Metabolite ID and relative quantification of oligonucleotides in plasma

Identify, quantify, and confirm the structure of oligonucleotide metabolites using the SCIEX ZenoTOF 7600 system and Molecule Profiler software

Remco van Soest, Kerstin Pohl, Todd Stawicki and Elliott Jones
SCIEX, USA

This technical note describes the identification, relative quantification and structural confirmation of the chain-shortened metabolites of a phosphorothioated oligonucleotide. Relative quantification was achieved at levels as low as 0.1% (*w*/*w*), while consecutive sequence coverage was realized at levels down to 1%.

Oligonucleotide therapeutics and gene therapies are rapidly gaining attention as their potency improves and delivery challenges are addressed. Modalities such as antisense oligonucleotides (ASOs) are becoming more important due to their high specificity and ability to reach formerly untreatable targets. To ensure safe drugs, methods for the identification and characterization of the full length product (FLP) and its metabolites are critical. High resolution mass spectrometry (HRMS) can be used for the identification of potential metabolites, by comparing the measured accurate masses and isotope patterns with those calculated. However, there is a lack of powerful yet intuitive processing software, and manual interpretation is cumbersome and time consuming. Furthermore, structural confirmation leveraging MS/MS adds an additional level of complexity.

Using the Molecule Profiler software to overcome these challenges, this technical note shows the identification and relative quantification of the 5' and 3' (*n*-1, 2 and 3) metabolites of an ASO spiked into a rat plasma extract, in the presence of the FLP, at levels between 0.1% and 10% (*w*/*w*) of the FLP. The software can perform relative quantification based on TOF-MS, and assign fragment ions of the potential metabolites to confirm their structures, facilitating metabolism studies of drugs in development.

Key features of Molecule Profiler software for oligonucleotide metabolite analysis

- Excellent quality and high mass accuracies for MS and MS/MS data allow for confident assignment of oligonucleotide FLP and metabolites in Molecule Profiler software
- Straightforward relative quantification based on TOF-MS can be achieved by grouping of charge states or, alternatively, UV data can be leveraged for quantification
- Significant improvement of identification of low abundant metabolites by improved S/N of MS/MS spectra using the Zeno trap of the ZenoTOF 7600 system

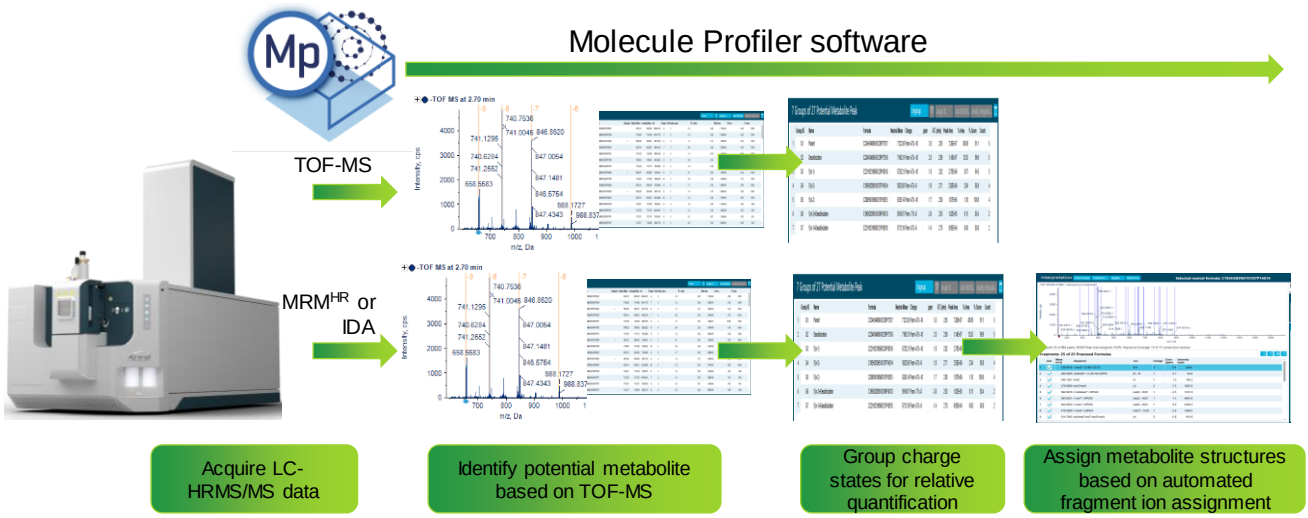

Figure 1. Workflow representation for relative quantification and structural confirmation of metabolites using the Molecule Profiler software.

