS data, such as data generated by time-of-flight (Tof) systems, as well as low-resolution MS data, such as data generated by quadrupole systems. ProMass can only be used to process low-resolution MS data.*

MassPREP OST Standard	Expected mass	Observed mass	Mass accuracy (ppm)
15 nt	4498.7348	4498.7300	-1.07
20 nt	6018.9650	6018.9730	1.33
25 nt	7539.1952	7539.1990	0.50
30 nt	9059.4254	9059.4210	-0.49
35 nt	10579.6560	10579.6260	-2.79
		Average	1.24

Table 1. Summary table of monoisotopic mass accuracy for the MassPREP OST Standard, containing a mixture of five polyT oligonucleotides.

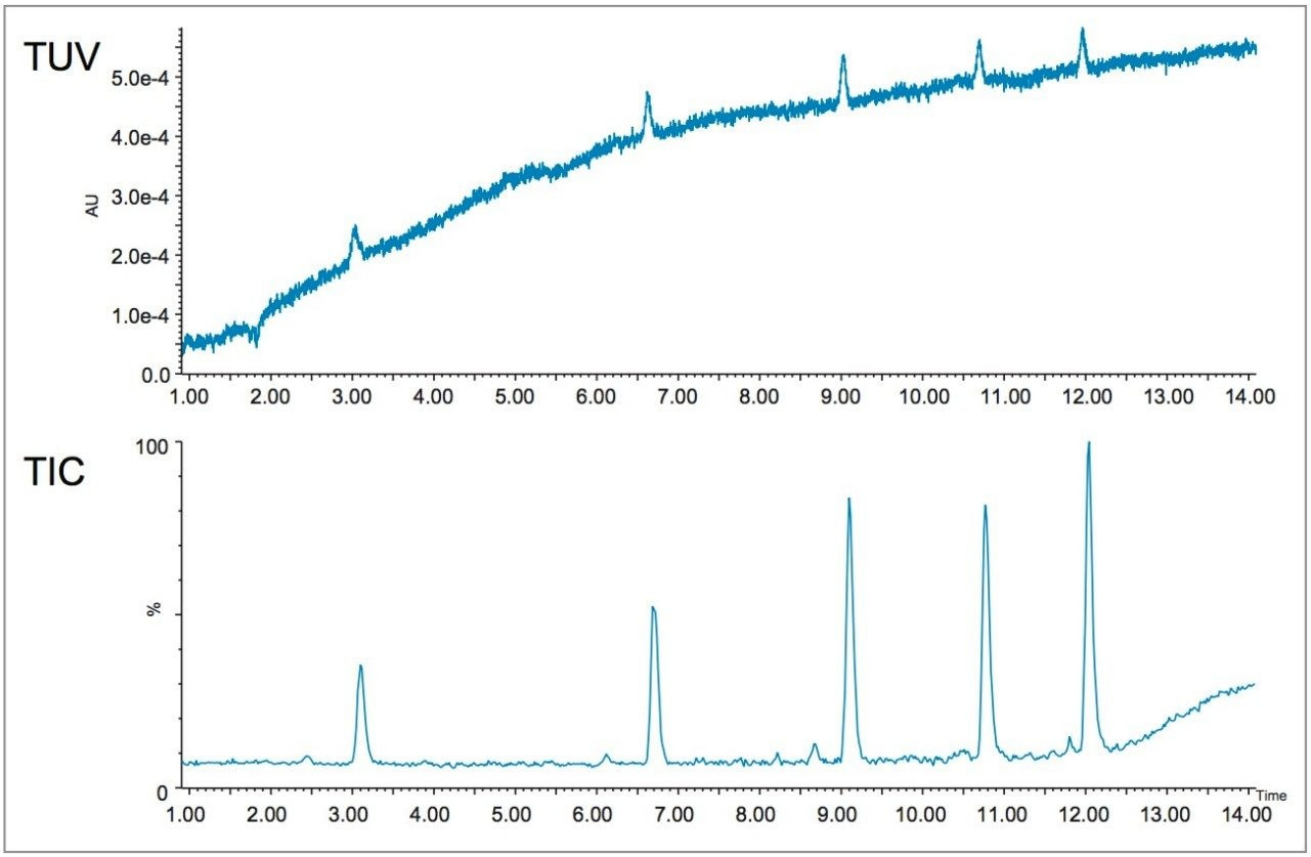

Figure 4. TUV (top) and TIC (bottom) chromatograms for an injection of 20 fmol of the MassPREP OST Standard, containing a mixture of five polyT oligonucleotides.

