Detection of Phosphorothioated Oligonucleotides in Equine Serum

By

EMILY MAE HELMES

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Approved:

Dr. Benjamin Moeller, Chair

Dr. Robert Poppenga

Dr. Carrie Finno

Committee in Charge

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Abstract

The ability to manipulate the genome and its expression products is of great concern in both human and animal sports due to potential improvements in athletic performance. There are a variety of approaches that have been developed that may allow for alterations of genetic material, including the use antisense oligonucleotides (ASOs) and small interfering RNAs (siRNA), which can interfere with mRNA prior to protein expression. This thesis focused on developing a sensitive, non-targeted Liquid Chromatography – High Resolution Mass Spectrometry (LC-HRMS) method to detect phosphorothioated oligonucleotides in equine serum. Sample preparation involved using solid phase extraction on a mixed mode sorbent, followed by evaporation and concentration steps prior to analysis by LC-HRMS. Extracted oligonucleotides were chromatographically separated using a reverse-phase gradient with ion-pairing reagents prior to introduction to a hybrid quadrupole orbitrap mass spectrometer using negative mode electrospray ionization and all-ion-fragmentation (AIF) and parallel reaction monitoring (PRM) scan modes. The method was validated with percent difference, precision, matrix effects, recovery, limits of detection and quantification, and stability assessed using a representative 13mer synthetic oligonucleotide (PS-1) containing phosphorothioate modifications. The limits of detection (LOD) for the PS-1 oligonucleotide ranged from 10-50 ng/mL, and the limits of quantification (LOQ) ranged from 25-50 ng/mL based on the scan mode with acceptable percent difference and precision. The method was then applied for the detection of two phosphorothioated oligonucleotide sequences targeting either myostatin or EGL9 transcripts that represent gene targets. This LC-MS method successfully detected phosphorothioated oligonucleotides and has potential to be used as a screening method for modified ASOs in equine serum.

Abbreviations

AIF: All ion fragmentation

ASO: Antisense oligonucleotide

CRISPR/Cas9: Clustered regularly interspaced short palindromic repeats associated with (Cas) protein 9

crRNA: CRISPR RNA

DI: Deionized

DIA: Data independent acquisition

DIEA: N, N – Diisopropylethylamine

DMSO: Dimethyl Sulfoxide

HFIP: Hexafluoro-2-propanol

LC – MS: Liquid chromatography – mass spectrometry

LC-ESI-MS: Liquid chromatography-electrospray ionization-mass spectrometry

LLE: Liquid-liquid extraction

MALDI-TOF-MS: matrix-assisted laser desorption ionization-time-of-flight mass spectrometry

miRNA: micro-RNA

MW: Molecular weight

PCR: Polymerase chain reaction

PRM: Parallel Reaction Monitoring

PS: phosphorothioate

PSO: Phosphorothioated oligonucleotide

QC: Quality control

RISC: RNA-induced silencing complex

siRNA: Small interfering ribonucleic acid

SPE: Solid phase extraction

SRM: Selected Reaction Monitoring

UHPLC: Ultra high-performance liquid chromatography

1.0 Introduction

In the past 20 years, there have been many advances in therapies designed to either modify DNA or impact the expression of genes for several disease states including cancer, diabetes mellitus, and Duchenne muscular dystrophy (*1*). While these technologies hold great promise in the treatment of patients for a variety of diseases or conditions, they also offer opportunities for misuse in sport, with the goal to gain a competitive advantage. The use of these agents is of great concern in horseracing as they may pose threats to racing safety and integrity. Some proposed genes targeted for manipulation are focused on impacting the ability to gain muscle mass, facilitate repair from injury, increase oxygen carrying capacity, or altering the perception of pain or mental states by altering the expression of specific proteins associated with those pathways. Some of the likely protein targets are related to erythropoietin, insulin-like growth factor, growth hormone, myostatin, vascular endothelial growth factor, fibroblast growth factor, endorphin, and enkephalin (*1*). Since there are many potential gene products that can be targeted, developing methodological approaches to detect the use of agents capable of altering these genes or their products is highly challenging.

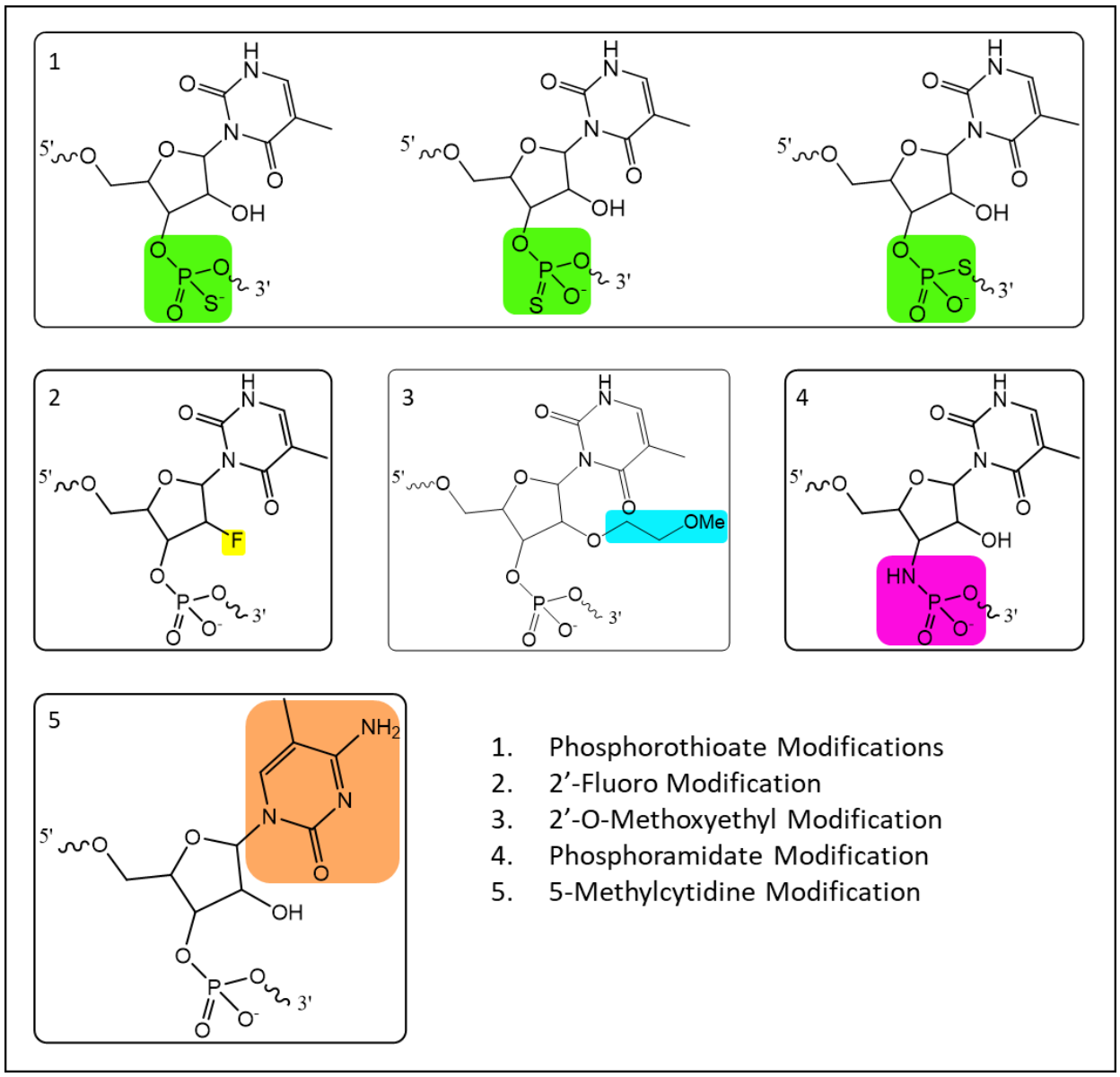
There are three main types of approaches used to manipulate the genome that may be leveraged in gene doping, including gene silencing, gene transfer, and gene editing (*2*). Gene silencing involves suppressing genes through modified ASOs, siRNA, or aptamer oligonucleotides. In horseracing, this would be targeting genes that could potentially affect a horse's athletic performance. Gene transfer introduces exogenous genes, called transgenes, into living targeted cells that will express the desired protein. Gene therapies for erythropoietin and insulin-like growth factor are two examples of gene transfer that have been explored for treating disease. By inserting these transgenes, they will continuously produce the protein inside the

body. Gene editing involves altering the genome through modifications to sequences using methods such as CRISPR/Cas9 or other gene editing systems (*2*). CRISPR/Cas9 is a robust genome editing tool, which contains a sequence specific CRISPR RNA (crRNA) that guides Cas9 to cleave DNA leaving double-stranded breaks at target sites, which facilitates the repair process that leads to the desired modification to the gene (*3*).

