Enhanced protein precipitation with ammonia enables rapid, universal extraction of oligonucleotides for bioanalysis

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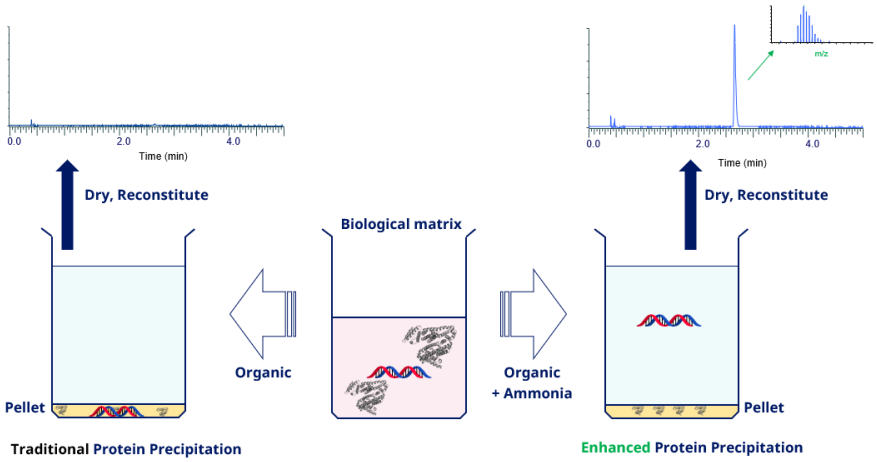

ABSTRACT: Extracting oligonucleotides from biological matrices for mass spectrometry (MS) using current methodologies is time-consuming and costly. Accelerating the discovery, characterization, and development of oligonucleotides (ONTs) requires a universal method that is low-cost, efficient, and that does not need extensive method development for each new test article. Protein precipitation using organic solvents meets these criteria for small molecules but has historically failed for oligonucleotide therapeutics due to poor recovery arising from their tendency to co-precipitate with proteins. This study introduces a novel approach utilizing small amines dissolved in organic solvents to significantly boost extraction recovery of ONTs from biological matrices .We first present the method's development and then analytically qualify it for the bioanalysis of ASOs and small interfering RNAs (siRNAs) extracted from various tissues, using ion-pairing reverse phase (IPRP) liquid chromatography coupled to tandem MS and high resolution MS (HRMS) detection. This method, Enhanced Protein Precipitation (EPP), achieves near-complete recovery across multiple ONT classes from biological matrices without need of sample digestion, costly solid phase extraction plates, or custom-designed hybridization probes. The method has proven to be more versatile and sustainable than conventional approaches.

Oligonucleotide therapeutics are a swiftly advancing drug modality. [1-3] ONTs include antisense oligonucleotides (ASOs), small interfering RNAs (siRNAs), and short hairpin RNAs (shRNAs). [4, 5] Successful discovery and development of any drug is contingent, in part, on the ability to measure, from biological samples, its absorption, metabolism, distribution, and excretion (ADME) properties by bioanalytical assays. [6] For ONTs, the predominant bioanalytical techniques used in the field are quantitative hybridization enzyme linked immunosorbent assay (hELISA),[7, 8] quantitative polymerase chain reaction (qPCR) [9] or chromatographic separation coupled to either fluorescence detection or mass spectrometry.[10-15] The preference for MS-based analysis is attributed not only to its capability for quantification but also to its utility in metabolite identification, enabling differentiation between the absolute or relative concentrations of full-length products (FLPs) and their metabolites.[13] Matrix proteins interact with nucleotides through a variety of interaction types involving diverse amino acid

residues.[16-21] The MS-based quantification of ONTs derived from biological matrices necessitates the elimination of interfering components, particularly proteins, present within these matrices.[22, 23] This challenge is also prevalent in the analysis of small molecule therapeutics; however, for this modality typically the introduction of organic solvents for protein precipitation, commonly referred to as "protein precipitation" or "crashing", serves as a straightforward extraction method.[24-26] Protein precipitation has not been widely used for ONTs due to their proclivity to co-precipitate with proteins in the presence of organic solvents, resulting in diminished recovery rates.[27, 28]

Contemporary extraction methods for ONTs employ strategies to mitigate interference from matrix proteins via protein digestion and lysis reagents. Notable examples include solid-phase extraction (SPE)[29, 30], liquid-liquid extraction (LLE),[29, 31] and hybridization techniques in which complementary sequences of peptide nucleic acid (PNA) or locked nucleic acid (LNA) probes conjugated to biotin/streptavidin beads selectively hybridize with the target compound, thereby facilitating its extraction from the biological matrix. [29, 30, 32] Designing dedicated probes for large screening experiments involving multiple test articles can be expensive and time consuming, slowing down the discovery process. Also, all mentioned extraction methods frequently incorporate a pre-digestion step utilizing proteolytic enzyme or cocktail of enzymes, such as Proteinase K, typically requiring sample processing at temperatures of 55 ºC or higher for durations ranging from 1 to 2 hours.[29, 30] While each of these techniques presents distinct advantages, they often entail substantial costs, labor-intensive procedures, and a strong reliance on automation. Furthermore, the current strategies do not guarantee sufficient recovery of oligonucleotide therapeutics for the constantly emerging novel conjugated ONTs.[29, 33, 34] Previous investigations into the use of protein precipitation for the recovery of oligonucleotide therapeutics have been documented; however, these efforts have not achieved efficient recovery comparable to those obtained through contemporary solid-phase extraction (SPE) and hybridization techniques.[27, 29] Moreover, their limited success was primarily observed with specific classes of oligonucleotide chemistries or backbones.[29, 35, 36] In this study we present a novel method that enables high recovery for multiple classes of oligonucleotide therapeutics from biological matrices through protein precipitation, which involves extraction via addition of an organic solvent modified with small quantities of ammonia or other volatile amines, such as triethylamine (TEA) and diisopropylethylamine (DIPEA). There are several existing use cases for amines with nucleic acids that serve to corroborate the mechanistic underpinnings of this extraction method. For example, (1) the stabilization of mRNA therapeutics within lipid nanoparticles (LNPs) through the use of ionizable lipids,[37, 38] (2) the application of short-chain amines as ion-pairing reagents in reversed-phase (IPRP) chromatography for oligonucleotides,[39-41] and (3) the fact that commonly used solid-phase extraction (SPE) cartridges for ONT extraction are functionalized with ionizable or cationic amine groups and utilize elution buffers alkalinized with ammonia or other amines.[42, 43]Additionally, such interactions have been even leveraged on a class of oligonucleotide therapeutic where amines with aliphatic chains, covalently bound to amidite as modifications contribute to the stabilization of ONTs and better uptake of the therapeutic due to such neutralizaiton.[44] A more in-depth review of the literature reveals sporadic reports highlighting the advantages of utilizing amines or their salts in various extraction strategies for ONTs. For instance, Zhang et al. noted that the use of tetrabutylammonium hydrogen sulfate enhanced SPE recovery.[34] Johnsson et al. reported below 25% recovery of protein precipitation for ASO compounds by using acetonitrile with incorporation of triethyl amine (TEA).[27] Given that traditional protein precipitation techniques utilizing organic solvents result in low recoveries, we refer to this novel approach as "enhanced protein precipitation" (EPP), which leads to a substantial improvement in oligonucleotide recovery post-protein precipitation.

