Poster Reprint

ASMS 2025
Poster number ThP 565

High-throughput Ion-Pairing Free Reversed Phase Analysis of Oligonucleotides using RapidFire Quadrupole Time-of-Flight Mass Spectrometry

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Introduction

Oligonucleotides are commonly analyzed by LC/MS in negative ion polarity mode using ion-pair reverse-phase (IPRP) methods. However, because many ion-pairing reagents persist in the analytical system long after their use and present a very strong MS response in positive ion polarity, using the same analytical system for multiple applications can be challenging. In this work, we present a high-throughput, ion-pairing-free method for oligo characterization using Agilent RapidFire 400 and AdvanceBio LC/Q-TOF system. The method uses MS-friendly, ammonium bicarbonate-based mobile phases and achieves a 12-second cycle time. The method is effective for both unmodified and heavily modified oligos (Table 1).

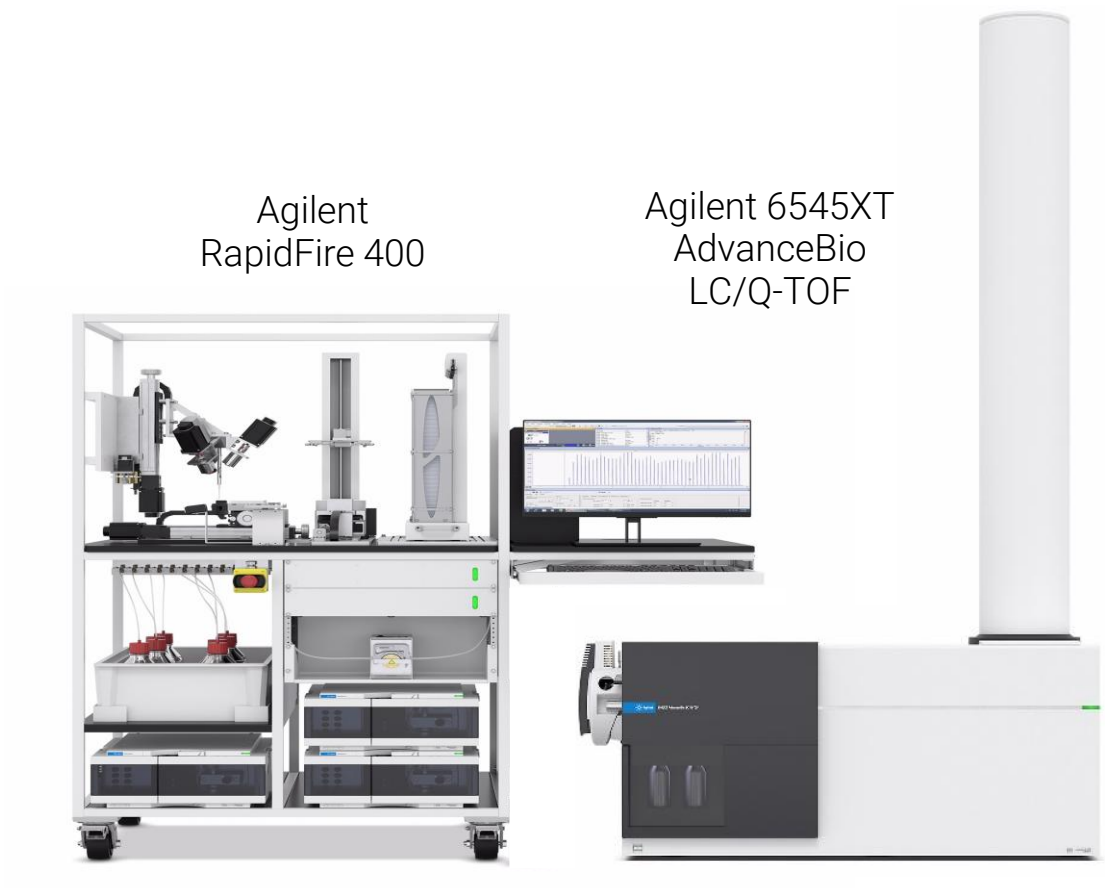

Figure 1. Instrument configuration

Table 1. Oligonucleotides used in this study

Oligo Name	Length	Sequence
20mer	20	TGCATGCATGCATGCATGCA
30mer	30	CATGCATGCATGCATGCATGCATGCATGCA
40mer	40	TGCATGCATGCATGCATGCATGCATGCATGCATGCA
60mer	60	TGCATGCATGCATGAATGCATGCATACAAGCATGCATACAAGAATGAATACATGCAAGCA

