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Application Note

Improved Oligonucleotide SPE-LC-MS Analysis Using MaxPeak High Performance Technology

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Abstract

The demand for highly selective and sensitive LC-MS bioanalytical assays in support of research and development of next-generation oligonucleotide therapies has greatly increased. The work described herein uses SPE, RP-UPLC with the sub-2-µm ACQUITY Premier Oligonucleotide C_{18} Column, and tandem-quadrupole MS for detection and quantification of oligodeoxythymidines and the fully phosphorothioated oligonucleotide antisense therapy, GEM91 (Figure 1).

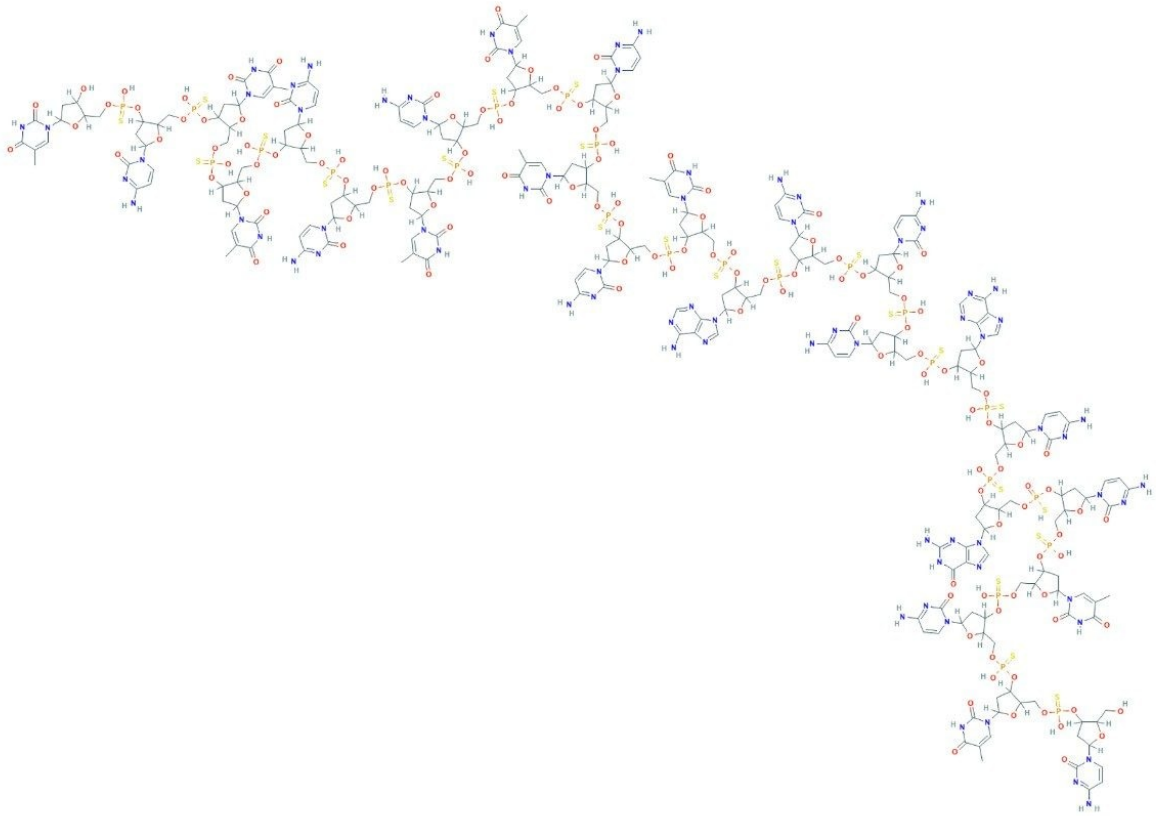

Figure 1. GEM91 (Trecovirsen) oligonucleotide structure.[1]

Benefits

- The ACQUITY Premier Oligonucleotide C_{18}, sub-2-µm column, improved oligonucleotide chromatographic recovery, improved LLOQs, and reduced lengthy column passivation.
- A simple sample preparation and UPLC-MS/MS analytical method was developed for detection and quantification of oligodeoxythymidines and GEM91, a phosophorothioated antisense oligonucleotide.
- A selective RP and mixed-mode SPE extraction method was developed, which achieved high oligonucleotide recovery.
- Use of 96-well SPE plates in the µElution format eliminated the need for sample evaporation, reducing oligonucleotide losses due to adsorption.
- System suitability check with the Waters MassPREP OST Standard, pre- and post-sample analysis, ensured

overall system health and performance.

- Quantification limits of GEM91, following LLE-Oasis WAX SPE extraction, was 50 ng/mL with LODs ≤2.5 ng/mL.

Introduction

With improved target specificity and stability of next-generation oligonucleotide therapies (ONTs), demand for LC-MS bioanalytical assays in support of their research and development has greatly increased in recent years. Developing robust, sensitive, and selective sample preparation and LC-MS methods for ONTs remains quite challenging due to their size, physiochemical diversity, poly-anionic nature, and known issues with protein and non-specific binding (NSB). Additionally, obtaining LC-MS sensitivity and selectivity remains a challenge due to limited ionization/fragmentation, poor RP chromatographic retention, and need for resolution from endogenous matrix interferences.

This work described herein provides a single, analytical method for the extraction and quantification of various oligonucleotides (15–35T). Using reversed-phase (RP) and mixed-mode ion-exchange µElution solid phase extraction (SPE) sample preparation provided high oligonucleotide recovery. RP UPLC chromatographic separation with the novel sub-2-µm ACQUITY Premier Column, provided fast analysis, recovery, and high-resolution of the oligonucleotides. Use of the ACQUITY Premier Column, specifically designed to prevent analyte non-specific adsorption, significantly increased oligonucleotide analyte recovery by minimizing ionic analyte/surface interaction. This developed SPE-LC-MS (ACQUITY UPLC I-Class PLUS System with Xevo TQ-XS Tandem Quadrupole Mass Spectrometer) method achieves high recovery, selectivity, and sensitivity, achieving low ng/mL lower limits of quantification (LLOQs) from neat and extracted samples.

Experimental

Oligonucleotide SPE Method Development

Solutions of the Waters MassPREP OST Standard (15–35T; p/n: 186004135 <

https://www.waters.com/nextgen/in/en/shop/standards--reagents/186004135-massprep-oligonucleotide-standard.html>) and the oligodeoxynucleotide phosphorothioate (GEM91) were prepared at various concentrations in proteinase (RNAse) free water. A 50 nmol/mL concentrated stock solution of Waters MassPrep OST 5 nmol Standard (OST standard) was prepared by adding 100 µL of RNAse free water to the vial, followed by subsequent mixing. Concentrated stock solutions of GEM91 (2.50 mg/mL) and GEM132 (4.00 mg/mL) were prepared in RNAse free water. GEM132 was used as an internal standard (IS). Working stock solutions of the OST standard (50 nmol/mL) and Gem 91 (500 µg/mL) were used to prepare various oligonucleotide samples during SPE method development and final analysis. The various SPE protocols, using the Waters Oasis HLB and WAX µElution 96-well SPE Extraction Plates, are shown in Figures 2A and 2B, respectively. All SPE steps were performed using a Waters positive pressure manifold.

