

## HILIC as an Alternative Separation Mode for Intact Mass Confirmation of Oligonucleotides on the BioAccord System

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### Abstract

HILIC chromatography provides a clean, cost-effective separation method that can be easily incorporated into automated workflows for intact mass confirmation of oligonucleotides. As shown in this study, the BioAccord LCMS System enables waters\_connect users to perform rapid and accurate intact mass confirmation of small and medium size oligonucleotides (up to 50-mers).

### Benefits

- An automated, compliance-ready HILIC LC-MS workflow is shown that provides good mass accuracy (better than 15 ppm) for intact mass confirmation of oligonucleotides analyzed with a HILIC LC-MS assay
  - HILIC separation of oligonucleotides offers three main advantages in terms of mobile phase considerations when compared to traditional ion-pair reversed phase (IP-RP) separations: 1) at least 10X or more reduction in mobile phase cost, 2) significantly reduced toxicity and 3) at least a 10x improvement in mobile phase stability (up to two weeks) for LC-MS operation
  - The ACQUITY Premier BEH Amide Column with MaxPeak High Performance Surfaces resulted in no column
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conditioning as compared with the traditional ACQUITY BEH Amide Column in stainless-steel hardware, saving time in column passivation right out of the box

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## Introduction

Oligonucleotide therapeutics have emerged in recent years as a powerful alternative to small molecule and protein therapeutics.<sup>1,2</sup> Manufacturing and quality control of oligonucleotide therapeutics requires highly selective and sensitive LC-MS methods. One of the well-accepted mass spectrometry-based methods for oligonucleotide analysis is using ion-pairing reversed phase chromatography (IP-RP) for oligo separation and detection by MS in negative ESI-MS mode. Using this method for oligonucleotide analysis, an automated workflow employing the BioAccord system operating under waters\_connect for compliance-ready data acquisition, processing and reporting was recently described.<sup>3,4</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 used here is comprised of an ACQUITY UPLC I-Class PLUS System, a Tunable Ultraviolet (TUV) Detector and an ESI-Tof ACQUITY RDa Mass Detector, as shown in Figure 1.



*Figure 1. BioAccord LC-MS System.*

Here we investigated the capabilities of the BioAccord LC-MS system for intact mass confirmation of oligonucleotides using hydrophilic-interaction chromatography (HILIC) as an alternative separation technique to IP-RP. Two recent publications<sup>5,6</sup> indicate that, while not as popular as IP-RP chromatography, HILIC can provide some unique capabilities for oligonucleotide analysis. HILIC mobile phases do not use ion pairing reagents or toxic and expensive volatile modifiers (like the 1,1,1,3,3,3 -hexafluoro-isopropanol-HFIP) and this seems to be the major consideration behind the adoption of this alternative separation technique.<sup>7</sup> The LC-MS data presented in this application note was acquired for three different types of compounds: oligonucleotide polyT standards (OSTs), a modified oligonucleotide (a fully phosphorothioated 25-mer oligonucleotide), and a larger 57-mer oligonucleotide. All datasets were acquired in full scan MS mode and processed in waters\_connect using the BayesSpray mass spectral charge deconvolution algorithm to produce accurate intact mass measurements for each compound.

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## Experimental

### Reagents and Sample Preparation

Ammonium acetate reagent (LiChropur, catalogue number 5.33004.0050) was purchased from Millipore Sigma (St Louis, MO). Acetonitrile (LC-MS grade, catalogue number 34881-1L) was obtained from Honeywell (Charlotte, NC). HPLC grade deionized (DI) water was purified using a MilliQ System (Millipore, Bedford, MA). The MassPREP OST (Oligonucleotide Separation Technology) standard (Waters p/n: [186004135 <https://www.waters.com/nextgen/us/en/shop/standards--reagents/186004135-massprep-oligonucleotide-standard.html>](https://www.waters.com/nextgen/us/en/shop/standards--reagents/186004135-massprep-oligonucleotide-standard.html) ) was dissolved in Solvent A (10 mM ammonium acetate in 75% acetonitrile) to prepare a solution with a concentration of 10  $\mu$ M. The 25-mer fully phosphorothioated oligonucleotide (25-mer PS oligo with the sequence: 5'-C\*T\*C\*T\*C\* G\*C\*A\*C\*C\* C\*A\*T\*C\*T\* C\*T\*C\*T\*C\* C\*T\*T\*C\*T\*-3') and the 57-mer oligonucleotide with the sequence: 5'-CAA TAT TTT ACA TGA ACT GGA GGT CCG TCA ATG ACA GTG TAG GCT GGA GCT GCT TCG-3' were both purchased from Integrated DNA Technologies (Coralville, IA). Both oligos were dissolved in Solvent A (10 mM ammonium acetate in 75% acetonitrile) at a concentration of 10  $\mu$ M. The injection volume was 2  $\mu$ L for all oligo samples.

