DEVELOPING NOVEL INTEGRATED LC-MS WORKFLOWS FOR OLIGONUCLEOTIDE CHARACTERIZATION, HIGH THROUGHPUT MASS CONFIRMATION AND IMPURITY PROFILING

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INTRODUCTION

For therapeutic oligonucleotide development, 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.

In this study, two novel integrated LC-MS workflows for oligonucleotide analysis are developed; one for oligonucleotide characterization and high resolution monitoring, and another for high throughput mass confirmation and impurity profiling. Both workflows include automated data acquisition, processing, and reporting, with results shown for both LC-MS and LC-MS/MS analyses.

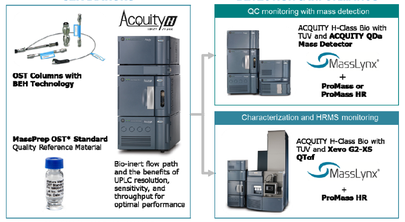


***Figure 1**. Two fit-for-purpose LC-MS workflows for Oligonucleotide analysis*

METHODS

LC Conditions:
LC System: ACQUITY UPLC® H-Class
Detectors: ACQUITY UPLC® TUV, Ti flow cell
Absorption Wavelength: 260 nm
Column: OST BEH C18 130 Å 1.7, 2.1x50 mm
Column Temperature: 60 °C
Injection Volume: 5 µL (50 pmol mass load)

Mobile phase:
A: H2O, 15mM TEA, 400 mM HFIP, pH 8.0
B: MeOH, 15mM TEA, 400 mM HFIP
Poly T oligo standards were run with a gradient of 19% B to 26.5% B in 15 mins. ssRNAs were run with a gradient of 13% B to 23 B% in 15 mins.

ACQUITY® QDa Settings:
Sample rate: 2 points/sec
Mode: Negative
Mass range: 410 – 1250 Da.
Cone voltage: 20 V
Capillary voltage: 0.8 kV
Probe Temperature: 600 °C

Xevo G2-XS QTof Settings:
Mode: ESI Negative
Mass range: m/z=400—3000
Capillary: 2.0kV
Sample Cone voltage: 80 V
Source offset: 80 V
Source Temp: 125°C
Desolvation Temp: 350 °C
Desolvation Gas Flow: 800 L/h

Informatics:
MassLynx 4.1, MaxEnt1 and 3
ProMass HR (Novatia, Newtown, PA)

RESULTS AND DISCUSSION

Robust Chromatography

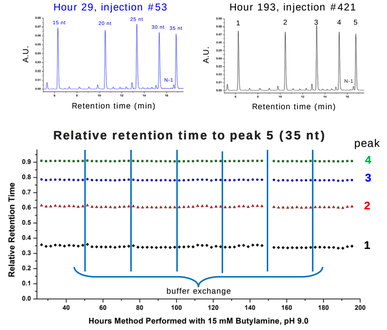


***Figure 2**. Robustness of BEH Particles. Waters OST columns using patented BEH technology designed to tolerate aggressive separation conditions of high temp and pH were evaluated for their applicability in the current study. The relative retention times of the polyT standard are stable and consistent over 200 hours of use at an elevated pH (pH 9.0) and temperature (60 ° C) resulting in consistent chromatographic performance.*

Improving Productivity Using MS

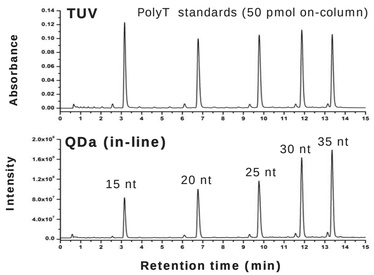


***Figure 3**. In-line orthogonal TUV and QDA MS detection. The ACQUITY® QDa combines straightforward mass spectral data with optical data. The compact footprint of the ACQUITY® QDa allows for convenient integration into laboratories for improved productivity. showing excellent separation of the PolyT standards mixtures with LC-MS grade TEA, HFIP and H_2O.*

Oligonucleotide QC monitoring with ACQUITY QDA detection

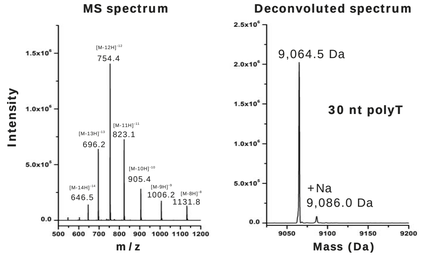


***Table 1.** Deconvolution Mass Accuracy (average mass in Da.)*

N=3	15 nt	20 nt	25nt	30 nt	35 nt
Expected	4500.9	6021.9	7542.9	9063.8	10584.8
Observed average	4500.9	6022.5	7543.5	9064.5	10585.5
Δ mass	0.0	0.6	0.6	0.7	0.7

***Figure 4.** Combined raw spectrum and deconvoluted MaxEnt1 spectrum demonstrate high spectrum quality (e.g. low sodiated ions), charge state coverage and good mass accuracy. The mass accuracy of the deconvoluted spectra for the PolyT standards are shown in Table 1.*