Oligonucleotide SPE and LC-MS/MS Quantification

Calibrators and quality control (QC) samples: To prepare calibrators and QCs, OST standard and GEM91 working stock solutions were added to water or commercially available plasma/sera at various concentrations, 0.0025–50 nmol/mL (OST standard) and 0.005–100 µg/mL (GEM91). GEM132 IS solution was added to each prepared sample. Final IS concentration was 20 µg/mL. Human plasma (K2EDTA treated, non-stripped) was purchased from BIOIVT (New York, NY).

Sample Extraction

Neat Standard Samples:

Samples were prepared in 50 mM ammonium acetate buffer (pH 5.5). Extraction of the prepared samples was performed using the Waters Oasis WAX µElution 96-well SPE Plate (p/n: 186002500 < https://www.waters.com/waters/partDetail.htm?partNumber=186002500>) and protocol shown in Figure 2C. Addition of homogenizing buffer was omitted due to lack of need to disrupt protein binding.

Plasma Samples:

A 2-step extraction was performed using a phenol-chloroform liquid-liquid extraction (LLE) followed by Oasis WAX SPE of the resulting plasma supernatant. For this, 400 µL aliquots of the prepared plasma samples were diluted with 1000 µL of denaturation/lysis buffer and vortexed. A 200 µL aliquot of phenol:chloroform:isoamyl alcohol (25:24:1) was subsequently added. Samples were vortexed for 30 minutes and centrifuged at 14,000 RPM for 10 minutes. The top layer of the LLE supernatant (2 x 650 µL) was loaded to a conditioned and equilibrated Oasis WAX SPE Plate and extracted using the optimized protocol shown in Figure 2C.

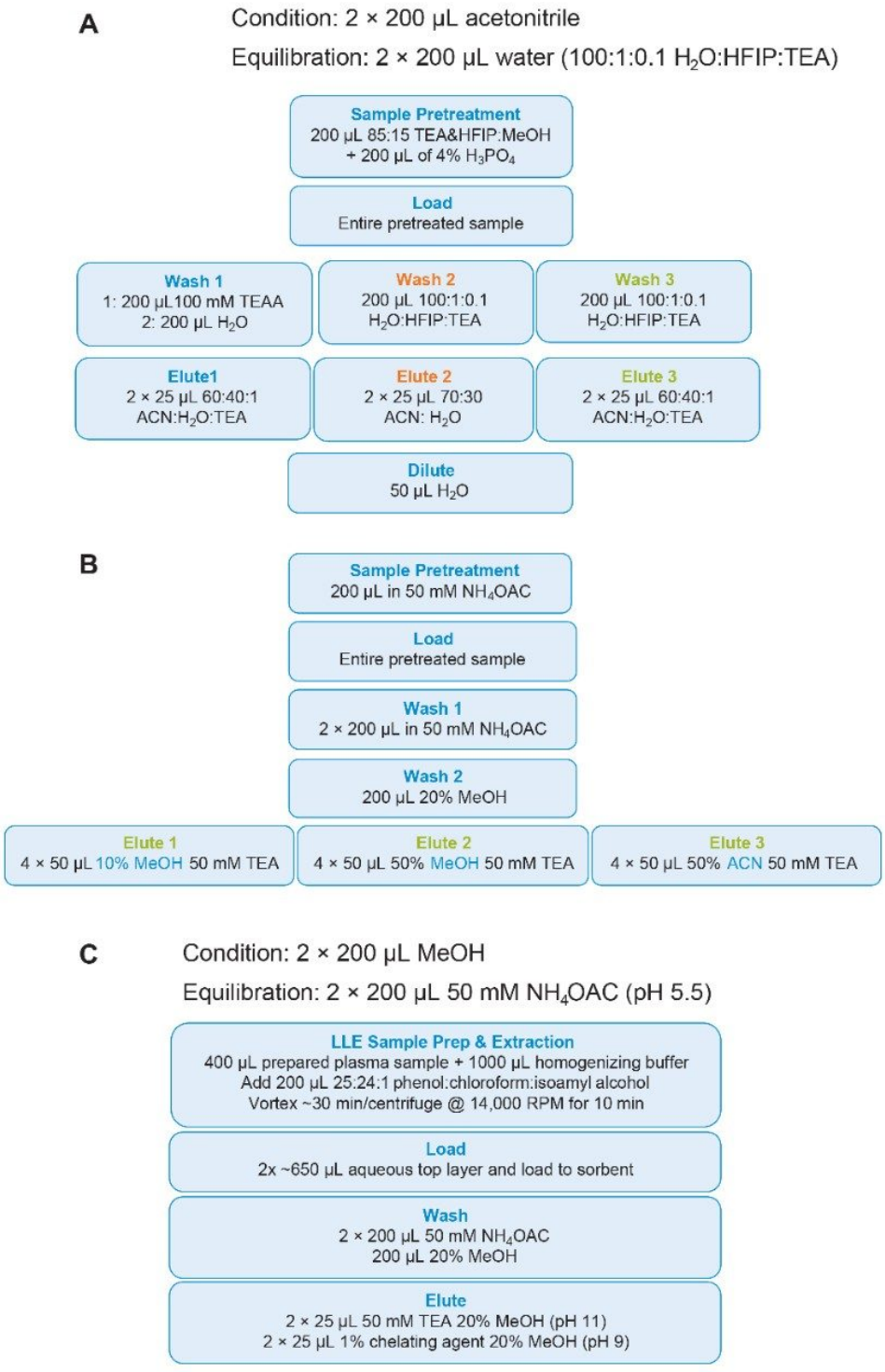

Figure 2. SPE sample preparation and purification protocols: (A) Oasis HLB, (B) Oasis WAX, and (C) final

optimized LLE-Oasis WAX SPE. All SPE was performed using the µElution 96-well plate format.