Methods

Samples and reagents: A 18-mer 2'-O-methoxyethyl phosphorothioated oligonucleotide with the same sequence as nusinersen, a drug developed for the treatment of spinal muscular atrophy, was obtained from IDT (desalted). In addition, 5'(*n*-1, 2, and 3) and 3'(*n*-1, 2, and 3) shortmers were ordered (desalted) from IDT to mimic metabolites of the FLP. The ion-pairing reagents 1,1,3,3,3-hexafluoroisopropanol (HFIP, ≥ 99.8%) and diisopropylethylamine (DIEA, ≥ 99.5%,), and ethylenediaminetetraacetic acid (EDTA), were purchased from Sigma Aldrich.

Sample preparation: 1 mL plasma was extracted using Clarity OTX solid phase extraction (SPE) cartridges (Phenomenex) following the manufacturer's protocol for extracting oligo therapeutics from biological samples. After drying with nitrogen gas at 40°C, the plasma extract was reconstituted in 1 mL mobile phase A containing 100 µM EDTA.

Samples of 10 µg/mL FLP in the extracted plasma containing 100 µM EDTA were spiked with the six related shortmers at 0.1, 0.3, 1, 3 and 10% (*w*/*w* relative to FLP) in order to mimic a sample from an *in-vitro* metabolism study. The FLP was used as a control sample.

Chromatography: A Shimadzu LC-20 series HPLC system was used with water as mobile phase A and 90:10 methanol/water (*v*/*v*) as mobile phase B, both with 15 mM N,N-diisopropylethylamine and 35 mM hexafluoroisopropanol. A gradient from 20-40% B in 5 min with a 1.5 min wash step at 90% B was used at a flow rate of 0.25 mL/min. The column was a Waters ACQUITY PREMIER Oligonucleotide C18 (2.1 × 50 mm, 1.7 µm, 130 Å) at 70ºC, and the injection volume was 10 µL.

Mass spectrometry: A SCIEX ZenoTOF 7600 system was used in negative polarity using an MRM^{HR} method (method details available on request). To determine the precursor masses, the data from a TOF-MS scan of the 10% spiked sample was processed using the Molecule Profiler software to extract the *m*/*z* values for the most abundant charge states for the FLP, the spiked-in metabolites, and the desulfurization products (back-exchange of one S to O) of each of these. Collision induced dissociation (CID) was used, and collision energies (CE) were selected that ensured the generation of fragment-rich MS/MS spectra. The parameters for the final MRM^{HR} method are summarized in Table 1. Data was acquired both with the Zeno trap on and off, to determine the effect of the Zeno trap functionality on MS/MS data quality The Zeno trap is located before the TOF pulser and accumulates ions during each TOF pulse, resulting in up to 90% duty cycle.

Table 1. MS parameters.

Parameter	MS	MS/MS
Scan mode	*TOF-MS*	*MRM^{HR}*
Polarity	*negative*	
Gas 1	*70 psi*	
Gas 2	*70 psi*	
Curtain gas	*30 psi*	
Source temperature	*350°C*	
Ion spray voltage	*-4000 V*	
Declustering potential	*-80 V*	
CAD gas	*7*	
Start mass	*600 m/z*	*150 m/z*
Stop mass	*2,000 m/z*	*3,000 m/z*
Q1 resolution	*NA*	*Low*
Accumulation time	*0.1 s*	*0.03 s*
Collision energy	*-10 V*	*available upon request*
CE spread	*0 V*	*3 V*
Zeno	*NA*	*ON/OFF*
ZOD threshold (CID)	*NA*	*40,000 cps*
Time bins to sum	*6*	*12*
QJet RF amplitude	*190 V*	*190 V*

Data processing: Data was processed using the SCIEX Molecule Profiler software. Considering the structure of the oligonucleotide, the number of bonds to break in the parent structure, and a comprehensive list of 83 possible standard transformations, the software identifies the different charge states of potential metabolites. This assignment is based on the accurate mass match—a 20 ppm tolerance was used for the work presented in this application note—and the match between the measured and theoretical isotope patterns. Additional transformations can be added, while also custom nucleotide residues can be used by defining the 5' and 3' linkers, sugar, base and phosphate groups (Figure 2). The list of found potential metabolites can be manually curated, and the different charge states of each potential metabolite are grouped together for relative quantification based on the TOF-MS data.

