

## Intact Mass Confirmation Analysis on the BioAccord LC-MS System for a Variety of Extensively Modified Oligonucleotides

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## Abstract

This application note demonstrates an automated compliance-ready LC-MS workflow for mass confirmation of unmodified and extensively modified oligonucleotides.

### Benefits

- An automated compliance-ready LC-MS workflow for mass confirmation of unmodified and extensively modified oligonucleotides
- The Bayes Spray algorithm embedded in waters\_connect provides accurate results for charge deconvolution of heavily modified oligonucleotides

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## Introduction

Oligonucleotide therapeutics have emerged in recent years as a powerful alternative to small molecule and protein therapeutics.<sup>1</sup> Manufacturing and quality control of oligonucleotide therapeutics requires highly selective and sensitive LC-MS methods. The method most often used for mass spectrometry-based oligonucleotide analysis has been reversed phase chromatography employing a variety of ion-pairing reagents and modifiers in negative ESI-MS mode. Integrated LC-MS workflows for oligonucleotide analysis have been recently implemented on quadrupole and QToF LC-MS platforms.<sup>2-4</sup>

The BioAccord LC-MS System featured in Figure 1 was introduced in 2019 as a compact, robust, easy-to-use platform for routine biopharmaceutical analysis. The fully integrated BioAccord LC-MS System is comprised of an ACQUITY UPLC I-Class PLUS System, a Tunable Ultraviolet (TUV) Detector and the ESI-ToF based ACQUITY RDa Mass Detector, as shown in Figure 1. A streamlined, compliance-ready workflow for oligonucleotide analysis employing the BioAccord System was recently introduced.<sup>5</sup> Both data acquisition and processing capabilities are incorporated in waters\_connect, a recently introduced compliance-ready informatics platform.



Figure 1. BioAccord LC-MS platform.

The capabilities of this LC-MS platform were evaluated here for the analysis of extensively modified oligonucleotides. Unlike the regular (unmodified) oligonucleotides, the modified ones can be challenging to characterize by LC-MS due to their increased hydrophobicity, poor solubility in organic solvents or poor response in electrospray mass spectrometry. Several classes of oligonucleotide modifications were investigated in this study. The same LC-MS system equipped with a standard 2.1 x 50 mm C<sub>18</sub> OST (Oligonucleotide Separation Technology) Column and using the same mobile phase (see Experimental section) was used for ion-pairing RP analysis of all oligonucleotides. The oligonucleotide mixtures analyzed contained an unmodified oligonucleotide sequence (labeled as CTRL-control) along with 1-3 extensively modified oligonucleotides (labeled as TEST compounds). All the LC-MS data presented in this application note was acquired in full scan MS mode and was processed in waters\_connect. Spectral deconvolution was performed simultaneously using the combined ESI-MS spectra of the unmodified and the modified oligonucleotides to obtain the intact oligonucleotide mass measurement for each compound.

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## Experimental

### Reagents and Sample Preparation

Triethylamine (TEA, 99.5% purity, catalogue number 65897-50ML) and methanol (LC-MS grade, catalogue number 34966-1L) were obtained from Honeywell (Charlotte, NC), while 1,1,1,3,3,3-hexafluoro-2-propanol (HFIP, 99% purity, catalogue number 105228-100G) was purchased from Sigma Aldrich (St Louis, MO). HPLC grade deionized (DI) water was purified using a MilliQ system (Millipore, Bedford, MA). Mobile phases were prepared fresh and used on the same day. Seven different types of oligonucleotide modifications were investigated. Please see the Results section for a detailed description of each oligonucleotide sequence. In addition to the modified oligonucleotide sequence (labeled as a TEST compound), an unmodified oligo with identical sequence (labeled as the CTRL compound) was purchased from the same oligonucleotide manufacturer (ATDBio, Southhampton, UK). Both TEST and CTRL oligonucleotide samples were dissolved in DI water in the same sample vial at a concentration of 1  $\mu$ M, from which a 10  $\mu$ L volume was injected.

### LC-MS System

BioAccord System incorporating the ACQUITY RDa Detector, ACQUITY UPLC I-Class Plus and ACQUITY UPLC TUV

### LC Conditions

Column: OST Column, 2.1 x  
50 mm, packed  
with 1.7  $\mu$ m C<sub>18</sub>  
particles (P/N:  
186003949)

Column temperature: 60 °C or 75 °C as  
indicated in the  
Results section

Flow rate: 300  $\mu$ L/min

Mobile phases: Solvent A: 80 mM

HFIP  
(hexafluoroisopropanol),  
7 mM TEA  
(triethylamine) in  
DI water

Solvent B: 40 mM  
HFIP, 3.5 mM TEA  
in 50%  
methanol/50% DI  
water

Gradient table: The optimized  
gradient  
conditions are  
indicated in the  
Results section for  
each type of  
oligonucleotide  
modification

