Application Note

BioPharma/Pharma

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Eliminate Metal Ions from Your Oligonucleotide LC/MS Analysis

Avoid undesirable metal interactions with Agilent Ultra Inert HPLC column hardware and fully biocompatible LC instrumentation

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Abstract

Metal ions are ubiquitous in regular HPLC columns and instruments that contain stainless steel components. Iron ions can cause a wide range of problems from absorption of analytes to sample oxidation. Avoiding or overcoming these problems can be time consuming and costly, involving unnecessary conditioning steps which may only prove temporary. Replacing stainless steel components with ultra-inert coated materials or alternative metals with a substantially reduced iron content such as MP35N can improve your chromatography and increase your productivity. Oligonucleotides can also form adducts with alkali metal ions, particularly sodium and potassium. This can complicate mass spectrum analysis unless care is also taken to eliminate or reduce these ions in the workflow.

Introduction

Analysis of oligonucleotides by liquid chromatography (LC) with mass spectrometry (MS) can be achieved by several approaches. Most commonly, ion pair reversed-phase (IP RP) chromatography is used. Since oligonucleotides are strongly negatively charged due to the phosphate backbone, suitable ion pair reagents are hydrophobic amines.[1]

Traditionally, amines such as triethylamine have been paired with acetic acid for protonation, but that causes extremely high ion suppression in MS detectors. Instead, hexafluoroisopropanol (HFIP) is used. This is still sufficiently acidic for protonation but is also volatile and greatly reduces ion suppression. HFIP is not miscible with acetonitrile (ACN) therefore it is necessary to use methanol as organic modifier. Unfortunately, HFIP is among the group of compounds called PFAS (per- and polyfluoroalkyl substances), but more commonly known as "forever chemicals." In this application note we compare the results obtained using an ion-pair reversed-phase method with an ion- pair-free alternative that does not require the use of HFIP.[2] Alternative MS-compatible ion-pair-free approaches such as HILIC were not considered here but may be useful in situations where large injection volumes are not necessary.[3]

Even with the use of ion pair reagents, there can be undesirable secondary interactions between negatively charged oligonucleotides and positively charged metal ions. The consequences of such interactions vary, but can include poor sample recovery, peak tailing, or simply complex analysis results due to adduct formation. These concepts are explored in more detail in the remainder of this document.

Experimental

Reagents and chemicals

Fresh ultrapure water was obtained from a Milli-Q Integral system equipped with a 0.22 µm membrane point-of-use cartridge (Millipak). All other reagents (including ion-pair reagents and buffer salts) were HPLC-grade or higher where possible MS-grade solvents or reagents were employed.

Bio solvent bottles, 1 L (polypropylene), (part number 9301-6028), were also used to reduce alkali metal ion adduct formation.

Sample preparation

The custom synthesized oligonucleotide included: 25 mer DNA, 50 mer DNA, and a fully thiolated DNA/RNA hybrid molecule (Table 1).

Samples were dissolved in ultrapure water to a concentration of 1 mg/mL prior to analysis.

Instrumentation

- Agilent 1290 Infinity II high-speed pump (G7132A)
- Agilent 1290 Infinity II multisampler (G7167B) with Agilent InfinityLab thermostat
- Agilent 1290 Infinity II multicolumn thermostat (G7116B)
- Agilent 1290 Infinity II diode array detector (G7115A) with a standard flow cell
- Agilent 6545XT AdvanceBio quadrupole time-of-flight LC/MS system (G6545BA)

Software and data processing

- Agilent MassHunter Acquisition software for instrument control, version 10 or later versions
- Agilent MassHunter Qualitative Analysis software, version 12

Table 1. Oligonucleotides investigated in this application.

Name	Formula	Monoisotopic Mass (Da)	Average Mass (Da)
25 mer DNA	$C_{244}H_{310}N_{86}O_{152}P_{24}$	7,619.287	7,622.927
50 mer DNA	$C_{481}H_{612}N_{179}O_{295}P_{49}$	15,131.553	15,138.743
Fully Thiolated Hybrid	$C_{188}H_{243}N_{65}O_{104}P_{18}S_{18}$	6,207.597	6,216.012

Method conditions

Ion-pair reversed phase

LC Parameters	
Columns	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)
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
Column Temperature	60 °C
Injection Volume	0.5–2.0 µL
Standard Gradient	Flow rate: 0.6 mL/min; 0.21 mL/min Time (min) A% B% 0 50 50 1 50 50 11 20 70 12 50 50 16 50 50
Q-TOF Parameters	
Ionization Mode	Negative
Gas Temperature	300 °C
Drying Gas	8 L/min
Nebulizer	35 psi
Sheath Gas Temperature	350 °C
Sheath Gas	8 L/min
Capillary Voltage	3,500 V
Nozzle	1,000 V
Fragmentor	200 V
Skimmer	65 V
Octopole RF Voltage	750 V

Ion-pair-free reversed phase

LC Parameters	
Columns	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)
Mobile Phase	A) 20 mM ammonium bicarbonate B) Methanol
Column Temperature	75 °C
Injection Volume	0.5–1.0 µL
Gradient (0.4 mL/min)	Flow rate: 0.4 mL/min Time (min) A% B% 0 95 5 1 95 5 21 65 35 23 10 90 25 10 90 27 95 5 32 95 5
Gradient (0.8 mL/min)	Flow rate: 0.8 mL/min Time (min) A% B% 0 95 5 0.5 95 5 5 65 35 6 10 90 7 10 90 8 95 5 10 95 5
Q-TOF Parameters	
Ionization Mode	Positive
Gas Temperature	300 °C
Drying Gas	12 L/min
Nebulizer	30 psi
Sheath Gas Temperature	400 °C
Sheath Gas	12 L/min
Capillary Voltage	3,000 V
Nozzle	1,000 V
Fragmentor	180 V
Skimmer	65 V
Octopole RF Voltage	750 V

Results and discussion

Figures 1 and 2 illustrate the challenges that can be faced during ion-pair reversed-phase analysis of oligonucleotides. Figure 1 shows the peak area observed using UV detection with repeated injections of dilute oligonucleotide solutions. In particular, initial injections onto stainless steel columns may show greatly reduced peak area due to absorption of negatively charged oligonucleotides onto exposed metal ions sites. Although peak area can increase with repeated injections, Figure 2 shows the MS peak area response across a range of injection volumes. It is apparent that the peak area is improved when an ultra-inert coated column is used, improving sensitivity and increasing accuracy. This may be particularly important when trying to identify minor components or impurities present in a sample.

