Application Note

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Mastering HILIC-Z Separation for Polar Analytes

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Abstract

This application note presents recommended practices when using an Agilent InfinityLab Poroshell 120 HILIC-Z column in the separation of polar metabolites. An example methodology demonstrates that, even during a multiday study and with a complex matrix, consistent peak shapes and reproducible retention times can be achieved with the HILIC-Z column.

Introduction

Hydrophilic interaction liquid chromatography (HILIC) is a method that can use various hydrophilic stationary phases to retain polar analytes during liquid chromatography (LC) and can separate some isobaric analytes without the use of ion-pair reagents. The Agilent InfinityLab Poroshell 120 column line features three HILIC stationary phase chemistries: HILIC, HILIC-Z, and HILIC-OH5. Each has unique hydrophilic and ion exchange properties that yield different selectivities.[1] The HILIC-Z column phase is a great option for retaining polar and acidic small molecules, such as metabolites.[2,3] The column also works with a wide pH range (pH 2 to 12) and temperatures (5 to 80 °C), offering the flexibility to use different mobile phases. Its unique zwitterionic functionality bonded to a robust hybrid silica particle provides a complete water layer, allowing polar analytes to partition between the water layer and mobile phase. Meanwhile, the charged zwitterionic group provides further interactions with acidic and basic moieties, providing enhanced retention and improving the selectivity of the stationary phase. By increasing the percent aqueous and ionic strength (low aqueous starting conditions) of the mobile phases, the polar analytes are eluted, and analyte elution is typically from least to most polar.

Though polar metabolites can be analyzed with a robust column phase such as C18 using ion-pair reagents, this approach requires dedicated LC instrumentation, as the ion-pairing reagents are difficult to remove from the LC system. Some ion-pair reagents can also limit the ion polarity of an experiment to negative mode only, and in certain global markets are considered a health hazard and are banned.

As previously mentioned, the real benefit of HILIC is that it does not use ion-pair reagents, and thus does not require dedicated instrumentation or special maintenance. This aspect makes it more flexible for other LC/MS analyses in positive and negative ion mode. However, the use and success of deploying any HILIC column can be tricky for new users. The biggest challenge to new users is obtaining reproducible retention times over time and across manufactured lots, maintaining a stable pressure trace, and increasing column lifetime.

This application note provides best practices for operating a HILIC-Z column to achieve stable retention times, robust separation of polar analytes, and consistent pressure traces. A methodology is also described for a global polar metabolomics separation and tested for retention time stability.[4] This application note shares tips and tricks to achieving success with this method. While biological metabolites are heavily discussed, using these tips and tricks, the HILIC-Z column may be tested for any small polar analytes that cannot be retained on a reversed-phase column.

Experimental

Materials and methods

Table 1. Materials and consumables.

Item	Vendor	Part Number	Specification and Notes
Agilent InfinityLab Stay Safe Purging Bottle	Agilent	5043-1339	Quantity: three minimum, four recommended
Agilent InfinityLab Thread Adapter, GL45(M) to GPI 38(F), PTFE	Agilent	5043-1192	– Quantity: two minimum, four recommended – Used for attaching the Safe LC caps to the narrow neck Nalgene bottles.
Agilent InfinityLab Stay Safe Cap Starter Kit	Agilent	5043-1222	Quantity: one
Nalgene FEP Bottle	Sigma	Z266167-1EA	Narrow-mouth, quantity: two minimum, four recommended
Agilent InfinityLab Poroshell 120 HILIC-Z Column, 2.1 × 150 mm, 2.7 µm	Agilent	683775-924	Quantity: one
Benchtop pH Meter with pH Probe and Stir Plate	VWR	89231-664	– This model has a stir plate attached to it. If your pH meter does not, add a stir plate to it. pH resolution >0.01 – **pH paper is not sufficient. A pH meter is required.** – Calibrated daily, or for each use.
Analytical Balance, Weigh Paper, Spatula			Milligram setting on balance needed
1 mL Pipette and Pipette Tips			Calibrated
100 and 1,000 mL Graduated Cylinder			Clean and dedicated for HILIC
250 mL Glass Beaker			Clean and dedicated for HILIC
1 in Magnetic Stir Rod			Clean and dedicated for HILIC

