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A Software Workflow Using Wide Mass Range Single Quadrupole Mass Spectrometry Data Stream Applied to Oligonucleotides Confirmation

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Overview

- LabSolutions Insight Biologics software has been developed to accelerate data processing for charactering chemically synthesized oligonucleotides for nominal mass and high mass resolution mass spectrometers.
- To extend the scope of detection capabilities in oligonucleotide characterization in release and stability testing a production prototype single quadrupole LC-MS instrument has been developed with an extended mass range up to m/z 3000.
- The extended mass range single quadrupole LC-MS was been applied to the analysis of a 30-mer anti-sense oligo.

1. Introduction

The development of novel oligonucleotide therapeutics increasingly relies on solid-phase phosphoramidite chemistry, a method characterized by iterative cycles of coupling, capping, oxidation, and deprotection to elongate nucleotide sequences. This synthetic complexity necessitates comprehensive analytical characterization to confirm full-length product integrity and to elucidate impurity profiles. As therapeutic modalities evolve, incorporating diverse backbone chemistries, modifications, and delivery strategies, analytical technologies need to adapt to changing needs. As a result, a production prototype single quadrupole LC-MS has been developed with an extended mass range and applied to oligonucleotide characterization using LabSolutions Insight Biologics software for data processing.

2. Instruments and Methods

2.1 LC-MS system

Liquid Chromatography: Shimadzu Nexera XS ™ inert LC-40 was selected, and its bio-inert coating is expected to reduce adsorption.

Mass Spectrometer: A production prototype single quadrupole mass spectrometer based on Shimadzu LCMS™-2050 was modified to scan up to m/z 3000 without changing the instrument footprint or performance. The principal modification reduced the RF frequency from 1.2 to 1.0 MHz on the existing RF power supply unit resulting in an extended mass range and unit mass resolution across the entire mass range without affecting signal intensity.