### LC-MS System

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

### LC Conditions

Column:	ACQUITY Premier BEH Amide 1.7 $\mu$ m, 130 Å, 2.1 x 50 mm, p/n: 186009504
Column temperature:	60 °C
Flow rate:	300 $\mu$ L/min
Mobile phases:	Solvent A: 10 mM Ammonium acetate, 75% acetonitrile in DI water

Solvent B: 10 mM Ammonium acetate, 25%  
acetonitrile in DI water

Sample temperature: 6 °C

Sample vials: QuanRecovery MaxPeak Vials (p/n: 186009186)

Injection volumes: 1 µL

## Gradient Table

Time (min)	Flow rate (mL/min)	Solvent A composition (%)	Solvent B composition (%)	Curve profile
0.00	0.3	100	0	Initial
1.00	0.3	100	0	
16.00	0.3	50	50	6
20.00	0.3	50	50	6
21.00	0.3	10	90	6
22.00	0.3	10	90	6
23.00	0.3	100	0	6
30.00	0.3	100	0	6

## Wash solvents

Purge solvent: 10 mM Ammonium acetate, 25% acetonitrile in DI water

Sample manager wash solvent: 10 mM Ammonium acetate, 25% acetonitrile

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

Lockmass: waters\_connect lockmass solution (p/n: 186009298)

Data acquisition software: waters\_connect

Data processing software: waters\_connect

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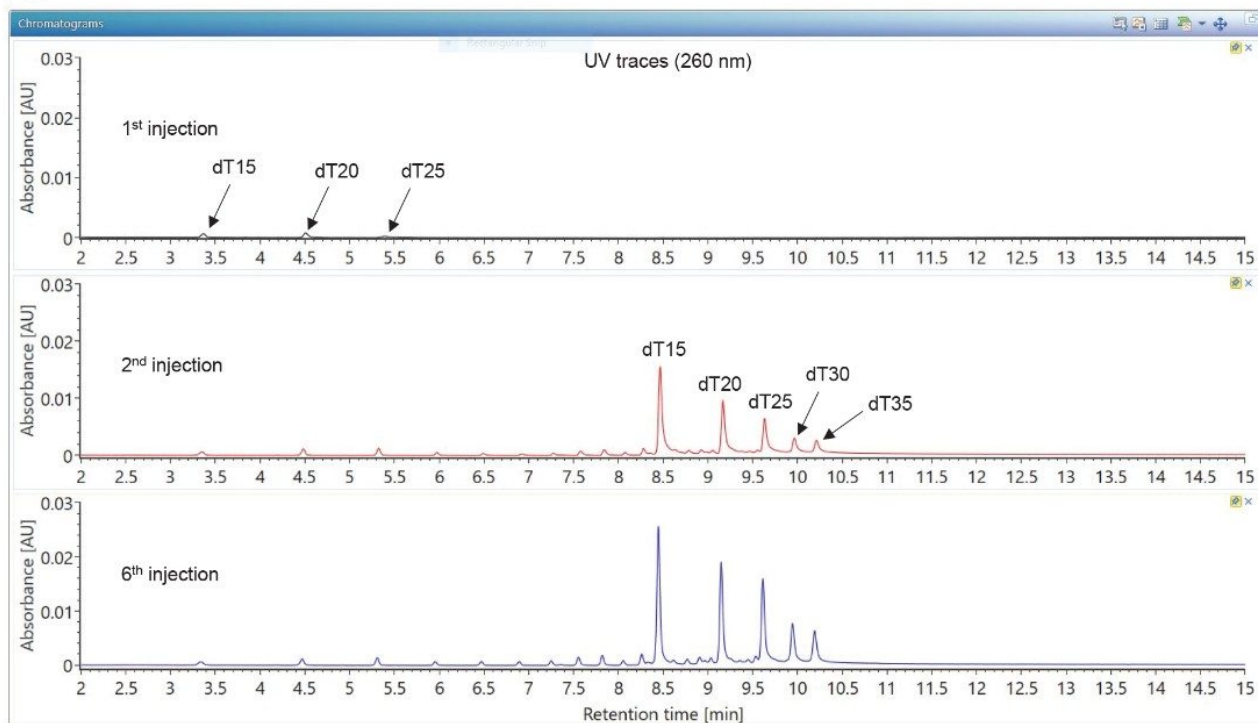
## Results and Discussion

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HILIC separations of the Waters MassPREP oligonucleotide standard mixture (OST standard) were performed on a regular ACQUITY UPLC BEH Amide Column (p/n: [186004800 <https://www.waters.com/nextgen/us/en/shop/columns/186004800-acquity-uplc-beh-amide-column-130a-17--m-21-mm-x-50-mm-1-pk.html>](https://www.waters.com/nextgen/us/en/shop/columns/186004800-acquity-uplc-beh-amide-column-130a-17--m-21-mm-x-50-mm-1-pk.html) ) and on a recently introduced ACQUITY Premier BEH Amide Column (p/n: [186009504 <https://www.waters.com/nextgen/us/en/shop/columns/186009504-acquity-premier-beh-amide--column-17--m-21-mm-x-50-mm-1-pk.html>](https://www.waters.com/nextgen/us/en/shop/columns/186009504-acquity-premier-beh-amide--column-17--m-21-mm-x-50-mm-1-pk.html) ). The latest column belongs to a family of columns packed with sub 2  $\mu$ m particles, featuring the MaxPeak High Performance Surfaces (HPS) Technology.<sup>8-12</sup> Oligonucleotides contain a negatively charged phosphate backbone known to interact with metal surfaces (like stainless-steel casing or frits) typically used in conventional UPLC/HPLC columns. These interactions are often responsible for oligonucleotide losses (poor column recovery), poor chromatographic peak shapes, or poor data reproducibility. Typically, a series of injections onto a newly installed column with a highly concentrated oligonucleotide sample (>20 picomoles on-column), are needed to passivate the column and to mitigate the unwanted interactions between the analyte and the metal surfaces along the fluidic pathway.

