Optimisation of LC-MS/MS conditions for characterization of RNA and large oligonucleotides and automated detection of clipping variants using OligoQuest

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Introduction

Although oligonucleotides have traditionally been sequenced by Sanger sequencing and NGS, mass spectrometry (MS) is now seen as critical due to the need to validate highly modified molecules and characterize impurities. Interest in RNA sequencing has led to mapping methods being adopted for larger molecules, whereby the parent molecule is enzymatically digested into smaller fragments prior to LC-MS/MS analysis.

Here we investigate the optimal conditions for characterization of RNA and larger oligonucleotides (>70mers) using RP-UPLC-ESI-MS/MS and the role of different collision energies in yielding internal or terminal fragmentation, without enzymatic digestion. An MS screening workflow was also used to automatically detect clipping variants.

Methods

As a model oligonucleotide, the 76mer tRNA-Phe (Sigma) was analyzed by LC-MS and LC-MS/MS using a timsTOF-Pro 2 (Bruker). MS and MS/MS (MRM) data were acquired and processed in the OligoQuest workflow of the BioPharma Compass software (Bruker). To optimize the fragment ion yield, experimental parameters were optimized including collision energy (CE) in the range from 16 to 60 eV. For MS/MS, charge states 26-28 (910 m/z selected with isolation width 120 m/z) were jointly isolated and fragmented and the monoisotopic MS/MS peaklist was calculated using the SNAP algorithm. The monoisotopic fragment ion list was matched against the theoretical fragment ions calculated from the RNA sequence using OligoQuest.

Results

- LC-MS analysis of the 76mer tRNA-Phe (Sigma) revealed several degradation or clipping products in addition to the intact oligonucleotide, which were automatically annotated in OligoQuest (*Fig. 1*).

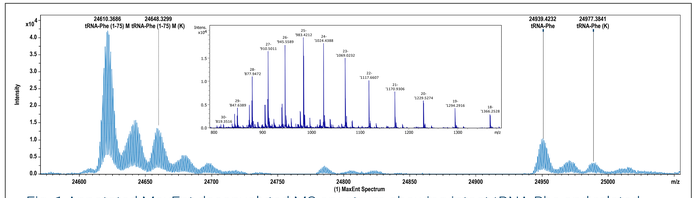

Fig. 1 Annotated MaxEnt deconvoluted MS spectrum showing intact tRNA-Phe and related products including tRNA-Phe (1-75) excerting an A76-loss. Insert: original MS spectrum

- MS/MS analysis of charge states 26-28 with 19 or 34 eV CE resulted in very different MS/MS spectra: the 19 eV spectra predominantly exhibited high Mr and charge state fragments, whilst lower charge state fragments were observed with 34 eV (*Fig. 2*).

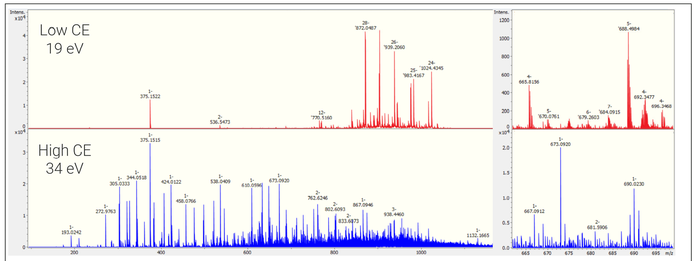

Fig. 2 MS/MS spectra acquired using 19 and 34 eV CE. Right: Zoom view of 660-700 m/z region

- MS/MS spectra for the most abundant form, tRNA Phe (1-75), were matched to the sequence in OligoQuest with 15 ppm tolerance. Low CE resulted in better terminal coverage, whilst High CE resulted in better internal fragment coverage(*Fig. 3*).
- As a result of the wide isolation width, the chimeric MS/MS spectra also matched to the full-length tRNA-Phe 76mer with different matches at the 3' terminal, reflecting dominant 3' clipping (data not shown).
- Analysis of the charge state of the matching fragments in OligoQuest revealed that Low CE fragmentation predominantly involved terminal fragments. The mean average matched charge state was z=-5.5 (*Fig. 4*).
- Conversely, fragmentation with High CE predominantly produced internal fragment matches, with a mean average matched charge state of z=-2.7 (*Fig. 4*).

