Application Note

Agilent
Trusted Answers

High-throughput, Ion-Pairing-Free, HILIC Analysis of Oligonucleotides Using Agilent RapidFire Coupled to Quadrupole Time-of-Flight Mass Spectrometry

Author

Peter Rye, PhD
Agilent Technologies, Inc.

Abstract

This application note describes a high-throughput, ion-pairing-free method for oligonucleotide characterization using the Agilent RapidFire high-throughput MS system. The HILIC-based method achieves a 12-second cycle time, and demonstrates high robustness and reproducibility. Results include the identification of impurities less than 0.5% of the target, detection limits in the single-digit nanomolar range, and a linear concentration response over more than three decades. Analysis of nine unique oligonucleotides, comprising both unmodified and heavily modified components, illustrates that the method is highly versatile for samples with unique chemistries.

Introduction

LC/MS methods for the analysis of oligonucleotides (oligos) have traditionally been based on ion-pairing reverse-phase (IPRP) chromatography, because this approach generally delivers good separation and MS response in negative mode. However, considering that many ion-pairing reagents can present a memory effect which can diminish the performance of the system in positive mode, IPRP methods can be burdensome for mixed-use systems, pushing many laboratories to seek ion-pairing-free alternatives. In this work, a high-throughput, ion-pairing-free method for oligo characterization using an Agilent RapidFire 6545XT MS system is presented. This method leverages the Agilent HILIC-Z resin and MS-friendly, ammonium acetate-based mobile phases, which allow for subsequent positive mode use of the system without flushing or hardware changes. The method achieves a 12-second cycle time, along with the robustness, reproducibility, dynamic range, and sensitivity that are sought after for high-quality oligo characterization. Tests also demonstrate that the method is equally effective for unmodified and heavily modified oligos, including antisense (ASO) and aptamer samples.

Experimental

Analytical methods and samples

The RapidFire/Q-TOF instrument consists of an Agilent RapidFire 365 high-throughput MS system coupled to an Agilent 6545XT AdvanceBio LC/Q-TOF equipped with an Agilent Jet Stream source. A HILIC cartridge (type H6, 4 µL bed volume, G9527) was used for online solid phase extraction. Data acquisition was performed with RapidFire Acquisition software, version 6.1, and MassHunter acquisition software for LC/MS systems, version 10.1. The RapidFire and MS methods used for this study are detailed in Table 1. LC/MS grade acetonitrile was sourced from Agilent. Water was sourced from a Milli-Q system. Mobile phase A (MPA) and mobile phase B (MPB) were prepared without any pH adjustments. All injection volumes in this study were 10 µL.

Following sampling by the RapidFire, samples were delivered to the cartridge and desalted using MPA at 1 mL/min for 5,000 ms (20 cartridge volumes of wash). The desalted oligo mixture was then eluted to the MS for measurement using MPB at 0.5 mL/min for 4,000 ms (eight cartridge volumes). The resulting chromatographic peaks were approximately 6 seconds wide and composed of 24 unique spectra.

The cartridge was then re-equilibrated with MPA at 1 mL/min for 500 ms (two cartridge volumes) before introduction of the next sample. There was insignificant benefit to longer load/wash, elute, or re-equilibration times (data not shown). The optimized method, including plate movements, sustained a 12-second cycle time.

Following acquisition, the MS data files were automatically parsed by the RapidFire software into individual injection files. Extracted ion chromatogram and Maximum Entropy deconvolution techniques were used in MassHunter BioConfirm software, version 10.0, for analysis.

Table 1. RapidFire and 6545XT MS methods used in this study.

RapidFire Conditions		
Cartridge	HILIC (PN G9527)	
Cartridge Temperature	Room temperature	
Injection Volume	10 µL	
Pump 1	MPA = 85% acetonitrile + 15 mM ammonium acetate	1.0 mL/min
Pump 2	MPB = 50% acetonitrile + 15 mM ammonium acetate	1.25 mL/min
Pump 3	MPB = 50% acetonitrile + 15 mM ammonium acetate	0.5 mL/min
State 1	Aspirate sample (sip sensor on)	600 ms
State 2	Load/wash (desalt)	5,000 ms
State 3	Extra wash	0 ms
State 4	Elute (inject)	4,000 ms
State 5	Reequilibrate	500 ms
6545XT Q-TOF Conditions		
Ion Polarity	Dual AJS Negative	
Data Storage	Both (Centroid and Profile)	
Gas Temperature	300 °C	
Drying Gas Flow	11 L/min	
Nebulizer Gas	35 psi	
Sheath Gas Temperature	350 °C	
Sheath Gas Flow	11 L/min	
Capillary Voltage	3,500 V	
Nozzle Voltage	2,000 V	
Fragmentor	175 V	
Skimmer	65 V	
Oct 1 RF Vpp	750 V	
Mass Range	100 to 3,200 *m/z*	
Acquisition Rate	4 spectra/sec	

Oligos used in this study (Table 2) were purchased from Integrated DNA Technologies (Coralville, Iowa) with standard desalting purification. Products were resuspended in water to make 1 mM stocks and diluted in MPA for analysis. See the individual experimental sections for the final concentrations used.