For confirmation of the structure of each potential metabolite, MS/MS spectra can be automatically annotated with *a*, *a*-B, w, *b*, *x*, *c*, *y* and *d* fragments (see Figure 2). Sequence coverage by consecutive fragments is automatically calculated by the software.

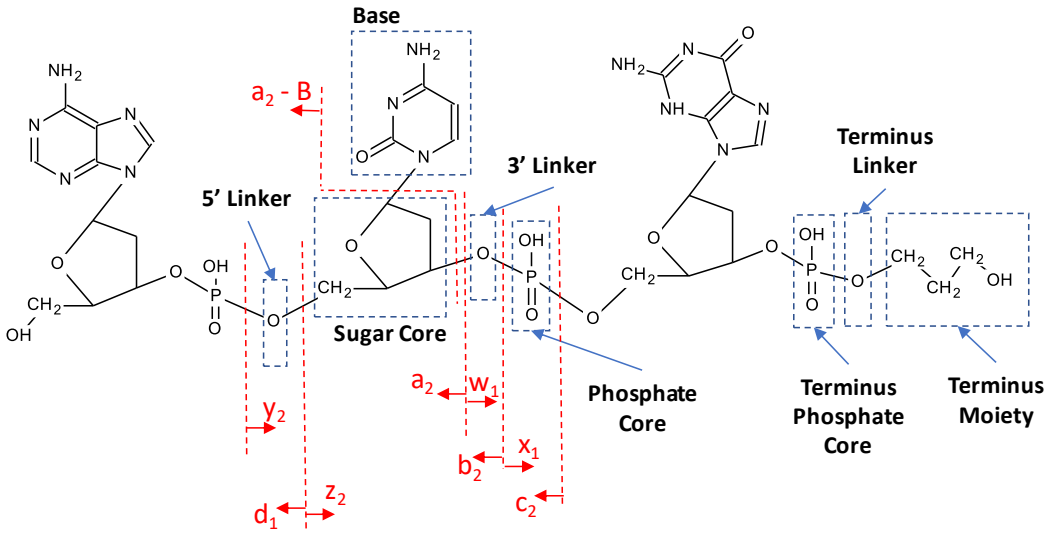

Figure 2. Representation of an oligonucleotide showing the different groups that can be defined for custom nucleotides. Also the nomenclature of the MS/MS fragments used for sequence confirmation is shown in this figure. (Image taken from the Molecule Profiler software.)

Chromatography

Metabolites of oligonucleotides are difficult to baseline separate from the main product with reversed phase LC while providing medium to high throughput, especially if they differ only by one or two nucleotides. Modified backbones such as phosphorothioated species are essential for improved pharmacokinetic properties and binding to the target, but complicate separation even further because they are mixtures of diastereomers, resulting in peak broadening. Figure 3 shows the separation achieved for the different metabolites; only the 5'(*n*-3) showed (partial) separation from the FLP.

