Application Note

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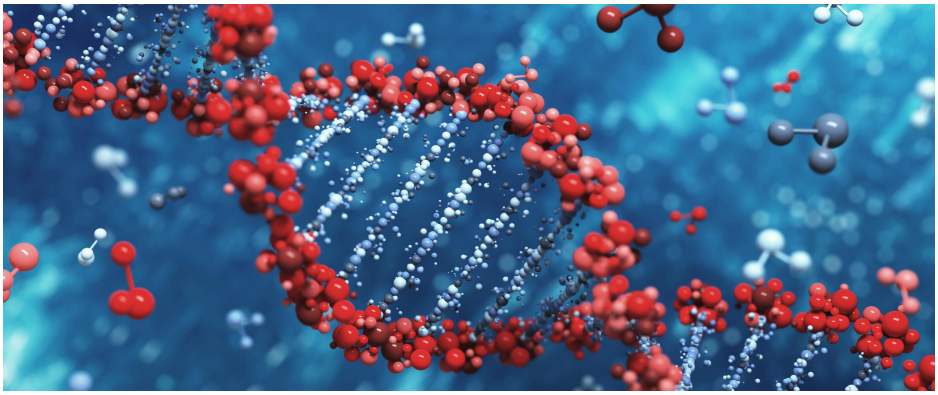
MS1 Oligonucleotide Characterization Using LC/Q-TOF with HILIC Chromatography



Authors

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Introduction

The synthesis of RNA and DNA oligonucleotides (oligos) is an iterative process that, despite highly optimized chemistry, results in impurities that must be characterized for research and quality assurance and quality control (QA/QC) efforts. While many techniques exist for analyzing complex oligo samples, LC/MS offers both efficient chromatographic separation and identification capabilities through high-resolution accurate mass information.

IP-RP chromatography has traditionally been used for LC/MS analysis of oligos for sensitivity and exceptional chromatographic resolution.[1–7] However, these methods often require a dedicated negative mode polarity system, since the memory effect of the ion-pairing reagents can suppress ionization in positive mode and decrease performance in that polarity. For mixed-use systems where positive mode data collection is required, the use of positively charged ion-pairing reagents is often not acceptable.

In this application note, LC separation and MS1 mass identification of a variety of oligos without the use of ion-pairing reagents is demonstrated. The LC separation allows subsequent positive mode use with little to no flushing or hardware changes. This HILIC-based method uses an Agilent InfintyLab Poroshell 120 HILIC-Z column and MS-friendly ammonium acetate-based mobile phases. The samples were analyzed on an Agilent 1290 Infinity II LC system and a 6545XT AdvanceBio quadrupole time-of-flight mass spectrometer (LC/Q-TOF).

Experimental

Instrumentation

- Agilent 1290 Infinity II LC including:
  - Agilent 1290 Infinity II high-speed pump (G7120A)
  - Agilent 1290 Infinity II multisampler (G7167B) with the optional sample cooler (G7167-60005)
  - Agilent 1290 Infinity II multicolumn thermostat (G7116B)
  - Agilent InfintyLab Poroshell 120 HILIC-Z column, 2.1 × 100 mm, 1.9 µm (part number 685675-924)
- Agilent 6545XT AdvanceBio LC/Q-TOF equipped with an Agilent dual spray Jet Stream source

Materials

Oligos used in this study (Table 1) were purchased through Integrated DNA Technologies (Coralville, Iowa) and purified by standard desalting. InfinityLab Ultrapure LC/MS solvents were provided by Agilent Technologies (Santa Clara, CA) and LC/MS-grade ammonium acetate, ammonium hydroxide, and acetic acid were purchased through Sigma-Aldrich (St. Louis, MO).

Sample preparation

Oligo samples were resuspended to 100 µM in water and stored at –80 °C. For analysis, oligo aliquots were diluted to 5 to 10 µM (experiment dependent) in polypropylene vials using mobile phase A (see Table 2) and stored in the chilled autosampler for up to 2 days.

LC/MS analysis

The LC/MS methods used in this study are described in Table 2. Mobile phases were prepared by first adding the desired amount of ammonium acetate to the water component and then adding acetonitrile to the final composition. These solutions were prepared just before acquisition and were used for up to 2 days.

Table 1. Oligos used in this study and their associated code notations. All sequences are written in the 5' to 3' orientation.

