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Improving Recovery and Quantitation of Oligonucleotide Impurities using MaxPeak HPS Technology

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Abstract

Ion-pair reversed-phase chromatography is a commonly used technique for the separation of synthetic oligonucleotide impurities. Analysis of oligonucleotides is notoriously difficult due to their high affinity for metallic surfaces when using traditional ion-pairing method conditions. ACQUITY PREMIER Columns and LC systems were designed with MaxPeak High Performance Surfaces Technology to address the variability in the analysis of metal-sensitive analytes such as oligonucleotides. The ACQUITY PREMIER Solution offers out-of-the-box performance without the need for conditioning or passivation, as would be required by stainless-steel columns and LC systems having a metal-containing flow path. Furthermore, ACQUITY PREMIER Solutions are demonstrated to improve recovery for greater sensitivity and limits of detection and offer more reliable quantitation of trace-level impurities in optical workflows.

Benefits

- Out-of-the-box performance without the need for conditioning or passivation
- Extended dynamic range with increased linearity
- Enhanced repeatability and quantitation of trace-level impurities
- Increased recovery across all oligonucleotide types evaluated

Introduction

Nucleic acid therapeutics continue to generate interest as new modalities are explored and brought to market. This includes synthetic oligonucleotides, which are synthesized through a series of coupling reactions that link one nucleotide to the next in a growing chain. Due to the nature of chemical synthesis, impurity types are often predictable, but the impurity profile becomes more complex as the length of the oligonucleotide increases. Separation and purification of the analyte of interest from these impurities is critical to development and manufacturing activities but is not without its challenges.

Ion-pair reversed-phase chromatography (IP-RP) is the method of choice for oligonucleotide analysis. Typical method conditions are carried out at approximately pH 7 or 8, but oligonucleotides are extremely acidic and are

deprotonated when using conventional conditions. The negatively charged phosphate backbone of the oligonucleotide is susceptible to non-specific adsorption through ionic interactions with the metallic surfaces of stainless-steel columns and the LC flow path. This can lead to poor peak shape and recovery, making reliable quantitation difficult, which is especially true for trace-level species. Non-specific adsorption can be even more problematic when conjugates or modifications to the base, sugar, or backbone are incorporated into the oligonucleotide structure, where these alterations can increase the affinity for metal surfaces. Laboratories employ various strategies to mitigate this phenomenon, including conditioning or passivating the column and LC system or adding metal chelators the mobile phase. Conditioning or passivation most generally refers to repeated injections of an analyte to block active sites on the metal surfaces. Chemical passivation of an LC system that uses a phosphoric acid solution is a more aggressive approach that is sometimes used when working with analytes that are especially sensitive. While these techniques may offer a temporary solution, they can be cumbersome and are not permanent.

MaxPeak High Performance Surfaces (HPS) Technology was developed specifically to reduce variability in the analysis of metal-sensitive analytes by providing an organic/inorganic barrier between the metal surface and the analyte.[1] ACQUITY PREMIER Columns and the ACQUITY PREMIER System were designed with MaxPeak HPS Technology and together form the ACQUITY PREMIER Solution. This work evaluates the ACQUITY PREMIER Solution compared to conventional stainless-steel columns and LC systems having a metal-containing flow path for UV-based analysis of oligonucleotides to address the challenges associated with non-specific adsorption. The ACQUITY PREMIER Solution provides greater sensitivity and recovery leading to increased reproducibility in quantitation. Ultimately, greater confidence in results can be achieved while bypassing the need for additional time spent conditioning or passivating surfaces.

Experimental

Sample Description

All analytes and their respective sequences are reported in Table 1. Highly concentrated stock solutions of each analyte were prepared and further diluted as reported in the corresponding figures.

Analyte	Sequence
OST Standard	15, 20, 25, 30, and 35 mer oligodeoxythymidines
FAM-25mer	FAM-TTT GAC TTA GAC TTA GAC TTA GTT T
Cy3-25mer	Cy3-TTT GAC TTA GAC TTA GAC TTA GTT T
GEM91	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*

Table 1. Oligonucleotide analyte and sequence information. The Waters MassPREP Oligonucleotide Standard (p/n: 186004135), which contains approximately 1 nmol each of 15, 20, 25, 30, and 35 nucleotide (nt) long oligodeoxythymidines, was reconstituted in 250 µL to yield a concentration of 4 µM. Stock solutions of the fluorescein dye conjugate (FAM-25mer) and the cyanine dye conjugate (Cy3-25mer) were prepared at 100 µM. GEM91, a fully phosphorothioated antisense oligonucleotide, was prepared at 130 µM. Stock solutions were further diluted for analysis.

