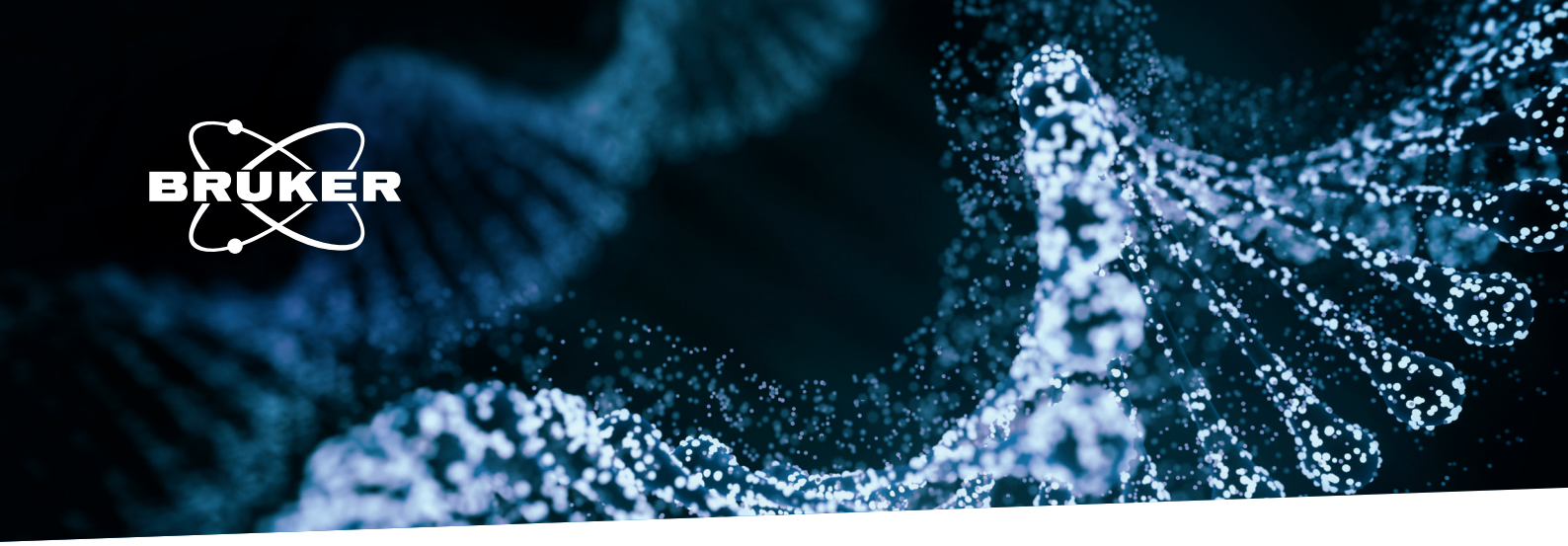

Oligonucleotide characterization with a timsTOF Pro 2 QTOF mass spectrometer and the OligoQuest workflow of the BioPharma Compass software

Oligonucleotides are becoming increasingly vital research tools due to their ability to inhibit genes or to function as aptamers to interact with protein targets (1).

Introduction

Keywords: Oligonucleotide Characterization, timsTOF Pro 2, BioPharma Compass

Oligonucleotides are single- or double-stranded small synthetic nucleic acid polymers about 20-mer in size and can target pre-mRNA, mRNA, or non-coding RNA to induce degradation, modulate splicing events, or interfere with protein translation (2). Accurate analytical characterization of oligonucleotides is necessary to confirm their identity, to determine their purity, and to identify possible synthesis side products.

Determining molecular weight and confirming the nucleotide sequence of an oligonucleotide are fundamental criteria for establishing the molecule's identity, which is a regulatory requirement. Oligonucleotide synthesis is a complex process that requires more than 100 sequential chemical reactions to make a single 25-base sequence (3), and the quality of each synthesized oligonucleotide must be evaluated prior to use to ensure that the correct sequence was made, and that purity meets regulatory standards.

This technical note describes the use of the Bruker timsTOF Pro 2 QTOF System with the VIP-HESI ion source operated in negative ion mode for the analysis of oligonucleotides. A Bruker Elute HPLC system was used to efficiently separate impurities from the target compound and to provide unambiguous confirmation of sequences with minimal adduct formation or analysis induced modifications. Complete characterization of the oligonucleotides was accomplished by the OligoQuest workflow within Bruker´s BioPharma Compass® software.