Name	Length	Approximate Average Molecular Weight (Da)	Sequence
PRL20	20	6,108	CTAGTTACTTGCTCAGCGGA
PRL40	40	12,278	CTAGTTACTTGCTCAGCGGACTAGTTACTTGCTCAGCGGA
PRL60	60	18,448	CTAGTTACTTGCTCAGCGGACTAGTTACTTGCTCAGCGGACTAGTTACTT-GCTCAGCGGA
PRL80	80	24,617	CTAGTTACTTGCTCAGCGGACTAGTTACTTGCTCAGCGGACTAGTTACTT-GCTCAGCGGACTAGTTACTTGCTCAGCGGA
PRL100	100	30,787	CTAGTTACTTGCTCAGCGGACTAGTTACTTGCTCAGCGGACTAGTTACTT-GCTCAGCGGACTAGTTACTTGCTCAGCGGACTAGTTACTTGCTCAGCGGA
PR1	20	6,148	AGAGTTTGATCCTGGCTCAG
PR2	20	6,103	GGCCACGCGTCGACTAGTAC
PR3	20	6,007, 6,031, 6,047	TTTTTTTTTTTTTTTTTTTV
PR4	38	11,564, 11,588, 11,604	GGCCACGCGTCGACTAGTACTTTTTTTTTTTTTTTTTTV
PR5	24	7,289	CGCCAGGGTTTTCCCAGTCACGAC
PR6	20	6,204	CTTGGGTGGAGAGGCTATTC
PR7	21	6,102	/5Phos/TTTTTTTTTTTTTTTTTTTT
PR8	18	5,505	CTAGTTATTGCTCAGCGG
PR9	23	7,145	CCGGGAGCTGCATGTGTCAGAGG
Aptamer	28	9,116	/52FC/mGmGrArA/i2FU//i2FC/mAmG/i2FU/mGmAmA/i2FU/mG/i2FC//i2FU//i2FU/mA/i2FU/mA/i2FC/mA/i2FU//i2FC//i2FC/mG/3InvdT/
siRNA sense	18	8,111	mGmCrAmGrUmGrUrUrArArUmArUmCrGmCrUmUrUrGrUrGrAmAG
siRNA antisense	18	8,633	rCrUmUrCrArCrArAmArGmCrGmArUrArUrUrArArCrArCmUrGmCmCmU

Code	Description
A	2'-deoxyribose adenine
C	2'-deoxyribose cytosine
G	2'-deoxyribose guanine
T	2'-deoxyribose thymine
mA	2'-O-methyl A
mC	2'-O-methyl C
mG	2'-O-methyl G
mU	2'-O-methyl U
rA	Ribose adenine
rC	Ribose cytosine
rG	Ribose guanosine
rU	Ribose uradine
V	Mixed C, A, and G
/3InvdT/	3' Inverted T
/32MOErG/	3' Methoxyethoxy G
/5Phos/	5' Phosphate
/52FC/	5' 2'-Fluoro C
/52MOErT/	5' 2-Methoxyethoxy T
/i2FC/	Internal 2'-fluoro C
/i2FU/	Internal 2'-fluoro U

Table 2. LC/MS methods used in this study.

Agilent 1290 Infinity II LC Conditions	
Column	InfinityLab Poroshell 120 HILIC-Z, 2.1 × 100 mm, 1.9 µm (p/n 685675-924)
Column Temperature	30 °C
Injection Volume	2 to 5 µL
Autosampler Temperature	4 °C
Needle Wash	Methanol/water 50/50
Mobile Phase	A) 70% acetonitrile : 30% water + 15 mM ammonium acetate B) 30% acetonitrile : 70% water + 15 mM ammonium acetate
Flow Rate	0.4 mL/min
Gradient Program	**Time (min) B (%)** 1.00 15 11.00 40 13.00 60 14.00 15 15.00 15
Stop Time	15.00 min
Post Time	9.00 min

6545 XT AdvanceBio LC/Q-TOF Conditions	
Ion Polarity	Dual AJS Negative
Data Storage	Both (centroid and profile)
Gas Temperature	350 °C
Drying Gas Flow	13 L/min
Nebulizer Gas	35 psi
Sheath Gas Temperature	400 °C
Sheath Gas Flow	12 L/min
Capillary Voltage	4,500 V
Nozzle Voltage	2,000 V
Fragmentor	180 V
Skimmer	65 V
Oct 1 RF Vpp	750 V
Mass Range	400 to 3,200 *m/z*
Acquisition Rate	1 spectra/sec

The gradient consisted of a 1 minute hold at 15% mobile phase B (MPB), then ramping to 40% MPB at 10 minutes, then increasing to 60% at 13 minutes. The gradient was returned to starting conditions at 14 minutes and held for 1 minute. The column was equilibrated for a total of 10 minutes (1 minute in the gradient plus 9 minutes of post time) before the next injection. This 15 minute gradient proved suitable for chromatographic resolution of our oligo ladder (20 to 100-mer range) but could easily be modified based on separation needs.

HILIC separations were performed on a 1290 Infinity II LC system. The column used was an InfinityLab Poroshell 120 HILIC-Z. Mass spectrometric detection was performed on a 6545XT AdvanceBio LC/Q-TOF with a dual spray Jet Stream source. Results were analyzed using Agilent MassHunter BioConfirm software 12.0 and Qualitative Analysis software 11.0.