Here, we initially present establishment of the EPP workflow followed by a brief optimization of some key parameters. Finally, we demonstrate a proof of concept applicability of EPP for bioanalysis of oligonucleotides in various biological matrices such as mouse plasma and tissue.

EXPERIMENTAL SECTION

Chemicals and Reagents. All ASOs and siRNA molecules were designed in-house and synthesized by Bio-Synthesis Inc. (Lewisville, TX). The type of chemistry and/or conjugation utilized in these compounds is detailed in Table 1. Their identity and purity were verified through liquid chromatography and mass spectrometry, while the reference quantities were determined by measuring the optical density at 260 nm. LC-MS grade methanol, acetonitrile, water, formic acid, and 1,1,1,3,3,3-Hexafluoro-2-propanol (HFIP) were procured from Fisher Scientific (Fisher Chemical, Fairlawn, NJ, USA). DNase/RNase-free water was obtained from Invitrogen (Austin, TX). LC-MS grade ammonia was sourced from Honeywell (Muskegon, MI), and N,N-Diisopropylethylamine (99.5%, biotech grade), along with various quaternary amine chloride and bromide salts (including tetrabutylammonium and tributylmethylammonium chloride), sodium chloride, ammonium formate, and other reagents, were purchased from Sigma-Aldrich (St. Louis, MO). CD-1 mouse plasma (K_2EDTA), gender-pooled, unfiltered, along with blank tissues, were acquired from Bio-IVT (Westbury, NY).

Solutions and Standard Preparation. The EPP extraction solution is comprised of 1:1 (v/v) mixture of acetonitrile and methanol, 1% ammonia (w/v) . In cases where a reagent other than ammonia is evaluated, this is explicitly stated, and concentrations of the alternative reagent are adjusted to be equimolar to 1% (w/v) ammonia (approximately 600 mM) or other values justified for given experiments. For quantitative analyses, an internal standard (IS) was incorporated at a concentration of 100 ng/mL into the EPP extraction solution. Analyte reference materials were individually reconstituted in DNase/RNase-free distilled water to a final concentration of 10mg/mL. For qualitative, mechanistic, and recovery studies, working solutions of ASO1, ASO2, ASO3, siRNA1, and siRNA2 were spiked into plasma or other media to achieve a final concentration of 1000 ng/mL.

Quantitative assessment. The MS/MS-based quantitative evaluation of plasma samples was conducted for ASO2, utilizing ASO4 as an internal standard (IS), with calibration standards prepared at concentrations of 1.00, 2.00, 5.00, 10.0, 20.0, 50.0, 100, 200, 500, 1000, and 2000 ng/mL. The lower limit of quantitation (LLOQ) and quality controls (QCs) were prepared at 1.00 ng/mL (LLOQ), 3.00 ng/mL (LQC), 750 ng/mL (MQC), and 1500 ng/mL (HQC) in plasma. HRMS-based quantitation of plasma samples was performed for siRNA3, with siRNA4 serving as the IS. Calibration standards in plasma were prepared at concentrations of 5.00, 10.0, 20.0, 50.0, 100, 200, 500, 1000,

2000, and 5000 ng/mL. The LQC, MQC, and HQC for siRNA3 were prepared at 15.0, 750, and 3500 ng/mL, respectively, in plasma. Additionally, HRMS quantitation for siRNA3 was conducted on liver homogenized 1:9 (w/v) in blank plasma. Calibration standards were prepared at 50, 100, 200, 500, 1000, 2000, 5000, 10000, 20000, and 50000 ng/g $_{tissue}$ (5.00 to 5000 ng/mL $_{Homogenate}$) with 3 QC levels at 750 ng/ g $_{tissue}$ (LQC), 15000 ng/ g $_{tissue}$, and 35000 ng/ g $_{tissue}$. Cross-matrix quantitation of kidney and heart homogenates vs calibration curve made in liver homogenate was demonstrated at LQC level for each tissue type.

Sample Extraction. In this report, the extraction solvent-to-sample ratio is maintained at 4:1 (v/v) unless indicated otherwise (see Results and Discussions). Typically, a 100 µL aliquot of plasma or liver homogenate (called 1X volume) is extracted with 400 µL of the pre-chilled EPP extraction solution (4X volume). The mixture is vigorously agitated for a minimum of 5 minutes at room temperature and subsequently centrifuged at 18,000 rcf for at least 20 minutes. The supernatant is then collected and evaporated under a nitrogen flow using a plate-based evaporator (SPE Dry™ Biotage, Salem, NH). Following evaporation, the lyophilized sample resuspended in 100uL of 10% methanol (aq) followed by a 60sec vortex at 2500 rpm. The reconstituted sample is subjected to an additional centrifugation step for 5 minutes at 18,000rcf, with 90 µL of the clarified supernatant transferred to injection plate. 5 µL final sample was analyzed by ultra-high-performance liquid chromatography (UHPLC) coupled with either a high-resolution mass spectrometer (HRMS) operated in full scan mode or a triple quadrupole mass spectrometer (MS/MS). Additional details regarding EPP extraction using other reagents can be found in the Supporting Materials.

UHPLC-HRMS and MS/MS. Chromatographic separation of oligonucleotides (ONTs) was achieved using reversed-phase ion-pairing chromatography. A 2.1 mm x 50 mm Waters Premier BEH C18 Oligonucleotide column was used. A binary mobile phase system was used including mobile phase A consisted of 10% methanol in water, and mobile phase B consisted of 70% methanol and 20 % acetonitrile in water, both mobile phases supplemented with 1% (v/v) 1,1,1,3,3,3-Hexafluoro-2-propanol (HFIP), 0.25% N,N-Diisopropylethylamine (DIPEA), and 2.5 µM EDTA. The flow rate of the mobile phase was set to 0.3 mL/min at a temperature of 75 °C. Further information is available in the Supporting Materials. For mechanistic studies, HRMS-based analyses were performed using either a Q-Exactive HF or an Orbitrap Exploris 120 (both Thermo Fisher Scientific, San Jose, CA), both instruments equipped with an H-ESI source operating in negative ion mode. MS/MS-based quantitation of ASO2 was conducted using the QTRAP 6500+ (Sciex, Framingham, MA) with an electrospray ionization (ESI) source operating in negative ion mode and employing multiple reaction monitoring (MRM) optimized for ASO2 and its internal standard (IS), ASO4 (Supporting Table S.1). HRMS-based quantitation of siRNA3 was executed using a Q-Exactive HF mass spectrometer operated in full scan mode. Additional details regarding these methodologies are provided in the Supporting Materials.