Material and Methods

A 24-mer RNA with 2'-O-methylation of the ribose at each position was purchased from Axolabs GmbH (product number X119083K2; sequence: **5OH c a c g c g u g c u u u u g c a c g g c g u g c 3OH**). The sample was diluted with Eluent A to 0.1 µg/µL prior to analysis and 0.4 µg was injected onto the UHPLC system for subsequent MS analysis in the TIMS off mode. A Bruker Elute UHPLC - equipped with an Azura UVD 2.1S UV detector (KNAUER) recording the 260 nm chromatogram - was connected to Bruker timsTOF Pro 2 QTOF System with the VIP-HESI ion source to separate side-products, concentrate target compounds and to remove salts.

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Chromatography	
UHPLC column	Waters XBridge Oligonucleotide BEH C_{18}, 130Å 2.5 µm, 2.1 x 50 mm operated at 60°C
Eluent A (aqueous phase) in water	0.24% (v/v) Triethylamine (TEA) 1.00% (v/v) Hexafluoro-2-propanol (HFIP) 1.00% (v/v) Methanol (MeOH)
Eluent B (organic phase) in Acetonitrile	10% (v/v) Isopropanol (IPA)

Time [min]	Flow [mL/min]	% A	% B
0	0.250	99	1
1	0.250	99	1
3	0.250	96	4
16	0.250	90	10
16,2	0.250	5	95
16,8	0.250	5	95
17	0.250	99	1
23,5	0.250	99	1

timsTOF Pro 2 parameters – MS

VIP-HESI Source				
Nebulizer	Dry gas	Dry temp	Probe gas temp	Probe gas
4 bar	8 L/min	220°C	450°C	4 L/min

TIMS		
Scan range	Scan mode	Spectra rate
240-3500 *m/z*	AutoMsMs	1 Hz

Sweeping				
	Time (%)	Collision RF (Vpp)	Transfer time (µs)	Collision energy (%)
1	0	800	90	100
2	25	3500	140	100

Transfer	
Deflection 1 delta	-70 V
Funnel 1 RF	350 Vpp
isCID	0
Funnel 2 RF	400 Vpp
Multipole RF	600 Vpp
Quadrupole ion energy	4 eV
Quadrupole low mass	500 *m/z*
Collision energy	10 eV
Pre-pulse storage	10 µs

timsTOF Pro 2 parameters – MS/MS	
Total cycle time	1 s
MS spectra rate	2 Hz
MS/MS spectra rate (fixed)	2 Hz
No. precursors	1
Normalized threshold	31 counts/1000 scans
Average scans	5
Scan range, isol. width, collision energy	500 -3000 *m/z*, isol. width 3 *m/z*, 15.5-97.6 eV

For a pure sample such as the 24-mer it is beneficial to use a low-speed auto MS/MS cycle with only one precursor being selected. This ensures that the charge states with the highest intensity will be selected for MS/MS.

Data processing

The raw LC-UV-MS(/MS) data were processed in BioPharma Compass® using the OligoQuest (autoMS/MS) tutorial workflow method based on a user defined sequence of the 24-mer RNA, which includes residue-specific modifications. Here, 2'-O-methylation nucleotides were used, abbreviated by a, c, g and u. OligoQuest enables the automated rapid verification of molecular mass, sequence and the assessment of purity by quantifying chromatographic peaks using the UV and MS signal intensities. In addition, it can annotate variant sequences and synthetic impurities based on the input of the target sequence and further workflow parameters, which allow for the screening for failure sequences, addition of nucleotides or nucleotide exchange variants.

Main View | Processing View | Chemical Calculator

Info | Result

Row	Result	Position	Base Peak Mr [Da]	ΔBase Peak Mr [Da]	Mr Ref	Mr Sample	Δ Mr [ppm]	Rt [min]	Purity [%]	Int. [a.u.]	MSMS Score	Sample Name	Sequence
1		5	7969.3735	-0.0129	7969.3865	7969.3735	-1.62	9.25	86.0	9.796E+05	61.04	mod3	5OH c a ...

