

Analysis of Oligonucleotide Impurities on the BioAccord System with ACQUITY Premier

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Abstract

This application note demonstrates an automated, compliance-ready LC-MS workflow for purity analysis and intact mass confirmation of extensively modified oligonucleotides and their impurities.

Benefits

- A category of products incorporating the MaxPeak High Performance Surfaces (HPS), including the ACQUITY Premier UPLC BSM System and the ACQUITY Premier OST Columns, provide critical advantages for oligonucleotide impurity analysis
 - An automated, compliance-ready workflow is shown to provide good mass accuracy (better than 15 ppm) for intact mass confirmation of modified oligonucleotides and their impurities analyzed with an ion-pairing reversed-phase (IP-RP) LC-MS assay
 - In addition, this automated, compliance-ready workflow provides purity information for all the sample components, down to 0.2% abundance levels
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Introduction

Oligonucleotide therapeutics have emerged in recent years as a powerful alternative to small molecule and protein therapeutics.^{1,2} 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 ion-pairing reversed-phase chromatography (IP-RP) separation followed by ESI-MS detection in negative ion mode. Using this method, an automated workflow for oligonucleotide analysis employing the BioAccord System – operating under compliance-ready waters_connect data acquisition and processing software – was recently described.^{3,4} A fully integrated LC-MS system comprised of an ACQUITY Premier UPLC BSM System, a Tunable Ultraviolet (TUV) Detector, and an ESI-Tof ACQUITY RDa Mass Detector (system shown in Figure 1) was used in this application note for the analysis of low-level oligonucleotide impurities. This is a challenging analysis because oligonucleotides contain many negatively charged phosphate groups prone to interact strongly with metal surfaces. To address these challenges, Waters has developed a family of new technologies containing a more inert surface specifically designed to address difficult to analyze analytes, MaxPeak High Performance Surfaces (HPS).⁵⁻¹⁰ The ACQUITY Premier UPLC BSM System has this technology implemented across the entire fluidic path in order to provide a very effective barrier that significantly reduces analyte interactions with all types of metal surfaces. Along with the ACQUITY Premier OST Columns introduced in 2020, the entire UPLC System was recently tested for bioanalysis related LC-MS applications of oligonucleotides.⁹ Here we investigated the capabilities of this UPLC system for intact mass confirmation of oligonucleotides and their associated impurities. All datasets were acquired in full scan MS mode on the BioAccord System with ACQUITY Premier and processed in waters_connect using the BayesSpray mass spectral charge deconvolution algorithm to produce accurate intact mass measurements for each compound.



Figure 1. Components of the BioAccord LC-MS System with ACQUITY Premier.

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. A 21-mer heavily modified oligonucleotide, containing a 2'-OMe modification on 19 of its nucleosides, having the sequence GUA ACC AAG AGU AUU CCA UTT and the elemental composition $C_{229}H_{306}N_{76}O_{143}P_{20}$ was purchased from ATDBio (Southampton, UK). Stock solutions were prepared in DI water at a concentration of 1 μ M (or 2.34 μ g/mL), from which a 10 μ L volume was injected, which corresponds to loading 10 picomoles of the 21-mer oligonucleotide on-column.

LC Conditions

LC-MS system:	BioAccord System with ACQUITY Premier
Columns:	1) ACQUITY Premier OST Column 1.7 μm , 130 \AA , 2.1 x 100 mm, (p/n: 186009485) 2) Conventional ACQUITY OST Column 1.7 μm , 130 \AA , 2.1 x 100 mm, (p/n: 186003950) 3) ACQUITY Premier OST Column 1.7 μm , 130 \AA , 2.1 x 50 mm, (p/n: 186009484)
Column temperature:	60 $^{\circ}\text{C}$
Flow rate:	300 $\mu\text{L}/\text{min}$
Mobile phases:	Solvent A: 40 mM HFIP (hexafluoroisopropanol), 7 mM TEA (triethylamine) in DI water Solvent B: 20 mM HFIP (hexafluoroisopropanol), 3.5 mM TEA (triethylamine) in 50% methanol
Sample temperature:	6 $^{\circ}\text{C}$
Sample vial:	Certified Clear Glass Vials (p/n: 186000327C)
Injection volume:	10 μL
Wash solvents:	Purge solvent: 50% MeOH Sample Manager wash solvent: 50% MeOH Seal wash: 20% acetonitrile in DI water