Recovery calculations and data processing. In all cases a recovery is presented as ratio of chromatographic peak area for a given compound in extracted sample versus that in neat aqueous (Aq) solution at same concentration. All recovery measurements are performed as minimum duplicate measurements. Other data processing was done with the corresponding softwares from each MS vendor (supporting material). Graphs were plotted with GraphPad™ Prism 10 software.

RESULTS AND DISCUSSIONS

Establishing EPP. Development of a successful extraction method for ONTs via protein precipitation necessitates recognition of two distinct factors: (1) When biological matrix is absent, such as in an aqueous solution, the addition of a 4X organic solvent results in chromatographic peak splitting rather than precipitation, in addition to lower signal due to dilution; (2) Conversely, in the presence of a biological matrix, ONTs undergo actual precipitation upon the introduction of an organic solvent. Figure 2A presents the combined extracted ion chromatogram (combined XIC, explained in Supporting Materials) of ASO1 in a neat Aq solution, serving as a reference signal. As depicted in Figure 2B, when a 4X 1:1 acetonitrile:methanol mixture (termed organic solvent) is added to a 100 µL aqueous solution of ONTs, resulting in an 80% organic content, a notable peak splitting of ASO1 during the IPRP chromatography is observed. This process results in elution of the majority of ASO1 at the void volume (0.4 min) and a minor peak the expected retention time (RT) of 2.7 min. Mass spectrometry confirmed that the early eluting peak at 0.4 minutes (z=-5 charge state) was indeed ASO1, and not due to crosstalk with background signals. Common strategies of peak shape improvement such as lowering the injection volume or increasing Aq content did not fully resolve this peak splitting, while it would decrease the sensitivity. If the typical practice of diverting void eluents to waste is followed, the unretained portion of ASO1 would not be detected, potentially leading to the erroneous conclusion of "precipitation." This interpretation was confirmed by drying the supernatant and reconstituting it to the original 100 µL volume using 10% methanol in water (termed reconcentration), where ASO1 nearly fully recovered at 90% at the anticipated retention time of 2.7 min (Figure 2C). In contrast, ONTs did truly precipitate within biological matrices upon the addition of organic solvent. When 100 µL plasma solutions of ASO1 were treated with 4X organic solvent, negligible recovery was observed both before (Figure 2D) and after (Figure 2E) reconcentration. Comparisons between these and the findings when matrix was neat Aq solution suggests that ONT loss through "precipitation" in biological samples is significantly associated with the presence of biological matrix components, likely proteins, rather than an inherent insolubility in solutions at 80% (v/v) organic content.

Incorporating ammonia or other small volatile amines in the extraction solution enhanced the recovery of ONTs from biological matrices. Figure 2F illustrates that the inclusion of 1% (w/v) ammonia (approximately 590 mM) in this organic solution (termed EPP extraction solution) facilitated the recovery of ASO1 from mouse plasma. Similarly enhanced recoveries were observed with the same molar concentrations of other small amines in extraction solution, such as triethylamine (TEA) (Figure 2G) and diisopropylethylamine (DIPEA) (Figure 2H), both achieving recoveries exceeding 90%. These trends were consistent across ASO2, ASO3, and various siRNAs, as described in subsequent discussions. Additional small volatile amines, including trimethylamine (TMA), tripropylamine (TPA), and hexylamine (HA), were also evaluated with satisfactory outcomes (Figure S.1). Ammonia was ultimately chosen as the EPP reagent due to its lower boiling point, a more manageable

odor than certain alternatives like TMA, and better solubility than other amines. Other qualitative observations further justified the selection of ammonia (data not shown), for example that DIPEA resulted in reduced pellet thickness and less clean extracts, or tributyl amine (TBA) forming immiscible droplets.

Recovery does not depend on the order in which ammonia and organic solvent are added to the sample. Adding ammonia either before or after the organic solvent yielded comparable results with the addition of a combined solution, highlighted in Figure 3. Furthermore, when a solid pellet obtained post-centrifugation of a plasma sample exposed to 4X volume of organic solvent, where low recovery in supernatant is already established, was extracted by EPP the ONTs were recovered at near 100% recovery. These observations suggest that ammonia might act as disruptor of the interactions between ONTs and matrix, at any step of the precipitation and "rescue" ONTs from the matrix and keep them soluble in the supernatant. It is worth pointing out that although extraction from precipitated pellet involves additional steps than direct EPP, since biological samples are often limited, this approach potentially offers parallel extraction of molecules of different classes from the same biological sample (i.e. small molecule therapeutics/endogenous metabolite/lipid biomarkers) in supernatant of the organic extraction step, and ONTs from its pellet. This would be beneficial for studies where such comprehensive analysis is needed while it is not as easily achieved in other ONT extraction strategies such as SPE or Hybridization.

EPP extraction for both strands of siRNAs were effective. EPP allows recovery of both Sense and Antisense strands from siRNAs from mouse plasma (Supporting Figure S2).

Ammonia Optimization. The effect of ammonia concentration in the extraction solution was examined (Figure 4). An optimal 1% (w/v) ammonia enhanced recovery for ASOs. For both siRNAs addition of even 0.1% (w/v) ammonia increased the recovery to 70% which after addition of 1% (w/v) was increased to above 80%. Elevated concentrations of ammonia above 2% (w/v) resulted in formation of less desirable gel-like samples, and lack of clear supernatants after centrifugation, impeding further processing. For universality of future experiments, we chose 1% (w/v) ammonia to be included in EPP extraction solution.

Evaluation of Organic Solvents. The combination of acetonitrile and methanol (1:1) with 1% (w/v) ammonia demonstrated superior results in plasma recovery compared to using either solvent alone (Figure 5). While methanol showed reasonable recovery across different ONTs, it muddled supernatant clarity and introduced background noise, eventually impacting column integrity. Post-extraction spiking of ASO4 and siRNA4 confirmed that methanolic extracts are showing suppressed signals which are more extensive for GalNAc conjugated siRNA4. In contrast with methanol with 1% (v/v) ammonia, using acetonitrile with ammonia rendered negligible recovery.

Solvent-to-Sample Ratio Optimization. Different EPP extraction solvent volumes were tested (Figure 6). Excessive solvent (10X) diminished recovery, denoting a true precipitation at very high organic contents. Less than 4X volumes caused elevated column back pressure and more ion suppression over the extended periods of application. Opting for 4X volume avoided these issues.