Glass LC solvent bottles are acceptable for cleaning the LC and prepping the column. The bottles used here have multiple necks to safely purge several solvent lines with the same solvent at once. For the analysis, the buffers should be prepared in Nalgene FEP bottles to reduce the sodium in the mobile phase. This step is intended to maintain low background ions and keep the ionic strength of the solution equal to what is measured in the buffer. It is also important to have a tight seal on solvent bottles to reduce atmospheric exposure, and so maintain buffer pH and stability. The Nalgene bottles have a narrow neck, so the traditional LC solvent bottle caps will not fit. Instead, LC bottle solvent caps will need to be replaced with the Agilent InfinityLab Stay Safe caps and an Agilent InfinityLab thread adapter will need to be used.

To maintain the quality and consistency of results, it is highly recommended that all necessary glassware is dedicated to this methodology. It should be cleaned with isopropyl alcohol (IPA), water, and methanol individually, then stored with foil between uses and lightly rinsed with the used solvent before each measurement. The IPA, water, and methanol wash should be repeated periodically.

All bench-top instruments, including pipettes and pH meter, should be calibrated and well maintained to deliver a precise and accurate measurement, enabling reproducible retention times.

The Agilent InfinityLab deactivator additive is a modifier that actively chelates metal ion contamination within the LC system, reducing metal-analyte interactions and sharpening the peak shapes with use. This additive should never be added into acetonitrile-based solvents, as it can promote polymerization, which may clog the pump heads. Instead, use the additive only with aqueous mobile phase A. When using a mass spectrometer (MS)-based detector, it is recommended that ion polarities are switched in the method used, either by incorporating positive and negative ion transitions in a multiple reaction monitoring (MRM) method or by switching the polarity of the MS to the opposite polarity during the column re-equilibration.

Table 2. Chemicals.

Item	Vendor	Part Number	Specification and Notes
InfinityLab Deactivator Additive	Agilent	5191-4506	Medronic acid, no substitution, 50 and 25 mL volume available
Ammonium Hydroxide	Honeywell	44273-10x1mL	Alternate, Sigma 221228-100ML-A
Milli-Q Water, 0.22 µm Filter	Millipore	MPGP04001	HPLC-grade water is an alternate
Acetonitrile (ACN)	Sigma	34851	HPLC grade, >99.9%, 1 L × 4 vol. recommended
Isopropyl Alcohol (IPA)	Sigma	34863	HPLC grade, 99.9%, 1 L × 2 vol. recommended
Methanol	Sigma	646377	HPLC grade, 99.9%, 1 L × 2 vol. recommended
Pyrophosphoric Acid	Sigma	433314	Technical grade, 100 g recommended
Ammonium Acetate	Sigma	431311	≥99.99% trace metals basis
Acetic Acid	Sigma	A6283	Glacial, ≥99%
pH 7.0 Calibration Buffer	Sigma	BX1632-1	Replace the aliquot used for calibration monthly
pH 10.0 Calibration Buffer	Sigma	BX1633-1	Replace the aliquot used for calibration monthly

Hardware and software

The Agilent 1290 Infinity II bio LC is preferred for this work, because its MP35N alloy construction provides low interaction potential with metal-sensitive analytes and thus gives improved peak shapes and lower detection limits. The bio LC stack used in this method included an Agilent 1290 Infinity II bio high-speed pump (G7132A), an Agilent 1290 Infinity II bio multisampler outfitted with a thermostat and multiwash (G7137A), an Agilent 1290 Infinity II multicolumn thermostat (G7116B), and an Agilent InfinityLab Quick Change 2-Pos/10-port Bio Valve (G5641A). All the capillaries from the sampler onward were 0.12 mm id. Alternatively, a standard Agilent 1290 Infinity II LC may be used. However, the LC should be passivated to remove free metal ions from the system, as these impair metabolite chromatographic peak shape. A 1290 Infinity II high-speed pump is preferred over other LC models because of the low delay volume, and it delivers solvent very precisely. Precise delivery of the mobile phase is key to reproducible retention times; any variation in the delivery of percent mobile phase A between injections can cause significant variation, especially with HILIC.[1]

Column washing

Any new HILIC-Z column should be prepared with a series of solvents to optimize background and peak shape.

