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Enhanced LC-MS/MS Platform to Address Common Challenges During the Analytical Evaluation of Modern Peptide and Oligonucleotide Therapeutics

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1. Introduction

Oligonucleotide and peptide therapeutics have become increasingly important analytes in pharmaceutical research. Advancements have led to chemical modification and additions to these polymeric molecules dramatically improving their pharmacological properties. These advancements have also increased analytical challenges for LCMSMS bioanalysis. For highly charged oligonucleotides and lipophilic peptides, the typical stainless steel sample flow path UHPLC has seen increased non-specific binding and sample carryover. These molecules are also more sensitive to ESI in-source fragmentation as well as increased collision cell fragmentation complexity. These enhanced therapeutics are also more potent, which reduces the dosing requirement and ultimately lowers biological sample concentrations. This introduces a constant need for sensitive analytical workflows. In this study, we developed an LC-MS/MS workflow to address these challenges.

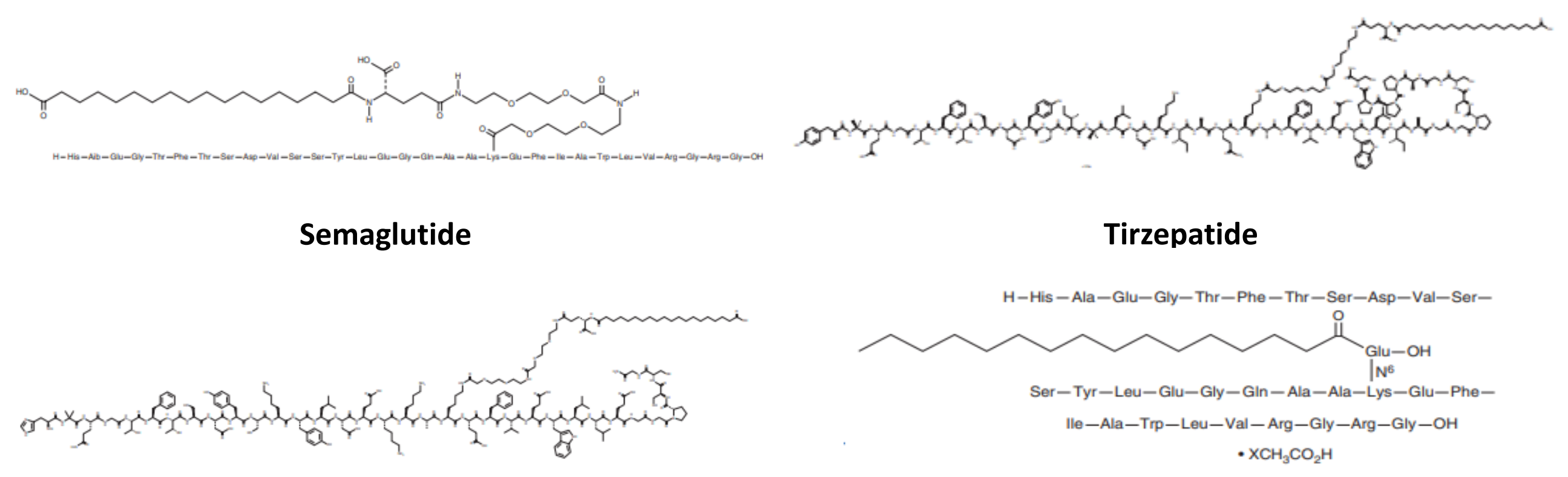
2. Methods

LC Methods

Both the LC XSInert and LC-40 series were coupled with the same Shimadzu LCMS-8060RX Triple Quadruple MS for each analysis. Two analytical methods were developed, one for analyzing 4 lipophilic peptides and another for oligonucleotides. For the oligonucleotide method SiRNA was analyzed, and parameters were optimized to separate and quantify both the Sense and Antisense. For each application, the same LC method parameters were used to compare each system and can be viewed in Table 1.