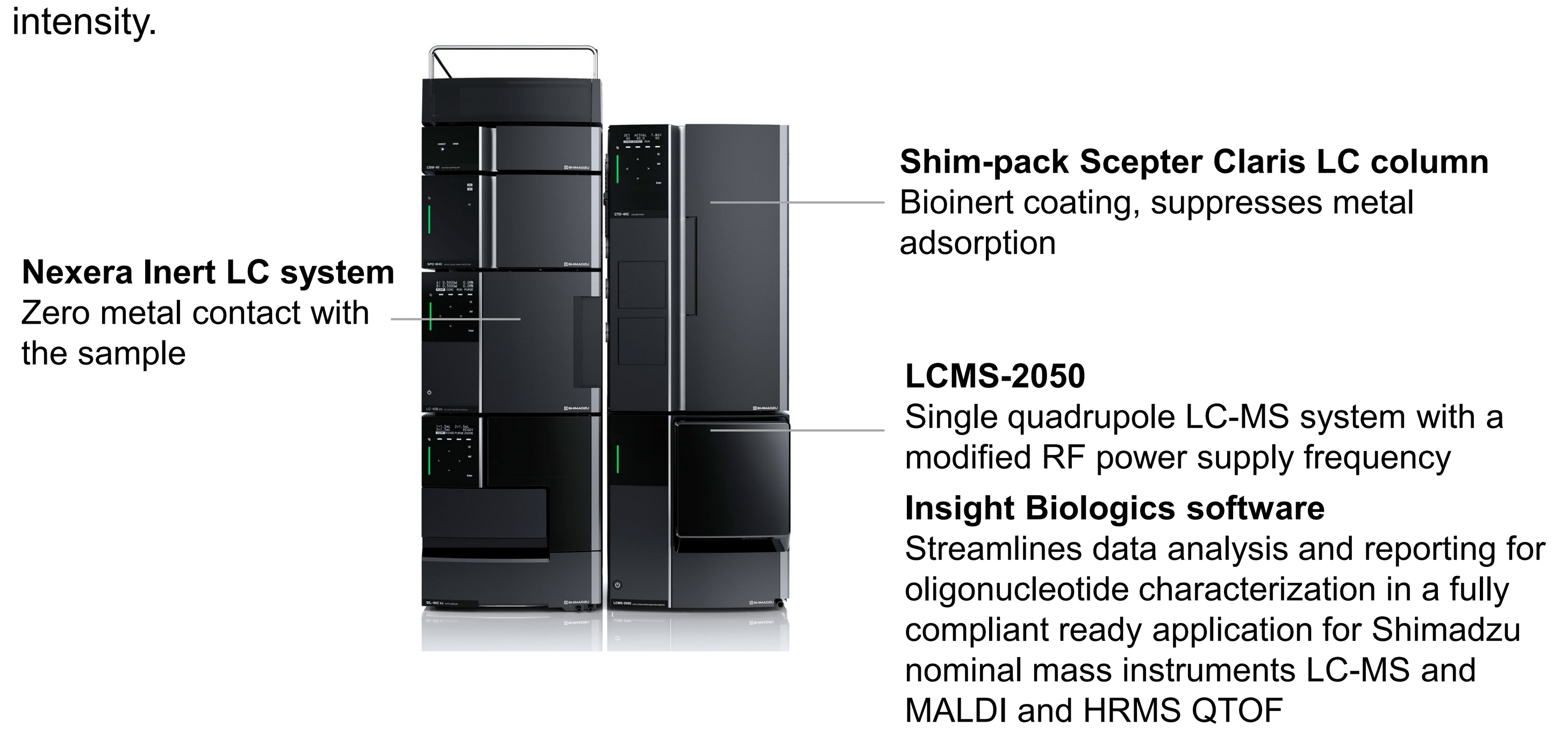

Figure 1. LC-MS configuration for characterising chemically synthesized oligonucleotides

2.2 LC-MS method

Aa phosphorothioate oligonucleotide (20-mer anti-sense oligo; C230 H324 N67 O122 P19 S19; average mass 7177.11; Ge-smCe-smCe-smUe-smCe-sAd-sGd-sTd-smCd-sTd-sGd-smCd-sTd-sTd-smCd-sGe-smCe-sAe-smCe-smCeA polyT30 oligonucleotide (30-mer anti-sense oligo; C300 H391 N60 O208 P29; averge mass 9063.88; Td-pTd-pTd-pTd-pTd-pTd-pTd-pTd-pTd-pTd-pTd-pTd-pTd-pTd-pTd-pTd-pTd-pTd-pTd-pTd-pTd-pTd-pTd-pTd-pTd-pTd-pTd-pTd-pTd-pTd) and).

LC Separation

- A Shim-pack Scepter™ Claris column using ion pair additives was used. Mobile phase A consists of 10% Acetonitrile (ACN), 5mM tributylammonium acetate (TBuAA), and 1μM ethylenediaminetetraacetic acid (EDTA) free acid in water. Mobile phase B consists of 80% ACN, 5mM TBuAA, and 1μM EDTA. Optimized 6 minutes gradient condition focusing on FLP detection in a short run time.

LC-MS detection and data processing workflow

- Data acquired in profile mode. MS mass scan *m/z* 600-3000; event time 1 sec; negative ion mode; interface voltage -3.0 kV; desolvation temperature 450℃
- Acquired data was processed using LabSolutions Insight Biologics using the defined full-length product (FLP) sequence of the 30-mer anti-sense oligo as the target input.

3. Results

3.1 20-mer mipomersen FLP confirmation

Mipomersen as a model ASO was separated using ion pair chromatography and charge state deconvolution using LabSolutions Insight Biologics resulted in a calculated molecular weight 7176 Da.