Sample  
temperature: 6 °C

Sample vials: QuanRecovery  
Max Peak, 300 µL  
vials (P/N:  
186009186)

Injection volumes: 10 µL

Wash solvents: 50% MeOH

Sample Manager  
wash solvent: 50% MeOH

Seal wash: 20% acetonitrile in  
DI water

## MS Conditions

Ionization mode: ESI(-)

Capillary voltage: 0.8 kV

Cone voltage: 40 V

Source temperature: 120 °C

Desolvation temperature: 400 °C

Desolvation gas (N<sub>2</sub>)  
pressure: 6.5 bar

TOF mass range: 400–5000

Acquisition rate: 2 Hz

Lock-mass: waters\_connect lockmass  
solution (P/N: 186009298)

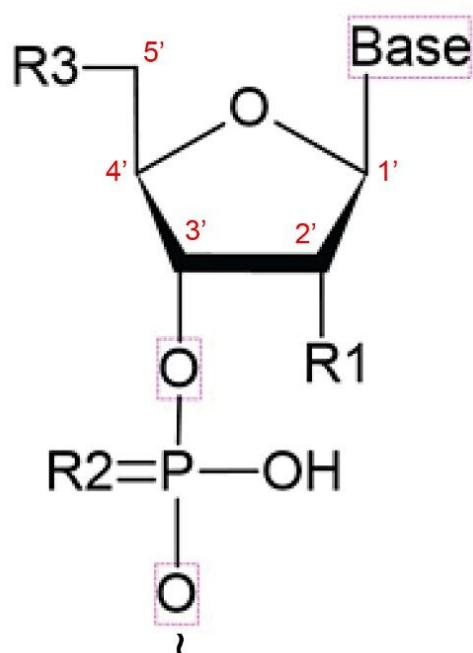
IDC setting: On

Data acquisition and  
processing software: waters\_connect

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## Results and Discussion

Therapeutic oligonucleotides often incorporate chemically modified nucleotides in order to resist degradation by naturally occurring nucleases. Three major types of chemical modifications including backbone, sugar and nucleobase modifications are observed. While therapeutic oligos are mostly modified on backbone and sugar groups, the nucleobase modification is the preferred target for oligonucleotides used in molecular diagnostics. Oligonucleotides with seven common modification types were analyzed in this study, including 2'-O-methoxyethyl (2'-MOE), N-Acetylgalactosamine (GalNac), 2'-O-Methyl (OMe), 2'-Fluorine (F), phosphorothioates (PPT), 6-Fluorescein amidite (FAM), as well as the locked nucleic acid structures (LNA). Although the PS abbreviation is frequently used in the scientific literature when referring to phosphorothioated oligonucleotides, this application note and the isotopic model used for such compounds in waters\_connect uses the PPT abbreviation. Figure 2 illustrates these sites of modifications via the R1, R2 and R3 functional groups attached to the oligonucleotide chemical structure. Three of these functional groups, generally labeled as R1, are typical modifications employed by the pharmaceutical industry which are introduced in the 2'-position of the ribose structure. Two other modifications (R2: GalNac or FAM) were selected as typical groups attached to the 5'-end of oligonucleotides. Finally, two modifications of either the backbone (PPT, R3) or the ribose moiety (LNA) were also investigated. Overall, these seven different types of modifications cover a wide range of the most common modifications currently employed by the pharmaceutical industry.



R1: F, OMe or MOE replacing OH

R2: S replacing O

R3: GalNac or FAM replacing OH

Figure 2. Oligonucleotide chemical structure displaying six types of modifications investigated in this study. In addition, oligonucleotide structures containing the LNA (locked nucleic acid) configuration – which contains an extra methylene group linking the 2' with the 4' position, were also analyzed.

The first oligonucleotide sample analyzed was a tri-component mixture containing a 20-mer unmodified oligonucleotide with the sequence 5'-GCC TCA GTC TGC TTC CAC CT-3' labelled as Oligo1 CTRL, along with two modified versions of the oligonucleotide. The first modified oligonucleotide contained a GalNac modification attached via a C3-linker to the 5'-end of the Oligo1 CTRL (labeled as Oligo 1 GalNac). The second modified oligonucleotide (a 21-mer labeled Oligo1 TEST) consists of 8 unmodified nucleotides from the original sequence, and 13 modified nucleotides. These modifications include the 2'-MOE modification on six cytidines (C), two guanosines (G), one adenosine (A), one 5-Me uracil (5-Me U) and three 5-Me deoxycytidines (dC) modifications. The detailed sequences of each Oligo1 oligonucleotides are displayed in Table I, along with their elemental compositions, the calculated average masses as well as the mass accuracies recorded for each intact mass measurement. The conjugation of GalNac moiety to therapeutic oligonucleotides is typically used to increase the ability of these modified oligonucleotides to penetrate the cellular membranes.<sup>1</sup> The 2'-MOE derivatization is commonly used for antisense oligonucleotides (ASO), aptamers and siRNA oligonucleotides to increase their resistance to nuclease degradation, reduce non-specific protein binding and to increase the binding affinity to targeted complementary DNA.<sup>1</sup> Figure 3A displays the UV and TIC chromatogram recorded for the separation of the three oligonucleotide mixture