Due to the rapid degradation of RNA molecules in biological matrices, which ranges from minutes to a few days, synthetic modifications have been made with the aim of increasing stability by increasing resistance to nucleases (*4*). The main mechanisms of degradation for ASOs are cleavage via exonucleases and endonucleases cleaving the phosphodiester bonds, leading to metabolites being excreted primarily through the urinary system (*5*). In turn, the presence of more than ten PS modifications in an oligonucleotide results in decreased nuclease activities, allowing the molecule to reach their targets without significant degradation (*6*). Multiple modifications in the same nucleotide sequence have provided more enhanced stability for siRNA and ASOs, with five of the most common oligonucleotide modifications used to increase the stability, pharmacodynamics, and pharmacokinetics of the compounds within the body shown in **Figure 1** (*7,8*). Sulfur modifications have been more extensively studied in tRNA for their functions in preserving structural stability, aiding in the codon recognition process, and reproducibility during translation (*9*).

While there are a number of non-natural modifications that have been developed, the use of PS modifications is commonly found in most ASO or siRNA molecules under development (*10*). Because the PS modification is not naturally occurring in high abundance, this creates an opportunity to develop methodologies capable of detecting their presence in biological matrices for racehorses and other performance athletes where use of these technologies is prohibited.

Figure 1: Five of the most common oligonucleotide modifications



The phosphorothioate modification involves sulfur replacing an oxygen group in the phosphate backbone (section 1). The 2'-fluoro modification involves a fluorine replacing the 2' alcohol group on the ribose group (section 2). The 2'-O-methoxyethyl modification involves a methoxyethyl group at the 2'-oxygen (section 3). The phosphoramidate modification involves an amine group replacing the oxygen group that links the ribose to the phosphate backbone (section 4). The 5-methylcytidine modification involves the methylation of the 5' position of cytosine (section 5).

2.0 Background

Many of the gene silencing technologies utilize heavily modified versions of RNA to interfere with gene expression and these modifications are done to improve stability, pharmacokinetics, pharmacodynamics, and target sequence recognition functionality (*7*). Typically, ASOs are 15-20 bases long, while siRNAs are slightly longer between 21 and 27 bases (*1*). ASOs target RNA sequences to induce protein down regulation or restoration, while protecting itself from degradation, enhancing its bioavailability and increasing RNA affinity (*11*). Small interfering RNA is short double-stranded RNA that breaks into single-stranded RNA with the intention of binding to specific messenger RNA sequences to result in the cleavage or degradation of the mRNA to prevent translation from occurring (*12*). Aptamer oligonucleotides are short RNA or single-stranded DNA oligonucleotides that are sought after for their high specificity and affinity for their targets, acting as either a drug or a drug carrier (*4*). Antisense oligonucleotides are pharmacologically active and can change the behavior of the targeted RNA by recruitment of endogenous proteins that degrade RNA. The difference between ASOs and siRNA is the way they target RNA and how that affects the expression of proteins for these kinds of therapies. RNA-induced silencing complex (RISC) is an important binding protein complex that can silence RNA, preventing it from being cleaved by enzymes (*1*). The micro-RNA (miRNA) that binds to the targeted mRNA, along with the Argonaut proteins complex in RISC behaves as a gene repressor. By binding to the targeted mRNA, RISC can recruit epigenetic modifiers or cleave antisense transcripts that allow for RISC to induce gene silencing (*13*).

Sulfur, being a key component of the phosphorothioate modification, is one of the most crucial elements present in the body, being present in amino acids, vitamins like biotin and thiamine, and many secondary metabolic products (*14*). The sulfur atom present in cysteine and

glutathione is often utilized as a strong nucleophile in enzymatic reactions and plays a critical role in controlling cellular homeostasis (*14*). For therapeutic oligonucleotides, there are some limitations when it comes to production and analysis of these compounds. The phosphates that make up the backbone of the oligonucleotides potentially have many diastereomers which makes analysis more complicated and may alter physiochemical properties (*15*). The physiochemical properties of the chiral phosphorus atoms could affect solubility, target affinity, and cellular uptake (*15*). Therefore, having a strong detection method to detect the impurities in the synthesized oligonucleotides is vital to verify the impurity levels to eliminate unwanted physiochemical activity. When it comes to separating the diastereomers, it has proved complicated and can contribute to peak broadening, which makes analysis of phosphorothioated compounds complicated (*15*).

There are many technologies available to detect modified oligonucleotides in biological matrices including mass spectrometry, matrix-assisted laser desorption ionization-time-of-flight mass spectrometry (MALDI-TOF-MS), liquid chromatography-electrospray ionization-mass spectrometry (LC-ESI-MS), or polymerase chain reaction (PCR) based approaches (*16, 17, 18*). Previously, phosphorothioated oligonucleotides (PSO) were analyzed using MALDI-TOF-MS to determine their sequences using the combination of the observed precursor and product ions and extensive post-acquisition analysis (*16*). Recently, an LC-MS method utilizing a quadrupole time-of-flight mass spectrometer was reported using the phosphorothioate product ion (94.9358 m/z) to differentiate oligonucleotides that contained the modification (*19*). With oligonucleotides having a high molecular weight (>3500 Da), it is important to have an instrument with high mass accuracy and sufficient mass resolution to resolve potential interferences and improve sensitivity and selectivity.

With the potential abuse of gene doping technologies being of great concern in horseracing, ability to detect these agents is crucial. There are several approaches that one could use to conduct gene doping with the use of ASO or siRNA-based compounds being a way to modify gene products in a more controllable fashion as compared to the use of transgenes or CRISPR/CAS based approaches. Since ASO and siRNA-based therapeutics commonly utilize the phosphorothioate medication to improve pharmacokinetics and pharmacodynamics, developing sensitive methods to allow for its unambiguous detection are essential. Accordingly, this thesis describes the development of an LC-MS based analytical method to target the non-natural phosphorothioate modification of ASOs and siRNA in equine serum using its unique product ion to show exogenous administration.

3.0 Materials and Methods

3.1 Chemicals, Reagents, and Equipment

The oligonucleotides were purchased from Integrated DNA Technologies (San Diego, CA, USA). The 13mer (PS-1) was a custom random phosphorothioated sequence, while the 20mers were custom designed and synthesized by IDT with sequences and modification shown in **Table 1.**

The Clarity OTX SPE cartridges (100 mg/3 mL columns) and Clarity 2.6 µm Oligo-MS 100A LC column 100 x 2.1 mm purchased from Phenomenex® (Torrance, CA, USA). The Avantor ACE C8, 3µm, 100A HPLC column, 100 x 4.6 mm was manufactured by Avantor, Inc. (Radnor, Pennsylvania, USA). The DIEA, HFIP, HPLC grade water, Nuclease-free water, and HPLC grade ethanol purchased from Sigma Aldrich Inc. (Milwaukee, WI, USA). Tetrahydrofuran was manufactured by Supelco® (Bellfonte, PA, USA).

Table 1. Targeted Oligonucleotide Sequences

Base Length	Name	Sequence
13	PS-1	5’ – rA*rU*rC*rA*rG*rG*rU*rC*rA*rC*rU*rG*rC – 3’
20	MYO	5’ – 2MOErC/*/i2MOErT/*/i2MOErT/*/i2MOErC/*/i2MOErA/*C*A*T*C*A*A*T*G*C*T*/i2MOErC/*/i2MOErT/*/i2MOErG/*/i2MOErC/*/32MOErC – 3’
20	EGL9	5’ – 2MOErT/*/i2MOErT/*/i2MOErA/*/i2MOErC/*/i2MOErC/*T*T*G*G*C*A*T*C*C*C* /i2MOErA/*/i2MOErG/*/i2MOErT/*/i2MOErC/*/32MOErT – 3’

Abbreviation: r –Ribose, * -Phosphorothioate modification, A – Adenine, G – Guanine, C – Cytosine, T – Thymine, U – Uracil, 52MOErC - 5’ 2-MethoxyEthoxy MeC, 52MOErT - 5’ 2-MethoxyEthoxy T, i2MOErT - Internal 2-MethoxyEthoxy T, i2MOErC - Internal 2-MethoxyEthoxy MeC, i2MOErA - Internal 2-MethoxyEthoxy A, i2MOErG - Internal 2-MethoxyEthoxy G, 32MOErC - 3’ 2-MethoxyEthoxy MeC, 32MOErT - 3’ 2-MethoxyEthoxy T

Glassware necessary for this method was 12 x 75 mm KIMBLE® Mark-M ™ borosilicate glass disposable culture tubes which were manufactured by Duran Wheaton Kimble (Millville, NJ, USA) and crimp vials with fixed inserts manufactured by Agilent Technologies (Waldbronn, Germany). The Nuclease-Free water was purchased and manufactured by Sigma-Aldrich, Co. (St. Louis, MO, USA). The TipOne® sterile pipette tips were manufactured by Starlab International (Hamburg, Germany). TipOne® pipettes are certified RNase, DNase, DNA, and pyrogen free. 15 mL centrifuge tubes with Centristar™ caps were manufactured by Corning® (Reynosa, Tamaulipas, Mexico). Non-Stick RNase-Free 1.5 mL Microfuge tubes were manufactured by Life Technologies Corporation (Austin, TX, USA). The TurboVap® LV Concentration Workstation was manufactured by Caliper Life Sciences (Hopkinton, MA, USA). The Agilent 1100 Series Capillary LC System was manufactured by Agilent Technologies (Santa Clara, CA, USA). The Finnigan TSQ Quantum Ultra was manufactured by Thermo Electron Corporation (San Jose, CA, USA). The Q Exactive HF Mass Spectrometer was manufactured by Thermo Scientific (Bremen, Germany). The Large Capacity Mixer 120V was manufactured by Glas-Col® (Terre Haute, IN, USA). AMICON® Ultra – 0.5mL Centrifugal Filters Ultracel® - 3k were manufactured by Merck Millapore Ltd. (Tullagreen, Carrigtwohill Co Cork, IRL). Negative control serum was obtained from BioIVT (Westbury, NY, USA).