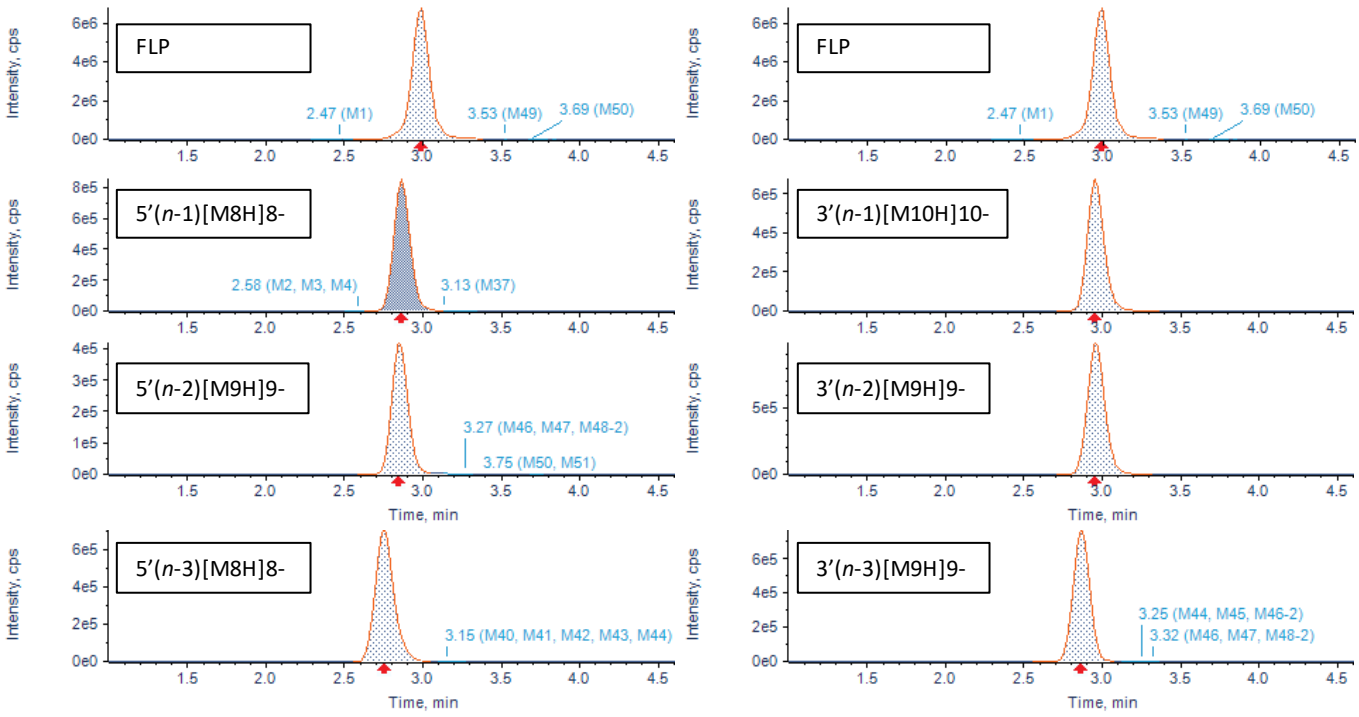

Figure 3. XICs of one charge state for the FLP and each of the spiked-in metabolites generated in Molecule Profiler software. (Partial) separation from the main product was only achieved for the 5'(*n*-3) metabolite.