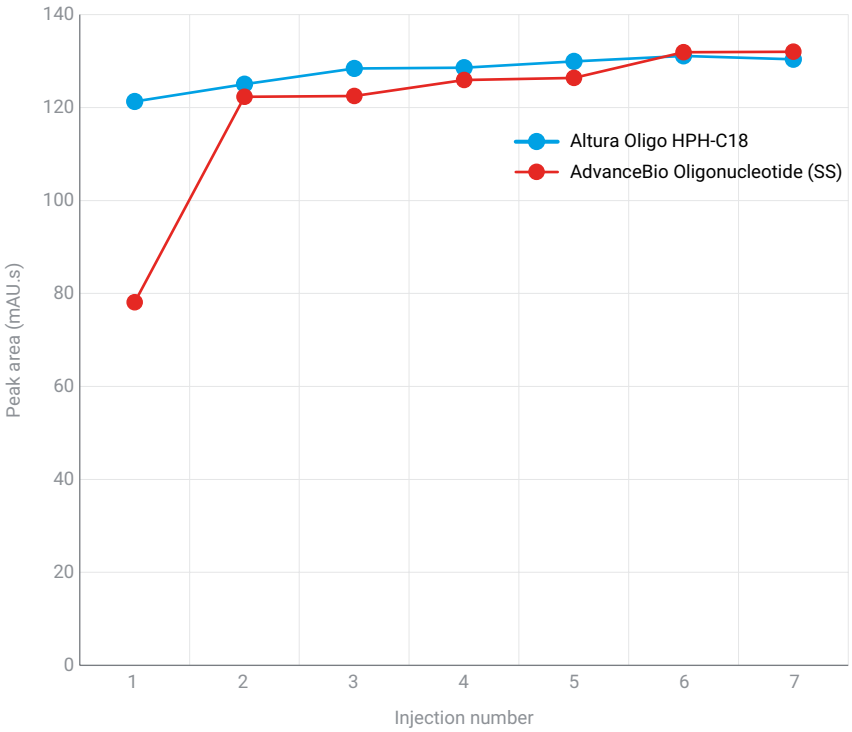

Figure 1. Oligonucleotide recovery: Agilent Altura Ultra Inert coated column versus Agilent AdvanceBio stainless steel column.

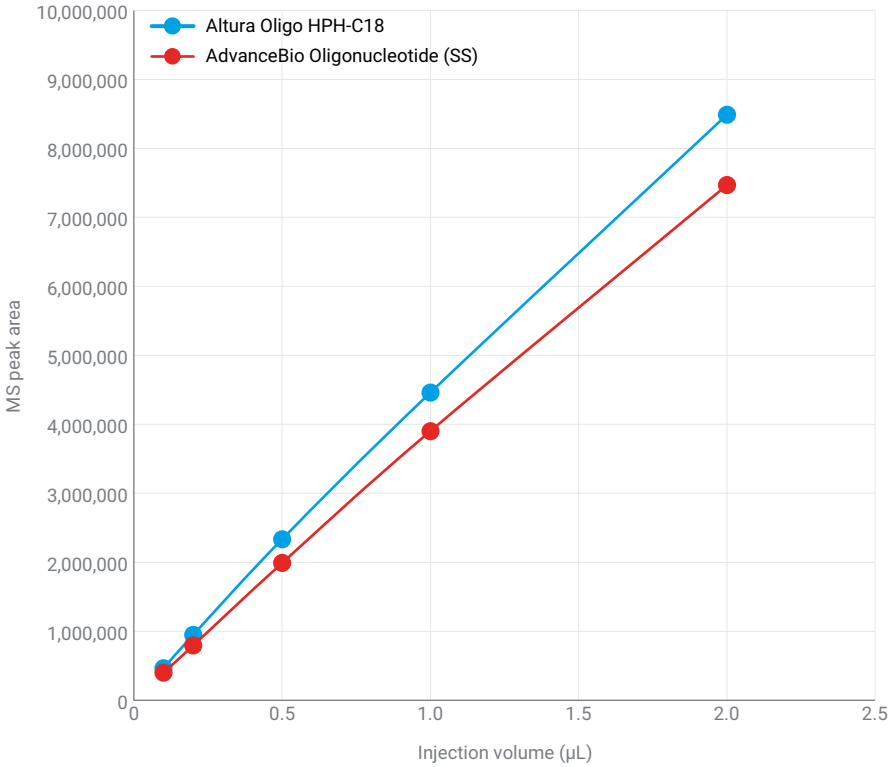

Figure 2. Oligonucleotide peak area vs. injection volume: Agilent Altura Ultra Inert coated column versus Agilent AdvanceBio stainless steel column.

Initial ion-pair reversed-phase experiments were conducted using a short gradient time and high flow rate 0.6 mL/min (see "Method conditions"). Although this was successful, the peak shape was not ideal (data not shown) and the MS results demonstrated a further type of metal interaction, namely metal adduction (Figures 3 and 4).

Instead of a number of peaks corresponding to individual charge states (the largest peak corresponds to $[M-4]^{4-}$), both the Agilent Ultra Inert (UI) coated Altura Oligo HPH-C18 column and the stainless steel Agilent AdvanceBio oligonucleotide column demonstrated significant levels of sodium and potassium adducts replacing some protons, including individual Na^+ (+22) and K^+ (+38), as well as various combinations such as $[2\ Na^+]$ (+44), $[Na^+\ K^+]$ (+60) and $[2\ K^+]$ (+76).

This demonstrates that alkali metal ions may be present in samples that have been purified by ion exchange, dissolved in solutions that contained alkali metal ions, or they may simply arise from the use of glass components (bottles, bottle filters).