LC Conditions

System:	ACQUITY UPLC I-Class PLUS, FTN with single column heater
Detection:	MS
Vials/plate:	QuanRecovery with MaxPeak 700 µL Plate (p/n: 186009185) with round polypropylene cap mat (p/n: 186002483)
Column(s):	AQUITY Premier Oligonucleotide C_{18}, 1.7 µm, 2.1 x 50 mm (p/n: 186009484)
Column temp.:	50 °C
Sample temp.:	8 °C
Injection volume:	20 µL
Flow rate:	0.6 mL/min
Mobile phase A:	150 mM hexafluoroisopropanol (HFIP), 5 mM hexyl amine (HA) in water
Mobile phase B:	150 mM HFIP, 5 mM HA in methanol

Purge solvent:	90:10 water:methanol
Wash solvent:	90:10 water:methanol

Gradient

Time (min)	Flow (mL/min)	%A	%B	Curve
Initial	0.6	90	10	6
1.0	0.6	90	10	6
1.5	0.6	50	50	6
3.0	0.6	45	55	6
3.5	0.6	40	60	6
4.0	0.6	30	70	6
4.1	0.6	5	95	6
4.5	0.6	5	95	6
4.6	0.6	90	10	6
5.0	0.6	90	10	6

MS Conditions

System:	Xevo TQ-XS Tandem Quadrupole
Ionization mode:	ESI-
Acquisition range:	MRM
Capillary voltage:	2.00 kV
Desolvation temp.:	500 °C

Desolvation flow:	1000 L/Hr
Cone gas flow:	150 L/Hr
Collision gas flow:	0.2 mL/min
Nebulizer gas flow:	7 Bar
Collision energy:	See Table 1
Cone voltage:	See Table 1

Compound	Charge	Precursor (*m/z*)	Products	Collision energy (eV)	Cone voltage (V)
15T	(-4)	1123.8	302.8 382.6 606.8	40 40 40	40 40 40
20T	(-4)	1504.2	303.2 382.9 607.2	45 45 45	40 40 40
25T	(-4)	1884.7	302.6 382.8 624.7 929.4	50 50 45 40	40 40 40 40
30T	(-5)	1811.5	302.5 382.8 606.7 928.4	45 45 45 45	40 40 40 40
35T	(-6)	1762.9	302.7 383.1 624.5 705.1	50 50 50 50	40 40 40 40
GEM 91	(-5)	1553.7	512.8 722.0	30 30	40 40
GEM 132	(-5)	1319.2	94.5 357.3 807.6	40 40 25	40 40 40

Table 1. Final MS conditions used for oligonucleotide analysis.

Data Management

Instrument control software:	MassLynx, v4.2
Quantification software:	TargetLynx

Results and Discussion

Several multiply charge precursors were observed for the 15, 20, 25, 30, and 35T OST standard. Full scan MS and MS/MS spectra were obtained for all OST mers, GEM91, and GEM132 (data not shown). A representative full scan MS spectrum, showing the dominant precursor charge states for the OST MassPREP Standard (15–35T), is shown in Figure 3A. While Figure 3B shows representative MS/MS spectra for the most abundant -6 and -12 precursor charge states for the OST 35 mer. Final MRM transitions used for detection and quantification of the OST 15–35 mers, GEM91, and GEM132 are shown in Table 1. Like most large molecule biologics, many fragments are produced with most intense fragments seen below *m/z* 200. The low mass *m/z* fragments often result in high background in extracted samples due to their lack of specificity. In this assay, use of highly specific ion fragments above *m/z* 200 yielded significantly improved specificity, facilitating the use of simpler LC and SPE methodologies.

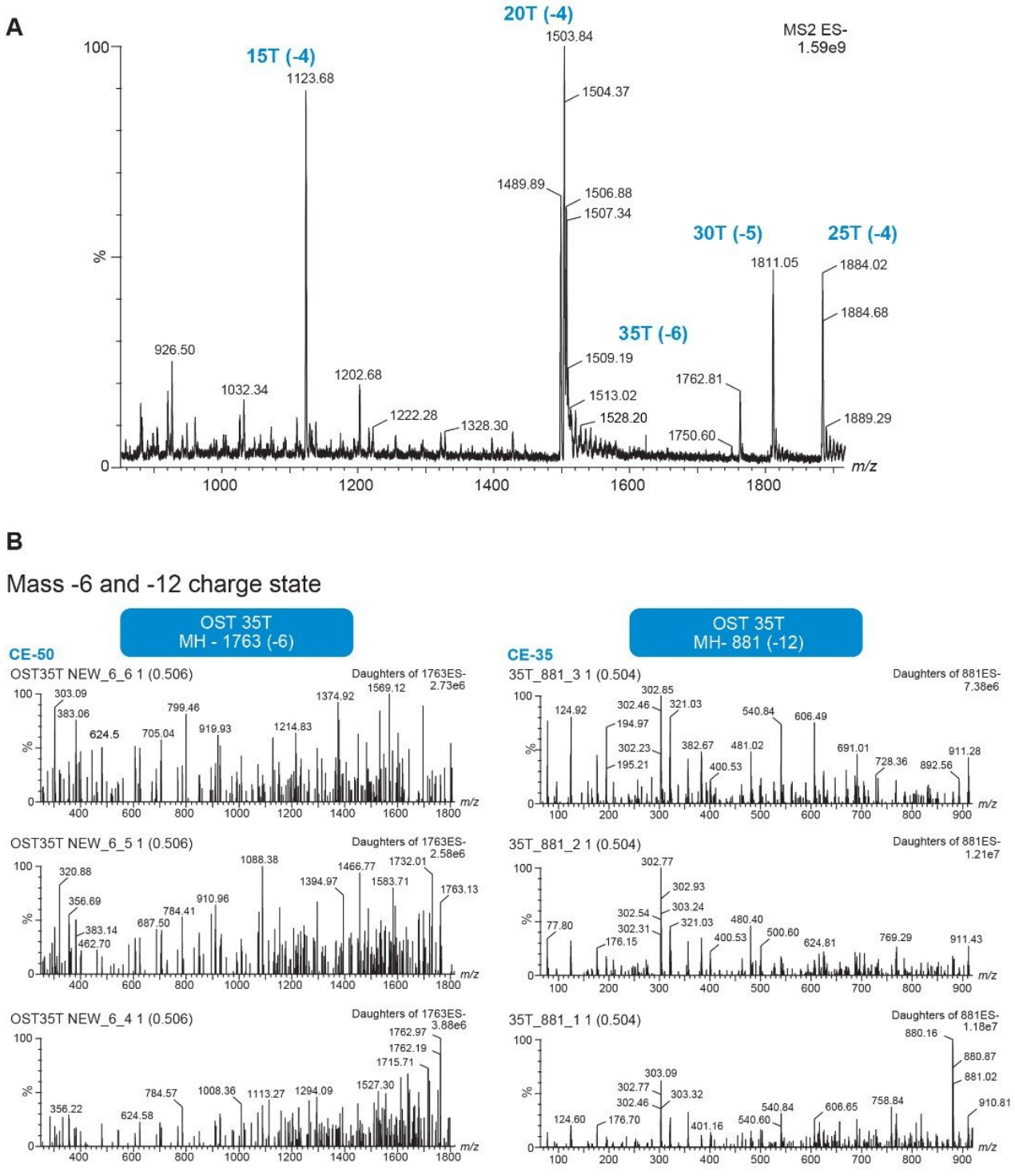

Figure 3. Representative MS full scan and product ion spectra (PIS) for the Waters MassPREP OST Standard.