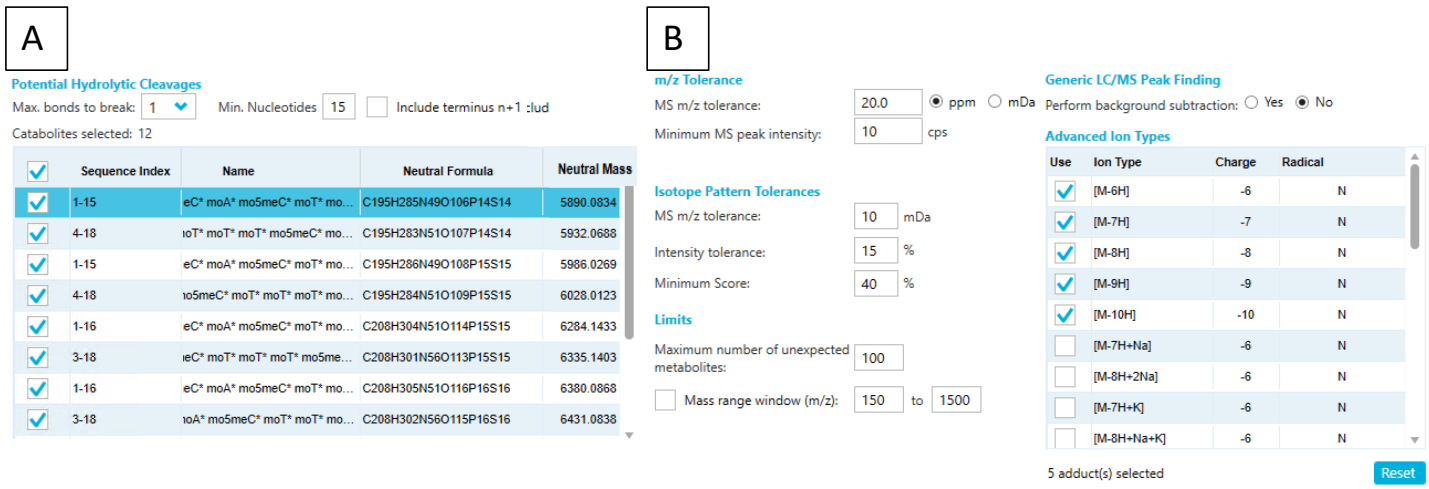

Figure 4. Main settings used for the identification of potential metabolites based on the TOF-MS data. A:The search space was limited to metabolites with a maximum of one bond broken and a minimum length of 15 nucleotides (the FLP contained 18 residues). No internal *n*-1 or terminal *n*+1 metabolites were sought during the search, as the main objective of this study was to demonstrate the capability of the software to find the spiked-in 5' and 3' *n*-1 to *n*-3 shortmers. B: An MS *m/z* tolerance of 20 ppm was used, and charge states -6 to -10 were considered.

Relative quantification

Nusinersen is reported to mainly be metabolized via exonuclease-mediated hydrolysis at the 5' and 3' ends.[1] Therefore, extracted plasma was spiked with 10 µg/mL FLP and the six 5'(*n*-1,2 and 3) and 3'(*n*-1,2 and 3) deletion metabolites at 0.1, 0.3, 1, 3 and 10% (*w/w*) to mimic a metabolism sample. In addition, the defined spike-in amounts allowed for an estimation of the sensitivity of the presented workflow . All samples and a control spiked with only 10 µg/mL FLP were measured and subsequently analyzed using the Molecule Profiler software. Processing parameters (Figure 4) were selected to allow for the identification of the FLP and metabolites. Based on the TOF-MS data, the FLP and potential metabolites are being matched to the different charge states (see 3'(*n*-3) metabolite matching in Figure 5). All identified charge states can be easily grouped for an automatic calculation of the % area for the main component and each potential metabolite.

