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Improved Oligonucleotide LC–MS Performance through Inert Column Hardware and Alternative Separation Strategies

Andrew Coffey[1]; Daniel Meston[1]; Chae-Young Ryu[2]; Anne Blackwell[2]; Jordy Hsiao[4]

[1]Agilent Technlogies, Church Stretton, United Kingdom; [2] Agilent Technologies, Seoul, South Korea; [3]Agilent Technologies, Wilmington, DE; [4]Agilent Technologies, Santa Clara, CA

Introduction

Liquid chromatography–mass spectrometry (LC–MS) is a critical analytical tool for oligonucleotide characterization; however, analyte interactions with metal surfaces and mobile-phase additives can compromise recovery, peak shape, and mass spectral clarity. When paired with MS detection, ion-pair reversed-phase (IP-RP) LC often relies on hexafluoroisopropanol (HFIP) to enhance ionization efficiency. Unfortunately, HFIP is a perfluoro alkyl substance (PFAS), or "forever chemical," associated with significant environmental concerns. This work evaluates ion-pair-free reversed phase (RP) chromatography and hydrophilic interaction liquid chromatography (HILIC) as alterative separation modes and evaluates data improvements gained by minimizing metal interactions using inert LC column hardware, an inert LC flow path, and polypropylene solvent bottles.

Experimental

Oligonucleotides were analyzed under conventional IP-RP conditions, as well as ion pair-free RP and HILIC conditions. All methods were compared on conventional stainless steel HPLC columns and Ultra Inert HPLC columns loaded with the same stationary phases.

Experimental

Agilent RNA resolution standard (p/n 5190-9028) and Agilent DNA ladder (p/n 5190-9029) were run in addition to the following samples:

- 25-mer DNA
- 50-mer DNA
- Fully thiolated hybrid DNA
- 2-MeO RNA

Samples were analyzed using an Agilent 1290 Infinity II Bio HPLC and Agilent 6545XT AdvanceBio QTOF.

MS Conditions			
Parameter	**IP-RP**	**IP-Free RP**	**HILIC**
Ionization mode	Negative	Positive	Negative
Gas temp.	300 °C	300 °C	275 °C
Drying gas	8 L/min	12 L/min	12 L/min
Nebulizer	35 psi	30 psi	35 psi
Sheath Gas Temp	350 °C	400 °C	350 °C
Sheath Gas	8 L/min	12 L/min	12 L/min
Capillary	3,500 V	3,000 V	3,500 V
Nozzle	1,000 V	1,000 V	2,000 V
Fragmentor	200 V	180 V	175 V
Skimmer	65 V	65 V	65 V

LC Conditions									
Parameter	**IP-RP**			**IP-Free RP**			**HILIC**		
Column	Agilent AdvanceBio Oligonucleotide, 2.1 × 50 mm, 2.7 µm (p/n 659750-702) Altura Oligo HPH-C18, 2.1 × 50 mm, 2.7 µm (p/n 227205-702)			Agilent AdvanceBio Oligonucleotide, 2.1 × 50 mm, 2.7 µm (p/n 659750-702) Altura Oligo HPH-C18, 2.1 × 50 mm, 2.7 µm (p/n 227205-702)			Agilent AdvanceBio Glycan Mapping, 2.1 × 150 mm, 2.7 µm (p/n 683775-913) Prototype Ultra Inert Glycan Mapping, 2.1 x 150 mm, 2.7 µm		
Column temp.	60°C			75°C			30°C		
Inj. vol.	0.5 – 2.0 µL			0.5 – 1.0 µL			0.5 µL		
Mobile phase	A) 10 mM hexylamine + 50 mM HFIP in 5:95 methanol/water B) 10 mM hexylamine + 50 mM HFIP in 90:10 methanol/water			A) 20 mM ammonium bicarbonate B) Methanol			A) 10:90 100 mM ammonium acetate, pH 6.8 : H_2O B) 10:90 100 mM ammonium acetate, pH 6.8 : ACN		
Flow rate	0.6 or 0.21 mL/min			0.4 mL/min			0.25 mL/min		
Gradient program	Time 0 min 1 min 11 min 12 min 16 min	A 50% 50% 30% 50% 50%	B 50% 50% 70% 50% 50%	Time 0 min 1 min 21 min 23 min 25 min 27 min 32 min	A 95% 95% 50% 10% 10% 95% 95%	B 5% 5% 50% 90% 90% 5% 5%	Time 0 min 2 min 9 min 11 min 12 min 13 min	A 20% 20% 40% 40% 20% 20%	B 80% 80% 60% 60% 80% 80%

Results and Discussion

Column Equilibration

For all chromatographic methods, new columns with Ultra Inert hardware gave reproducible results faster than their stainless steel counterparts, reducing the need for time-, solvent-, and sample-consuming conditioning injections.