Experimental

Table 1. Oligonucleotides used in this study (Continued)

Oligo Name	Length	Sequence
Nu ASO	18	dU*/i2MOErC/*/i2MOErA/*/i2MOErC/*dU*dU*dU*/i2MOErC/*/i2MOErA/*dU*/i2MOErA/*/i2MOErA/*dU*/i2MOErG/*C*dU*/i2MOErG/*G
Ep ASO	20	dU*/i2MOErC/*dU*dU*/i2MOErG/*T*T*/i2MOErA/*/i2MOErC/*/i2MOErA/*/i2MOErT/*/i2MOErG/*/i2MOErA/*/i2MOErA/*/i2MOErA/*dU*/i2MOErC/*/i2MOErC/*/i2MOErC/*C
Givosiren sense	21	mC*mA*mGmAmAmAfGmAfGmUfGmUfCmUfCmAmUmCmUmUmA/L96/
Givosiren antisense	23	mU*mG*mGfUmCfUmUfUfCmUfCfAmCfAmGfAmGfUmAmGfA*fA*mU
Fomivirsen	21	G*C*G*T*T*T*G*C*T*C*T*T*C*T*T*C*T*T*G*C*G

Code	Description
*	Phosphorothioate bond
A	2'-deoxyribose adenine
C	2'-deoxyribose cytosine
G	2'-deoxyribose guanine
T	2'-deoxyribose thymine
U	Uridine
dU	2’-deoxyribose uridine
/L96/	C78H139N11O31

Code	Description
/i2MOErA/	Internal 2-methoxyethoxy A
/i2MOErC/	Internal 2-methoxyethoxy C
/i2MOErG/	Internal 2-methoxyethoxy G
mA/C/G/U	2'-O-methyl A/C/G/U
fA/C/G/U	2'-fluoro A/C/G/U

High-throughput oligonucleotide analyses were conducted on a RapidFire 400 system coupled with a 6545XT AdvanceBio LC/Q-TOF equipped with an Agilent Dual Jet Stream ESI source (Figure 1). A PLRP cartridge was used for online solid phase extraction. Following sampling by the RapidFire, samples were delivered to the cartridge and desalted using 98% 30 mM ammonium bicarbonate in water and 2% acetonitrile (Mobile phase A, MPA) at 1 mL/min for 5,000 ms. The desalted sample was then eluted to the MS for measurement using 50% 30 mM ammonium bicarbonate in water and 50% acetonitrile at 0.5 mL/min for 4,000 ms. The cartridge was then re-equilibrated with MPA at 1 mL/min for 500 ms. Data was acquired with a positive MS scan from 400 to 3200 m/z. (Table 2). Oligo samples were diluted with water to 10 µM without further purification. The resulting data were processed in Agilent MassHunter BioConfirm 12.1 software.

Experimental

Table 2. RapidFire and 6545XT MS methods

Agilent RapidFire 400 Conditions		
Column	PLRP-S, 30µm, 1000Å, 4µL bed volume (custom-packed)	
Cartridge temperature	Room temperature	
Injection volume	10 µL	
Pump 1	MPA = 98% 30 mM ammonium bicarbonate in water + 2% acetonitrile	1.0 mL/min
Pump 2	MPB = 50% 30 mM ammonium bicarbonate in water + 50% acetonitrile	1.25 mL/min
Pump 3	MPB = 50% 30 mM ammonium bicarbonate in water + 50% acetonitrile	0.5 mL/min
State 1	Aspirate sample (sip sensor on)	600 ms
State 2	Load/wash (desalt)	4000 ms
State 3	Extra wash	0 ms
State 4	Elute (inject)	5000 ms
State 5	Re-equilibrate	500 ms

