

Accurate mass spectral deconvolution of multiply-charged oligonucleotides using unit resolution single quadrupole LC/MS

Patrick M Batoon¹; Lee Bertram²; Yongdong Wang³

¹Agilent Technologies Inc., Santa Clara, CA;

²Agilent Technologies, Santa Clara, CA;

³Cerno Biosciences, Las Vegas, NV

ASMS 2024
We 480



Introduction

The success of nucleic acid based therapeutics such as CRISPR-Cas, mRNA vaccines, and oligonucleotides has ignited rapidly growing interest for bio-pharmaceutical development. Supporting this growth is the need for detection, confirmation, and analysis of quality attributes via LC/MS. A typical workflow involves the generation of ions with multiple charges represented by multiple mass spectral peaks. By knowing m/z and z (charge), it is possible to compute an *averaged Molecular Weight* of the oligonucleotide molecule.

Improved specificity can be obtained by using a high-resolution accurate mass (HRAM) LC/MS to obtain accurate mass molecular weight assignment and provide unambiguous assignment of nucleotide sequence. In certain cases, accessibility to this type of technology is challenging often due to their prohibitive cost or high technical barrier to entry.

LC/MSD quadrupole mass spectrometry is a cost-effective and easy-to-use technology but is known to produce unit resolution, "nominal mass" assignments. However, by augmenting the system with in-spectrum calibration software such as Cerno MassWorks, it is possible to produce accurate mass assignments.

Here we present a methodology that provides a spectrally deconvoluted accurate mass assignment for oligonucleotide sequences using an easily accessible unit resolution LC/MS system.

Experimental

An oligonucleotide standard containing a 40-mer synthetic oligodeoxythymidine (p/n 5190-9029) as dissolved in solution. A generic HPLC gradient run on an Infinity II 1290 HPLC was used to produce a chromatographic peak while the 6135C LC/MSD XT was used to detect eluting analytes using Full Scan from m/z 700-3000 in profile mode. For proper analysis using Cerno MassWorks, MS abundance thresholding was set to zero (Threshold = 0).

Data for external calibration in Cerno MassWorks was carried out via infusion of ESI-L calibrant through diverter valve programming and as separate lines in the OpenLab CDS acquisition sequence.

Length	Sequence	Molecular Weight (Da)
40mer	TTTTTTTTTT TTTTTTTTTT TTTTTTTTTT TTTTTTTTTT	12106.6