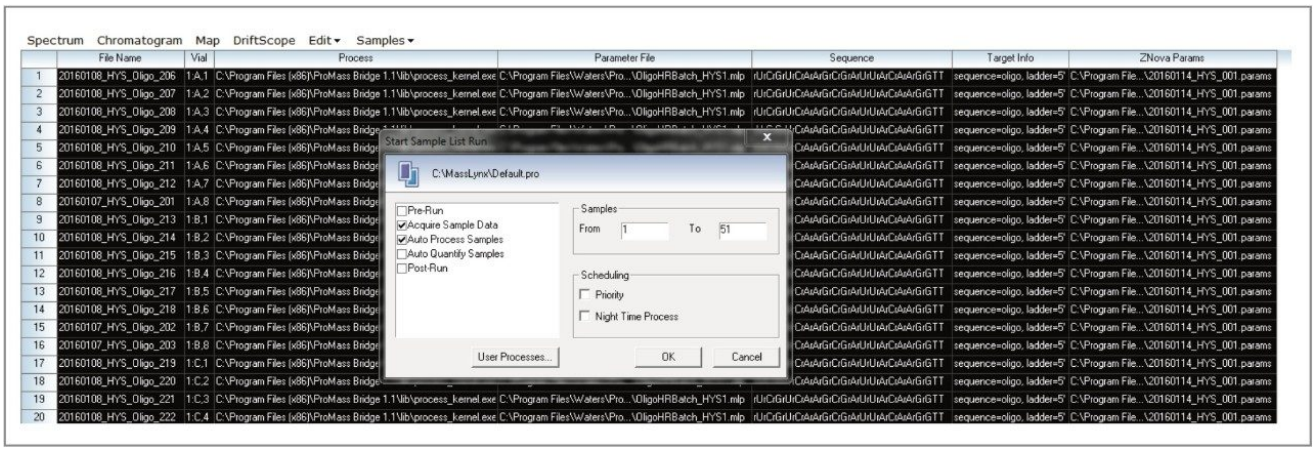

Figure 5. The MassLynx sample list shows the fields required for the automated data acquisition, processing, and analysis process. They include the sample-specific file names where data is stored, the designated injection vial, the process and parameter file locations, the sequence(s) of the targeted molecule(s), the molecule types and their prime information, and the ZNova file that defines ProMass HR parameters.

Figure 6 displays the TUV chromatogram of one of the targeted ssRNA, which has the sequence of 5'-UCGUCAAGCGAUUACAAGGTT-3' with a double thymine overhang (upper strand). The chromatogram shows that the targeted ssRNA was well separated from its single base deletion (N-1) and single base insertion (N+1) forms.

