Application Note

Biopharma/Pharma

High-Sensitivity Oligonucleotide Analysis Using Ion-Pairing-Free HILIC and TOF LC/MS

Author

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Abstract

Laboratories often perform ion-pairing reversed-phase (IPRP) chromatography for oligonucleotide analysis, which requires the use of expensive and environmentally harmful ion-pairing reagents. This application note presents an alternative method using time-of-flight liquid chromatography/mass spectrometry (TOF LC/MS) and hydrophilic interaction chromatography (HILIC) that enables accurate and repeatable analysis of diverse oligonucleotides without using ion-pairing reagents.

Introduction

Oligonucleotides have emerged as a pivotal class of biomolecules that hold immense promise in revolutionizing advances in pharmaceutical research and development of drug products. These short, single- or double-stranded nucleic acid sequences play crucial roles in the regulation of gene expression and many cellular processes. Oligonucleotides can be designed to provide highly specific and targeted treatments. This targeted specificity demonstrates the importance of this class of pharmaceuticals because of their potential to address previously challenging medical conditions and diseases. Oligonucleotides will continue to facilitate valuable progress in precision medicine, gene therapy, and personalized pharmaceutical approaches. By harnessing the power of these versatile biomolecules, researchers and clinicians aim to develop groundbreaking therapies that have the potential to transform patient outcomes and usher in a new era of safer, more effective, and targeted pharmaceutical treatments.

Oligonucleotide analysis enables researchers to gain valuable insights into the structure, composition, and function of these essential biomolecules. Among the diverse analytical techniques available, TOF LC/MS has emerged as a powerful tool for characterizing oligonucleotides. The Agilent 6230B TOF LC/MS performing a HILIC method using Agilent MassHunter BioConfirm software allows the separation and identification of oligonucleotide sequences, modifications, and impurities. HILIC effectively resolves oligonucleotides based on their hydrophilicity before MS analysis. Analytical results from the HILIC method run on a TOF LC/MS facilitate advances in gene therapy, personalized medicine, and the development of innovative nucleic-acid-based therapeutics.

This application note describes a sensitive and ion-pairing-free HILIC LC method for the separation and identification of oligonucleotides using the 6230B TOF LC/MS. One advantage to this HILIC application is that users can switch polarity in a subsequent analytical method and run in positive mode with little downtime. The HILIC method provides a useful alternative to traditional IPRP chromatography, which can have cost- and burden-related issues.

Experimental

Chemicals and reagents

The chemicals and reagents used in this study are listed as follows:

- Acetic acid, glacial, >99% (Sigma-Aldrich)
- Acetonitrile, LC/MS grade (Supelco, Inc.)
- Ammonium acetate, LiChropur, LC/MS grade (Supelco, Inc.)
- Agilent InfinityLab deactivator additive (5 mM medronic acid, part number 5191-3940)
- Agilent DNA ladder standard (part number 5190-9029)
- Water, LC/MS grade (Honeywell)

Instrument configuration

This experiment was carried out using the following instrument configuration:

- Agilent 6230B TOF LC/MS
- Agilent MassHunter acquisition software for LC/TOF, version 11.0
- Agilent MassHunter BioConfirm software, version 12.0
- Agilent 1290 Infinity II high-speed pump (G7120A)
- Agilent 1290 Infinity II multisampler (G7167B)
- Agilent 1290 Infinity II multicolumn thermostat (G7116B)
- Agilent 1260 Infinity II diode array detector HS (G7117C)

Sample preparation

All oligos were resuspended in 1 mL of water and stored at –80 °C. Before analysis, samples were transferred to polypropylene vials and stored in the temperature-controlled autosampler for up to 2 days.

Liquid chromatography/mass spectrometry analysis

HILIC separations were performed on the Agilent 1290 Infinity II bio LC system with an Agilent InfinityLab Poroshell 120 HILIC-Z column. Before analysis, the column was equilibrated overnight with 10 mM ammonium acetate at pH 10 at a flow rate of 0.4 mL/min. The mobile phases used in this study were mixtures of 30 mM ammonium acetate buffer adjusted to pH 5.5 and acetonitrile. InfinityLab deactivator additive was added to the aqueous phase to increase the overall sensitivity of the instrument and reduce instrument preparation time. The gradient consisted of a 0.5-minute hold at 70% mobile phase B, then to 35% mobile phase B at 12 minutes, then decreasing to 20% at 12.5 minutes for cleaning. The gradient was returned to

starting conditions at 14.6 minutes and the column was equilibrated for a total of 3 minutes before the next injection. This 12-minute gradient proved suitable for chromatographic resolution of the oligo ladder (15- to 40-mer range), but it can easily be modified for oligos up to 130-mers. Mass spectrometry was performed on the 6230B TOF LC/MS with a dual-spray Agilent Jet Stream Electrospray ionization source. Results were analyzed using Agilent MassHunter BioConfirm software 12.0 and MassHunter Qualitative Analysis software 11.0. Source parameters for the 6230B TOF LC/MS are shown in Table 1, and HPLC parameters are shown in Table 2.