Results and discussion

Several factors were considered for the preliminary chromatographic method including the ionic strength of the mobile phases as well as the HILIC column choice. Previous work has established that the concentration of ammonium acetate (AA) used in HILIC-based mobile phases can affect oligo retention, peak shape, and MS signal.[8] Review of these findings led us to conclude that 15 mM AA provides a favorable balance of multiple LC/MS performance criteria. Our own experiments corroborated these results (data not shown) and 15 mM AA, in both mobile phase A (MPA) and B (MPB), was chosen for subsequent studies in the future. The InfinityLab Poroshell 120 HILIC-Z material, which uses a novel zwitterionic phase, was chosen because of its excellent tolerance to a wide pH range beyond what traditional silica

particles normally provide. This wide range was considered advantageous because recent literature[9] indicates that mobile phases with elevated pH (e.g., pH 9) promote oligos of different chemical makeup to have similar charge state distributions and subsequent column separation. In support of these findings, mobile phases at pH 9 yielded better MS sensitivity and chromatographic performance than those at pH 7 for a number of oligos. Ultimately, even though the column could tolerate these high pHs, neutral pH mobile phases were chosen for subsequent studies. The experiments showed that the results at pH 7 were more than acceptable and easier to reproduce.

Chromatographic resolution and spectral deconvolution of oligo ladder species

To evaluate the ability of the optimized method to separate oligos over a wide mass range and provide high-quality data for impurities analysis, an oligo ladder standard was injected. The oligo standard consisted of a 20, 40, 60, 80, and 100-mer DNA (25 pmol/each on column). Baseline separation of the first four components was achieved, and the 80 and 100-mer exhibited slight overlap (see Figure 1). This overlap proved not to be problematic for intact mass determination or impurities assessment of any oligo. The unique extraction and deconvolution of all five species was easily accomplished by automatic peak spectrum background subtraction, defined by the average of spectra at peak start and end. As expected, low-abundance depurination and depyrimidination impurities were easily identified across each oligo in the series. Figure 2 shows the deconvoluted results for the 20 and 100-mer, showing intact mass determinations and a multitude of low-abundance impurities in these samples. A deeper dive into impurities assignments was subsequently conducted and is described in more detail in the section titled “Identification of low abundance impurities".

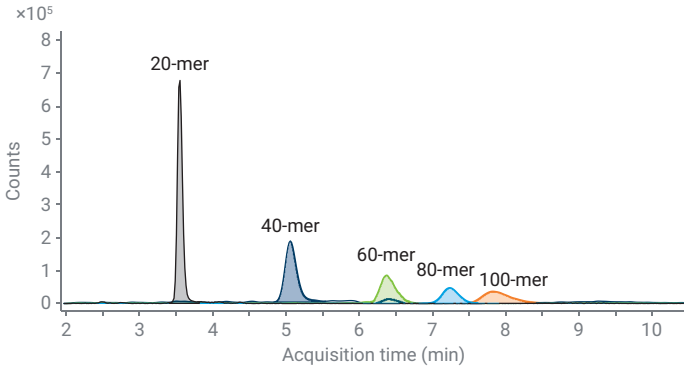

Figure 1. Extracted ion chromatogram overlay for a 20, 40, 60, 80, 100-mer ladder.

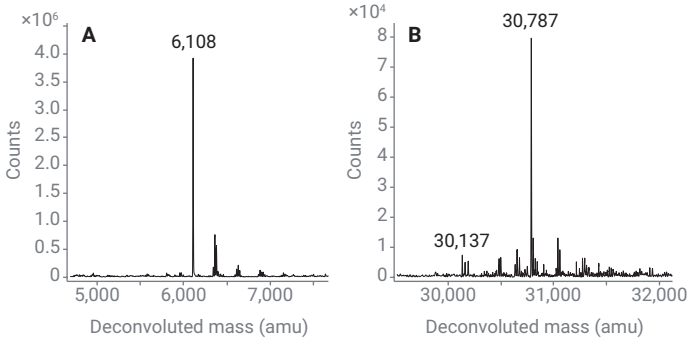

Figure 2. Spectral deconvolution of a 20-mer (PRL20, left) and 100-mer (PRL100, right), showing the applicability of the LC/MS method for intact mass and impurity determinations for a wide distribution of oligo lengths.

MS data from oligos of different size

While the LC/MS method was able to chromatographically resolve a wide size range of oligos, a decreasing response was observed with increasing analyte length. Figure 3 shows how the deconvoluted peak height decreases significantly with oligo size, beyond what is expected based on comparing the number of molecules on column.

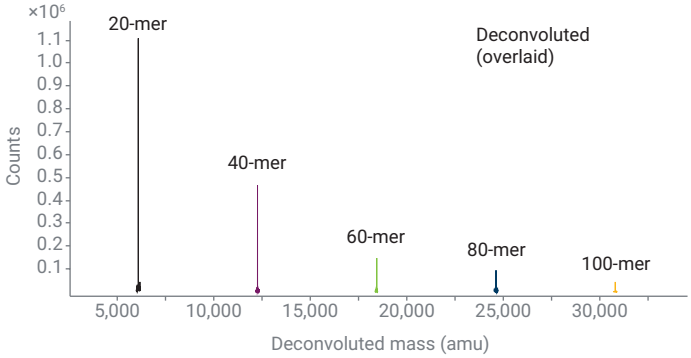

Figure 3. Overlay of deconvoluted spectra from a 20, 40, 60, 80, and 100-mer DNA strand.

