Application Note

Small Molecule Pharma

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Retaining and Separating Polar Molecules—A Detailed Investigation of When to Use HILIC Versus a Reversed-Phase LC Column

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Abstract

This work examines when to use hydrophilic interaction LC (HILIC) and when to use reversed-phase LC (RPLC) columns for analyzing difficult-to-retain small polar and ionized molecules. The data presented demonstrate the impact of analytes/samples, mobile phases, modes of detection, and other method suitability requirements (retention, resolution, sensitivity, and peak shape).

Introduction

Adequately retaining and separating small polar molecules with reversed-phase liquid chromatography is a challenging task. Alkyl phase LC columns, such as C18, are a common starting point for LC method development. However, highly polar analytes have a low affinity for, and are poorly retained on, nonpolar C18 stationary phases. Several techniques may be explored to retain these compounds in reversed-phase mode, such as adding an ion pairing reagent to the mobile phase, or derivatizing the analytes. Ion pairing reagents can contaminate LC columns and systems, and are generally not suitable for LC/MS. Derivatization is often avoided, as it is a cumbersome extra step that leads to longer sample preparation times, and introduces the possibility for errors and issues with reproducibility.

A better practice to successfully retain and separate polar analytes is to explore the wide variety of stationary-phase chemistries available. Several chemistries are well suited for troublesome polar analytes. In the reversed-phase mode, there are alternate chemistries that are more polar, allowing for stronger interactions between polar analytes and the stationary phase. Many of these phases can also be used with very weak mobile phases, such as 100% aqueous, to allow for better retention of polar compounds. In addition to reversed-phase chemistries, there are also several hydrophilic interaction LC (HILIC) column phases on the market. HILIC mode uses polar stationary phases to retain polar analytes. Which column and which mode of LC is optimally used for a given analysis may be determined by other method factors, including analyte solubility, mobile phase, and preferred mode of detection.

Modern LC columns are typically packed with superficially porous particles, known for their ability to generate high efficiency with low backpressure. High efficiency can contribute to resolving closely eluting peaks, while low backpressure allows for flexibility with LC instrumentation.

This work demonstrates a logical, stepwise methodology to enable chemists to optimally retain, separate, and detect their polar analytes with superficially porous particle LC columns.

Experimental

An Agilent 1290 Infinity II LC with an Agilent Ultivo LC/TQ was used in this experiment. The system was modified from its standard configuration to have lower system volume and dispersion. Table 1 shows the configuration details.

Table 1. System configuration.

Agilent 1290 Infinity II LC System Configuration	
Agilent 1290 Infinity II Flexible Pump (G7104A)	• Degasser • Seal wash pump • 35 µL solvent mixer: Agilent Jet Weaver, 35 µL/100 µL (p/n G4220-60006) • Firmware: B.07.23 [0009]
Agilent 1290 Infinity II Vialsampler (G7129B)	• Sample thermostat (p/n G7167-60101) • Metering parameter: seat assembly PEEK 0.12 mm, sample loop 20 µL, analytical head 20 µL • Autosampler → heater: capillary, stainless steel, 0.12 × 105 mm, SL/SL (p/n 5500-1238) • Vial, screw top, amber with write-on spot, certified, 2 mL, 100/pk (p/n 5182-0716) • Cap, screw, blue, PTFE/red silicone septa, 100/pk (p/n 5182-0717) • Vial insert, 250 µL, glass with polymer feet, 100/pk (p/n 5181-1270) • Firmware: D.07.23 [0009]
Agilent InfinityLab LC Series Integrated Column Compartment (G7130A)	• Integral type: G7129B • 3.0 µL heat exchanger • Heater → column: A-Line quick-connect assembly, 105 mm, 0.075 mm (p/n 5067-5961) • Column → flow cell: capillary, stainless steel, 0.075 × 220 mm, SV/SLV (p/n 5067-4784) • Firmware: B.07.23 [0009]
Agilent Ultivo LC/TQ (G6465A)	• Agilent Jet Stream ESI Source
Agilent 1290 Infinity II Diode Array Detector (G7117B)	• Ultralow dispersion Max-Light cartridge flow cell, 10 mm, 0.60 µL (p/n G4212-60038) • UV lamp (5190-0917) • Firmware: D.07.23 [0009]
Agilent LC Columns	• Agilent InfinityLab Poroshell 120 HILIC-Z, 2.1 × 100 mm, 2.7 µm (p/n 685775-924) • Agilent InfinityLab Poroshell 120 PFP, 2.1 × 100 mm, 2.7 µm (p/n 695775-408) • Agilent InfinityLab Poroshell 120 Phenyl-Hexyl, 2.1 × 100 mm, 2.7 µm (p/n 695775-912) • Agilent InfinityLab Poroshell 120 HILIC-OH5, 2.1 × 100 mm, 2.7 µm (p/n 685775-601) • Agilent ZORBAX RRHD Eclipse Plus C18, 2.1 × 100 mm, 1.8 µm (p/n 959758-902) • Agilent ZORBAX RRHD HILIC Plus, 2.1 × 100 mm, 1.8 µm (p/n 959758-901)

Figure 1 shows the Agilent stationary phases used in this study. All columns were used in a 2.1 × 100 mm format. Table 1 details specific column part numbers.

