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應用手冊

Improved Separation of RNA Nucleotides, Nucleosides, and Nucleobases on Atlantis Premier BEH Z-HILIC Columns

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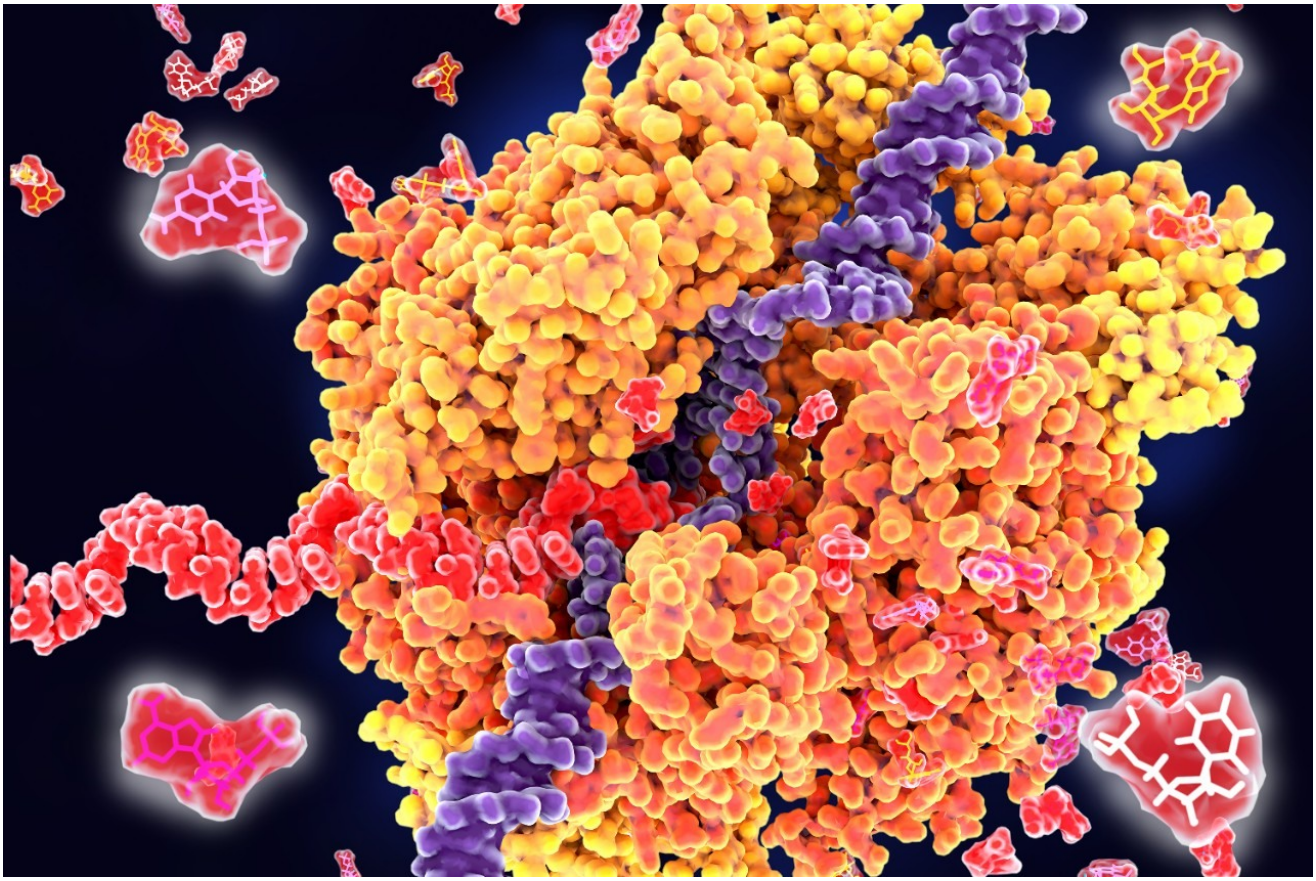