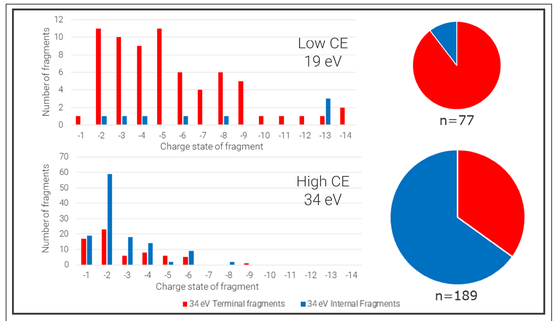

Fig. 4 The number of unique annotated fragment ions and their charge states after fragmentation of tRNA-Phe (1-75) with 19 or 34 eV CE

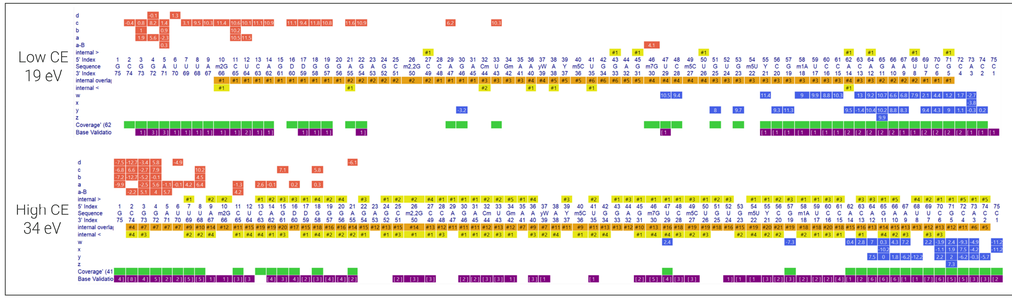

Fig. 3 Annotation of MS/MS spectra in OligoQuest.
Terminal 5' (red), Terminal 3' (Blue) and Internal (yellow) fragment ion matches are shown. Sequence coverage for terminal fragments: Low CE 62.7 %, High CE 41.3 %, rising to 92.0 % and 100 % respectively when internal fragments are also considered. The sequence was further validated according to the number of adjacent cleavages (purple).

Summary

- The MS analysis of 76nt tRNA-Phe revealed that in this particular analysis the dominant species was the 3´-truncation variant t-RNA-Phe (1-75).
- tRNA-Phe sample comprised of 80.1% 75mer and 19.9% 76mer based on the MS peak intensities (*Fig. 1*).
- The 75mer was analyzed by MRM under different conditions. At 34 eV, internal fragments provided a wealth of sequence matches of the 75mer (*Fig. 4*).
- At 19 eV, fragmentation was less efficient and yielded fewer but higher mass fragment ions with higher charge states (avg. z=5.5) (*Fig. 2*).
- 19 eV are typically used to fragment smaller oligonucleotides, which are predominantly MS/MS analyzed by terminal fragment ions (*see poster WP 390*).
- The sequence coverage calculation included terminal as well as internal fragment ions. SC for 19 eV was 92 % and for 34 eV was 100 %. (*Fig. 3*).
- The terminal fragments allowed to confirm 5' and 3' terminal sequences, whilst internal fragments validated the core sequence.

Conclusion

- OligoQuest is a dedicated workflow in the BioPharma Compass software to validate oligonucleotide and RNA sequences, quantify side products and identify sequence aberrations such as truncations
- OligoQuest supports the visualization of internal fragment ion matches and can use them to calculate the sequence coverage
- For mid-sized RNA such as tRNA-Phe, the inclusion of internal fragments increased the sequence coverage significantly vs. only terminal fragment ions

Technology