Even when stainless steel columns have achieved equilibrium, sample recovery remains 13-17% lower than with an Ultra Inert column.[1]

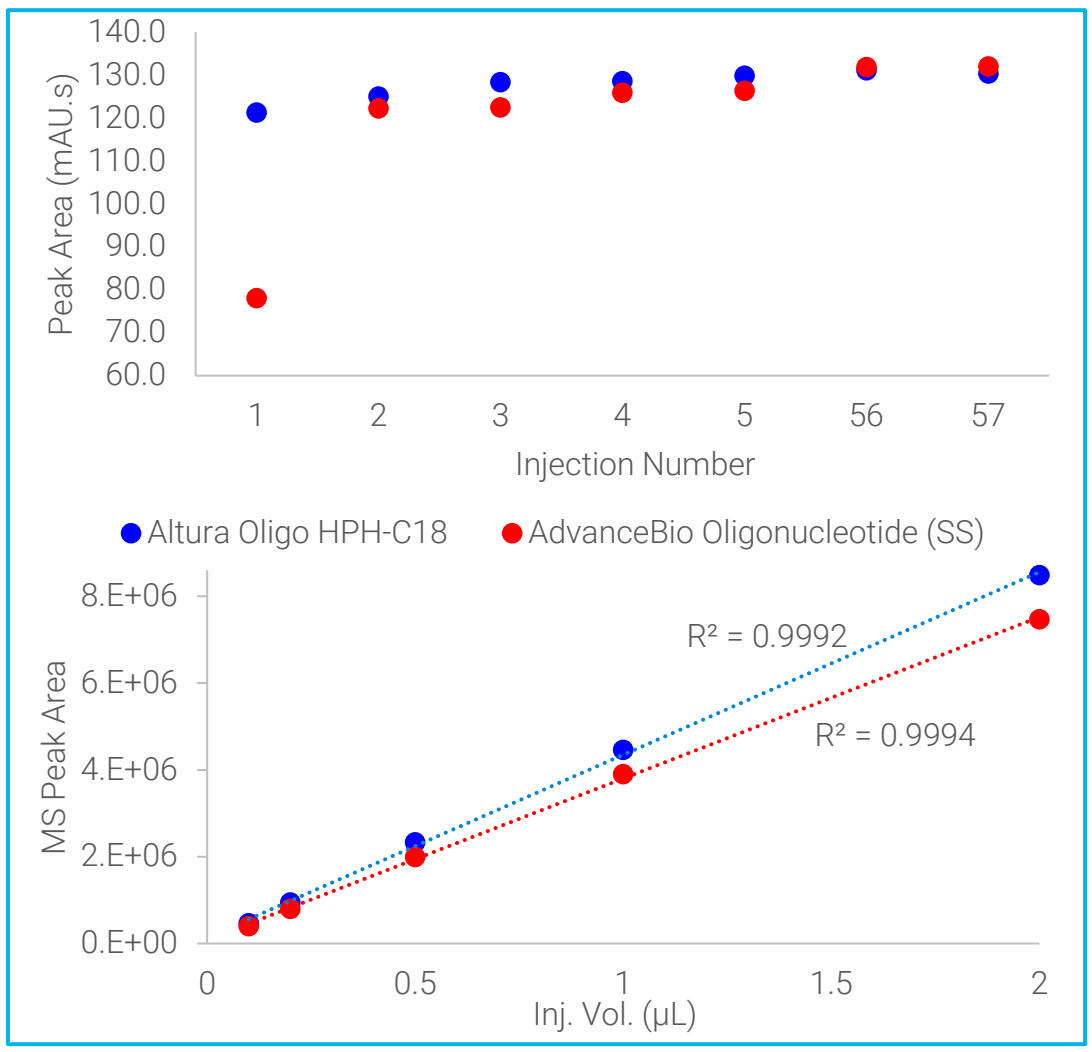

Figure 1. Oligonucleotide signal for Altura Ultra Inert columns versus conventional stainless steel.