Inspection of the mass to charge (*m/z*) spectra from the HILIC method (Figure 4A) shed some light on this trend, especially when compared to *m/z* data collected on the same samples using IP-RP conditions (Figure 4B). The *m/z* data from the IP-RP conditions show a characteristic wide distribution of charge states for each oligo that include both relatively high and low charged species (at low and high *m/z*, respectively). Comparison of the most predominant ion intensity for each sample shows a difference of 4-fold (5^4 for the 100-mer to 2^5 for the 60-mer). The results indicate relatively little difference in desolvating these samples under IP-RP conditions.

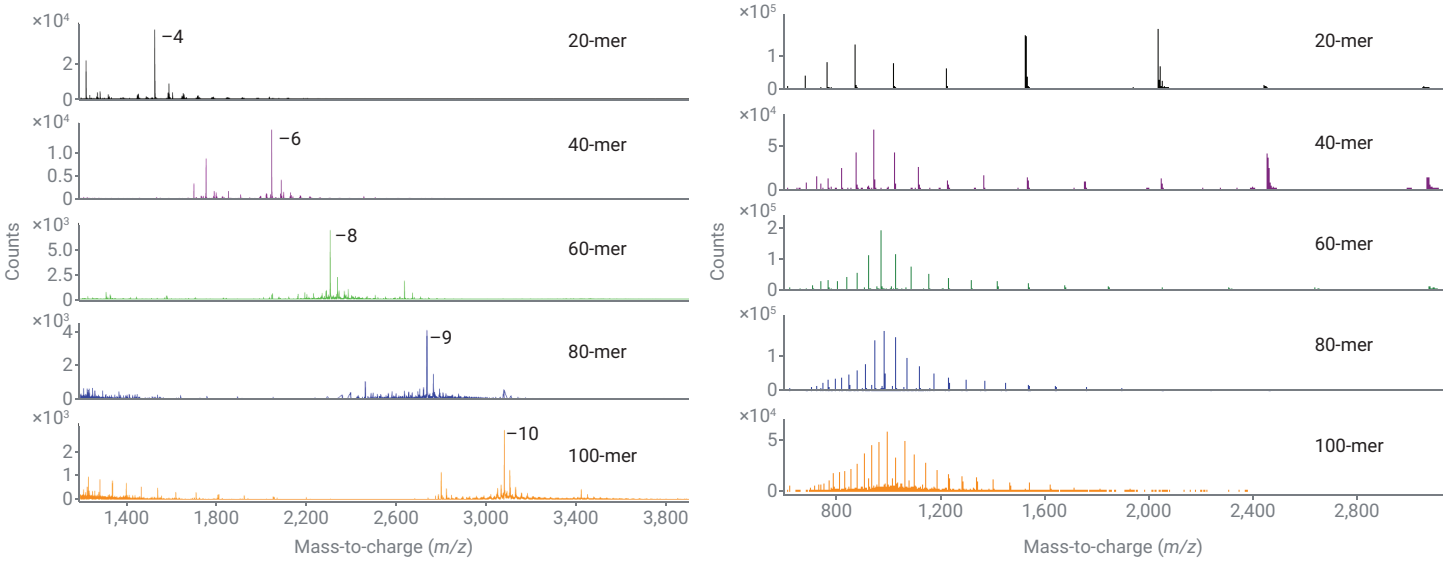

Figure 4. Raw *m/z* spectra for 20, 40, 60, 80, and 100-mers, run under HILIC conditions (left) and IP-RP conditions (right).

In contrast, the *m/z* spectra from the HILIC method display a narrow distribution of charge states that are shifted heavily towards the lower charge states (higher *m/z*). These observations are discussed in depth in the following section. Also, comparison of the most predominant ion intensity for each sample shows a difference of greater than a 10-fold (3^3 for the 100-mer to 4^4 for the 20-mer). These results indicate that larger oligos are harder to desolvate under these conditions. The combination of low ion number and dwindling intensity for the larger oligo ions helps explain the significantly lower deconvoluted heights for the larger oligos. These behaviors may challenge the utility of this specific method on longer samples. Separately, the right shifting of the ion *m/z* range for oligos of increasing size highlights the importance of an extended *m/z* range on the mass spectrometer.

Evaluation of secondary structure with the HILIC method

Interestingly, the *m/z* results from the HILIC method are consistent with what is often observed for biomolecules under native conditions. These results show a narrower charge state distribution focused around lower charged species (as compared to denaturing conditions). Based on these observations, the HILIC LC/MS data for three samples with significantly different potential for forming higher-order structures were compared. The comparison included a poly dT oligo (PR7, with low potential for self-annealing), an oligo containing a variety of building blocks (PR1, with some potential for self-annealing), and an siRNA duplex composed of complementary 18-mers (siRNA, with high potential for self-annealing). Several data features were compared for each of these samples.