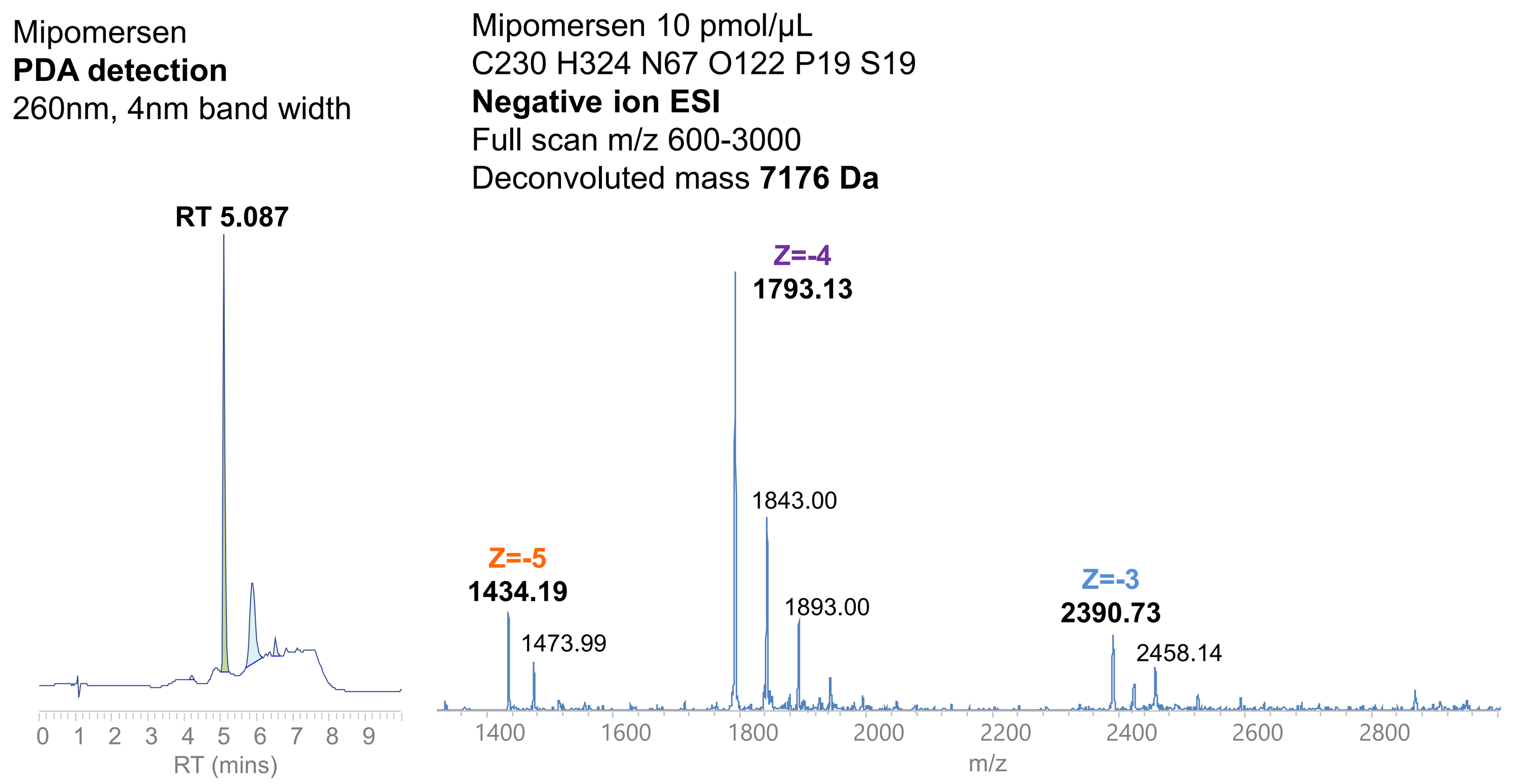

Figure 2. Analysis of mipomersen generating a negative ion spectrum with charge states above m/z 2000 detected using a production prototype single quadrupole mass spectrometer.

3.2 Charge state selection

Oligonucleotide-based therapeutics, including antisense oligonucleotides (ASOs; MW ~7,000 Da) and small interfering RNAs (siRNAs; MW ~15,000 Da), are typically amenable to analysis by single quadrupole mass spectrometry for molecular weight confirmation and impurity profiling. When ionized via electrospray ionization (ESI), these molecules exhibit multiple charge states, generating a complex pattern of peaks distributed across a broad m/z range. Due to the trade-off between scan range and data quality, analytical workflows often target one or two charge states that yield optimal signal intensity, based on prior molecular knowledge. This approach, however, often requires iterative optimization and reanalysis.