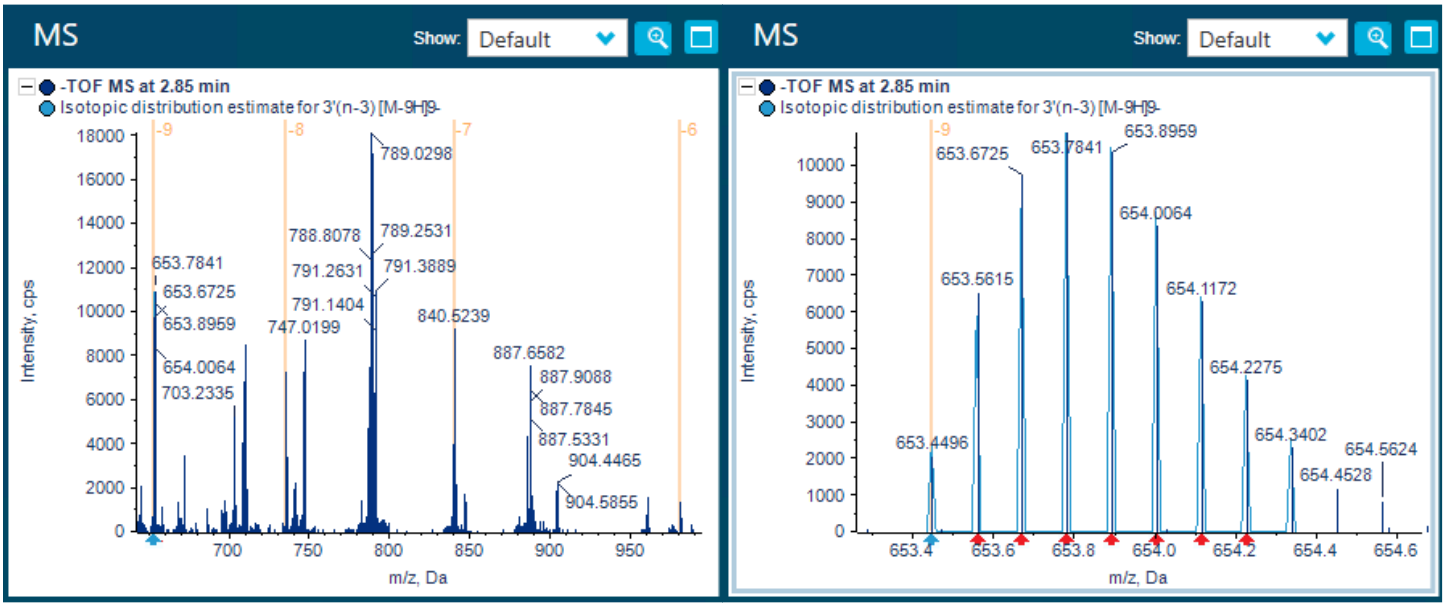

Figure 5. Identification of potential metabolite based on the TOF-MS data. A:TOF-MS showing identified charge states (orange labeling) and suggested ID for the 3'(*n*-3) at 3% spike-in level. The peaks without annotation represent coeluting charge states from the FLP and other spiked in shortmers. B: Zoom-in to TOF-MS data of charge state -9 for 3'(*n*-3). Blue arrow indicates the theoretical monoisotopic mass and the first seven isotopes are indicated with red arrows. An overlay of the theoretical isotopic distribution can be used for confidence in the correct assignment.

Table 2. Relative quantification of the spiked-in metabolites. The %areas are based on the summed areas of all identified charge states of a metabolite relative to the area of all identified peaks.

Spike-in (% w/w of FLP)	Spike-in (% w/w of total)	FLP (% area)	*5'(n-1)* (%area)	*5'(n-2)* (%area)*	*5'(n-3)* (%area)	*3'(n-1)* (%area)	*3'(n-2)* (%area)	*3'(n-3)* (%area)
0	*0.00*	*81.5*	*NA*	*NA*	*0.26*	*NA*	*NA*	*0.02*
0.10	*0.10*	*79.0*	*0.12*	*NA*	*0.31*	*0.08*	*NA***	*0.08*
0.30	*0.29*	*79.0*	*0.25*	*0.10*	*0.46*	*0.11*	*NA***	*0.18*
1.00	*0.94*	*75.0*	*0.69*	*0.27*	*0.99*	*0.52*	*0.38*	*0.56*
3.00	*2.54*	*70.0*	*2.20*	*0.88*	*2.50*	*1.70*	*1.50*	*1.70*
10.00	*6.25*	*52.0*	*6.10*	*4.60*	*6.10*	*5.10*	*5.50*	*4.80*

* Because of an overlap of the -8 charge state isotopes of the 5'(*n*-2) spike-in with the -9 charge state of the FLP (no chromatographic resolution), the charge state was excluded for relative quantification, leading to lower %area values for the 5'(*n*-2).
** No signals were observed for any of the charge states of the 3'(*n*-2) metabolite at the 0.1 and 0.3% spike-in level. Potential causes for this are losses due to sequence-based adsorption or ion suppression.

The % area is the sum of the TOF-MS areas of all found charge states of each identified analyte as a percentage of the total area of all analytes found. In Table 2 the % areas of the spiked-in metabolites are listed for each of the spike-in levels. The purity found for the control sample is consistent with the information from the manufacturer for products that have not been purified with HPLC.

The main impurities found in the control sample were desulfurization (16.1%), and di-desulfurization (1.37%). Correlation between the spiked-in amounts, and the reported areas was good for the 5'(*n*-1), 5'(*n*-3), 3'(*n*-1) and 3'(*n*-3) metabolites, with small amounts found in the control sample (Table 2). For the 5'(*n*-2) metabolite the correlation was found to be poor (Table 2). Upon further inspection of the data this could be attributed to an overlap of the -8 charge state isotopes of the metabolite with those of the -9 charge state of the FLP. For relative quantification of this compound based on MS data, a better chromatographic separation will be required. Alternatively the Analytics module in SCIEX OS software can be used to perform quantification based on reconstruction of the TOF-MS data, or by quantification based on fragment masses using MRM^{HR} data. Note that the Molecule Profiler software also supports relative quantification based on UV data (not shown). No signal was found for any of the charge states of the 3'(*n*-2) metabolite at the 0.1 and 0.3% spike-in levels. Possible causes for this could be sequence dependent adsorption losses or ion suppression.

Correlation

The Molecule Profiler software also allows for comparing the peak areas of metabolites between different samples. The software can be used to compare samples taken at various times after administering a therapeutic, or compare samples taken from different test animals. In Figure 6, this function of the software was used to graph the peak areas for several of the metabolites at the different spike-in levels.