SiRNA: *Duplex: sense and anti-sense hybridized:

Sense	5′-rGmUrA rAmCmC rArArG rArGmU rAmUmU mCmCrA mUTT-3′
AntiSense	5′-rArUrG rGrArA mUrArC rUrCrU rUrGrG rUmUrA rCTT-3′



Semaglutide **Tirzepatide**

Mazdutide **Liraglutide (acetate)**

Figure 1. Analytical structure of compounds analyzed

Method Parameter	Peptide Analysis	Oligonucleotide Analysis
Flow Rate	0.3 mL/min	0.2 mL/min
Mobile Phase A	Water + 1% FA	50mM HFIP, 16.3mM TEA in Water
Mobile Phase B	Acetonitrile + 1% FA	25mM HFIP, 8.15mM TEA in 50:50 MeOH:Water
Gradient	0B - 100B, 9min	20-80B, 10min
	100B, 9 - 11min	98B, 10.01 - 11.01min
	0B, 11 - 13min	20B, 11.02 - 15min
Oven Temperature	60C	42C
Rinse Settings	Port Only, 2s	Port Only, 2s
Column	Shim-pack Velox C18, 2.7um, 2.1x100mm (P/N 227-32009-03)	Shim-pack Scepter Claris C18-300, 1.9um 2.1x150mm, (P/N 227-31209-03)
Injection Volumen	5uL	25uL

Table 1. LC method parameters

Mass Spec Method

For each compound, all interface and MRM parameters were optimized utilizing LabSolutions Connect MRM software. Optimized interface parameters for each method an be viewed in Table 2. Interface and focus voltages varied for each compound and can be found in Table 3, along with corresponding MRM parameters. For all analyses, the ESI probe position was set to +2mm.

Method Parameter	Peptide Analysis	Oligonucleotide Analysis
Nebulizing Gas Flow	3	3
Heating Gas Flow	13	15
Interface Temperature	350	250
Desolvation Line Temperature	200	250
Heat Block Temperature	400	400
Drying Gas Flow	6	4
CID Gas Pressure	360	330
Nebulizing Gas Flow	3	3
Heating Gas Flow	13	15

Table 2. MS interface parameters

Data processing

Chromatographic and LC-MS data was collected by LabSolutions software, and all data processing was done by LabSolutions Insight LCMS.

3. Results and Discussion

Automated Optimization of MRMs & Interface Parameters

Connect MRM software was used to automate the optimization of all MRMs and interface parameters for each peptide and oligonucleotide. For each compound, transitions for multiple charge states were optimized. Results for optimization of MRMs as well as interface parameters were displayed graphically, making it easy to determine parameters of importance. An example of both for the +3charge state of Tirzepatide can be viewed in Figure 2. For all compounds CID Gas Pressure, Interface Voltage, and Focus voltage had the largest impact on signal intensity. Final MRMs used for quantitation can be viewed in Table 3.