Gradient

Time (min)	Flow rate (mL/min)	Solvent A composition (%)	Solvent B composition (%)	Curve profile
0.00	0.3	75	25	Initial
25.00	0.3	65	35	6
30.00	0.3	65	35	6
30.50	0.3	15	85	6
32.50	0.3	15	85	6
33.00	0.3	75	25	6
40.00	0.3	75	25	6

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 ₂) pressure:	6.5 bar
Tof mass range:	400–5000
Acquisition rate:	2 Hz
Lock-mass:	waters_connect Lockmass solution (p/n: 186009298)
Data acquisition software:	waters_connect
Data processing software:	waters_connect

Results and Discussion

A 21-mer oligonucleotide containing a variety of low-level oligonucleotide impurities was separated on two C₁₈ columns, a regular 2.1 x 100 mm OST column (p/n: 186003950 <<https://www.waters.com/nextgen/us/en/shop/columns/186003950-acquity-uplc-oligonucleotide-beh-c18-column-130a-17--m-21-mm-x-1.html>>) and a recently introduced ACQUITY Premier 2.1 x 100 mm OST Column (p/n: 186009485 <<https://www.waters.com/nextgen/us/en/shop/columns/186009485-acquity-premier-oligonucleotide-c18-column-130a-17--m-21-x-100-m.html>>). For the 21-nt oligomer, a significant portion of the molecule (19 nucleosides) contained modified nucleobases (as illustrated in the sequence listed in the experimental section and in Figure 2). The 2'-OMe modification was attached to three guanosines (G) labeled in blue and seven adenosines (A) labeled in green in the oligonucleotide sequence. Besides the attachment of the same 2'-OMe functional group to uridines and cytidines, the nucleobases of these two nucleosides were further modified by the attachment of a 5-Methyl group to produce five 2'-OMe 5-Me uridines (U, labeled in purple) and four 2'-OMe 5-Me cytidines (C, labeled in red). The only nucleosides left unmodified are the two deoxythymidines (TT) at the 3'-end of the 21-mer. All the oligonucleotide modifications can be summarized in the following sequence: OMEG OME5mU OMEA OMEA OME5mC OME5mC OMEA OMEA OMEG OMEA OMEG OME5mU OMEA OME5mU OME5mU OME5mC OME5mC OMEA OME5mU dT dT, which uses a specially designed nomenclature. When analyzed on the ACQUITY Premier OST Column, the separation of the 21-nt oligonucleotide reveals a rather complex impurity profile as shown in Figure 2A. Fourteen oligonucleotide impurities were separated and detected by both TUV and MS detectors. Analysis of the same sample on a conventional column (with stainless-steel casing), having the same dimensions and packed with the same stationary phase (C₁₈ 1.7 μ m particles, 130 Å pores), resolved only half (seven) of the same impurities, completely missing a significant portion of the early eluting impurities, as indicated in Figure 2B. A recent publication⁷ indicated that untreated column frits are mainly responsible for analyte adsorption on metal surfaces. The results presented in Figure 2B can be explained by considering the adsorption effects of oligonucleotides on the inlet and outlet frit of a conventional column. When the sample is loaded at the inlet of the column, the major oligonucleotide component can be used to passivate the inlet frit, such that minor oligonucleotide impurities are not adsorbed to this frit. However, after undergoing the IP-RP separations, because most of the oligonucleotide impurities elute before the major component, there is a great possibility that the outlet frit would retain some of these impurities until it gets fully passivated. It is very likely that in the example shown in Figure 2B, the first seven early eluting impurities were not detected because they were adsorbed to the outlet frit.