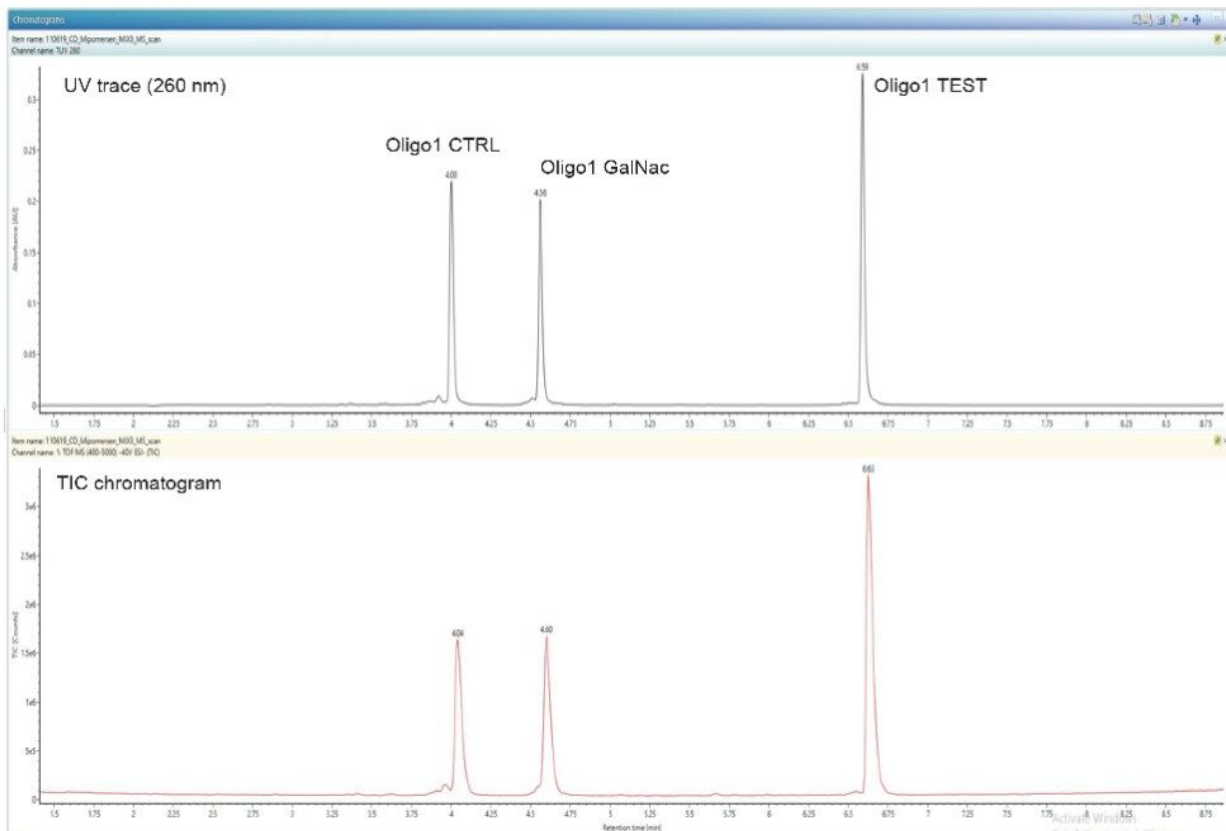
using an initial 5-min gradient from 10 to 30% Eluent B, followed by another linear gradient from 30 to 60% B over 4.9 min. The ESI-MS spectra of the three Oligo1 compounds present in the mixture are presented in Figure 3B. All spectra display a bimodal distribution for the observed charge states, with the lower charge states (from -3 to -5) and the higher charge states (between -6 and -10) reaching two distinct maxima for each oligo. This spectral feature is typical for ion-pair reversed phase separations of modified and unmodified oligos and provides a wide mass range ( $m/z = 600 - 3,000$ ) and a relatively large number of charge states (6-12) for deconvolution. The combined ESI-MS spectra of all three oligonucleotides were processed automatically using the Bayes Spray deconvolution algorithm<sup>6</sup> from waters\_connect software, using the standard oligonucleotide isotopic model and the results are displayed in a screenshot shown in Figure 3B. For all analyzed components, regardless of their modifications and size, a mass accuracy of 15 ppm or better was achieved, allowing for a fast and accurate intact mass confirmation.