Panel A highlights the dominant precursors for the OST 15, 20, 25, 30, and 35 mers; Panel B demonstrates the representative PIS spectra for the 35 mer of the OST standard and its dominant -6 and -12 precursor charge states.

For this work, the novel ACQUITY Premier Oligonucleotide C_{18} Column was selected for analysis. This column is packed with 1.7 µm hybrid-silica particles that are well suited for separations performed at neutral to moderately basic pH and high temperatures, which are required for retention and adequate resolution of oligonucleotides. ACQUITY Premier Columns incorporate MaxPeak High Performance Surface (HPS) Technology to column hardware which is critical for improving oligonucleotide recovery and assay limits of detection. HPS Technology was developed specifically to minimize metal interactions with analytes such as oligonucleotides, phosphopeptides, small molecule organophosphates, and other analytes that have historically shown strong affinity towards metal surfaces. Narrow peak widths for the OSTs, GEM91, and GEM132 were obtained using the LC conditions described in the experimental section. The resulting separation is shown in Figure 4A for the OST standard (15–35T) and Figure 4B for GEM91 and GEM132 (IS). Improvements in oligonucleotide analyte out-of-the-box recovery that are afforded with the use of ACQUITY Premier Oligonucleotide C_{18} Column vs the standard ACQUITY UPLC Oligonucleotide BEH C_{18} Column is shown in Figure 5 for the OST 20mer (A) and GEM91 (B), respectively. In addition to improved oligonucleotide recovery, the need to passivate the MaxPeak Premier Column with oligonucleotide standards was greatly reduced (data not shown). This greatly improved instrument assay up-time and saved on costly mobile phase reagents.

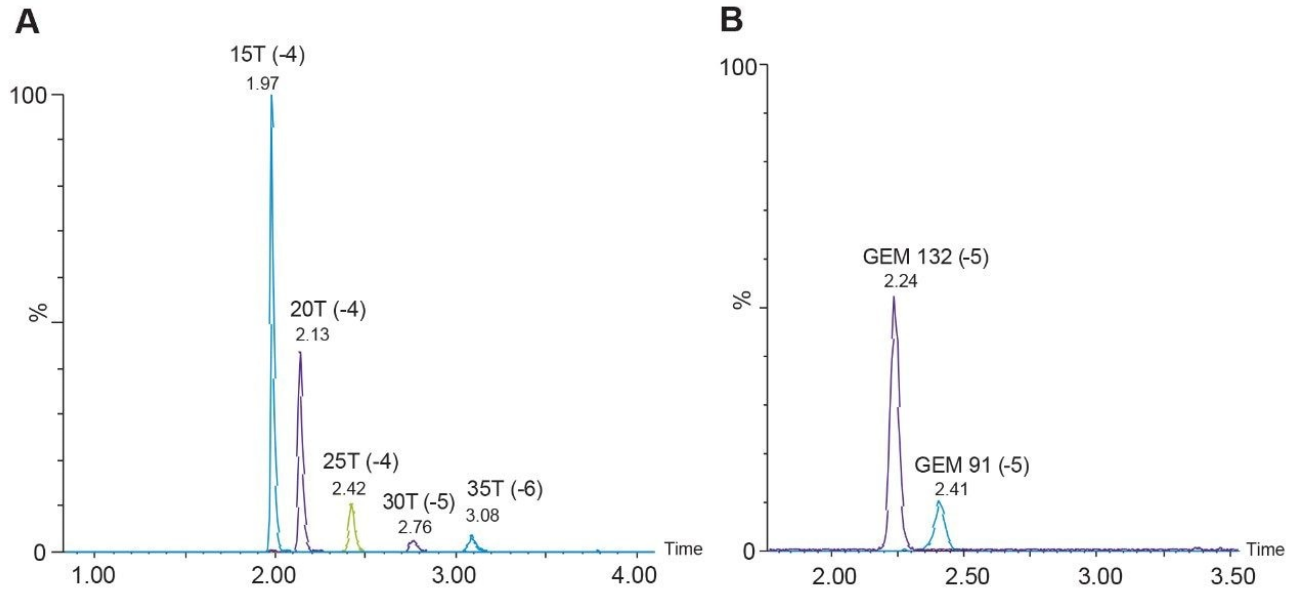

Figure 4. UPLC chromatographic separation of the (A) MassPREP OST 15–35 mers and the (B) fully phosphorothioated oligonucleotides GEM91 and GEM132 using the AQUITY Premier Oligonucleotide C_{18}, 1.7 µm, 2.1 x 50 mm Column and LC conditions described in the experimental section.