Table 1. Source parameters for the Agilent 6230B TOF LC/MS.

Parameter	Value
Ion Source	Dual Agilent Jet Stream Electrospray ionization source
Polarity	Negative
Gas Temperature	350 °C
Drying Gas Flow	12 L/min
Nebulizer	30 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	750 V
Mass Range	700 to 3,000 *m/z*
Acquisition Rate	1.25 spectra/s

Table 2. HPLC parameters used in this study.

Parameter	Value
Column	Agilent InfinityLab Poroshell 120 HILIC-Z, 2.1 × 100 mm, 1.9 µm
Sampler Temperature	8 °C
Mobile Phase A	90:10 ammonium acetate (30 mM, pH 5.5) in water with deactivator:acetonitrile
Mobile Phase B	10:90 ammonium acetate (30 mM, pH 5.5) in water with deactivator:acetonitrile
Flow Rate	0.2 mL/min
Injection Volume	5 µL
Wash	50:50 water:acetonitrile; flush port; 5 s
Column Temperature	50 °C
Post-Time	3.5 min
Gradient Program	Time (min) %B 0.0 70 0.5 70 12.0 35 12.5 10 14.5 10 14.6 30

Results and discussion

Chromatographic development was carried out by assessing factors such as mobile phase pH and ionic strength, and stationary phase particle size and chemistry. The HILIC-Z stationary phase was selected for this method due to its stability at a wider temperature range and shorter equilibration time. Previously published literature on the topic has shown that improving the ionic strength of the mobile phase improves peak shape.[1] During testing, buffers with higher ionic strength and lower pH showed an improved peak shape over buffers with higher pH and lower ionic strength. Therefore, mobile phases containing 30 mM ammonium acetate were used for both mobile phases A and B.

For method evaluation, a DNA ladder standard consisting of 15-, 20-, 25-, 30-, 35- and 40-mer oligos was used. The ladder standard was separated with near-baseline resolution (Figure 1). There was some overlap between the fifth and sixth oligonucleotide; however, this resolution enabled accurate deconvolution. The deconvolution and intact mass determination were accomplished by automatic peak spectrum background subtraction, defined by the average of spectra at peak start and end (Figures 2 and 3).

HILIC methods usually suffer from instability of peak retention times (RT), which can be a result of inadequate re-equilibration time in the given method.[2] The InfinityLab Poroshell 120 HILIC-Z column has comparatively fast re-equilibration times because the particles are superficially porous—constructed of a solid silica core and a porous outer layer. The superficially porous particles shorten the diffusion path of the particles, thus enabling higher resolution and faster re-equilibration.[3]

To evaluate the RT stability while using a short re-equilibration time, 15 replicate injections (10 pmol on column) of the ladder standard were performed. The method showed an RSD of <0.2% for the RTs of all six oligonucleotides in the standard mixture. These results demonstrated excellent peak RT stability (Figure 4).