Abstract

The use of novel HILIC stationary phases is critical to obtaining the best separation possible for highly polar analytes. Method development activities in HILIC have been limited by the uniqueness of the available stationary phases. While a lot of difference can be seen between manufacturers of a column chemistry (i.e., unbonded silica) the use of different stationary phases, including bonded ligand type or particle type, can drive selectivity even further. In this work, four columns were used to separate thirteen RNA components, including nucleobases, nucleosides, and nucleotides in a single analytical run. An ACQUITY UPLC BEH Amide Column and Atlantis Premier BEH Z-HILIC Column, as well as two other commercially available polar retention stationary phases, were used in 2.1 x 50 mm column hardware with an ACQUITY Premier System. Detection was achieved using multiple reaction monitoring with a Xevo TQD Triple Quadrupole Mass Spectrometer. A separation of all thirteen components was achieved on the Atlantis Premier BEH Z-HILIC Column. Selectivity differences between the three zwitterionic phases tested were observed. Out of the four columns evaluated, the unique column chemistry of BEH Z-HILIC particles coupled with ACQUITY Premier Column and System hardware allowed for the separation of these structurally similar and biologically relevant compounds.

Benefits

- Separation of thirteen biologically relevant and structurally similar RNA components
- Unique selectivity of Atlantis Premier BEH Z-HILIC Column compared to other zwitterionic stationary phases
- MaxPeak High Performance Surfaces provide peak shape improvements for nucleotides

Introduction

The analysis of RNA components is an important and vital aspect of many different workflows including, but not limited to, food testing and metabolomics.[1-5] RNA components include nucleobases, nucleosides, and nucleotides as well as those RNA compounds with modifications that can occur during different biological functions. In addition, RNA constitutes important structural elements in the ribosome and spliceosome. The residues of RNA and their potential modifications are often examined as biomarkers for different diseases or to track treatment progress.

One of the challenges of this analysis is the highly polar nature of these components. Reversed-phase LC can be used but often requires ion-pairing agents to increase retentivity at the cost of decreased MS ionization. Additionally, ion-pairing agents require significant time to equilibrate in the system and can linger in the LC after analysis. Often, even if using ion-pairing agents, retaining these analytes with reversed-phase LC columns can

still be difficult.

An alternative technique for analyzing these compounds is hydrophilic interaction liquid chromatography (HILIC), which is designed to retain polar analytes using MS-friendly mobile phases and polar stationary phases. With HILIC, RNA components can be readily retained but may still be not well separated due to the structural similarities of the RNA components. In this work, the separation of thirteen RNA components is performed using four different stationary phases. The nucleobases, nucleosides, and nucleotides along with ribose phosphate were found to be most effectively separated on the Atlantis Premier BEH Z-HILIC Column.

Experimental

Sample Description

Analytes were purchased from Sigma Aldrich and stock solutions were made at 1.0 mg/mL using 50:50 acetonitrile:water with 0.1% formic acid. A stock solution of guanine (0.5 mg/mL) was made in a 1:1 solution of acetonitrile:100 mM potassium hydroxide to allow for proper dissolution. Component structures are shown in Figure 1. Stock solutions were combined to create a sample with the following concentrations: adenine, adenosine, cytosine, cytidine, uracil, uridine, guanine, guanosine (2.0 µg/mL each), adenosine monophosphate (30 µg/mL), cytidine monophosphate, uridine monophosphate, guanosine monophosphate, and ribose phosphate (50 µg/mL each).

Method Conditions

LC Conditions

LC system:	ACQUITY Premier with Quarternary Solvent Manager
Detection:	Multiple reaction monitoring (see Table 1)
Vials:	LCMS TruView Max Recovery Vials (p/n: 186005668CV)
Column(s):	ACQUITY UPLC BEH Amide (p/n: 186004800),

	Atlantis Premier BEH Z-HILIC (p/n: 186009978), Competitor A: HILIC-z, Competitor B: Polar x; all in 2.1 x 50 mm hardware
Column temp.:	50 °C
Sample temp.:	10 °C
Injection volume:	0.5 µL
Flow rate:	0.5 mL/min
Mobile phase A:	Water
Mobile phase B:	Acetonitrile
Mobile phase D:	200 mM ammonium formate pH 3.0
Gradient:	A water and buffer gradient was employed for this separation. Water content was changed from 5–40%, while buffer concentration was changed from 10–50 mM. *See gradient table for details.*

