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Accurate Mass Spectral Deconvolution of Multiply-charged Oligonucleotides using Unit Resolution Single Quadrupole LC/MS

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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.

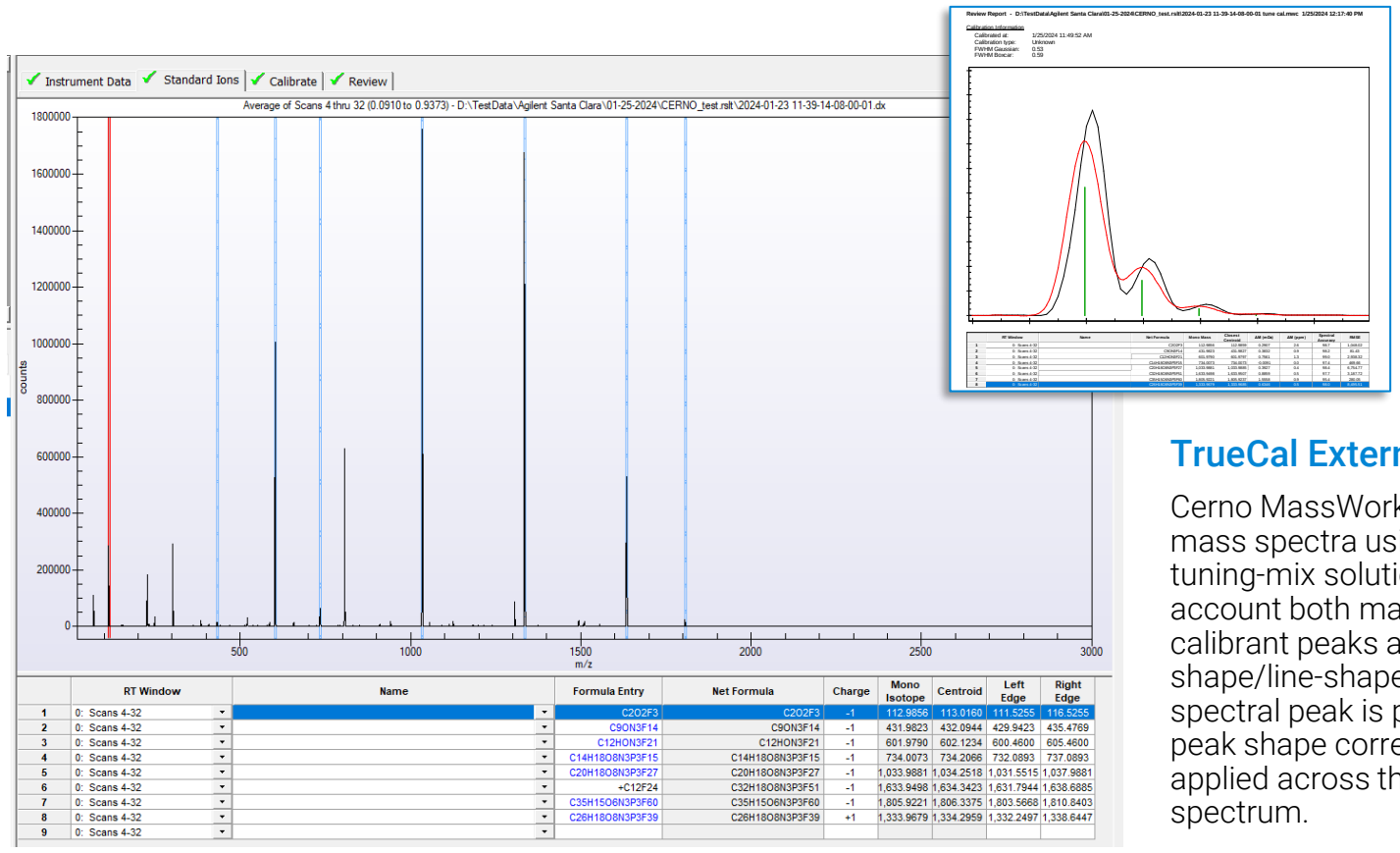
Length	Sequence	Molecular Weight (Da)
40mer	TTTTTTTTTT TTTTTTTTTT TTTTTTTTTT TTTTTTTTTT	12106.6

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.