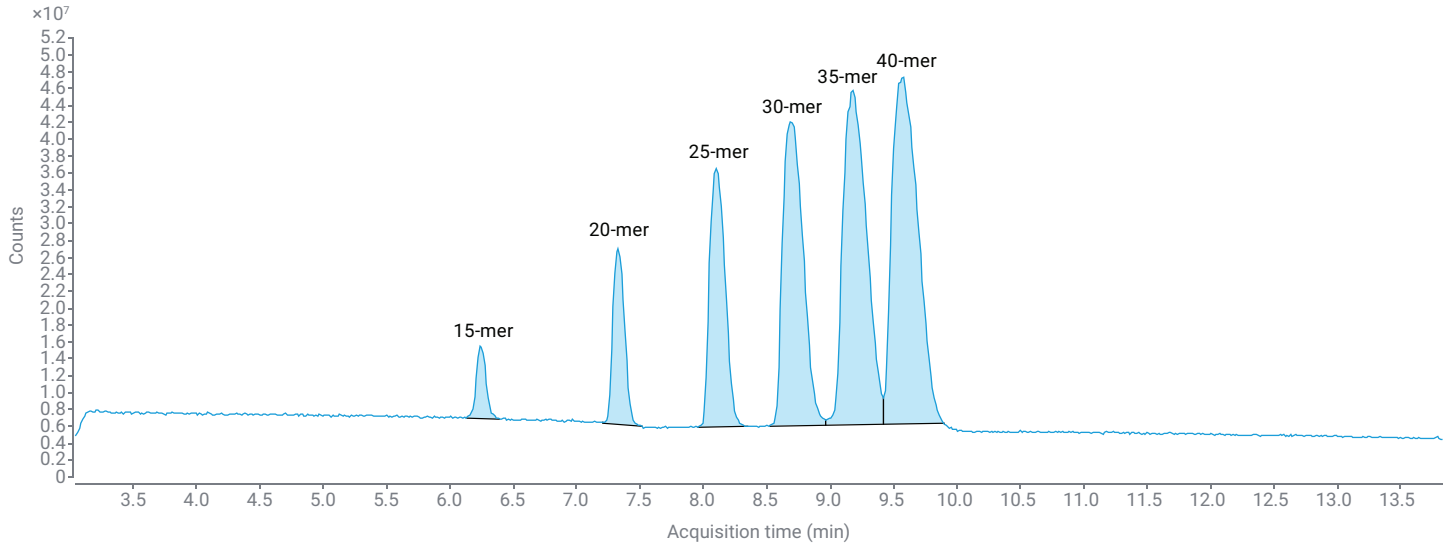

Figure 1. Total ion chromatogram (TIC) of the DNA ladder standard analyzed by TOF LC/MS.