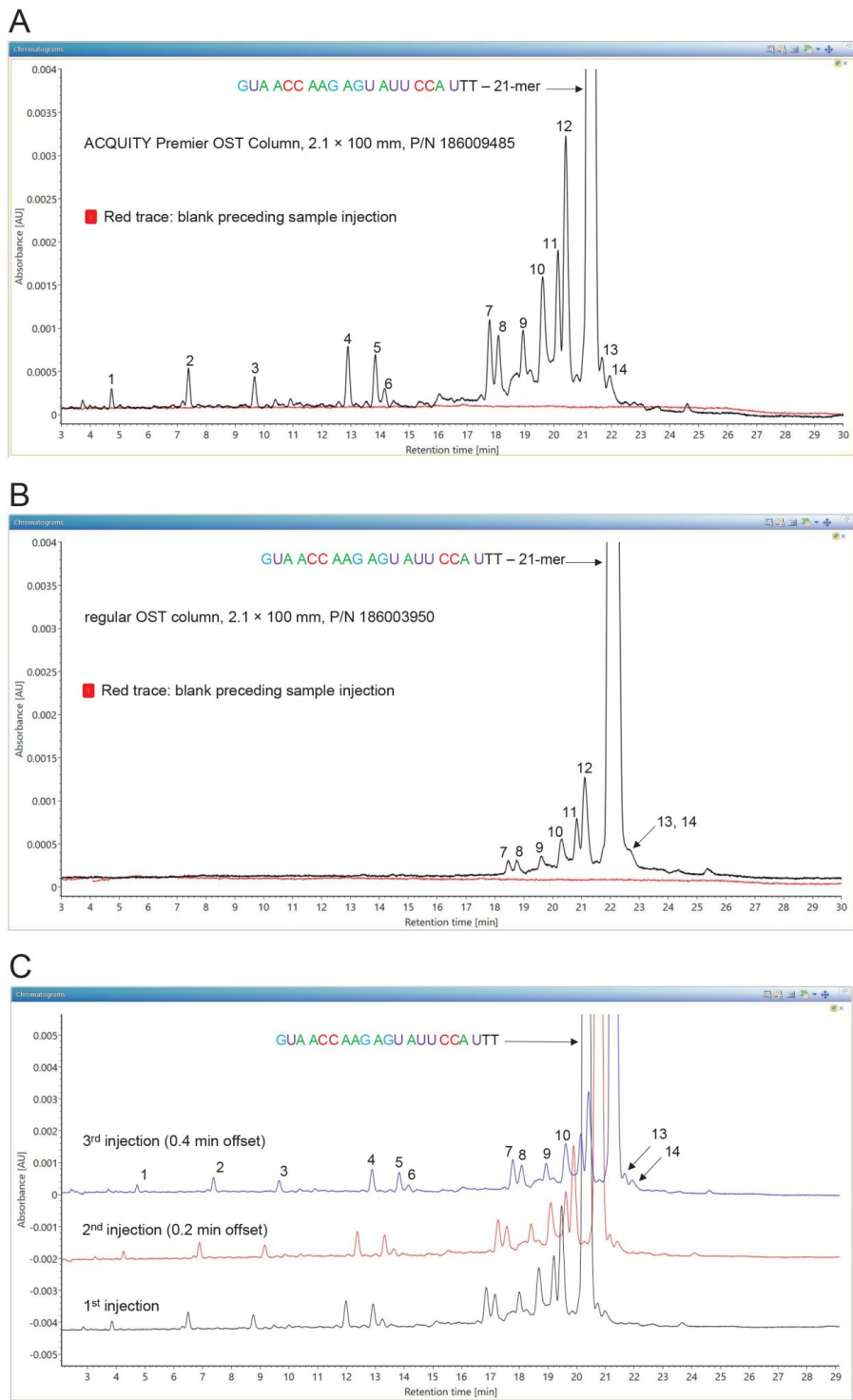


Figure 2. TUV chromatograms showing the separation of oligonucleotide impurities from a 21-nt sample: (A) UV chromatogram recorded on an ACQUITY Premier OST Column; (B) UV chromatogram recorded on an extensively conditioned conventional column. The ACQUITY Premier OST Column belongs to a family of columns packed with sub 2 μm particles, featuring the MaxPeak High Performance Surfaces (HPS) Technology.⁸⁻¹⁰ Oligonucleotides contain a negatively charged phosphate backbone known to interact with metal surfaces (like stainless-steel, titanium, or MP35N—a Ni-Co alloy). Three replicates injections performed on the ACQUITY Premier OST Column. For better clarity, the red and blue traces are offset by +0.2 min from the previous trace.