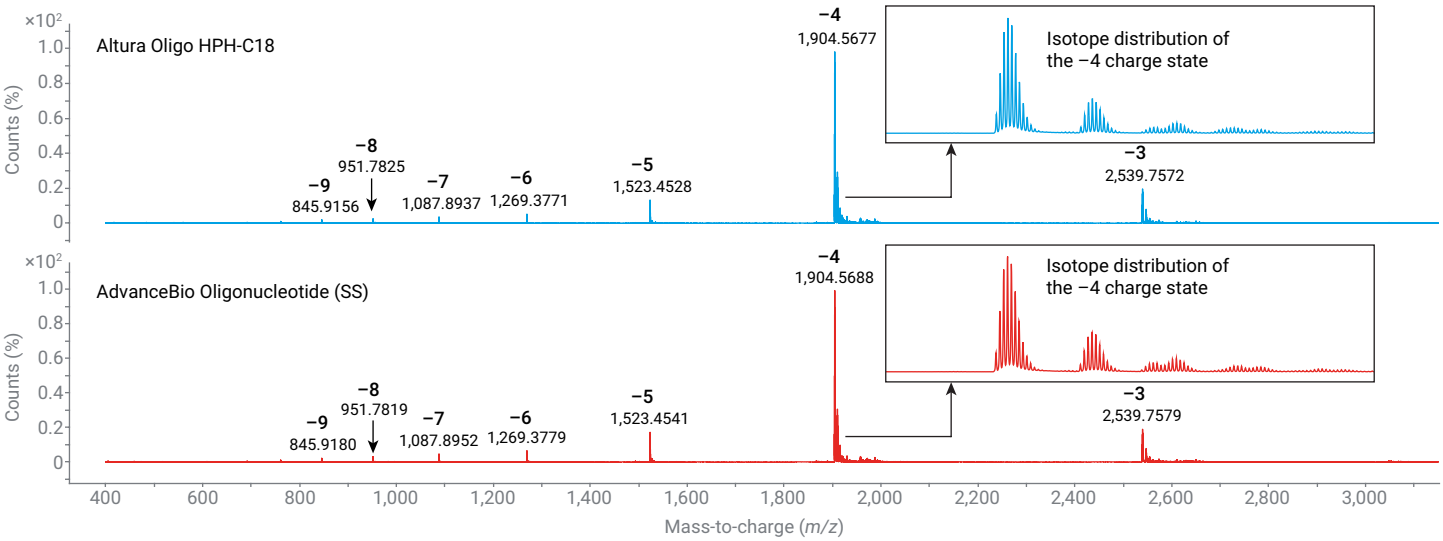

Figure 3. Mass spectra of 25 mer DNA.

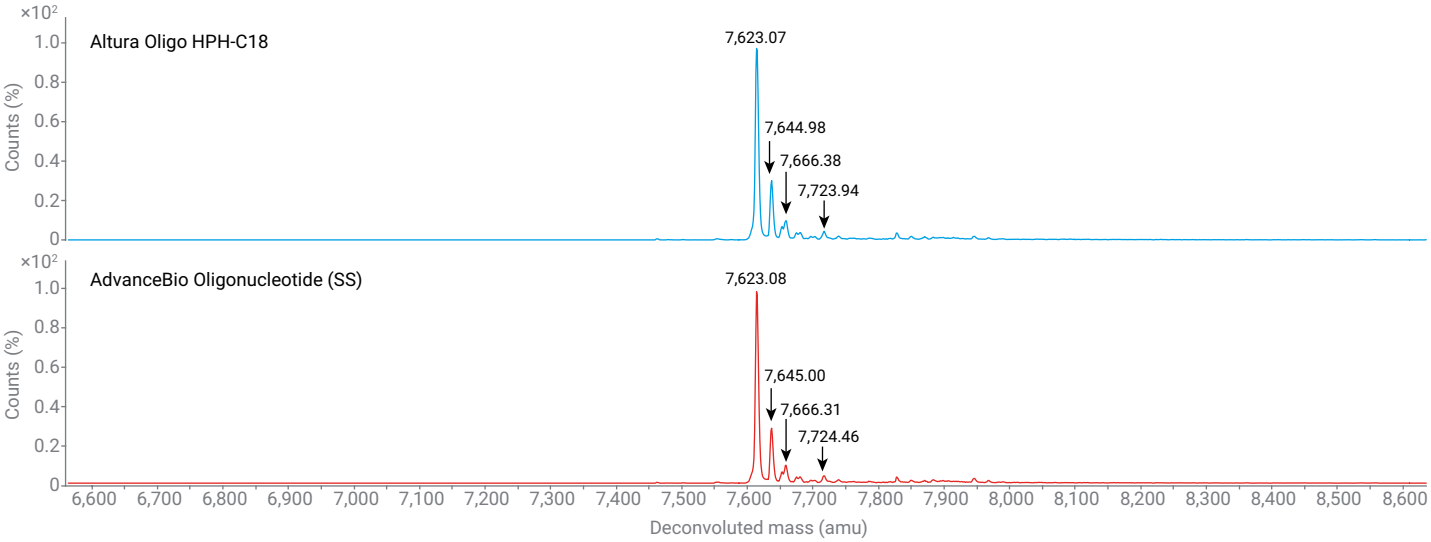

Figure 4. Deconvoluted mass spectra of 25 mer DNA showing average mass.

Taking additional care to prepare the samples without introducing additional alkali metal ions can help reduce metal adducts, as well as switching to polypropylene solvent bottles.

Furthermore, using a lower flow rate was found to improve peak shape and impurity resolution (Figure 5A). It was also noted that the charge state distribution was altered slightly and helped reduce the level of adducts observed (Figure 5B).

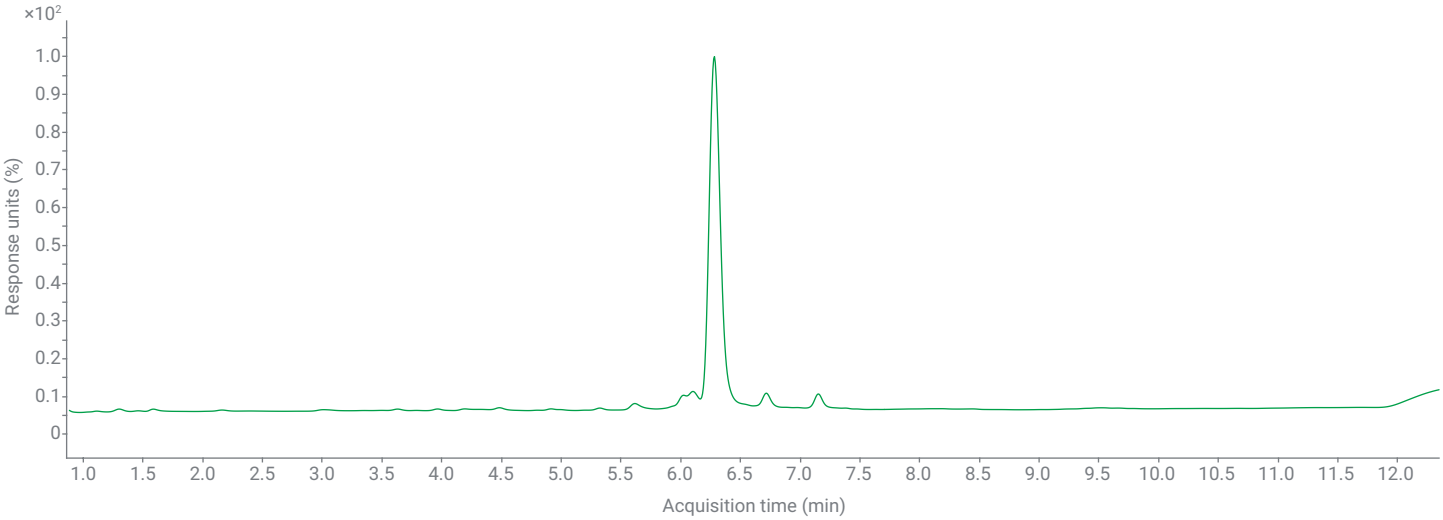

Figure 5A. UV Chromatogram of 25 mer DNA by ion-pair reversed-phase (0.21 mL/min).