Minimizing System Sodium

Replace glass solvent bottles with polypropylene significantly reduced sodium adducts, simplifying MS data analysis.

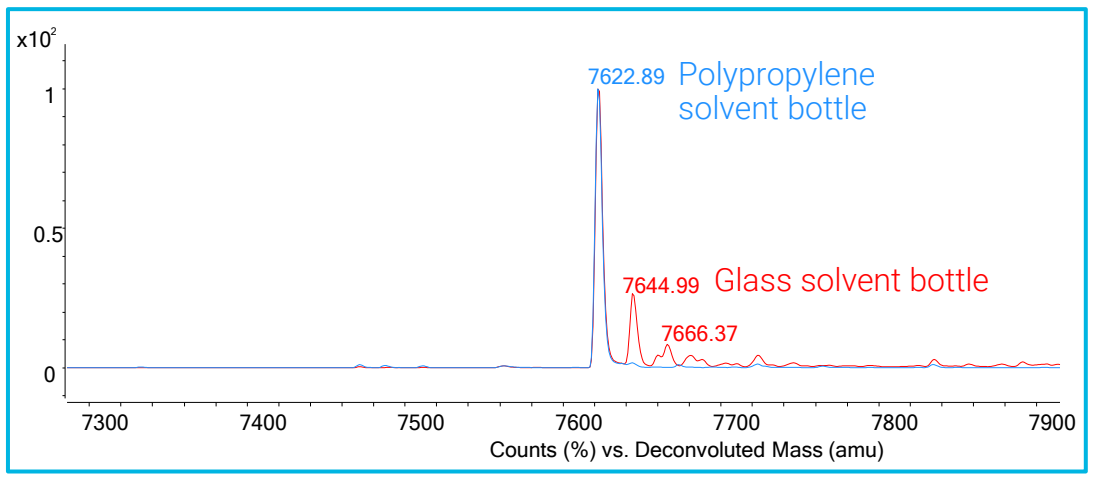

Figure 2. Oligonucleotide signal for Altura Ultra Inert columns versus conventional stainless steel.

Inert Hardware Enables Less IP Reagent

Ion pair reagent blocks interactions with stationary phase active sites, but also with metal active sides in column hardware. Less IP reagent is preferable for reducing costs and instrument contamination. Under a longer gradient[2], IP-RP separations were carried out with IP reagent concentration reduced to 15 mM hexylamine and 25 mM HFIP from 100 mM hexylammonium acetate. Either column resolves the 39-mer from the 40-mer under high IP reagent conditions, but only the Ultra Inert column resolves the same pair under low IP reagent conditions.