alloy) typically found in the fluidic path of the UPLC system. These interactions are often responsible for oligonucleotide losses, poor chromatographic peak shapes, or poor data reproducibility. The MaxPeak HPS Technology implemented along the UPLC fluidic path and the OST Column significantly reduced these unwanted interactions, as demonstrated by the number and % abundance (related to UV peak areas) of impurities detected. The conventional OST Column showed much more modest results in terms of impurity recovery, even after extensive passivation. The separations performed on the ACQUITY Premier Column are highly reproducible, as shown in Figure 2C where three replicate injections are overlaid. The ESI-MS spectrum of the major oligonucleotide component is presented in Figure 3A. The lowest abundance impurity (0.18% according to the UV data, see Table 1), confidently identified based on its ESI-MS spectrum, is an 11-mer oligonucleotide missing all 10 nucleosides from the 5'-end of the molecule. The ESI-MS spectrum of this impurity showing two major charge states (doubly and triply charged ions) is displayed in Figure 3B in the bottom panel. There was no signal detected for this impurity in UV (see Figure 2B) and the ESI-MS spectrum recorded at the expected elution time (top panel of Figure 3B) indicates that, very likely, this impurity was trapped (irreversibly adsorbed) by metal surfaces inside the regular column (outlet column frit very likely), as the same ACQUITY Premier UPLC System was used in both experiments. The full list of oligonucleotide impurities detected in the 21-mer analyzed here is displayed in Table 1, along with their sequences, elemental compositions, accurate average masses, and percent abundances calculated using the UV peak areas. The data presented in Table 1 was used for setting up an automated processing using the BayesSpray charge deconvolution algorithm from waters_connect and Figure 4 illustrates the processing results obtained. All fourteen oligonucleotide impurities were detected across a relatively wide dynamic range (~500 fold) with deconvoluted mass accuracies under 15 ppm.

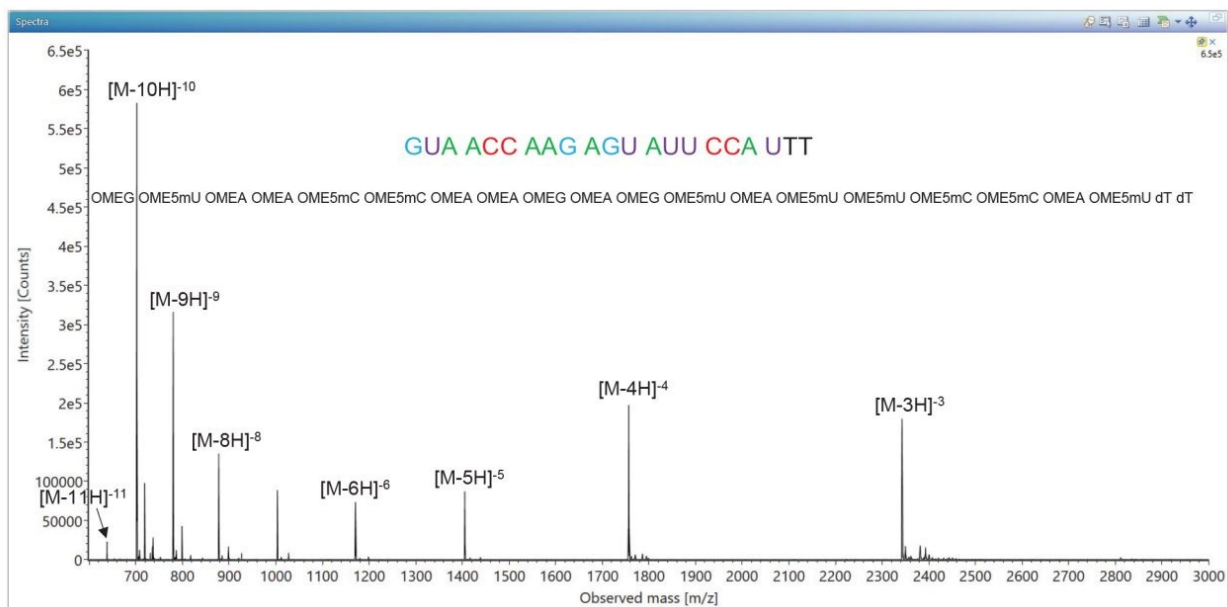
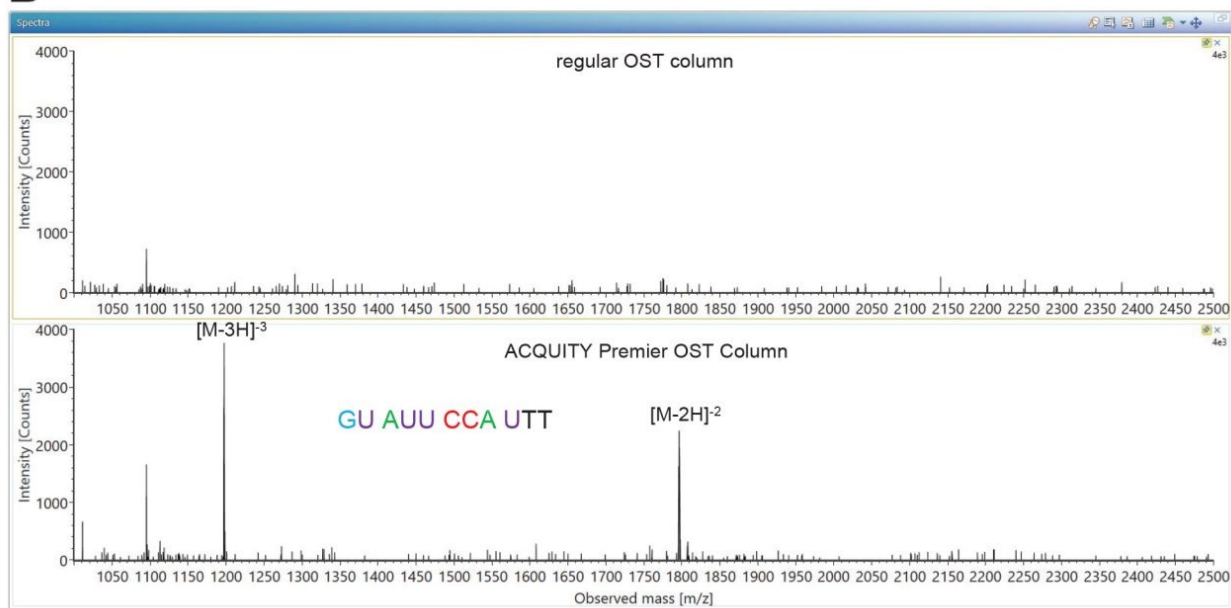
A**B**