Warning: all the column preparation should be directed to waste, ideally by replacing the postcolumn line with a line headed to a waste container (hard plumb to waste and not the detector). Do not run the column flush solvents into the MS during the procedure, as they can contaminate and damage the detector.

First, pressure-equilibrate the column with a step-wise flow rate increase from 0.05 to 0.3 mL/min. Use 100% water that is purged through both channels A and B with 50% A and 50% B. After equilibration, flow water at 0.3 mL/min for approximately 1 hour. Next, replace the water with IPA and purge both channels. Adjust the flow to 0.1 mL/min and direct the flow to the column for 1 hour. Finally, replace the IPA with 50 mM pyrophosphoric acid in 90% acetonitrile,

purge the lines, and flow 0.1 mL/min for more than 6 hours and up to 72 hours; 12 to 18 hours is recommended. After the acid flush, place water on channel A and B and flow it through the column at 0.1 mL/min for 1 hour.

This acid flush phosphonates the system and is directly correlated with improved peak shape of analytes containing acidic moieties such as phosphate or carboxylic acid groups. If analytes used do not have these functional groups, then a shortened acid wash could be tested. If cross-column retention time alignment is required, the timing of the washing steps should be kept consistent or automated with set methods, because large variations in the wash procedures will lead to variations in observed retention time from column to column. This acid flush is highly recommended when beginning to use a new column. The flush can also be repeated if peak shape for analytes with phosphate and carboxylic acid groups are broadening, but it may decrease the chromatographic separations of other analytes such as leucine and isoleucine.

In the example method (Table 3), buffer salts are not added to mobile phase B. This omission improves retention time reproducibility by reducing the possibility of error in complex buffer preparation. Instead, the LC mixer is used to precisely mix water and acetonitrile together. The starting conditions for this column are best between 80% to 90% acetonitrile.

The percent water reaches 90% in the example gradient, which is considered much higher than what other HILIC columns can handle. This high percent water is advantageous to the HILIC-Z because washing at a higher percent water removes any water-soluble material that builds up in the LC or on the column frits, enhancing the longevity of the column. Without this, HILIC columns have the tendency to have significant pressure increases over the lifetime of the column, leading to faster analytical performance degradation. The HILIC-Z column's lifetime is extended because of the column's high-water tolerance and its ability to recondition to initial conditions more quickly. This gradient also does not have any isocratic holds for separation. Although isocratic holds can be a good way to achieve novel separation of isomers, reproducible retention times over large studies are often difficult to achieve.

The temperature of the column in the example method is set at 15 °C. Lower temperatures increase viscosity of the solvents and retention of early eluters, and thus their separation. However, lower temperatures also increase the peak width across the whole method. Alternatively, for a method that does not require the separation of analytes that elute at 10 to 15% water, increasing the temperature is recommended. **Note:** the HILIC-Z column can be heated up to 35 °C at pH 2 to 12 or 80 °C at pH 7.

Gradient

Table 3. An example method for global polar metabolomics chromatography using an Agilent InfinityLab Poroshell 120 HILIC-Z column.

Parameter	Value
Column	Agilent InfinityLab Poroshell 120 HILIC-Z, 2.1 × 150 mm, 2.7 µm (p/n 683775-924)
Column Temperature	15 °C
Injection Volume	1 to 5 µL
Autosampler Temperature	5 °C
Needle Wash	Standard wash, 10 s, IPA:ACN:H_2O 1:1:1
Mobile Phase*	A) 20 mM ammonium acetate, pH 9.3 + 5 µM medronic acid in water B) Pure ACN
Flow Rate	0.400 mL/min
Gradient Program	Time %B 0.00 90 1.00 90 8.00 78 12.00 60 15.00 10 18.00 10 19.00** 90 23.00 90
Total Run Time	24 min

* Mobile phase preparation and pH accuracy are key to reproducible retention times. Using a standard operating procedure for preparation is highly recommended.

** Increase the flow rate to 0.5 mL/min during re-equilibration to rebuild the water layer faster and decrease total run time.