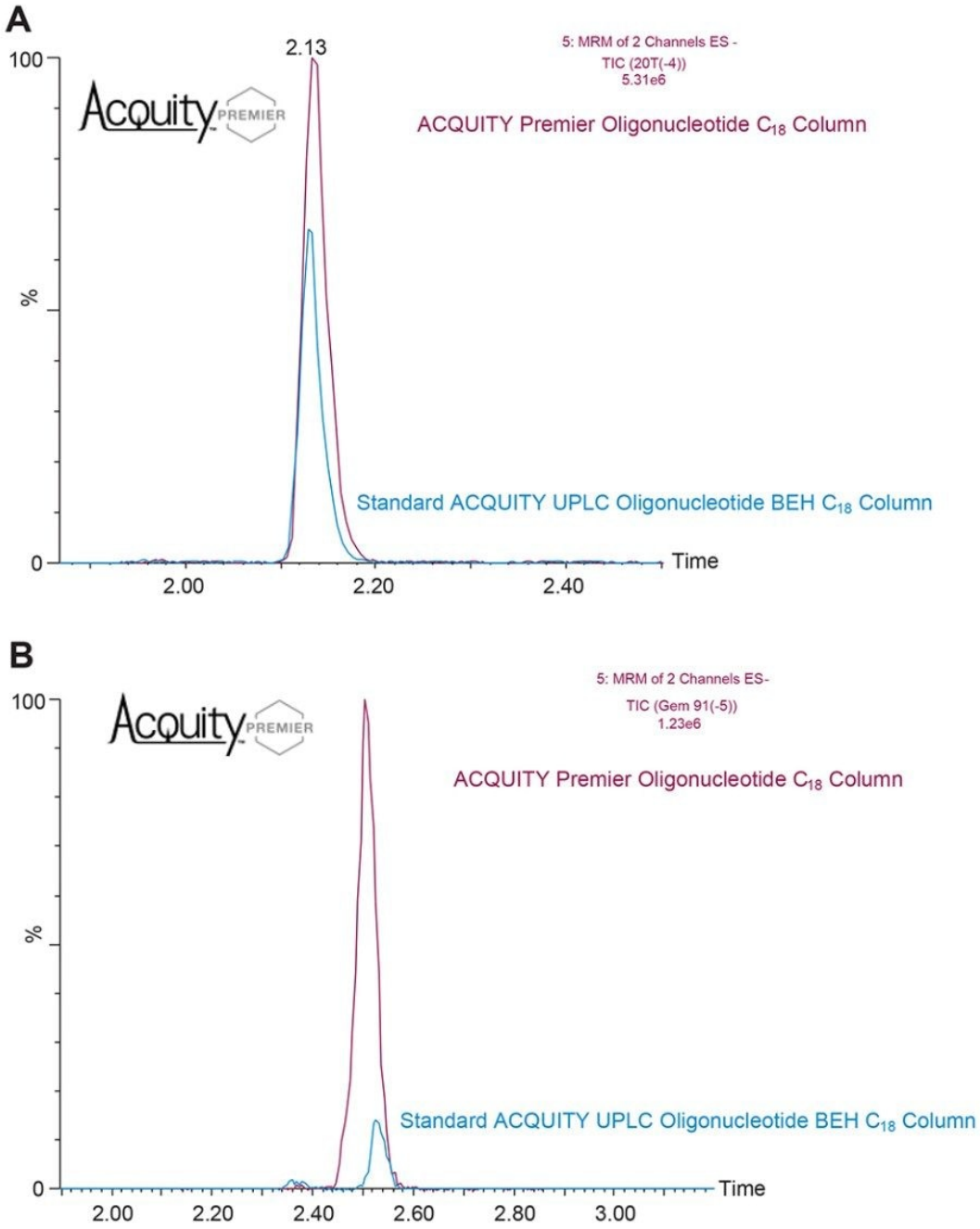

Figure 5. Demonstration of improved out-of-the-box (injection 2) chromatographic performance (oligonucleotide

recovery) for the (A) Waters MassPREP OST Standard 20 mer and (B) GEM91 using the ACQUITY Premier Oligonucleotide C_{18} vs standard ACQUITY UPLC Oligonucleotide BEH C_{18} Column.

During sample preparation method development, poor recovery and reproducibility issues of the oligonucleotides were found to be related to non-specific adsorption, protein binding, and solubility which are common issues to most large molecules. Careful and systematic evaluation of various pretreatment options, as well as wash and elution SPE solutions, was critical to improving SPE recovery and specificity of this method. SPE protocols used during sample preparation development are shown in Figure 2 for the Waters Oasis HLB (A) and WAX (B) SPE sorbents. For each protocol, oligonucleotide recovery was evaluated using neat solution standards. Various SPE conditions screened were load, wash, and elution steps. Oligonucleotide SPE recovery results are highlighted in Figures 6A (HLB) and 6B (WAX). For both the Oasis HLB and WAX sorbents, with all protocols (HLB and WAX), SPE recovery generally decreased with oligonucleotide size. Best overall HLB SPE recovery was realized when pretreatment of the sample was performed with a TEA:HFIP:MeOH solution, followed by dilution with H_3PO_4 sorbent, wash with a H_2O:HFIP:TEA solution, and subsequent elution with an ACN:H_2O:TEA solution. Due to their strong anionic nature, mixed-mode SPE, with RP and anionic exchange separation, is ideal for oligonucleotide purification. In addition to providing adequate recovery, use of Oasis WAX SPE generally affords improved selectivity allowing neutral interferences to be washed away during SPE purification. It also provides orthogonality to the overall assay, as LC separation is being performed with reversed-phase. Best overall recovery using Oasis WAX SPE was realized when the elution solution contained a combination of a 50 mM TEA and 50% ACN or MeOH.

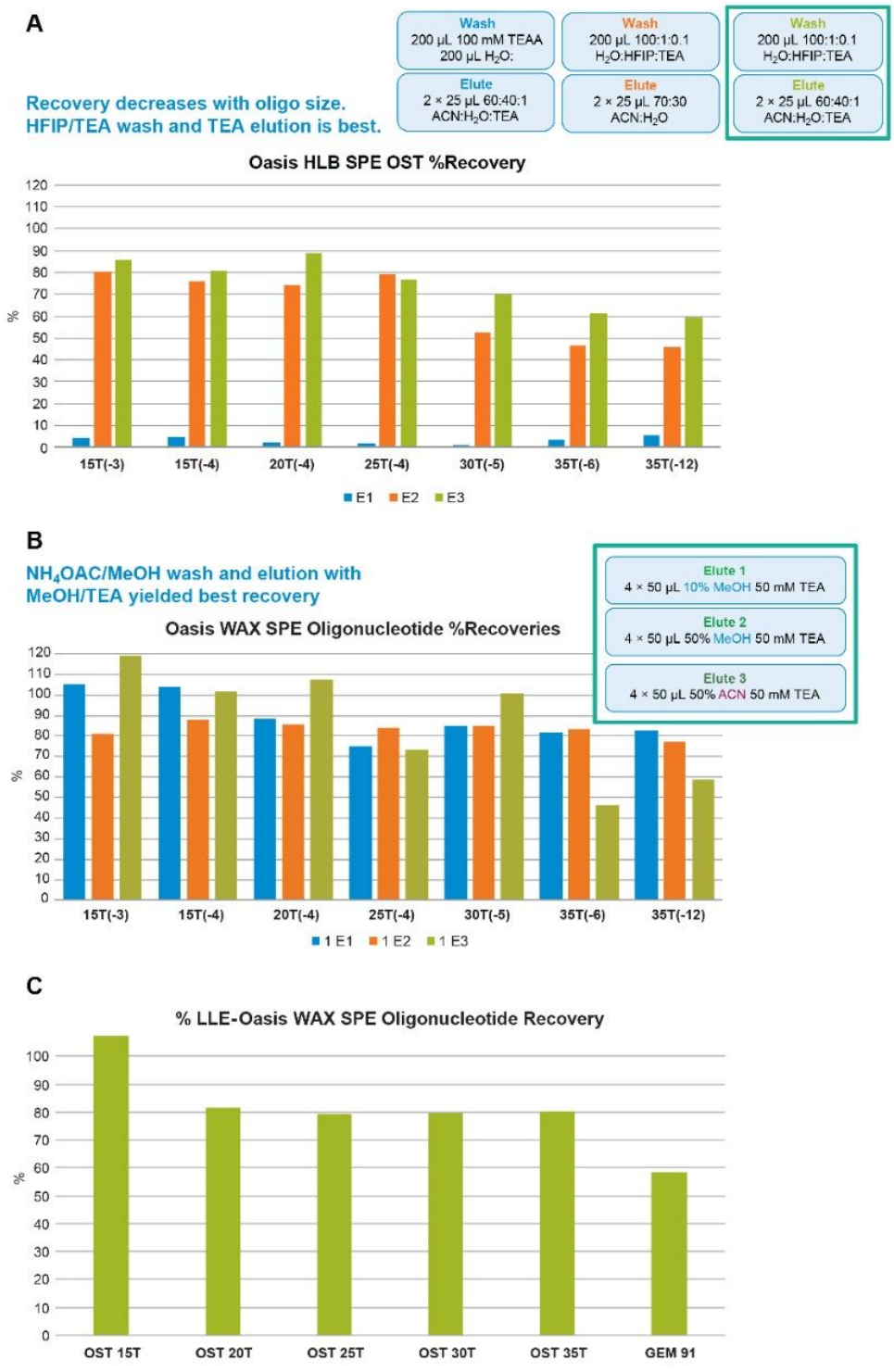

Figure 6. Oligonucleotide sample preparation recovery using (A) Oasis HLB SPE, (B) Oasis WAX SPE, and (C)