First, since chromatographic peak widths can be influenced by molecules with structural diversity, the total ion chromatograms were compared. As can be seen in Figure 5, the peak widths increase from PR7 (top left) to the siRNA (top right). Interestingly, even though PR1 is one building block shorter than PR7, its peak width is larger because the mixed base composition allows for higher-order structures (e.g., hairpins and homodimers). This composition produces a structural mixture that spreads on column. Next, it was not a surprise that the siRNA peak is broad and nonsymmetrical because it is composed of an array of single and double stranded formations.

Second, the *m/z* spectra for the three samples were compared. The spectrum for the oligo sample with low potential for higher-order structures (PR7, middle left) displayed a wide charge state distribution composed of highly charged ions. In contrast, the spectra for PR1 and siRNA, which can form higher-order structures, shows narrow charge state distributions of lesser relative charge. These observations are consistent with higher-order structures being preserved and have been described elsewhere on linear versus hairpin forming strands.[10]

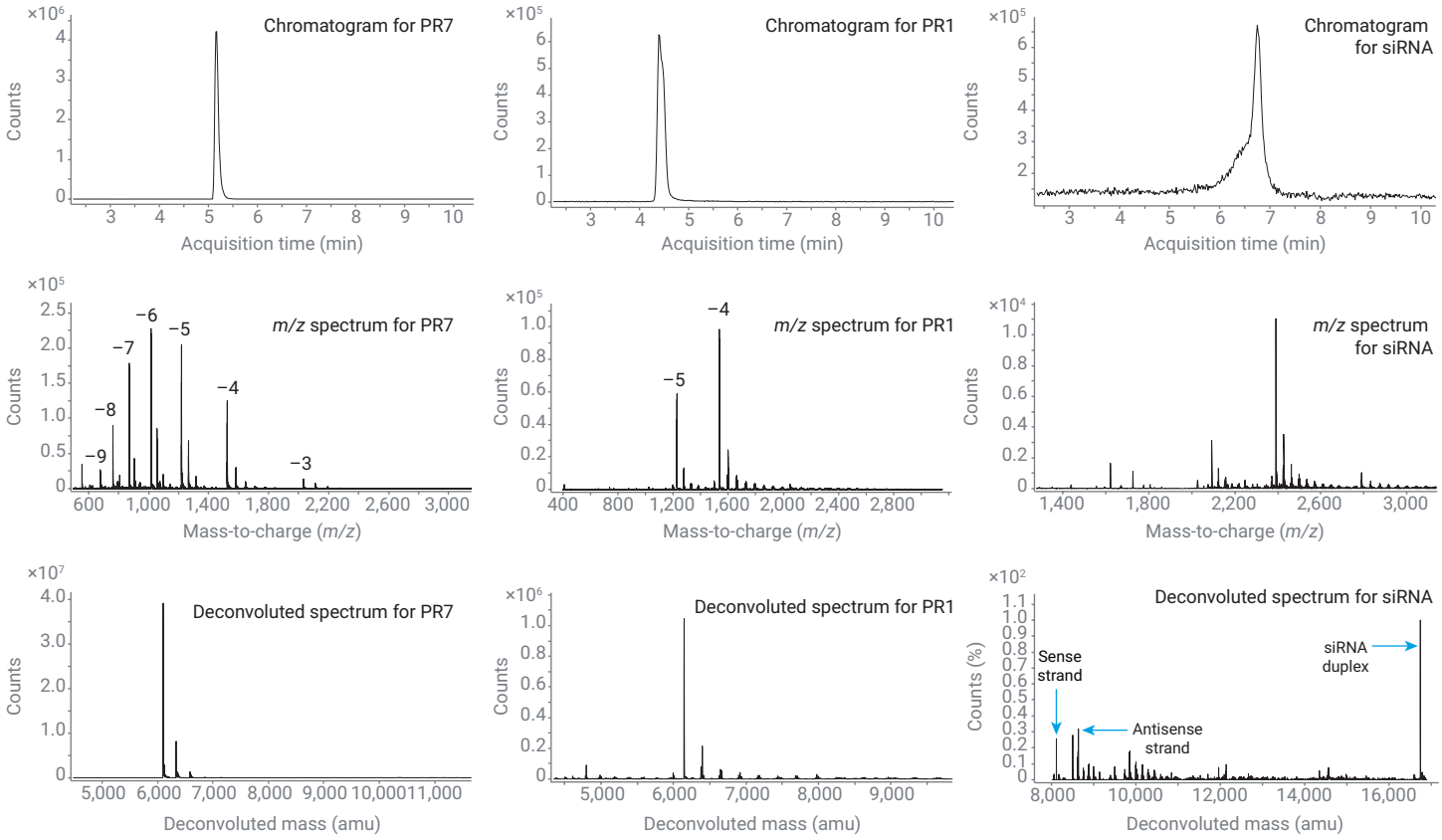

Figure 5. Comparison of LC/MS data for samples (organized by column) with different potentials for higher-order structures.