LC Conditions

LC systems:	ACQUITY UPLC H-Class PLUS Bio Binary System ACQUITY PREMIER System
Columns:	ACQUITY UPLC Oligonucleotide BEH C_{18} Column, 130 Å, 1.7 µm, 2.1 mm x 50 mm (p/n: 186003949)

	ACQUITY PREMIER Oligonucleotide C_{18} Column, 130 Å, 1.7 µm, 2.1 mm x 50 mm (p/n: 186009484)
Wavelength:	TUV detection at 260 nm
Injection volume:	4 µL
Column temp.:	60 °C
Flow rate:	0.200 mL/min
Figure 1:	
Mobile phase A:	100 mM TEAA, pH ~7
Mobile phase B:	Acetonitrile
Gradient:	FAM-25mer: 9–19% B in 10 minutes
Figures 2, 3, and 4:	
Mobile phase A:	8.6 mM TEA, 100

	mM HFIP, pH ~8.25
Mobile phase B:	Methanol
Gradients:	OST Standard: 12–22% B in 10 minutes FAM-25mer: 14–24% B in 10 minutes Cy3-25mer: 21–31% B in 10 minutes PS: 14–24% B in 10 minutes

Data Management

Empower 3 Chromatography Data Software FR4 (unless otherwise indicated)

Results and Discussion

Bypassing conditioning and passivation requirements: Evaluating stainless-steel versus ACQUITY PREMIER Column technology

When working with metal sensitive analytes such as oligonucleotides, passivation or conditioning both the column and LC system are important considerations for achieving desired results. Without proper conditioning or passivation, analytes interact with active sites on the metal surfaces which can lead to distorted peak shape and poor recovery. When using a new stainless-steel column for the first time, peak area is expected to increase over

an injection series until these active sites are saturated, after which, peak area becomes more reproducible.

In Figure 1, a TEAA ion-pairing system was used to separate FAM-25mer impurities using a stainless-steel column and a well-seasoned LC system that had been used for high throughput routine analysis of proteins, peptides, and oligonucleotides. As expected, peak area was observed to increase over the injection series until surfaces were effectively conditioned or passivated with analyte (Figure 1A, inset). However, even after the column was properly conditioned, recovery of the FAM-25mer n-1 impurity was still not observed (Figure 1A). When using this same LC system with a PREMIER column, the n-1 impurity and an additional unidentified impurity on the front shoulder of the main peak could be resolved (Figure 1B). The identity of the n-1 impurity was confirmed through a spike-in study as TEAA is not amenable to MS detection (data not shown). The n-1 impurity was calculated to be present at approximately 0.1% and had a percent RSD of 2.4% over six injections, which indicates low-level impurities can be reliably quantitated from the first injection. From the inset of Figure 1B, peak area of the main peak was also shown to be maintained across the injection series without the conditioning effect observed when using the stainless-steel column. This demonstrates the out-of-the-box performance of the ACQUITY PREMIER Column, which bypasses the need for conditioning with sacrificial analyte as was required by the stainless-steel column.