Results and discussion

Oligos of different sizes

To assess the applicability of the HILIC RapidFire/Q-TOF method to oligos of different sizes, an 18-mer (PR8), a 40-mer (PRL40), and a 60-mer (PRL60) were analyzed. A 10 µM sample (100 pmol on cartridge) of each oligo was analyzed, and results were compared to data previously collected using the IPRP technique.[1] Figure 1 shows ions for several expected charge states which were observed in the HILIC data (A) and the IPRP data (B) for each sample. Furthermore, several unique spectral qualities were observed in the RapidFire data.

First, the IPRP conditions resulted in a much wider charge state envelope for each oligo. In some cases, a bimodal distribution was observed; this is best exemplified by the 40-mer data in Figure 1B. These wide, bimodal distributions are thought to stem from portions of the oligo remaining in native conformation (leading to lower-charged species), while other portions are in a denatured conformation (facilitating formation of the higher-charged species). Spectra collected under HILIC conditions showed a much narrower charge state distribution shifted towards the less-charged species, suggesting that these oligos were maintained in their native state. This behavior would be consistent with observations from other mass spectrometry techniques, for example, analysis of native proteins in which ammonium acetate is commonly used.

Second, the IPRP conditions resulted in the larger oligos showing more charges than their smaller counterparts, resulting in a relatively consistent *m/z* range for spectral ions from oligos of different sizes. In fact, the most abundant charge state for the 18-mer (−4 at *m/z* ~1,375) had a higher *m/z* value than the most abundant charge state for the 60-mer (−19 at *m/z* ~970). In the case of the HILIC conditions, the *m/z* value for the most predominant charge state of each oligo trended higher as the oligo size increased. Again, this result is consistent with the HILIC conditions preserving a native folded state of the oligo, and charge-charge repulsion deterring the formation of higher-charged species. Strategies to mitigate this effect are required for the analysis of larger oligos on mass spectrometers with limiting *m/z* range. These studies are underway and will be described elsewhere.

Table 2. Oligonucleotides used in this study and their associated code notations. All sequences are written in the 5' to 3' orientation.

Name	Length	Approx. Molecular Weight	Sequence
PR1	20	6148	AGAGTTTGATCCTGGCTCAG
PR3	20	6007, 6031, 6047	TTTTTTTTTTTTTTTTTTTV
PR5	24	7289	CGCCAGGGTTTTCCCAGTCACGAC
PR7	21	6101	/5Phos/TTTTTTTTTTTTTTTTTTTT
PR8	18	5505	CTAGTTATTGCTCAGCGG
PRL40	40	12278	CTAGTTACTTGCTCAGCGGACTAGTTACTTGCTCAGCGGA
PRL60	60	18448	CTAGTTACTTGCTCAGCGGACTAGTTACTTGCTCAGCGGACTAGTTACTTGCTCA GCGGA
ASO	18	7127	/52MOErT/*/i2MOErC/*/i2MOErA/*/i2MOErC/*/i2MOErT/*/i2MOErT/* /i2MOErT/*/i2MOErC/*/i2MOErA/*/i2MOErT/*/i2MOErA/*/i2MOErA/* /i2MOErT/*/i2MOErG/*/i2MOErC/*/i2MOErT/*/i2MOErG/*/32MOErG/
Aptamer	28	9116	/52FC/mGmGrArA/i2FU//i2FC/mAmG/i2FU/mGmAmA/i2FU/mG/i2FC// i2FU//i2FU/mA/i2FU/mA/i2FC/mA/i2FU//i2FC//i2FC/mG/3InvdT/

Code	Description
*	Phosphorothioate bond
A	2'-deoxyribose adenine
C	2'-deoxyribose cytosine
G	2'-deoxyribose guanine
T	2'-deoxyribose thymine
mA	2'-O-methyl A
mG	2'-O-methyl G
rA	Ribose adenine
rG	Ribose guanosine
V	Mixed C, A, and G
/3InvdT/	3' inverted T

Code	Description
/32MOErG/	3' methoxyethoxy G
/5Phos/	5' phosphate
/52FC/	5' Fluoro C
/52MOErT/	5' 2-methoxyethoxy T
/i2FC/	Internal Fluoro C
/i2FU/	Internal Fluoro U
/i2MOErA/	Internal 2-methoxyethoxy A
/i2MOErC/	Internal 2-methoxyethoxy C
/i2MOErT/	Internal 2-methoxyethoxy T
/i2MOErG/	Internal 2-methoxyethoxy G

To compare the MS signal intensities for oligos of different sizes, scaling was removed by linking their Y-axes. Figure 2A shows that the HILIC conditions resulted in a significant drop in *m/z* ion intensities as the size of the oligo increased. More specifically, the height of the most predominant charge state for the 60-mer was approximately 25-fold less than that of the 18-mer. Similarly, comparison of the deconvolution peak heights (Figure 2B) shows an approximate 25-fold drop from the 18 to 60-mer. By comparison, the deconvolution peak heights for the 18-, 40-, and 60-mer, when run by IPRP, were within 2-fold of the lowest (data not shown).