In the example shown in Figure 2A, up to six injections were necessary to stabilize the UV response for the five major oligonucleotides contained in the OST mixture in the case of using a regular new ACQUITY BEH Amide Column. Noticeably, the first injection displayed very poor retention and significant analyte loss, even with a high sample load (20 picomoles OSTs injected on-column). In subsequent injections, the interactions between analytes and metal surfaces impacted the separation to a lesser extent. However, even after the column is considered to be well passivated, (e.g. after six consecutive injections of 20 picomoles OSTs on column), the recovery of the larger oligonucleotides (dT25, dT30, and dT35) was still poor. In contrast, the first series of injections performed on the ACQUITY Premier BEH Amide Column, displayed in Figure 2B, indicate that all five major oligonucleotides have consistent UV responses. In addition, the ACQUITY Premier BEH Amide Column was able to resolve in reproducible fashion twenty low-level oligonucleotide impurities (failed sequences), ranging from dT3 to dT24. The stability of the UV response, illustrated by the reproducibility of these chromatograms, indicates that no column passivation was required for achieving these separations. The MaxPeak HPS layer acts like a barrier between the metal surfaces and the oligonucleotides prone to chelate to these surfaces, significantly reducing these unwanted interactions. As demonstrated here, the ACQUITY Premier BEH Amide Column is capable of performing high-resolution oligonucleotide separations from the first injection without column conditioning.

2A



2B

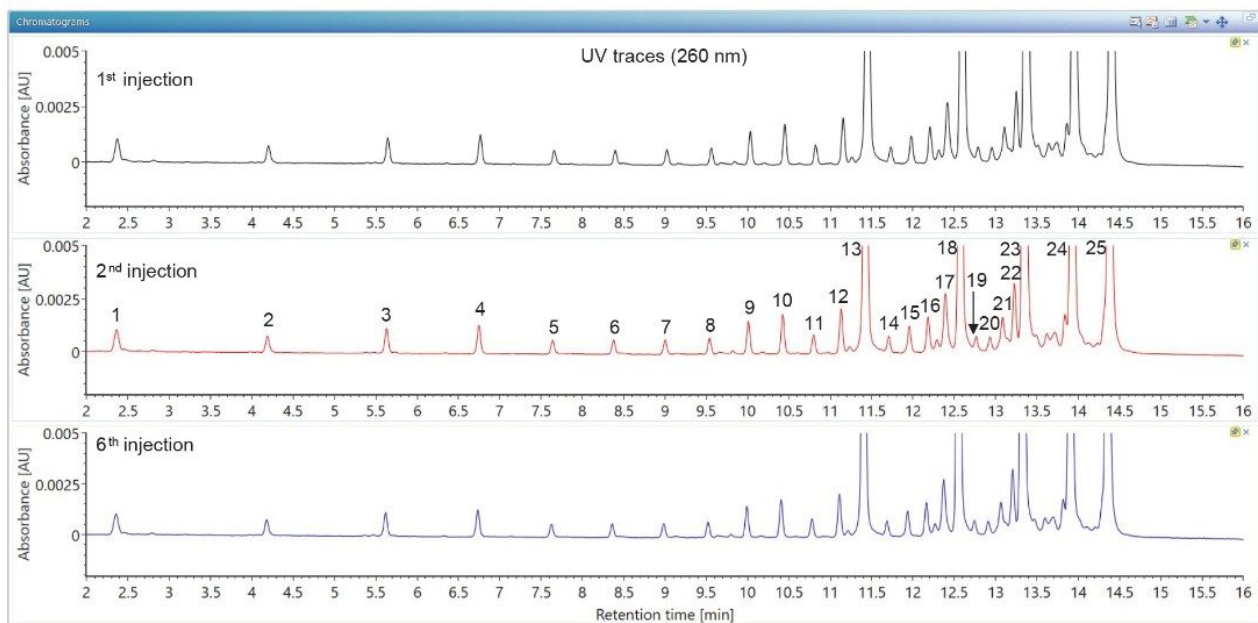


Figure 2. TUV chromatograms showing the first consecutive injections of the OST mixture performed on 2.1 x 50