6545XT AdvanceBio LC/Q-TOF Conditions	
Ion Polarity	Dual AJS Positive
Data Storage	Both (Centroid and Profile)
Gas temperature	350 ºC (250 ºC for siRNA)
Drying gas flow	12 L/min
Nebulizer gas	45 psi
Sheath gas temperature	400 ºC (250 ºC for siRNA)
Sheath gas flow	12 L/min
Capillary voltage	3500 V
Nozzle voltage	2000 V
Fragmentor	175 V (130 V for siRNA)
Skimmer	65 V
Oct 1 RF Vpp	750 V
Mass Range	400 – 3200 *m/z*
Acquisition Rate	4 spectra/sec

Results and Discussion

Oligos of Different Sizes

To assess the applicability of the method to oligos of different sizes, a 20mer, a 30mer, a 40mer and a 60mer were analyzed. A 2-fold drop in m/z ion intensities as the size of the oligo increased was observed (Figure 2). The deconvolution peak heights of longer oligos decreased slightly (Figure 3).

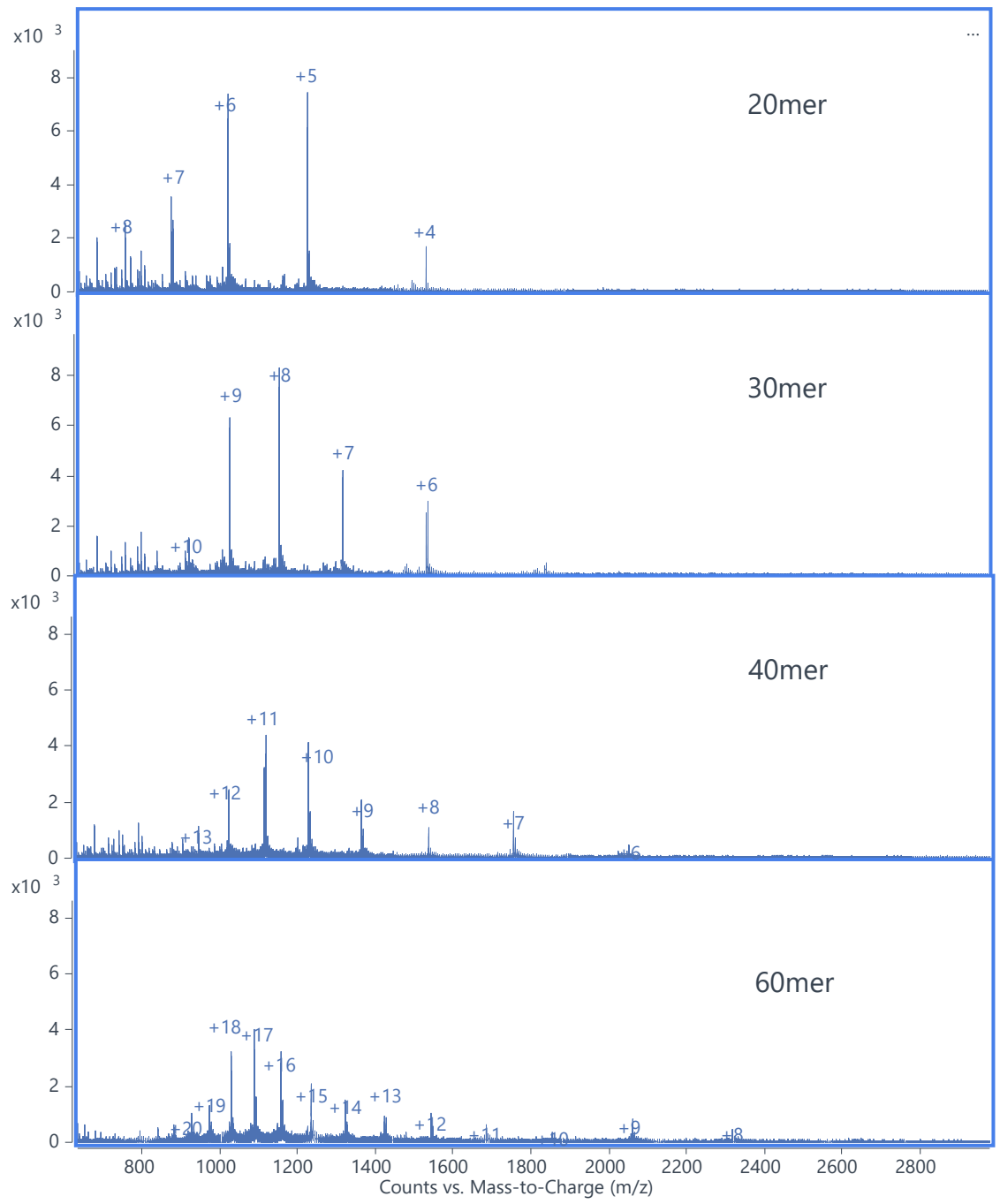

Figure 2. Raw spectra of 20mer, 30mer, 40mer and 60mer with the same Y scale

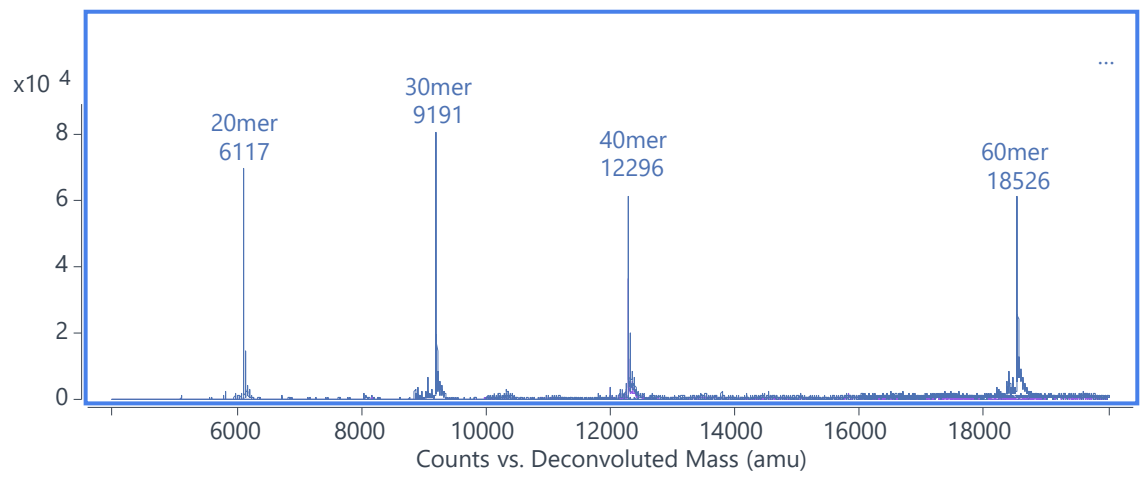

Figure 3. Overlaid deconvolution results of 20mer, 30mer, 40mer and 60mer

Results and Discussion

Oligos of Different Types

To evaluate the applicability of the method to oligos with different base compositions, linker types, and modifications. ASO and siRNA samples were analyzed. Raw spectra and overlaid deconvoluted results were shown in Figure 4 and 5 respectively. Source temperatures and fragmentor voltages were lowered for siRNA samples to reduce in-source fragmentation.