Detailed LC method parameters are shown with their respective figures throughout this document.

All analytes and mobile phase additives used in this work were purchased from Sigma-Aldrich (St. Louis, MO, USA). Acetonitrile was purchased from Honeywell (Burdick and Jackson, Muskegon, MI, USA). Water was 0.2 µm filtered 18 MW from a Milli-Q system (Millipore, Burlington, MA, USA).

Results and discussion

Hydrophilic interaction LC (HILIC) and reversed-phase LC (RPLC) are complimentary chromatographic techniques. Table 2 highlights a few key characteristics between the two LC methodologies. RPLC columns use mostly nonpolar stationary phases (C18, C8, and so forth) while HILIC columns use polar phases (silica, amide, and so forth). Mobile phases for both techniques are often comprised of acetonitrile and water, which allows the flexibility to easily switch between the two LC modes.

The primary difference in mobile phase between HILIC and RPLC is solvent elution strength. For RPLC, acetonitrile is the strong eluting solvent. However, for HILIC, water is the strong eluting solvent. The resulting chromatography is typically polar to nonpolar analytes for RPLC, with the opposite being true for HILIC. This reversal of elution order is what makes HILIC a nice complement to the more commonly used RPLC. This is especially true for polar and ionized analytes, which will be retained longer in HILIC mode.

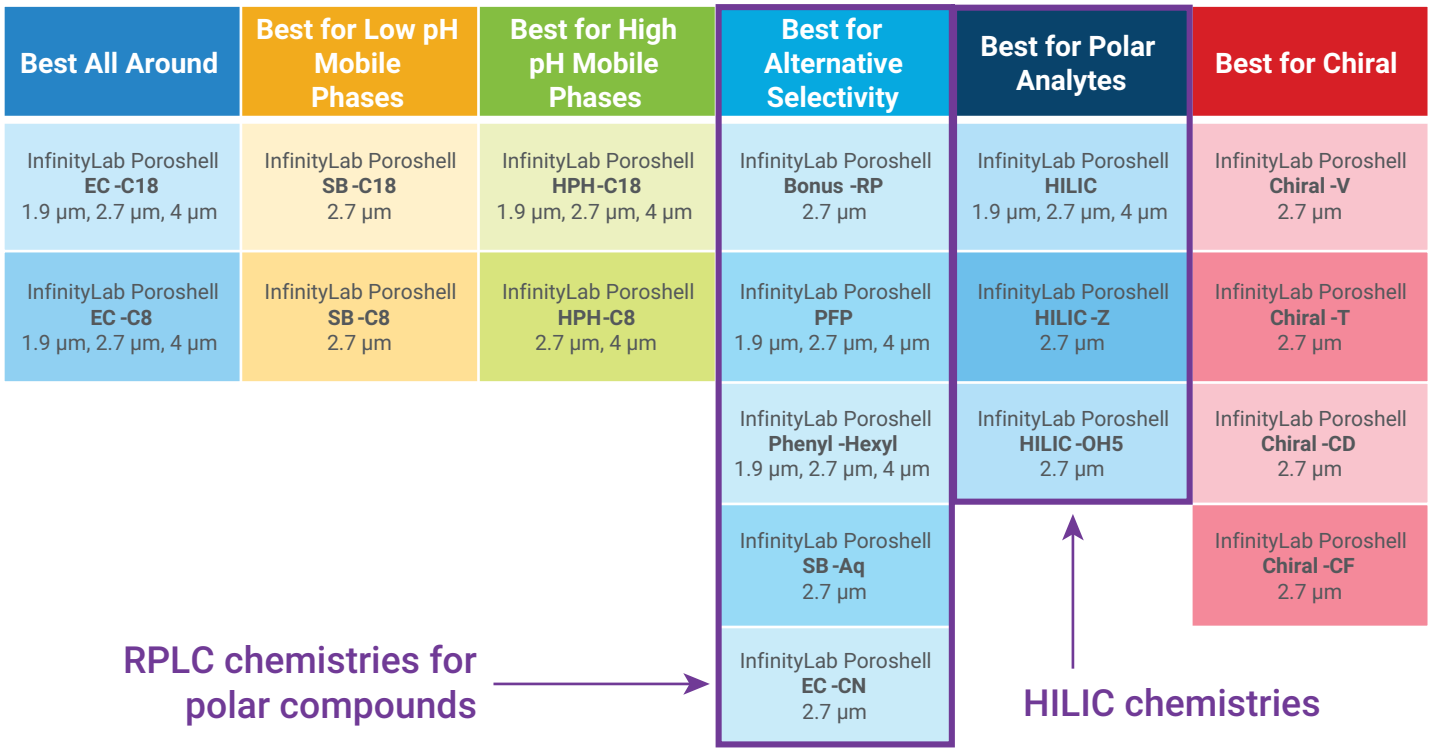


Figure 1. Agilent InfinityLab Poroshell 120 portfolio for small molecule separations.

Table 2. HILIC complements RPLC.