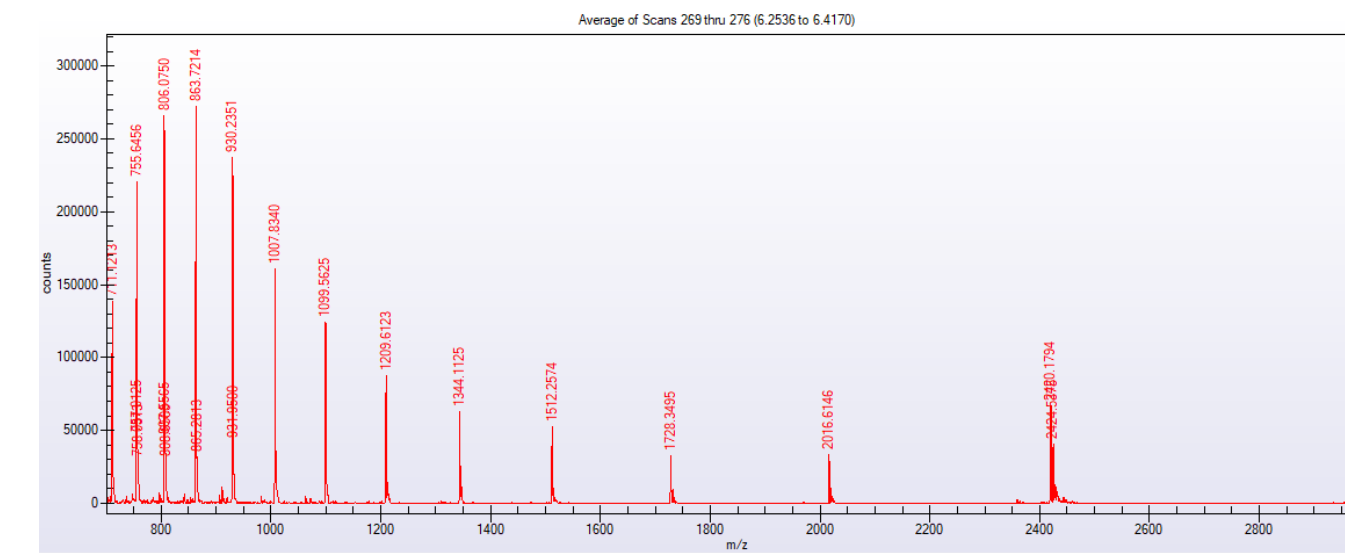
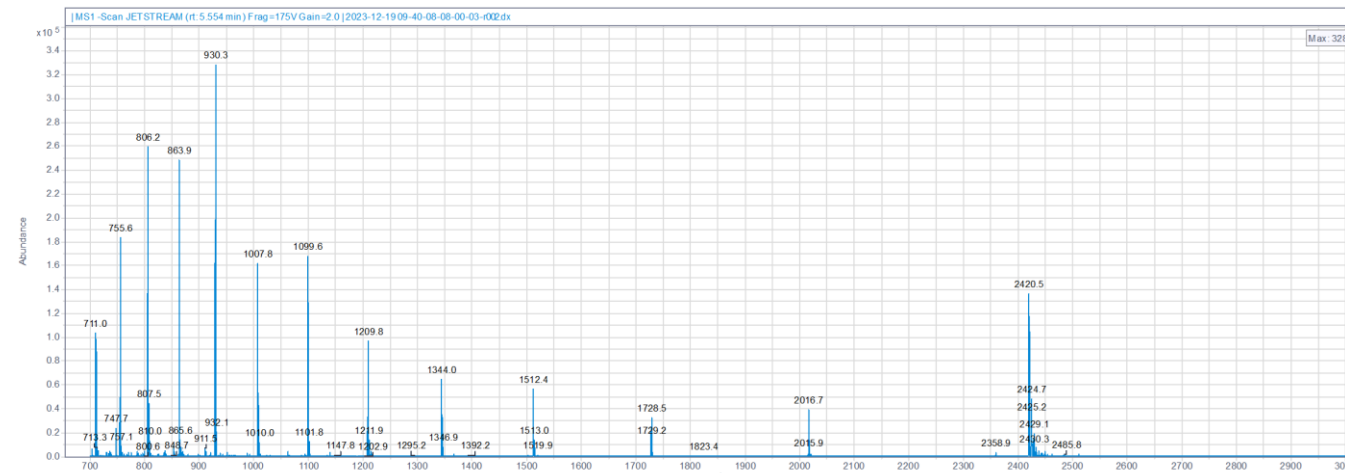
Data Analysis and Software Input

Data files were imported into Cerno MassWorks software for analysis. First, external calibration of the data was carried out using known mass assignments for ESI-L (the instrument's standard tuning and calibration mixture).

Then, Spectrally Accurate Modeling of Multiply charged Ions (SAMMI) algorithm accepted externally calibrated accurate mass assignments for spectral deconvolution of multiple charged species.

Results and Discussion

Mass Spectra for Poly-deoxythymidine 40-mer



By-hand Mass Spectral Deconvolution // Molecular Weight Calculations

Molecular Weight (MW) calculations were first carried out by reviewing a tabulated form of the mass spectrum and assigning charge (z) based on the equation shown. m_1 and m_2 are the m/z values for two adjacent mass spectral peaks, while X is the mass of the adduct or neutral loss, depending on charge polarity. When comparing the result of the raw Nominal Mass spectrum and Accurate Mass spectrum for the 40mer oligonucleotide, the uncertainty of the averaged MW is dramatically reduced and is very close to the expected MW.

$$z = \frac{m_1 - 1}{m_2 - m_1} \quad MW = (z * m_1) \pm (X * z)$$

Nominal Mass Calculated MW

m/z	z	MW
711.0	17.0	12104.0
755.6	16.0	12105.6
806.2	15.0	12108.0
863.9	14.0	12108.6
930.3	13.0	12106.9
1007.8	12.0	12105.6
1099.6	11.0	12106.6
1209.8	10.0	12108.0
1344.0	9.0	12105.0
1512.4	8.0	12107.2
1725.5	7.0	12085.5
2016.7	6.0	12106.2
2420.5	5.0	12107.5