Oligo ID	Oligo length	RT (min)	Oligonucleotide sequence	Elemental composition	Average mass (Da)	Mass accuracy (ppm)	
						Oligo model	PPT/PS Oligo model
Oligo1 CTRL	20-mer	4.04	GCC TCA GTC TGC TTC CAC CT	C191 H247 N64 O122 P19	5979.8619	1.6	-
Oligo1 GalNAc	20-mer	4.60	XGCC TCA GTC TGC TTC CAC CT	C216 H291 N67 O134 P19	6589.4655	-3.9	-
Oligo1 TEST	21-mer	6.63	GmCmC mUmCA GTmdC TGmdC TTmdC GmCA mCmCT	C241 H341 N69 O147 P20	7176.1356	-15.2	-
Oligo2 CTRL	28-mer	2.18	CGG AAT CAG TGA ATG CTT ATA CAT CCG T	C274 H345 N104 O166 P27	8587.5598	6.5	-
Oligo2 TEST	28-mer	3.13	5'-FAM -CGG AAT CAG TGA ATG CTT ATA CAT CCG T	C318 H384 N108 O178 P28 F13	9681.3129	-11.8	-
Oligo3 CTRL	16-mer	2.83	TGG CAA GCA TCC TGT A	C156 H197 N60 O94 P15	4881.1854	-0.2	-
Oligo3 TEST	16-mer	3.18	TGG CAA GCA TCC TGT A	C162 H197 N60 O100 P15	5049.2460	-4.0	-
Oligo3 PPT CTRL	16-mer	5.40	T*G*G* C*A*A* G*C*A* T*C*C* T*G*T* A*	C156 H197 N60 O79 P15 S15	5122.1694	-38.1	1.3
Oligo3 PPT TEST	16-mer	5.82	T*G*G* C*A*A* G*C*A* T*C*C* T*G*T* A*	C162 H197 N60 O85 P15 S15	5290.2300	-38.7	7.2

*Table 1. Oligonucleotide sequences of the modified and unmodified compound analyzed in this study, along with their retention times, elemental formulas, expected accurate average masses and mass accuracies resulted after measuring their corresponding intact masses on the BioAccord LC-MS platform.*

*Note: The commonly used acronym for phosphorothioated oligonucleotides is PS, but throughout this application note we used the PPT acronym.*