Reversed-Phase LC	Hydrophilic Interaction LC
Nonpolar stationary phase (for example, C18)	Polar stationary phase (for example, silica)
Polar mobile phase • Water/acetonitrile, water/methanol	Polar mobile phase • Water/acetonitrile
Decrease retention by decreasing polarity of mobile phase • Increase acetonitrile in mobile phase to decrease retention	Decrease retention by increasing polarity of mobile phase • Increase water in mobile phase to decrease retention
Elution order: polar to nonpolar	Elution order: nonpolar to polar

Deciding which LC technique to use for polar compound analyses can be challenging. Careful consideration should be given to which type of method will meet separation requirements, will work best with the sample, and which types of detection are feasible.

Column choices

When analyzing polar compounds, consider a wide variety of stationary phase chemistries. For simplification within this paper, one family of LC columns was studied; the phases are shown in Figure 1. The Agilent InfinityLab Poroshell 120 family of LC columns contains 18 unique column chemistries on three scalable superficially porous particle sizes. The phases highlighted in the boxes should be evaluated for polar compound analysis. HILIC chemistries are often thought to be the default for polar compounds. However, there are several good RPLC choices as well. The RPLC phases highlighted in Figure 1 can be run with 100% aqueous mobile phase, without the risk of dewetting; this can help with polar or ionized compound retention.

In most cases, the best column for an analysis will provide suitable retention, resolution, and peak shape for the compounds of interest; additional method suitability criteria may also be required. Resolution is particularly critical when using nonselective detections, such as diode array (DAD), fluorescence (FLD), evaporative light scattering (ELSD), or refractive index (RID). With a mass spectrometer (MS), chromatographic resolution may still be necessary if isobars are present.

Consider the example separation of amino acids in Figure 2. The HILIC column provides good retention and resolution of the analytes, including the leucine and isoleucine isobaric pair. However, the RPLC column is unable to retain most of these free amino acids. Most amino acids elute near the void with RPLC, providing no