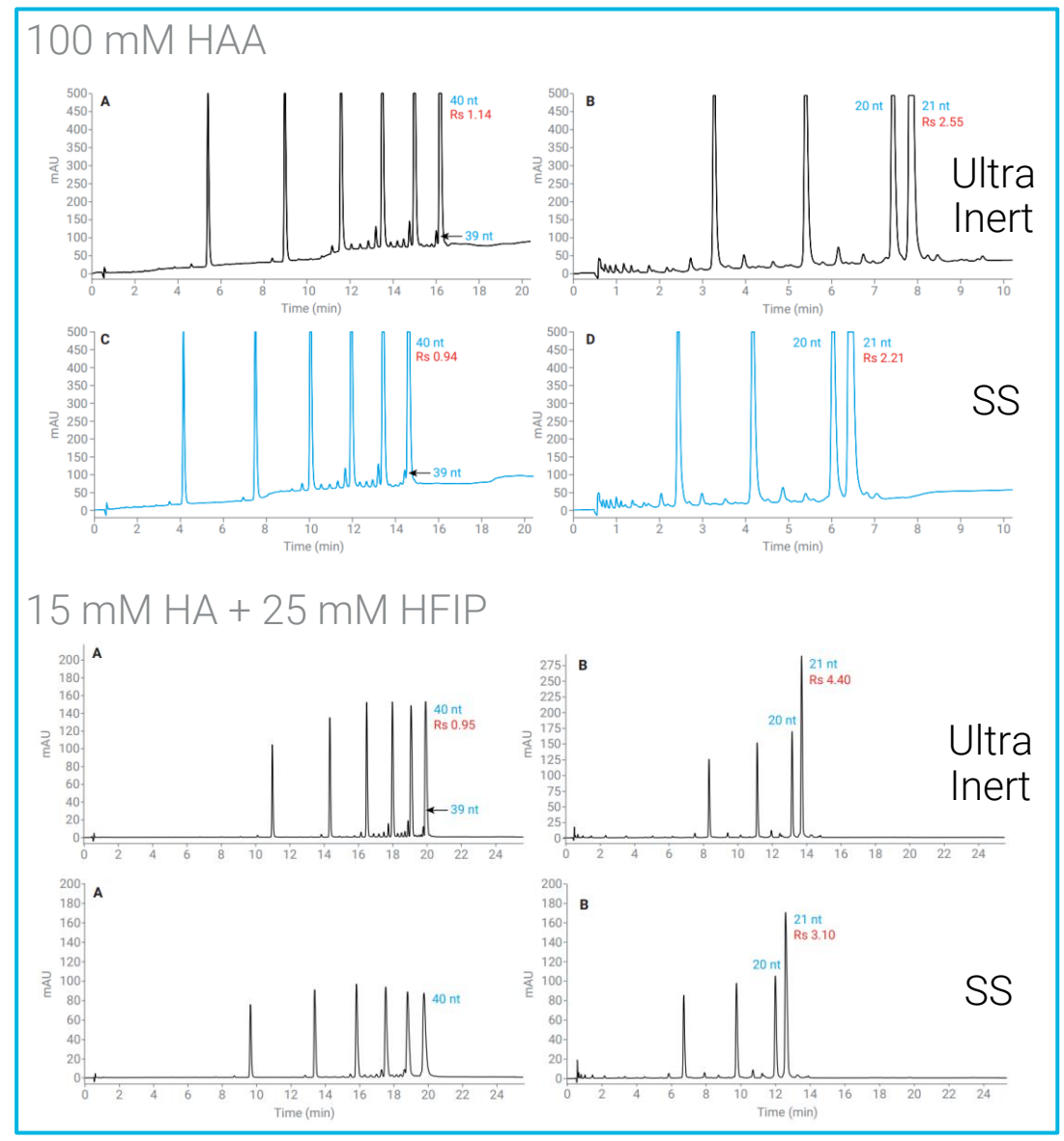

Figure 3. UV chromatograms for DNA and RNA standards under high (top) and low (bottom) IP reagent conditions.

Alternate Chromatography Techniques

Evaluation of the same RNA resolution standard with each separation method gives an idea of the relative resolving power of each method.[2-4]

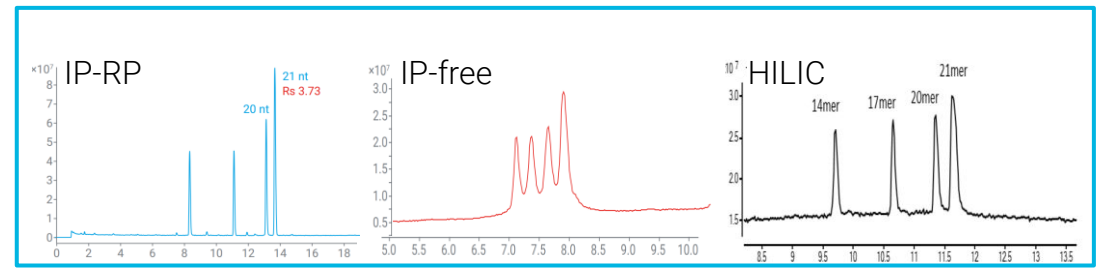

Figure 4. Separation of Agilent RNA resolution standard (p/n 5190-9028) by IP-RP, IP-free RP, and HILIC.

Results and Discussion

Reversed Phase, with and without IP

IP-free RP gives better signal in positive ion mode rather than negative ion mode used for IP-RP analysis. Comparable MS results can be achieved with either method. Fewer ion adducts were observed using IP-free RP.