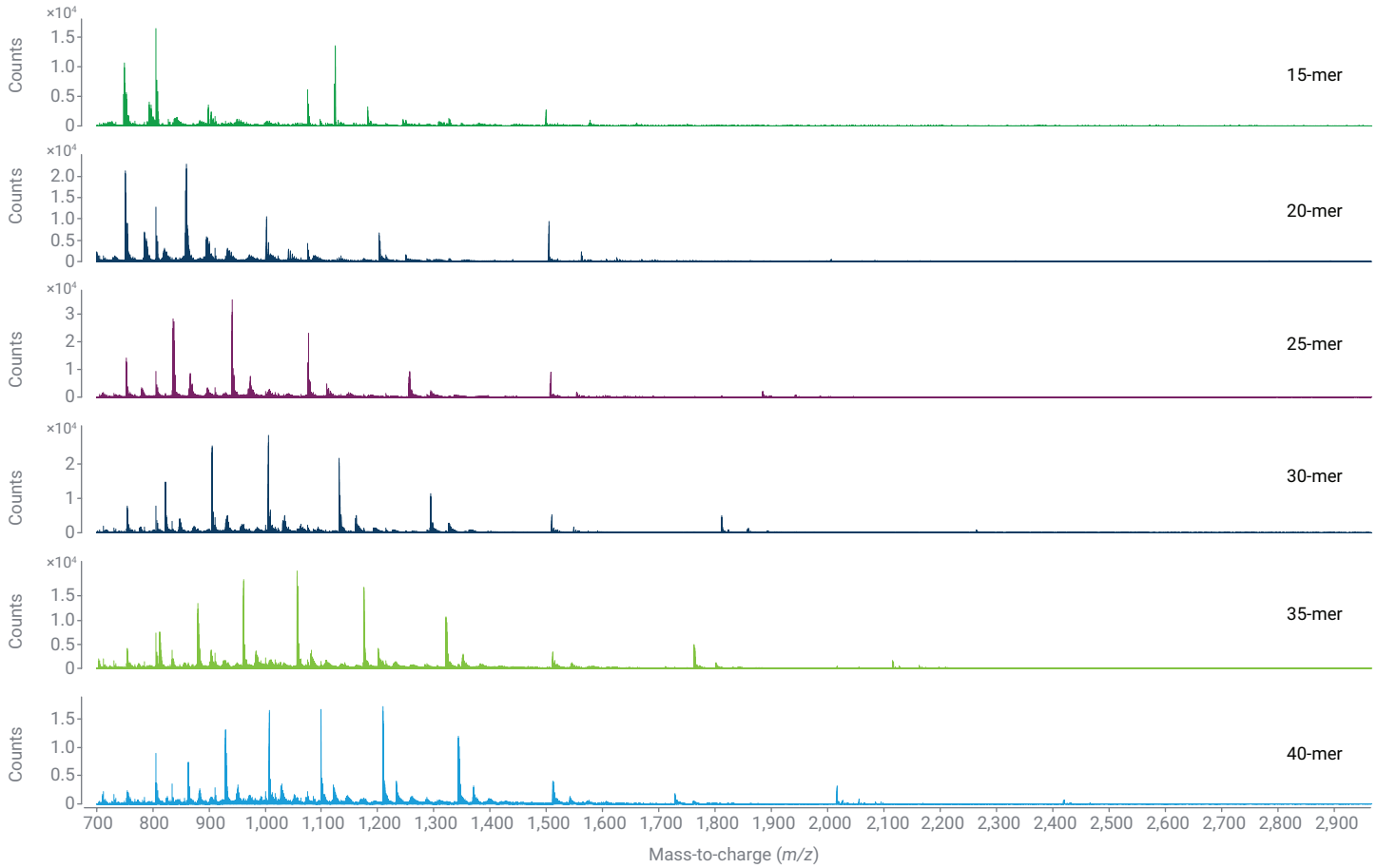

Figure 2. Raw *m/z* spectra for 15-, 20-, 25-, 30-, 35-, and 40-mer DNA analyzed in this study.

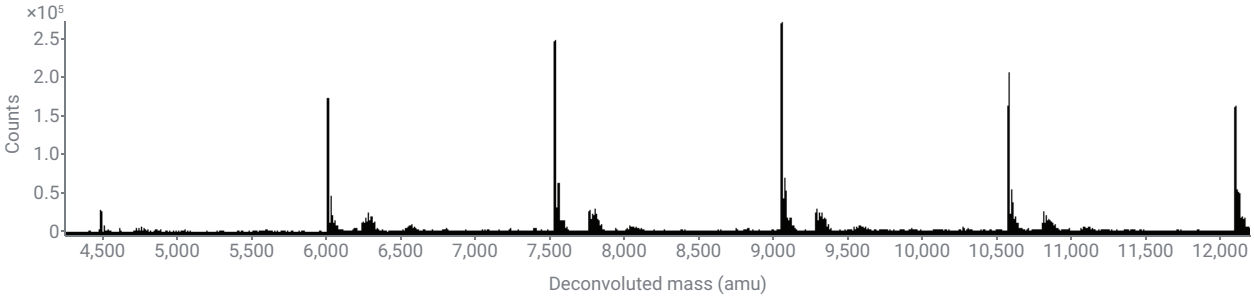

Figure 3. Overlaid deconvolution spectra of the ladder standard.

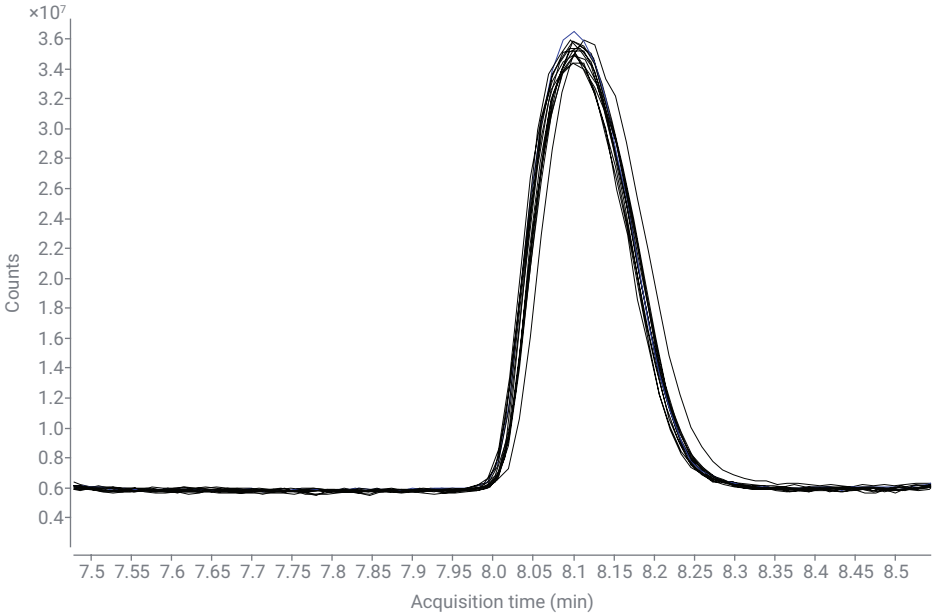

Figure 4. Overlay of 15 injections of the 30-mer standard to show the repeatability of the retention times.

Conclusion

The described method allows accurate and repeatable analysis of diverse oligonucleotides using TOF LC/MS and HILIC. This HILIC method enables laboratories to move away from the expensive and environmentally harmful ion-pairing reagents commonly used in oligonucleotides analysis. This improved method provides users the opportunity to easily switch between LC/MS methods in both negative and positive mode with little-to-no LC/MS system downtime.

References

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