Using 30-mer anti-sense oligo sample, we evaluated the performance of a limited m/z scan window (600 Da width) relative to a full-range acquisition extending to m/z 3000, while maintaining a constant data acquisition rate (1 point/sec). Both approaches produced comparable results for FLP identification using the Insight Biologics platform. Nevertheless, the full-range method provided more comprehensive and unbiased data by obviating the need for predefined charge state input.

To further assess the robustness of full-range acquisition, varying sample injection amounts were analyzed, generating signal intensities ranging from detector saturation to the lower detection limit. Saturated signals, which adversely affected peak width and mass accuracy, were mitigated by the presence of alternative charge states captured within the full-range data.

These findings underscore the advantages of employing data acquisition strategies and analysis software capable of leveraging the complete m/z range in oligonucleotide characterization. Such an approach minimizes dependence on a priori knowledge of ionization behavior or injection optimization and enhances the accuracy of compound identification. Ongoing investigations aim to extend these insights to larger oligonucleotides, such as single guide RNAs (sgRNAs), with particular focus on their quantitative analytical performance.

Charge state response evaluation

- A 30-mer ASO calibration curve was constructed including concentrations at 0.5, 1, 2, 5, 10 and 20 pmol/μL and Insight Biologics was used for charge deconvolution.
- 30-mer ASO nominal mass negative ion MS spectrum results in 3 principal charge states ranging from -4 to -6.