MW (Nominal) =

$$\bar{x} = 12104.97$$

$$\sigma = \pm 5.99$$

$$RSD = \pm 4.9\%$$

Accurate Mass Calculated MW

m/z	z	MW
711.1213	17.0	12106.186
755.6456	16.0	12106.446
806.0750	15.0	12106.234
863.7214	14.0	12106.201
930.2351	13.0	12106.151
1007.8340	12.0	12106.095
1099.5625	11.0	12106.268
1209.6123	10.0	12106.196
1344.1125	9.0	12106.078
1512.2574	8.0	12106.117
1728.3495	7.0	12105.497
2016.7000	6.0	12106.244
2420.1794	5.0	12105.933

MW (Accurate Mass) =

$$\bar{x} = 12106.127$$

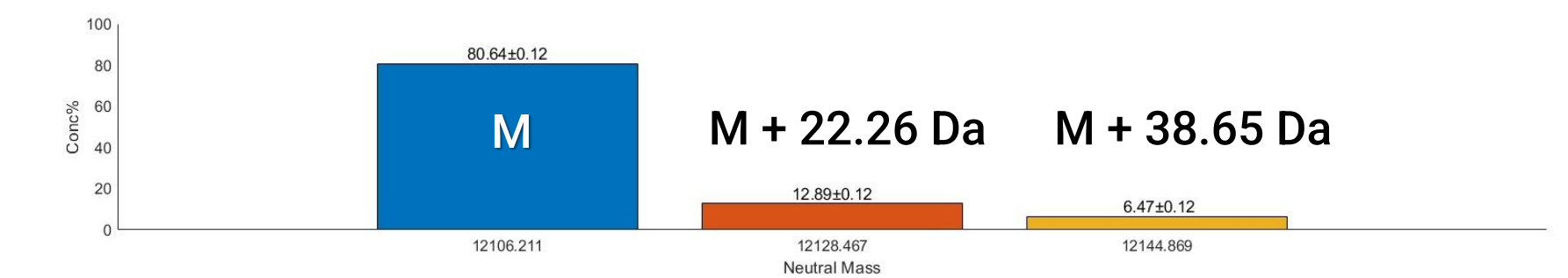
$$\sigma = 0.22$$

$$RSD = \pm 0.18\%$$

Results and Discussion