Third, the deconvoluted spectra for the three samples were compared. The predominant peaks for PR7 and PR1 matched expectations (6,102 and 6,148 Da, respectively). The most striking result was observed for the siRNA (bottom right) in which the main peak matched the mass of the duplex (16,744 Da). Results for this sample also showed peaks for the individual sense and antisense strands at 8,111 and 8,633 Da, respectively. However, the height of the peak at 16,744 Da was approximately 3-fold higher than those for the individual strands, consistent with higher-order structures being preserved throughout the entire analysis.

Column equilibration and retention time reproducibility

For HILIC methods, inadequate stability of peak retention times and areas can result from inadequate re-equilibration or poor column properties.[11,12] Moreover, some have described HILIC re-equilibration times as exceptionally long.[13] It has been hypothesized that the InfinityLab Poroshell 120 HILIC-Z column would afford relatively fast re-equilibration time because the particles are superficially porous, constructed of a solid silica core and a porous outer layer. This design shortens the diffusion path in/out of the particle and enables higher resolution, faster resolution, and faster re-equilibration.

To evaluate the retention time stability that could be achieved with a relatively short re-equilibration time, 10 replicate injections (20 pmol on column) of PR1 were conducted. The injections were run with just 10 minutes of initial condition time between each. The method resulted in excellent peak retention time stability with an RT RSD of <0.1% (Figure 6).

In addition, shorter equilibration times were possible after two initial preconditioning runs, which likely allowed a dynamic equilibrium to be achieved. The effect can be observed in Figure 6 where the baseline is initially elevated but rapidly decreases. These preconditioning runs avoid the need for a full equilibration of the column, which can take a long time depending on column size and flow rate.[12] By including these initial preconditioning injections, it was found that that further decreasing the equilibration time to a total of 5 minutes only marginally increased the RSD of the RT over five injections to <0.2% (data not shown). The peak area reproducibility for both the standard and short equilibration times was calculated to a <2% RSD following the two preconditioning runs.

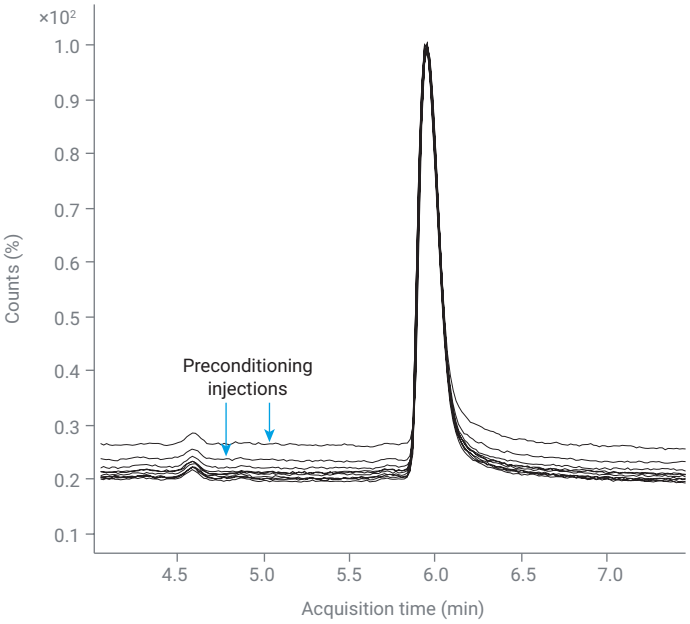

Figure 6. Retention time stability for repeat injections following two initial preconditioning injections.

For more information regarding HILIC method development and optimization, please refer to technical overview 5991-9271EN: Hydrophilic Interaction Chromatography Method Development and Troubleshooting.

Identification of low abundance impurities

A common technique for the identification and relative quantification of product-related impurities takes advantage of the high dynamic range offered by modern mass spectrometers, such as the 6545XT AdvanceBio LC/Q-TOF. This system displays up to five orders of in-spectra dynamic range. To evaluate the ability of the method to detect low abundance impurities in the presence of a highly abundant target, PR7 was injected (10 µM, 50 pmol on column). The resulting data were processed in Agilent MassHunter BioConfirm software 12.0 using both targeted and untargeted methods. The untargeted method was optimized to match and label truncations, depyrimidations, and loss of the terminal phosphate group from a single deconvolution result (from a single chromatographic peak). The deconvolution results are shown in Figure 7. The full-length product (FLP) was determined at 6,102 Da and multiple low-abundance impurities were identified. The impurities included products with loss of 3' T (−304 mu, 0.52% of FLP), loss of the 5' phosphate (−81 mu, 0.28% of FLP), and the gas phase depurination of T (−126 mu, 0.26% of FLP). The targeted method was optimized to interrogate depyrimidations, loss of the terminal phosphate, and all possible truncations using Find-by-Formula. In this case, 49 impurities were found in the *m/z* data, some of which had abundances well under 0.1% of the FLP (data not shown). These studies illustrate how even low-level impurities or degradation products can be characterized and quantified relative to the full-length product, even when they coelute.