3.3 Preparation of Standard and Quality Control Samples

The standards for the modified oligonucleotide (PS-1) were prepared at a 1 mg/mL concentration in nuclease free water. The dilutions were made in nuclease-free water from the 1 mg/mL standards to 100, 10, and 1 ng/µL and solutions stored at -20°C until use. A calibration curve was prepared for PS-1 oligonucleotide at 5, 10, 25, 50, 100, 500, and 1000 ng/mL in negative control serum. Quality control samples (n=6/level) were prepared at low (75 ng/mL)

and high (750 ng/mL) levels. The QC high level was used to assess stability at room temperature, refrigeration (2–8℃) and frozen (-20℃) storage conditions. For the MYO and EGL9 oligonucleotides, serum was spiked at 50, 100, 250, 500, 1000, 1500 ng/mL and quality control samples were prepared at 150 and 1250 ng/mL. Serial dilutions in serum were used for all calibrators and quality control samples. Negative controls were run with each set of data.

3.4 Solid Phase Extraction

All sample preparation steps were performed using 1.5 mL RNase free microcentrifuge tubes. A 500 µL of serum was aliquoted into the microcentrifuge tubes followed by the addition of 500 µL of lysis-loading buffer, containing guanidine hydrochloride and Triton X, and the mixture was vortexed and incubated in the 4℃ refrigerator for 15 minutes. Following sample pretreatment, the serum was extracted using solid phase extraction on a Clarity OTX weak anion exchange cartridge (Phenomenex®, Torrance, CA, USA) on a positive pressure extraction manifold. The cartridges were conditioned with 1 mL of methanol, then equilibrated with 1 mL of 50 mM ammonium acetate (pH 5.5) followed by the addition of the pre-treated serum samples. After the samples were loaded, the cartridges were washed twice with 3 mL of 50 mM ammonium acetate (pH 5.5) with 50% acetonitrile (ACN). The extract was then eluted from the cartridges with the addition of 1 mL of 100 mM ammonium bicarbonate (pH 9.5) with 40% ACN and 10% tetrahydrofuran (THF) into new microcentrifuge tubes under gravity conditions. The samples were then placed in the TurboVap to evaporate to about ~500 µL for 16 minutes at 35℃. The samples were then transferred to centrifugal filters and were microcentrifuged for 30 minutes at 14,000 x g. The concentrated samples were then pipetted into autosampler vials, brought up to volume at 60 µL, and capped. Lastly, they were mixed on the large capacity mixer for one minute and ready for analysis by LC-MS.

3.5 Mobile Phase Preparation

Mobile phase A was composed of HPLC grade methanol with 15 mM DIEA and 20 mM HFIP. Mobile phase B was composed of HPLC grade water with 15 mM DIEA and 20 mM HFIP. The reverse phase gradients used for the methods on the Quantum Ultra and the Q Exactive HF are presented in **Table 2**. The column temperature was 50℃ for the Quantum Ultra method and 35℃ for the Q Exactive HF method.

Table 2. Liquid Chromatography Gradient Conditions

Quantum Ultra Settings				**Q Exactive HF Settings**			
Time (Minute)	Flow Rate (mL/min)	%A	%B	Time (Minute)	Flow Rate (mL/min)	%A	%B
0	0.30	5	95	0	0.40	5	95
1	0.30	5	95	1	0.40	5	95
2	0.30	40	60	2	0.40	40	60
4	0.30	90	10	4	0.40	90	10
5.5	0.30	98	2	5.5	0.40	98	2
9	0.30	98	2	10	0.40	98	2
9.01	0.30	5	95	10.01	0.40	5	95
15	0.30	5	95	20	0.40	5	95

3.6 LC-MS Analysis

To begin the method development, an Agilent 1100 Series LC System and the Finnigan TSQ Quantum Ultra mass spectrometer were utilized. To start, the PS-1 oligonucleotide was infused via syringe pump, prepared in the respective mobile phase at a concentration of 10 ng/µL. The Q Exactive HF mass spectrometer was used for the final method, and the PS-1 oligonucleotide was infused via syringe pump to optimize sensitivity for the 94.935 m/z product ion. The 94.935 m/z ion is pictured in **Figure 1**, showing the structure of the fragment ion.

The instrument method on the Quantum Ultra was performed in negative mode with a HESI ion source and a spray voltage of 3500V, vaporizer temperature at 475℃, sheath gas of 40 arbitrary unit (a.u.), sweep gas of 10 a.u., aux gas of 40 a.u., capillary temperature of 240℃, capillary offset of -35 eV, and source CID of 8 a.u. There were 2 scan events for this method. Scan event 1 was run as a selected reaction monitoring (SRM) method focusing on the PS-1 oligonucleotide. This scan looked for parent ions 612.3, 714.6, and 857.6 m/z, charge states 5, 6, and 7, respectively, to find product ions of 94.9 and 192.9 m/z. The collision energy for 94.9 m/z was 33 eV, and 50 eV for 192.9 m/z. Scan event 2 was a single ion monitoring (SIM) method with center masses at 612, 714, and 857 m/z.

The instrument method on the Q Exactive HF was performed in negative mode with a HESI ion source and spray voltage of 4500V, capillary temperature of 350℃, sheath gas of 60 a.u., aux gas of 20 a.u., spare gas of 2 a.u., and probe heater temperature of 350℃. This method acquired 3 different scan modes: all ion fragmentation (AIF), parallel reaction monitoring (PRM), and Full-MS – SIM. For the AIF scan, the resolution was set at 30,000, AGC target of 1e6, collision energy of 40 eV, source CID of 60 eV, scan range of 90-1350 m/z. For the PRM acquisition, an in-source CID of 0 eV, the resolution was set at 60,000, an AGC target of 2e5,

loop count of 1, default charge state of 8, isolation window of 601 m/z, fixed first mass of 90 m/z, and a stepped collision energy of 20 to 40 eV. The inclusion list for the PRM lists 900 m/z, default charge state of 5, 1500 m/z, default charge state of 3, and 2100 m/z, default charge state of 3. For the Full MS – SIM acquisition, an in-source CID of 0 eV, resolution set at 60,000, AGC target of 3e6, and a scan range of 600-6000 m/z. The data collected was stored using the Xcaliber data system and processed using the Thermo Qual browser and Quan browser.

3.7 Statistics

The percent difference, precision, and stability (using QCs) were calculated from the data. The linear range of the assay was determined through linear regression analysis ($y = mx + b$) of the peak area and known concentrations of spiked samples in negative control equine serum. The limits of detection were described as the smallest amount distinguishable from the baseline across all scans for the method. The limits of quantification were determined for each scan type as the amount with less than 30% variability from the theoretical concentration. Percent difference was calculated as $\% = \frac{\bar{x} - True\ Value}{True\ Value} \times 100$. Precision was calculated $s = \sqrt{\frac{\sum(x_i - \bar{x})^2}{n-1}} \div \bar{x} \times 100$. Matrix effects was calculated as a percentage $\%ME = \left[\frac{\text{Response}_{\text{Post Extracted Spiked Sample}}}{\text{Response}_{\text{Neat Standard}}}\right] \times 100$. Recovery was calculated as a percentage $\%R = \frac{\text{Response}_{\text{Spiked Sample}}}{\text{Response}_{\text{Post Extracted Spiked Sample}}} \times 100$.

4.0 Results

4.1 Chromatography and MS Spectra

The chromatographic and mass spectrometry conditions were optimized for detection of the phosphorothioate product ion using AIF and PRM (**Figures 4-9**). The product ion spectra are in **Figures 10-13**.