Although a systematic comparison of EPP with other extraction methods for ONTs was not performed in this report, we observed that EPP offered improved recovery for lipid-conjugated siRNAs (above 75%) where SPE lagged under 50%. Other ONT classes performed similarly across EPP and SPE. Summarized qualitative observations and matrix interference are detailed in supplementary material. (Supplementary Figure S.3)

We propose that biological matrix exposure to organic solvents results in co-precipitation of ONTs with proteins, which ammonia or similar bases can mitigate. Understanding how amines perform this requires further experiments. However, 3 other notable observations may provide a starting point for future research: (1) lack of recovery when weak acids were used in extraction solution, (2) partial recovery when extraction solution contained small amounts of strong base like sodium hydroxide, and increasing recovery with increasing sodium hydroxide content from 3 to 5 mM, and (3) partial recovery when sample was pre-incubated with large amount of soluble inorganic salts like NaCl and ammonium formate at same molarity as 4X volume of 1% ammonia would provide; roughly 2.4 Molar (Supplementary Figure S.4). Although ammonium from EPP solution and phosphate from the ONT backbone create ionic pair, in contrast with other amines used here, ammonia, is not able to form ion-pairs as strong as other amines [45] hence, EPP is not driven solely by ion-pairing. However, in addition to ionic interactions amines and their ammonium ions can interact with nucleotides through variety of other forces like hydrogen bonding with different moieties of ONTs.[41, 46-49] One other factor that drives interaction with proteins is the extent of phosphorothioate (PS) in the backbone instead of phosphate (PO). As observed in Figure 4, ASOs require higher amounts of ammonia than siRNAs to be recovered from plasma. This agrees with higher unbound fractions observed for siRNAs[50] than those reported for ASOs.[51] Due to high PS content, ASOs are known to bind tightly to proteins.[52] The amount of ammonia in EPP (~600 mM) is in drastic excess compared to most abundant proteins in mouse plasma such as albumin (~600 µM). Elevation of pH impacts cationic residues of proteins, such as lysine and arginine, which leads to decreased charge interaction of those residues with negatively charged backbone of ONTs. In that situation, ONTs can interact with other components in solutions such as ionic interaction with ammonium ions and hydrogen bond with water and ammonia thus being freed from proteins that are meanwhile being precipitated. Lastly, the solubility of ammonia in aqueous and organic solvents, while it increases the pH and being able to provide ionic and H-bond interactions makes it a desirable molecule having all factors needed to rescue ONTs from matrix proteins (Supplementary Figure S.5).

Analytical Method Qualification:

A preliminary qualification of the EPP extraction method for the bioanalysis of oligonucleotide therapeutics (ONTs) was conducted. Analytical method qualification for two categories of ONTs, ASOs and siRNAs, is presented here while a different MS detection strategy was used for each category to showcase compatibility of EPP with different MS platforms commonly used for small and large molecule bioanalysis. For ASOs, we conducted MS/MS-based quantitation in MRM mode on a triple quadrupole mass spectrometer, while for siRNAs we chose full scan HRMS for the analysis. In each case calibration curves and associated quality control (QC) samples were analyzed and their accuracy and precision values assessed. For ASO2 in mouse plasma, the precision and accuracy of QC samples were assessed through inter-day and intra-day analyses, utilizing two calibration curves and four QC levels, each with six replicates.

For siRNA3 assessment was conducted in mouse plasma and liver homogenates, each with two calibration curves and 3 QC levels within a single-day evaluation. Additionally, since other tissue types are often limited, cross-tissue quantitation of siRNA3 in kidney and heart homogenates was examined via use of liver homogenate calibration curve. Despite EPP compatibility with automation, all spike-in samples, calibration curves, and QC samples were manually prepared.

Analytical Method Qualification for ASO2. The precision and accuracy of ASO2 QC samples are documented in Table 2, using ASO4 as the internal standard (IS). MS/MS parameters for ASO2 and ASO4 are outlined in Table S.1 (Supplementary Materials). For both ASO2 and the IS, the phosphorothioate fragment ion corresponding to m/z 95.0 was monitored. The response factor, expressed as the ratio of ASO2 to ASO4 peak areas, was plotted against the concentration of ASO2 utilizing linear regression with $1/x^2$ weighting, covering a range from 1 ng/mL to 2000 ng/mL (2000x). This methodology achieved linearity with R^2 values of 0.9972, 0.9929, and 0.9914 across three distinct runs. On-column carryover caused by upper limit of quantitation (ULOQ) samples in the calibration curve (2000 ng/mL) presented a notable challenge. This issue was effectively mitigated by injecting four matrix blanks, which reduced carryover signal to 20% of the lower limit of quantitation (LLOQ, 1 ng/mL). Figure 7 illustrates representative extracted ion chromatograms (XICs) for ASO2 and the IS in blank mouse plasma (Figure 7A), as well as at LLOQ (Figure 7B) and ULOQ levels (Figure 7C) accompanied by stability of IS across the run (Figure 7D) an ASO2 calibration curve in mouse plasma (Figure 7E). Although IS crosstalk was noted in the analyte channel (Figure 7B), the opposite crosstalk was not observed. Due to the chromatographic resolution achieved, this crosstalk did not impede accurate quantification.

Analytical Method Qualification for siRNA3. The evaluation of siRNA3 was conducted using HRMS detection following EPP extraction from plasma and tissue homogenate samples. While the focus was on the antisense strand, it is noteworthy that HRMS detection allows for potential detection of both antisense and sense strands and their potential metabolites. Liver samples were homogenized in blank plasma, as detailed in the Methods section.

Figure 8 shows the combined XICs for siRNA3 and its corresponding IS in blank mouse plasma (Figure 8A), at LLOQ (Figure 8B), and ULOQ levels (Figure 8C), and the calibration curve for siRNA3 in both mouse plasma (Figure 8D) and liver homogenate (Figure 8E). IS crosstalk was observed in the analyte channel (Figure 8B) however this posed no challenge the chromatographic resolution of the peaks. Using HRMS for siRNAs, in range of 5 to 5,000 ng/mL a linearity of 0.998 was achieved for plasma samples and 0.997 for liver homogenates between 5 to 5,000 ng/mL$_{homogenate}$ (50 to 50,000 ng/g$_{tissue}$). Table 3 summarized the analytical qualification outcomes for siRNA3 in mouse plasma and liver homogenate after extraction with EPP. Cross tissue quantitation was successful for kidney and heart tissues.

Interestingly, the calculated molar equivalents of the obtained LLOQs were approximately 0.15 nM for ASO2 and 0.13 nM for siRNA3, underscoring the similar molar sensitivity achieved for EPP across various compounds and across different MS workflows for quantitation.