3A



3B

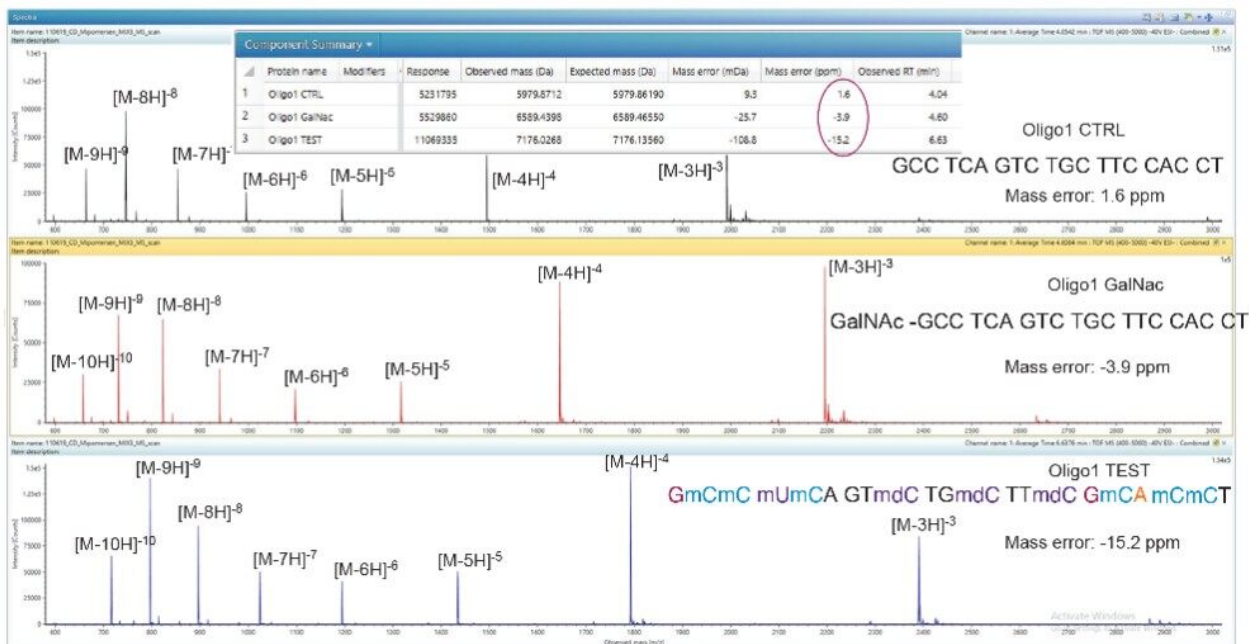


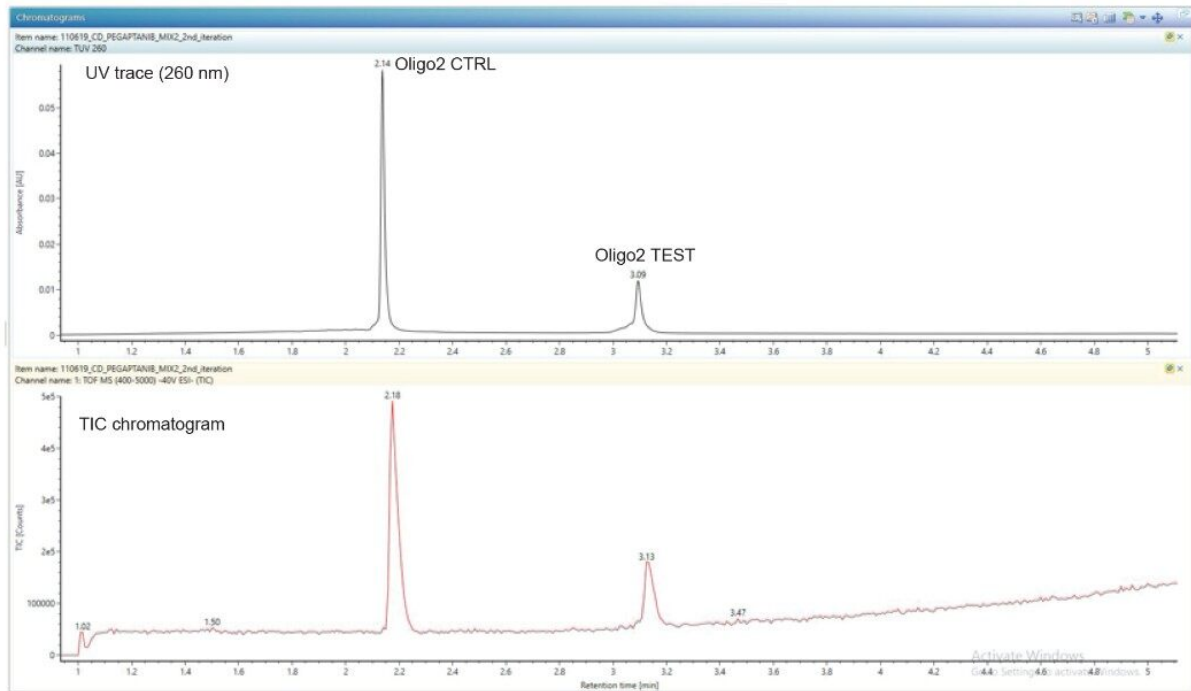
Figure 3. (A) TUV and TIC chromatograms showing the separation of the Oligo1 mixture; (B) ESI-MS spectra

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*recorded for the three components of the Oligo1 mixture. The figure inset displays the waters\_connect results produced after processing the three ESI-MS spectra. The mass accuracy obtained for all three compounds was better than 15 ppm.*

The second oligonucleotide mixture analyzed on the BioAccord LC-MS platform contained two 28-mer oligonucleotides. The unmodified oligonucleotide with the sequence 5'-CGG AAT CAG TGA ATG CTT ATA CAT CCG T-3' is labeled as Oligo2 CTRL. The other component is an extensively modified oligonucleotide labeled as Oligo2 TEST containing 27 modified nucleotides. In addition to the 6-Fluorescein amidite (FAM) moiety attached to the 5'-end of the Oligo2 TEST, two other common modifications are incorporated in the 2'-position of the nucleobase: 2'-O-Methyl (2'-OMe) or 2'-Fluorine. The detailed oligonucleotide structure is displayed in Table I and Figure 4B. It contains eight 2'-OMe adenosines, six 2'-OMe guadenosines, six 2'-F cytosines, as well as seven 2'-F uridines.