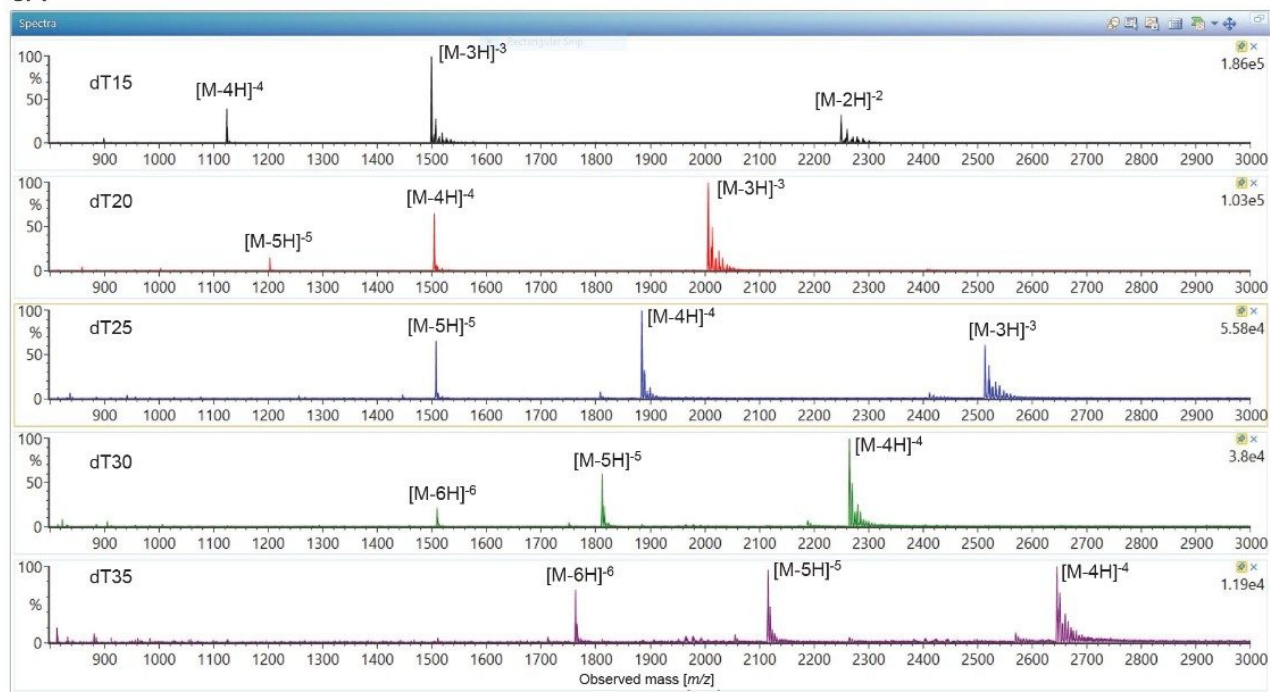
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*mm columns: (A) conventional stainless-steel ACQUITY UPLC BEH Amide Column (p/n: [1860004800](#)); (B) ACQUITY Premier BEH Amide Column (p/n: [186009504](#)). The conventional column required extensive conditioning before a stable UV signal could be produced, while the ACQUITY Premier Column does not require any conditioning. Reproducible chromatographic separations can be obtained even for low-level impurities on the ACQUITY Premier Column without any need for column conditioning/passivation. The oligonucleotides separated in Figure 2B correspond to the following failure sequences of deoxythiamidates: Peak 1-dT3, 2-dT4, 3-dT5, 4-dT6, 5-dT7, 6-dT8, 7-dT9, 8-dT10, 9-dT11, 10-dT12, 11-dT13, 12-dT14, 13-dT15 (major component), 14-dT16, 15-dT17, 16-dT18, 17-dT19, 18-dT20 (major component), peaks 19-22 correspond to dT21-24 according to their elution order, and peaks 23-25 belong to the major components dT25, dT30, and dT35.*

The HILIC ESI-MS spectra recorded for the five major oligonucleotides present in the MassPREP OST Standard sample are displayed in Figure 3A. While the IP-RP ESI-MS spectra of the same compounds display a bimodal distribution with a variety of charge states<sup>3</sup>, the HILIC ESI-MS spectra show significantly less charges, with mainly three abundant charge states observed for each oligonucleotide. IP-RP chromatographic separations rely on positively charged alkyl amines dissolved in high pH mobile phases (pH 8–10) to form strong ion pairs with the negatively charged phosphate backbone of oligonucleotides. While these non-covalent interactions are beneficial and essential for achieving oligonucleotide separations (because the alkyl amines subsequently interact with the C<sub>18</sub> hydrophobic chains of RP columns), they can also affect the oligonucleotide structure as demonstrated by the bimodal distribution of the ESI-MS oligonucleotide spectra recorded in IP-RP chromatography.<sup>3</sup> IP-RP chromatography partially denatures the OST oligonucleotides and produces two charge state distributions: one containing the lower charge states (from -3 to -5) corresponding to a native conformation and a denatured oligo conformation containing the higher charge states (from -7 and -15). In the case of HILIC separations there is no ion pairing reagent needed, and the oligonucleotide retention is based on a partitioning mechanism between the mobile phase and a water-rich layer, partially immobilized on the stationary phase.<sup>13–14</sup> It seems that HILIC interactions preserve the native state conformation of the OSTs as reflected in the corresponding ESI-MS spectra displayed in Figure 3A which contain a limited number of charge states (only three), all detected at a relatively high mass range ( $m/z = 1000–3000$ ). BayesSpray deconvolution of these much-simplified ESI-MS spectra (contrasting with the 7–12 charge state spectra typically observed in the IP-RP spectra) produces very accurate intact mass measurements as illustrated by the processing results shown in Figure 3B. The mass accuracy obtained for all major OSTs was better than 15 ppm, as previously observed for IP-RP separations as well.<sup>3</sup> The HILIC ESI-MS spectra displayed in Figure 3A contained a significantly higher level

of Na and K adduct ions (20–40% of the MS response of the naked oligonucleotide signal) probably indicating a tight association between native oligonucleotides and trace levels of metals present in the mobile phase. As a result, the HILIC LC-MS assay is not as sensitive as the denaturing IP-RP LC-MS assay.