Gradient Table

Time [min]	Flow [mL/min]	%A	%B	%D	Curve
0.00	0.5	0	95	5	6
10.36	0.5	15	60	25	6
11.57	0.5	15	60	25	6
11.63	0.5	0	95	5	6
15.00	0.5	0	95	5	6

MS Conditions

MS system:	Xevo TQD
Ionization mode:	Positive and negative mode (see Table 1)
Acquisition range:	Multiple reaction monitoring (see Table 1)
Capillary voltage:	3.0 kV
Collision energy:	Varies by analyte (see Table 1)
Cone voltage:	Varies by analyte (see Table 1)

Data Management

Chromatography software:	MassLynx v4.2
MS software:	MassLynx v4.2
Informatics:	MassLynx v4.2

Results and Discussion

The analysis of RNA components is critical in many fields of study including food and environmental applications as well as metabolomics workflows. Achieving high selectivity for the separation of these compounds is necessary for accurate and reliable quantitation and improved characterization. Hydrophilic interaction liquid chromatography (HILIC) is ideally suited to retain these small polar analytes and was employed for this work. The thirteen analytes examined are shown in Figure 1. Detection using multiple reaction monitoring (MRM) on a Xevo TQD was used with the conditions shown in Table 1.

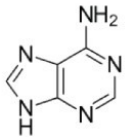
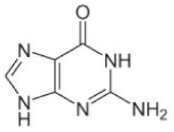
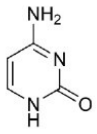
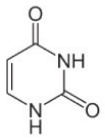
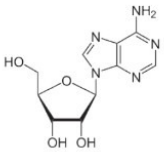
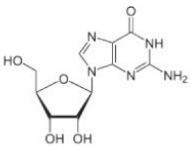
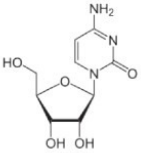
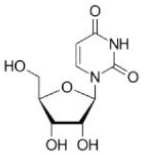
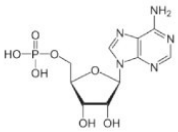
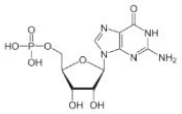
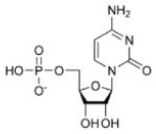
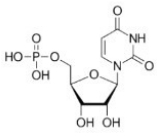
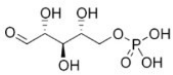
	Adenine	Guanine	Cytosine	Uracil	Ribose
Nucleobase					N/A
Nucleoside					N/A
Nucleotide					

Figure 1. Analyte structures of the nucleobases, nucleosides, and nucleotides. Structures are organized by the base component, and ribose phosphate was also analyzed.

Compound	Ionization mode	Precursor ion (*m/z*)	Cone voltage (V)	Product ions (*m/z*)	Collision energy (V)
Adenine	ESI+	136.0	30	119.0	20
Adenosine	ESI+	268.0	30	136.0/119.0	10/45
AMP	ESI+	348.0	30	136.0/119.0	20/55
Cytosine	ESI+	112.0	30	95.0	20
Cytidine	ESI+	244.0	30	112.0/95.0	15/45
CMP	ESI+	324.0	30	112.0/95.0	15/55
Uracil	ESI-	111.0	20	N/A	N/A
Uridine	ESI-	243.0	20	200.0/110.0	10/15
UMP	ESI-	323.0	20	111.0	30
Guanine	ESI+	152.0	30	135.0	20
Guanosine	ESI+	284.0	30	152.0/135.0	15/35
GMP	ESI+	364.0	30	152.0/135.0	15/50
Ribose phosphate	ESI-	229.0	20	97.0	15

Table 1. MRM conditions for the detection of the thirteen analytes. Additional MS conditions are listed in the Experimental Section.

Four HILIC stationary phases were evaluated for this separation. ACQUITY UPLC BEH Amide, Competitor HILIC-z, Competitor Polar X, and Atlantis Premier BEH Z-HILIC Columns were screened, and a method was optimized for these analytes. Figure 2 shows the separations achieved using a water and ammonium formate gradient.

