Auto MS/MS and targeted MS/MS in-depth qualitative and quantitative analysis of oligonucleotide synthesis products and side products

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Introduction

- Whilst confirmation of the full-length product (FLP) in oligonucleotide analysis is usually straightforward, characterization of low abundant side products can be more challenging
- We describe a method and software for highly specific and sensitive characterization of synthetic oligonucleotides, which combines UV-based quantitation of side products with automated MS/MS and subsequent targeted MSMS analysis.
- The approach can identify and quantify alterations of the FLP with high accuracy.

Methods

A 2´-O-permethylated RNA 24mer was synthesized (Axolabs). 0.4-1.6 µg were analyzed by LC-UV-QTOF MS and MS/MS using 2 scan modes: auto MS/MS datasets were used to quantify side products by UV and MS, and the sequences of the FLP, side products were identified by data dependent MS/MS. Targeted MSMS analyses were used to further increase sequence coverage and specificity for the identification of side products.

Datasets were analyzed by the OligoQuest workflow in the BioPharma Compass software (Bruker). Input were FLP sequences and respective datasets, output was the quantitative composition of the sample based on UV (260 nm) alone - or in combination with MS to address coeluting peaks.

Auto MS/MS analysis of the mod3 24mer FLP

Full sequence coverage was determined for the FLP. However, there is residual uncertainty for the a/g exchange variant – a16g being the more likely candidate.

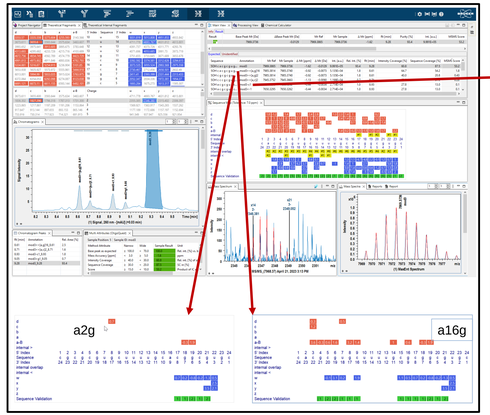

Fig. 1 Top; analysis overview with mod3, annotated MS and MS/MS peaks overlayed with theoretical isotope patterns. Bottom: Annotation of the variant sequences a2g and a16g

Auto MS/MS Analysis of the mod3-c1 23mer

The identification of the variant with loss of c1 was more challenging (**Fig. 2**).The isotope pattern of the precursor was heterogeneous and indicated the presence of a coeluting 7649.3 Da species in addition to the annotated mod3-c1 impurity.

The MS/MS spectrum was weak and only sporadic fragment ion matches were observed.

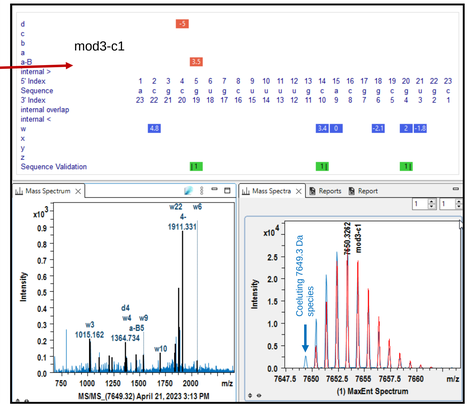

Fig. 2 Auto MSMS analysis of the mod3-c1 variant. The precursor ion isotope pattern indicates a coeluting species with 1 Da mass difference, the weak MS/MS spectrum was inconclusive.

Targeted MS/MS analysis of mod3-c1

The z=-4 ion of mod3-c1 was selected and a targeted MSMS analysis yielded a much improved Sequence Map (**Fig. 3, top**) and an MS/MS score of 12.58 vs. 0.1 from the autoMSMS dataset. An even higher score was obtained for a mod3-c1 variant with an additional exchange of (u|c)9 resulting in an MS/MS score of 38.82 (**Fig. 3, bottom**) . This partial exchange of u9 to c9 also explained the isotopic pattern of the parent ion (**Fig. 2**).

Fig. 3 Targeted MSMS analysis of mod3-c1 provided a 56.5 sequence coverage (top). Another species (mod3-c1, (u|c)9) scored even higher (bottom), indicating a partial contribution of c9 instead of u9.

Summary

The analysis of the 24mer FLP allowed the detailed identification and quantification of its side products using the timsTOF and the OligoQuest workflow in BioPharma Compass.

The True Isotope Pattern capability of the orthogonal TOF analyzer allowed to safely detect a partial u-to-c conversion (-1 Da) by MS.

Targeted MSMS allowed to localize the likely conversion site to u9.

Conclusions

- Auto MSMS and targeted MSMS were used to identify the FLP and side products with greater confidence
- (a|g)16 and (a|c)2 variants were IDed and quantified at the 2 % level.
- A mod3+g variant was detected (0.7%)
- At 1.8%, a mod3-c1 variant was proposed based on MS, which coeluted with an additional (u|c) conversion product presumably at pos. 9
- The true isotope pattern of the precursor and the high sequence coverage in the targeted MSMS spectrum allowed to propose that partial residue “conversion”

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