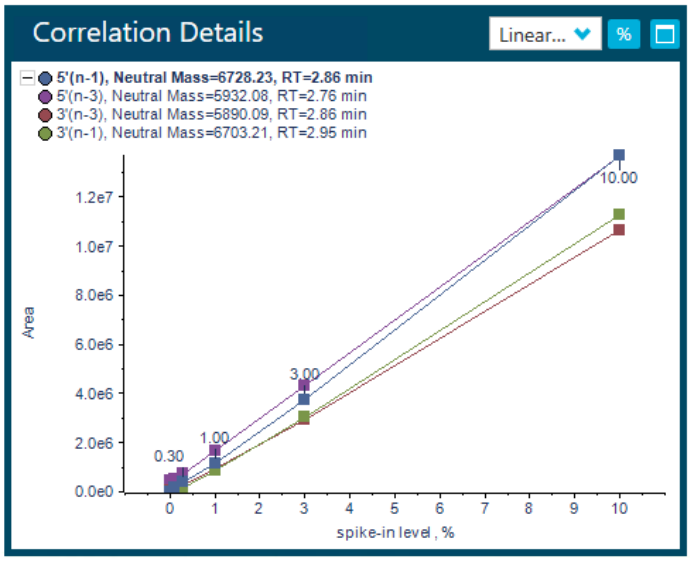

Figure 6. Correlation plot of area vs %spike-in level. Plots for the 5'(*n*-1) 5'(*n*-3), 3'(*n*-1) and 3'(*n*-3) metabolites with good linearity are shown.

Structural confirmation

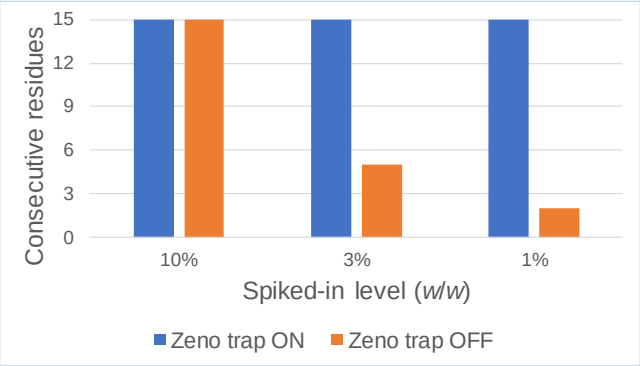

Figure 7. Consecutive residues for different spiked-in levels of the 3'(*n*-3) metabolite, with and without use of the Zeno trap. The metabolite has a length of 15 nucleotides.

The potential metabolites suggested by the software are based on accurate mass and isotope pattern matching of the TOF-MS data, which does not provide information for the localization of a modification, or the sequence of a potential metabolite. As the structure of a metabolite can be important for determining its toxicity, understanding and confirming the correct structures is critical. The Molecule Profiler software can help in confirming the structures of each of the potential metabolites by annotating the MS/MS spectra. Allowing for *a*, *w*, *c*, *y* and *d* terminal fragments, and allowing for the loss of one base or water molecule, the Molecule Profiler software was used to annotate the MS/MS spectra of the different charge states and calculate the consecutive sequence coverage. An *m*/*z* tolerance of 10 ppm was used. Figure 7 shows the number of consecutive residues covered in the MS/MS spectra of the -9 charge state for the 3'(*n*-3) metabolite at the various spike-in levels, and Figure 8 shows the number of identified fragments. Consecutive coverage was found down to the 1% level when the Zeno trap functionality was employed. Without the Zeno trap, the coverage was significantly lower, as expected. When allowing for two bonds to break, consecutive coverage was seen at the 0.3% and 0.1% levels, as well, with the Zeno trap on (data not shown). Figure 8 illustrates how much more information-rich MS/MS spectra with higher S/N can be acquired using the Zeno trap compared to traditional MS/MS analysis. The higher quality data enabled the assignment of many more fragments with great S/N by the software. The high-quality MS/MS information can be used to confirm the identification based on MS even further.