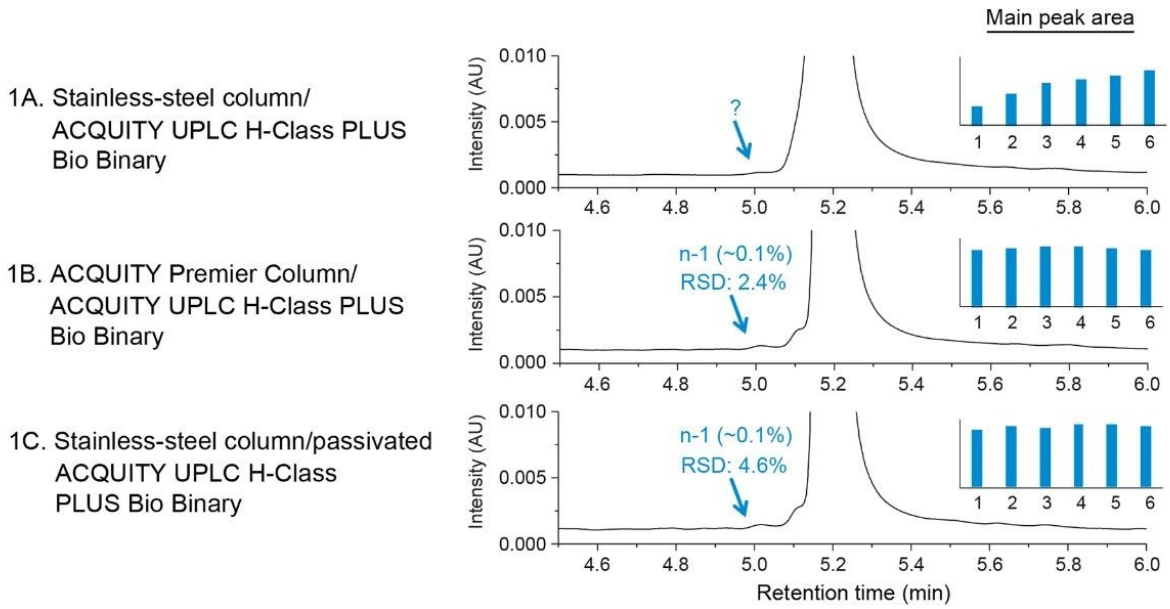


Figure 1. Stainless-steel columns and conventional LC systems require proper conditioning and passivation to achieve optimal results for separation of the n-1 impurity of FAM-25mer. 1A. Over an injection series, peak area of the main peak is shown to increase using a stainless-steel column until active sites on the metal surface are blocked (inset). From the chromatogram, the n-1 impurity cannot be recovered even after subsequent injections to condition the column. 1B. The ACQUITY PREMIER Column offers out-of-the-box performance and bypasses the need for conditioning (inset). Over six injections, the n-1 impurity can be quantitated at approximately 0.1% with 2.4% RSD. 1C: The n-1 impurity can only be recovered after passivating the LC system, suggesting that analyte is lost to both the column and the LC system. From the inset, peak area of the main peak is maintained over the injection series as the column has been conditioned prior to acid passivation.

Method conditions: Stock solution of FAM-25mer was diluted to 2.22 pmol/µL. MPA: 100 mM TEAA, pH ~7; MPB: acetonitrile; gradient conditions: 9–19% B in 10 minutes; column temp.: 60 °C, detection: 260 nm. Data was collected in MassLynx v4.2 SCN 993.

In an effort to recover the n-1 impurity when using the conventional stainless-steel column, the LC system was passivated using a 30% phosphoric acid solution. It should be noted that although phosphoric acid is frequently

used for LC surface passivation, that this is an extremely aggressive treatment and should be carried out with caution. It is also essential that the LC system be flushed with water up to 24 hours to ensure removal of trace acid from the system. It was only after both proper column conditioning and the acid flush that the n-1 peak (and additional shoulder peak) could be recovered (Figure 1C). Similar to results achieved using the PREMIER column, the n-1 peak was present at approximately 0.1% and had a percent RSD of 4.6% over the injection series. Because the results on the stainless-steel column were enhanced through LC system passivation, this indicates that there is observable analyte loss to the metal LC components as well as to the column, even after both the system and column surfaces had been passivated with analyte. This suggests that recovery using the ACQUITY PREMIER Column can be further enhanced when used in combination with an ACQUITY PREMIER System.

Expanding the dynamic range and enhancing linearity with MaxPeak HPS Technology: Understanding column and LC system contribution to sample loss

Although the surface area of the analyte flow path is significantly smaller than that of the stainless-steel column, the previous example suggests that optimal recovery can be achieved by taking advantage of the combination of an ACQUITY PREMIER Column used with an ACQUITY PREMIER System. To evaluate the impact of both the column and system contribution, a dilution series of GEM91 in TEA-HFIP was evaluated under three sets of conditions: using a stainless-steel column and conventional system, an ACQUITY PREMIER Column and conventional system, and an ACQUITY PREMIER Column and ACQUITY PREMIER System. Stock solution of GEM91 was further diluted to 2.6 pmol/µL and triplicate injections were carried out until the analyte was no longer detectable (SNR<3). All columns were conditioned prior to use. Although the ACQUITY PREMIER Column does not require conditioning, injection sets were kept consistent between the stainless-steel column and ACQUITY PREMIER Column. The dilution series using the stainless-steel column and conventional system and the ACQUITY PREMIER Column and System were run on the same day using the same sample preparation and mobile phase. The ACQUITY PREMIER Column on a conventional system was run Day 2 using a fresh sample preparation and mobile phase to avoid any potential degradation. Table 2 reports the average peak area over a triplicate injection series and the corresponding percent RSD under each of the three sets of conditions. The stainless-steel column and conventional system are shown to have the lowest recovery (as determined by peak area) over the entirety of the dilution series. Recovery increases when using an ACQUITY PREMIER Column on a conventional system but is the greatest when using both an ACQUITY PREMIER Column and System. The greater recovery also leads to a lower limit of detection and lower percent RSDs throughout the dilution series when using the entire ACQUITY PREMIER Solution.