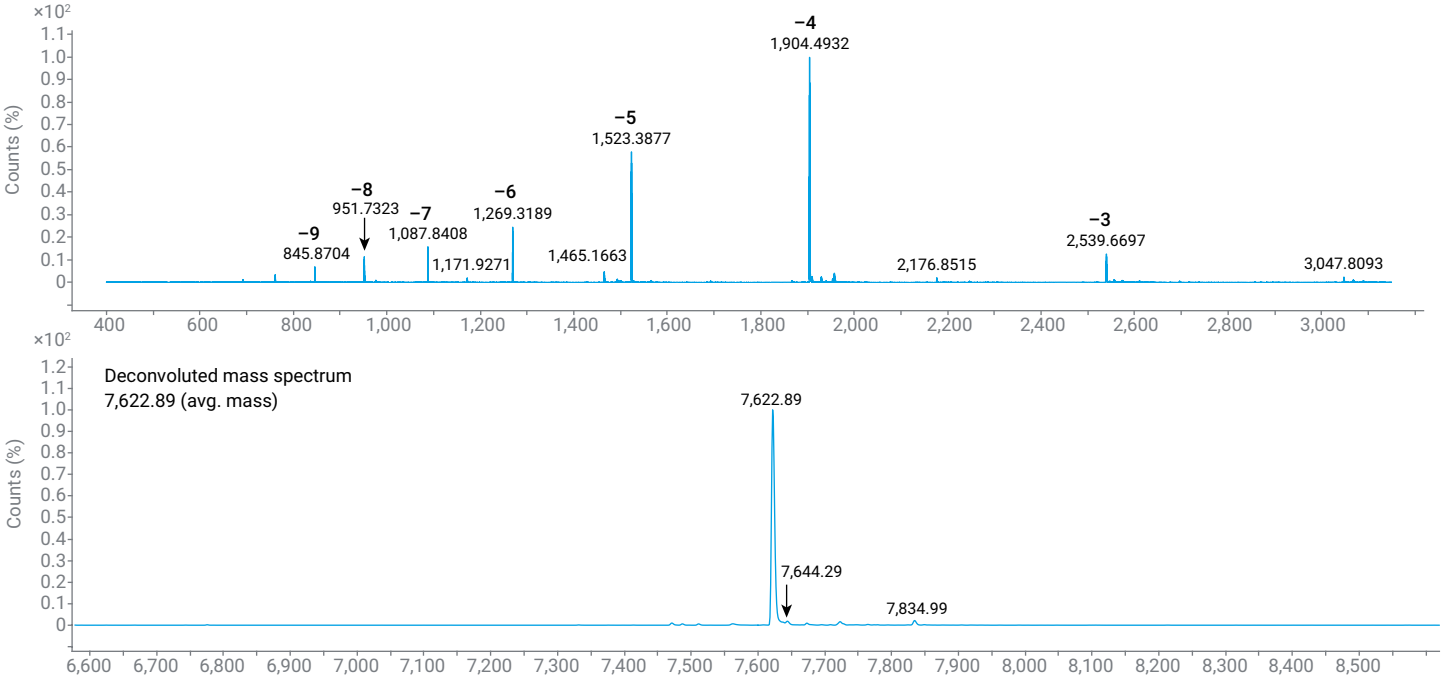

Figure 5B. Mass spectrum (FBF) and deconvoluted mass spectra of 25 mer DNA by ion-pair reversed-phase (0.21 mL/min).

The same gradient and lower flow rate were applied to the analysis of a larger DNA oligonucleotide (Figure 6A).

The number of charge states is greatly increased compared to the smaller oligonucleotide and there is evidence that the lower (−5 and −6) charge states have higher levels of adducts than the higher charge states (Figure 6B). Additionally, the deconvoluted mass also gives fewer adducts.

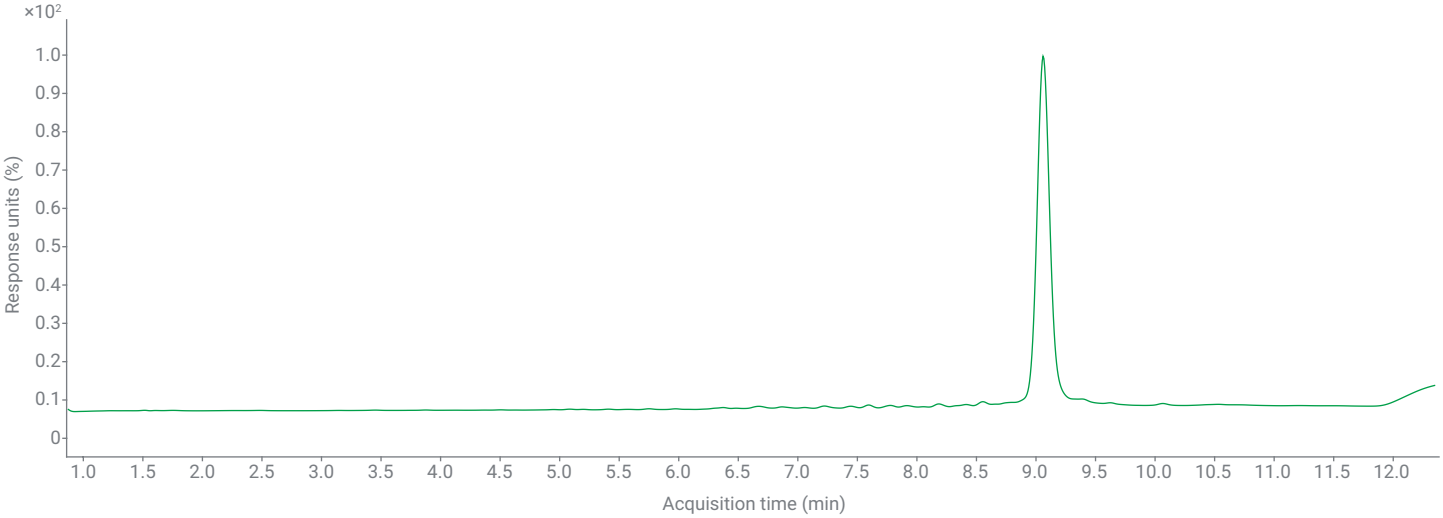

Figure 6A. UV chromatogram of 50 mer DNA by ion-pair reversed-phase (0.21 mL/min).