Expected | Unidentified

Row	Confir...	Sequence	Annotation	Mr Ref	Mr Sample	Δ Mr [ppm]	Δ Mr [Da]	Int. [a.u.]	Rel. Int. [%]	Rt [min]	Intensity Cov...	Sequence Cov...	MSMS Score
1	☑	5OH c a c g...	mod3	7969.3865	7969.3735	-1.62	-0.0129	9.796E+05	86.0	9.25	61.0	100.0	61.04
2	☑	5OH c a c g...	mod3>>[a,g]16	7985.3814	7985.3737	-0.97	-0.0077	2.397E+04	1.4	8.58	63.7	45.8	29.18
3	☑	5OH c g c g...	mod3>>[a,g]2	7985.3814	7985.3737	-0.97	-0.0077	2.397E+04	1.4	8.58	42.3	20.8	8.81

Figure 1
Result and Expected tables showing a summary of the analysis and all identified species of the selected sample.

Results and discussion

The Result table of the OligoQuest (autoMS/MS) tutorial workflow (Figure 1) provides a quick confirmation that the base peak in the dataset matches the expected molecular weight of the oligo with good mass accuracy and that the sample purity is above the expected minimum. The Expected table shows the analysis result for the currently selected sample, listing all identified molecular species, including sequence aberrations defined in the method´s matching parameters. Here we see, besides a 100% sequence coverage (SC) for the target sequence mod3, two side product candidates (mod3>> [a,g]16 and mod3>>[a,g]2), in which an a/g nucleotide exchange is suggested either at positions 2 or 16. The higher MS/MS score of the first side product suggests that the nucleotide exchange more likely happened at position 16.

For each sample multiple quality attributes are determined, which were previously defined in the analysis method. For this sample, as seen in Figure 2, all reporting fields are shown in green, indicating that all Narrow definitions of acceptance criteria were matched.

The Chromatogram (Figure 3) shows the target sequence mod3 as base peak in the UV chromatogram at 9.25 min. It also shows the mod3>>[a,g]_8.58 peak with a chromatographic UV peak area of 2.6% and a mod3 target molecule peak area of 94.9%. Thus, this value reflects the peak areas within the UV chromatogram.

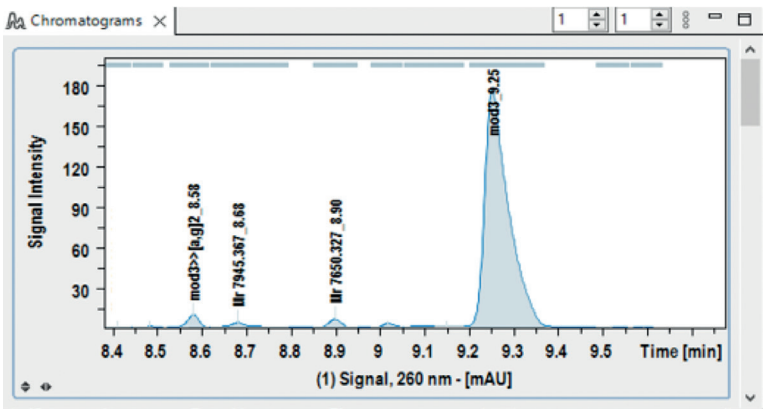
The overall Purity provided in the Result table (Figure 1) was calculated with 86.0%, which also includes MS peaks within the target molecule's chromatographic peak. These coeluting side products cannot be detected by LC-UV analysis alone.

Multi Attributes (OligoQuest)

Sample Position: 5 | Sample ID: mod3

Method Attribute	Narrow	Wide	Sample Result	Unit
Base peak as expected	≥ 100.0	< 70.0	100.0	Rel. int. [%] vs. base peak
Mass Accuracy [ppm]	< 4.0	≥ 6.0	-1.6	ppm
Intensity Coverage	≥ 40.0	< 30.0	61.0	Rel. int. [%] of all target signals
Sequence Coverage	≥ 30.0	< 20.0	100.0	SC in [%]
Score	≥ 15.0	< 10.0	61.0	Product of IC x SC in [%]

Figure 2
Multi Attributes view, displaying the Narrow and Wide acceptance criteria for 5 quality attributes and the values determined as Sample Result.



Chromatogram Peaks

Rt [min]	Int.	Area	Annotation	Rel. Area [%]	Rel. Area BPI [%]
8.58	9.520E+00	1.751E+01	mod3>>[a,g]2_8.58	2.6	2.7
8.68	2.813E+00	5.593E+00	Mr 7945.373_8.68	0.8	0.9
8.90	6.035E+00	1.148E+01	Mr 7649.326_8.90	1.7	1.8
9.25	1.770E+02	6.447E+02	mod3_9.25	94.9	100.0

Figure 3
UV 260 nm Chromatogram and the Chromatogram Peaks table used for quantitative assessment of side products.