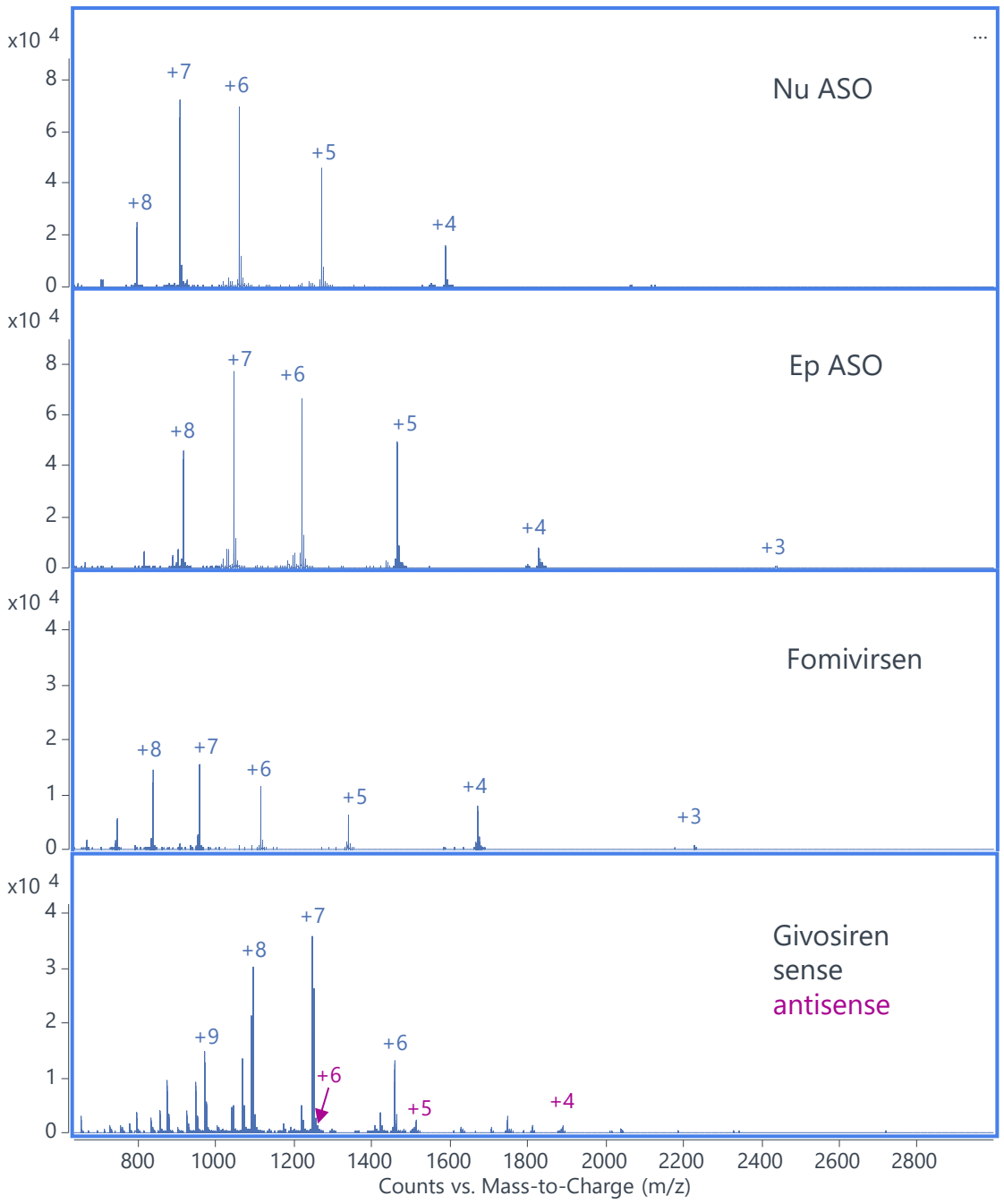

Figure 4. Raw spectra of Nu ASO, Ep ASO, Fomivirsen and Givosiren

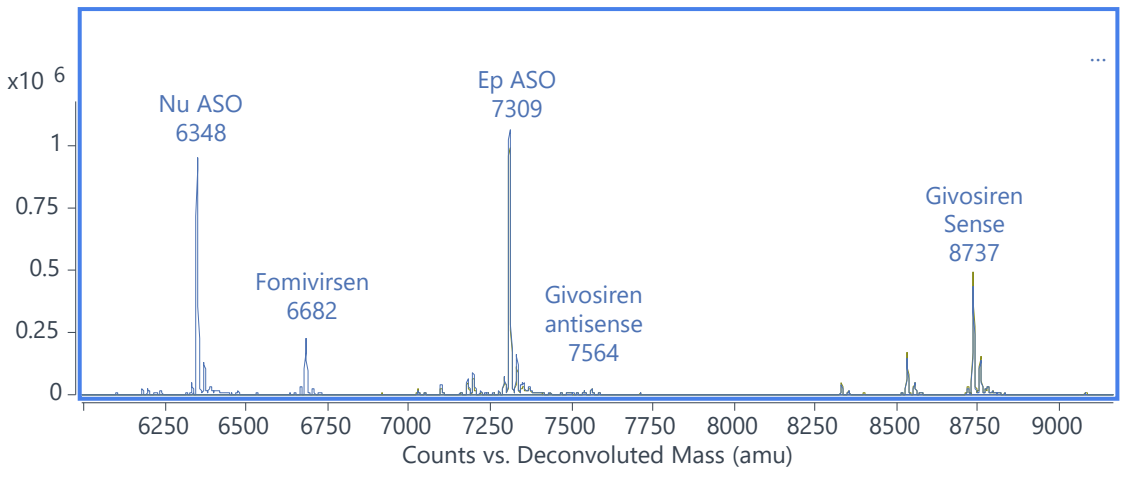

Figure 5. Overlaid deconvolution results of Nu ASO, Ep ASO, Fomivirsen and Givosiren