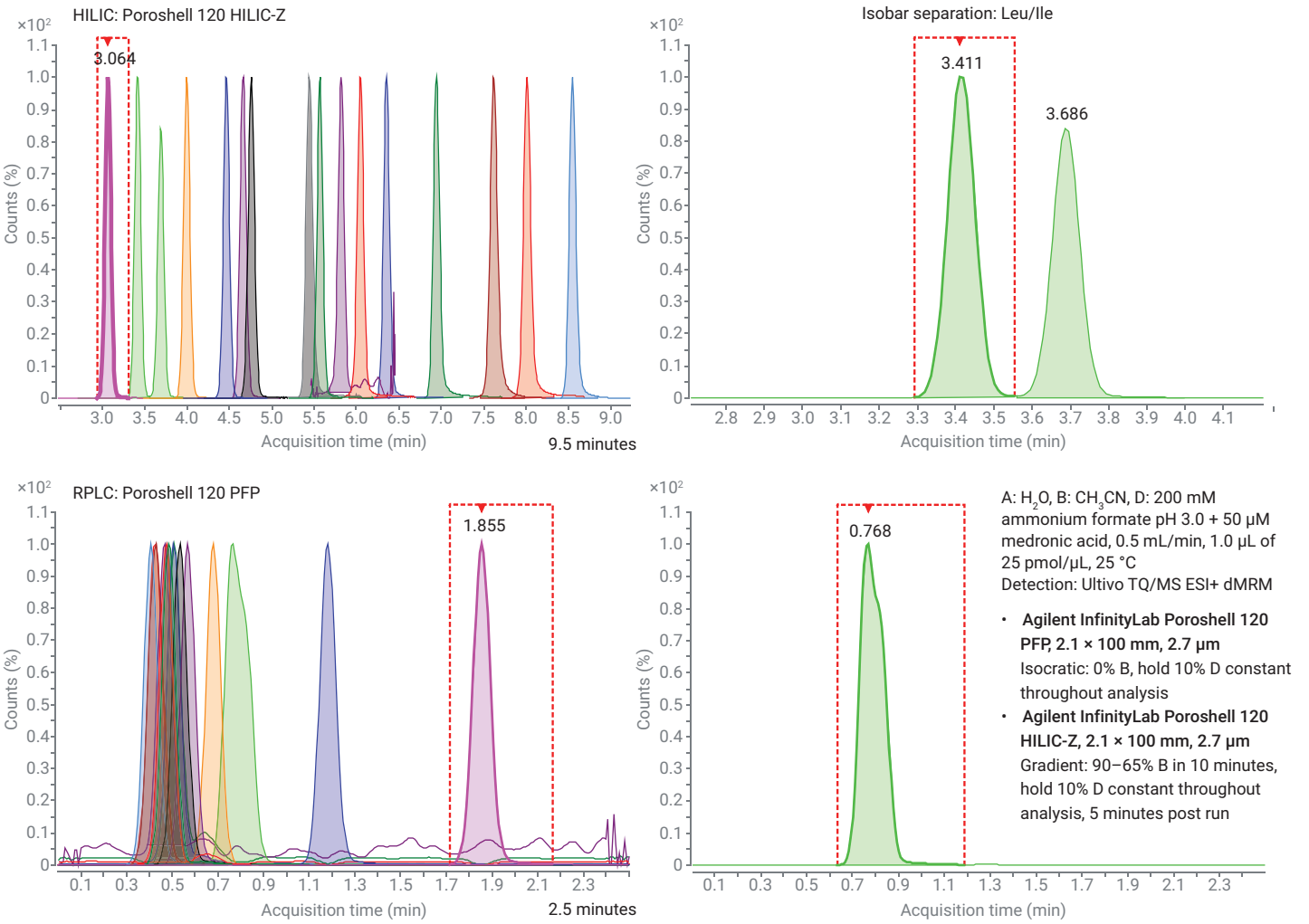


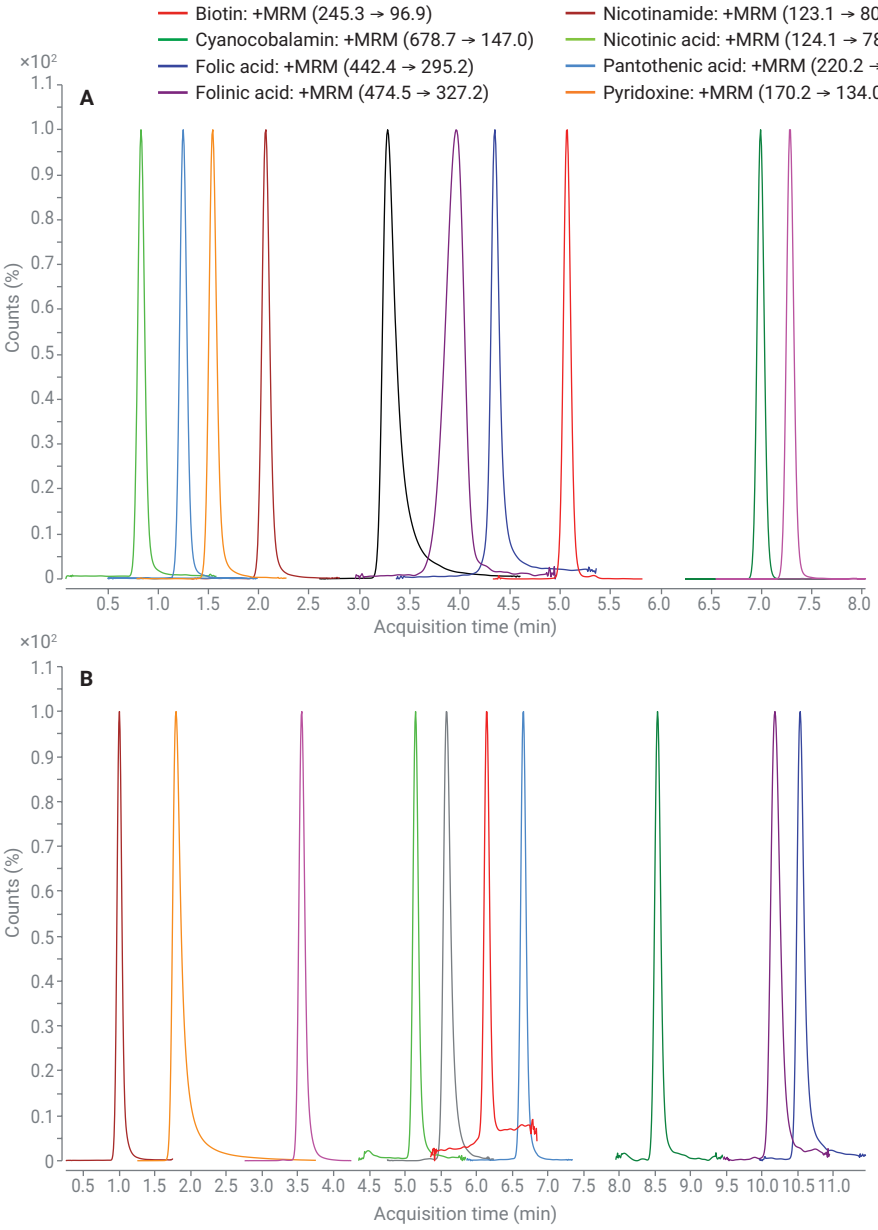
Figure 2. HILIC retains amino acids and separates isobars, while RPLC can't.

chromatographic resolution. Because this analysis was performed with an MS, these compounds can still be isolated and integrated; however, it is possible that ion suppression could occur with all the coeluting species. Moreover, the isobars leucine and isoleucine are not well resolved. In the case of these free amino acids, and other samples where RPLC cannot retain the analytes, HILIC would be preferred over RPLC.

Note: Amino acids can be analyzed by RPLC/UV after undergoing OPA/FMOC derivatization, as described in Agilent publication 5991-5571EN.[1]

For samples such as amino acids, it might be obvious which mode of LC to choose. However, for some samples, either RPLC or HILIC may provide good separations. See the analyses of water-soluble vitamins in Figure 3.

All 10 vitamin compounds are baseline resolved with RPLC and HILIC columns. In cases such as this, where either technique can provide the separation, it might be desirable to consider the sample and which mode of detection is preferred to help determine whether RPLC or HILIC would be best.