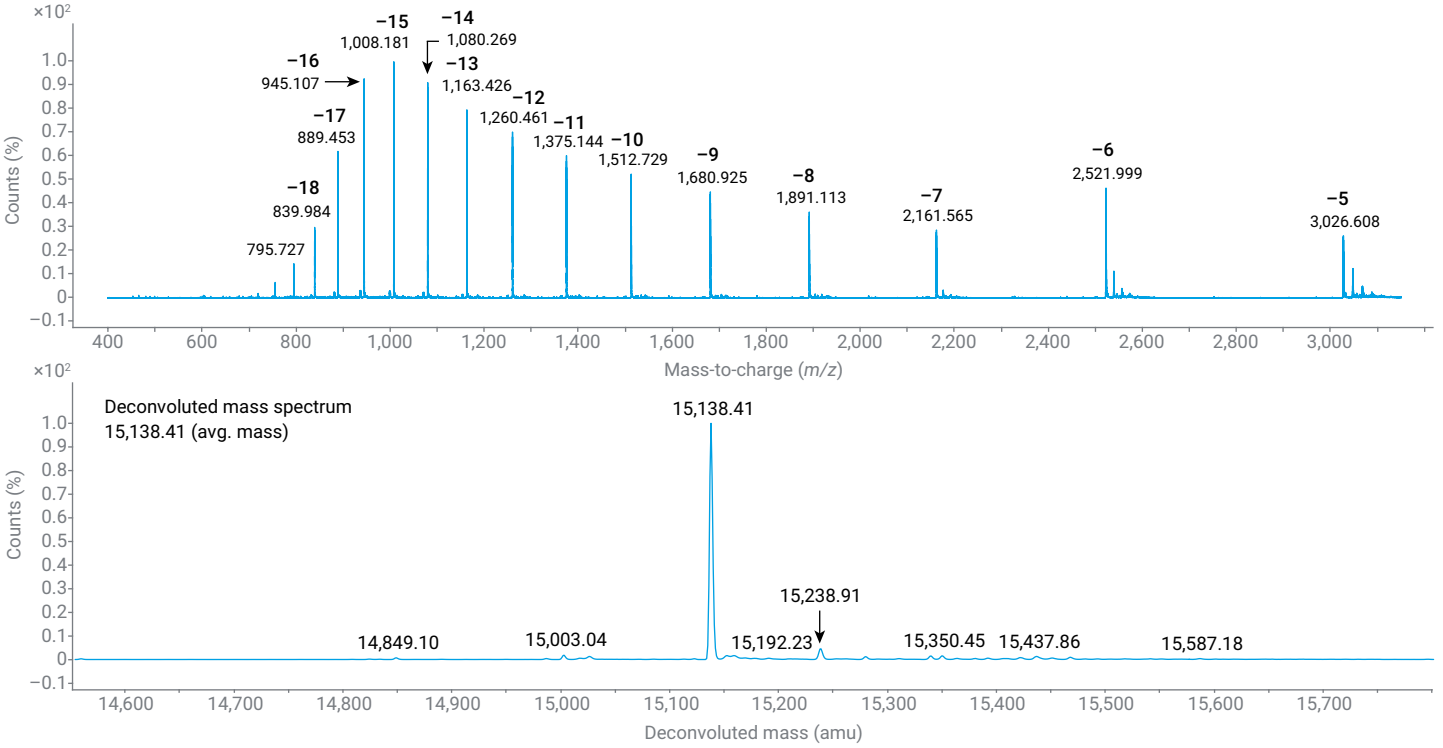

Figure 6B. Mass spectrum (FBF) and deconvoluted mass spectra of 50 mer DNA by ion-pair reversed-phase (0.21 mL/min).

The third oligonucleotide tested comprised a mixture of deoxy- as well as ribonucleotide bases. In addition, each phosphate group was thiolated which causes the formation of enantiomeric species.

A fully thiolated oligonucleotide containing twenty nucleotides has 19 phosphorothioate groups and 2^{19} diastereoisomers. This leads to a much broader peak compared to a nonthiolated molecule (Figure 7A), but each diastereomer has the same mass so the mass spectrum and deconvoluted mass is not made more complicated.

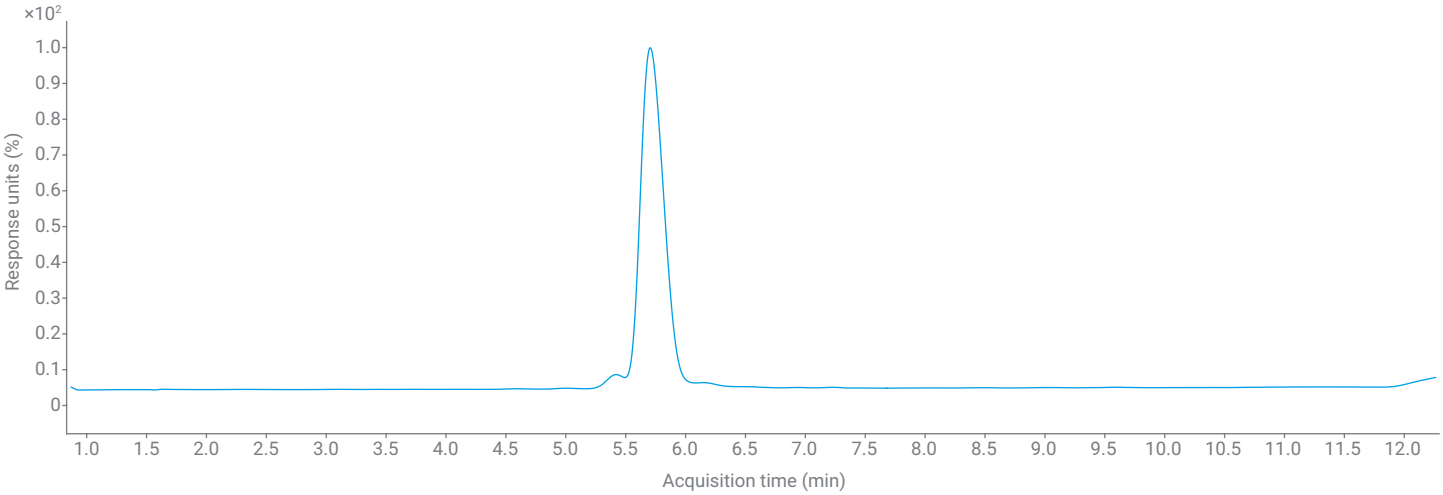

Figure 7A. UV chromatogram of fully thiolated hybrid by ion-pair reversed-phase (0.21 mL/min).