4A



4B

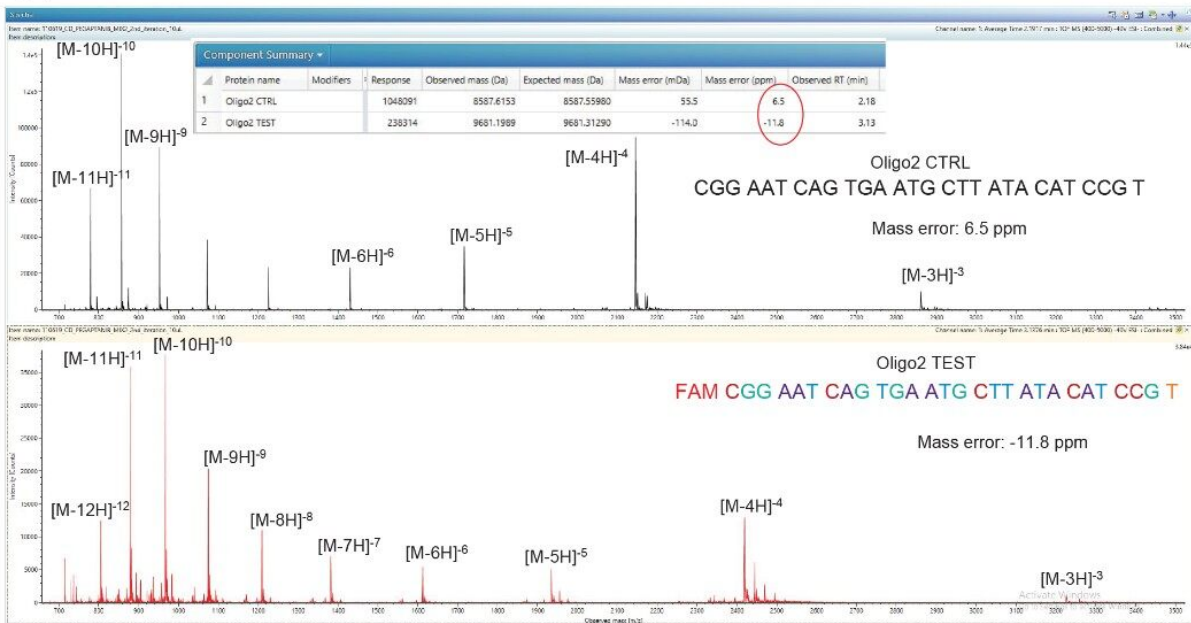


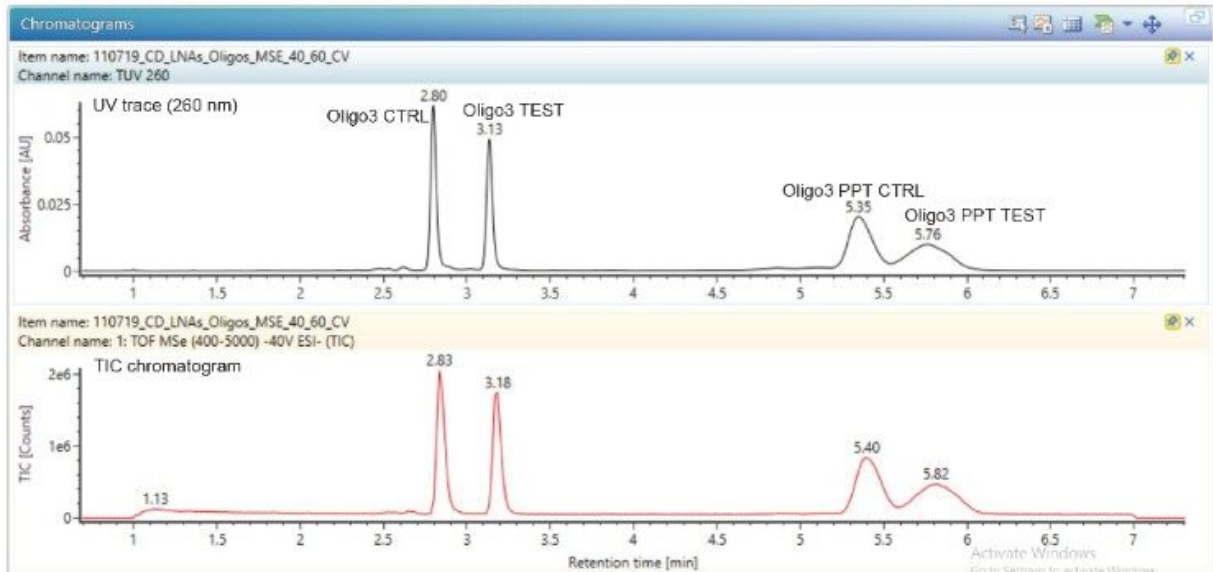
Figure 4. (A) TUV and TIC chromatograms showing the separation of the Oligo2 oligonucleotides; (B) ESI-MS spectra recorded for both components of the Oligo2 mixture; The inset screenshot shows the waters\_connect processing results. The mass accuracy obtained for both compounds was less than 15 ppm.