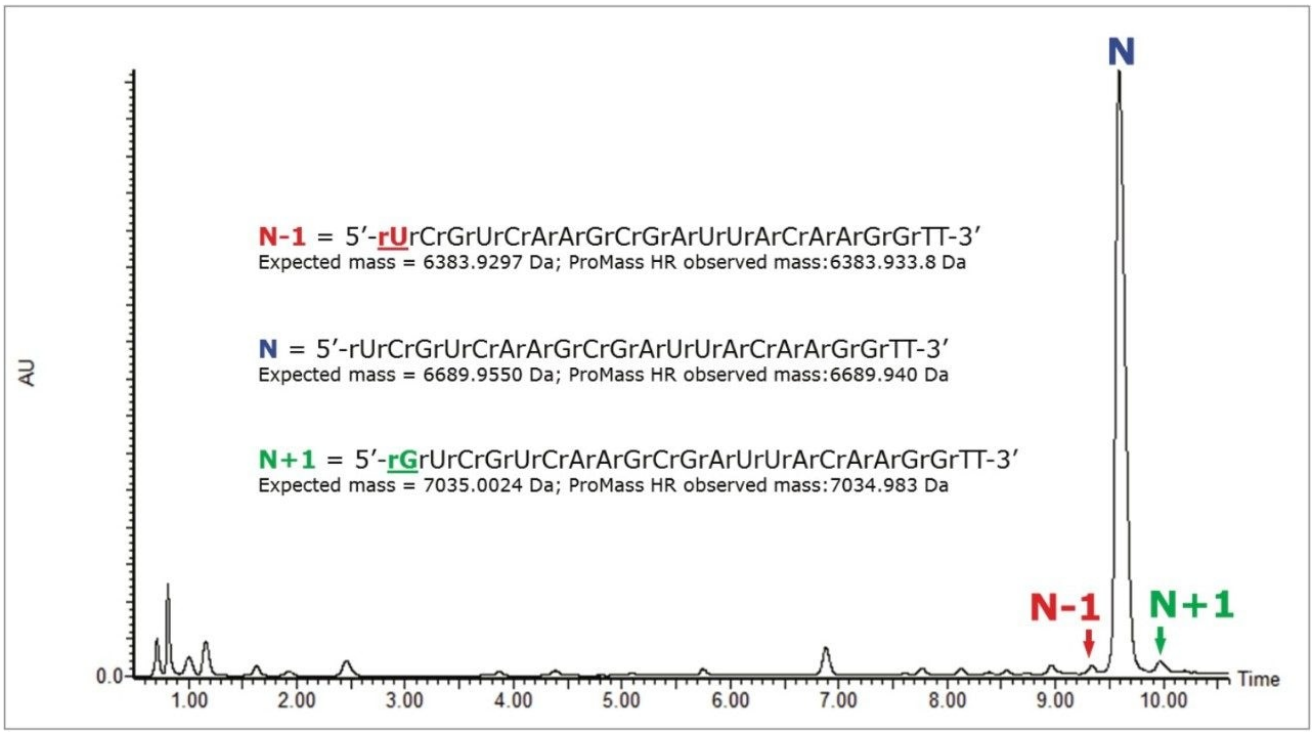

Figure 6. The TUV chromatogram of ssRNA sequence 5'-UCGUCAAGCGAUUACAAGGTT-3' with a double thymine overhang (upper strand of siRNA) shows good separation between the target sequence and the single base deletion (N-1) and single base insertion (N+1) forms using a ten minute LC gradient from 13% B to 23% B.

The batch-processed screening and profiling results for 48 samples, including eight blanks, are reported in Figure 7. The report displays a color-coded LC sample tray where samples containing the target oligonucleotide (upper strand) are marked in green, samples containing a different oligonucleotide (lower strand) are marked in red, and samples containing no oligonucleotide are marked in white. Each well position in the plate format acts as a hyperlink through which one can easily access the detailed experimental results for each sample, such as: raw ESI spectra, chromatographic retention times, variations between expected and observed masses (with mass accuracy in ppm), relative percent area of the targeted oligonucleotide(s), and associated impurities – which, when automatically identified, are labeled.

For instance, as shown in the lower table in Figure 7, the targeted ssRNA (upper strand), its deletion (N-1) of uracil, and the insertion (N+1) of guanine impurities are clearly identified and labeled, and the mass accuracies of 0.5 ppm, 2.2 ppm, and 2.8 ppm and retention times of 9.08, 9.28, and 9.73 minutes are reported for each, respectively. Additionally, from this data an estimate of the percent purity was calculated at 84.26%. The color-

coded HTML report interface makes it easy to assess the results.

Figure 7. The top image is a high-level summary of batch-processed, experimental results using ProMass HR with MassLynx. Also shown, successive drill-down results for an individual sample, accessed through embedded HTML hyperlinks.

To test the suitability of the LC-MS system for oligonucleotide sequence confirmation, a targeted LC-MS/MS experiment was performed using the siRNA upper strand. Figure 8 shows the MS full scan spectrum of the 21 nt ssRNA sample on the top and the deconvoluted MS/MS spectrum on the bottom. An individual charge state (M4- in this case) was chosen as the precursor ion and then fragmented in the Xevo G2-XS QTof collision cell with collision energy (CE) of 35 V. The MS/MS spectrum was then deconvoluted using MaxEnt3. Formulas composed in an Excel file were used to generate the list of predicted fragment ions based upon the oligonucleotide sequence. Next, the deconvoluted spectrum was manually matched against the list of predicted fragment ions. By finding most of all the predicted ions within the deconvoluted MS/MS spectrum, the sequence of the oligonucleotide can be confirmed. For the siRNA upper strand 21 nt sample and its complementary lower strand (data not shown), all of the C and Y ions were successfully matched to achieve a full ladder sequencing of the samples.