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

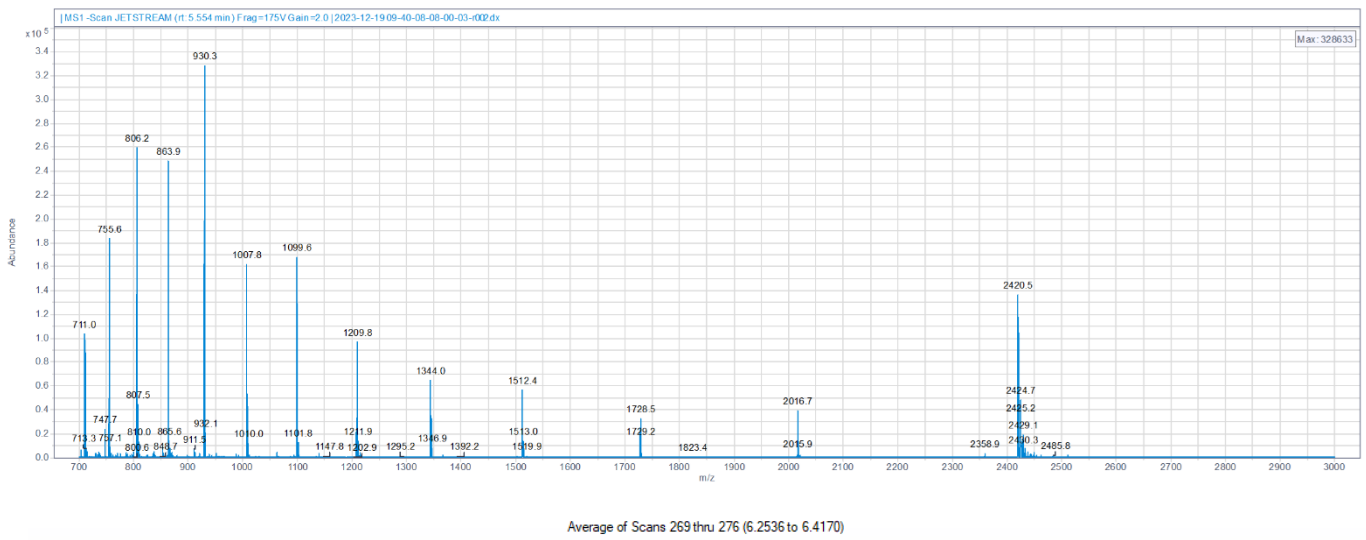


	RT Window	Name	Formula Entry	Net Formula	Charge	Mono Isotope	Centroid	Left Edge	Right Edge
1	0: Scans 4-32		C2O2F3	C2O2F3	-1	112.9856	113.0160	111.5255	116.5255
2	0: Scans 4-32		C9ON3F14	C9ON3F14	-1	431.9823	432.0944	429.9423	435.4769
3	0: Scans 4-32		C12HON3F21	C12HON3F21	-1	601.9790	602.1234	600.4600	605.4600
4	0: Scans 4-32		C14H18O8N3P3F15	C14H18O8N3P3F15	-1	734.0073	734.2066	732.0893	737.0893
5	0: Scans 4-32		C20H18O8N3P3F27	C20H18O8N3P3F27	-1	1,033.9881	1,034.2518	1,031.5515	1,037.9881
6	0: Scans 4-32		+C12F24	C32H18O8N3P3F51	-1	1,633.9498	1,634.3423	1,631.7944	1,638.6885
7	0: Scans 4-32		C35H15O6N3P3F60	C35H15O6N3P3F60	-1	1,805.9221	1,806.3375	1,803.5668	1,810.8403
8	0: Scans 4-32		C26H18O8N3P3F39	C26H18O8N3P3F39	+1	1,333.9679	1,334.2959	1,332.2497	1,338.6447
9	0: Scans 4-32								