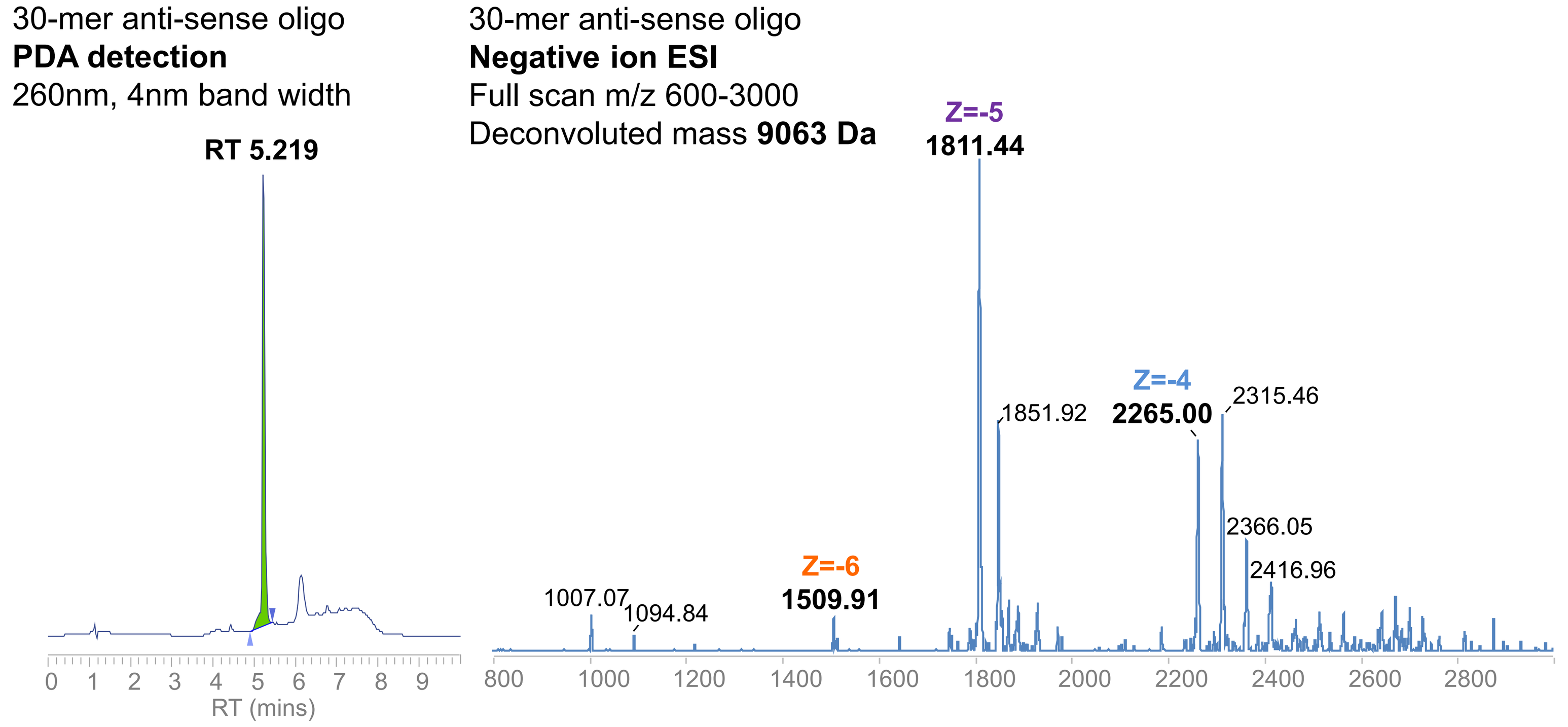

Figure 3. Analysis of PolyT30 with charge states z-4 to z-6 using a production prototype single quadrupole mass spectrometer.

Charge state linearity evaluation

- The linearity of response is affected by ion suppression and becomes more marked with higher concentrations and approximates to a quadratic curve.
- Summating the principal charge states typically enhances the lower limit of quantitation, negates the impact of variability of low intensity charge states resulting in greater precision.