3A



3B

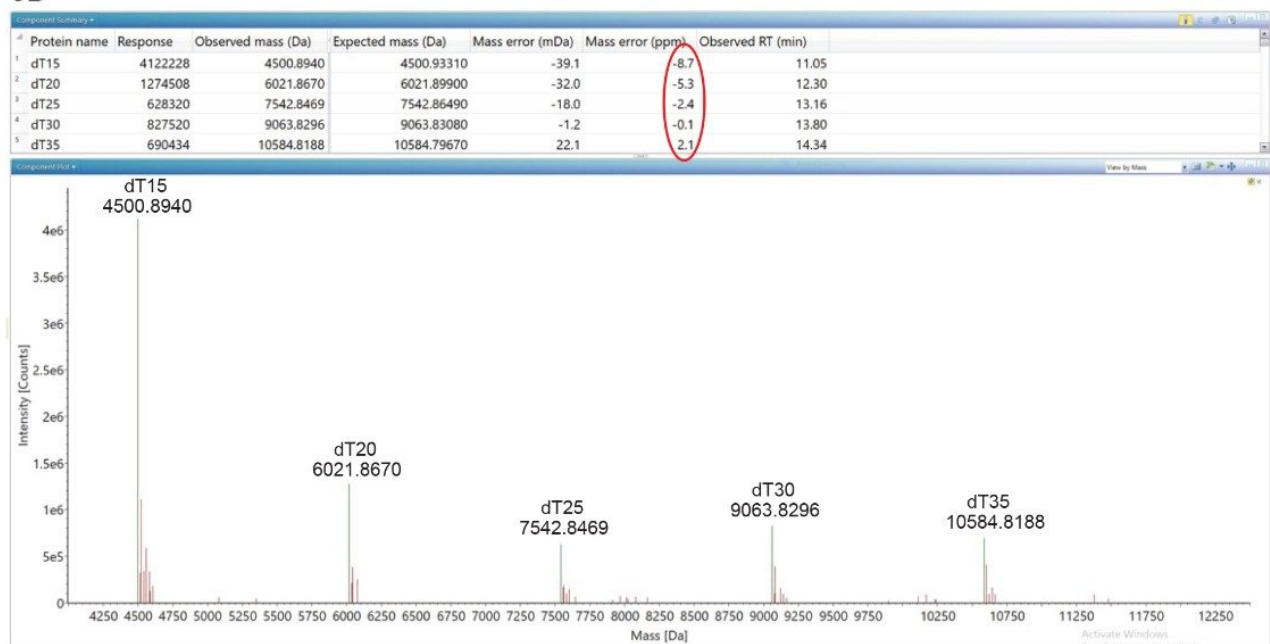


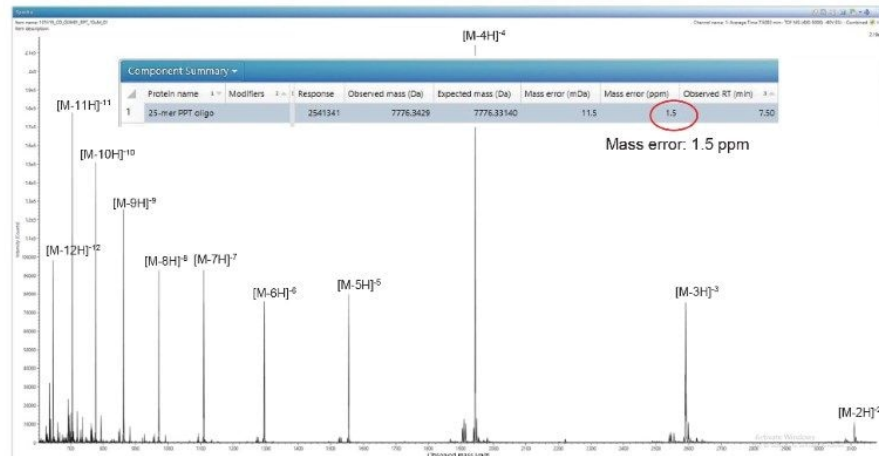
Figure 3. (A) HILIC ESI-MS spectra recorded for the five components of the OST mixture. Only three abundant charge states were detected for all oligonucleotides, suggesting that all OSTs adopted a native state

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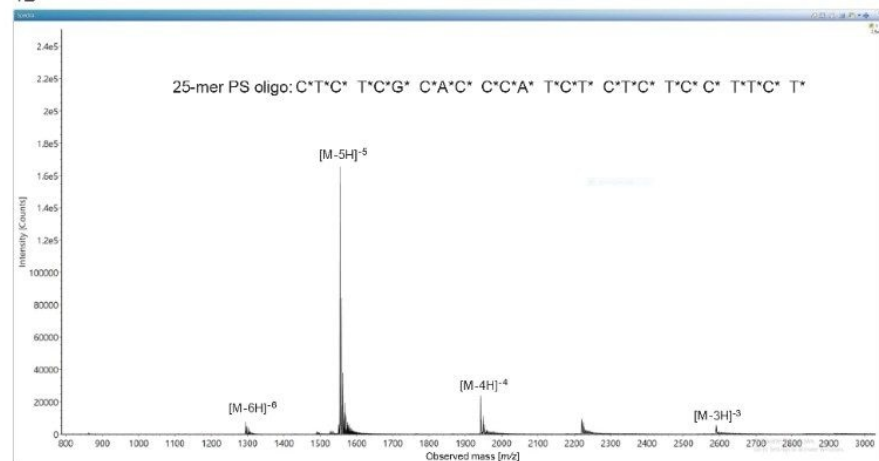
*configuration in the gas phase; (B) Charge deconvoluted OST spectra produced after BayesSpray processing of the OST spectra in waters\_connect Software. The mass accuracy obtained for all five compounds was better than 15 ppm.*