Figure 3. Ion pairing reversed-phase (IP-RP) ESI-MS spectra recorded for: (A) the most abundant sample component, the 21-mer heavily modified oligonucleotide; (B) the least abundant sample component, an 11-mer oligonucleotide impurity (see Table 1 for its sequence), present at 0.18% according to the UV peak area measurement. All the oligonucleotide modifications are summarized using a specially designed nomenclature as shown in Figure 3A.

Impurity label	Oligonucleotide length	Oligonucleotide modification (Da)	Retention time (min)	Oligonucleotide sequence	Elemental composition	Most abundant monoisotopic mass	Charge state	Accurate average molecular weight
1	11-mer	—	4.73	GU AUU CCA UTT	C119 H161 N33 O77 P10	1795.8464	2	3595.4641
2	12-mer	—	7.39	AGU AUU CCA UTT	C130 H175 N38 O83 P11	1311.2512	3	3938.6967
3	14-mer	—	9.67	AG AGU AUU CCA UTT	C152 H203 N48 O96 P13	1545.2950	3	4641.1611
4	15-mer	—	12.89	AAG AGU AUU CCA UTT	C163 H217 N53 O102 P14	1659.6510	3	4984.3936
5	16-mer	—	13.83	C AAG AGU AUU CCA UTT	C174 H233 N56 O109 P15	1770.6752	3	5317.6280
6	Modified 17-mer	+19 Da	14.15	CC AAG AGU AUU CCA UTT	C184 H261 N59 O116 P16	1881.7307	3	5650.9471
7	18-mer	-10 Da	17.78	ACC AAG AGU AUU CCA UTT	C196 H263 N64 O122 P17	1996.0555	3	5994.0950
8	Modified 21-mer	+19 Da	18.08	GUA ACC AAG AGU AUU CCA UTT	C228 H299 N76 O143 P20	2335.0998	3	7011.7123
9	Modified 21-mer	+19 Da	18.94	GUA ACC AAG AGU AUU CCA UTT	C228 H299 N76 O143 P20	2335.0998	3	7011.7123
10	Modified 20-mer	+343 Da	19.62	UA ACC AAG AGU AUU CCA UTT	C220 H284 N71 O136 P19	2227.0762	3	6687.5045
11	Modified 20-mer	+334 Da	20.15	UA ACC AAG AGU AUU CCA UTT	C220 H293 N71 O136 P19	2230.0997	3	6696.5760
12	Modified 20-mer	+333 Da	20.41	UA ACC AAG AGU AUU CCA UTT	C220 H294 N71 O136 P19	2230.4356	3	6697.5839
MAIN PEAK	21-mer	—	21.25	GUA ACC AAG AGU AUU CCA UTT	C229 H306 N76 O143 P20	2341.4514	3	7030.7786
13	Modified 21-mer	+1 Da	21.65	GUA ACC AAG AGU AUU CCA UTT	C229 H307 N76 O143 P20	2341.7874	3	7031.7865
14	Modified 21-mer	+1 Da	21.93	GUA ACC AAG AGU AUU CCA UTT	C229 H307 N76 O143 P20	2341.7874	3	7031.7865

Table 1. Fourteen oligonucleotide impurities identified in a 21-mer extensively modified oligonucleotide.