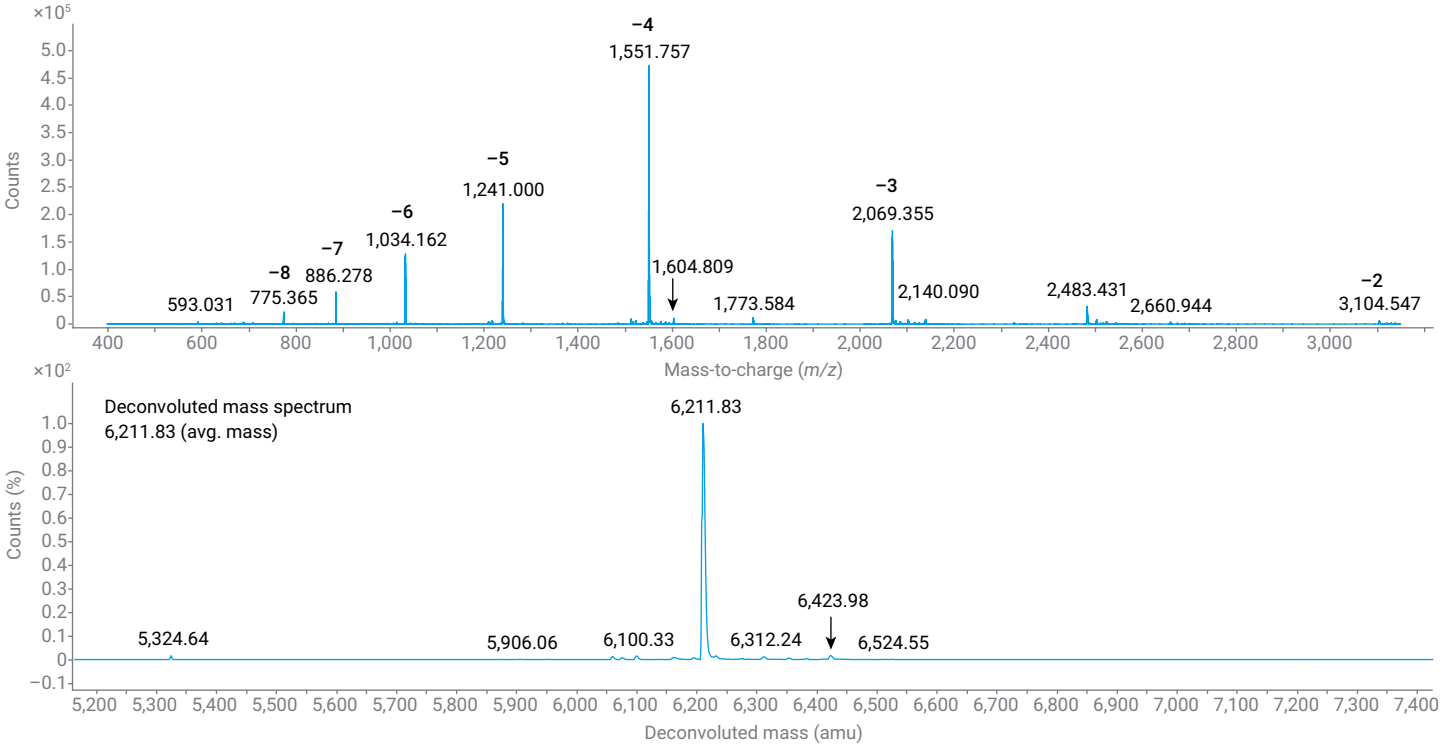

Figure 7B. Mass spectrum (FBF) and deconvoluted mass spectra of fully thiolated hybrid by ion-pair reversed-phase (0.21 mL/min).

Following the ion-pair reversed-phase analysis, the same samples were subjected to analysis using the same column but under ion pair-free conditions.

Instead of using triethylamine/HFIP, the mobile phase comprised a dilute ammonium bicarbonate buffer. It was still necessary to use methanol as the organic modifier to get sufficient retention on the column.

Several blank gradients were applied to remove all traces of ion pair reagent used previously before performing the analysis.

There may be advantages to using ion pair-free conditions in some situations, particularly if it is desirable to avoid using HFIP.

Under ion pair-free conditions, the ionic species detected are positively charged rather than negatively charged for better sensitivity. This causes a slightly different charge state envelope as shown in Figure 8.

In addition, the level of adducts observed is reduced (compared to the higher flow rate ion-pair reversed-phase method).

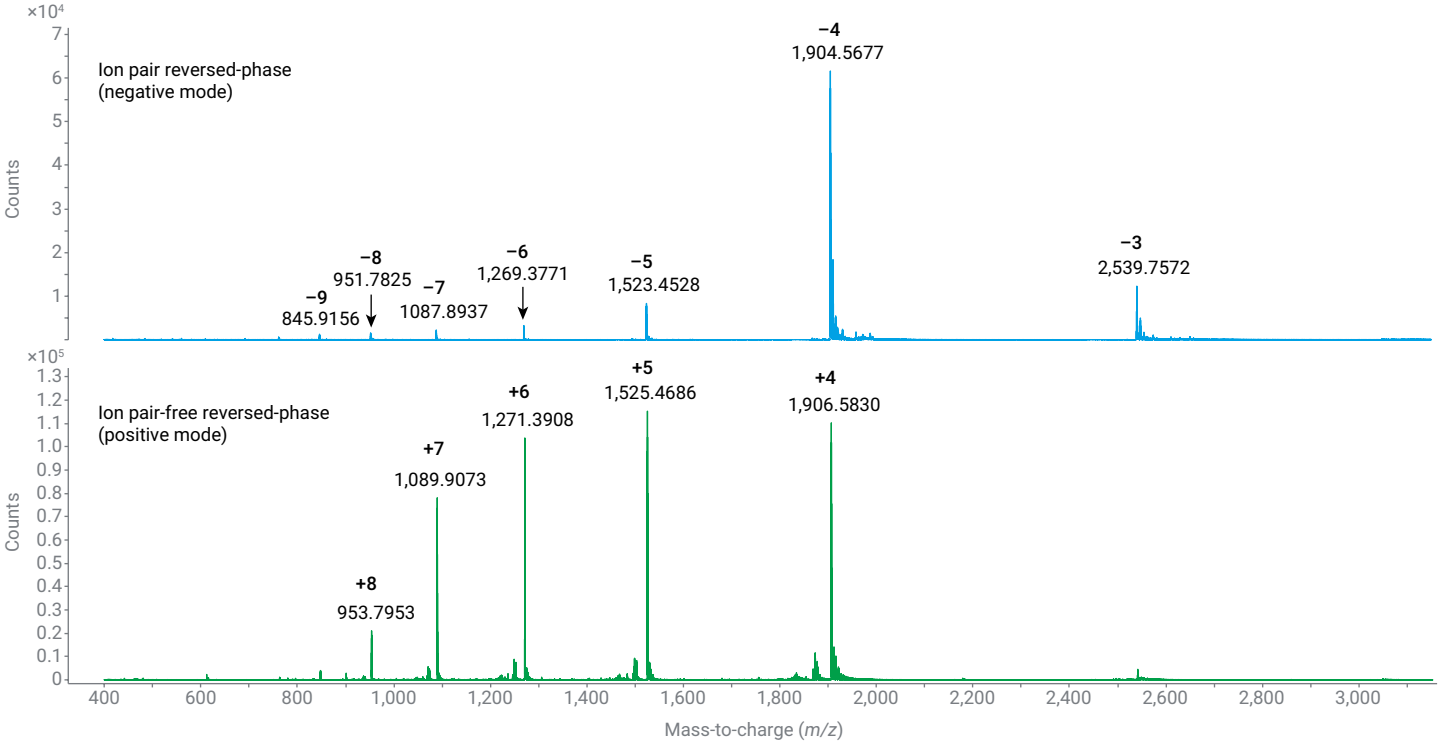

Figure 8. Mass spectra of 25-mer DNA: ion-pair reversed-phase versus ion-pair–free reversed-phase using Altura Oligo HPH-C18 column.