CONCLUSIONS

In this study we developed and qualified enhanced protein precipitation (EPP), a novel method for the bioanalysis of oligonucleotides which overcomes many limitations of existing approaches, including high cost, lengthy assay time, and intensive method development. EPP is protein precipitation with the addition of ammonia or other low molecular weight amines such as TEA or DIPEA. This unique optimization in the standard precipitation procedure boosts recovery of ONTs when extracted from biological matrices enabling a bioanalytical extraction method that is rapid, cost-effective, and scalable. EPP is applicable for chemically diverse oligonucleotide therapeutics, including ASOs and siRNAs and associated novel chemical conjugates of these molecules. We report that ammonia is ideally suited as an additive for this application due to several practical advantages, including ease of evaporation, cost, and post-precipitation extract consistency. We speculate that the enhancement of ONT recovery by amines is via disruption of interactions between proteins and oligonucleotides, leading to rescue of ONTs from matrix during the extraction step. While some properties of ammonia like hydrogen bond donor/acceptor activity as well as high pH may have other critical roles in the success of EPP; more evidence on this matter is needed and will be evaluated in future studies. Finally, we evaluated the applicability of EPP for the bioanalysis of biological samples, demonstrating excellent limits of quantitation, linear dynamic range, and good reproducibility. We anticipate that EPP will become a core extraction method for oligonucleotide bioanalysis, facilitating ONTs drug discovery and development.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge. Detailed experimental section, data processing, recovery when other amines used, comparison of recovery for sense and antisense strands for siRNAs, extraction with inorganic salts and sodium hydroxide, MRM optimized parameters for ASO2 and IS, and schematic view of proposed interactions between proteins and ONTs as well as amines and ONTs (PDF)

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Figures and Tables

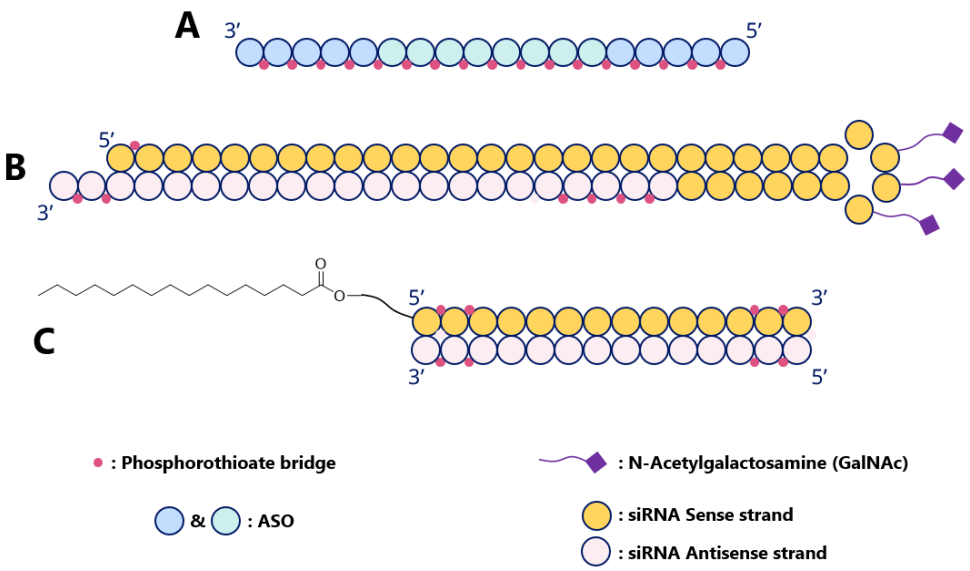

Figure 1. Representative structures for (A) ASO, (B) GalNAc conjugated siRNA, and (C) lipid conjugated siRNA

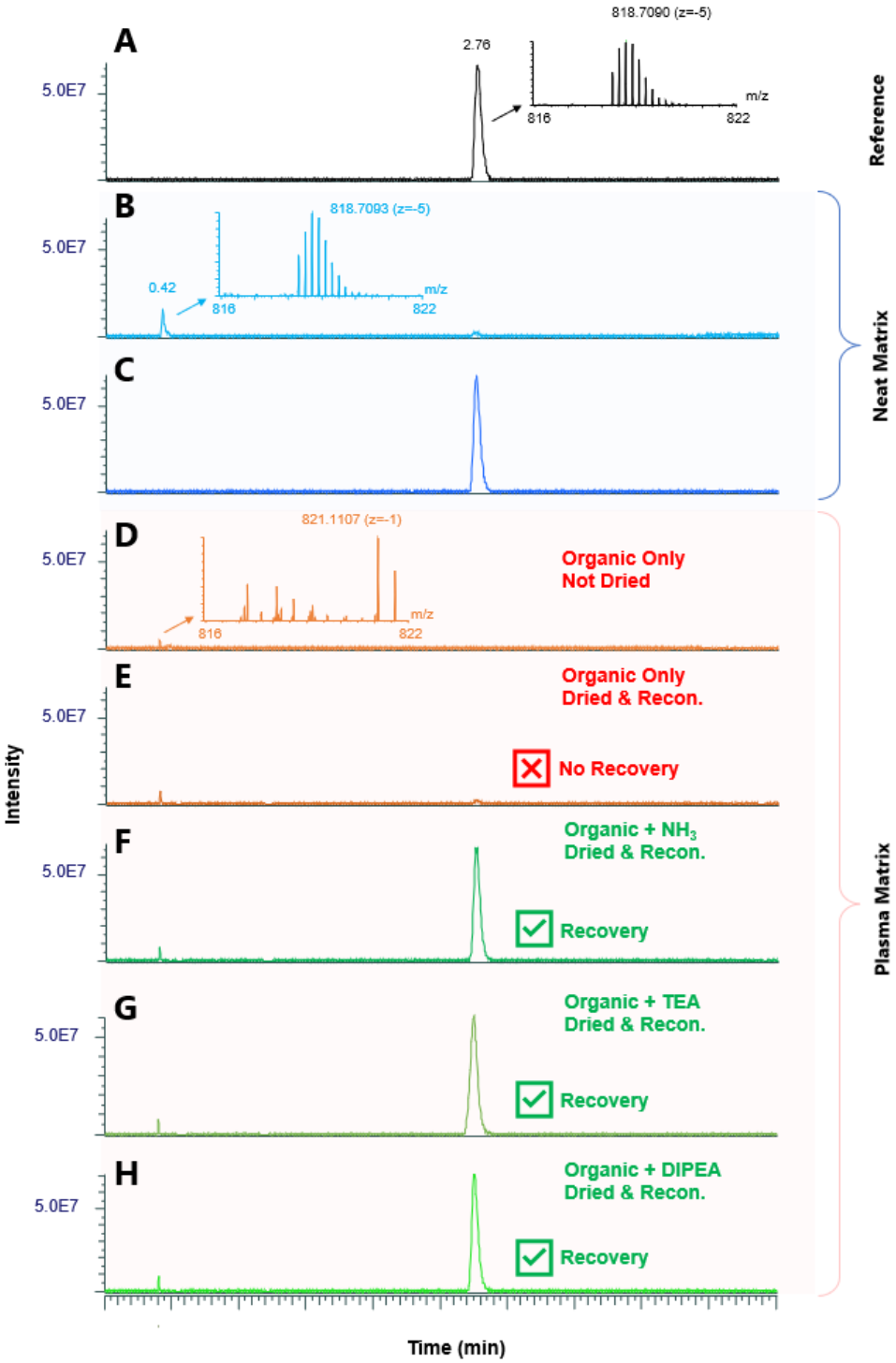

Figure 2. Combine Extracted Ion Chromatograms (XICs) of ASO1 by combining XIC of all charge states ranging from 650-2500 m/z. (A) ASO1 peak at RT 2.6 mins in neat aqueous solution, (B) ASO1 peak post organic only EPP extraction in water observed as retaining in the void volume due to presence of 80% organic in the injection, (C) ASO1 peak observed at the expected RT of 2.6mins post drying and re-constitution in 10% MeOH, (D) No ASO1 peak in void volume post organic only EPP extraction in plasma with 80% organic in the injection, (E) No ASO1 peak at expected RT post organic only EPP extraction in plasma with sample drying and reconstitution, (F) ASO1 peak at expected RT of 2.6mins post organic +NH3 EPP in plasma with sample drying and reconstitution, (G) ASO1 peak at expected RT of 2.6mins post organic +TEA EPP in plasma with sample drying and reconstitution, (H) ASO1 peak at expected RT of 2.6mins post organic +DIPEA EPP in plasma with sample drying and reconstitution