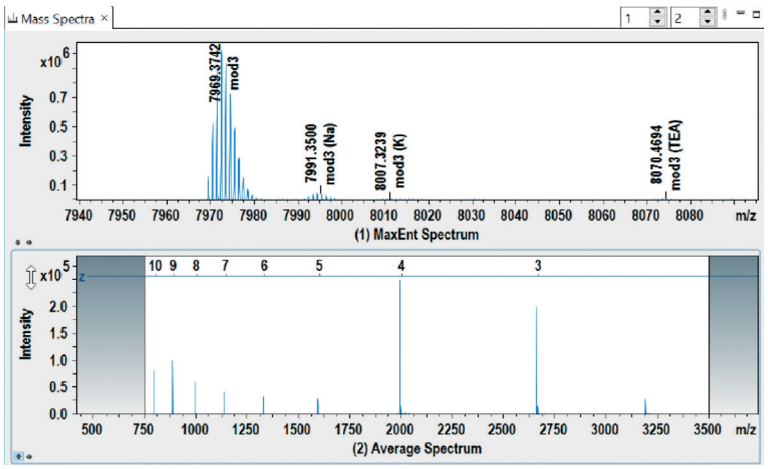


Figure 4
Average and Deconvoluted Spectrum of mod3.

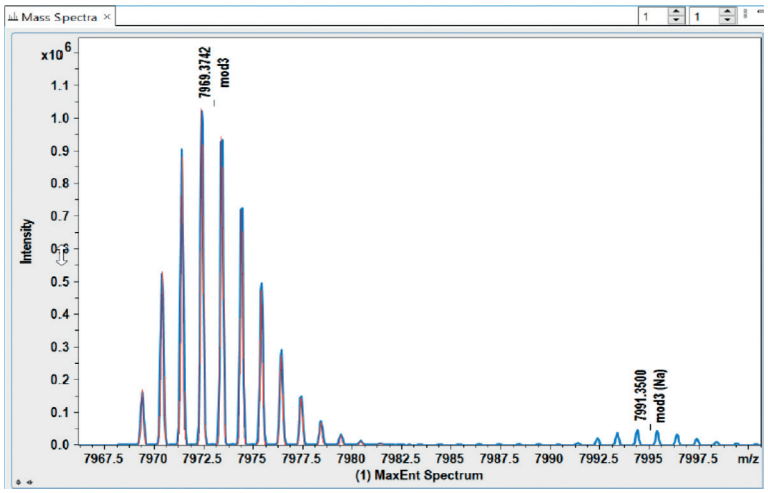


Figure 5
Overlay between experimental (blue) and calculated (red) isotope pattern of the intact oligonucleotide allows to assess, e.g., the absence or degree of C/U conversion.