Samples

When doing HILIC chromatography, it is important to recognize the impact of sample matrix and sample preparation on the robustness of the method used. Variations in salt, protein, or lipid content may lead to retention time shifts between different matrices or reduce column longevity. If the sample is complex, removing the proteins and lipids from the matrix is ideal for column lifetime and reproducibility. The evaluation of any new sample preparation should be expected when analyzing new or different sample matrices. Examples of good biological matrix sample preparation are provided in the References section.[5,6]

It is recommended that samples are prepared in 80% acetonitrile and 20% water for the best results for early eluters. With this solvent composition, a larger sample injection can be made. However, given various analyte solubilities, experiments can be performed that increase the water concentration, which in turn may increase the analyte solubility. With additional water, a smaller injection volume is necessary to minimize poor peak shape of early-eluting analytes resulting from sample solvent incompatibility.

Column cleaning and storage

The column can be cleaned by flushing with high percent water for an extended period (hours) and by heating the column. To store the column, routinely flushing a combination of pure water and acetonitrile (30% and 70%, respectively) is recommended.

Results and discussion

Many HILIC users may have previously experienced challenges with their methodology in terms of poor retention time reproducibility. However, with the example methodology provided here and by carefully following the guidelines in this document, it is possible to obtain reproducible retention times with HILIC-Z columns. This was demonstrated by preparing and testing three different lots of columns with six different batches of solvent and two different LC/MS users: one advanced and one novice. A standardized plasma sample extract that was spiked with additional compounds shows stable retention times across the gradient and across multiple column lots and batches of solvent (Figure 1).

Importantly, stable retention times were demonstrated across different manufactured lots of this column and solvent preparations, which have been historical pain points for HILIC. This shows that, regardless of the column or day of testing, it is possible to get reproducible retention times with HILIC columns, making data alignment and analysis easier. Intralot variation resulted in a maximum retention time difference of 0.8 minutes for serine, eluting in the center of the gradient. Because of the additional retention time error during the gradient, it is recommended that a wider retention time window is used for analytes during the gradient, so that the instrument does not miss the acquisition of the target analyte. More commonly, at the stable early or late portion of the gradient, the intralot retention time difference was <0.3 minutes. This reduced variation