Reproducibility

12 replicate injections of Nu ASO oligo were run and deconvoluted in BioConfirm. The resulting deconvolution spectra were overlaid. The results demonstrate excellent reproducibility. The total ion chromatograms for the replicates reveal consistent peak height and shape, illustrating that the absolute MS signals are stable across many injections (Figure 6).

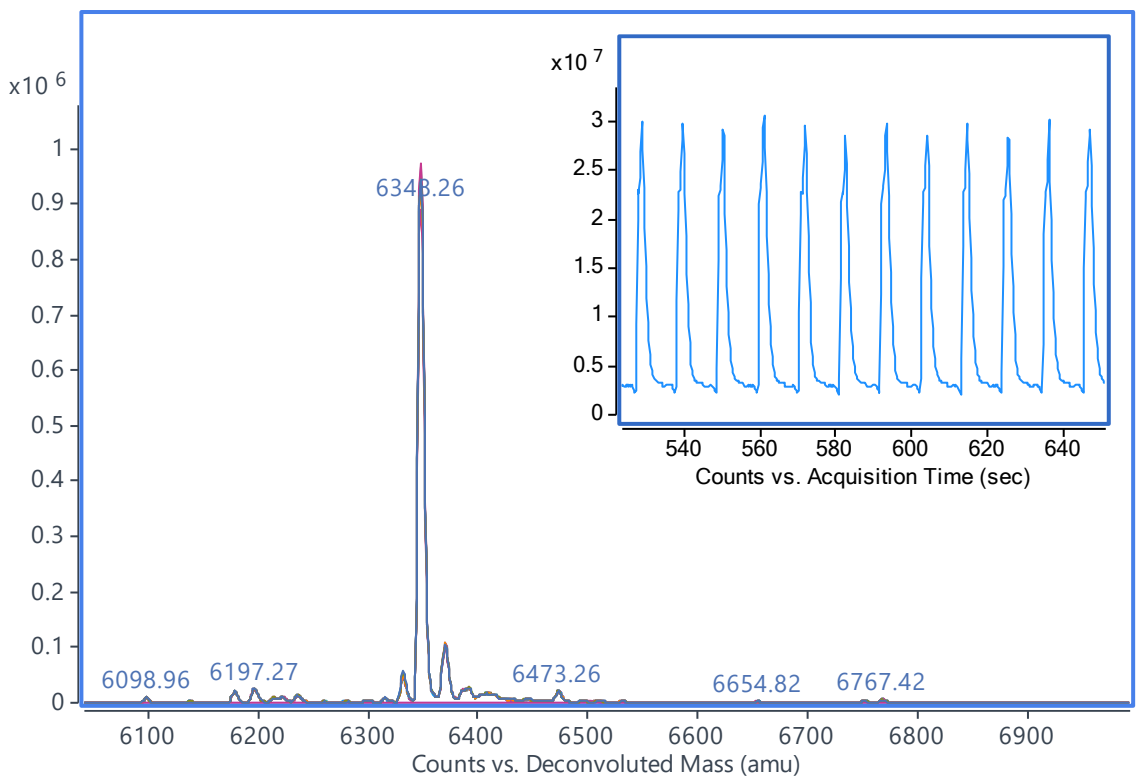

Figure 5. Reproducibility of total ion chromatogram (inset) and deconvolution results (main figure) for 12 replicate samples.

Conclusions

- This work describes an original high-throughput ion-paring free reversed phase method for oligonucleotide analysis.
- The method was successfully applied to oligos ranging from 18mer to 60mer.
- High quality data was generated for heavily modified oligos.
- Excellent reproducibility was demonstrated with 12 replicate injections.

References

[1]Rye, P.; Yang, Y., Agilent Technologies application note, publication number 5994-3753EN, 2022.

[2]Rye, P., Agilent Technologies application note, publication number 5994-4945EN, 2022

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Published in USA, May 15, 2025

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