Figure 4. PS-1 Chromatograms for Negative Control, LOD (50 ng/mL), and QC High (750 ng/mL) – AIF scan

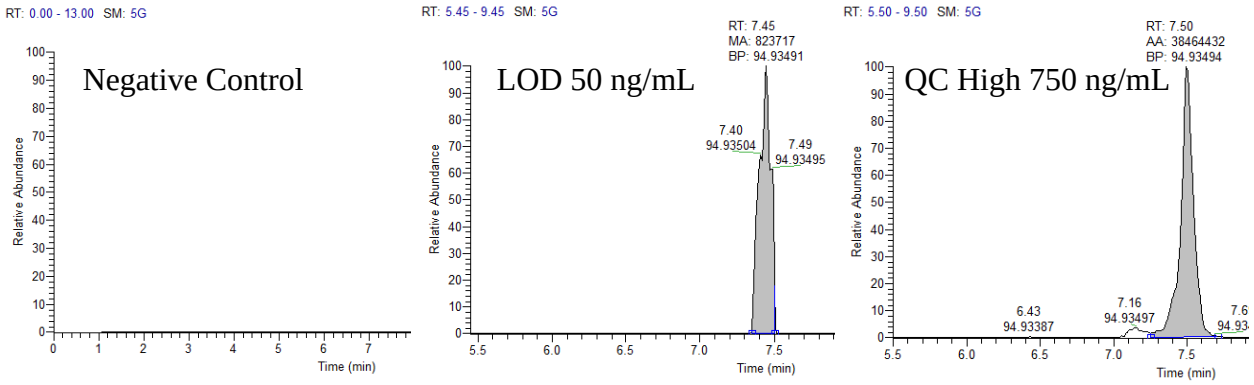


Figure 5. PS-1 Chromatograms for Negative Control, LOD (25 ng/mL), and QC High (750 ng/mL) – PRM-900 m/z

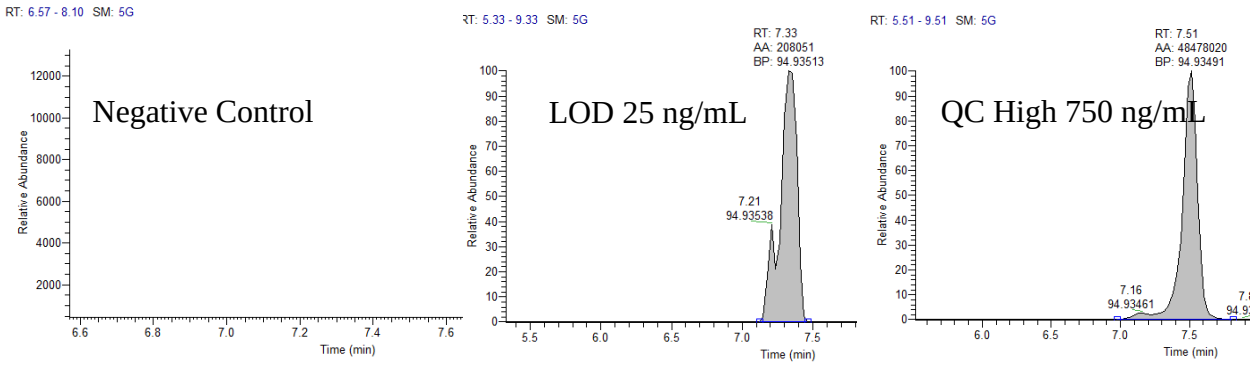


Figure 6. PS-1 Chromatograms for Negative Control, LOD (10 ng/mL), and QC High (750 ng/mL) – PRM-1500 m/z

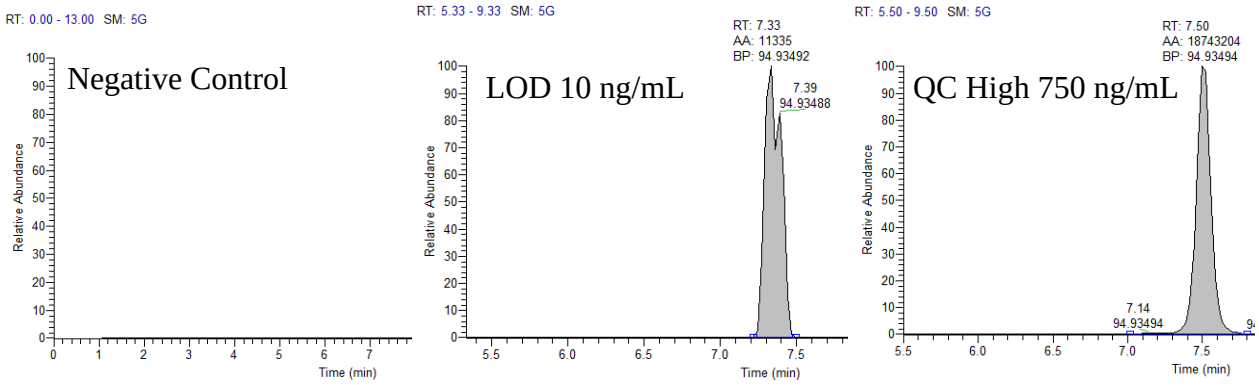


Figure 7. PS-1 Chromatograms for Negative Control, LOD (10 ng/mL), and QC High (750 ng/mL) – PRM-2100 m/z

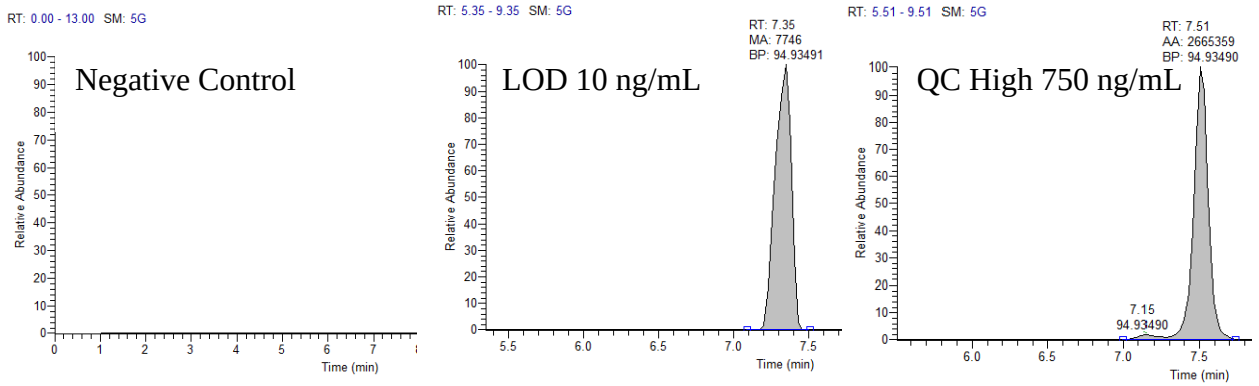


Figure 8. MYO Chromatograms for 100 ng/mL for PRM-900 m/z, 1500 m/z, 2100 m/z, and AIF Scans

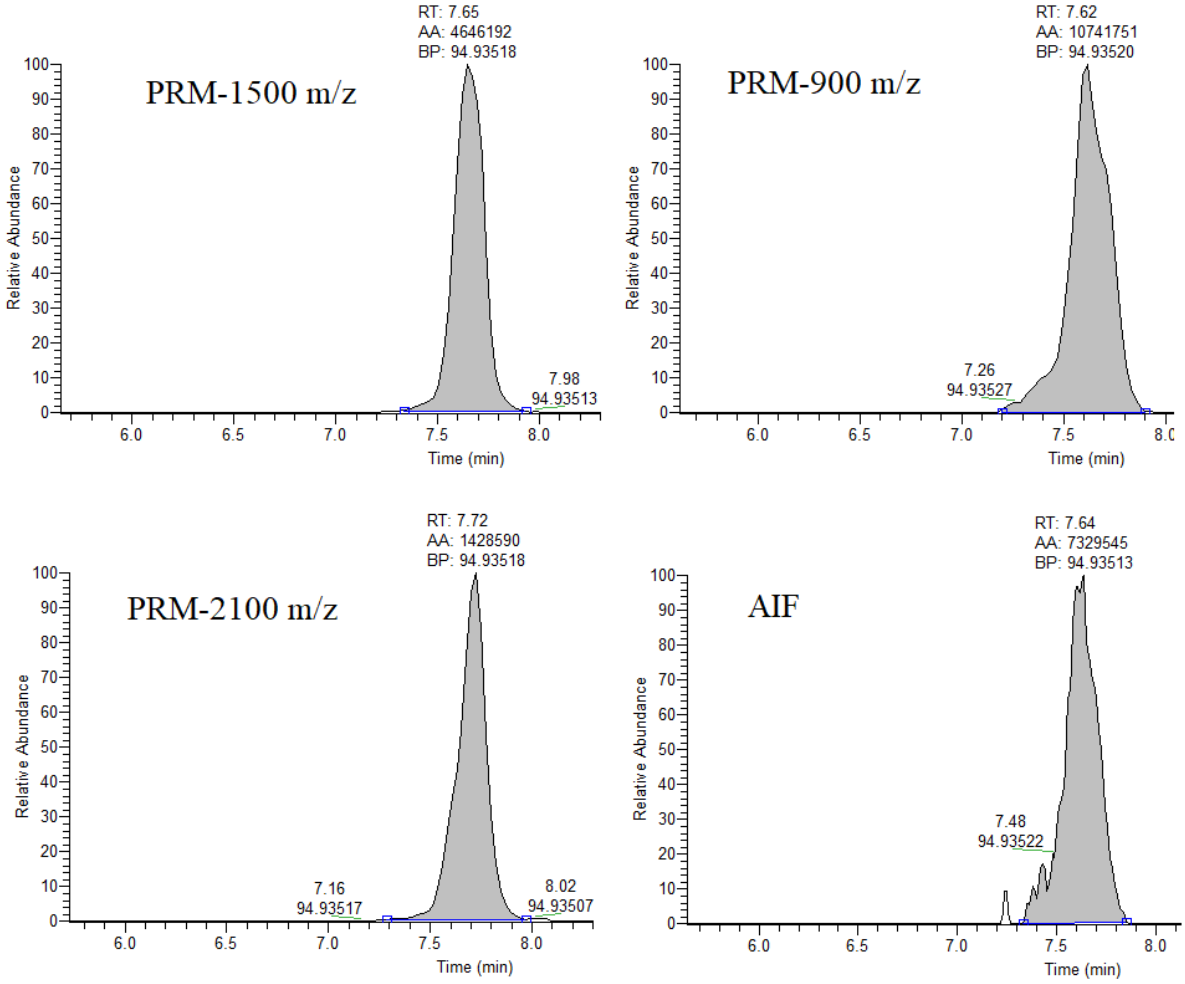


Figure 9. EGL9 Chromatograms for 50 ng/mL for PRM-900 m/z, 1500 m/z, 2100 m/z, and AIF Scans