LLE-WAX SPE in the 96-well µElution format. Using an HFIP/TEA wash and elution with ACN/TEA yielded best oligonucleotide HLB recovery, while an ammonium acetate/MeOH wash and elution with MeOH/TEA yielded best oligonucleotide WAX recovery for the OSTs and GEM91. LLE combined with WAX SPE ensured efficient oligonucleotide/plasma binding disruption in plasma.

Oligonucleotides bind very strongly to plasma proteins, and effectively disrupting this binding is critical for their recovery in serum/plasma. For this reason, a liquid-liquid extraction (LLE) of the prepared oligonucleotide plasma/sera samples was performed prior to the SPE. Using the LLE protocol described in the experimental section, recoveries were greater than 80% for all oligonucleotides evaluated (data not shown). Final LLE-SPE conditions are shown in Figure 2C. Using this total extraction method (lysis/neutralization pretreatment, LLE, and Oasis WAX SPE), ensured effective disruption of plasma protein binding, whilst providing improved recovery and selectivity. Final LLE-WAX SPE recovery of the OST standard and GEM91 is illustrated in Figure 6C. For overall improved recovery, use of a sequential elution with an 80% 50 mM TEA:20% MeOH (pH 9) solution and an 80% H_2O:20% MeOH solution containing 1% chelating agent (pH 11) was employed. In addition to high recovery, use of SPE in the µElution format successfully removed the non-MS friendly denaturation/lysis buffer and facilitated sample concentration, ultimately improving overall assay sensitivity and ensured system robustness. LC-MS system performance verification (retention time/peak area) for each analytical run was performed using the Waters MassPREP OST Standard pre- and post-sample. Illustration of this performance for the 15, 25, and 35 mer of the OST standard is highlighted in Figure 7.

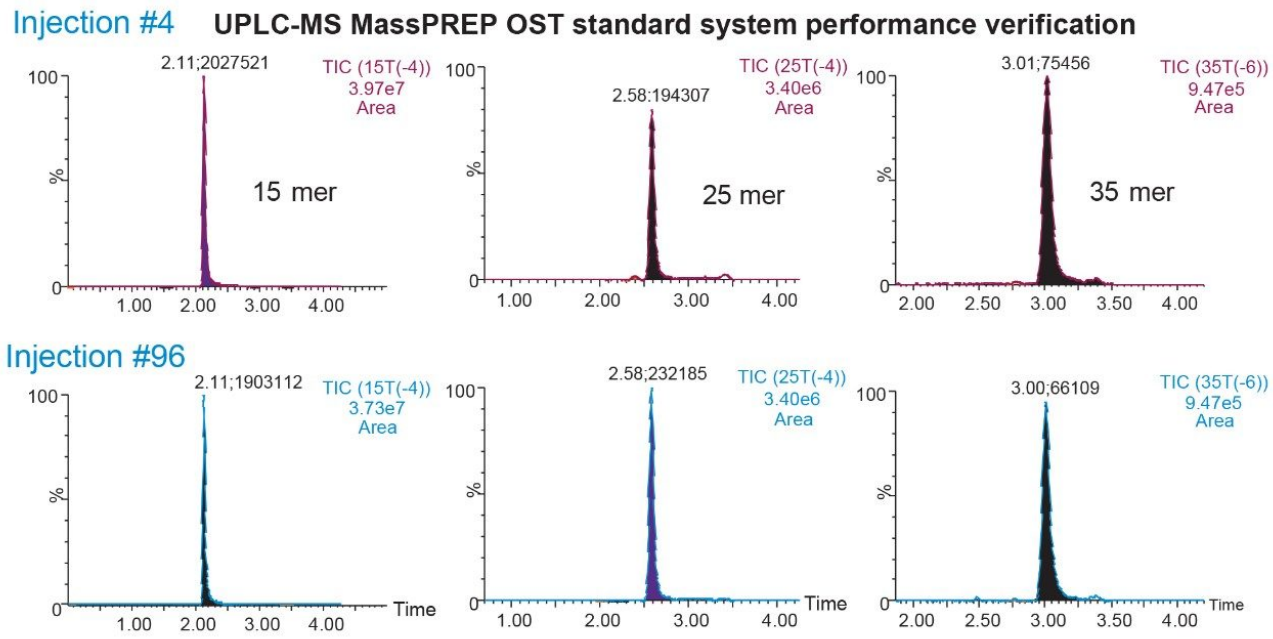

Figure 7. UPLC-MS system performance verification using the Waters MassPREP OST Standard (2 nmol/mL). Comparison of injection number 4 and 96, illustrating retention time/peak area (15, 25, and 35 mer) were maintained throughout analysis.

The combination of proper MS fragment choice improved recovery with the ACQUITY Premier Oligonucleotide C $_{18}$ Column, and selective SPE cleanup enabled limits of quantification of 0.0025 nmol/mL (OST 15 mer) and 50 ng/mL (GEM91) in neat and post-spiked LLE-WAX SPE prepared samples. A representative illustration of this OST quantification performance (linear dynamic range and LOQ) is highlighted in Figures 8 (neat solution) and 9 (post-spiked extracted plasma) for the 15 mer, Panels A and B, respectively. For GEM91, lower limit of detection achieved was 2.5 ng/mL (Figure 10). While representative illustration of GEM91 quantification performance (linear dynamic range and LOQ) is highlighted in Figures 11 (neat solution) and 12 (post-spiked extracted plasma), Panels A and B, respectively.