High Throughput Batch Processing

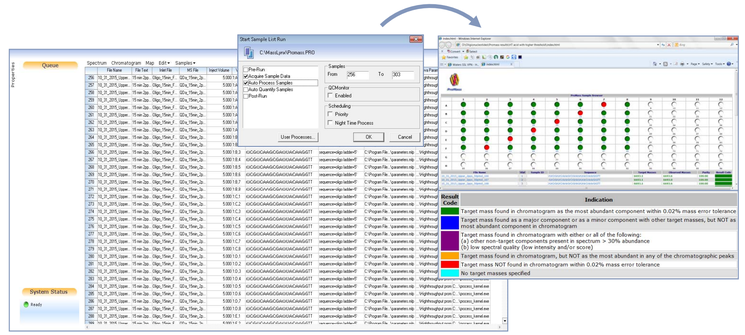
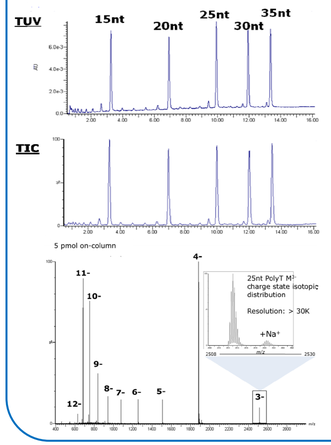


***Figure 5.** High Throughput Screening with ProMass HR. The addition of complementary mass information in a single workflow afforded by the ACQUITY® QDa provides analysts an efficient means in the identification and assessment of purity in synthetic therapeutic oligonucleotide screening assays for improved productivity. When coupled to programs such as Promass HR by Novatia, mass information from the ACQUITY® QDa (as shown) and Xevo G2-XS (data not shown) can be batch processed in an automated high-throughput manner for increased productivity and confidence in routine identification and purity assessments of synthetic oligonucleotides.* The interactively viewed color-coded results are user-friendly and offer straightforward data interpretation.

Oligonucleotide QC monitoring and characterization with Xevo G2-XS detection

Excellent LC-MS Data Quality



OST Standard	Expected Mass	Observed Mass	Mass Accuracy (ppm)
15nt	4498.7348	4498.7300	-1.07
20nt	6018.9650	6018.9730	1.33
25nt	7539.1952	7539.1990	0.50
30nt	9059.4254	9059.4210	-0.49
35nt	10579.656	10579.6260	-2.79
		Avg.	1.24

***Figure 6**. In-line orthogonal TUV and Xevo G2-XS HR MS detection (TIC) showing excellent separation of the PolyT standards mixtures. An example spectrum on the left shows the multiple charged species envelop of the 25nt polyT with minimum sodium adducts (usually less than 5%) in the blow-up region of the M^{3-} peak. The above table displays that the average exact mass mass accuracy for the 5 PolyT standard mixture was about 1.25 ppm (usually less than 5 ppm). The limit of detection (LOD) for the system was detected as 20 fmol on column (data not shown).*

High Throughput Impurity Profiling

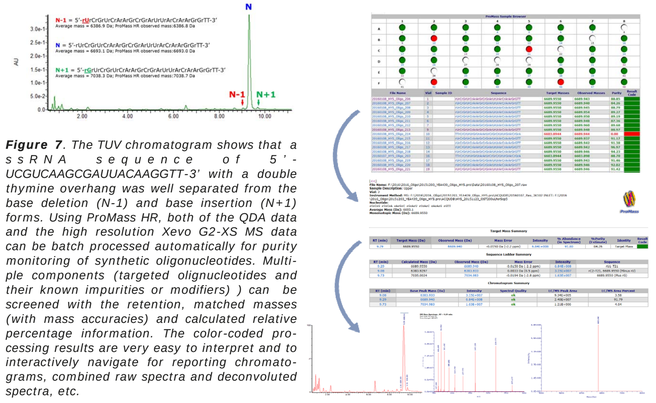


***Figure 7**. The TUV chromatogram shows that a ssRNA sequence of 5'-UCGUCAAGCGAUUACAAGGTT-3' with a double thymine overhang was well separated from the base deletion (N-1) and base insertion (N+1) forms. Using ProMass HR, both of the QDA data and the high resolution Xevo G2-XS MS data can be batch processed automatically for purity monitoring of synthetic oligonucleotides. Multiple components (targeted olignucleotides and their known impurities or modifiers)) can be screened with the retention, matched masses (with mass accuracies) and calculated relative percentage information. The color-coded processing results are very easy to interpret and to interactively navigate for reporting chromatograms, combined raw spectra and deconvoluted spectra, etc.*

LC-MS/MS Characterization

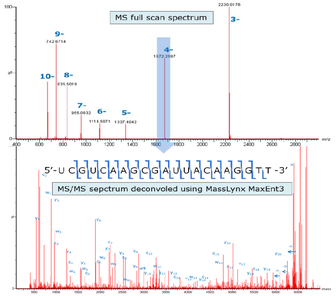


***Figure 8**. LC-MS/MS full ladder sequence confirmation for the ssRNA sequence of 5'-UCGUCAAGCGAUUACAAGGTT-3' was achieved. The experiment was conducted using the M^{4-} Peak as precursor ion. The fragmentation peak assignments were done by matching against the theoretical masses in an excel sheet after MaxEnt 3 deconvolutiond (5 ppm mass accuracy).*

CONCLUSION

- **LC-MS grade TEA, HFIP and H2O were needed to ensure the best separation and MS detection with the highest resolution and lowest salt adduct peak intensities (less than 5% in the MS spectra).**
- **QDA is an excellent MS detector for synthetic oligonucleotides Mass Confirmation.**
- **Using ProMass HR, high throughput LC-MS oligonucleotides impurity profiling with QDa and Xevo G2-XS MS systems are demonstrated. The excellent separation retention reproducibility and MS data quality confirm the robustness of the assays.**
- **LC-MS/MS experiments with Xevo G2-XS can be used to accomplish characterization (full ladder MS/MS sequencing) of the complex oligonucleotide molecules.**

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