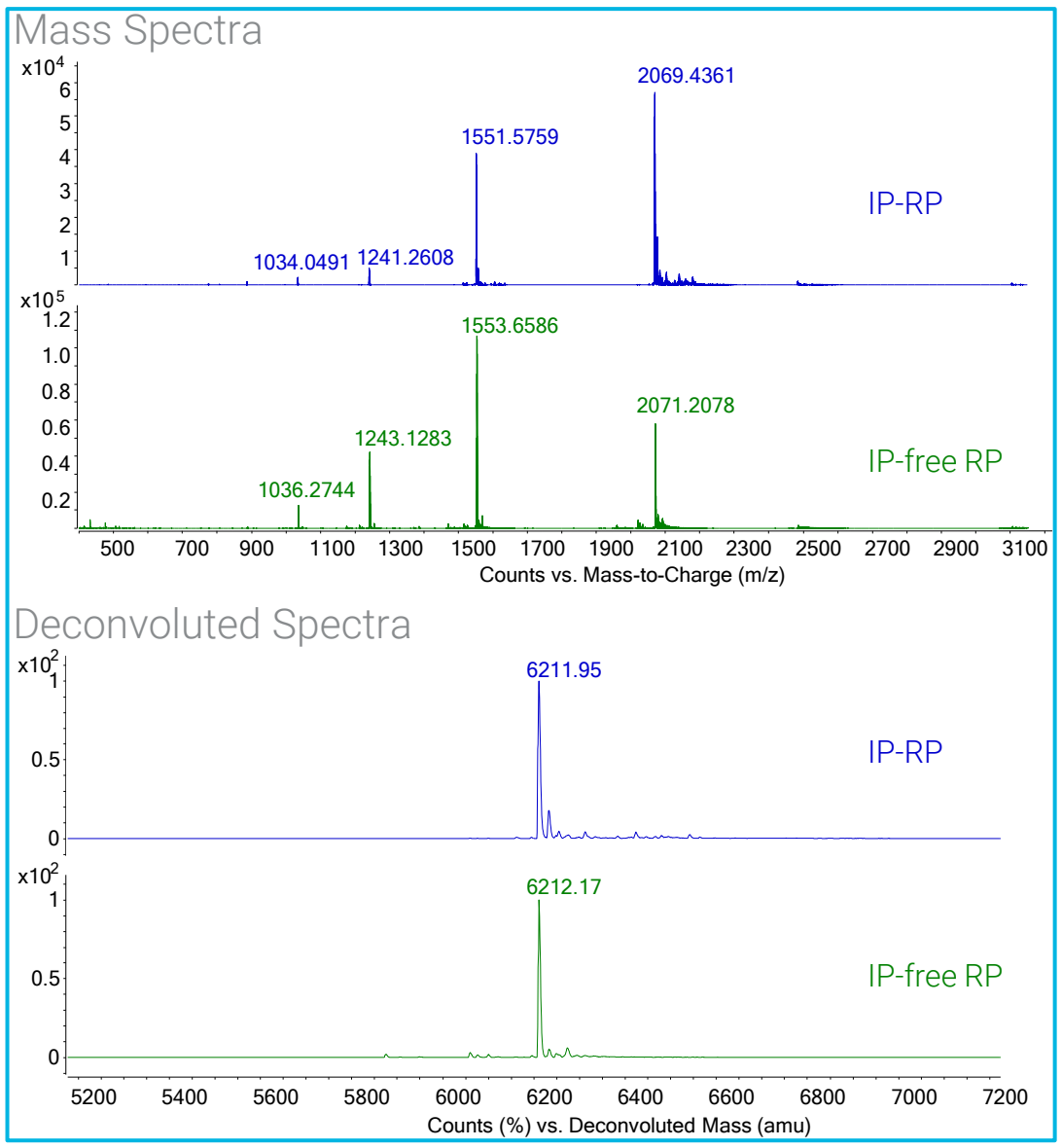

Figure 5. IP-RP and IP-free RP MS mass spectra (top) and deconvoluted average MW (bottom) for thiolated hybrid oligonucleotide

IP-RP	Monoisotopic Mass	Mass Observed	Error (ppm)
25-mer DNA	7619.2874	7619.2917	0.56
50-mer DNA	15131.5533	15131.5521	-0.08
Fully thiolated ON	6207.5974	6207.5629	-5.56
Fully thiolated ON (lower flow rate)	6207.5974	6207.5760	-3.52

IP-Free RP	Monoisotopic Mass	Mass Observed	Error (ppm)
25 mer DNA	7619.2874	7619.2927	0.69
50 mer DNA	15131.5533	15131.5605	0.48
Fully thiolated ON	6207.5974	6207.5988	0.22

HILIC for Oligos

HILIC separation reveals a complex impurity profile for 2-MeO RNA. With MS detection each of these impurities can be characterized and identified.