These general observations have been made before. Specifically, Lobue *et al.* previously demonstrated that, in comparison to IPRP, HILIC analyses of oligos can result in (1) a narrower charge state distribution, (2) a most predominant charge state of lower charge, and (3) a right-shifting of the most predominant charge state as the oligo size increases.

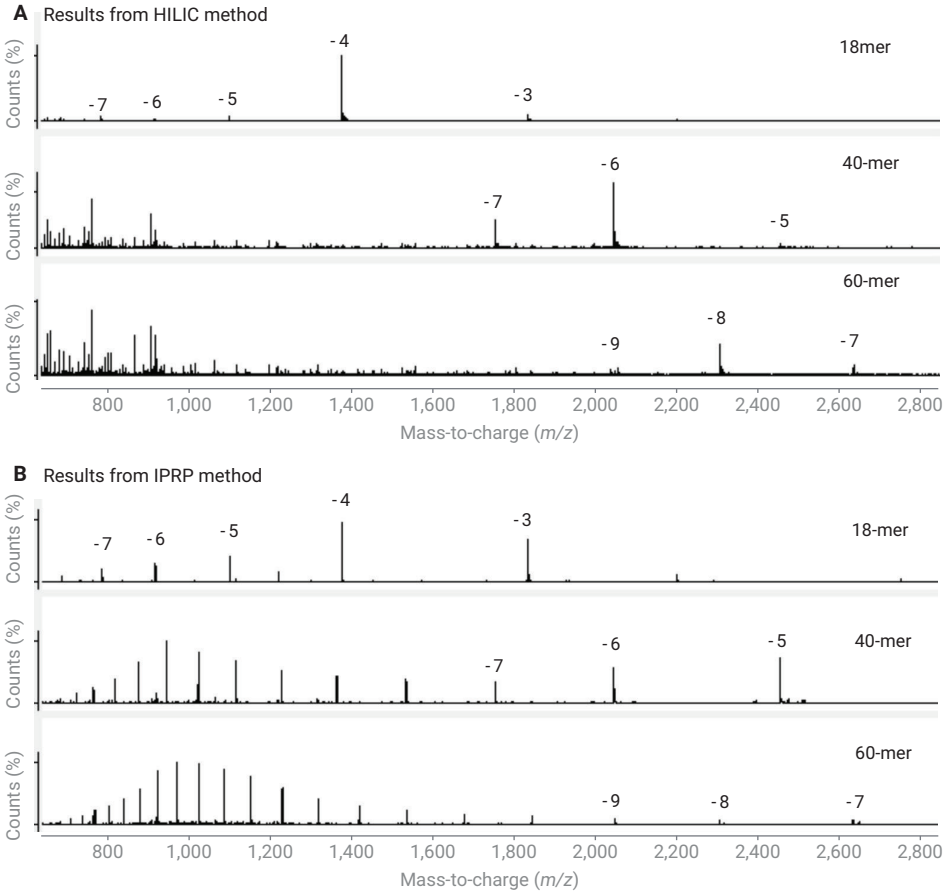

Figure 1. Raw *m/z* spectra for an 18-mer (top), a 40mer (middle), and a 60-mer (bottom) run by HILIC method (A) and, for comparison, an IPRP method (B). All spectra were each scaled to the largest peak within it. The predominant charge state clusters for HILIC method are labeled for each oligo.

Reproducibility

To test reproducibility of the HILIC RapidFire/Q-TOF method, 24 replicate injections of a poly-dT oligo with a 5' phosphate (PR7) were run and deconvoluted using an automated analysis method in BioConfirm. The resulting deconvolution spectra were then scaled to the largest peak in each spectrum and overlaid. The results demonstrate excellent reproducibility of the relative abundances within each sample, as the 24 spectra are superimposed near-perfectly (Figure 3). The total ion chromatograms for the replicates (Figure 3 insert) reveal consistent peak height and shape, illustrating that the absolute MS signals, in addition to the relative signals, are stable across many injections.

Determination of impurities

Oligo samples often contain a high number of low-abundance impurities, including truncated synthesis products, depyrimidations, and depurinations. It is therefore critical that analytical methods for oligo characterization demonstrate a wide dynamic range for impurity detection. This situation can be especially true for nonchromatographic methods, because the calculated purity can be overestimated if low abundance impurities are not detected in the presence of the highly abundant target oligo.