Mobile Phase A	H_2O
Mobile Phase B	CH_3CN
Mobile Phase D	200 mM ammonium acetate + 0.2% acetic acid, pH ~5.3
Flow Rate	0.5 mL/min
Gradient	0% B for 1 minute, 0 to 25% B in 8 minutes, hold 5% D constant throughout analysis, 3 minutes post run
Injection	0.5 µL of 0.4 µg/mL vitamin standard in H_2O
Column	25 °C, Agilent InfinityLab Poroshell 120 Phenyl-Hexyl, 2.1 × 100 mm, 2.7 µm
Detection	Agilent Ultivo TQ/MS ESI+ dMRM, DAD Sig = 260 nm, 80 Hz

Mobile Phase A	H_2O
Mobile Phase B	CH_3CN
Mobile Phase D	200 mM ammonium acetate (no pH adjustment), pH ~6.7
Flow Rate	0.5 mL/min
Gradient	95 to 65% B in 10 minutes, hold 5% D constant throughout analysis, 5 minutes post run
Injection	0.5 µL injection of 0.4 µg/mL vitamin standard in CH_3CN
Column	25 °C, Agilent InfinityLab Poroshell 120 HILIC-OH5, 2.1 × 100 mm, 2.7 µm
Detection	Agilent Ultivo TQ/MS ESI+ dMRM (parameters above), DAD Sig = 260 nm, 80 Hz

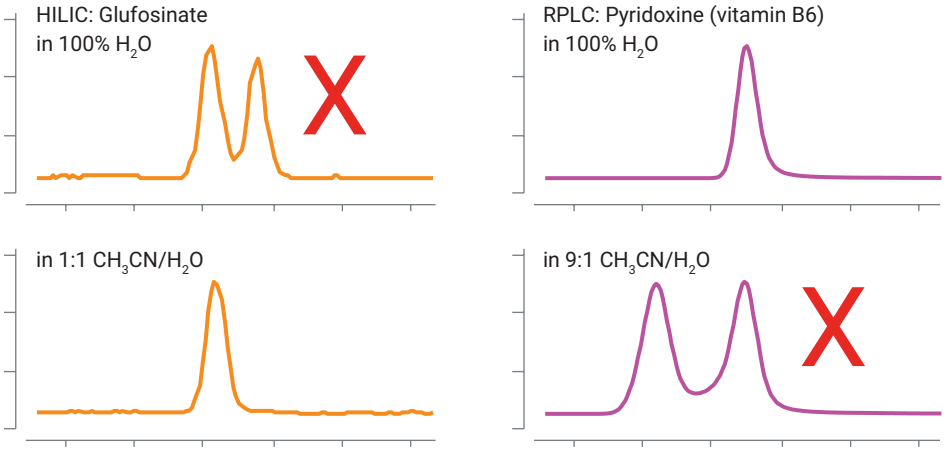
Figure 3. Both RPLC (A) and HILIC (B) are able to retain and separate water-soluble vitamins. RPLC: Agilent Poroshell 120 Phenyl-Hexyl, HILIC: Agilent Poroshell 120 HILIC-OH5.

Sample considerations

Strong solvent should not be injected onto the column with either RPLC or HILIC. For RPLC, a weak sample diluent will contain mostly water. For HILIC, a weak sample diluent will contain mostly acetonitrile. Figure 4 shows that both techniques suffer from undesirable peak shape if a strong solvent is injected. However, injecting a respectively weaker sample solvent results in a well defined peak with good symmetry.

The effect of injection solvent strength is more pronounced for early eluting compounds, and for isocratic analyses. Strong solvent effects are also greater with larger injection volumes, as shown in Figure 5. Performance can still be maintained with larger injection volumes, if weaker sample solvents are used. Balancing the sample solvent strength with injection volume is necessary to maintain chromatographic performance for both HILIC and RPLC.

While adjusting the strength of the sample solvent, pay careful attention to analyte solubility. In Figure 6, a HILIC analysis of three vitamins is shown. Each vitamin was prepared to a stock concentration of 4 µg/mL, then diluted 9:1 into either water or acetonitrile. Because this is an HILIC analysis, the samples would preferably be prepared in an acetonitrile-rich sample solvent. Looking at nicotinic acid first, there are comparable peak areas between the water sample and the acetonitrile sample. This indicates that the same amount of nicotinic acid was injected onto the column in each case. However, for thiamine and cyanocobalamin, the acetonitrile sample results in approximately half of the peak area, compared to the sample prepared in water. This loss of peak area is a result of poor solubility of thiamine and cyanocobalamin in acetonitrile.



Mobile Phase A	10 mM ammonium acetate, pH 9
Mobile Phase B	100 mM ammonium acetate, pH 9 in 90% ACN (final concentration: 10 mM)
Flow Rate	0.6 mL/min
Gradient	90 to 60% B in 10 minutes
Injection	4 µL of glufosinate, 100 ppb
Column	InfinityLab Poroshell HILIC-Z, 2.1 × 100 mm
Temperature	30 °C
System	Agilent 6490 LC/QQQ

Mobile Phase A	H_2O
Mobile Phase B	CH_3CN
Mobile Phase D	200 mM ammonium acetate + 0.2% acetic acid, pH ~5.3
Flow Rate	0.5 mL/min
Gradient	0% B for 1 minute, 0 to 25% B in 8 minutes, hold 5% D constant throughout analysis
Injection	0.5 µL of 13 µg/mL pyridoxine
Column	25 °C, Agilent InfinityLab Poroshell 120 Phenyl-Hexyl, 2.1 × 100 mm, 2.7 µm
Detection	Agilent Ultivo TQ/MS ESI+ dMRM

Figure 4. Strong injection solvents distort peak shapes for HILIC and RPLC.