	Stainless-steel column/ ACQUITY UPLC H-Class PLUS Bio Binary		ACQUITY Premier Column/ ACQUITY UPLC H-Class PLUS Bio Binary		ACQUITY Premier Column/ ACQUITY Premier LC System	
Mass load (ng)	Peak area (N=3)	%RSD	Peak area (N=3)	%RSD	Peak area (N=3)	%RSD
80.00	178627	1.27	221040	0.53	254558	0.26
40.00	84705	0.92	103585	1.30	125390	0.38
20.00	39348	1.97	51496	0.17	61893	0.72
10.00	16577	4.13	24547	1.17	29985	1.29
5.00	7857	3.10	12180	0.55	13757	1.90
2.50	2594	7.04	5818	0.89	6846	0.14
1.25	1002	9.16	2776	1.57	2958	2.07
0.625	348.03	12.16	1458	2.39	1596	0.30
0.313			596.82	8.57	689.76	2.30
0.156			Detectable but SNR <3		257.35	2.24
0.078					Detectable but SNR <3	

Table 2. Dynamic range of GEM91 dilution series. The lowest limit of detection is achieved when using the ACQUITY PREMIER Solution (an ACQUITY PREMIER Column and an ACQUITY PREMIER System). Furthermore, data quantitation is more repeatable using ACQUITY PREMIER Solutions, evidenced by the low percent RSDs present throughout the dilution series, even at low mass loads.

Method conditions: Stock solution of GEM91 was diluted to 2.6 pmol/µL and a two-fold dilution series was run until analyte could no longer be detected. MPA: 8.6 mM TEA, 100 mM HFIP, pH ~8.25; MPB: methanol; gradient conditions: 14–24% B in 10 minutes; column temp.: 60 °C, detection: 260 nm. Data was collected in Empower 3 FR4.

These results can be further interrogated through calibration curves of each dilution series to assess dynamic range (Figures 2 and 3). By first plotting calibration curves of each data set in a single plot, different slopes of each curve are observed (Figure 2). This indicates that there are differences in analyte recovery even when the column and system are conditioned and passivated with a sacrificial analyte. Although the curves appear linear and have R-squared values close to one, by plotting each data set independently and scaling the plots to show the lower mass loads, the stainless-steel column and system are not linear through the low mass data points (Figure 3). Recovery is improved by using an ACQUITY PREMIER Column and conventional system but is further improved by also incorporating the ACQUITY PREMIER System. The combination of ACQUITY PREMIER Column and System offers the best linearity throughout the dynamic range, lowest limits of detection, and best repeatability (lowest percent RSD). This becomes significant for impurity analysis as recovery can again be

correlated to detection at 0.1% level (area counts of 254,558 versus 257 for the highest and lowest reported peak areas) and detection covers almost three orders of magnitude compared to only two orders of magnitude with conventional systems and columns. This allows for more reliable detection and quantitation of trace level impurities, which could only be supported by the ACQUITY PREMIER Solution.