The 6545XT mass spectrometer used in this study was established to provide up to five orders of spectral dynamic range. Still, the dynamic range for this application was evaluated by comparing the relative deconvoluted peak heights of vastly different intensities for several samples.

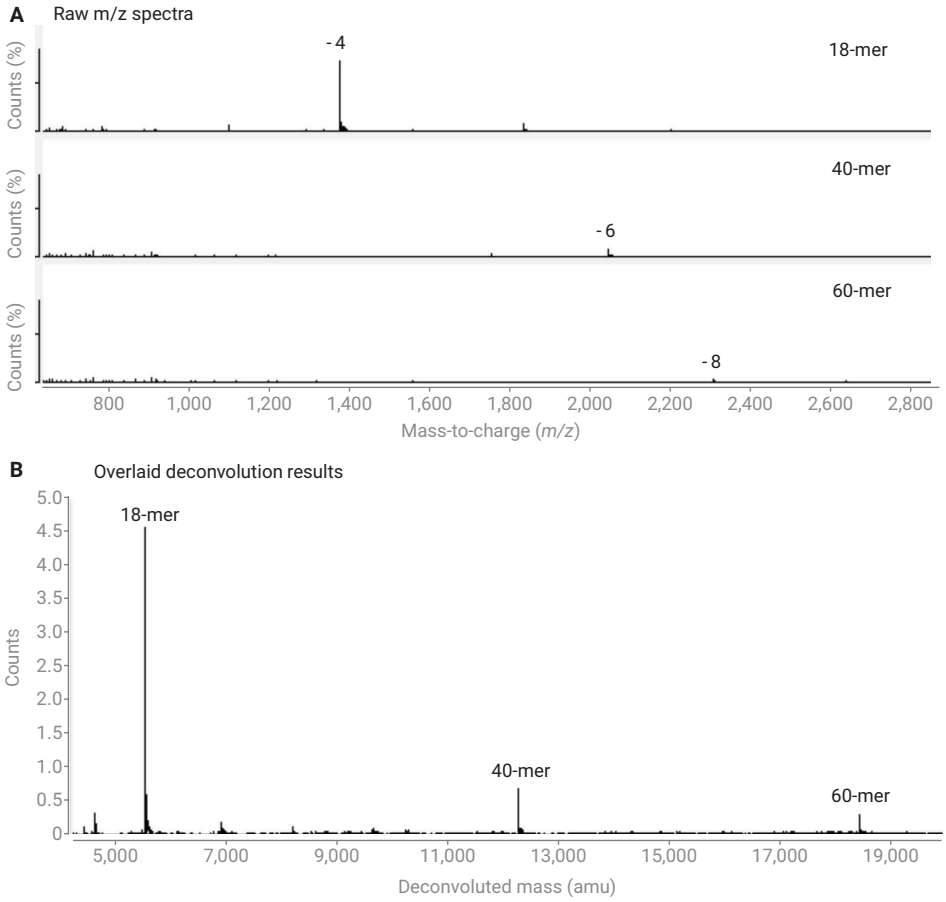

Figure 2. Comparison of MS signal intensities for an 18-mer, a 40-mer, and a 60-mer. Raw *m/z* spectra with linked Y-axis (A) and overlaid deconvolution results (B).