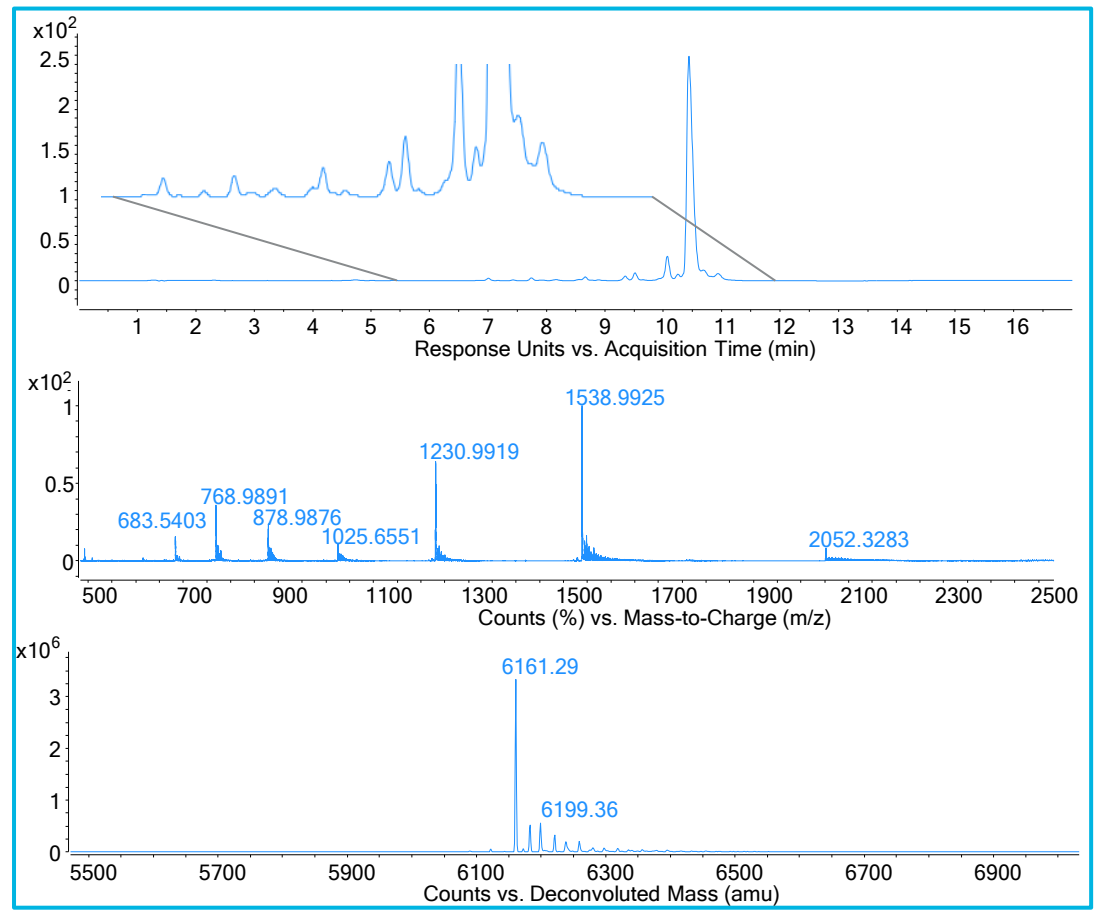

Figure 6. HILIC separation profile and mass spectra of the main peak with deconvolution.

Conclusions

- IP-free RP and HILIC methods are MS-friendly alternatives to conventional oligonucleotide methods reliant on contaminating amines and PFAS reagents.
- IP-RP still gives the best chromatographic resolution, though for shorter oligonucleotides IP-free RP and HILIC have adequate resolution, especially when paired with MS detection.

References

[1]Coffey, A. et al. Eliminate Metal Ions from Your Oligonucleotide LC/MS analysis. Agilent application note 5994-9018EN. 2026

[2]Ryu, C. Oligonucleotide Analysis Using the Agilent InfinityLab Pro iQ and Altura Oligo HPH-C18 Column. Agilent application note 5994-9057EN. 2026.

[3]Bertram, L. et al. Analysis of Oligonucleotides Using an Ion-Pairing-Free Reversed-Phase Method with TOF LC/MS. Agilent application note 5994-8013EN. 2024.

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