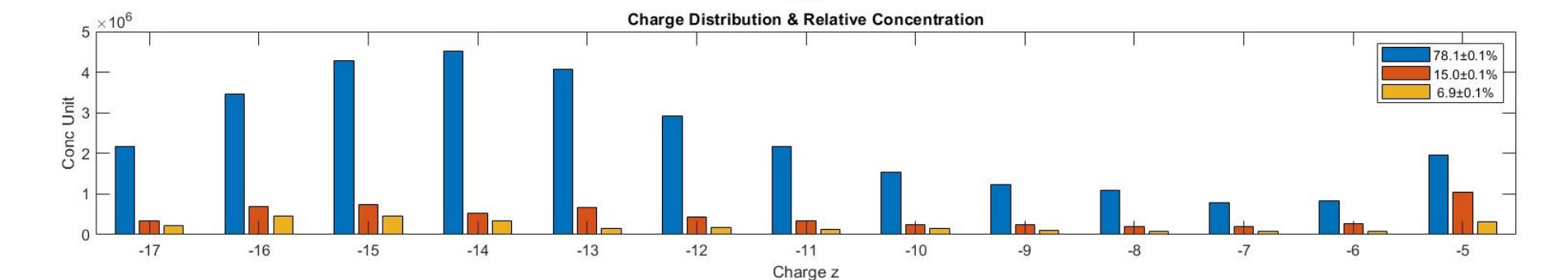
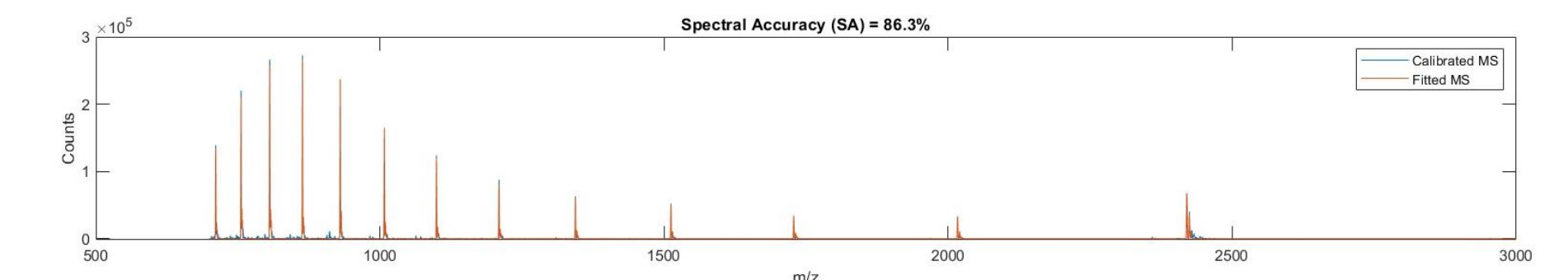
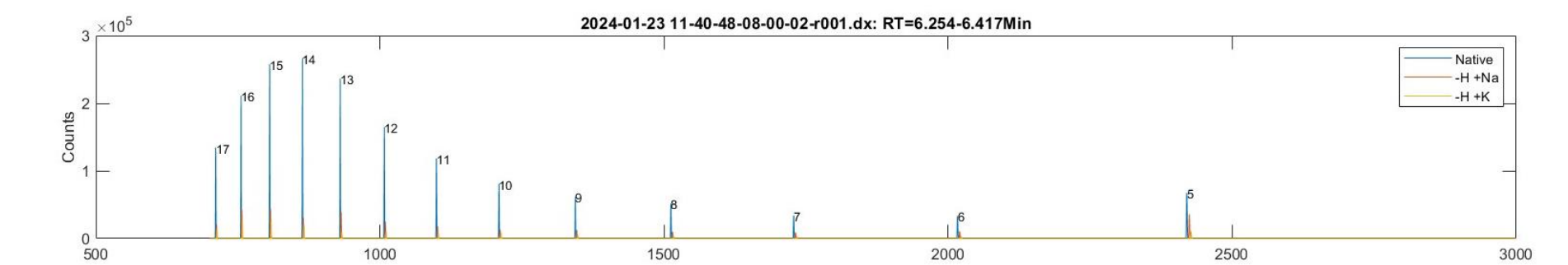
"Unknown Analysis" Molecular Weight Determination

Unknown Analysis in Cerno MassWorks carries out similar calculations within the data file itself, also examining smaller shoulder peaks. 3 unique species and their relative concentrations were detected. The principal species (80.64%) is the neutral form of the oligonucleotide. The other two species are shifted by +22.26 Da and +38.65 Da indicating Na+ and K+ adduct forms.



Spectrally Accurate Modeling of Multiply charged Ions (SAMMI)