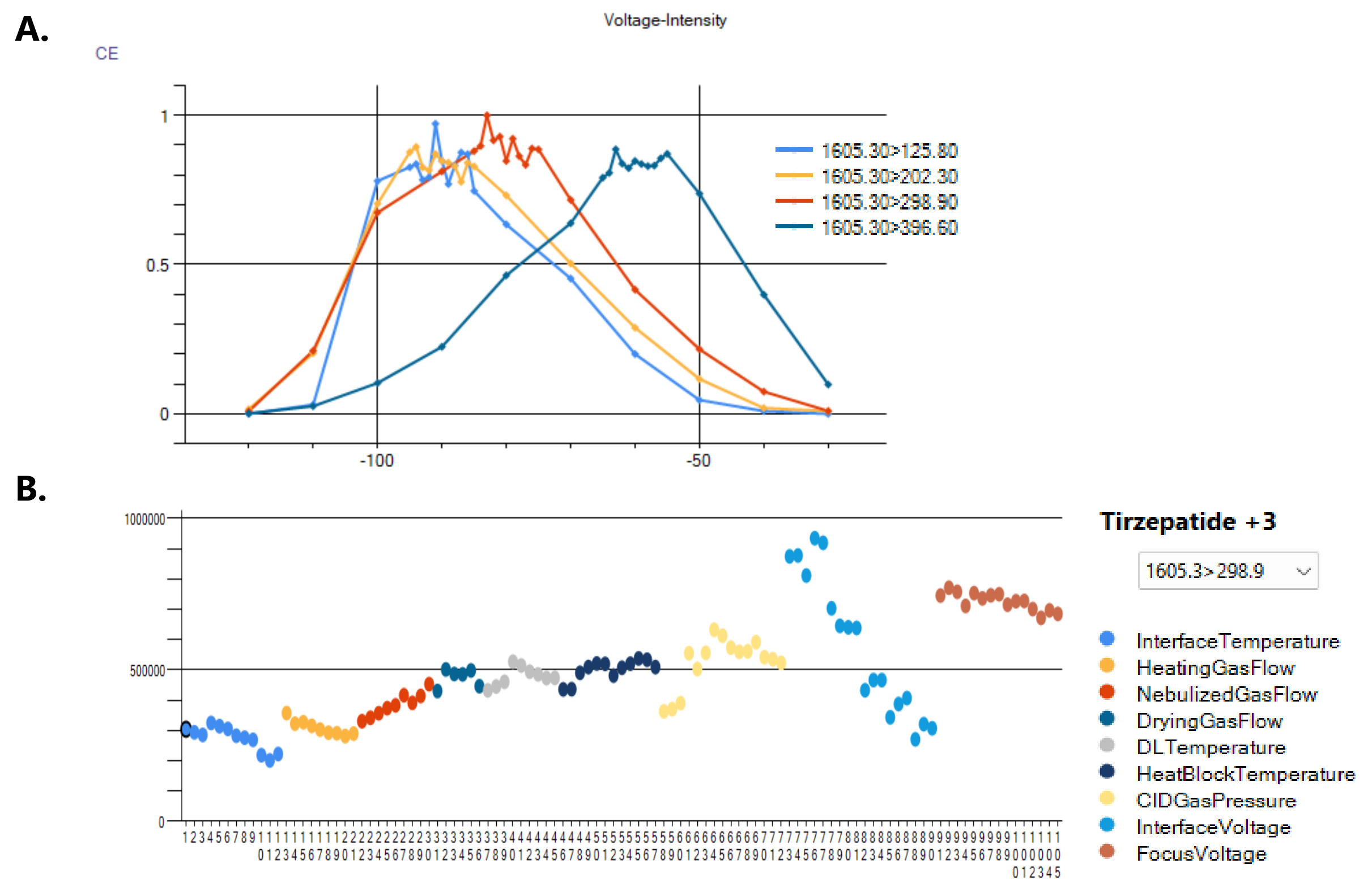


Figure 2: (A) Graphic visualization of results from MRM optimization of the +3 charge state of Tirzepatide. Collision energies ranging from -120V to -30V were simultaneously monitored for 4 transitions to maximize signal. (B) Results from optimization of source parameters for the +3 charge state of Tirzepatide. CID Gas Pressure and Interface Voltage had the highest effect on sensitivity.

Table 3: Target MRM Transitions used for quantitation. Additional MRMs (not shown) were monitored for confirmation.