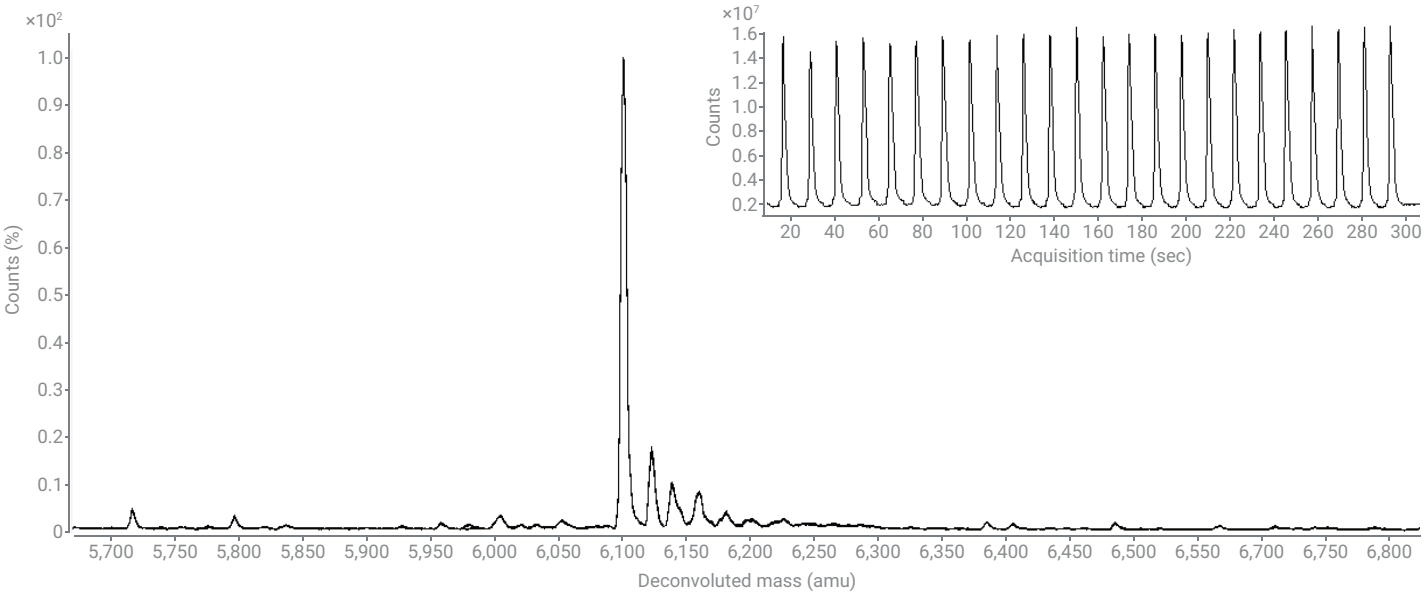

Figure 3. Reproducibility of total ion chromatogram (inset) and deconvolution results (main figure) for 24 replicate samples.

Figure 4 shows the deconvolution spectra from a 10 µM injection (100 pmol on cartridge) of a 20-mer DNA strand (PR1). The most abundant peak has a mass of 6,148 Da, consistent with the calculated mass of the target oligo. Several commonly observed metal adducts are present at masses larger than the target, and a large number of lower mass impurities are also observed. Close inspection of the mass ranges where commonly observed depurination (depur) and truncation (trunc) impurities were expected revealed several low-abundance peaks. Based on their mass differences from the target, 5' truncation of A, gas phase depurination of G, and hydrolytic depurination of G could all be assigned. The peak heights of the depurination impurities had a relative abundance of less than 1% of the target.

In some cases, it is necessary to analyze oligo mixtures containing individual components that are close in mass. To evaluate the ability of the HILIC RapidFire/Q-TOF method to mass resolve mixtures and their respective impurities of the components, 10 µM (100 pmol on cartridge) of a 20-mer poly-dT oligo containing a 3' variable base (C, A, or G) was injected (PR3). The *m/z* spectrum shown in Figure 5A illustrates multiple expected charge states, and the inset figure reveals good mass resolution of the isotopes for the –4 species. The deconvoluted result shown in Figure 5B clearly shows three predominant peaks that match the expected masses and relative abundances of the oligo with either C, A, or G on the 3' end. Moreover, for each of these species, the n-1 and n-2 impurities were observed. Loss of the 5'-T from the three species resulted in peaks of minus 304 Da, and loss of the 5'-TT from the three species resulted in peaks of minus 608 Da (304 + 304). For the oligo with a 3' G, comparison of the

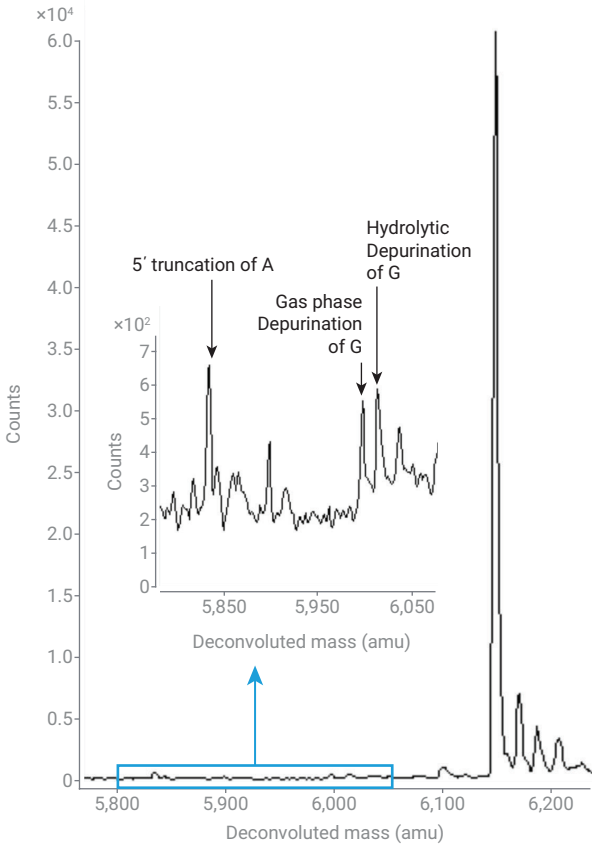

Figure 4. Identification of impurities in PR1.

peak heights corresponding to the target (2.5E5 counts) and loss of 5'-TT impurity (0.75E3 counts) demonstrates that 0.3% impurities are readily observed, despite the sample and spectral complexity.