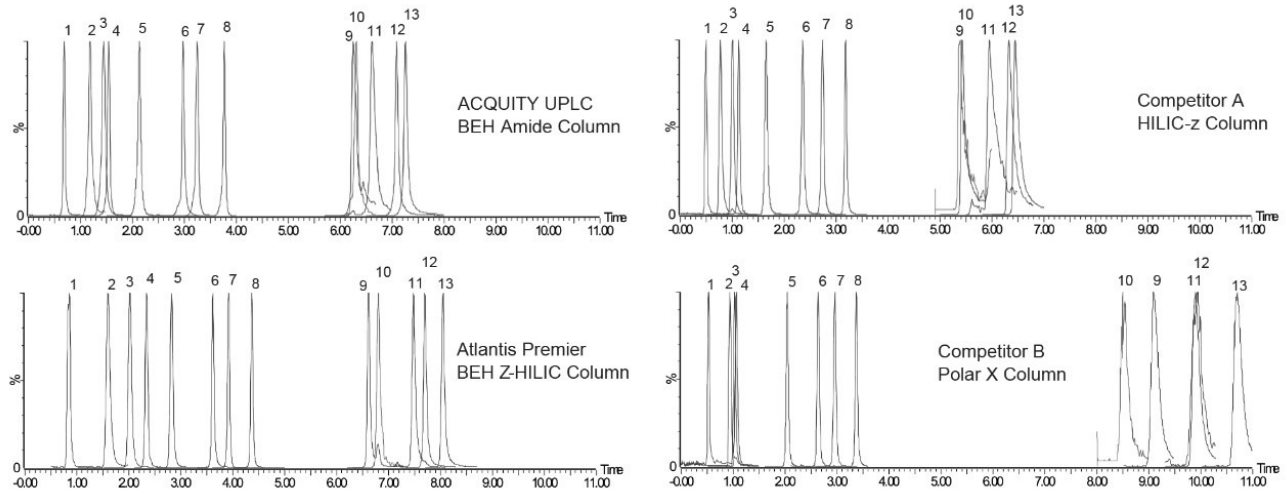


Figure 2. Separation of thirteen RNA components on four different HILIC stationary phases. Scheduled MRMs were used to increase sensitivity. Peak heights were normalized to show separation of the compounds and do not reflect the overall signal achieved. Peaks are labeled as: 1) uracil, 2) adenine, 3) adenosine, 4) uridine, 5) cytosine, 6) guanine, 7) cytidine, 8) guanosine, 9) adenosine monophosphate, 10) uridine monophosphate, 11) ribose phosphate, 12) cytidine monophosphate, and 13) guanosine monophosphate.
The only column to achieve resolution of all compounds was the Atlantis Premier BEH Z-HILIC Column. All other columns showed at least one set of co-eluting compounds. While the use of the Xevo TQD can still detect these peaks, separation of these co-eluting probes is preferred in order to provide better integration and characterization of these species.

Except for the BEH Amide column, all columns consisted of zwitterionic or mixed-mode stationary phases. Nevertheless, differences were still observed amongst these materials in terms of retention and selectivity. The HILIC-z stationary phase gave less retention than the other phases while the Polar X phase showed good retention for the monophosphate compounds with comparable retention for the nucleobases and nucleosides. Elution order for certain analytes also varied between the three phases, most notably the adenosine and uridine monophosphates (peaks 9 and 10). The differences in selectivity can be attributed to the nature of the different stationary phases. The HILIC-z material consists of a zwitterionic ligand attached to a superficially porous particle. Meanwhile, the Polar X column has a mixed-mode ion-exchange HILIC stationary phase attached to a superficially porous particle, and the BEH Z-HILIC uses a zwitterionic ligand attached to a fully porous BEH 95 Å particle. The differences between column chemistries is shown in Figure 3.

