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Optimizing LCMS Method Development for Oligonucleotide Separations: Advantages of Bio Compatible UHPLC Systems

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Introduction

Oligonucleotides (ONs) come in different sizes and forms and are often chemically modified to enhance therapeutic effects. ONs are manufactured in a multistep process where nucleotides are added one by one, giving rise to common impurities, called shortmers, in which the reaction was incomplete (e.g., n-1, n-2)[1]. Resolving such small differences remains an analytical challenge in IPRP-LC. Another difficulty in the analysis of ONs is the presence of trace metal salts commonly introduced by standard ultra-high performance liquid chromatography (UHPLC) components. These salts can significantly diminish spectral quality in IP-RPLC/MS-based analyses.[2] Low-adsorption UHPLC systems are the preferred choice to analyze metal-chelating components. Chromatographic data from a Biocompatible UHPLC system shows improved resolution, reproducibility, and precision over a stainless-steel system.

Experimental

Figure 1: Agilent 1290 Biocompatible UHPLC System & LCMSD XT Single Quad Mass Spec

UHPLC Systems were optimized with 0.12mm ID tubing of various lengths in MP35N or SS. The tubing lengths were the same across systems to ensure similar dispersion volumes. A Diode Array Detector was utilized with a LSS flow cell (10mm/1uL).

Experimental

IPLC is by far the most applied LC mode for ON analysis, when, a reversed-phase stationary phase is combined with a mobile phase containing an ion pairing reagent. These reagents are generally amines that interact with the anionic ONs to form a hydrophobic pair. This pair can then be retained on the reversed-phase column where separation will occur according to the ON length, type, and presence of chemical modifications [1]. Standard stainless steel LC components used in LCMS analysis of ON can introduce metal salts. The presence of trace alkali metal salts can significantly diminish spectral quality. The Agilent ON Resolution Standard was utilized to measure spectral quality and MS sensitivity. A synthetic 95Mer ON was injected 6 times in order to measure UHPLC system performance and a prep scale ON at a concentration of 2mg/mL was utilized in carryover studies.

HPLC Method Parameters:

Chromatographic Conditions			
Parameter	Value	Parameter	Value
Solvent	A) Water - 100mM HFIP 15mM TEA B)Methanol	Gradient	0 min: 95% A, 5% B 15 min: 55%A, 45%B 17 min: 20%A, 80%B 17.1 min: 95% A, 5% B Stop Time: 20 min
Flow Rate	0.500mL/min	Temperature	65°C with TED
Column	Poroshell HPH C18 2.1 x 150mm 1.9um	Injection	Injection Volume: 1uL Sample Temperature 4°C Needle Wash: Multiwash
Detection	260nm - 20Hz	Pump	1290 High Speed Bio & SS