Another noteworthy observation from this experiment was that the spectrum for the poly-dT oligo had a relatively wide distribution of charge states. Based on observations and discussion above, it appears poly-dT oligos do not readily adopt secondary structures that would otherwise reduce the charge states observed in the *m/z* spectrum.

Method sensitivity, linearity, and carryover

To evaluate the sensitivity and linearity of the HILIC RapidFire/Q-TOF method, triplicate injections for eight concentrations, plus a zero, of PR7 were analyzed. Two-fold serial dilutions starting at 1,250 nM were made down to 9.7 nM using MPA. A zero-concentration sample was injected between each replicate so that carryover could be studied at each concentration over the range. The resulting data for all 54 injections were analyzed two ways. First, for the targeted MS measurement, the extracted ion chromatogram for the –4 charge state (*m/z* ~1,524) was generated, smoothed, and integrated. The replicate areas for each concentration were averaged and plotted against their concentration. The standard deviations of the values were represented by error bars on that same plot, shown in Figure 6A and 6B. Second, for the untargeted deconvolution results shown in Figure 6C, the extracted ion chromatogram for the –4 charge state

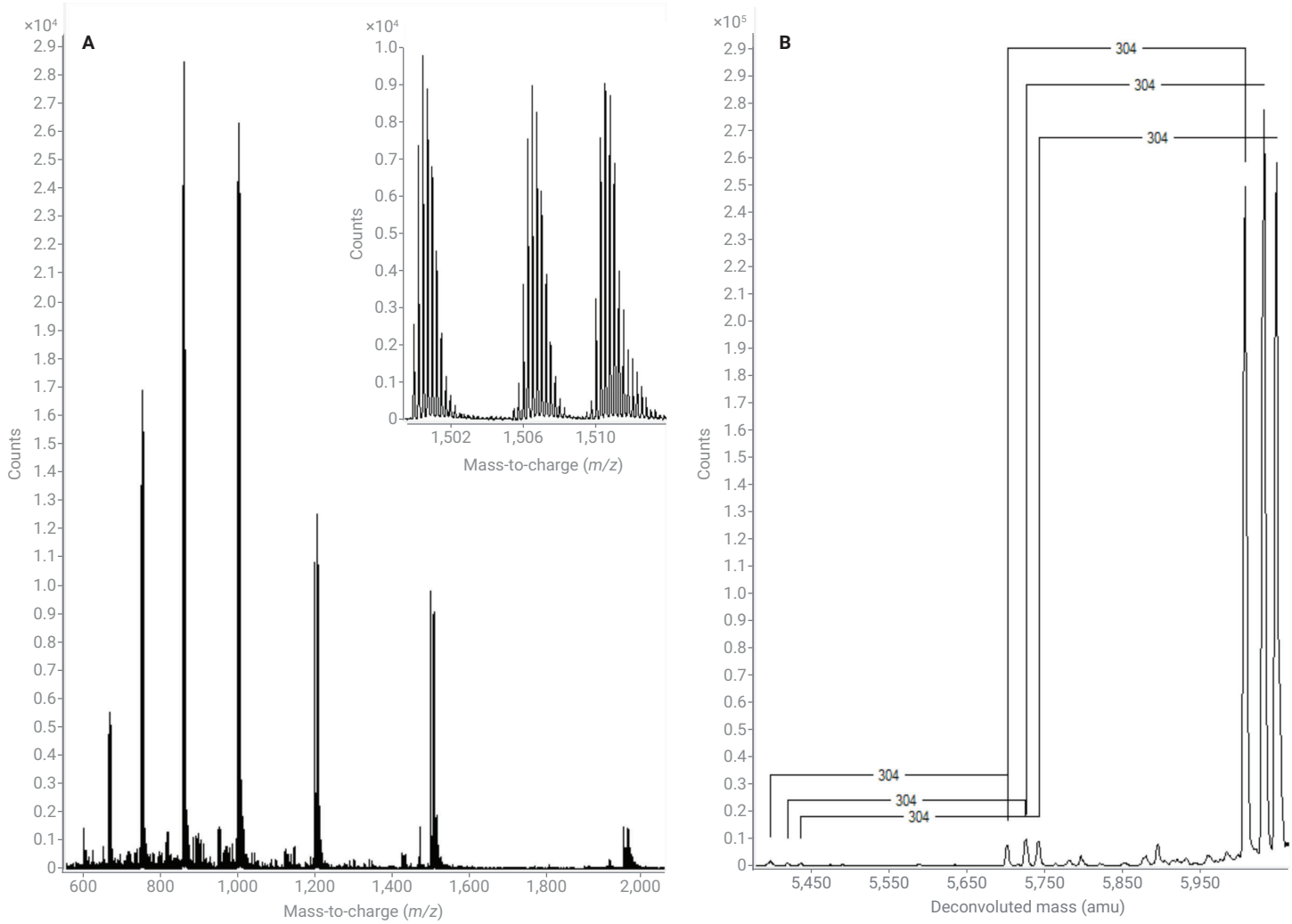

Figure 5. Identification of low abundance impurities in PR3 . Raw *m/z* spectra (A) and deconvolution results (B).