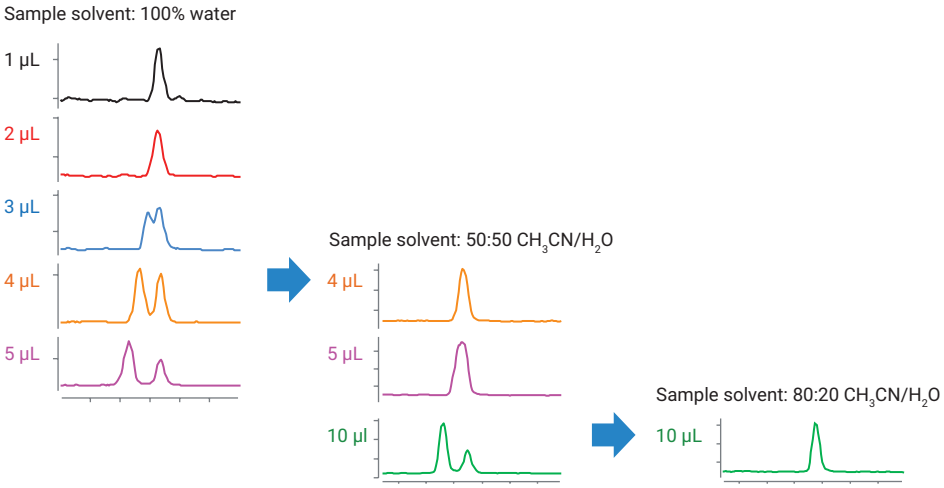
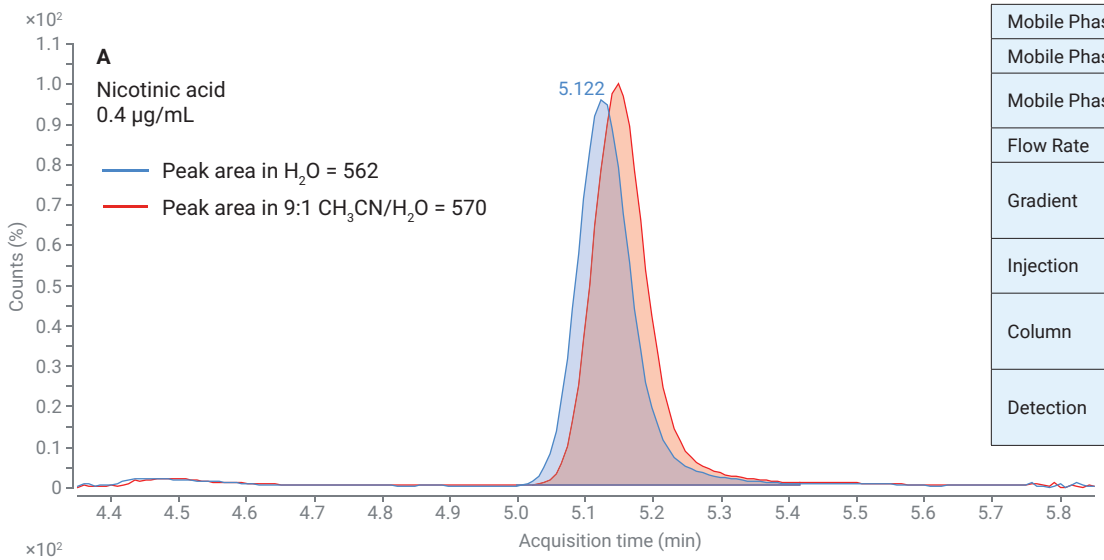
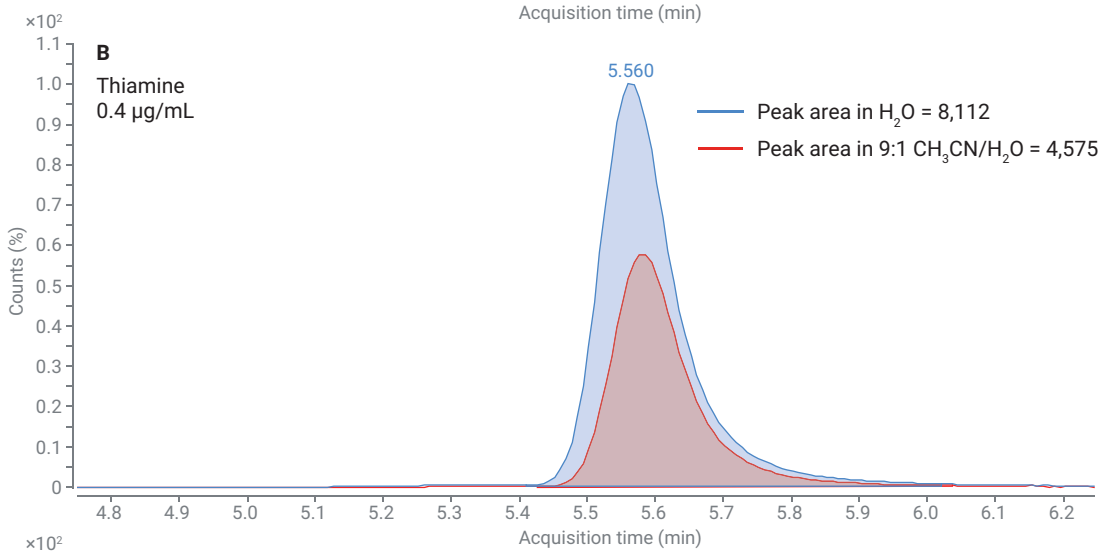
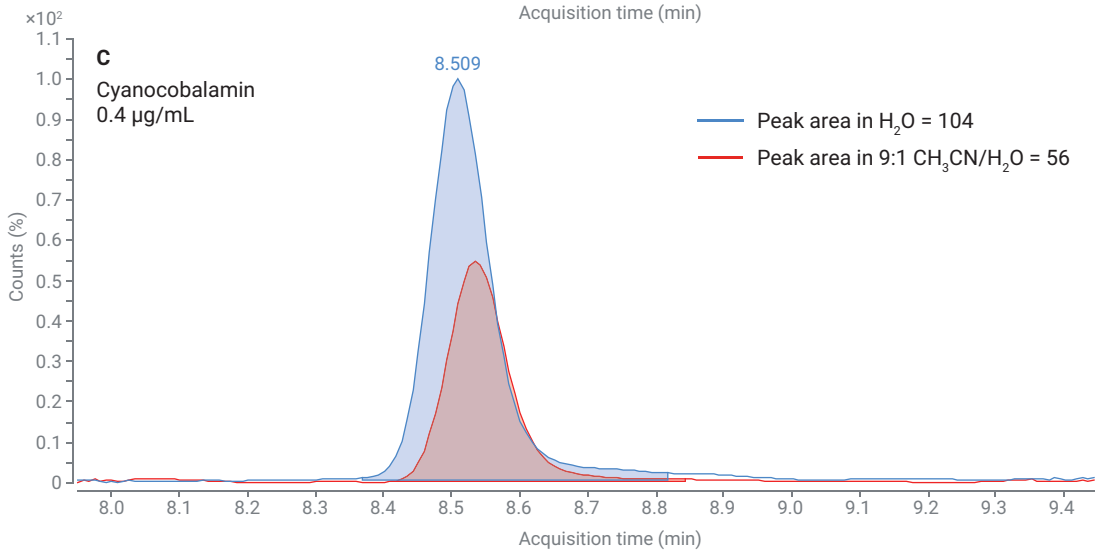