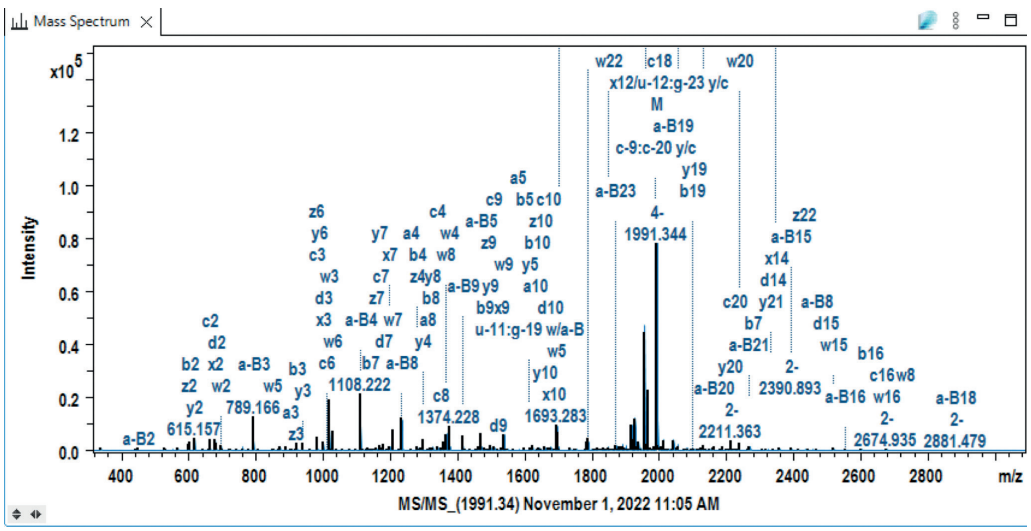
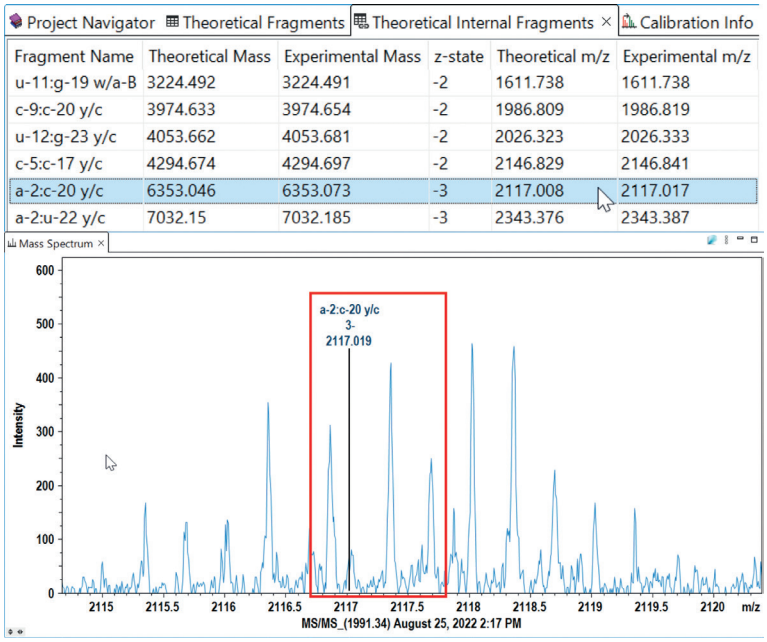


Figure 6
MS/MS spectrum of mod3 (z=-4).

The Average Spectrum (Figure 4) is obtained from a selected chromatographic peak or directly by clicking on the Expected table entry. The *m/z* range used for the MaxEnt (Maximum Entropy) deconvolution is marked as white spectra area and the charge states of the peaks are shown on top. The top window shows the MaxEnt charge-deconvoluted neutral mass spectrum of mod3 plus several adduct peaks.

The spectrum can be overlayed with the theoretical isotope pattern calculated based on the elemental composition of the sequence. This gives a visual comparison of the theoretical spectrum overlayed on the experimental spectrum and thus information about quality of the match. Partial C/U conversions (shift of +1 Da) can be detected sensitively by this comparison.

The MS/MS spectrum obtained from the best matching precursor charge state (here, the -4 state of mod3 at *m/z* 1991.34) is shown in Figure 6. For visual verification of individual matches, the MS/MS spectrum can automatically zoom in on the peak profiles of fragment ions in question. For that purpose, the spectrum display can be controlled from the Theoretical Fragments and the Theoretical Internal Fragments tables.



Fragment Name	Theoretical Mass	Experimental Mass	z-state	Theoretical m/z	Experimental m/z
u-11:g-19 w/a-B	3224.492	3224.491	-2	1611.738	1611.738
c-9:c-20 y/c	3974.633	3974.654	-2	1986.809	1986.819
u-12:g-23 y/c	4053.662	4053.681	-2	2026.323	2026.333
c-5:c-17 y/c	4294.674	4294.697	-2	2146.829	2146.841
a-2:c-20 y/c	6353.046	6353.073	-3	2117.008	2117.017
a-2:u-22 y/c	7032.15	7032.185	-3	2343.376	2343.387

Figure 7
Theoretical Internal Fragments table and MS/MS spectrum zoomed in to the fragment at 2343.3 *m/z*.

The Theoretical Internal Fragments table (Figure 7) lists the internal fragments assigned to the sequence. Matches are only reported if they are unique, i.e., the *m/z* values cannot be interpreted as a different internal ion or as a terminal fragment based on its mass. Theoretical and experimental masses (z=-1) are provided together with the z-states and theoretical and experimental *m/z* values. Selection of a table entry causes the MS/MS spectrum in the Mass Spectrum view to zoom to the respective *m/z* value to permit visual inspection. In Figure 7, clicking on the row for the mass of 6353.0 causes the MS/MS spectrum to zoom to 2117.0 *m/z* region of the spectrum for detailed inspection of the spectrum around the z=-3 peak.