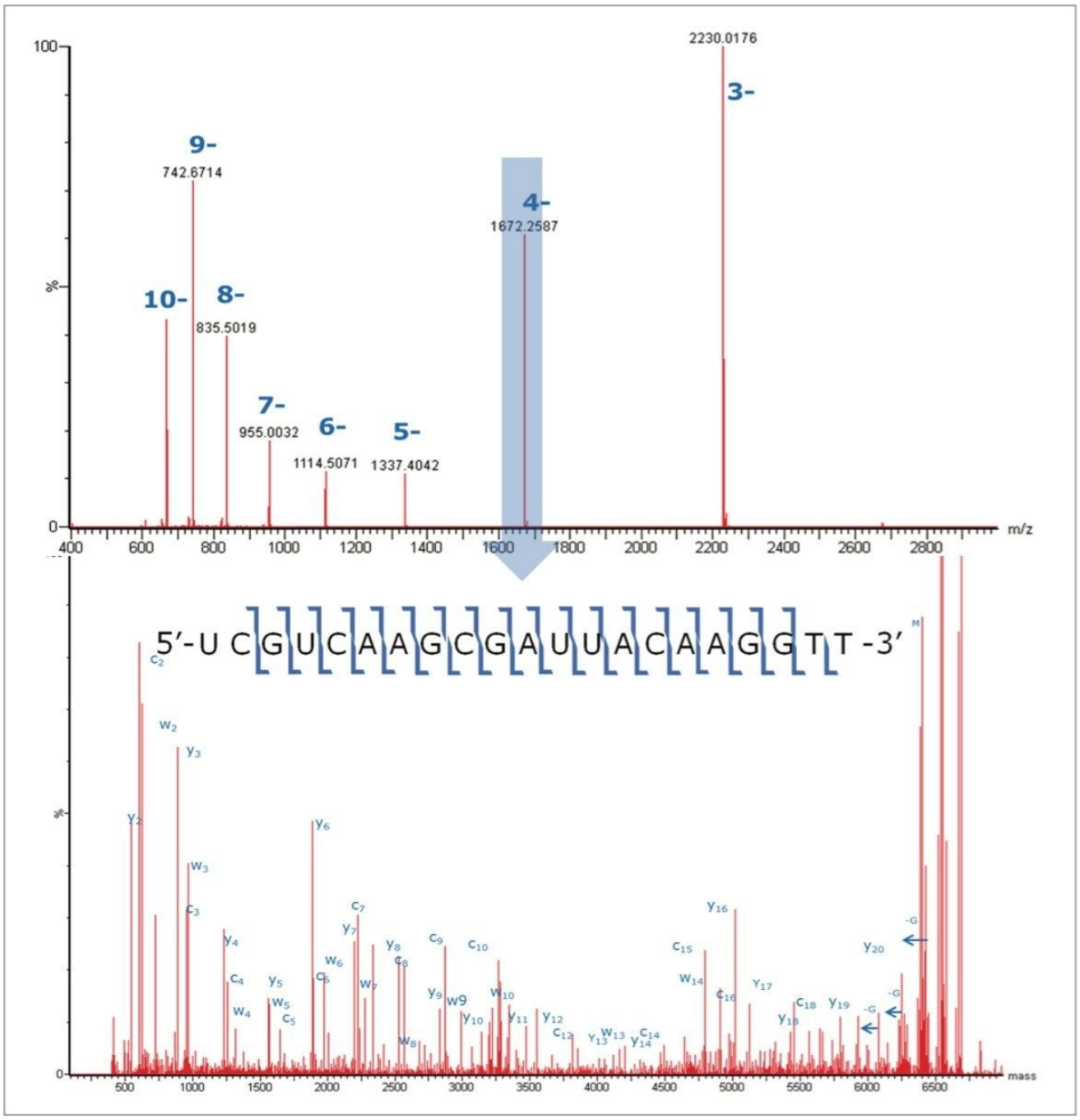

Figure 8. LC-MS/MS full ladder sequence confirmation for the ssRNA sequence of 5'-UCGUCAAGCGAUUACAAGGTT-3' was achieved. The experiment was conducted using the M4- peak as the precursor ion. The fragmentation peak assignments were done by matching against the theoretical masses using simple Excel file calculations. MaxEnt3 was used for charge deconvolution and deisotoping.

Conclusion

A newly-integrated LC-MS workflow for high-resolution mass spectrometry characterization and screening of oligonucleotides has been developed. To ensure the best LC separation and MS detection with the highest mass resolution and lowest salt adduct peaks (less than 5% intensity in the MS spectra), it is recommended to use LC-MS grade TEA, HFIP, and water. The system has been shown to have excellent sensitivity, with a LOD of 20 fmol on-column using the MassPREP Oligonucleotide Standard, containing a mixture of five polyT oligonucleotides. The integration of ProMass HR with MassLynx allows for automated deconvolution of high-resolution LC-MS data as well as automated target ID and impurity analysis of oligonucleotide samples using the Xevo G2-XS QTof MS instrument as demonstrated.

The LC component of the workflow demonstrates excellent and highly reproducible separation of oligonucleotides from their truncation (N-1) and insertion (N+1) sequences, and MS data quality confirm the robustness of the separation and detection workflow. The interactive HTML-based, color-coded summary report generated through ProMass HR makes it easy to review high-throughput experimental results and to navigate/drill down into sample-specific data, including chromatograms, combined raw spectra, deconvoluted spectra, impurity analysis, etc. LC-MS/MS experiments were also performed with the Xevo G2-XS QTof MS instrument, and demonstrate the ability of the system to accomplish full ladder MS-MS sequence confirmation of a 21 nt ssRNA oligonucleotide.

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MassPREP OST Standard <https://www.waters.com/186004135>

ACQUITY UPLC Oligonucleotide BEH C18 Column, 130Å, 1.7 µm, 2.1 mm X 50 mm < https://www.waters.com/186003949>

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