Figure 5. Strong solvent effects are greater with larger injection volumes. Method parameters are the same as in Figure 4.



Mobile Phase A	H_2O
Mobile Phase B	CH_3CN
Mobile Phase D	200 mM ammonium acetate (no pH adjustment), pH ~6.7
Flow Rate	0.5 mL/min
Gradient	95 to 65% B in 10 minutes, hold 5% D constant throughout analysis, 5 minutes post run
Injection	0.5 µL injection of 0.4 µg/mL vitamin standard in CH_3CN
Column	25 °C, Agilent InfinityLab Poroshell 120 HILIC-OH5, 2.1 × 100 mm, 2.7 µm
Detection	Agilent Ultivo TQ/MS ESI+ dMRM (parameters above), DAD Sig = 260 nm, 80 Hz





~50% sample lost due to poor solubility in CH_3CN

Figure 6. Ensure that samples are completely soluble. HILIC analysis of three vitamins at 0.4 µg/mL in water and 9:1 CH_3CN/H_2O.

To improve this analysis, thiamine and cyanocobalamin could be diluted to a soluble concentration in an acetonitrile-rich solution, or, if sensitivity necessitates a more concentrated sample, RPLC analysis of these compounds may be preferred. Polar compounds will generally be more soluble in water. So, if high concentration samples are needed, RPLC may be the preferred LC mode.

Modes of detection

There are several detector options for HPLC analyses. Table 3 lists some of the more common ones. Which detector you choose to work with may depend upon the properties of your analytes, as well as the sensitivity requirements of the analysis. Figure 7 shows an example of compounds that can be analyzed by MS, but not by UV. In this case, vitamins B7 and B5 cannot be detected at 260 nm, but they can be analyzed by MS. Therefore, if B7 and B5 are critical to the analysis, then MS detection would be necessary over UV.

Table 3. Common HPLC detectors.