Protein name	Observed mass (Da)	Expected mass (Da)	Mass error (mDa)	Mass error (ppm)	Observed RT (min)	TUV Peak Area	TUV Area Percentage (%)
Peak 1 11-mer oligo	3595.4240	3595.46410	-40.1	-11.1	4.78	1161.31	0.18
Peak 2 12-mer oligo	3938.6446	3938.69670	-52.1	-13.2	7.42	3243.55	0.51
Peak 3 14-mer oligo	4641.1368	4641.16110	-24.3	-5.2	9.67	2888.75	0.45
Peak 4 15-mer oligo	4984.3676	4984.39360	-26.0	-5.2	12.93	5690.73	0.89
Peak 5 16-mer oligo	5317.6013	5317.62800	-26.7	-5.0	13.89	5441.62	0.85
Peak 6 Mod 17-mer oligo	5650.8583	5650.94710	-88.8	-15.7	14.16	2183.12	0.34
Peak 7 18-mer oligo	5994.0855	5994.09500	-9.5	-1.6	17.82	8859.49	1.39
Peak 11 Mod 20-mer oligo	6696.5592	6696.57600	-16.8	-2.5	20.14	16066.20	2.52
Peak 12 Mod 20-mer oligo	6697.5438	6697.58390	-40.1	-6.0	20.41	29405.55	4.61
Peak 8 Mod 21-mer oligo	7011.7480	7011.71230	35.7	5.1	18.13	7615.09	1.19
Peak 9 Mod 21-mer oligo	7011.7467	7011.71230	34.4	4.9	18.95	6534.93	1.02
MAIN PEAK 21-mer oligo	7030.7784	7030.77860	-0.2	0.0	21.28	529488.11	83.03
Peak 13 Mod 21-mer oligo	7031.7655	7031.78650	-21.0	-3.0	21.70	1641.51	0.26
Peak 14 Mod 21-mer oligo	7031.7646	7031.78650	-21.9	-3.1	22.00	2175.47	0.34

Figure 4. Screenshot showing the waters_connect processing results obtained after BayesSpray charge deconvolution of the ESI-MS spectra recorded for the 21-mer major component and fourteen of its oligonucleotide impurities. The mass accuracy error for measuring the accurate average masses was better than 15 ppm for all sample components. The row corresponding to the main component is highlighted in blue and indicates a purity of 83.03%, while the abundance of the lowest abundant species (an 11-mer oligo) was 0.18% (highlighted by a red circle).

While it is important to be able to detect low-level oligonucleotide impurities with both UV and MS detectors, it is equally important to measure their abundances accurately. In other words, it is critical that these impurities produce a linear response in UV over the same dynamic range (~500 fold). To test the linearity of the UV assay, the 21-mer oligonucleotide was diluted and analyzed on a shorter ACQUITY Premier Column (2.1 x 50 mm, p/n: [186009484 < https://www.waters.com/nextgen/us/en/shop/columns/186009484-acquity-premier-oligonucleotide-c18-column-130a-17--m-21-x-50-mm.html>](https://www.waters.com/nextgen/us/en/shop/columns/186009484-acquity-premier-oligonucleotide-c18-column-130a-17--m-21-x-50-mm.html)) using faster (15-min) LC-MS runs. The UV chromatogram recorded for lowest

detected concentration of 0.5 nM, corresponding to a 1:2000 dilution of the stock sample (1 μ M), is shown in Figure 5A along with the preceding blank injection. Four replicate injections of the same solution (0.5 nM or 1.17 ng/mL) have peak area RSDs under 15%, demonstrating that low-level oligonucleotides can be analyzed reproducibly on the ACQUITY Premier Columns as highlighted in Figure 5B. A calibration plot, showing the UV response (peak area) for a wide range of concentrations of the 21-nt oligomer (eight concentrations in the range of 0.5 nM to 1000 nM), is presented in Figure 6. Taken together, the data presented in Figures 5A, B, and Figure 6 clearly proves that the 21-mer oligonucleotide displays a linear chromatographic behavior over a wide dynamic range (2000 fold) and has very good column recoveries even at the lowest concentration - 0.5 nM, which corresponds to 5 femtomoles (or ~12 picograms) of oligonucleotide loaded on-column. Finally, the carryover of the assay was evaluated by the injection of a Solvent A blank (10 μ L) right after the last replicate of the highest oligonucleotide concentration analyzed (1 μ M). The result is displayed in Figure 5C and it emphasizes the inertness of the ACQUITY Premier UPLC System and Column: there is no detectable signal for the 21-mer oligonucleotide following the injection of a large amount (10 picomoles) on-column.