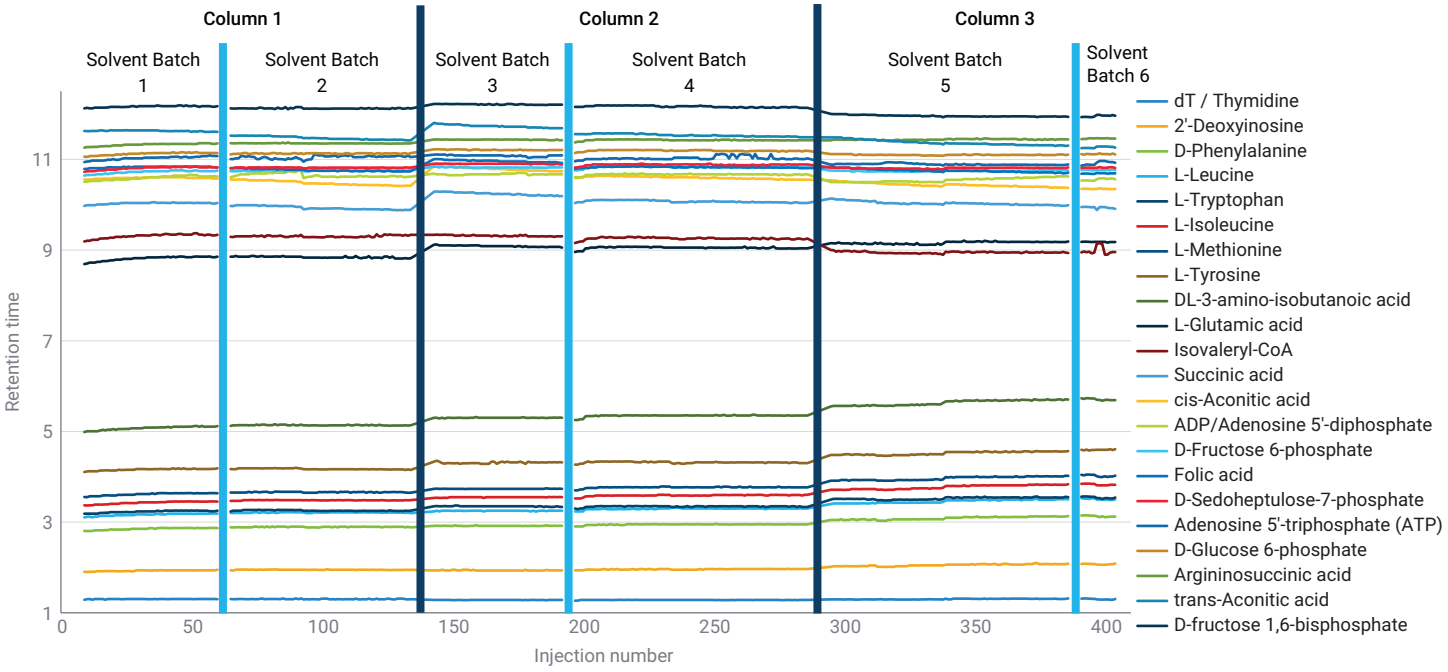

Figure 1. Multicolumn, 8-day, retention time study. Twenty-two analytes in plasma extract show the reproducibility of the Agilent InfinityLab Poroshell 120 HILIC-Z column. None of the analytes have a retention time relative standard deviation greater than 4%. **Note:** The columns and solvent batches last longer than what was tested here. They were changed more frequently to force error in the experiment.

allows for a smaller data alignment window with untargeted LC-Q/TOF studies and smaller dMRM windows with LC/TQ, improving dwell times in these more crowded regions.

The HILIC-Z column with a 24 minutes method can measure many different classes of analytes, including organic acids, amino acids, and nucleotides. Due to the method's stability, it can be coupled to a comprehensive dynamic MRM TQ or Q-TOF retention time database to help detect and identify compounds as part of a global polar metabolomics method. Figures 2 to 4 show how different metabolite classes are distributed over the gradient.

Over the course of a study, it is critical to maintain the peak shape, separation of critical isomeric/isobaric analytes, and stable retention times. Doing so will improve the overall quality and confidence in the data while reducing the data analysis and interpretation challenges. With the HILIC-Z column, the provided example gradient, and experimental considerations, it is possible to achieve stable chromatograms even when using a complex matrix (Figures 5A and 5B). This column and method are also resistant to pressure increases associated with using analyte extracts from complex matrices, which are a common problem with HILIC columns. No LC inline or precolumn filter was used with this data set, and the pressure trace for a biological extract did not significantly increase over the course of the study (Figure 5C).

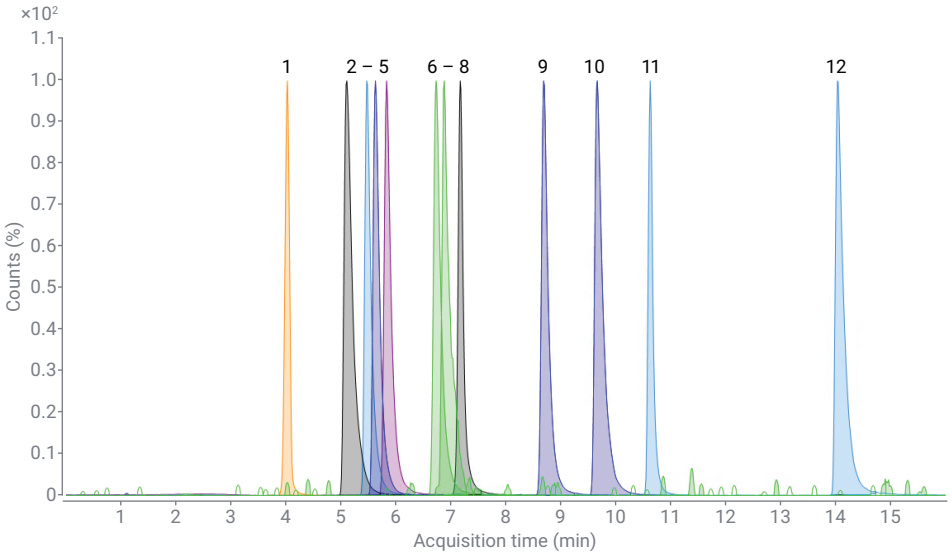

Figure 2. Amino acid chromatograms. These analytes elute over most of the chromatogram. 1. tryptamine; 2. phenylalanine; 3. leucine; 4. tryptophan; 5. isoleucine; 6. tyrosine; 7. valine; 8. proline; 9. alanine; 10. glutamine; 11. glutamic acid; 12. arginine. Some amino acids, such as histidine, have better peak shape at low pH, demonstrating that compromises need to be made when selecting buffer pH and detectable analytes.