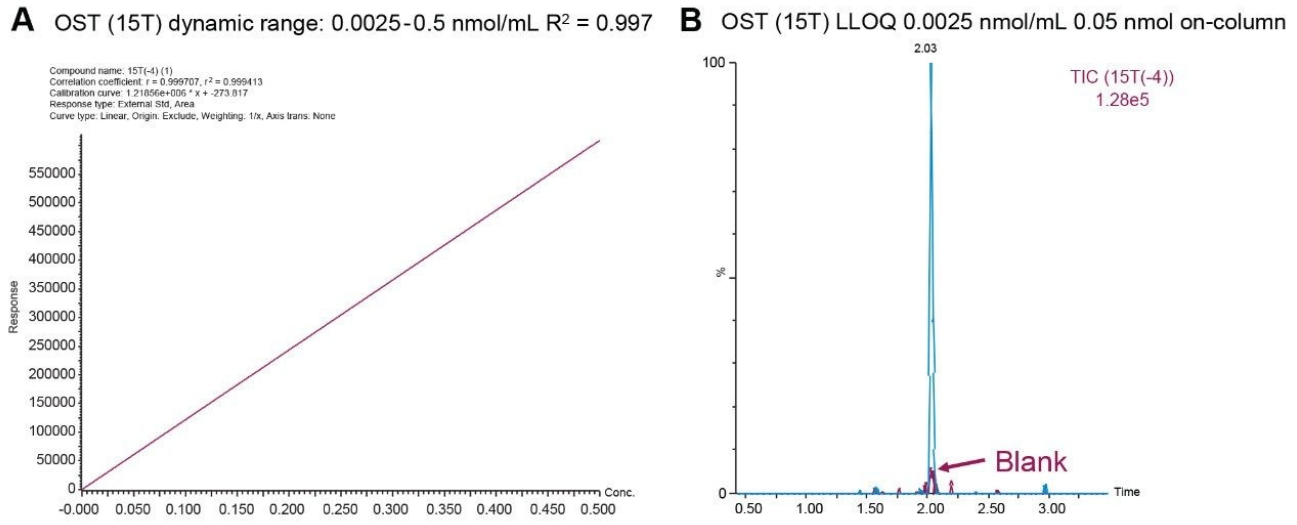

Figure 8. Representative standard calibration curve (0.025–0.5 nmol/mL) for (A) OST 15T standard and (B) LLOQ standard spiked in SPE eluate.

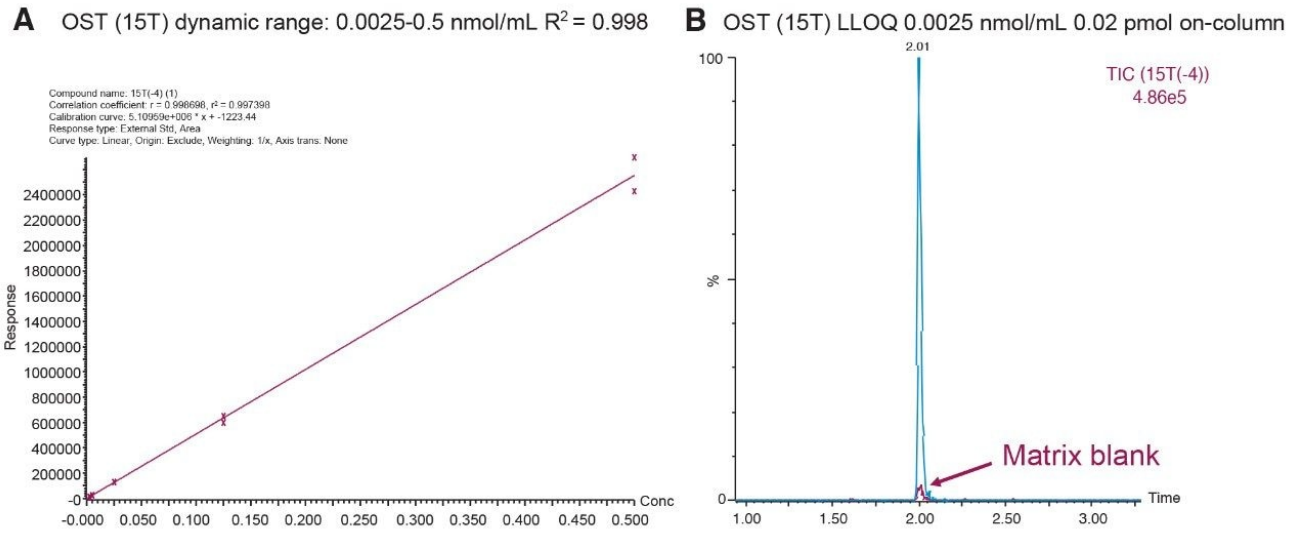

Figure 9. Representative standard calibration curve (0.025–0.5 nmol/mL) for (A) OST 15T standard and (B) LLOQ standard post-spiked in LLE-SPE extracted plasma.

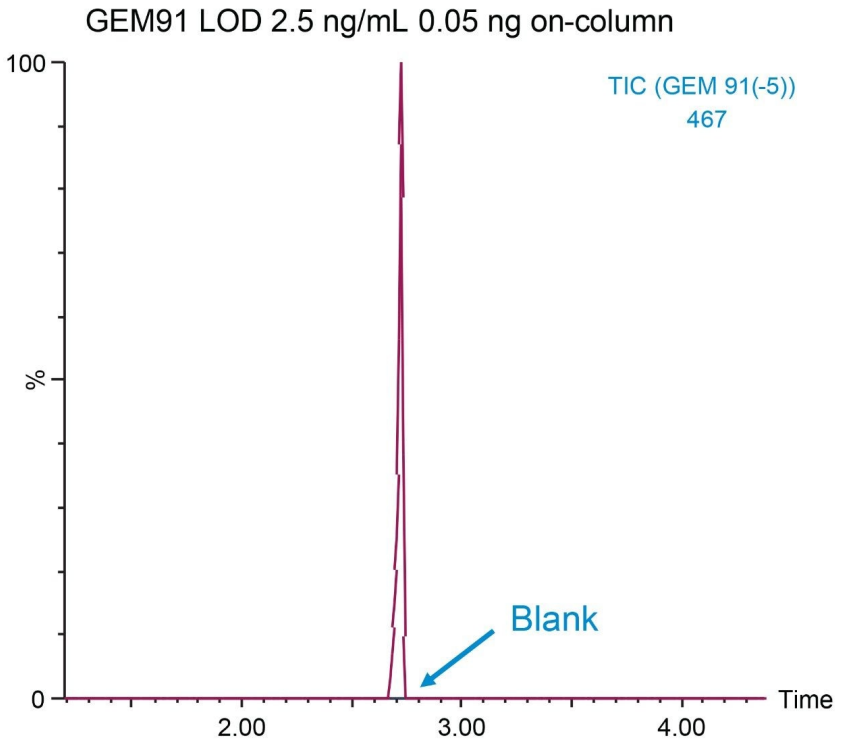

Figure 10. Chromatogram of GEM91 highlighting an LOD of 2.5 ng/mL in neat solution (0.05 ng on-column).