A 25-mer fully phosphorothioated oligonucleotide (25-mer PS oligo), that was a candidate therapeutic agent for human immunodeficiency virus type 1 (HIV-1)<sup>15,16</sup> was also analyzed by HILIC LC-MS using the same experimental conditions employed for the MassPREP OST Standard mixture. The corresponding ESI-MS spectrum displayed in Figure 4B, shows only four charge states, again pointing to a native state oligonucleotide conformation. This HILIC spectrum is very different from the much “busier” IP-RP spectrum of the same compound from Figure 4A, which contains a total of eleven charge states (see reference [3] for additional information). Nevertheless, the BayesSpray deconvoluted spectrum obtained following data processing in waters\_connect Software, provided very good mass accuracy (-4.0 ppm mass error) as indicated by the screenshot from Figure 4C. To achieve this mass accuracy, it was critical to select the phosphorothioated (PS) oligonucleotide isotopic model in the processing method, specifically designed for these types of oligonucleotides containing many sulfur atoms.

4A



4B



4C

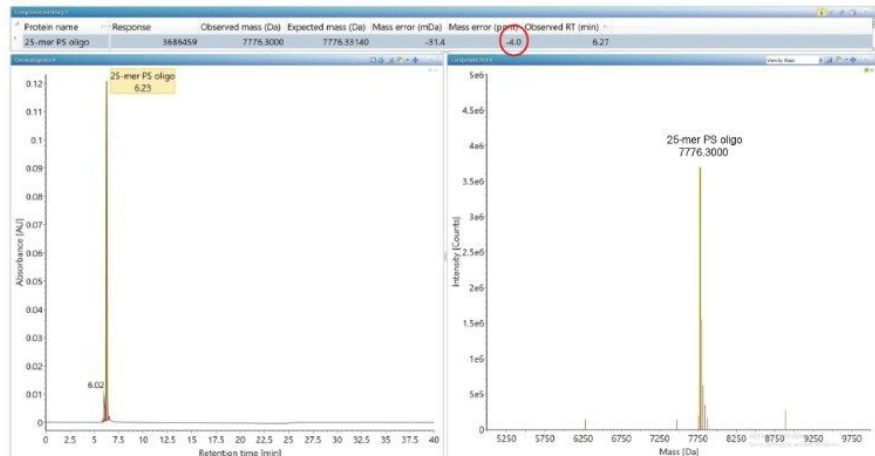


Figure 4. (A) IP-RP ESI-MS spectrum of the 25-mer PS (fully phosphorothioated) oligonucleotide. The inset of this

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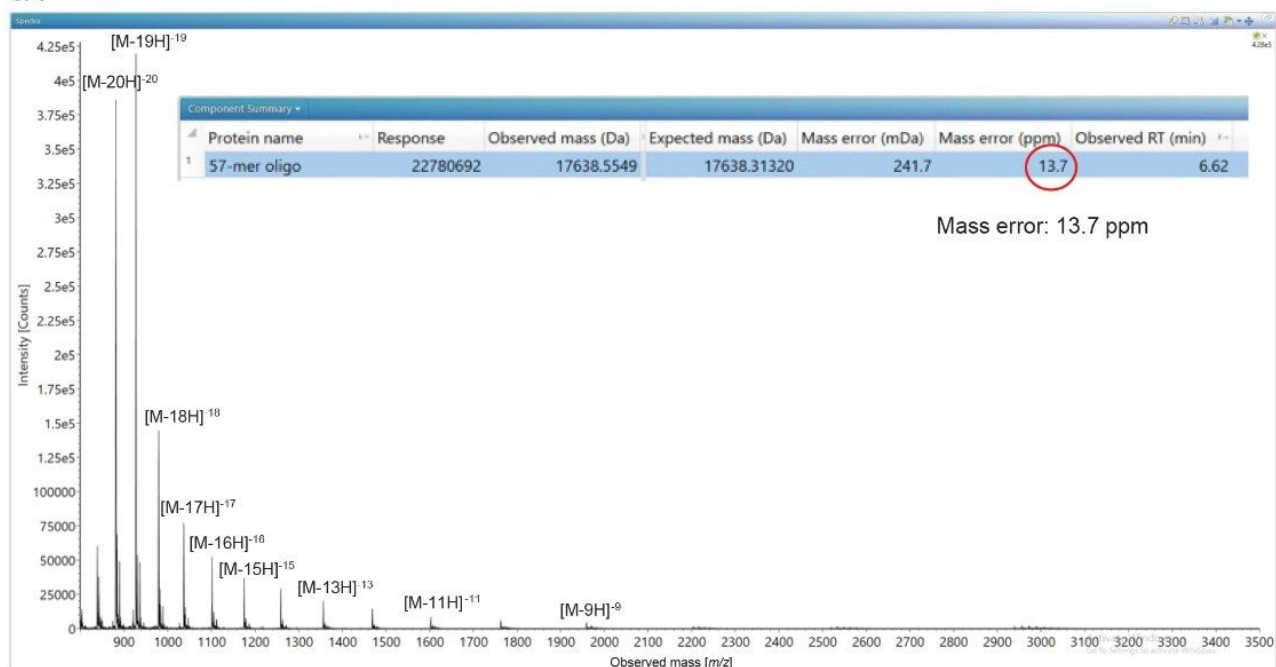
*figure contains a screenshot from the waters\_connect processing method indicating that the measured charge deconvoluted mass was only 1.5 ppm off from the expected mass. (B) HILIC ESI-MS spectrum recorded for the same oligonucleotide. Only four charge states were detected, indicating a native state conformation for this oligonucleotide. (C) Spectrum showing the charge deconvolution result for the HILIC ESI-MS spectrum.*