In other words, every C and U nucleotides are modified by 2'-F and every G and A nucleotides are modified by 2'-OMe. The only nucleotide that is left unmodified is located at the 3'-terminus and is an inverted deoxythiamidine. The Oligo2 TEST compound is an analogue of the Pegaptanib (Macugen) drug approved by FDA in 2004 and marketed by OSI Pharmaceuticals and Pfizer. While Pegaptanib contains a 5'-end modified PEG, the Oligo2 TEST oligonucleotide has a 5'-FAM modification. The 6-FAM conjugation is commonly used for real-time PCR applications involving a variety of oligonucleotide substrates. Both 2'-OMe and 2'-F nucleobases are frequently used in oligonucleotides that are developed for therapeutic or diagnostics applications in order to provide nucleoside resistance to degradation or to achieve a faster binding of these substrates to their targets.<sup>1</sup> In addition, both modifications are associated with good safety profiles for their therapeutic analogues.<sup>1</sup> The Oligo2 mixture was easily resolved on the OST column as illustrated by the UV and TIC chromatograms presented in Figure 4A. The corresponding ESI-MS spectra follow the typical bimodal charge state distribution observed for oligos shorter than 50-mers, as shown in Figure 4B. The processing result of this dataset with waters\_connect is presented in the screenshot shown in Figure 4B and summarized in Table I. As shown with the previous example (from Table I), the mass accuracy obtained for analyzing this set of unmodified and the modified oligonucleotides was better than 15 ppm.

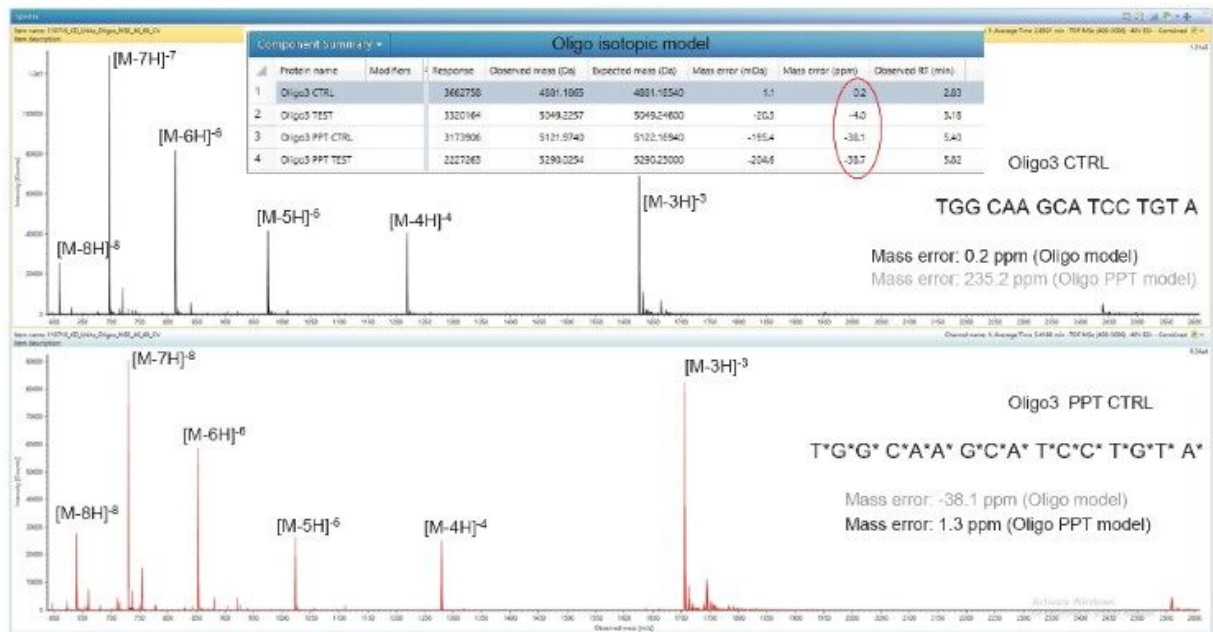
In another experiment, a mixture of four 16-mers with closely related structures was analyzed on the same BioAccord platform. One oligonucleotide pair contained the unmodified sequence 5'-TGG CAA GCA TCC TGT A-3', (labeled as Oligo3 CTRL) and a fully phosphorothioated (PPT) derivative with the same sequence (labeled as Oligo3 PPT CTRL). The commonly used acronym for phosphorothioated oligonucleotides is PS, but throughout this application note we used the PPT acronym. The second oligonucleotide pair contained a modified 16-mer bearing several locked nucleic acid (LNA) structures (Oligo3 TEST) and its corresponding fully phosphorothioated derivative (5'-T\*G\*G\* C\*A\*A\* G\*C\*A\* T\*C\*C\* T\*G\*T\* A\*-3' labeled as Oligo3 PPT TEST). The full sequences of all four oligonucleotides are presented in Table I, along with their elemental formulas and the expected accurate average molecular weights. One of the firstly introduced backbone modifications of oligonucleotides, involves the replacement of an oxygen atom with sulfur in the phosphate backbone to produce a phosphorothioated oligonucleotide. This modification confers significantly more resistance to nuclease degradation to its ASO substrates,<sup>1</sup> thus increasing the tissue elimination half-lives of therapeutic analogues. The LNA conformers of ASO are known to provide unprecedented binding affinity toward specific DNA/RNA targets.<sup>1</sup> This oligonucleotide mixture was resolved by an optimized gradient separation using a 3 min linear gradient from 20 to 28% Eluent B, followed by a 3 min shallow gradient from 28 to 30% B and a 2 min fixed mobile phase composition (set at 30 % B between 6 and 8 min). The UV and TIC chromatograms from Figure 5A indicate that both PPT oligos (Oligo3 PPT CTRL and Oligo3 PPT TEST) produced significantly broader chromatographic peaks compared to their non PPT analogues (Oligo3 CTRL and Oligo3 TEST) probably caused by multiple diastereoisomeric structures present after the replacement of