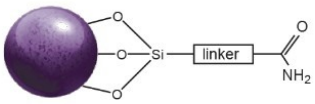
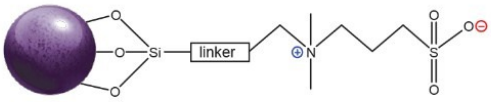
Column name	Base particle	Ligand chemistry
ACQUITY UPLC BEH Amide	BEH 130 Å Fully Porous Hybrid Silica pH tolerance: 2–11	
Atlantis Premier BEH Z-HILIC	BEH 95 Å Fully Porous Hybrid Silica pH tolerance: 2–12	
HILIC-z	100 Å Superficially Porous Silica pH tolerance: 2–12	Zwitterion, no additional details provided
Polar X	90 Å Superficially Porous Silica pH tolerance: 2–8	Mixed mode ligand, with HILIC and ion-exchange functionality

Figure 3. Description of base particle chemistry and attached ligand chemistry as described in the manufacturer's literature.

The change in selectivity for the BEH Amide compared to BEH Z-HILIC can be attributed to the differences in both the ligands and base particles. Both stationary phases use fully porous hybrid silica particles, however the BEH Z-HILIC uses a BEH 95 Å particle while the BEH Amide uses a BEH 130 Å particle. The distinctions in pore size as well as bonded phase ligand leads to these differences in apparent selectivity.

An added benefit observed when comparing the Atlantis Premier BEH Z-HILIC Column to other columns is the improved peak shape of the phosphorylated compounds. The differences are especially noticeable when comparing the Atlantis Premier BEH Z-HILIC to the other commercial zwitterionic columns, which show much wider peak shapes for the phosphorylated species. The ACQUITY UPLC BEH Amide Column also has slightly wider peaks compared to the BEH Z-HILIC column, which are still narrower than those of the alternative zwitterionic columns. The MaxPeak High Performance Surfaces (HPS) hardware used to pack the BEH Z-HILIC stationary phase mitigates secondary interactions between the phosphorylated compounds and the metal surfaces of the columns. Under certain conditions, these metal-analyte interactions can lead to peak shape differences, as seen here, and may even cause sample loss.[6] In this case, the loss of analyte to metal adsorption is not seen as peak areas are comparable across all four columns. Table 2 shows the peak shape differences for the phosphorylated compounds across the four investigated column technologies.

Compound	Peak width at 4.4% ACQUITY UPLC BEH Amide	Peak width at 4.4% Atlantis Premier BEH Z-HILIC	Peak width at 4.4% HILIC-z	Peak width at 4.4% Polar X
Adenosine monophosphate	0.208	0.163	0.384	0.460
Uridine monophosphate	0.258	0.164	0.374	0.329
Ribose phosphate	0.289	0.191	0.441	0.535
Cytidine monophosphate	0.186	0.159	0.510	0.468
Guanosine monophosphate	0.259	0.159	0.535	0.420
Average peak width at 4.4%	0.240	0.167	0.449	0.443

Table 2. Peak widths at 4.4% peak height (sec) for the phosphorylated compounds on all four stationary phases. Average peak widths for the columns calculated.

The average peak widths for the Atlantis Premier BEH Z-HILIC Column are 30% narrower compared to the ACQUITY UPLC BEH Amide Column, and approximately 60% narrower than either competitor column. The narrower peaks obtained on the BEH Z-HILIC column help to resolve critical pairs as well as improve sensitivity. By combining the unique particle-ligand combination with the MaxPeak HPS hardware, the thirteen analytes were resolved with good peak shape compared to other HILIC stationary phases.

Conclusion

Thirteen biologically relevant RNA components were analyzed on four different columns, including the Atlantis Premier BEH Z-HILIC Column. These compounds included RNA nucleobases, nucleosides, and nucleotides, which are structurally similar and thus have been historically very challenging to separate, especially on short 50 mm columns. The novel Atlantis Premier BEH Z-HILIC Column was able to separate all the compounds in a single run using a gradient of water, acetonitrile, and ammonium formate. Additionally, the Atlantis Premier BEH Z-HILIC Column provided better peak shape for the nucleotides compared to the other columns tested, in part due to the MaxPeak HPS Technology mitigating the interaction between the phosphoric acid moiety and the metal hardware of the columns. By using the Atlantis Premier BEH Z-HILIC Columns combined with the ACQUITY Premier System, quantitation of all nucleobases, nucleosides, and nucelotides as well as ribose phosphate. The accurate measurement of nucleotides and nucleosides is critical in the diagnosis of many

diseases including cancer and certain respiratory illnesses.

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