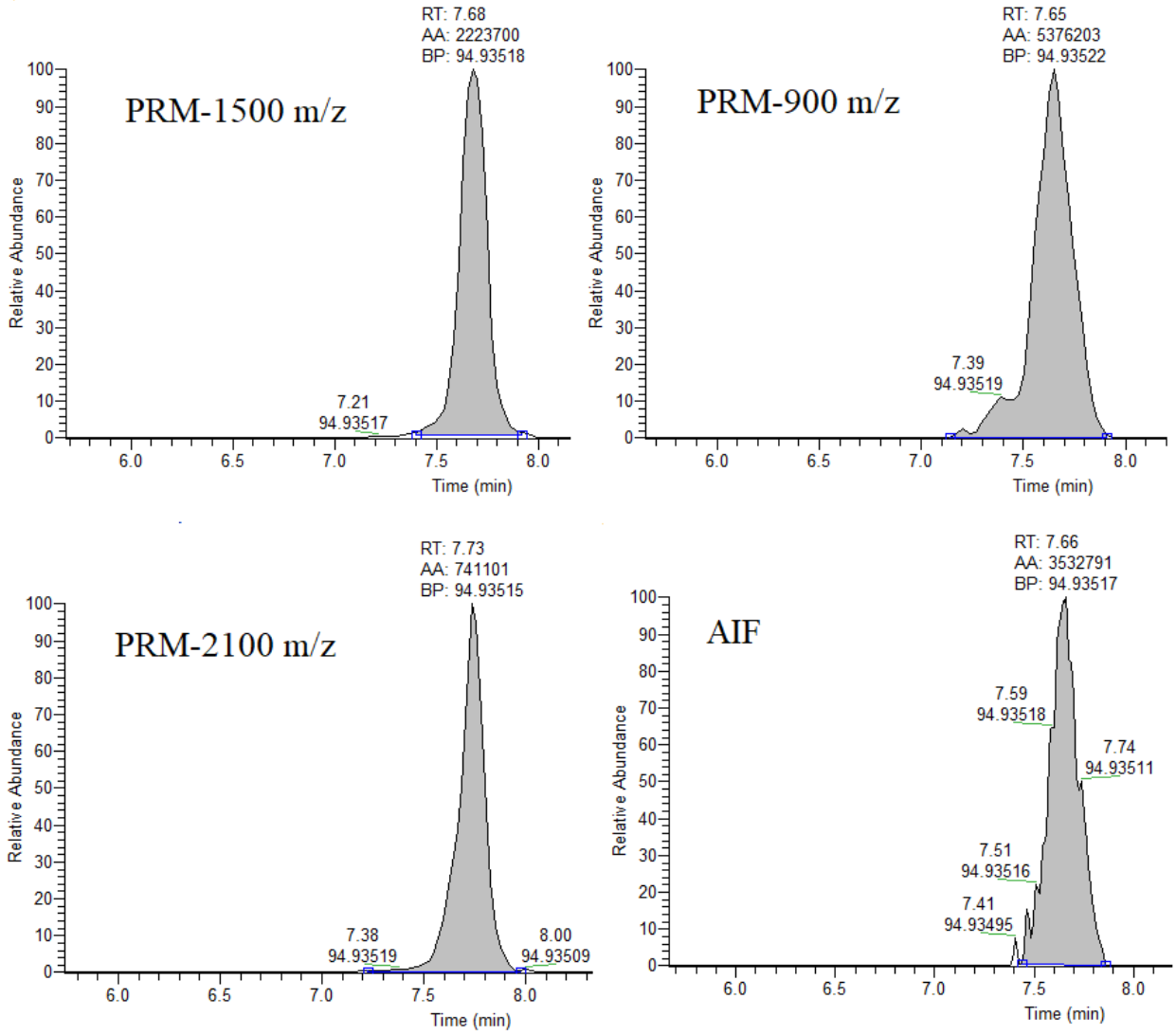


Figure 10. PS-1 Mass Spectra for Negative Control, QC High (750 ng/mL), and Neat Standard – AIF Scan

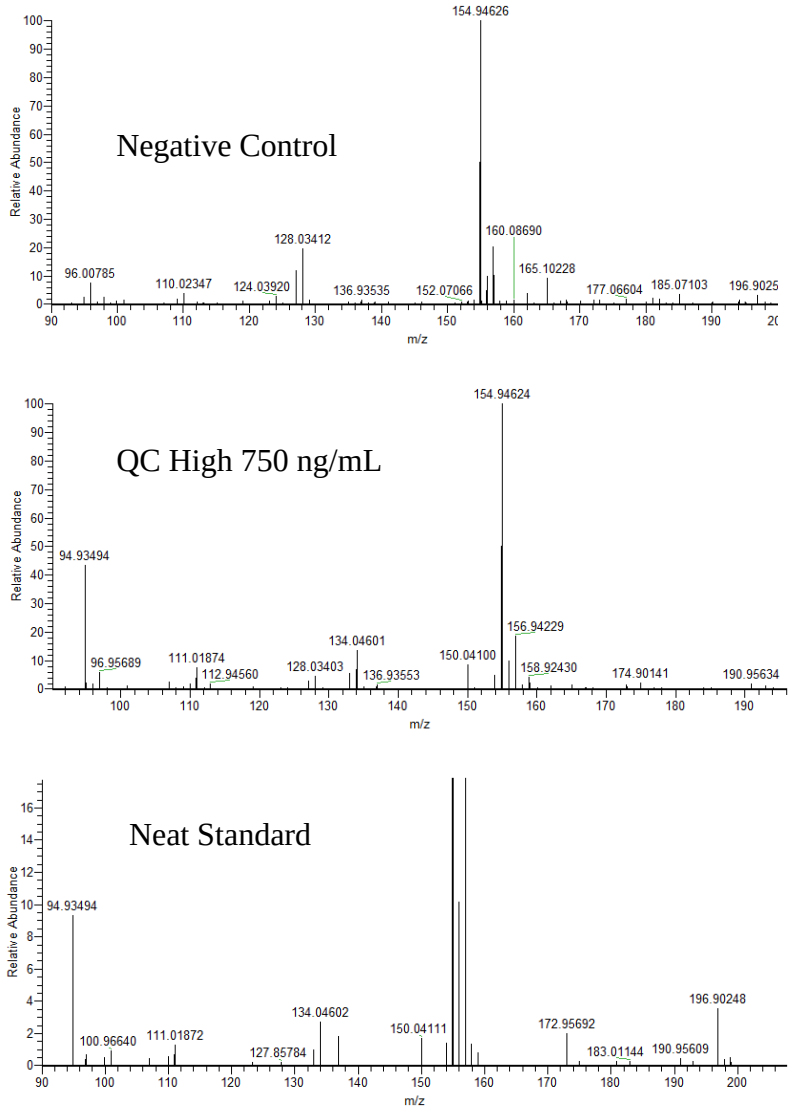


Figure 11. PS-1 Mass Spectra for Negative Control, QC High (750 ng/mL), and Neat Standard – PRM-900 m/z