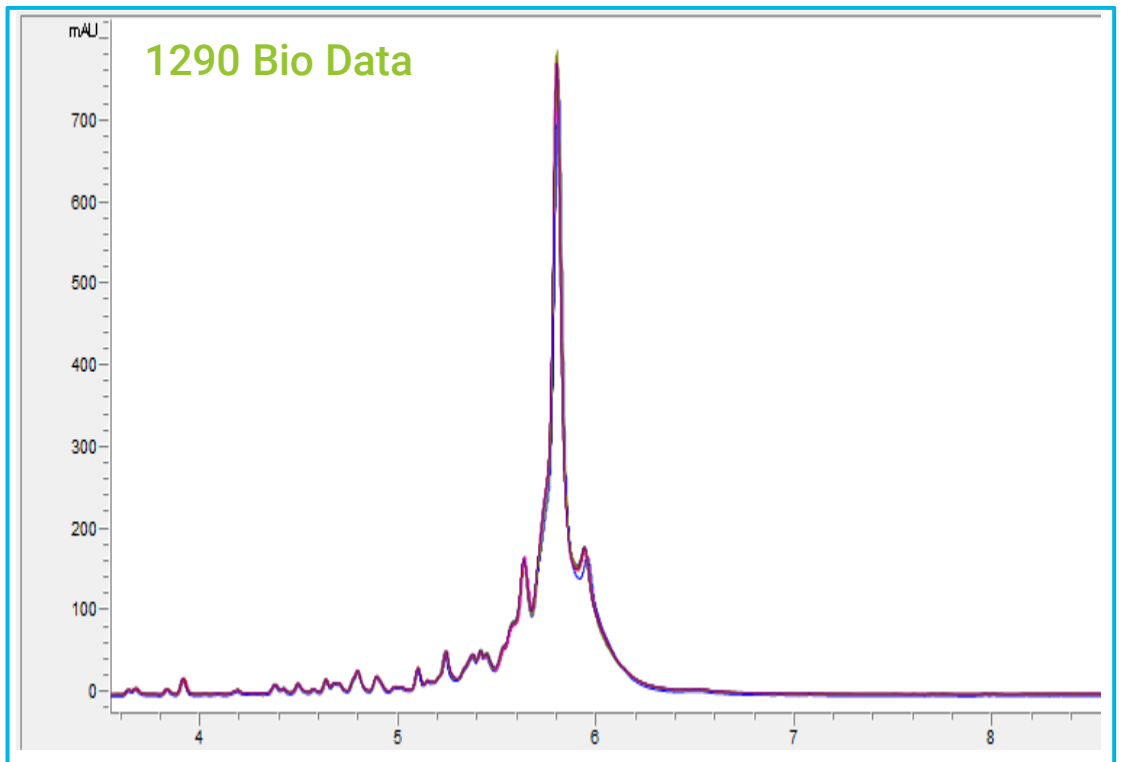
Multiwash Procedure for Carryover Studies:

Step	Solvent	Time [s]	Seat Back Flush	Needle Wash	Comment
1	S2	10	☑	☑	50:50 MeOH:Water
2	S1	10	☑	☑	90:10 Water: IPA
3	Off		☐	☐	
Start Cond.	S3		☑	☐	

LC MSD XT Method Parameters

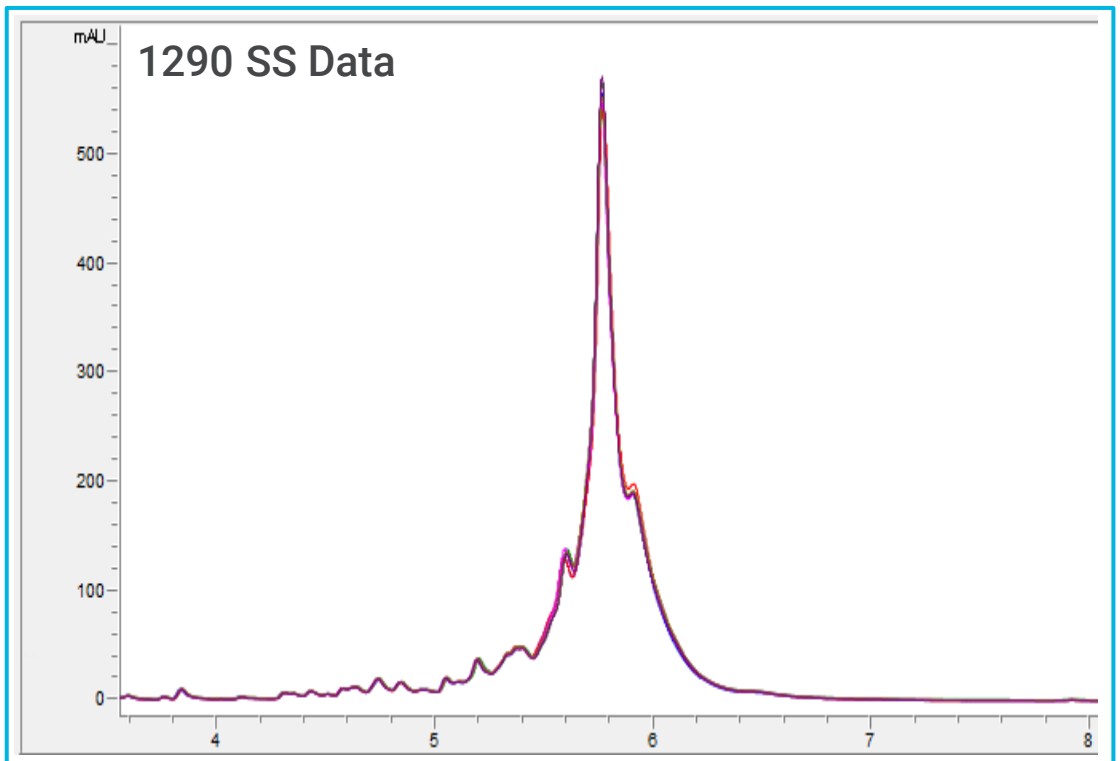
Agilent LCMSD XT Single Quad	
Source	Standard ESI
Gas Temp	350 °C
Gas Flow	12 L/min
Nebulizer	50 psi
VCap	3500 V
Fragmentor	150 V
Acquisiton Mode	Negative Standard
Mass Range	400-2500 m/z