(*m/z* ~1,524) was generated, smoothed, and integrated. The average *m/z* spectra over the integrated peak was then extracted and deconvoluted.

Figure 6A shows that the oligo used in this study had a linear response over the nine concentrations studied, with an R^2 = 0.9988 for the best fit line. The blanks data shows a slope of ~2.8, versus ~192 for the samples, revealing less than 1.5% carryover across the concentration range. In subsequent experiments (data not shown), this value dropped to below 0.1% when the "blank injection in between each sample" feature of the RapidFire was selected.

However, because blanks between each sample double the cycle time, and the carryover without them satisfied the acceptance criteria, the additional blanks were deemed unnecessary. Focusing on the low end of the concentration data (Figure 6B), a clear difference can be seen in the AUC between the 0 and 9.7 nM concentrations. The signal-to-noise ratio was over 4 at 9.7 nM, almost 6 at 19.5 nM, and 28 at 39 nM. While the slope of the concentration response was much greater for IPRP conditions (2,898, data not shown) the signal-to-noise values were nearly identical to those from the HILIC conditions.

To test the limitations of measuring the target oligo in an untargeted fashion, the spectra for each concentration were deconvoluted. Representative results for the low concentration injections are shown in Figure 6C, and easily allow the determination of the target peak from low double-digit nM samples. These results indicate, as expected, that while targeted extraction provides more measurement sensitivity, untargeted deconvolution is still quite powerful for target identification from low concentration samples.

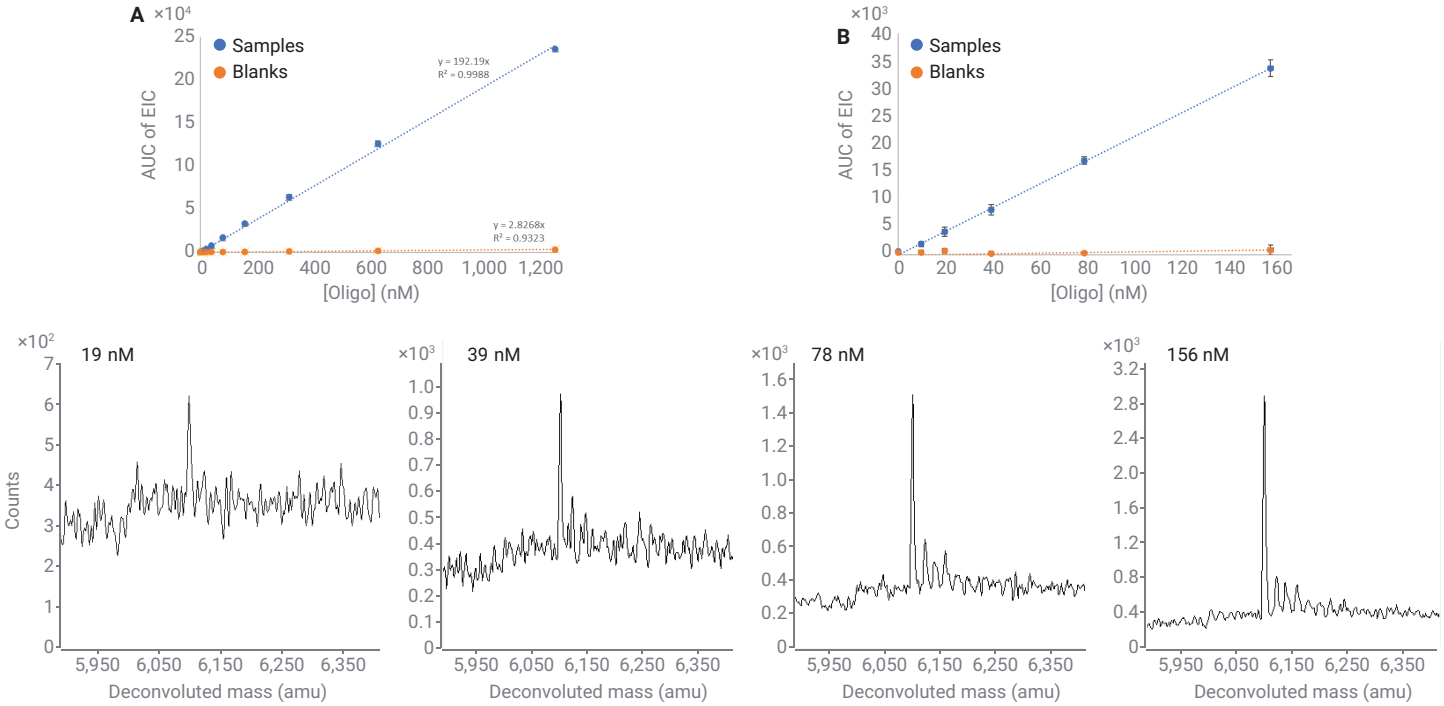

Figure 6. Concentration response of PR7 to evaluate method sensitivity, linearity, and carry over. The plot of signal against concentration (A), a zoom of the low concentration range (B) and deconvolution results (C).