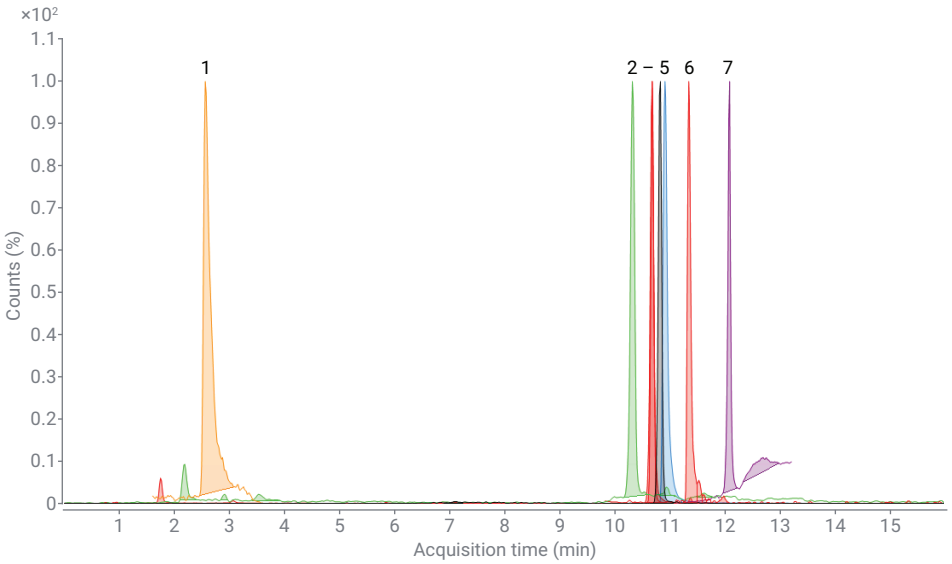

Figure 3. The TCA cycle analytes most detectable using a high pH buffer. 1. pyruvic acid; 2. oxaloacetic acid; 3. a-ketoglutartic acid and fumaric acid (overlapping peaks); 4. succinate; 5. acetyl CoA; 6. succinyl co-enzyme A; 7. citric acid. Not shown is malic acid, which elutes at 10.5 minutes and overlaps with compounds 2 to 5.

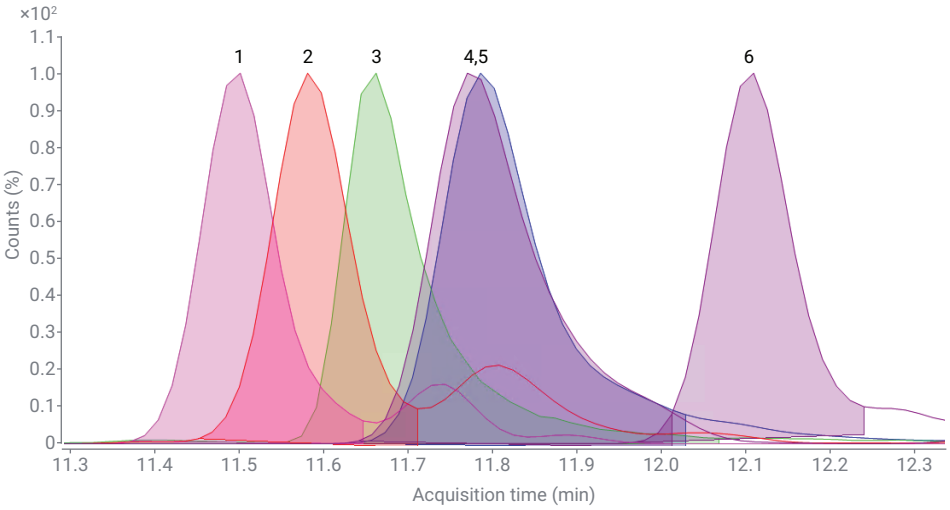

Figure 4. Phosphate-containing analytes elute later in the example method and with higher water content. The Agilent 1290 Infinity II bio LC is a preferred system for these metal sensitive analytes, as it is easier to maintain their peak shape. 1. N-acetyl-D-glucosamine 6-phosphate; 2. UDP; 3. glucose 6-phosphate; 4. UTP; 5. ATP; 6. GTP.