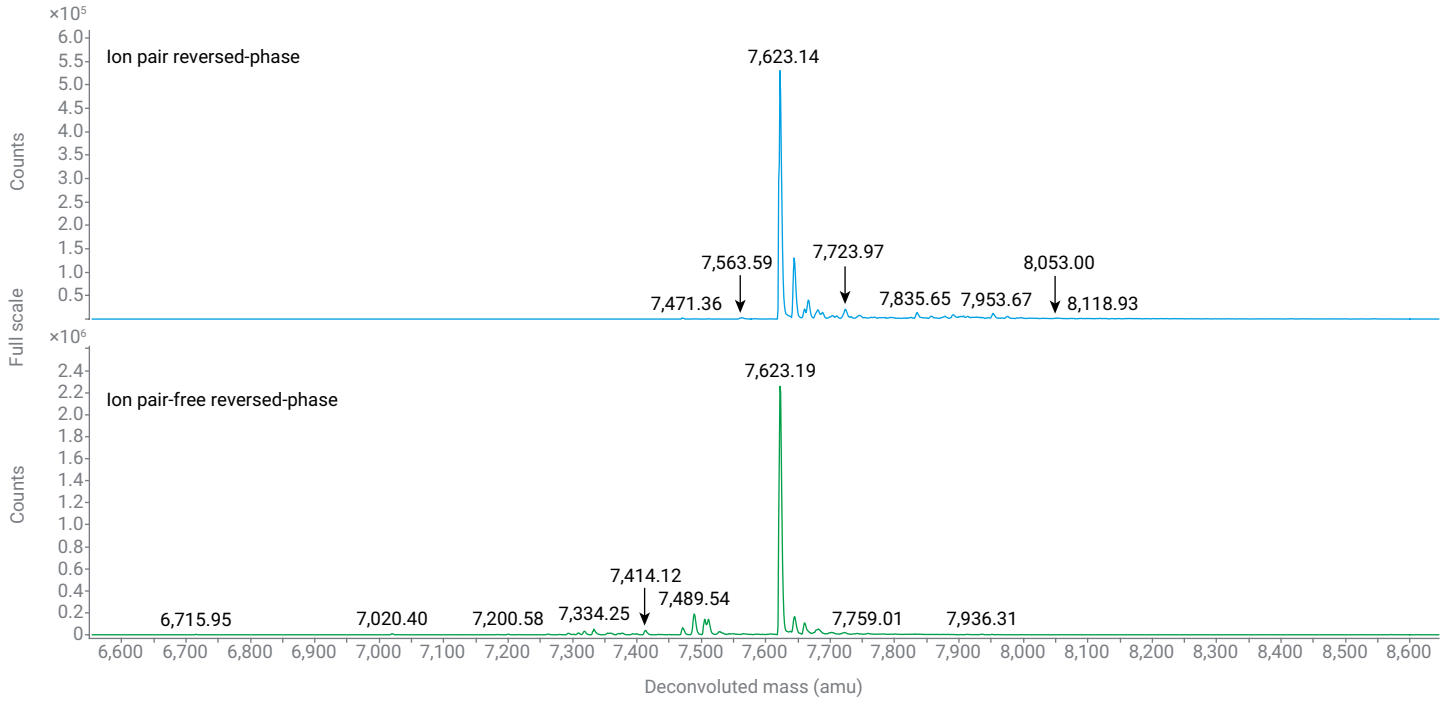

Figure 9. Average mass deconvoluted mass spectra of 25-mer DNA: ion-pair reversed-phase versus ion-pair–free reversed phase using an Altura Oligo HPH-C18 column.

The same behavior was observed with the fully thiolated hybrid oligonucleotide too. Increased levels of +4, +5, +6, +7, and +8 charge states were seen (Figure 10), with reduced levels of adducts observed.

As shown in Figure 11, the deconvoluted mass values were in close agreement with each other (taking into account the different negative and positive mode MS techniques employed).

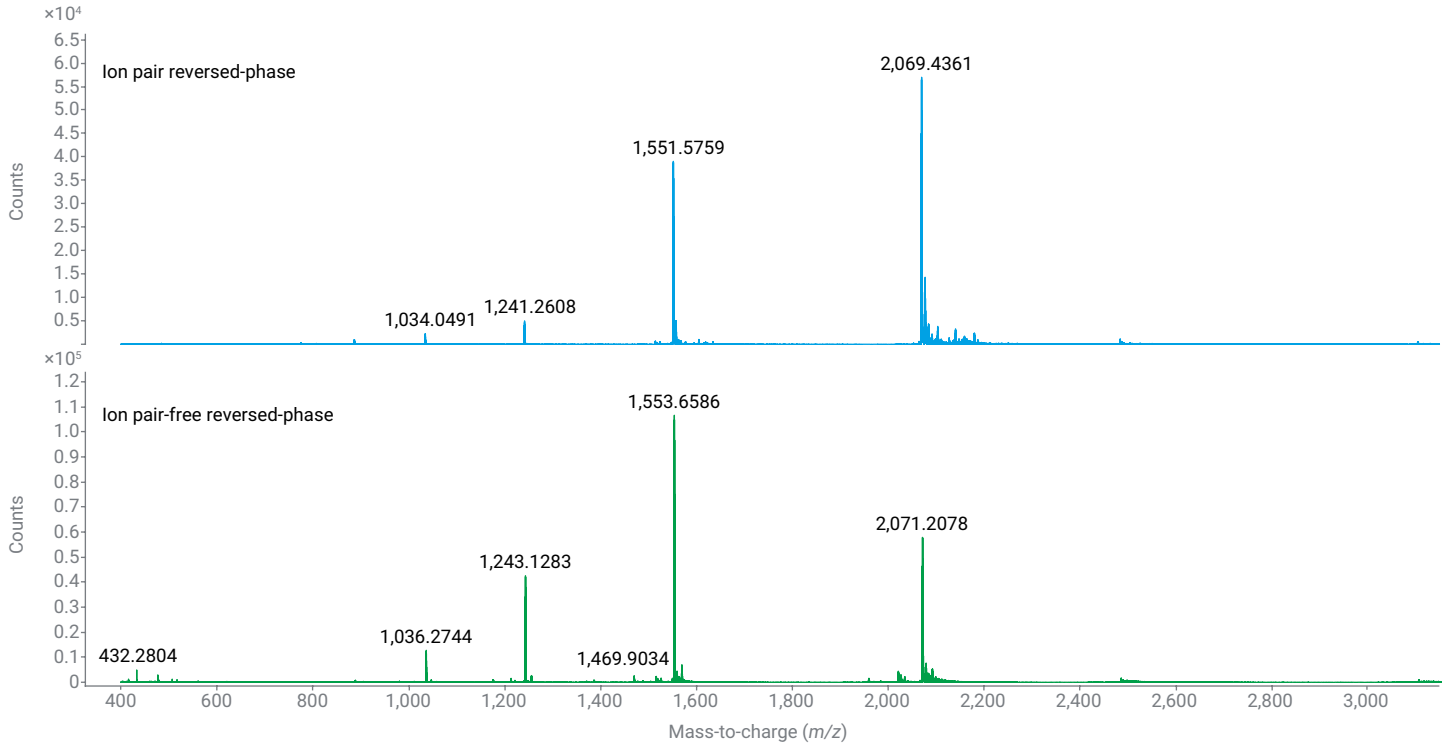

Figure 10. Mass spectra of fully thiolated hybrid: ion-pair reversed-phase versus ion-pair-free reversed-phase using an Altura Oligo HPH-C18 column.