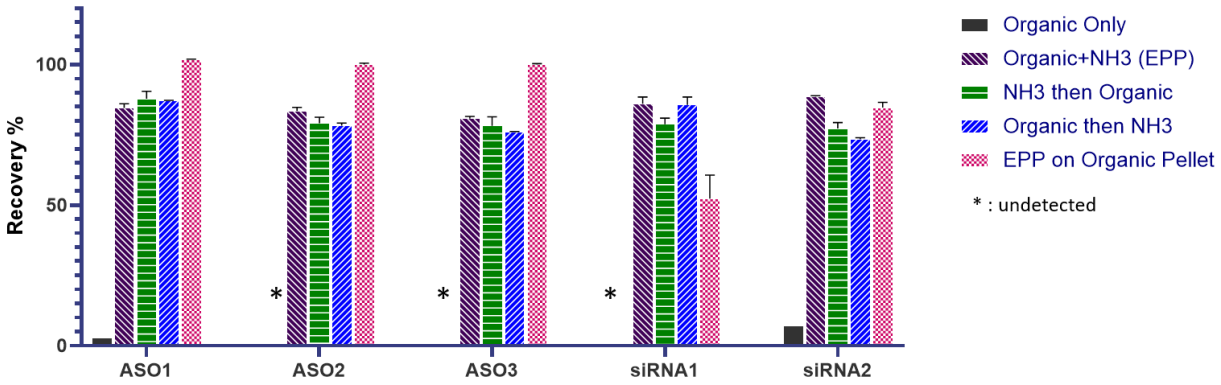

Figure 3. Effect of order of ammonia addition to different steps along the EPP extraction process to demonstrate no impact on recoveries for all 5 analytes. Furthermore, highlighting the role of ammonia in rescuing ONT from organic pellet (dotted bar plot)

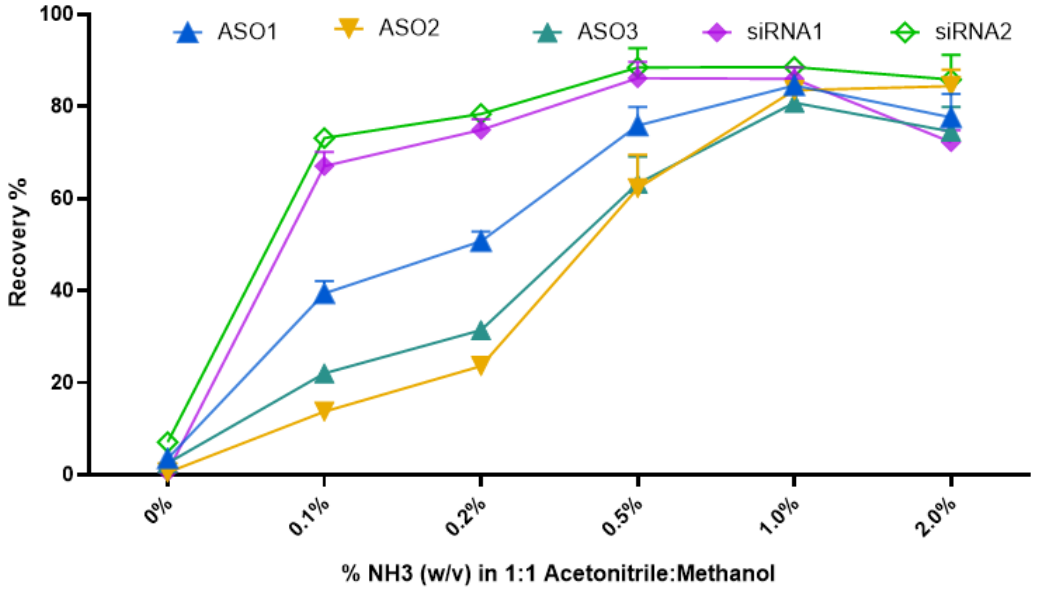

Figure 4. Assessment of extraction recoveries for all 5 analytes with increasing ammonia concentration in EPP extraction solution (Recovery % calculated as peak area ratio of combined analyte XIC pre-spiked samples/combined analyte XIC in neat solution)

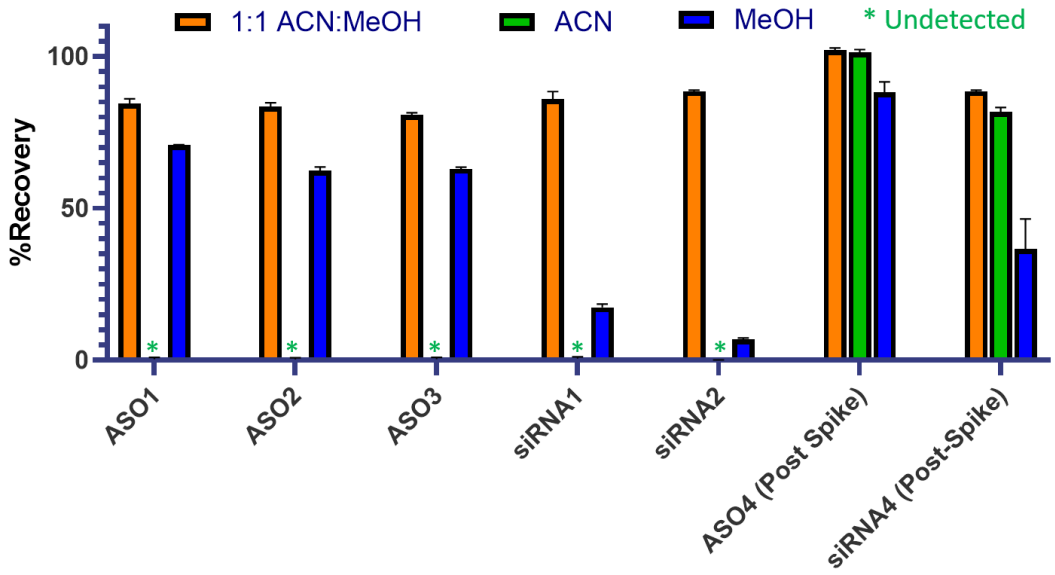

Figure 5. Selection of organic solvent as a choice for extraction solution by assessing recoveries of all 5 analytes in either 1:1 Acetonitrile: Methanol mixture, 100% Acetonitrile or 100% Methanol. Post-spike recoveries of ASO4 and siRNA 4 calculated after spiking known concentrations in EPP extracted and reconstituted mouse plasma. % recovery in post-spike samples lower for Methanol vs. Acetonitrile due to ion-suppression effects of an unclean sample.