The discrepancy between the charge states observed in IP-RP versus HILIC ESI-MS spectra was maintained even for larger oligonucleotides. A 57-mer oligonucleotide produced a wide variety of charge states (thirteen) in IP-RP chromatography (see Figure 5A), while HILIC chromatography generated only three completely different charge profiles (Figure 5B). Even with less charge states available for deconvolution, the HILIC ESI-MS spectrum produced a similar result following BayesSpray processing in waters\_connect: 10.2 ppm mass error vs 13.7 ppm for IP-RP ESI-MS spectrum. In both cases, the mass accuracy of the intact mass measurement was better than 15 ppm.

Besides the charge state difference in the ESI-MS spectra recorded in IP-RP vs HILIC modes, the latest chromatographic separation can bring some advantages to the operating costs of an LC-MS assay for intact mass confirmation of oligonucleotides. The typical cost of an IP-RP LC-MS mobile phase is about ten times higher than that of the HILIC mobile phase, assuming that an equivalent volume of mobile phase is prepared. This is mainly because of the high cost of LC-MS grade HFIP, a modifier with high toxicity required to increase the mass spectrometric response of oligonucleotides. This modifier is not required for HILIC mobile phases, which makes this separation mode even more attractive since the toxicity associated with IP-RP mobile phases can be avoided as well. Also, the LC-MS signal for the oligonucleotides analyzed by IP-RP starts to decrease about 24 hours after mobile phase preparation, requiring frequent preparation of small volumes of mobile phases (200–500 mL daily).<sup>17</sup> However, the stability of the UV oligonucleotide signal is not affected, so for applications using only optical detection, the mobile phase does not have to be prepared daily. To investigate the stability of the ESI-MS signal of oligonucleotides with HILIC separations, the peak area of the dT15 oligonucleotide recorded with both MS and UV detectors was monitored daily. Two large volume (1-L) mobile phases were prepared for Eluents A and B of the HILIC mobile phase and replicate injections (n=3) of the 10  $\mu$ M OST sample were performed daily for a duration of two weeks (14 days). The ESI-MS signal was monitored by the peak area of the extracted mass chromatogram generated for the triply charged monoisotopic mass  $[M-3H]^{-3}$  at  $m/z = 1498.57$  of the dT15 oligonucleotide present in the OST mixture. This area was compared with the peak area obtained from the UV chromatogram recorded at 260 nm for the same oligo and a ratio for MS vs UV signal was calculated by dividing the MS vs UV peak areas for each injection. This peak area ratio is plotted in Figure 6 and

it shows a very constant response over the entire two weeks period. This graph clearly indicates the stability of the HILIC mobile phase during this timeframe, highlighting one of the advantages of this HILIC LC-MS assay over IP-RP assays: the IP-RP analysis of oligos needs more frequent mobile phase preparation.