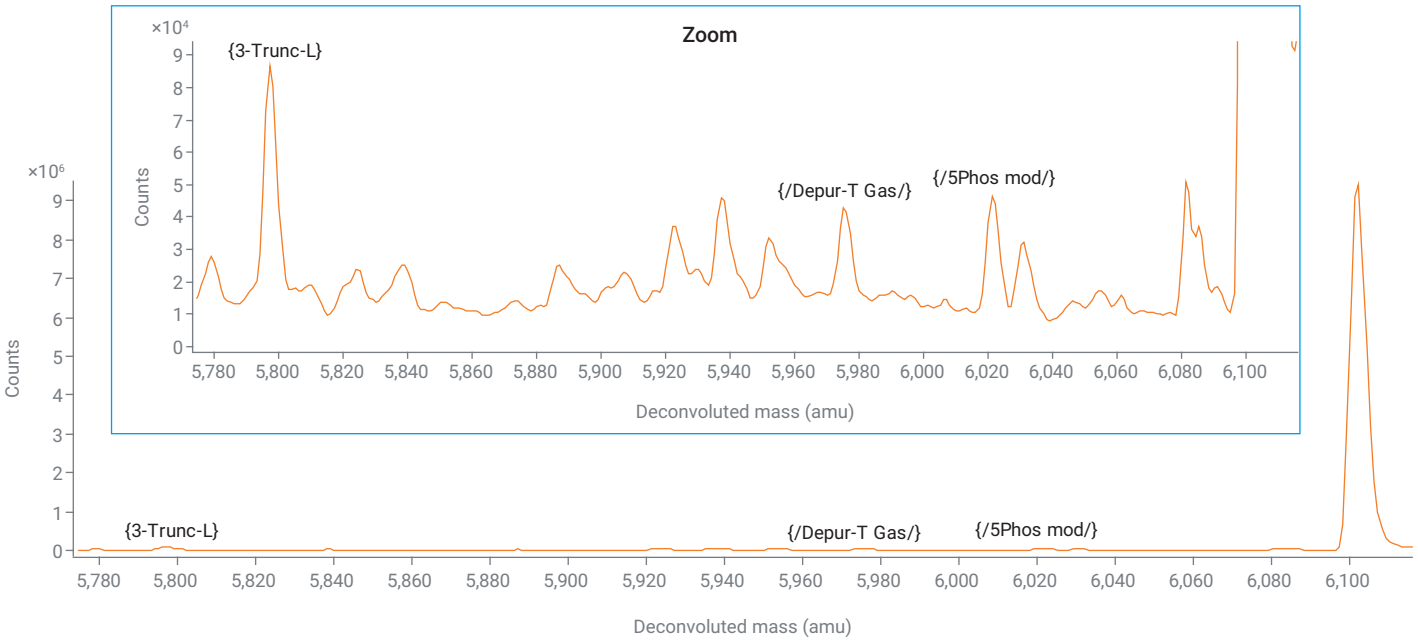

Figure 7. Identification of low-abundance impurities.

Evaluating the method on sample mixtures

Often, chromatographic separation of different oligo species is not possible because of insufficient chemical difference or method throughput needs. In these situations, resolution, and independent measurement of these species by the mass spectrometer is critical. To evaluate the ability of the method to mass-resolve multiple components without the need for chromatographic separation, two samples containing variable bases (V, indicative of a mixture of C, A, and G) at their 3' end were injected. In both cases, chromatographic separation of the mixtures was not observed (data not shown). First, a 20-mer poly dT strand containing a 3' V (oligo PR3) was analyzed. Deconvolution results (Figure 8A) show peaks at 6,007, 6,031, and 6,047 Da that perfectly match expectations, based on sequences ending in C, A, and G respectively. A second, a 38-mer oligo containing a 3' V (oligo PR4) was analyzed. Like the first sample, deconvolution results for PR4 (Figure 8B) show three peaks, at 11,564, 11,588, and 11,604 Da, which match calculations. In both cases, the relative abundances/heights match expectations, confirming the ability of the method to provide meaningful information about oligo mixtures without chromatographic separation of the individual components.

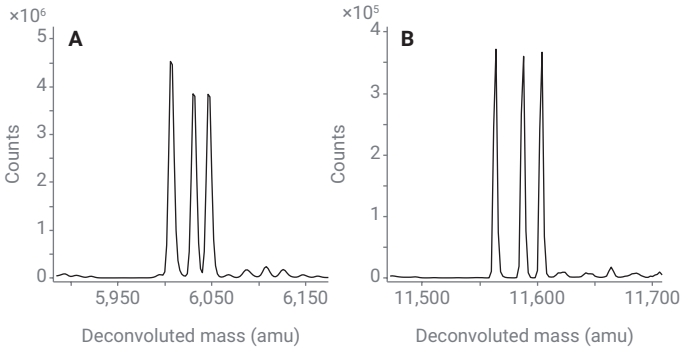

Figure 8 . Deconvolution results for 20-mer (A) and 38-mer (B) oligo mixtures.