TrueCal External Calibration

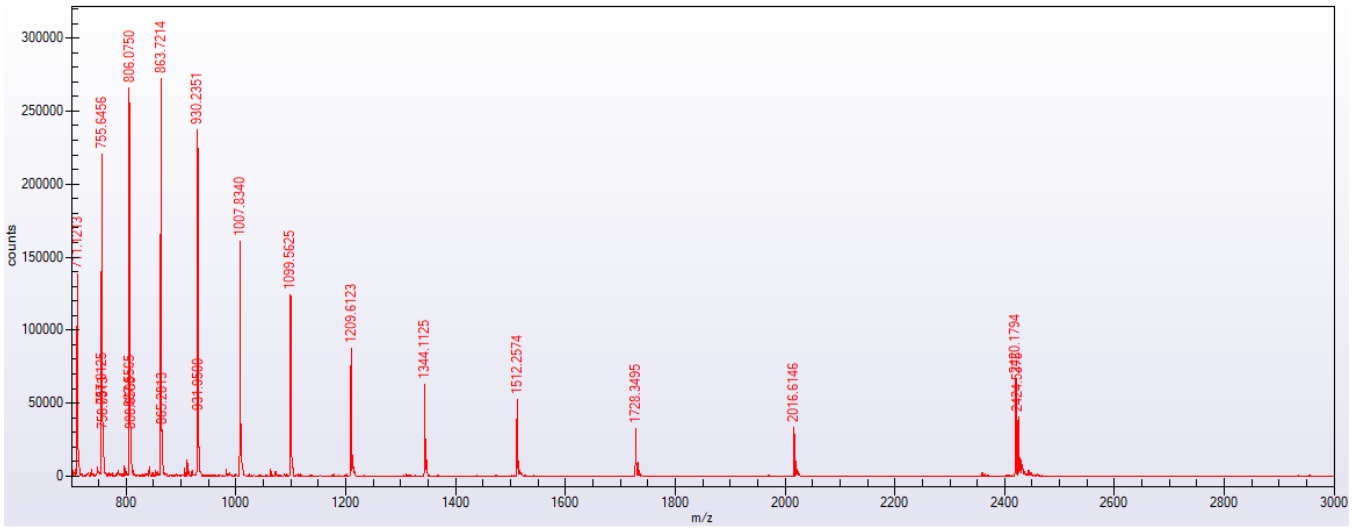
Cerno MassWorks externally calibrates mass spectra using acquisition of tuning-mix solution. This takes into account both mass axis assignment of calibrant peaks and “theoretical” peak shape/line-shape. A centroided mass spectral peak is produced using the peak shape corrected assignments, applied across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

Mass Spectra for Poly-deoxythymidine 40-mer



Initial Mass Spectrum from OpenLab CDS

Mass spectrum for raw, unit resolution, nominal mass. Each mass spectral peak reports an *m/z* to the tenths (0.1) decimal place.



Cerno MassWorks TrueCal calibrated Accurate Mass Spectrum

OpenLab CDS datafiles are imported into Cerno MassWorks for external mass axis and centroiding calibration using the references masses and peak shapes.

After calibration, each mass spectral peak reports an m/z to the ten-thousandth's (0.0001) decimal place

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. $\boldsymbol{m_1}$ and $\boldsymbol{m_2}$ 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.