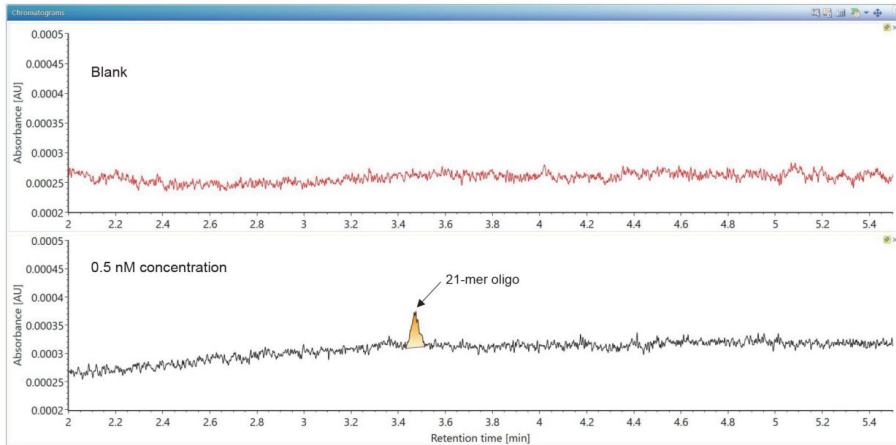
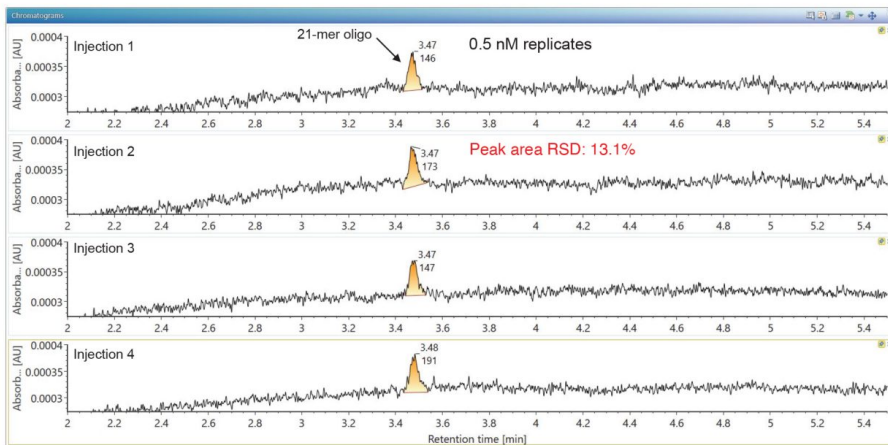
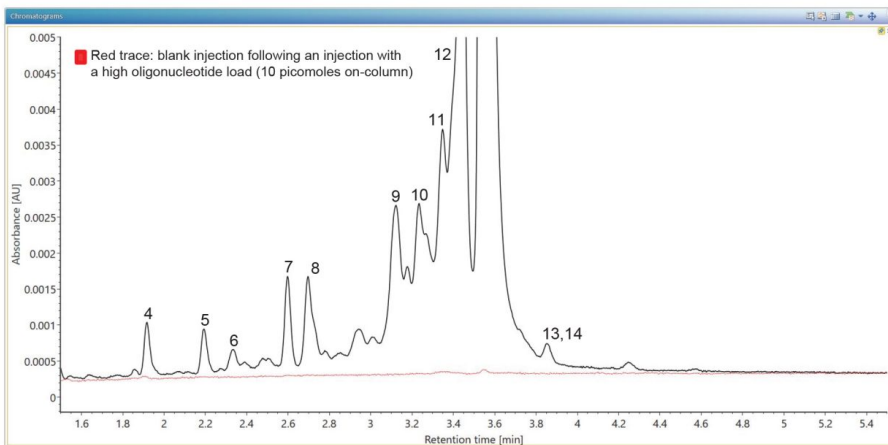
A**B****C**

Figure 5. Testing the inertness of the ACQUITY Premier UPLC System and ACQUITY Premier OST Column using the 21-nt oligomer: (A) UV chromatogram of the lowest detectable concentration (0.5 nM, or 5 femtomoles of the 21-mer oligonucleotide loaded on-column) compared against the preceding blank injection; (B) replicate UV chromatograms obtained for the lowest detectable concentration (0.5 nM), indicating that the UV peak areas had RSDs below 15%; (C) carryover evaluation, showing the UV chromatogram of a blank injection (red trace) following the injection of the highest sample concentration (1000 nM or 10 picomole oligonucleotide loaded on-column). There is no detectable signal from the 21-mer in the blank, suggesting that the UPLC system and column do not retain any analyte through non-specific adsorption to the various coated metal surfaces found in the fluidic flow-path.