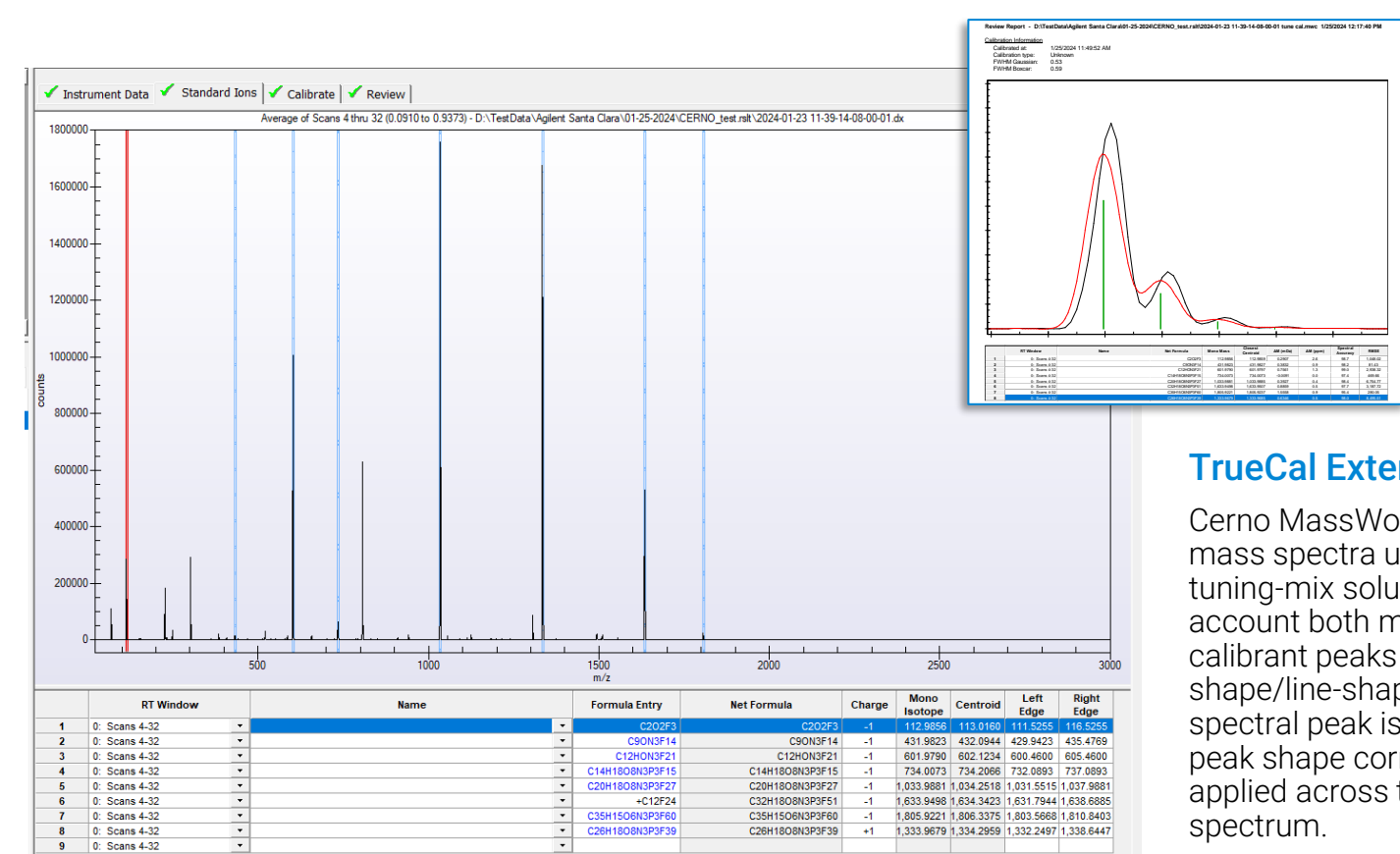
Since the oligonucleotide sequence is known, further confirmation can be carried out to consider mathematically defined peak shape/line shape and mass shift. This allows the assignment of other species such as adducts or modifications. In the example below, SAMMI was able to assign and account for the mass spectral peaks with 86.3% spectral accuracy (86.3% of all peaks can be explained by the native, sodiated, and potassiumated ion forms) and indeed confirms the presence of the Na+ and K+ from unknown analysis.



Conclusions

- The mass spectrum of Poly-deoxythymidine 40-mer was acquired on a Unit Resolution, Nominal Mass LC/MSD single quadrupole system. Cerno MassWorks can perform further calibration and transformation to produce Accurate Mass spectra.
- By-hand Molecular Weight calculations compare the results of using Nominal Mass vs Accurate Mass spectral peak assignments to known values. Accurate Mass assignments dramatically improve ID confidence.
- SAMMI unknown and known analysis confirms the presence of Na+ and K+ species with high spectral accuracy

Agilent 6135 LC/MSD XT quadrupole LC/MS coupled to an Infinity II 1290 HPLC



TrueCal External Calibration

Cerno MassWorks externally calibrates mass spectra using acquisition of tuning-mix solution. This takes into account both mass axis assignment of calibrant peaks and "theoretical" peak shape/line-shape. A centroided mass spectral peak is produced using the peak shape corrected assignments, applied across the overall mass spectrum.