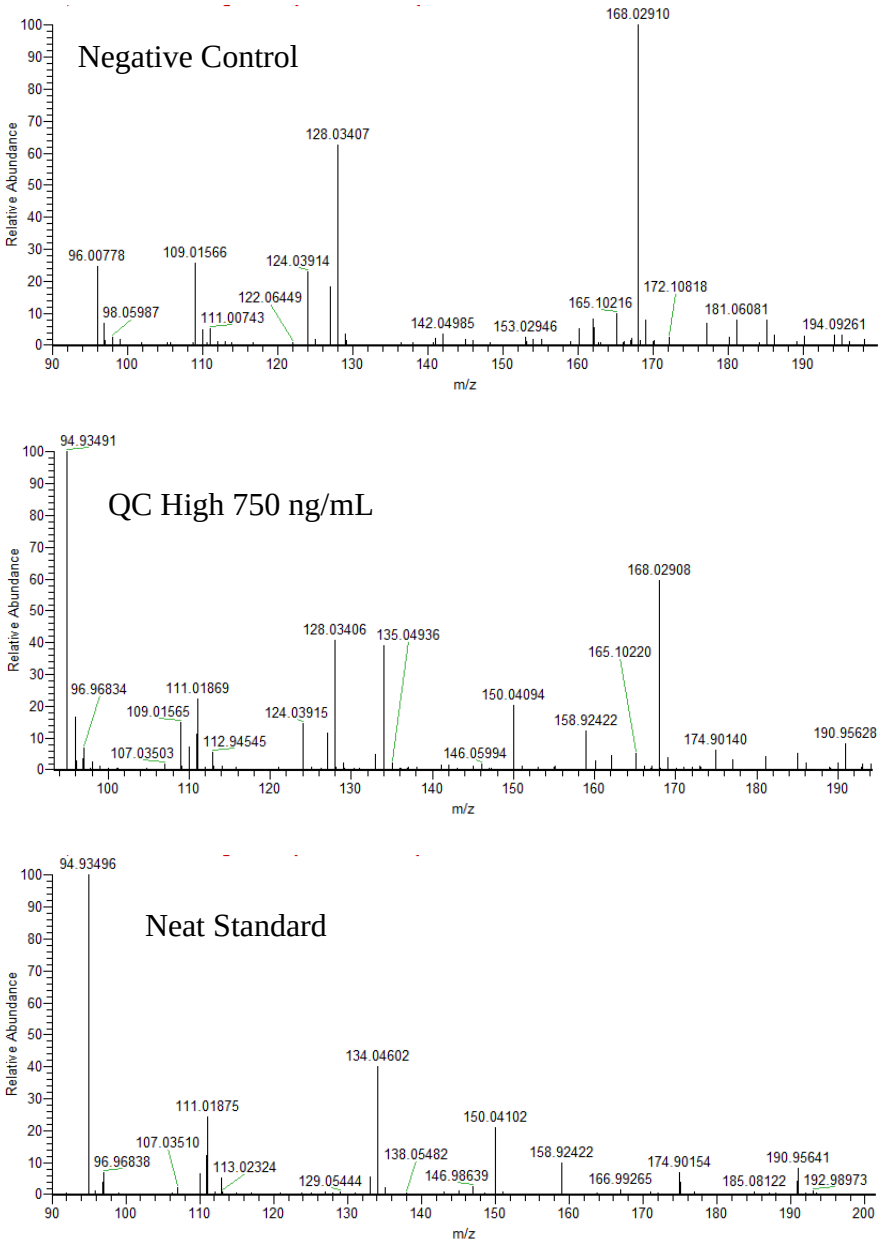


Figure 12. MYO Mass Spectrum (1500 ng/mL) for PRM-900 m/z Scan

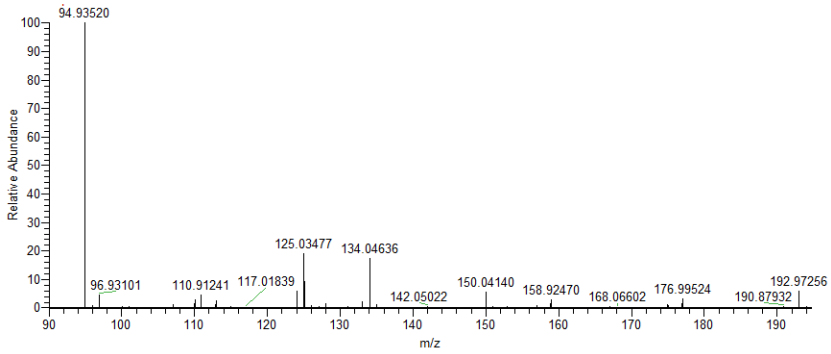
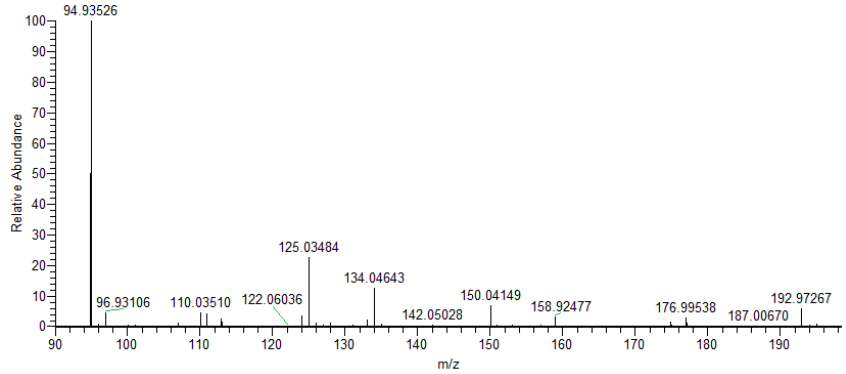


Figure 13. EGL9 Mass Spectrum (1500 ng/mL) for PRM-900 m/z Scan



4.2 Linearity and Detection Limits

The linearity of the data was assessed for three days of validation and a representative set of data for each scan from day two is shown in **Table 3**. The LOD and LOQ for the PS-1 oligonucleotide were assessed using different scan types. The LOD was 25 ng/mL for the PRM-900 m/z scan, 10 ng/mL for the PRM-1500 m/z scan, 10 ng/mL for the PRM-2100 m/z scan, and 50 ng/mL for the AIF scan. The LOQ was 50 ng/mL for the PRM-900 m/z scan, 25 ng/mL for the PRM-1500 m/z scan, 50 ng/mL for the PRM-2100 m/z scan, and 50 ng/mL for the AIF scan.

Table 3. Linear Regression Analysis for Calibration Curves

	PRM-900 m/z	PRM-1500 m/z	PRM-2100 m/z	AIF
Linear Fit	y = -670210+66804.4x	y = -404615+18892.7x	y = -61808.8+2904.56x	y = -1.13453e^6+62209.5x
R^2 Value	0.9974	0.9989	0.9990	0.9990

4.3 Percent Difference and Precision

Intra-day percent difference from expected concentration and precision was assessed by calculating the percent difference and relative standard deviation of the data each day for each scan type using the equations in **Section 3.7.** Inter-day percent difference and precision was assessed by taking the data from all three days and calculating the percent difference and relative standard deviation of all three days of data. The intra and inter-day percent difference and precision is summarized in **Table 4**.

Table 4. Intra and Inter-Day Percent Difference and Precision

		QCL (75 ng/mL)		QCH (750 ng/mL)	
	Scan Type	Percent Difference	RSD	Percent Difference	RSD
Day 1	PRM-900 m/z	39.6%	5.9%	15.5%	12.4%
	PRM-1500 m/z	46.6%	8.0%	58.7%	11.7%
	PRM-2100 m/z	37.6%	7.9%	50.8%	11.9%
	AIF	27.7%	5.0%	16.3%	12.3%
Day 2	PRM-900 m/z	17.9%	15.7%	1.1%	13.3%
	PRM-1500 m/z	24.1%	8.4%	9.7%	17.9%
	PRM-2100 m/z	17.4%	9.6%	4.4%	18.0%
	AIF	22.5%	10.6%	2.0%	12.9%
Day 3	PRM-900 m/z	37.0%	18.3%	10.2%	7.0%
	PRM-1500 m/z	31.1%	14.8%	11.1%	9.5%
	PRM-2100 m/z	30.9%	14.9%	12.0%	6.0%
	AIF	30.2%	10.5%	11.0%	7.0%
Inter-Day	PRM-900 m/z	31.5%	20.1%	2.1%	15.3%
	PRM-1500 m/z	34.0%	18.0%	12.6%	32.3%
	PRM-2100 m/z	28.6%	16.1%	11.5%	28.6%
	AIF	26.8%	9.7%	1.1%	15.8%

4.4 Matrix Effects and Recovery

Matrix effects and recovery were assessed and are summarized in **Table 5**. The loss of response due to matrix effects was between 30 and 55% and recovery averaged around ~61%.

Table 5. Matrix Effects and Recovery for PS-1 Oligonucleotide

Scan Type	Average Matrix Effects	Average Recovery
PRM-900 m/z	32%	66%
PRM-1500 m/z	48%	56%
PRM-2100 m/z	54%	60%
AIF	35%	64%

4.5 Stability

The stability was assessed at 25℃ for 24 hours, 2-8℃ for 24 hours and 27 days, and -20℃ for 27 and 28 days assessing one and two freeze/thaw cycles as well. Leaving the spiked serum at room temperature proved to be the least stable and not suitable for further analysis. The results are summarized in **Table 6**.

Table 6. Stability expressed as Percent of Targeted Concentration for PS-1 Oligonucleotide

	Stability at Various Storage Conditions					
Scan Type	24 hrs at ~25℃	24 hrs at 4℃	27 days at 4℃	27 days at -20℃	28 days at -20℃ 1x Frz/Thaw	28 days at -20℃ 2x Frz/Thaw
PRM-900 m/z	ND	51.7%	4.0%	61.1%	44.3%	53.0%
PRM-1500 m/z	26.9%	51.5%	4.7%	55.9%	50.3%	55.5%
PRM-2100 m/z	ND	62.8%	5.1%	53.9%	57.4%	65.0%
AIF	28.5%	54.4%	3.9%	59.9%	48.7%	54.8%

ND-Not Detected

5. Discussion

An HPLC-HRMS method was successfully developed and validated over three days with percent difference, precision, matrix effects, recovery, limits of detection and quantification, and stability determined. Sample preparation and extraction, chromatography and mass spectrometry optimization, validation, troubleshooting, and limitations will be discussed in this section.