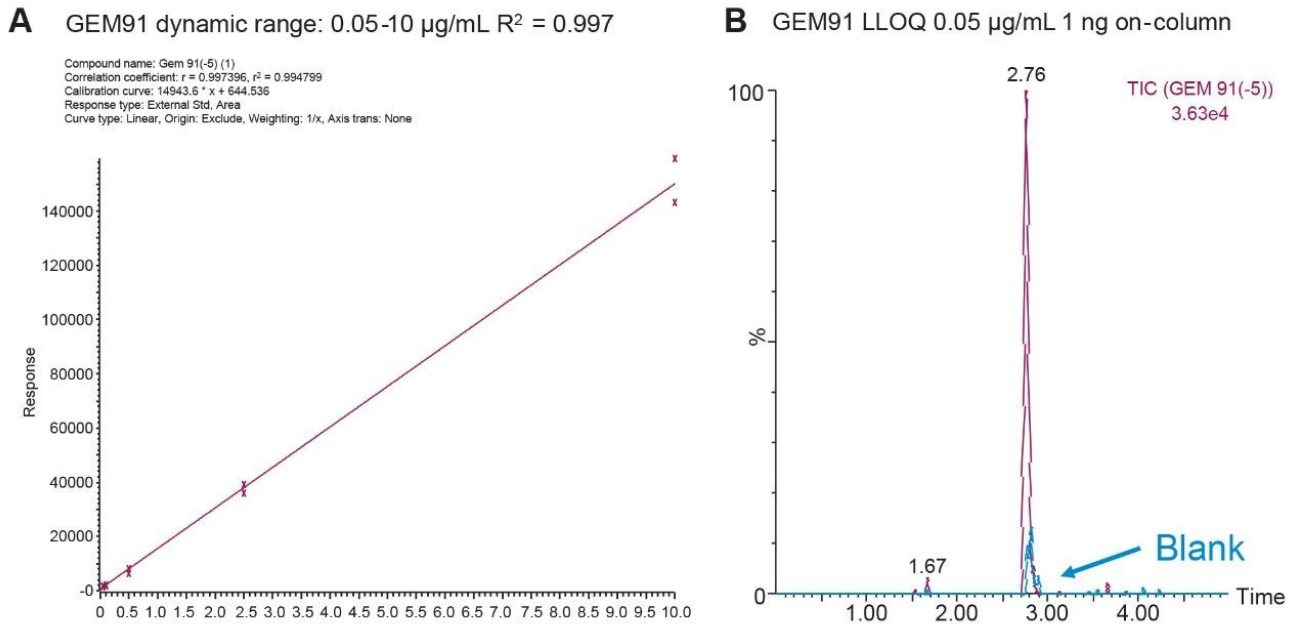

Figure 11. Representative standard calibration curve (0.05–10 µg/mL) for (A) GEM91 and (B) LLOQ standard spiked in SPE eluate.

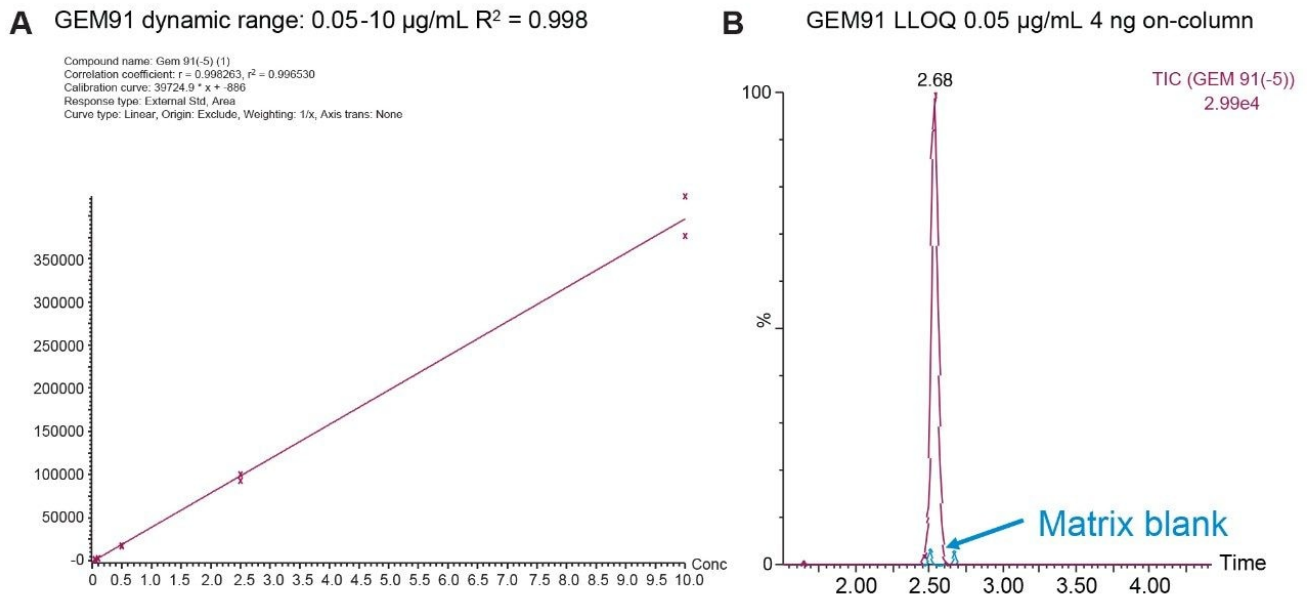

Figure 12. Representative standard calibration curve (0.05–10 µg/mL) for (A) GEM91 and (B) LLOQ standard post-spiked in LLE-SPE extracted plasma.

Conclusion

This application highlights the successful extraction and quantification of oligonucleotides from neat solution and extracted plasma. The developed method combined selective and sensitive UPLC-MS/MS analysis with LLE-SPE sample extraction using Oasis WAX SPE in the µElution plate format, providing sample concentration and high recovery. Use of the ACQUITY Premier Oligonucleotide C_{18} Column greatly improved chromatographic recovery and enabled improved LOD and LLOQs for the oligo-dT and antisense oligonucleotides that were studied. The analytical sensitivity of this developed method achieved an on-column LLOQ of 0.05 pmol for the deoxythymidine 15T OST standard and an on-column LOD of 0.05 ng for the fully phosphorothioated antisense oligonucleotide, GEM91.

References

1. GEM91 image, accessed 18 September 2020. <https://www.google.com/imgres?imgurl=https%3A%2F%2Fpubchem.ncbi.nlm.nih.gov%2Fimage%2Fimgsrv.fcgi%3Fcid%3D16204535%26t%3Dl&imgrefurl=https%3A%2F%2Fpubchem.ncbi.nlm.nih.gov%2Fcompound%2FTrecovirsen-sodium&tbnid=zslKeidmEWSzsM&vet=12ahUKEwj8066Bj_XrAhXLAN8KHQf5B6oQMygGegUIARCaAQ..i&docid=Y1CIm0B2xVZv6M&w=300&h=300&itg>

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720007019, September 2020