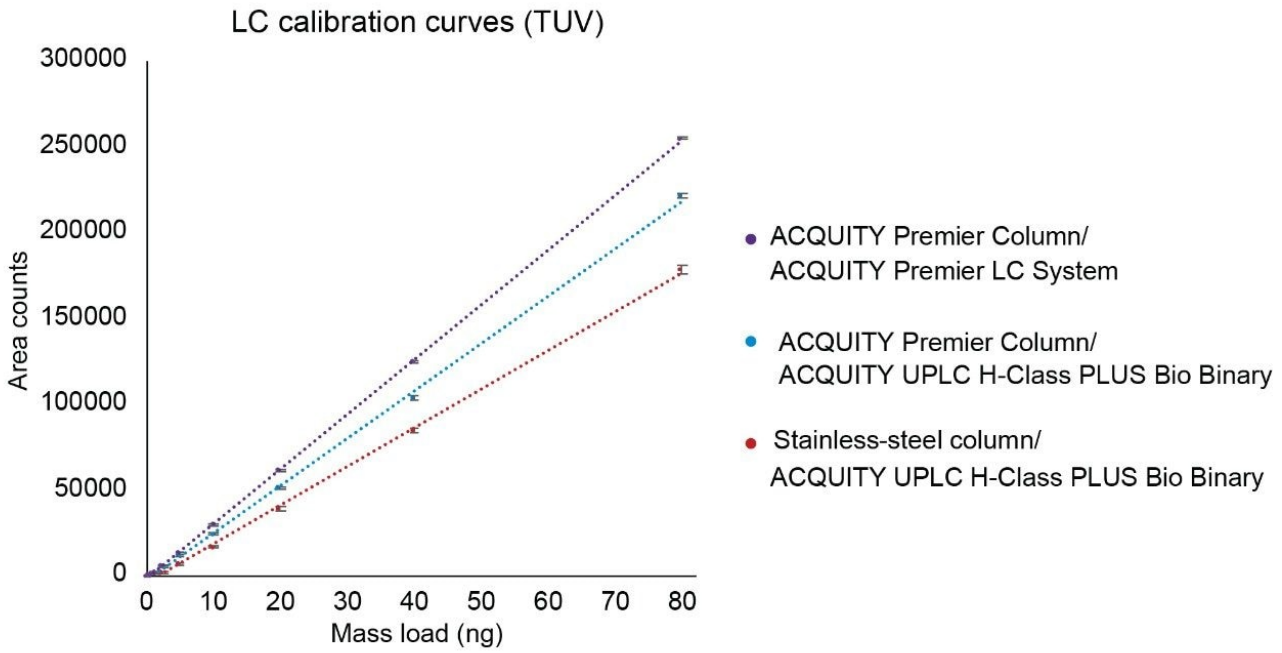
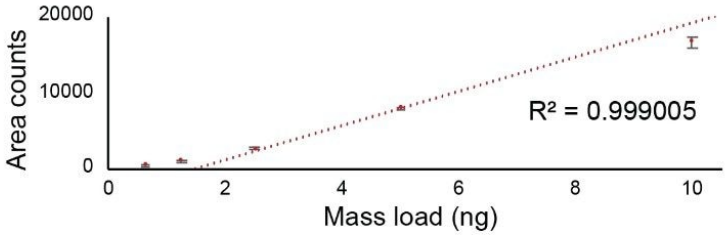


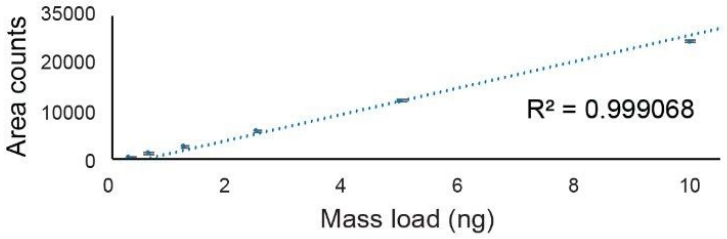
Figure 2. Calibration curves of GEM91 using a stainless-steel column and conventional system (red), an ACQUITY PREMIER Column and conventional system (blue), and an ACQUITY PREMIER Column and System (purple). Plots use peak area as reported in Table 2. The different slopes indicate distinct differences in recovery using the columns and systems tested.

Method conditions: Stock solution of GEM91 was diluted to 2.6 pmol/µL and a two-fold dilution series was run until analyte could no longer be detected. MPA: 8.6 mM TEA, 100 mM HFIP, pH ~8.25; MPB: methanol; gradient conditions: 14–24% B in 10 minutes; column temp.: 60 °C, detection: 260 nm. Data was collected in Empower 3 FR4.