UV Peak Performance:



Peak #	%rsd RT	%rsd Area	PWHH	Tailing
Im 1	0.047	0.488	0.105	0.64
FLP	0.051	0.244	0.055	0.91
Im 2	0.102	2.171	0.105	5.17

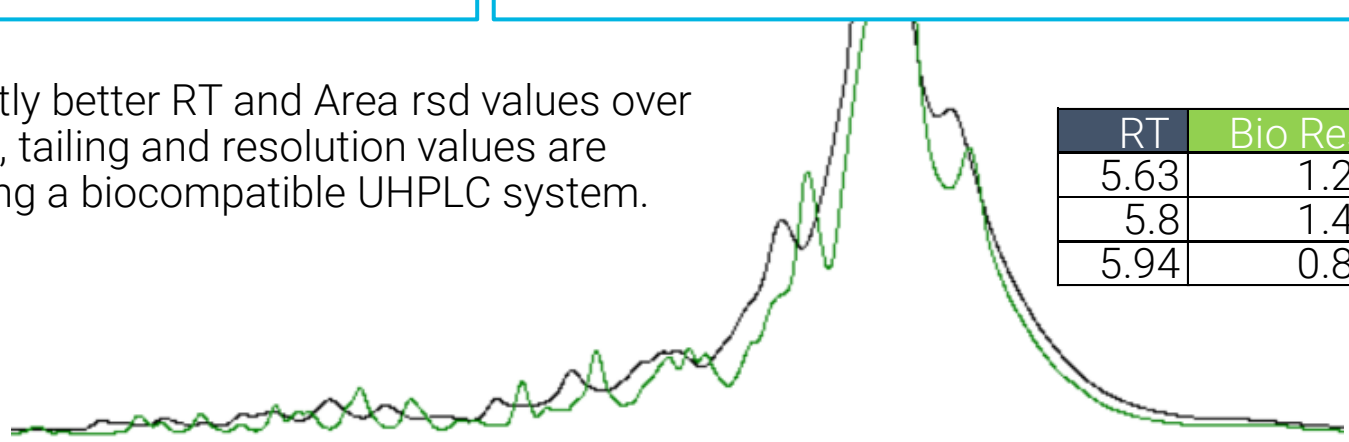
Figure 2: Replicate Injections of 95 Mer Oligo Sample (n =6) - Bio Compatible 1290 UHPLC System



Peak #	%rsd RT	%rsd Area	PWHH	Tailing
Im 1	0.109	0.984	0.115	0.61
FLP	0.036	0.576	0.101	0.94
Im 2	0.05	2.177	0.136	10.6