Method	Compound	m/z (1)	m/z (2)	Collision Energy (V)	Ion Mode	Interface Voltage (kV)	Focus Voltage (kV)
Peptides	Semaglutide	1029.2	1302.4	-35	+	4	1
	Liraglutide	938.5	1064.1	-29	+	4	4
	Tirzepatide	1605.3	298.9	-83	+	4	4
	Mazdutide	913.5	333.2	-45	+	4	4
Oligonucleotides	Sense	675.5	321.2	23	-	-2	0
	AntiSense	665.1	343.9	27	-	-1	-1

Increased Sensitivity with XSInert Flow Path

Standard curves were run on both the BioInert (XSInert) flow path and a traditional stainless-steel flow (SUS) path to evaluate estimated detection and quantitation ranges for both instruments. For the peptides, a standard mixture ranging from 0.1ppb – 1000ppb was prepared in 50:50, ACN:H2O. For the Oligonucleotides, the Duplex was serial diluted in water to a range of 0.015uMol – 1000uMol. Method conditions were determined to separate the Duplex into the Sense and AntiSense for analysis. When evaluating data, an increase in signal was observed for all peptides run on the XSInert flow path when compared to the SUS flow path, whereas the oligos analyzed had similar responses LODs were determined and reported in Table 4. Additionally, all calibration curves had a minimum of an R^2=0.99, and most peptides observed lower LOQs when using the XSInert flow path.