Comparison of the sensitivity from HILIC versus IPRP methods, by others, has resulted in mixed reports. Lobue reported a greater MS signal response from HILIC conditions versus IPRP[2], attributing the gains to the higher organic content of the mobile phase under HILIC conditions, leading to more efficient desolvation. In other cases, less intense target peak heights under HILIC conditions versus IPRP have been blamed on increased levels of Na and K adduct ions. Further investigation is therefore required to compare the sensitivity of these techniques on a multitude of oligo sizes and chemistries, controlling for a wide host of acquisition and analysis parameters which can affect the result.

Method versatility

The chemistry of oligo samples can vary significantly. To evaluate the applicability of the HILIC RapidFire/Q-TOF method to oligos with different base compositions, linker types, and modifications, the data for a host of samples were acquired with the optimized method. These 10 µM samples included DNA strands (containing phosphodiester linkers and 5' phosphates), an ASO (containing phosphorothioate linkers and 2-methoxyethoxy building blocks), and an aptamer (containing inverted T, 2-methoxyethoxy groups, and fluorinated bases). The resulting deconvoluted spectra were each scaled to the largest peak and overlaid with each other. The results shown in Figure 7 reveal highly abundant target peaks, with excellent mass accuracy, for each sample. Common impurities could also be assigned for each sample (data not shown). These results illustrated that the HILIC RapidFire/Q-TOF method can provide high-quality data for a wide range of oligo types and chemistries in the 18- to 28-mer range.

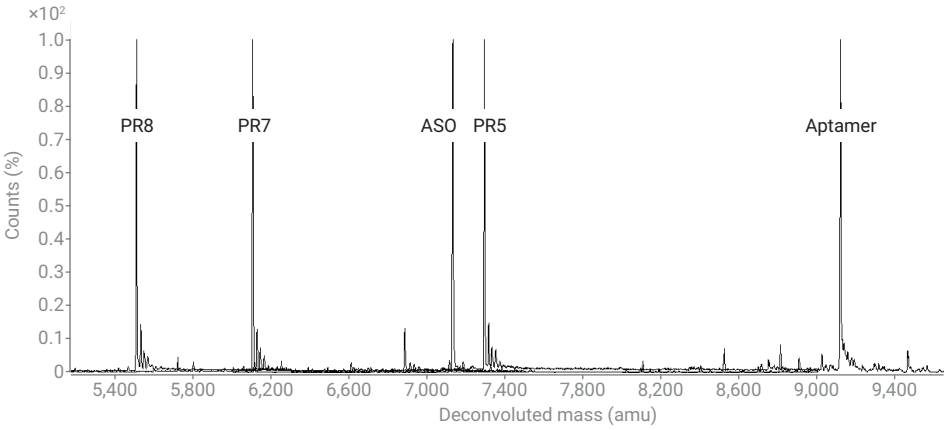

Figure 7. Overlaid deconvolution results for a wide variety of oligo chemistries run by RapidFire MS without ion pairing reagents.

Conclusion

The Agilent RapidFire high-throughput MS system, coupled to an Agilent 6545XT mass spectrometer, offers high-throughput oligo characterization by sustaining cycle times as fast as 12 seconds per sample during data acquisition. Acquisition methods include the previously described IPRP conditions[1], as well as the ion-pair-free HILIC conditions described here. The HILIC method was simple to set up and use, as it used standard Agilent products, and required no pH adjustments to the mobile phases.

The HILIC method displayed the robustness, reproducibility, dynamic range, and sensitivity that are sought after for high-quality oligo characterization. Tests on a variety of oligos illustrated high method performance on highly modified ASO and aptamer samples. Even though HILIC methods are commonly used for oligos approximately 25-mer in size, quality data on up to 60-mers were generated.

References

1. Rye, P. T.; Yang, Y. High-throughput Mass Spectrometry of Synthetic Oligonucleotides: A Comparison of Data from Fast LC and RapidFire Methods. *ASMS* **2020**. TP 434.
2. Lobue, P. A. *et al.* Oligonucleotide Analysis by Hydrophilic Interaction Liquid Chromatography-Mass Spectrometry in the Absence of Ion-Pair Reagents. *J. Chromatogr. A* **2019**, *1595*, 39–48.
3. Huang, M.; Xu, X.; Qiu, H.; Li, N. Analytical characterization of DNA and RNA oligonucleotides by hydrophilic interaction liquid chromatography-tandem mass spectrometry. *J. Chromatogr. A* **2021**, *1648*, 46–2184.

www.agilent.com

DE54527408

Printed in the USA, June 22, 2022
5994-4945EN

Agilent
Trusted Answers