3A. Stainless-steel Column/
ACQUITY UPLC H-Class PLUS
Bio Binary



3B. ACQUITY Premier Column/
ACQUITY UPLC H-Class PLUS
Bio Binary



3C. ACQUITY Premier Column/
ACQUITY Premier LC System

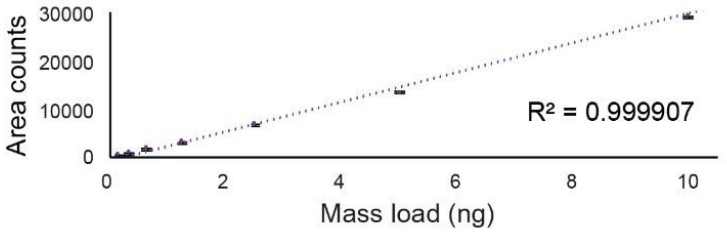


Figure 3. Dynamic range plots of GEM91 dilution series using a stainless-steel column and system (3A), an ACQUITY PREMIER Column and conventional system (3B), and an ACQUITY PREMIER Column and System (3C). Plots use peak area as reported in Table 2. The linear regression is through all data points, but data is scaled to highlight deviation at low mass loads.

Method conditions: Stock solution of GEM91 was diluted to 2.6 pmol/µL and a two-fold dilution series was run until analyte could no longer be detected. MPA: 8.6 mM TEA, 100 mM HFIP, pH ~8.25; MPB: methanol; gradient conditions: 14–24% B in 10 minutes; column temp.: 60 °C, detection: 260 nm. Data was collected in Empower 3 FR4.

Comparing MaxPeak HPS Technology to conventional technology for various oligonucleotide types

Analysis of GEM91 showed obvious benefits with the combination of an ACQUITY PREMIER Column and ACQUITY PREMIER System when compared to conventional technology. For this reason, it is of interest to understand if these same benefits can be observed across additional oligonucleotide types. Stock solutions of OST Standard, FAM-25mer, and Cy3-25mer were further diluted so that separations in TEA-HFIP using sub-nanogram mass loads on-column could be compared to GEM91 between conventional technology and MaxPeak HPS Technology. Chromatographic gradients were adjusted so that analytes eluted in approximately the middle

of the 10-minute gradient. The OST standard, FAM-25mer, and GEM-91 use similar gradients while Cy3-25mer required an almost 2-fold increase in methanol content to elute.

The OST Standard was selected as an oligonucleotide analyte expected to behave well because it is an oligomer of repeating dT units and does not contain modifications. Apart from the phosphate backbone, it was not expected to have an especially high affinity for metal surfaces. Although the fluorescently labelled oligonucleotides are more representative of oligonucleotides used for primers or probes in biological applications versus the therapeutic application space, they represent an additional class of unmodified oligonucleotides apart from their fluorescent labels. These labels are known to impart hydrophobicity to the otherwise hydrophilic oligonucleotide with the FAM conjugate being only slightly hydrophobic and the Cy3 conjugate being extremely hydrophobic. The degree of hydrophobicity of these dye conjugates is well documented in literature but is also apparent from the gradients required for elution. Additional modifications to the oligonucleotide base, sugar and backbone impact metal binding affinity.[2] GEM91 is a fully phosphorothioated oligonucleotide and is known to suffer from poor recovery, which can also be inferred from the previous example of the dilution series.

As with GEM91, like-samples were run on the same day on the stainless-steel column and conventional system and the ACQUITY PREMIER Column and System using the same sample preparation and mobile phase to minimize experimental variability. Sample loads were adjusted so that peak area was comparable (approximately 2000 area counts) for all samples analyzed using the ACQUITY PREMIER Solution, which assumes there is no analyte loss to the metal surfaces of the column or flow path. This allows for a fairer comparison across sample types as recovery suffers more greatly for trace-level species (i.e., lower area count).

As expected, recovery is analyte dependent (Figure 4). When comparing conventional technology to the MaxPeak HPS Technology, the OST Standard showed a general trend of shorter sequences having lower recovery. The difference in peak area between conventional technology and MaxPeak HPS Technology was approximately 2x for the 15mer. Although MaxPeak HPS Technology shows increased area counts for all species in the standard, the longer oligonucleotides were less susceptible to non-specific adsorption. The failed sequences throughout the chromatogram are also more distinguishable using MaxPeak HPS Technology when compared to conventional technology.