5.1 Sample Preparation and Extraction

Having a robust sample preparation procedure to effectively extract targeted compounds from a biological matrix is an important parameter for optimization prior to the instrumental analysis as the selected approach may have dramatic impacts on detection limits and overall robustness of the methodology. There are several properties to consider when choosing an extraction approach, while keeping in mind the effects certain reagents or chemicals might have on further analysis (*20*). Utilizing an extraction method that yields in high recovery and minimal matrix effects is an important consideration in bioanalytical method development and implementation. Different volumes and makeups of buffers and reagents, as well as number of wash steps done, may affect the recovery of the analyte (*20*). The development of extraction approaches of oligonucleotides from biological matrices is a relatively new area of investigation with phenol/chloroform liquid-liquid extraction followed by solid phase extraction being commonly used (*21*).

Several different extraction approaches were evaluated during initial method development to determine an optimal approach that balanced obtaining good recoveries, low background, and ease of the procedure. Protein precipitation was one method tested, and the results suggested that the oligonucleotides bind to proteins in the matrix and were lost, bound to the proteins following the addition of the organic solvent. Waters Oasis HLB and Mixed Mode WAX SPE cartridges were also tested with an extraction procedure that included using ion-pairing reagent in the equilibration and elution steps. These methods did not work well with the method on the Quantum Ultra, as the method was not sensitive enough to confidently detect the 94.935 m/*z* ion. The Clarity OTX SPE cartridges and extraction procedure ended up working the

best, resulting in recoveries between 56% and 66% and matrix effects between 32% and 54%, both depending on scan mode.

Previously, Chen and Bartlett created a one-step extraction method using Clarity OTX SPE cartridges for a phosphorothioated oligonucleotide and one of its metabolites in rat plasma via uHPLC-MS/MS (*20*). The results demonstrated increasing the elution buffer pH from 8.8 to 9.5 improved the recovery by 20%, resulting in a recovery between 70 and 80%. Any higher increase of the pH did not reduce the binding of oligonucleotides to the walls of the SPE cartridges (*20*). This study also found that when combining the plasma and the lysis-loading buffer using a 1:2 ratio resulted in higher recovery and overall response signal (*20*). The lysis-loading buffer lowers the binding of the oligonucleotides to plasma proteins and allows the analytes to be retained on the stationary phase instead of eluting directly through the column. This was confirmed by an experiment that did not include the lysis buffer that resulted in the oligonucleotide being non-detectable. For validation and method development a 1:1 ratio of lysis buffer was used, and further optimization may yield better results in the future. The recovery with this validated HPLC-MS/MS method was about 10-20% lower than the recovery achieved with Chen and Bartlett's uHPLC-MS/MS method (*20*).

Additionally, a study completed by Nuckowski et al. evaluated different extraction approaches by comparing results from four different automated SPE microextraction packed sorbents, the number of ideal vial injections for the conditioning and loading steps of the microextraction, the amine type and concentration, with 5 mM dimethylbutylamine (DMBA) being the best paired with 150 mM HFIP, and the volume and composition of the solvents used for the elution of the targeted compounds (*22*). The volume and composition of the elution solution was identified as being the most influential parameter for the recovery of the analytes

using nonpolar C8 and C18 sorbents for the microextraction (*22*). This extraction method is a miniature version of solid phase extraction with benefits being shorter extraction time and smaller sample volumes necessary (*22*).

5.2 Chromatography and Mass Spectrometry Optimization

Selection of the mobile phase solvents and modifiers is critical for good chromatographic performance and high sensitivity using mass spectrometry. The solvents utilized in this study were initially based on previous reports of the mixtures working the best with a strong signal intensity and good selectivity (*23*). Previous studies found that using fluorinated alcohols as an acidic modifier in the mobile phase increased the signal intensity greatly when paired with an ion-pairing agent (*23*). HFIP was found to work very well with less hydrophobic ion-pairing agents, and performed very well with DIEA, an alkylamine ion-pairing agent (*23*).

Previously, acetonitrile, ethanol, methanol, and isopropanol were compared as organic solvents in mobile phase mixtures with water to determine their effect on signal enhancement (*24*). To increase the signal, it was found pairing HFIP with DIEA resulted in a dramatic increase in ionization efficiency as compared to having only DIEA as a mobile phase modifier (*24*). This was because the oligonucleotides could enter the gas phase more easily because of the thermodynamics of this system. DIEA retains the oligonucleotides much better than less hydrophobic ion-pairing agents because it has a higher tendency to bind with the C18 or similar stationary phase. Paired with the HFIP, when the molecules are deionized, they become less water soluble and push the DIEA towards the stationary phase and increase the retention of the oligonucleotides (*24*). This makes for an ideal combination in the mobile phases.

The use of HFIP and DIEA mobile phase modifiers with a reverse phase column allowed for good retention of the targeted oligonucleotides with the retention times for the PS-1, MYO,

and EGL9 oligonucleotides eluting over a one-minute window between 7 and 8 minutes as shown in **Figures 4-9**. The instrumental parameters used the gradient compositions shown in **Table 2** to allow for the compounds to be highly retained and elution from the stationary phase required a high composition of organic solvent, replicating the results observed by Basiri and colleagues (*24*). The methodology utilized a 20-minute run time with starting conditions at 5% organic mobile phase followed by a gradient elution over 5 and a half minutes with 98% organic mobile phase being held for 4 and a half minutes. These chromatographic conditions were utilized to allow for broad acquisition of potential PS modified oligonucleotides. The MYO and EGL9 oligonucleotides eluted at a later retention time than the shorter PS-1, which could be due to a longer sequence or the presence of other structural modifications that were not present on the PS-1 molecule.

In addition to mobile phase concentration, the selection of the stationary phase has a large impact on chromatographic and mass spectrometry performance and should be carefully considered depending on the purpose of the methodology. The initial method development was accomplished with a 100 x 2.1 mm C18 column (2.6µm particle size) from Phenomenex and resulted in very strong interactions of the PS-1, with the stationary phase that required high composition of organic solvent for elution. The final methodology utilized a 100 x 4.6 mm C8 column (3µm particle size). While both columns utilized reverse phase chromatographic conditions coupled with the use of a fluorinated alcohol and DIEA, the two columns differed in particle size, internal diameter, and stationary phase composition. The larger particle size of the C8 column allowed for a higher flow rate and a lower back pressure as compared to the C18 column. Since the method utilized non-targeted MS/MS detection with minimal precursor ion selection, reduction in co-elution of targeted compounds was desired and thus the C8 was

utilized for final validation. In addition to the use of the C8 and C18 based stationary phases, others have used cholesterol bonded stationary phase with ion pairing reagents in the mobile phase (*25*). In addition to the use of reverse phase gradient elution with ion-pairing reagents, others have found chromatographic success using HILIC and anion exchange stationary phases without the use of HFIP and DIEA. Demelenne and colleagues accomplished coupling HILIC and capillary zone electrophoresis (CZE) to drift tube ion-mobility quadrupole time of flight mass spectrometry to obtain exact mass and information about number of nucleotides in the oligonucleotides that were assessed (*26*).

To successfully detect the oligonucleotide using mass spectrometry, the instrumentation must be sensitive and selective enough to detect trace levels in biological matrices. During initial sample preparation and instrument method development, the Thermo Quantum Ultra triple quadrupole mass spectrometer detector was utilized, though it was not the final mass spectrometer used for the results. The Quantum Ultra repeatedly showed lack of sensitivity towards the product ions for the PS modification. The signal response for the 94.9 m/z product ion was consistently low or undetectable using MS/MS based detection. To increase the signal for detecting the phosphorothioated sequence, data was acquired using full MS scans focusing on characteristic mass to charge ratios with charge states at 5, 6, and 7 being 857, 714, and 612 m/z, respectively. The product ions at 94.9 and 192.9 m/z were not as readily detectable likely due to poor ion transmission or MS/MS fragmentation on the triple quadrupole. Following initial method development on the triple quadrupole mass spectrometer, a high-resolution accurate mass Q Exactive HF system was evaluated, and detection of the 94.935 m/z product ion optimized using both AIF and PRM scan modes.

The phosphorothioate ion 94.935 m/z was detectable at limits of detection between 10 and 50 ng/mL for the PS-1 oligonucleotide on the Q Exactive. Previously, a limit of detection for sequence analysis via MALDI-TOF MS was determined at 70.8 ng, and 100 ng via LC/ESIQqTOF (*16*). Negative controls were also run without the oligonucleotides in the matrix, and the 94.935 m/z ion did not show up in those scans. The AIF scan had an in-source CID of 60 eV to fragment the ion pairing reagents. The regression correlation coefficients in **Table 3** were above 0.99 for the PS-1 oligonucleotide. The mass spectra in **Figures 10-13** display the 94.935 m/z ion intensities for the PS-1, MYO, and EGL9 oligonucleotides, showing detection of the phosphorothioate fragment ion, while the negative control spectra in the figures do not show it.