an oxygen with sulfur in the phosphate backbone. The corresponding ESI-MS spectra recorded for all Oligo3 compounds are displayed in side by side comparisons in Figure 5B and 5C. Because of the unique abundance distribution of the sulfur isotopes, the charge deconvolution of the data generated for PPT molecules needs to employ a different isotopic model than the isotopic model used for non-PPT oligonucleotides, in order to obtain accurate deconvolution results. The PPT Oligo isotopic model takes into account the natural abundances of the sulfur isotopes and fits better with the experimental data. The Oligo3 dataset was deconvoluted with Waters Bayes Spray deconvolution algorithm using either the regular oligo isotopic model or the PPT Oligo isotopic model and the comparison is displayed in Figure 5B and 4C and summarized in Table I. Clearly, accurate mass measurements can only be achieved when the appropriate isotopic model is selected for deconvolution of PPT and non-PPT (regular) oligonucleotides. When the suitable isotopic model is used, the mass accuracy obtained is again better than 15 ppm for both modified and unmodified oligonucleotides.

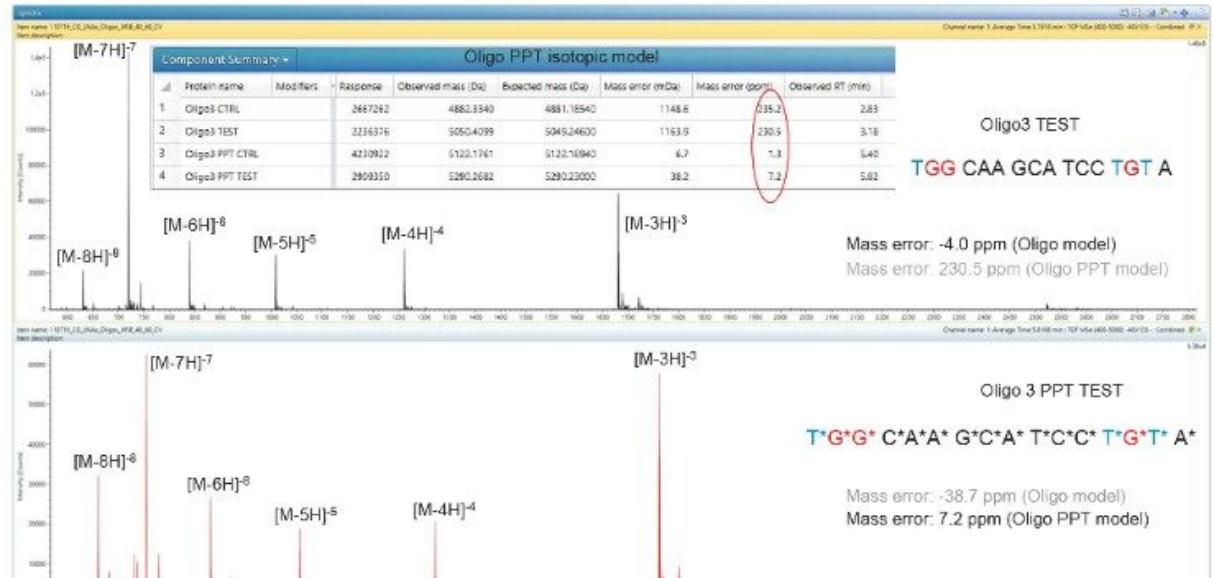
### 5A



### 5B



### 5C



- The Bayes Spray algorithm embedded in waters\_connect provides accurate results for deconvolution of heavily modified oligonucleotides

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