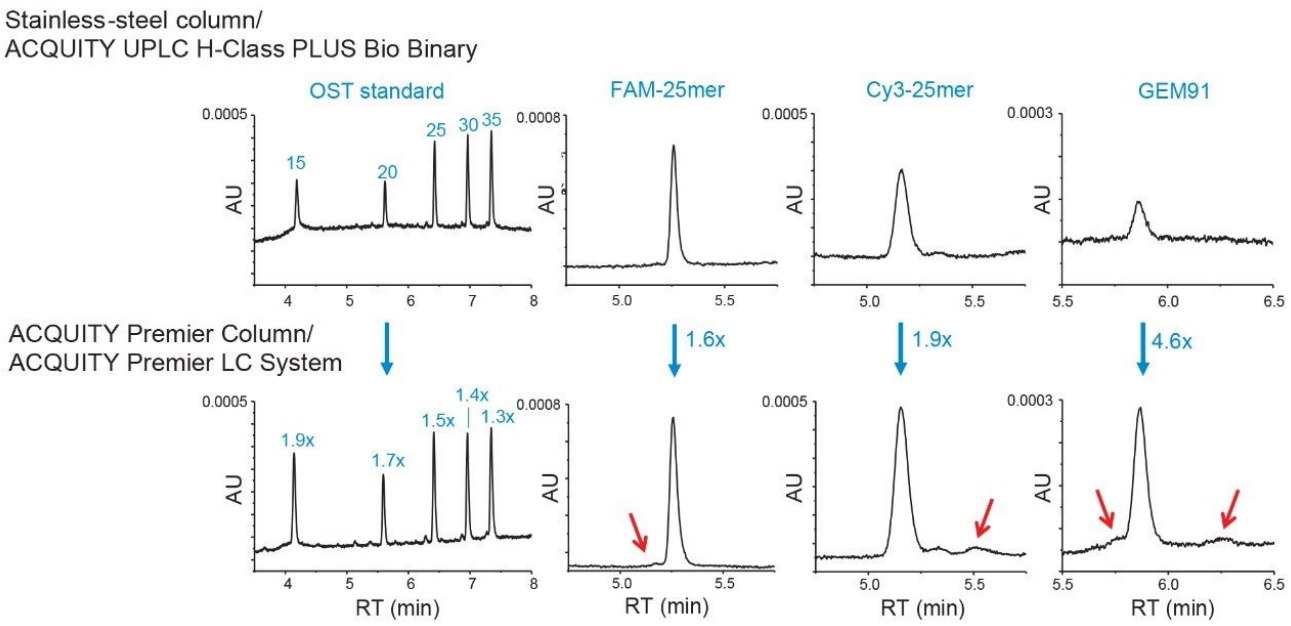


Figure 4. ACQUITY PREMIER Solutions show increased recovery of various oligonucleotide types at sub-nanogram mass loads. Sample loads were adjusted so that peak area was comparable (approximately 2000 area counts) across results using ACQUITY PREMIER Solutions for better alignment across sample types. Final mass loads were approximately 0.3 to 0.6 ng on-column. The red arrows on the ACQUITY PREMIER chromatograms highlight where additional impurities are present that are not visible using conventional technology. These are in addition to the OST standard, where failed sequences are also better recovered using ACQUITY PREMIER Solutions. (A blank subtraction was used for all conventional column and LC system data sets and RT is aligned for better visualization of the data.)

More notable differences can be seen across the modified oligonucleotide types. The FAM-25 mer shows a 1.6x improvement in recovery using MaxPeak HPS Technology, which is very similar to the un-modified poly dT standard of the same length, which showed a 1.5x improvement in recovery using MaxPeak HPS Technology. (It should be noted that the FAM-25mer is in TEA-HFIP and that the same chromatographic profile as shown in Figure 1 was not expected as it used TEAA.) The Cy3-25mer showed approximately a 2x increase in peak area using MaxPeak HPS Technology, indicating that this sample suffers from greater analyte adsorption using conventional technology. GEM91, as previously discussed, shows a 4x improvement in recovery when using MaxPeak HPS Technology. Furthermore, from visual inspection of the chromatograms, additional impurities can still be readily detected at these low mass loads when using MaxPeak HPS Technology compared to

conventional technology.

Conclusion

Oligonucleotides are notoriously difficult to characterize and quantify due to non-specific adsorption to metal surfaces, which is especially true of trace-level species and low sample loads. This work demonstrated that the ACQUITY PREMIER Solution could be used to enhance recovery for more reliable quantitation of impurities compared to a more traditional analysis using a stainless-steel column and an LC system containing a metal flow path. The best performance was achieved through the combination of an ACQUITY PREMIER Column and ACQUITY PREMIER System, which showed greater recovery, lower detection limits, and increased linearity and reproducibility.

References

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2. Zhou, W., Saran, R., Liu, J. Metal Sensing by DNA. ***Chem. Rev.*** 2017, 117, 12, 8272–8325.

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