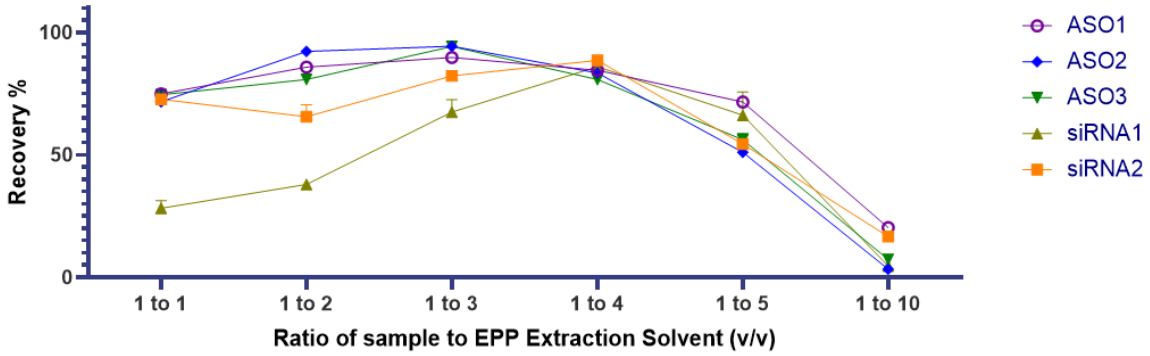

Figure 6. Assessing Solvent-to-Sample ratio by evaluating recoveries of all 5 analytes in plasma by EPP extraction using organic+NH3

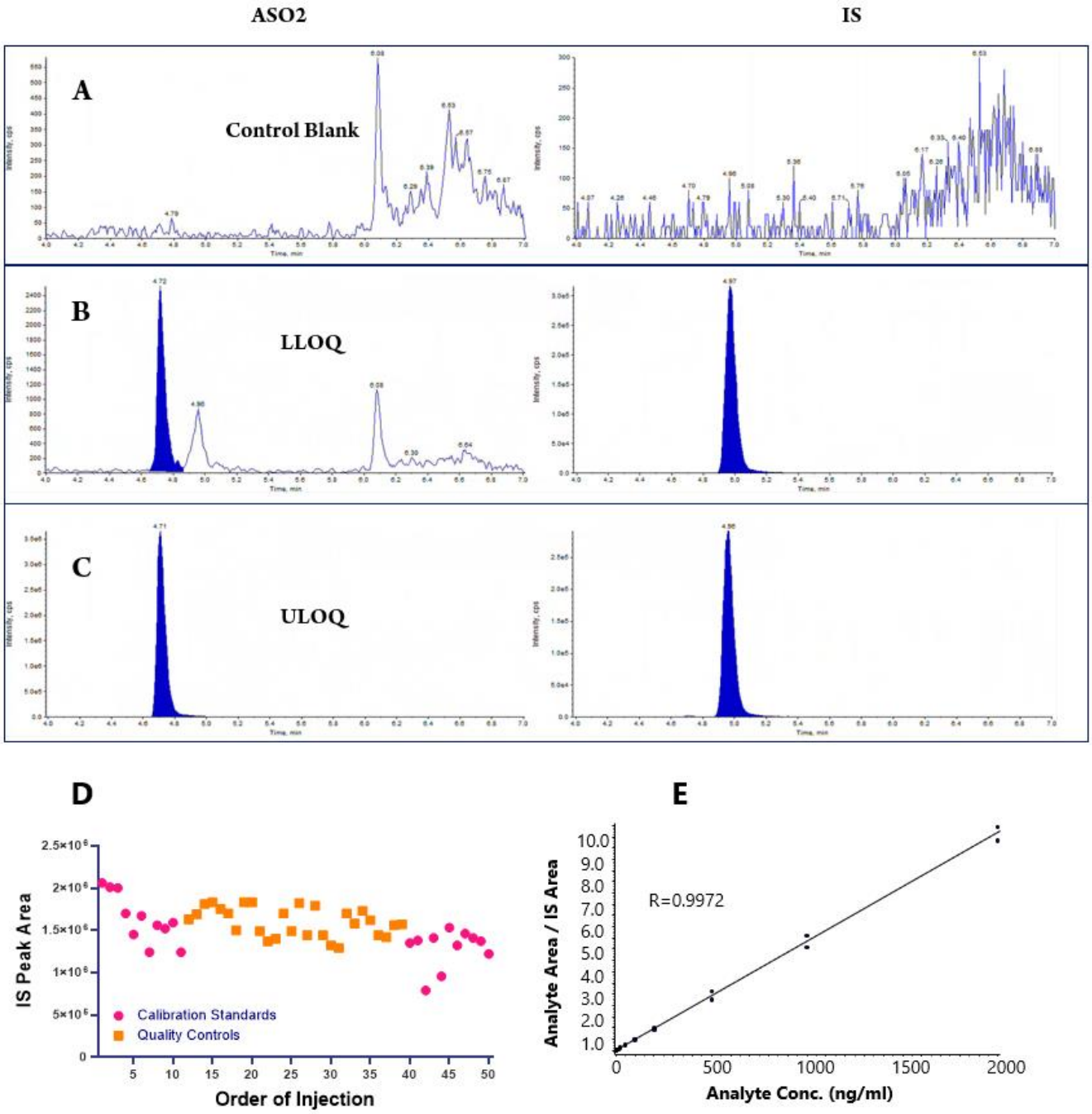

Figure 7. LC-MS/MS chromatograms of ASO2 and the IS from (A) blank mouse plasma sample, (B) spiked LLOQ sample at 1ng/mL in mouse plasma, (C) spiked ULOQ sample at 2000ng/mL in mouse plasma, (D) IS peak area response over the course of data acquisition (E) Calibration curve of ASO2 from 1ng/mL – 2000 ng/mL in mouse plasma.