Evaluating method performance on oligos of different sizes and chemistries

Many oligos studied by LC/MS, for confirmation of Target Plus Impurities, are constructed from chemically modified building blocks. Subsequently, the versatility of LC/MS methods for oligos of different size and chemical makeup is of interest. To evaluate the HILIC method in this regard, a variety of 18 to 34-mer oligo samples were analyzed individually for their LC/MS behavior. Overlaid deconvolution results for eight disparate samples (Figure 9) showed high-quality data across a simple to a complex sample group. Results show data for a relatively simple 18-mer sample (PR8, 5 µM, 25 pmol on column) to the more complex aptamer with multiple modified bases (50 µM, 100 pmol on column) through an siRNA duplex (50 µM, 100 pmol on column) where the duplex state of two 18-mer strands was maintained throughout. These results provide confidence in the ability of the method to perform well across a wide range of chemistries on 20 to 40-mer samples.

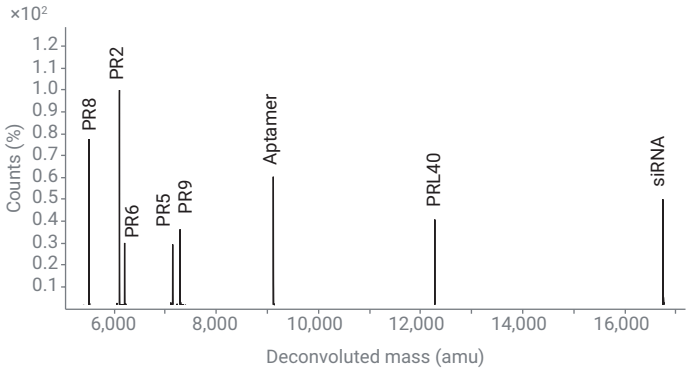

Figure 9. Overlaid deconvolution spectra from oligos of different sizes and chemistries.

Conclusion

- The analysis of oligos via HILIC chromatography allows for effective separation and high-resolution mass data collection without contaminating the instrumentation with ion-pairing reagents.
- The MS-friendly, ammonium acetate-based additive necessitated little system maintenance and source cleaning, allowing fast and easy LC/MS switching between negative and positive mode applications.
- The 15-minute LC/MS method described here provided chromatographic separation of a wide range of oligo samples including 20 to 100-mers, variable base mixtures, aptamers, and siRNA duplexes.
- The HILIC-Z column enabled excellent retention time stability with short re-equilibration times.
- The HILIC method was able to preserve higher-order oligo structures – in one case maintaining an siRNA duplex throughout the analysis.
- The native-like conditions observed did result in narrow charge state distributions (relative to IP-RP) and significantly lower signal intensity for the longer synthetic oligos.
- The HILIC method performed well for impurity analyses, enabling the determination of modifications over a wide dynamic range.

References

1. Wong, D.; Rye, P. An Integrated Workflow for the Analysis of Oligonucleotides and Their Impurities by Agilent High-Resolution LC/(Q-)TOF Mass Spectrometry. *Agilent Technologies application note*, publication number 5994-4817EN, **2022**.
2. Liau, B. Rapid Analysis of mRNA 5' Capping with High Resolution LC/MS. *Agilent Technologies application note*, publication number 5994-3984EN, **2021**.
3. Rye, P.; Yang, Y. High-throughput Mass Spectrometry Analysis of Synthetic Oligonucleotides. *Agilent Technologies application note*, publication number 5994-3753EN, **2022**.
4. 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, **2021**.
5. Duong, P. *et al.* Fast and High-Resolution Reversed-Phase Separation of Synthetic Oligonucleotides. *Agilent Technologies application note*, publication number 5991-6006EN, **2015**.
6. Wong, D. Rye, P. Comprehensive and Integrated Workflow for Oligonucleotide Sequence Confirmation by Agilent High-Resolution LC/Q-TOF. *Agilent Technologies application note*, publication number 5994-5071EN, **2022**.
7. Liau, B. Analysis of mRNA Poly-A Sequence Variants by High-Resolution LC/MS. *Agilent Technologies application note*, publication number 5994-3005EN, **2021**.
8. Lobue *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.
9. Guimaraes *et al.* The Critical Role of Mobile Phase pH in the Performance of Oligonucleotide Ion-Pair Liquid Chromatography–Mass Spectrometry Methods. *Future Science OA* **2021**, *FSO753*.
10. Guo *et al.* Secondary Structural Characterization of Oligonucleotide Strands Using Electrospray Ionization Mass Spectrometry. *Nucleic Acids Res.* **2005**, *33(11)*, 3659–3666.
11. Buszewski *et al.* Hydrophilic Interaction Liquid Chromatography (HILIC)--a Powerful Separation Technique. *Anal. Bioanal. Chem.* **2012**, *402(1)*, 231–247.
12. McCalley *et al.* A Study of Column Equilibration Time in Hydrophilic Interaction Chromatography. *Journal of Chromatography A* June **2018**, *1554*, 61–70.
13. Bell *et al.* Investigation of Reequilibration in Hydrophilic Interaction Liquid Chromatography. *LCGC Europe* **2016**, *29*, 687–692.

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