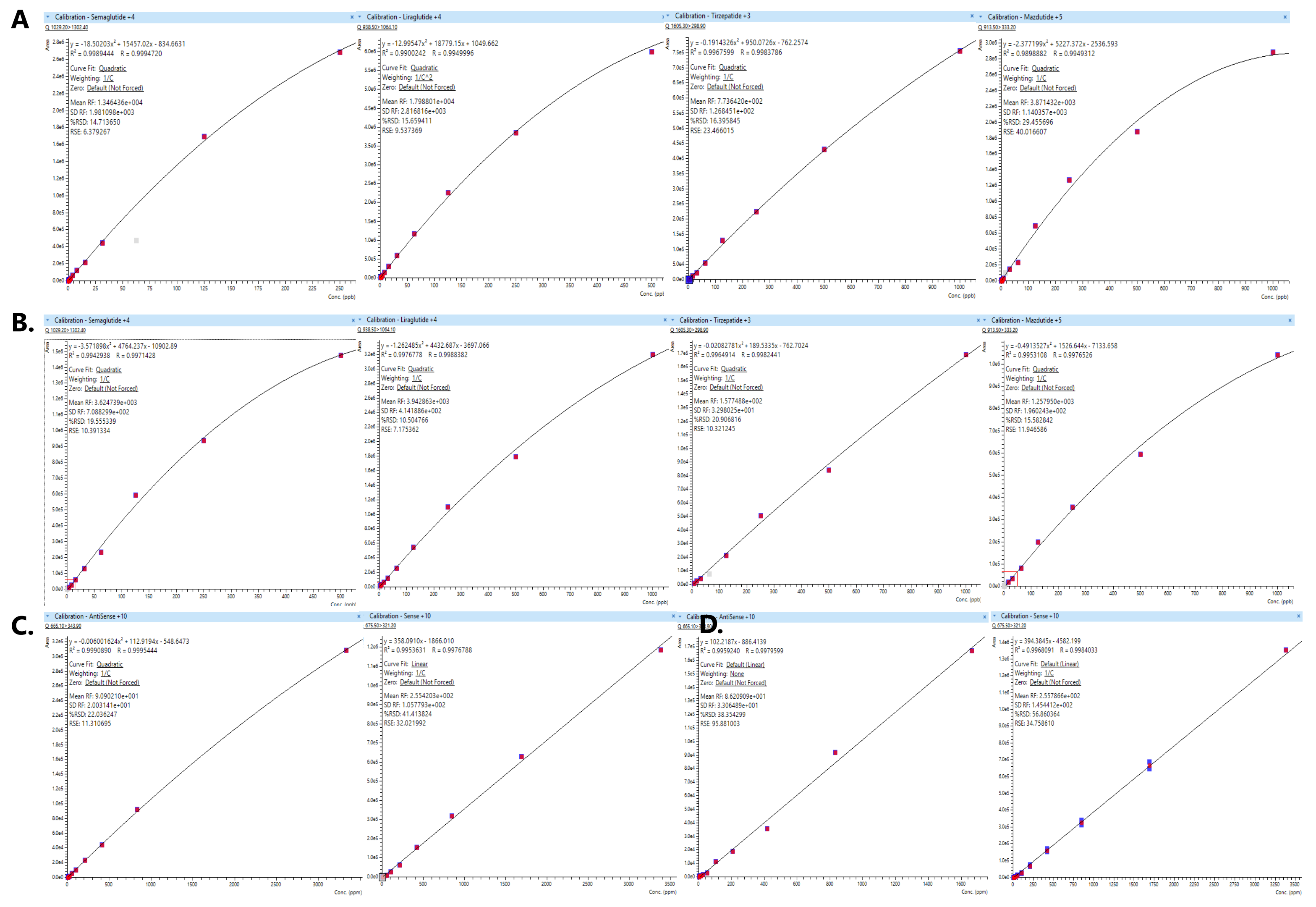


Figure 2: (A) Calibration curves for each lipophilic peptide on the BioInert LC. (B) Calibration curves for each peptide on a traditional stainless steel flow path, LC-40 series. (C) Calibration curves for each oligonucleotide on the BioInert LC (bottom left). (D) Calibration curves for each Oligonucleotide on the SUS LC (bottom right).

Method	Compound	BioInert		Stainless-steel	
		LOD	Quantitative Range	LOD	Quantitative Range
Peptides	Semaglutide	< 0.1 ppb	0.1-250ppb	0.1ppb	4-500ppb
	Liraglutide	0.1ppb	0.5-500ppb	0.25ppb	4-100ppb
	Tirzepatide	0.25ppb	1-1000ppb	4ppb	7.75-1000ppb
	Mazdutide	0.25ppb	0.5-1000ppb	0.5ppb	15-1000ppb
Oligos	Sense	0.12uMol	0.48-1000uMol	0.12uMol	0.97-1000uMol
	AntiSense	0.48uMol	0.97-1000uMol	0.48uMol	0.97-500uMol

Table 4. Comparison of LODs and quantitative ranges for the XSInert flow path vs. SUS flow path

Decreased Carryover with the XSInert Flow Path

To test carryover, the highest concentration standard was injected, followed by multiple blank injections. This was repeated on both the bioinert (XSInert) LC and a traditional stainless steel (SUS) LC. Both methods utilized solely the rinse pump for minimal rinsing. In the oligonucleotide analysis, little to no carryover was observed. For the peptides, carryover was decreased by nearly 50% in the first blank injection when utilizing the XSInert LC over the SUS LC. This was decreased to less than 0.5% after 3 blank injections, where a higher percent was still observed when utilizing the SUS flow path.