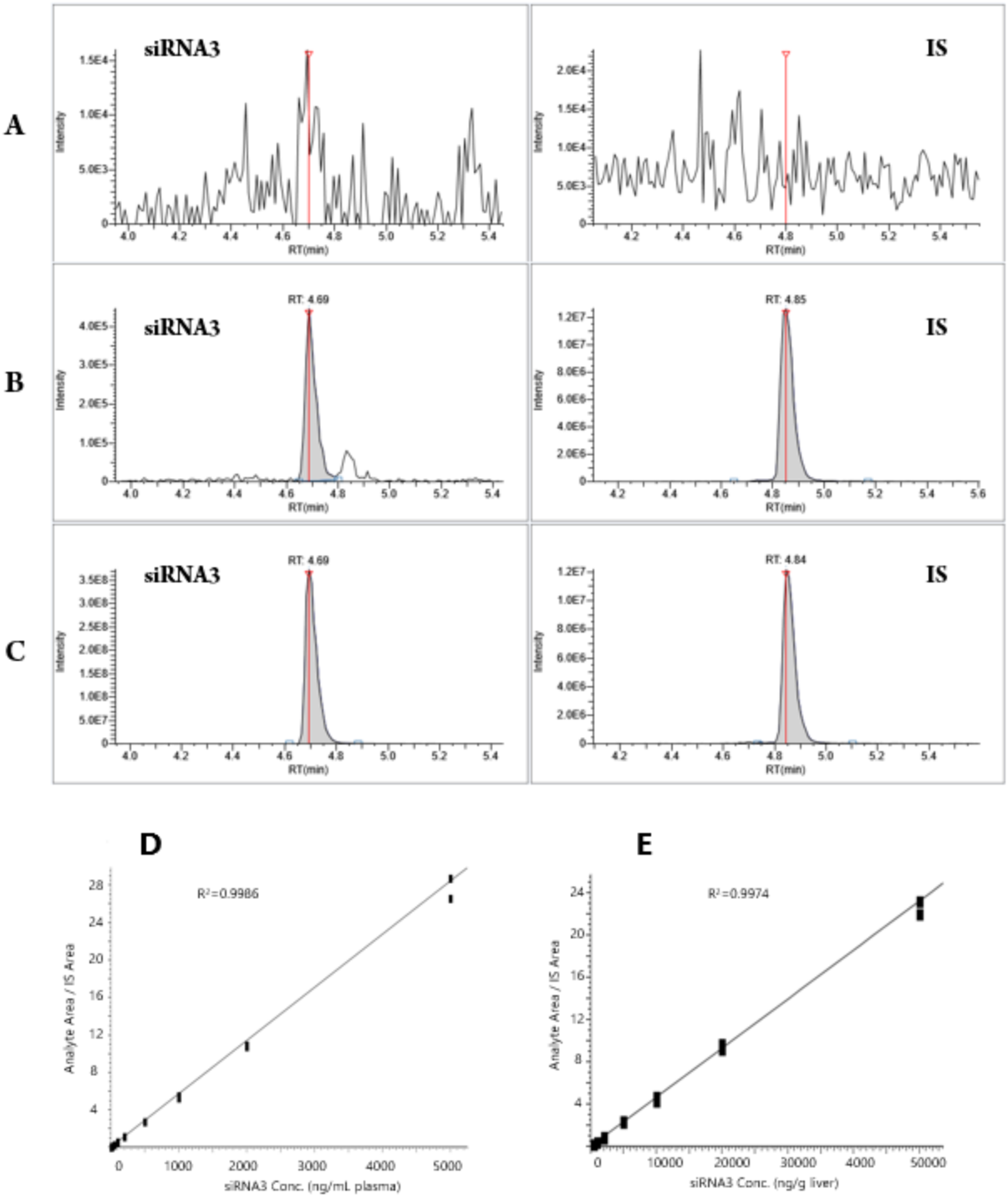

Figure 8. Combined Extraction Ion Chromatograms (XICs) of siRNA3 and the IS from (A) blank mouse plasma sample, (B) spiked LLOQ sample at 5ng/mL in mouse plasma, (C) spiked ULOQ sample at 5000ng/mL in mouse plasma, (D) Calibration curve of siRNA 3 from 5ng/mL – 5000 ng/mL in mouse plasma, (E) Calibration curve of siRNA 3 from 5ng/mL – 5000 ng/mL (equivalent to 50-50,000 ng/g tissue) in mouse liver homogenate

TABLE 1. ASO & siRNA Analytes and Internal Standards.

Name	MW (kDa)	Chemistry[a]	Use
ASO1	4.1	MOE/DNA gapmer with PS backbone	Analyte
ASO2	6.4	MOE/DNA gapmer with PS backbone	Analyte
ASO3	3.8	MOE/DNA gapmer with PS backbone	Analyte
ASO4	7.1	5-10-5 MOE Gapmer with PS backbone	Internal Standard
siRNA1	S: 13.3 AS: 7.4	GalNAc Conjugated siRNA, mixed backbone with OMe and F modifications	Analyte
siRNA2	S: 5.7 AS: 6.6	Lipids Conjugated siRNA, mixed backbone with OMe and F modifications	Analyte
siRNA3	S: 6.3 AS:7.4	Lipids Conjugated siRNA, mixed backbone with OMe and F modifications	Analyte
siRNA4	S: 13.9 AS: 8.0	GalNAc Conjugated siRNA, mixed backbone with OMe and F modifications	Internal Standard

aDNA, deoxyribonucleotides; MOE, 2'-O-(2-methoxyethyl)-oligoribonucleotides; OMe: 2'-O-methylated-oligonucleotide, F, 2'-Flouro-oligoribonucleotides; PS, phosphorothioate linkage; GalNAc, N-Acetyl galactosamine, S: Sense strand, AS: antisense strand

TABLE 2. Precision and Accuracy of ASO2 Quantitation in mouse plasma

#	QCs in Plasma LLOQ 1 measured conc (ng/mL)	QCL 3 measured conc (ng/mL)	QCM 750 measured conc (ng/mL)	QCH 1500 measured conc (ng/mL)
1	1.14	2.66	840	1610
2	1.17	2.75	785	1590
3	1.22	3.45	853	1550
4	1.15	2.96	837	1660
5	1.05	2.72	863	1540
6	0.851	2.91	764	1580
n	6	6	6	6
mean	1.097	2.91	823	1588
SD (n-1)	0.13	0.29	39.7	43.5
CV%	12.1%	9.9%	4.8%	2.7%
accuracy%	109.7%	96.9%	109.8%	105.9%
n	18	18	18	18
mean	1.055	2.85	779	1519
SD (n-1)	0.15	0.19	73.6	126
CV%	16.6%	6.7%	10.3%	9.1%
accuracy%	105.5%	95.0%	103.8%	101.3%

TABLE 3. Analytical method qualification for siRNA 3 with mouse plasma and mouse liver QC samples quantitated against calibration curves in matching matrices

Plasma QCs			
no	LQC 15	MQC 750	HQC 3500
	measured conc (ng/mL)	measured conc (ng/mL)	measured conc (ng/mL)
1	15.6	713	3381
2	15.2	701	3395
Mean Accuracy	-3.2%	-5.7%	2.4%

Tissue QCs					
no	Liver LQC 75	Liver MQC 750	Liver HQC 3500	Kidney LQC 75	Heart LQC 75
	measured conc (ng/mL)	measured conc (ng/mL)	measured conc (ng/mL)	LQC	LQC
1	74.5	826	3454	75.4	79.9
2	74.8	755	3399	71.6	79.3
3	80.5	824	3691	75.8	81.5
4	74.4	780	3628	74.1	78.1
Mean Accuracy	6.2%	1.4%	1.2%	-1.1%	6.2%
%CV	4.1%	4.4%	3.9%	4.1%	4.1%