5A



5B

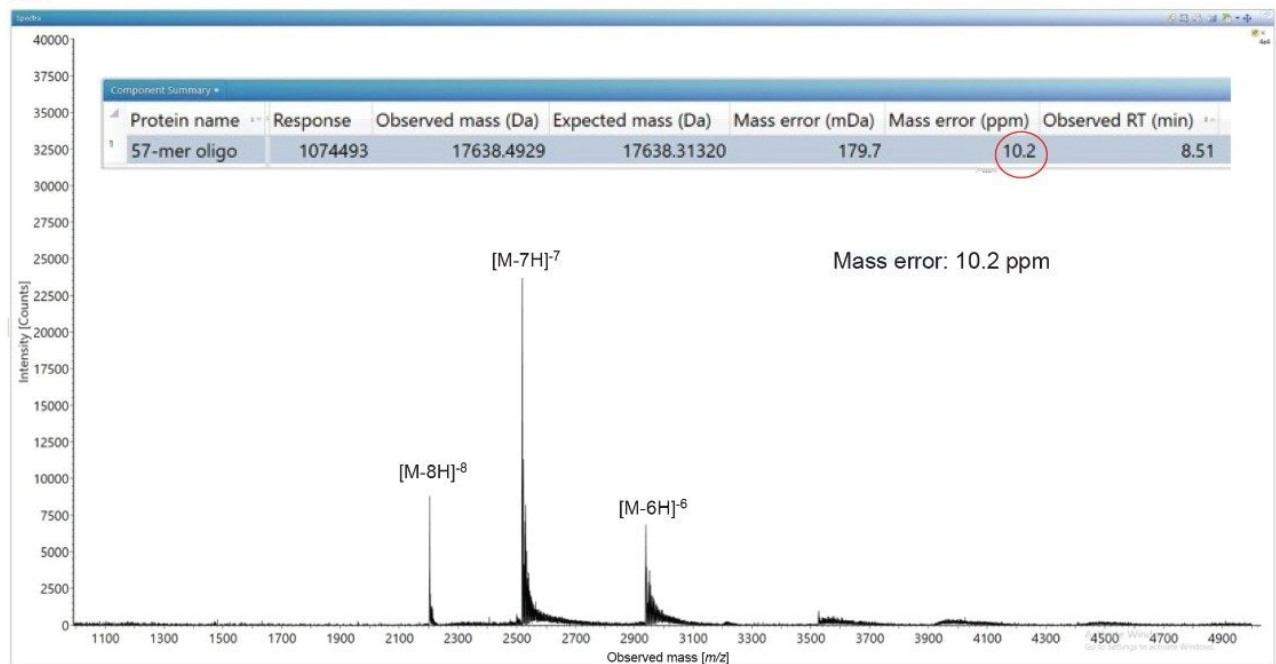
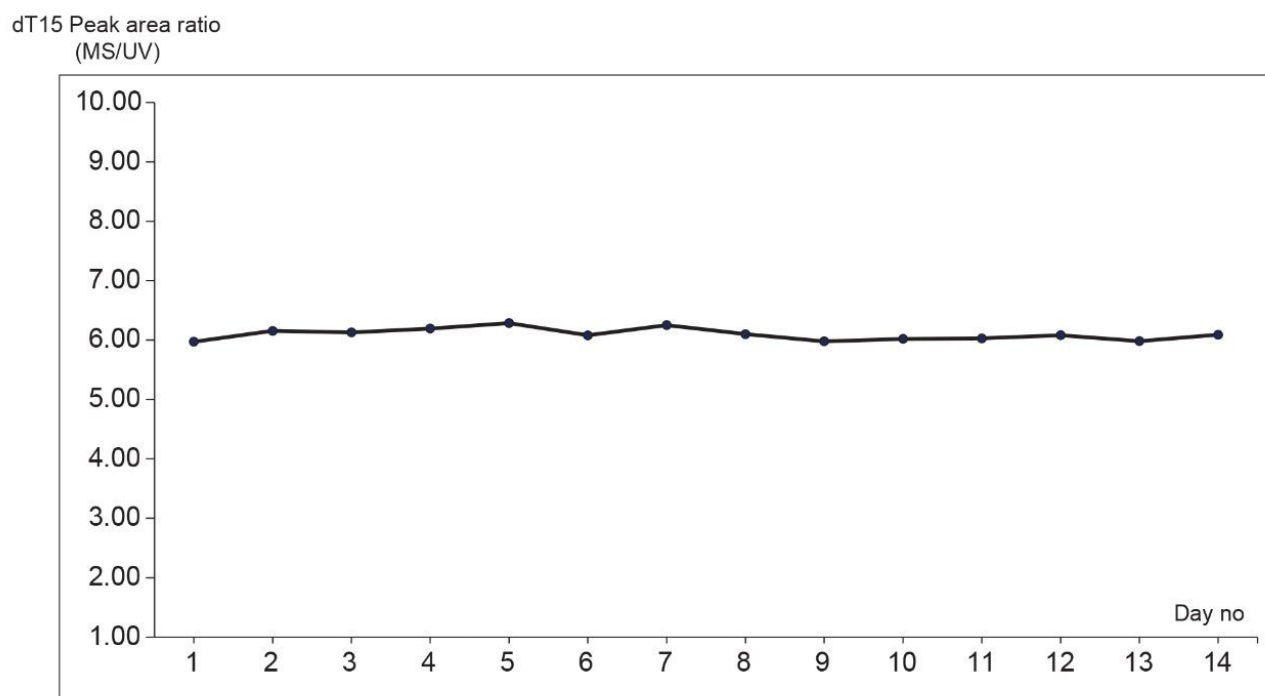


Figure 5. ESI-MS spectra of a 57-mer oligonucleotide: (A) IP-RP ESI-MS spectrum showing a wide distribution of charge states; (B) HILIC-ESI MS spectrum displaying only three unique charge states, not detected by IP-RP



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chromatography. The insets of each panel highlight the mass errors obtained after BayesSpray charge deconvolution, both measurements gave mass accuracies under 15 ppm.



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Figure 6. Trend plot showing the stability of the HILIC mobile phase during a two-week long experiment. The ratio between the peak area of the dT15 oligonucleotide response recorded in ESI-MS vs the UV peak area was plotted daily. For the MS response, the extracted mass chromatogram of the triply charge oligonucleotide precursor ion ( $[M-3H]^{-3}$  at  $m/z = 1498.57$ ) was used, while the TUV response at 260 nm was used for UV peak area measurements.

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## Conclusion

- A waters\_connect workflow is shown to provide good mass accuracy (better than 15 ppm) for intact mass confirmation of oligonucleotides analyzed with a HILIC LC-MS assay

- The ACQUITY Premier BEH Amide Column with MaxPeak High Performance Surfaces provided increased sensitivity and reproducibility for twenty low-level impurities without the need for column passivation.
- The main benefits of the HILIC based LC-MS assays are centered around the mobile phase composition: the HILIC mobile phases have low toxicity, low cost and longer LC-MS stability compared to IP-RP mobile phases.
- The HILIC LC-MS assay developed here can be considered as a viable alternative for intact mass analysis of oligonucleotides.

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720007395, Revised December 2021

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