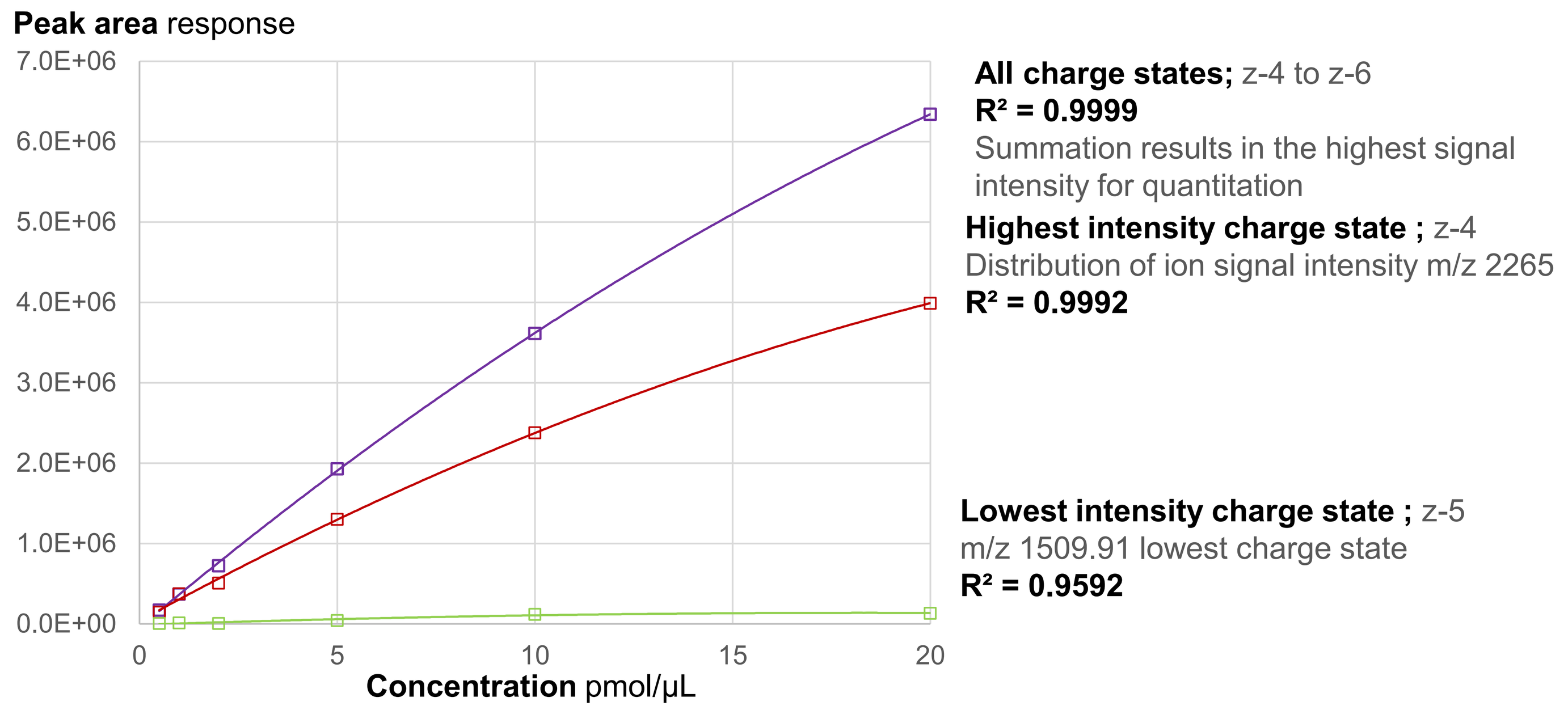

Figure 4. Quantitative analysis of PolyT30 highlighting the advantage of ion signal summation of all charge states in quantitation.

Affect of mobile phase selection on charge state distribution

- Marked differences in the distribution and intensity of charge states dependent on mobile phase. TBuAA and EDTA reagents resulted in higher intensity signal intensities and charge states compared to HFIP and TEA reagents.

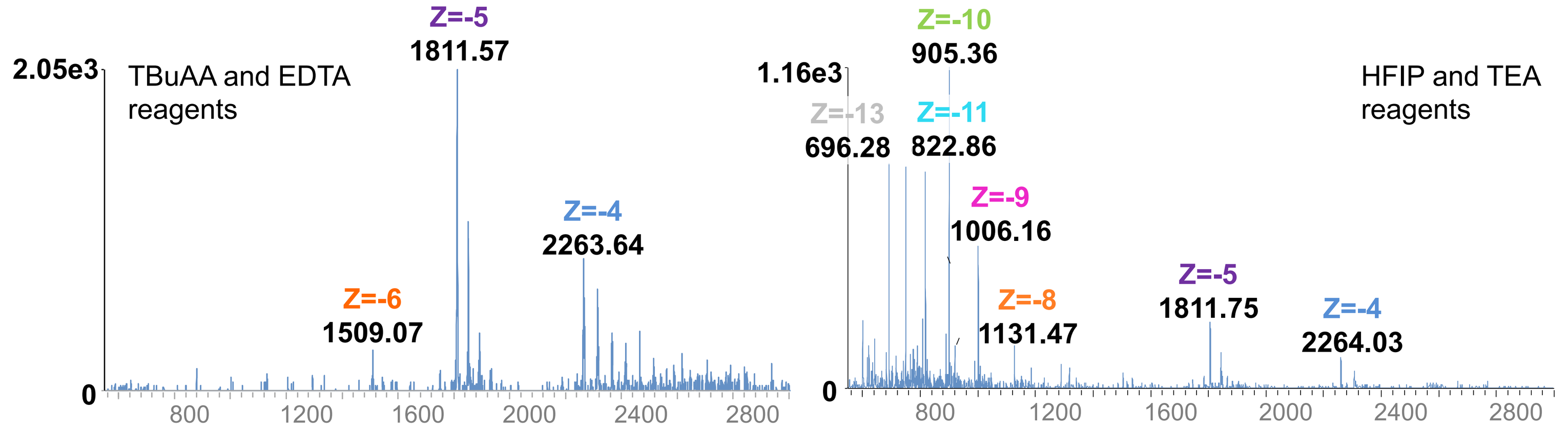

Figure 5. Distribution of charge states with different mobile phases; HFIP and TEA results in lower charge state signal intensities but with a greater number of charges compared to TBuAA and EDTA.

4. Conclusions

- Single quadrupole mass spectrometer has the capability for impurity analysis and its assay confirmation of FLP. It is also easier to handle than HRMS.
- Wide range mass spectrometer may affect the accuracy of abundance of ions in the high mass region depending on the sample and mobile phase conditions.
- It is suggested that the calculation of abundance considering all charge state peaks of its component improves the quantification.