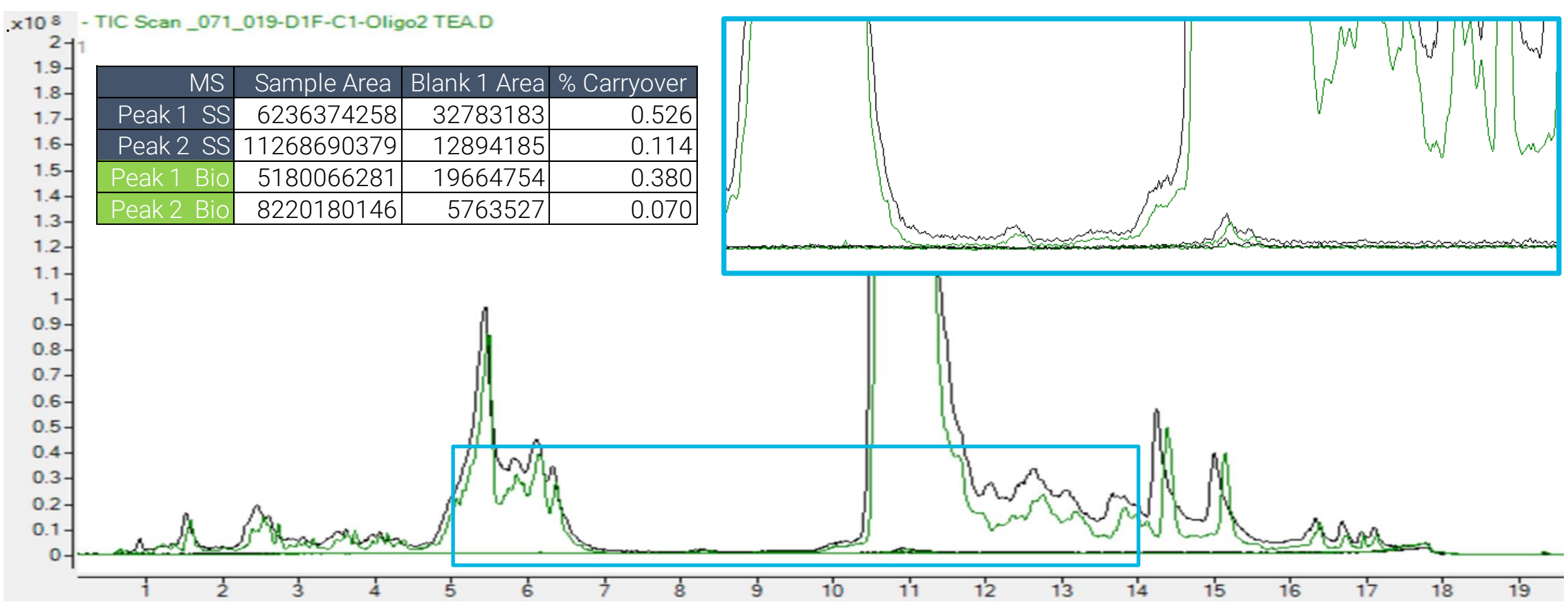
Figure 3: Replicate Injections of 95 Mer Oligo Sample (n =6) - Stainless Steel 1290 UHPLC System

Biocompatible Systems yield slightly better RT and Area rsd values over SS systems. However, peak width, tailing and resolution values are significantly improved when utilizing a biocompatible UHPLC system.



RT	Bio Res	SS Res
5.63	1.23	0.43
5.8	1.49	0.75
5.94	0.81	0.25

SQ MS Carryover Data:

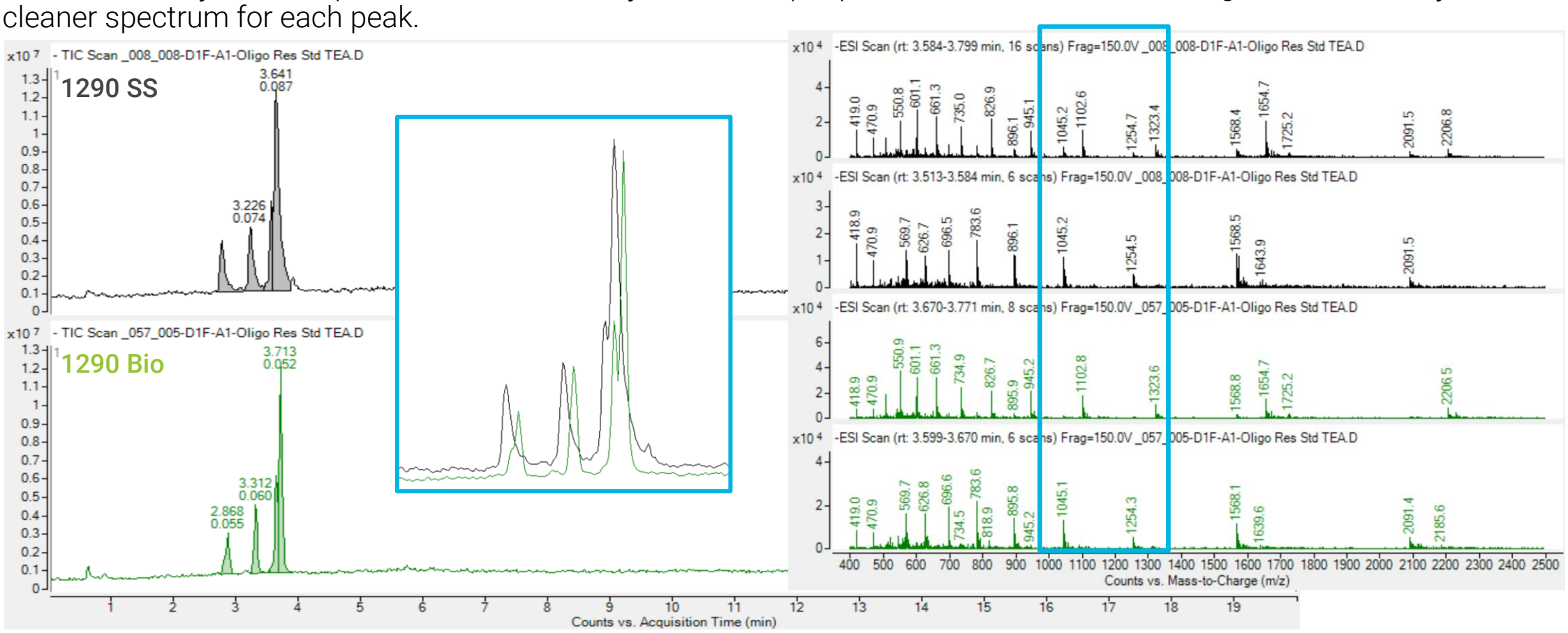


MS	Sample Area	Blank 1 Area	% Carryover
Peak 1 SS	6236374258	32783183	0.526
Peak 2 SS	11268690379	12894185	0.114
Peak 1 Bio	5180066281	19664754	0.380
Peak 2 Bio	8220180146	5763527	0.070

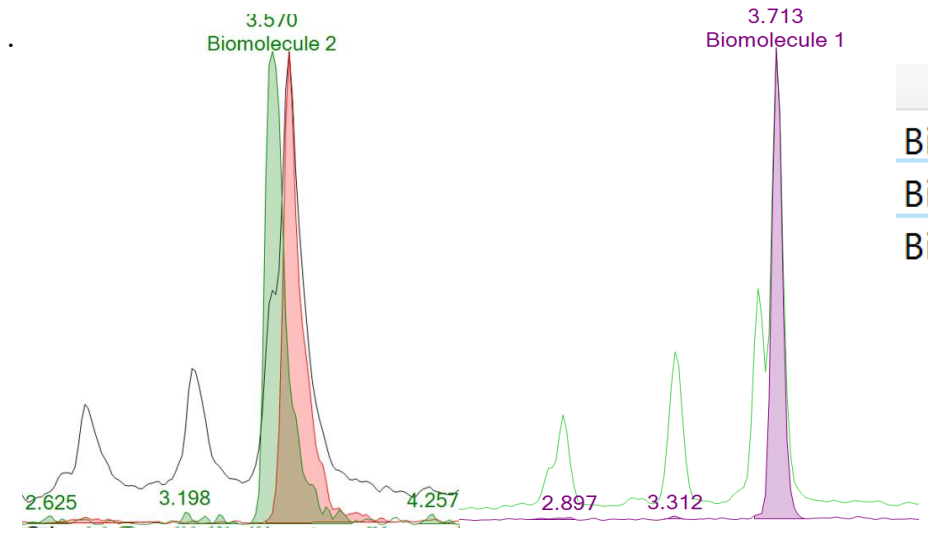
Results and Discussion

Mass Spectral Data:

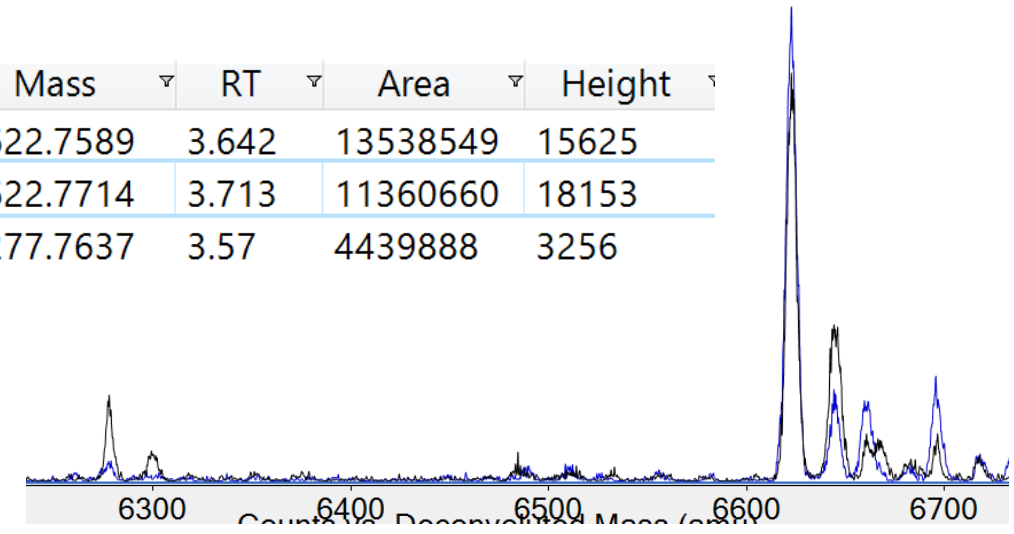
The 1290 Bio System coupled to the LCMSD XT yields a sharper peak which, in turn, results in greater sensitivity and a cleaner spectrum for each peak.



Deconvolution Results:



Label	Mass	RT	Area	Height
Biomolecule 1	6622.7589	3.642	13538549	15625
Biomolecule 1	6622.7714	3.713	11360660	18153
Biomolecule 2	6277.7637	3.57	4439888	3256



Conclusions

- The 1290 Infinity II Biocompatible UHPLC System yields more reproducible retention time and area results due to minimized secondary interactions between sample and MP35N tubing material. This in turn produces cleaner mass spectral data and a more accurate deconvoluted intact mass of the ON peak.
- Improved ON peak performance and resolution is observed with biocompatible materials
- Biocompatible systems prove to be more robust and lead to less carryover of concentrated ON samples as compared to a stainless-steel system

References

1. Vanhoenacker, G., Lecluyse, C., Debyser, G., Sandra, P., Sandra, K., RIC Biologics, Schipperges S., Huber, U., Agilent Technologies, Evaluation of Different Ion-Pairing Reagents for LC/UV and LC/MS Analysis of Oligonucleotides, Application Note 2021.
2. Birdsall RE, Gilar M, Shion H, Yu YQ, Chen W. Reduction of metal adducts in oligonucleotide mass spectra in ion-pair reversed-phase chromatography/mass spectrometry analysis. Rapid Commun Mass Spectrom. 2016 Jul 30;30(14):1667-1679. doi: 10.1002/rcm.7596. PMID: 28328039; PMCID: PMC5094505.

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