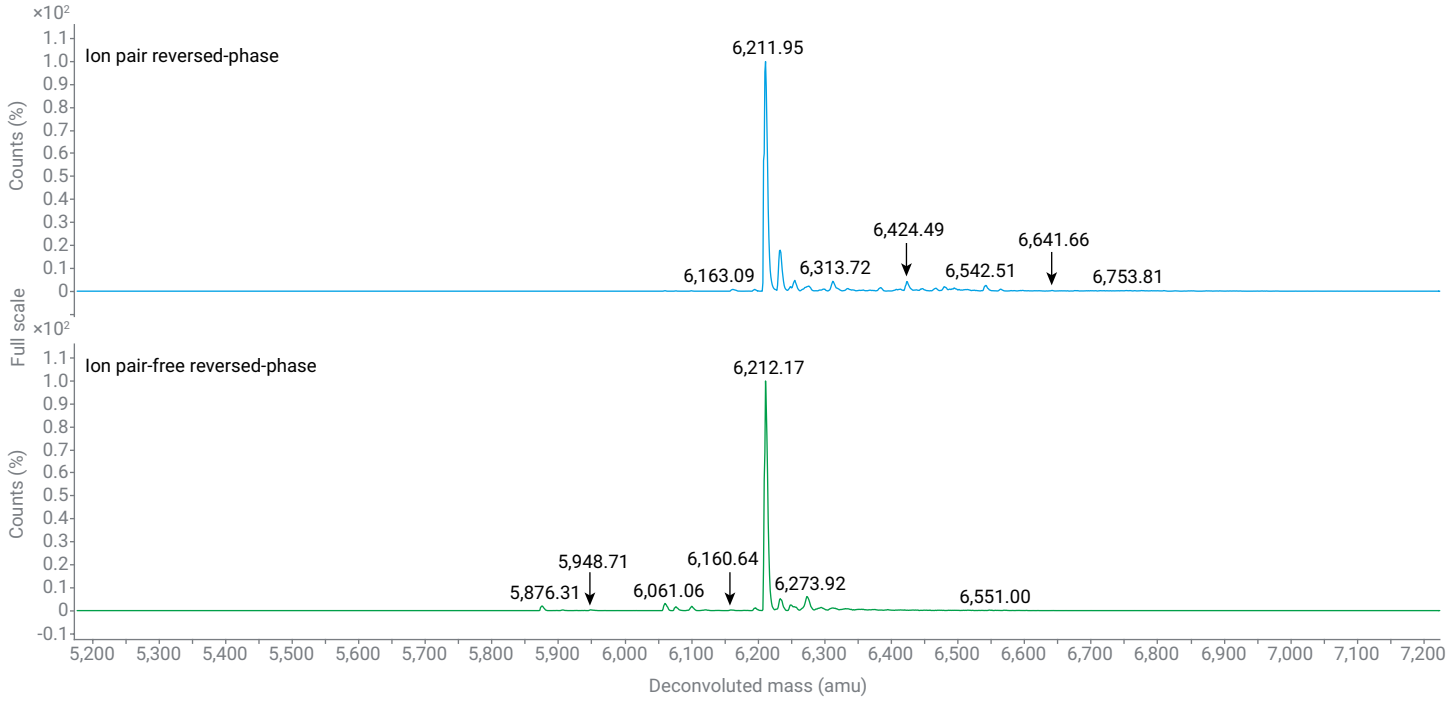

Figure 11. Average mass deconvoluted mass spectra of fully thiolated hybrid: ion-pair reversed-phase versus ion pair-free reversed-phase using an Altura Oligo HPH-C18 column.

Conclusion

In this application note we demonstrated that improved oligonucleotide peak shape and area can be achieved by switching from a stainless steel HPLC column to an Agilent Ultra Inert Altura Oligo HPH-C18 column. Furthermore, attention to other experimental details can further reduce the impact of alkali metal adducts. The monoisotopic Find by Formula results are summarized in Table 2, clearly indicating that improved MS accuracy is also ensured with Agilent Ultra Inert Altura Oligo HPH-C18 columns.

References

1. Vanhoenacker, G.; *et al.* Evaluation of Different Ion-Pairing Reagents for LC/UV and LC/MS Analysis of Oligonucleotides, *Agilent Technologies application note*, publication number 5994-2957EN, **2024**.
2. Bertram, L.; Hsiao, J. Analysis of Oligonucleotides Using an Ion-Pairing-Free Reversed-Phase Method with TOF LC/MS, *Agilent Technologies application note*, publication number 5994-8013EN, **2024**.
3. Hsiao, J.; Bertram, L.; *et al.* Evaluating HILIC Stationary Phases for Oligonucleotide Separation by LC/MS, *Agilent Technologies application note*, publication number 5994-8180EN, **2025**.

Table 2. Summary of MS results comparing different columns and techniques

Ion pair reversed-phase (hexylamine/HFIP/methanol)

	Monoisotopic Mass	Altura Oligo HPH-C18		AdvanceBio Oligonucleotide (SS)	
		Mass Observed	Error (ppm)	Mass Observed	Error (ppm)
25 mer DNA	7,619.2874	7,619.2917	0.56	7,619.2835	−0.51
50 mer DNA	15,131.5533	15,131.5521	−0.08	1,5131.5427	−0.70
Fully Thiolated ON	6,207.5974	6,207.5629	−5.56	6,207.2018	−63.72
Fully Thiolated ON (Lower Flow Rate)	6,207.5974	6,207.5760	−3.52		

Ion pair-free reversed-phase (ammonium bicarbonate/acetonitrile)

	Monoisotopic Mass	Altura Oligo HPH-C18 (IP FREE)	
		Mass Observed	Error (ppm)
25 mer DNA	7,619.2874	7,619.2927	0.69
50 mer DNA	15,131.5533	15,131.5605	0.48
Fully Thiolated ON	6,207.5974	6,207.5988	0.22

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