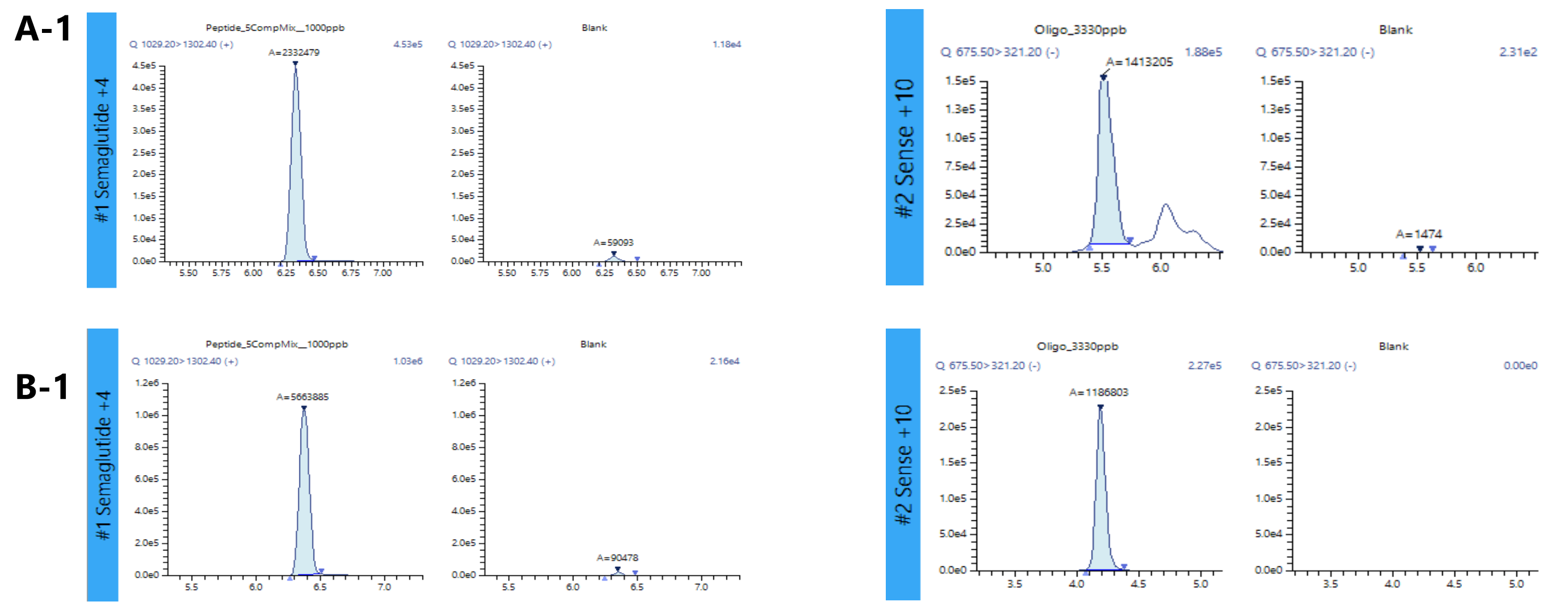


Figure 3: (A) Chromatograms showing typical carryover of a SUS UHPLC flow path for a peptide (A-1) and oligonucleotide (A-2). High standard injection (left) followed by blank injections (right) for Semaglutide and the Sense. (B) Chromatograms showing decreased carryover when utilizing the XSInert flow path, injecting the same peptide (B-1) and oligo (B-2).

Method	Compound	BioInert			Stainless-steel		
		High Std (area)	Carryover 1st Blank (%)	Carryover 3rd Blank (%)	High Std (area)	Carryover 1st Blank (%)	Carryover 3rd Blank (%)
Peptides	Semaglutide	5663885	1.6	0.3	2332479	2.5	1.1
	Liraglutide	9800316	1.7	0.4	3200687	2.8	1.4
	Tirzepatide	755087	0.3	0.0	169134	0.4	0.2
	Mazdutide	2879995	1.4	0.2	1042300	6.3	1.2
Oligos	Sense	1186803	0.0	0.0	1413205	0.1	0.0
	AntiSense	308441	0.1	0.0	268557	0.0	0.0

Table 5. Carryover for all compounds analyzed in both the BioInert flow path and stainless-steel flow path

4. Conclusion

- Connect MRM was used to automate the optimization for MRMs as well as all source parameters for 4 different lipophilic peptides: Semaglutide, Liraglutide, Tirzepatide, and Mazdutide. CID Gas pressure, Interface Voltage & Focus voltage had the largest impact on signal. This workflow was also applied to siRNA to optimize both the Sense and AntiSense, where focus voltage and CID gas pressure had the largest impact.
- Utilizing the BioInert XSInert flow path, our study demonstrated an increase in signal and therefore sensitivity when analyzing these 4 lipophilic peptides. Less of an impact was observed on these reference oligonucleotides.
- For these highly charged lipophilic peptides lower carryover associated with non-specific binding was also observed. This was due to the implementation of the BioInert XSInert flow path, minimizing the use of stainless steel in the flow path.

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