Inter- and intra-day percent difference and precision were calculated for the PS-1 oligonucleotide as shown in **Table 4**. The calculated values for the inter-day data did not vary more than 15% from the theoretical concentration of the QCH and 35% from the QCL theoretical concentration, and the intra-day variability was less than 60% for QCH data and less than 50% for QCL data. The intra-day data showed a relative standard deviation under 20% for both QCH and QCL data and the inter-day relative standard deviations were under 35%. Percent difference and precision varied likely due to varying mixing in the dilution preparation for the spiked standards, as well as spiking, which can fluctuate based on technique used, and weighting of the linear fit favoring the higher concentrations.

5.3 Past Method Development

Many previous methods use targeted methods because they are looking for specific oligonucleotides and their metabolites. A method introduced by Husser et al. is an example of this, where the capillary flow LC-HRMS method was targeting a GalNAc modified therapeutic oligonucleotide, allowing for analysis of its metabolites (*27*). This method was very slow due to

the trapping column it utilized. Another example of a targeted method would be the Zhang et al. study in 2007, where an LC-MS/MS method was developed to investigate an antisense phosphorothiote deoxynucleotide and its metabolites (*21*).

In Li et al., the UHPLC achieved its optimal separation at a 0.5mL/min flow rate, using a gradient elution, at 60°C. The ESI-MS/MS was operated in negative ion mode with the curtain gas at 40, collision gas at high, ion spray voltage at -4000, temperature at 550, ion source gas 1 at 80 and gas 2 at 80. They used a Phenomenex® Clarity 1.7 µm Oligo-XT 100 Å LC Column 50 x 2.1 mm. The complete method was finished in 6.5 minutes (*28*). This method is different from the non-specific method developed for this thesis, as the run time is about a third of the length. The spray voltage, temperature, and ion source gas are all higher in the Li et al. method than the method this thesis focuses on. The spray voltage was more stable at 3500V than 4000V when the MS parameters were being optimized for the Q Exactive non-specific method. For a method that is not looking for a specific sequence, rather a specific ion, 94.935 m/*z*, having a longer method was necessary to allow for longer oligonucleotides to be potentially analyzed.

Similar to reports in previous studies, the phosphorothioate fragment ion showed up at 94.936 m/*z* during AIF (*19*, *27*). A robust method was developed to detect the oligonucleotide and their metabolites in negative ion mode using similar mobile phases as this experiment, with ion-pairing reagents (*27*). The complete run took 42.5 minutes. When the MS was in all ion fragmentation mode, the characteristic fragment ions at 94.936 m/*z* and 192.973 m/*z* were determined as the PS ion and the ion with the PS bonded with the ribose, respectively. This method used SPE first for sample clean up, then an on-line trapping capillary column. The biggest difference between this method and the other published methods is that it introduces column switching. A trapping column is used, that then concentrates and elutes the sample onto

the analytical column utilizing a slower flow rate, which greatly improved the sensitivity (*27*). The downside of this is that it creates a longer run time, which is not usually ideal in routine analysis, but the goal of this method was to optimize the sensitivity.

5.4 Troubleshooting

During the method development phase of this project, there were issues that had to be addressed. The most pressing problem was the low recovery of the analytes in the beginning stages. There are many different places from the sample preparation process through the LC-MS analysis where the analytes can be lost. Some possibilities could be degradation in the freezer over time, analytes binding to glassware, proteins in the matrix degrading the RNA, loss during the extraction procedure, oxidation during the dry down step, and loss during the transfer after reconstitution.

To determine where the apparent losses were taking place, separate experiments were created to test which parameters were affecting the recovery the most. To prevent any nuclease activity, nuclease free water and nuclease free pipette tips were used in the sample preparation process. To minimize loss to the glassware, especially at trace concentrations, the test tubes were coated in the serum matrix for an hour prior to analysis so the active sites could be filled with proteins from the matrix. However, this did not seem to have significant effects on the concentration. The buffers from the extraction kit purchased were not the correct pH and this is critical to recovery due to the weak anion exchange column. However, once the method was reoptimized the recovery was good. The analytes may not have been eluting off the column, or even making it onto the column if the lysis-loading buffer was not working properly due to the incorrect pH. The loss could happen at multiple stages in the extraction. After elution, the next possible loss was the evaporation step. This was a definitive loss proved through repeated

smaller experiments. One minor solution to minimize loss during the dry down was adding 10 µL of DMSO to the eluted samples before extraction, since evaporating the samples to dryness resulted in complete loss of the analytes. DMSO was not used in the final procedure, as evaporating to ~500 uL had better results. The second action taken was eluting into Lobind tubes and placing these in 16 x 100 mm test tubes and drying the samples down in those to minimize contact with the glassware, which was used in the final preparation procedure.

5.5 Limitations

The development of a non-targeted method capable of detection of phosphorothioated oligonucleotides suitable for use as a preliminary screening approach in equine serum samples opens the possibility for the detection of an entire class of therapeutic agents that was previously non-detectable by methodologies commonly utilized in drug testing laboratories. While the developed methodology affords low detection limits with a relatively straightforward extraction approach, there are some limitations of the approach that open new possibilities for future research. One consideration is the data collection window on the mass spectrometer. The LC-MS method developed on the Q Exactive HF was 20 minutes in length, but MS data was only collected from 1 to 13 minutes and thus any compound that would elute outside this window would not be detectable. Just as the composition and number of nucleotides present on an oligonucleotide have large impacts on a compound's pharmacokinetics and pharmacodynamics, these properties also have impacts on the ability of the analytical approach to detect their presence in a biological matrix (*8*). The developed approach utilized a molecular weight cut-off filter to concentrate the extract prior to LC-MS analysis and thus compounds under 3,000Da are discarded during this concentration step. Compounds eluting after 13 minutes would not be detected either due to the method only collecting data in the range of 1 to 13 minutes. Per the

manufacturers' instructions, the Clarity OTX SPE cartridge was developed to extract oligonucleotides less than or equal to 40 bases long which provides an upper mass range for detection. In addition to challenges with extraction and concentration of oligonucleotides in a non-targeted fashion, there are instrumental challenges that make the analyses challenging. The upper mass ranges for the PRM scans were fixed at 2500 m/*z* on the Q Exactive series instrument, thus depending on the observed charge state following electrospray ionization an oligonucleotide with a mass range outside this upper range would be undetectable using this scan. For the AIF scan, the upper mass was set to 1350 m/*z* and the lower mass at 90 m/*z*, so any masses outside of this range would not be detectable. Both the PRM and AIF scans were optimized for the 94.935 m/*z* product ion and because of their high sensitivity were used in a non-targeted fashion to test equine serum. While the use of targeted product ion provides excellent sensitivity and the ability to detect exogenous administration it is only specific for the presence of the phosphorothioate bond and the use of the extracted ion chromatograms targeting this ion are unable to differentiate between co-eluting molecules that contain the modification. Accordingly, this made the incorporation of an internal standard problematic as the use of a 15N or13C stable isotope labelled oligonucleotide could not be differentiated using a PRM or AIF scan mode. The use of a surrogate non-isotopically labelled phosphorothioated oligonucleotide as the internal standard as commonly done in targeted analysis was not utilized due to the inability to differentiate the presence of the targeted product ion from an unknown oligonucleotide from that of the internal standard. The use of the extracted ion chromatogram targeting the 94.935 m/*z* product ion following either AIF or PRM acquisition would not be able to detect non-phosphorothioated oligonucleotides as their fragmentation would lack the diagnostic 94.935 m/*z* product ion. Lastly, while the MS/MS scan types employed may provide a

large amount of product ions following fragmentation of the targeted molecule using higher-energy C-trap dissociation, full characterization of the exact sequence of nucleotides is a more challenging endeavor without prior knowledge of the various modification and exact sequence utilized.

6.0 Conclusion

A liquid chromatography – high resolution mass spectrometry (LC-HRMS) method was developed and validated that can successfully detect phosphorothioated oligonucleotides in equine serum using the Agilent 1100 series HPLC and Thermo Q Exactive HF high resolution mass spectrometer. Sample clean-up was accomplished using solid phase extraction on a Phenomenex Clarity OTX SPE cartridge which utilized mixed mode weak anionic exchange sorbent to interact with the oligonucleotides following dissociation of the oligonucleotide using the lysis buffer. The methodology was able to achieve limits of detection from 10-50 ng/mL in equine serum for the PS-1 oligonucleotide and limits of quantification ranging from 25-50 ng/mL depending on the specific scan mode. Room temperature stability was tested, and its use was found to be unsuitable for analysis. Stability at 4℃ and -20℃ storage conditions had losses of ~50% or less. This method was validated with acceptable accuracy and precision and tested with 2 other modified oligonucleotides, proving the feasibility of using this method to detect unknown phosphorothioated oligonucleotides and could be eventually used as a screening method for anti-doping. The use of full scan MS/MS using either AIF or PRM based acquisitions may allow for further identification of other exogenous modifications and possibly permit the detection of other strategies utilized to improve the pharmacokinetics and pharmacodynamics oligonucleotide therapeutics.

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