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

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

σ = ±5.99

RSD = ±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

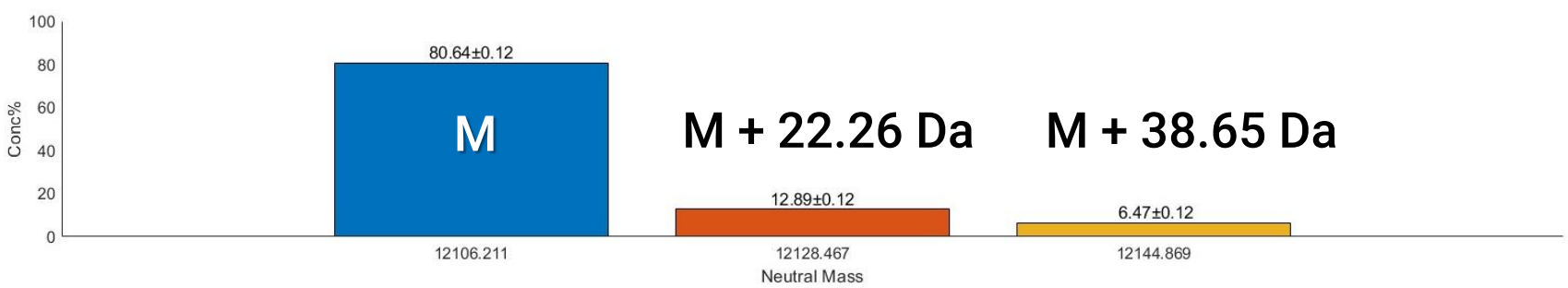
σ = 0.22

RSD = ±0.18%

Results and Discussion

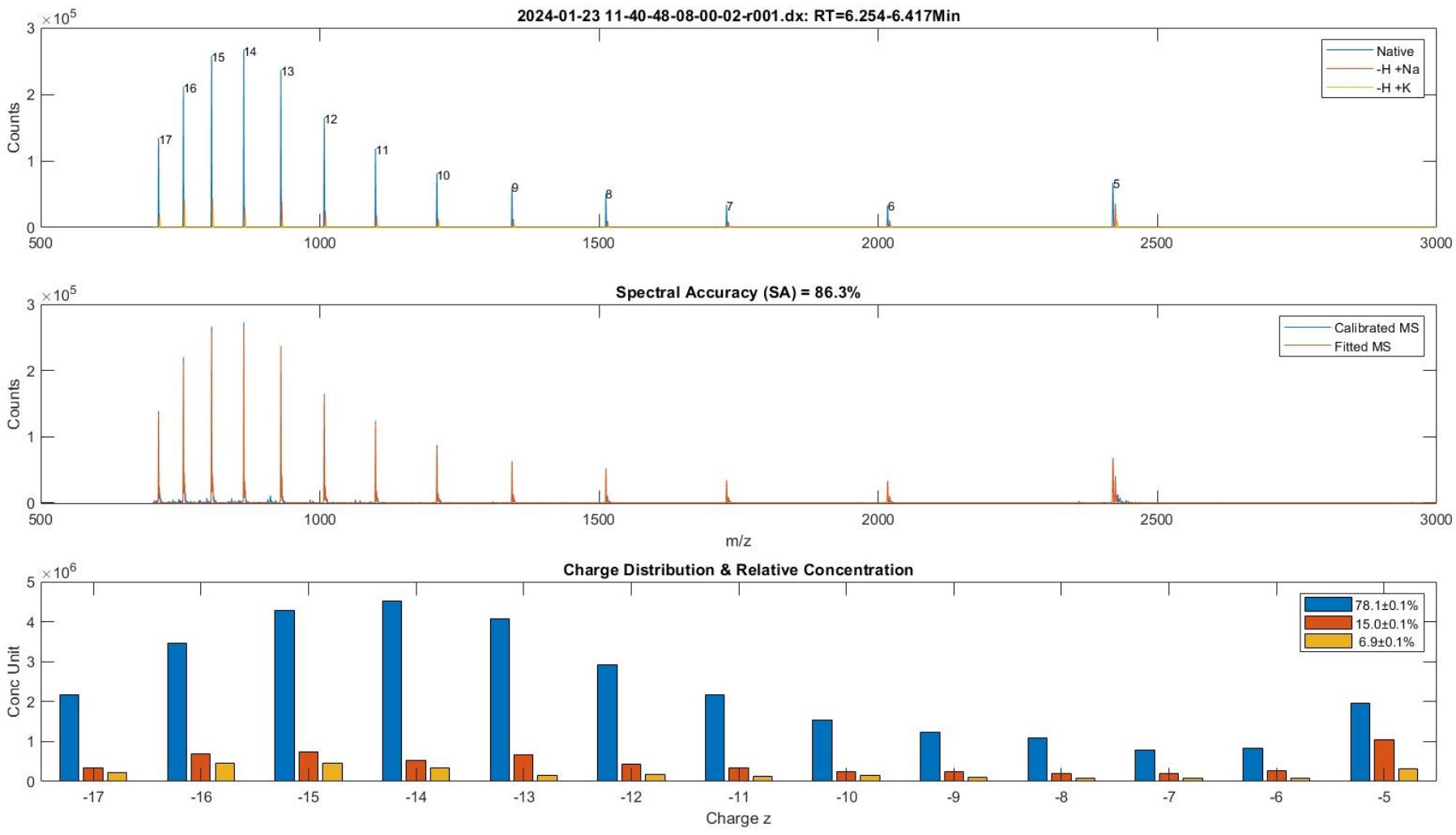
“Unknown Analysis” Molecular Weight Determination

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 potassiated 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

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