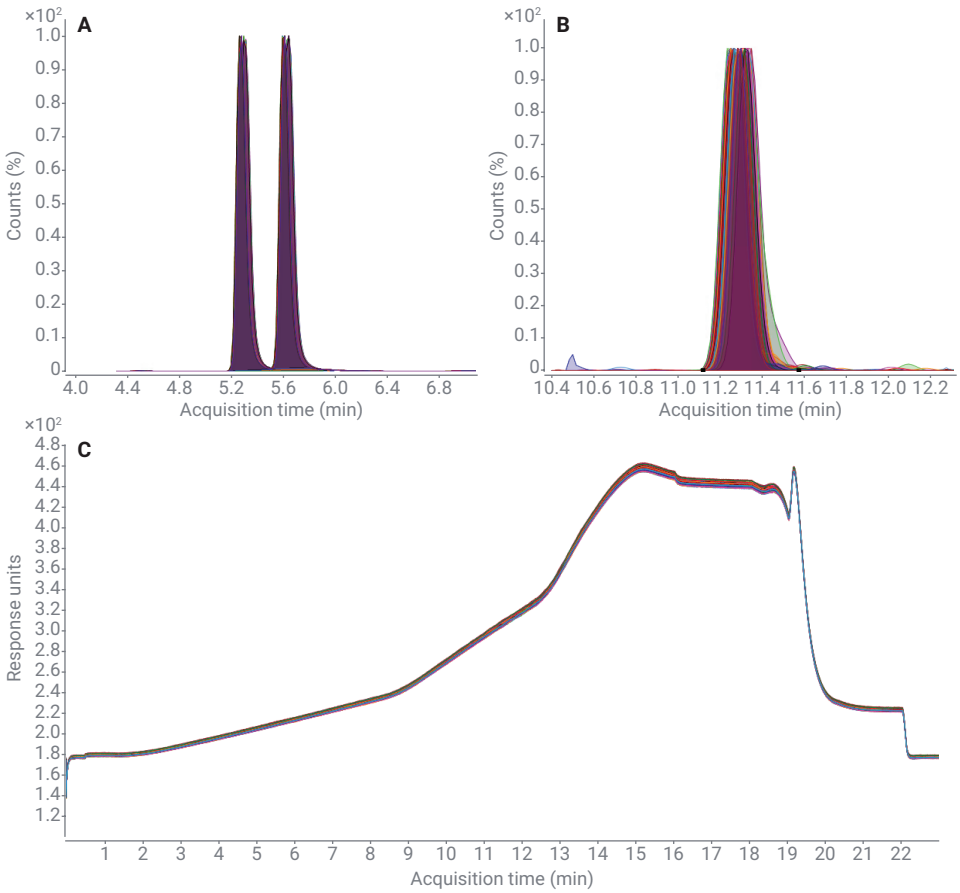

Figure 5. Stability of chromatogram with complex cell extract (n = 77). (A) Leucine (5.3 minutes) and isoleucine (5.6 minutes) are isobaric analytes that remain separated over a data set. (B) A late eluter such as acetyl-CoA is also stable over the same dataset. (C) The pressure trace of the method is also reproducible without a significant pressure increase when running a complex matrix.

Conclusion

Historically, polar analyte separation has required derivatization, ion-pair reagents, or an expert HILIC user to meet experimental goals. The derivatization and ion pair approach have had pain points such as complex sample preparations or the requirement for dedicated instruments, respectively. In the past, HILIC had a reputation for yielding poor reproducibility, especially regarding retention time stability of analytes when compared to reversed-phase chromatography. Here, we showed improvements in the hardware, chemistry, and protocols for using HILIC, making it more accessible to new users and providing great data quality. Acquiring reproducible retention times and good, consistent peak shape for polar metabolites can be accomplished with the HILIC-Z column, making it suitable for larger multiday studies as well as method standardization. As with any method, there are useful tips and tricks, and the recommendations described in this application note should be used to help achieve the best results.

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