The Sequence Map (Figure 8) provides a clear overview about the quality of the match between MS/MS data and the selected sequence in the Expected table. The 5'-fragment assignment matches are shown in red bricks, 3'-fragments are shown in blue bricks (ppm errors inside). The sequence with index numbers is counted from 5'-end above and from 3'-end below. The green bottom line provides some more insights about the sequence validation quality for each residue: the number inside counts the redundancy in the validation of an individual residue. It uses algorithms common in gene assembly. In Figure 8, residue 6 is framed by 5'-terminal fragments if for example a5 and c6 fragments are observed, similarly it is framed by 3'-terminal fragments if cleavages before and after residue 6 are observed.

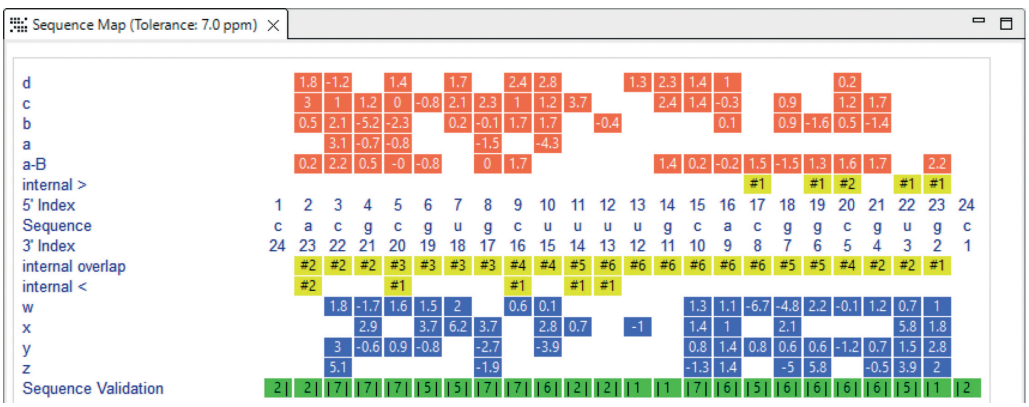


Figure 8
Sequence Map shows 100% sequence coverage (green bar) for the mod3 24mer.

Internal fragments can further add to confirming a sequence. The yellow bars in the Internal Fragments Map (Figure 9) visualize unique internal fragments and their mass errors in ppm. The fragment ion type is shown on the left: a-2:c-20 y/c codes for a fragment that covers all residues from a2 - c20 with 5' y-type and 3' c-type termini. The formation of internal fragments can be enhanced or attenuated by the collision energy and other instrument parameters.

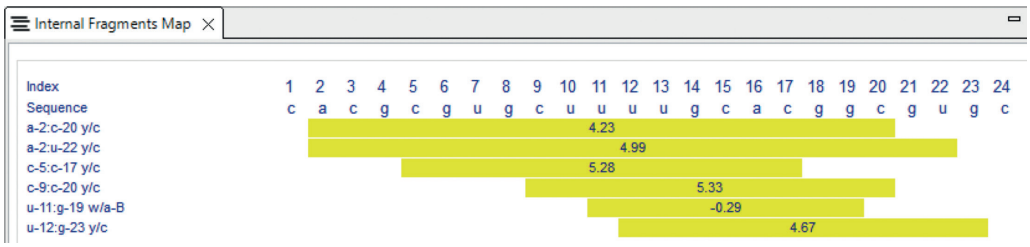


Figure 9
Internal Fragments Map.

Conclusion

An integrated LC-UV-MS/MS workflow for characterization of synthetic oligonucleotides has been developed on the timsTOF Pro 2 QTOF with an Elute UHPLC and UV-detector. The analyzed RNA 24-mer was well separated chromatographically from most impurities and purity information of the oligonucleotide was obtained from the UV chromatogram and the mass composition of its target peak. The intact mass of the target compound with mass accuracy of -1.6 ppm and the theoretical isotope patterns' excellent match to the mass spectrum confirmed the absence of any C-to-U conversion. The MS/MS spectrum yielded a sequence coverage of 100%. In addition, 2 isomeric side product candidates were proposed based on intact mass, one of them (mod3>> [a,g]16) was accepted based on its higher MS/MS score and the better matching fragment ions (data not shown).

The OligoQuest workflow in BioPharma Compass enables the fast identification and characterization of oligonucleotide samples based on LC-UV-MS and MS/MS data. The interactive interface makes it time efficient to review analysis results in both tabular and graphical forms and to generate meaningful reports and reducing analysis turnaround time.

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