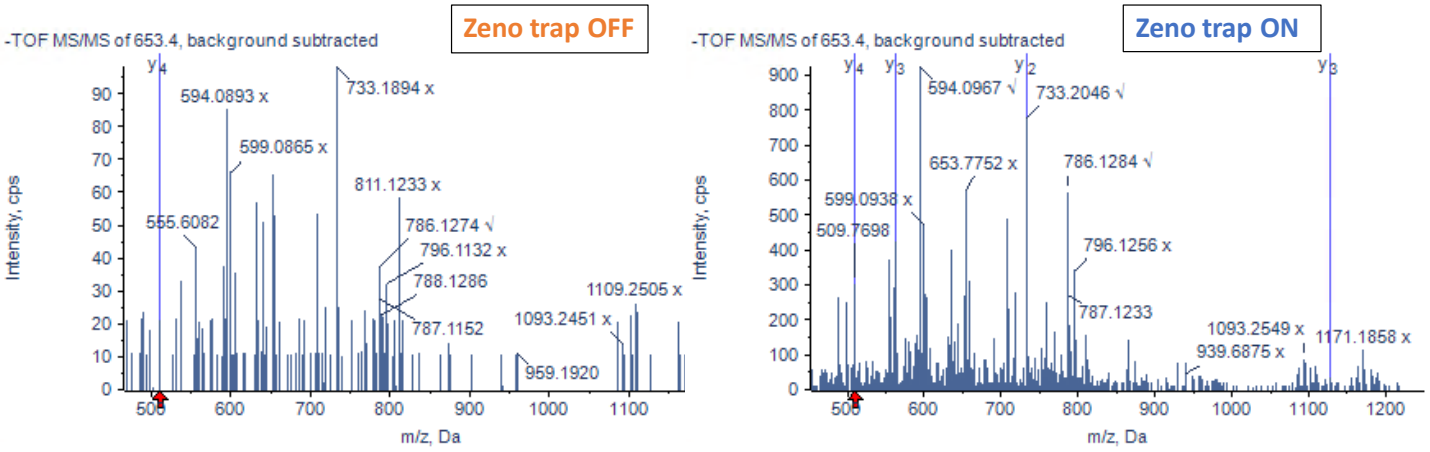

Figure 8. Zoom-in to the MS/MS spectrum of the 3'(*n*-3) metabolite -9 charge state at the 0.3% spiked-in level. Data was acquired without using the Zeno trap (left) and with using the Zeno trap (right). Only y fragments are annotated; just one y fragment ion was found in the MS/MS spectrum acquired without using the Zeno trap, while four were found when the Zeno trap was used. S/N of the spectrum with the Zeno trap on was approximately 10x better showing significantly more automatically assigned fragment ions in Molecule Profiler software.

Fragments: 7 of 34 Proposed Formulae

	Use	Mass (m/z)	Sequence	Ion	Charge	Error (ppm)	Intensity (cps)
4	✓	366.0989	moG*mo5meC	y2	2	3.9	161.6
5	✓	733.2009	moG*mo5meC	y2	1	-1.8	3853.2
6	✓	563.1275	moT*moG*mo5meC	y3	2	0.0	1287.2
7	✓	1127.2557	moT*moG*mo5meC	y3	1	-5.8	79.6
3	✓	764.6566	moA*moT*moG*mo5meC	y4	2	-8.7	398.2
1	✓	643.7970	moA*moA*moT*moG*mo5meC	y5	3	0.2	500.2
2	✓	681.8770	moA*moT*moA*moA*moT*moG*mo5meC	y7	4	-2.6	76.5

Figure 9. Example of the annotation table in the Molecule Profiler software. The table shows the proposed fragments for a particular *m/z*, their charge states, mass accuracies and intensities. The data is from the 3'(*n*-3) metabolite -9 charge state at the 3% spiked-in level, acquired using the Zeno trap .Only the *y* fragments are shown as an example.

Conclusions

- Excellent MS data quality and mass accuracy were leveraged for the identification of FLP and spiked-in metabolites down to a 0.1% level
- A fast and accurate relative quantification of oligonucleotides and their metabolites based on MS or UV data can be performed in Molecule Profiler enhancing product understanding and PK/PD and toxicology studies
- The annotation of MS/MS with the most commonly found fragment ions based on a proposed structures in Molecule Profiler software greatly reduces manual workload and speeds up the correct identification of oligonucleotide-based drugs and metabolites
- MS/MS data quality and fragment assignment can significantly be enhanced using the Zeno trap with the ZenoTOF 7600 system, which allows for the identification of even very low abundant metabolites

References

1. Committee for Medicinal Products for Human Use (*CHMP*) Assessment report EMA/289068/2017.

SCIEX

Headquarters
500 Old Connecticut Path | Framingham, MA 01701 USA
Phone 508-383-7700
sciex.com

International Sales
For our office locations please call the division headquarters or refer to our website at
sciex.com/offices