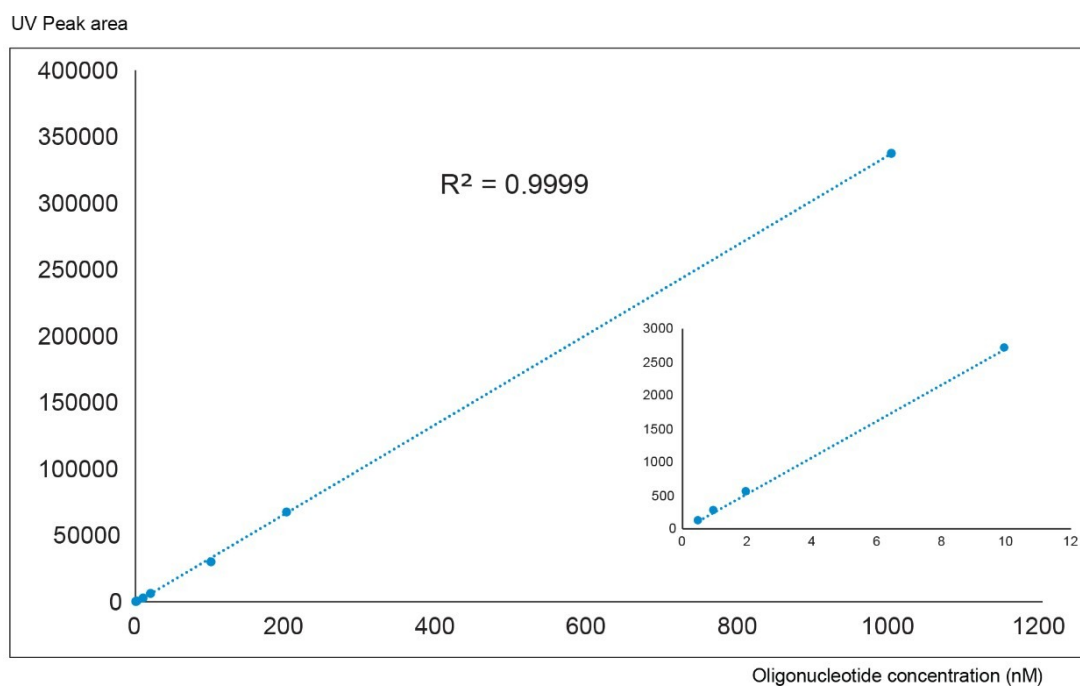


Figure 6. Calibration curve of the 21-mer oligonucleotide showing linearity over three orders of magnitude. Peak areas obtained from the TUV detector were plotted against a wide range of oligonucleotide concentrations, including 0.5, 1, 5, 10, 20, 100, 200, and 1000 nM. Excellent signal linearity was obtained for this assay, indicating that very low oligonucleotide concentrations can be recovered completely from a very inert LC system that does not interact in any way with the analyte. The inset shows the calibration curve in the 0.5–10 nM concentration range.

In conclusion, the BioAccord System with ACQUITY Premier is capable of measuring and accurately detecting oligonucleotide impurities down to 0.2% and also provides intact mass confirmation capabilities via an automated, compliance-ready workflow using the waters_connect Software.

Conclusion

- A category of products incorporating the MaxPeak High Performance Surfaces (HPS) – including the ACQUITY Premier UPLC BSM System and the ACQUITY Premier OST Columns – provide critical advantages for oligonucleotide impurity analysis
- Improved oligonucleotide analysis in terms of low detection limit and chromatographic reproducibility is

demonstrated using the BioAccord LC-MS System with ACQUITY Premier operated under compliant-ready software

- The oligonucleotide impurity analysis workflow provides mass confirmation for oligonucleotide impurities as well as their relative abundance. The results from our study indicate that the LC-MS platform provides good mass accuracy (better than 15 ppm) for intact mass confirmation of modified oligonucleotides and their impurities, while the LC-UV information allows for the measurements of all the sample components, down to 0.2% relative abundance levels

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