Detector Type	
UV, VIS Absorbance	For light-absorbing compounds
Refractive Index	Universal detection, but poor sensitivity; can only run isocratic
Evaporative Light Scattering	For nonvolatile analytes
Mass Spectrometer	Low limits of detection based on molecular weight
Fluorescence	For compounds that fluoresce or can be derivatized to do so

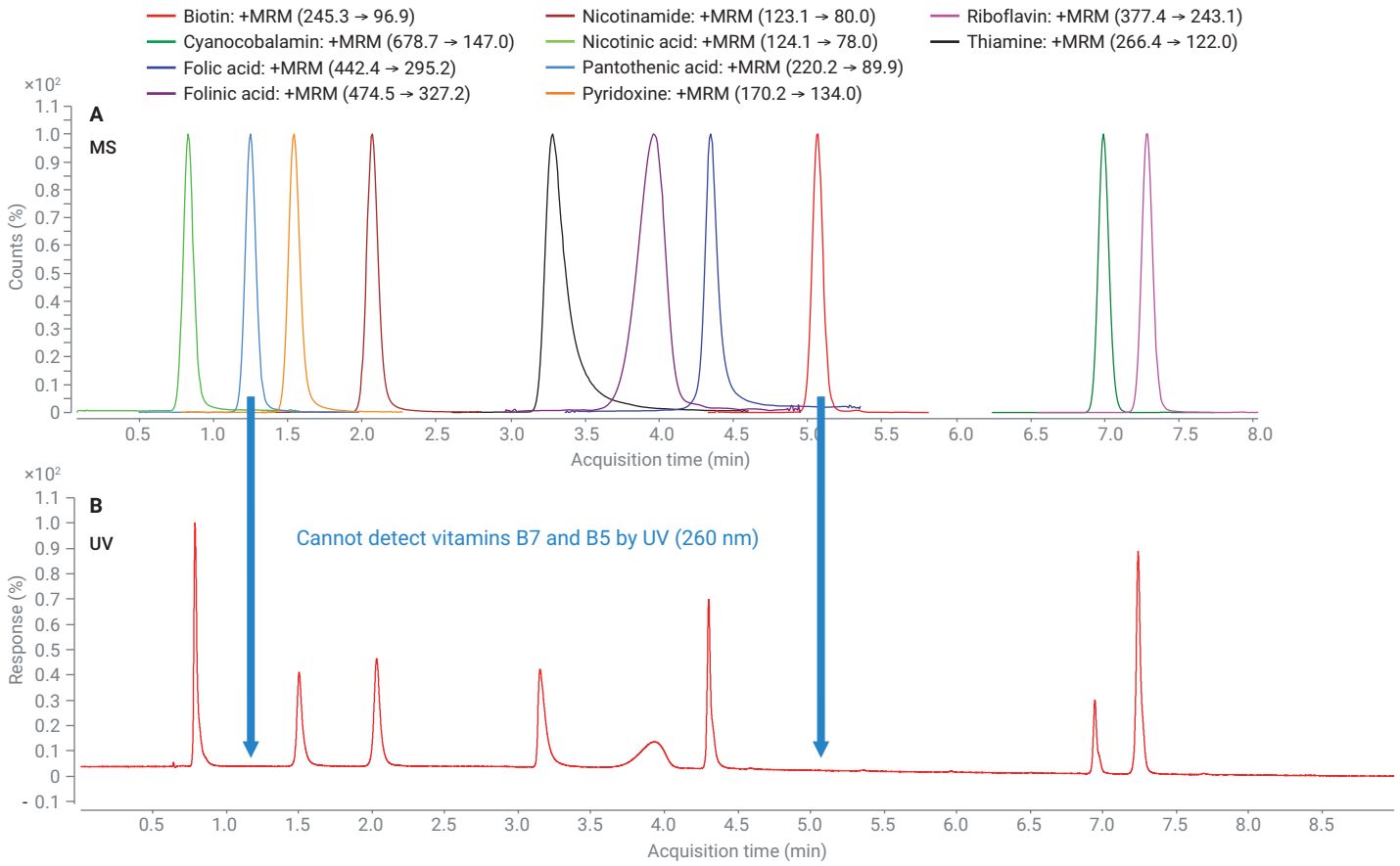


Figure 7. Choose a detector that can analyze compounds of interest. Method parameters are the same as in Figure 3.

If the analytes dictate a certain type of detection, this may suggest whether to use HILIC or RPLC for the analysis. HILIC pairs well with MS, as shown in Figure 8. Buffers used in HILIC mode must be fairly soluble in acetonitrile.

These buffers are often formates or acetates, which also happen to be volatile—a must for LC/MS. The synergy between the volatile HILIC buffers, paired with the high volatility of a mostly acetonitrile mobile phase, leads to better ionization for LC/MS analyses, as shown in Figure 8. In Figure 8, the HILIC analysis has less baseline noise, and a taller peak for the morphine metabolite, M6G.

Mobile Phase A	10 mM ammonium formate pH 3.2 in water
Mobile Phase B	Acetonitrile/100 mM ammonium formate pH 3.2 in water (9:1)
Flow Rate	0.4 mL/min
Elution	Isocratic
Injection	2 µL of 1 µg/mL each of morphine-3-β-D-glucuronide, and morphine-6-β-D-glucuronide
Columns	2.1 × 100 mm, 1.8 µm
Temperature	25 °C
MS Source	ESI+, 200 V, 250 °C, 11 L/min, 30 psi, 4,000 V
SIM	462, Frag 170 V
Agilent publication number 5991-0245	

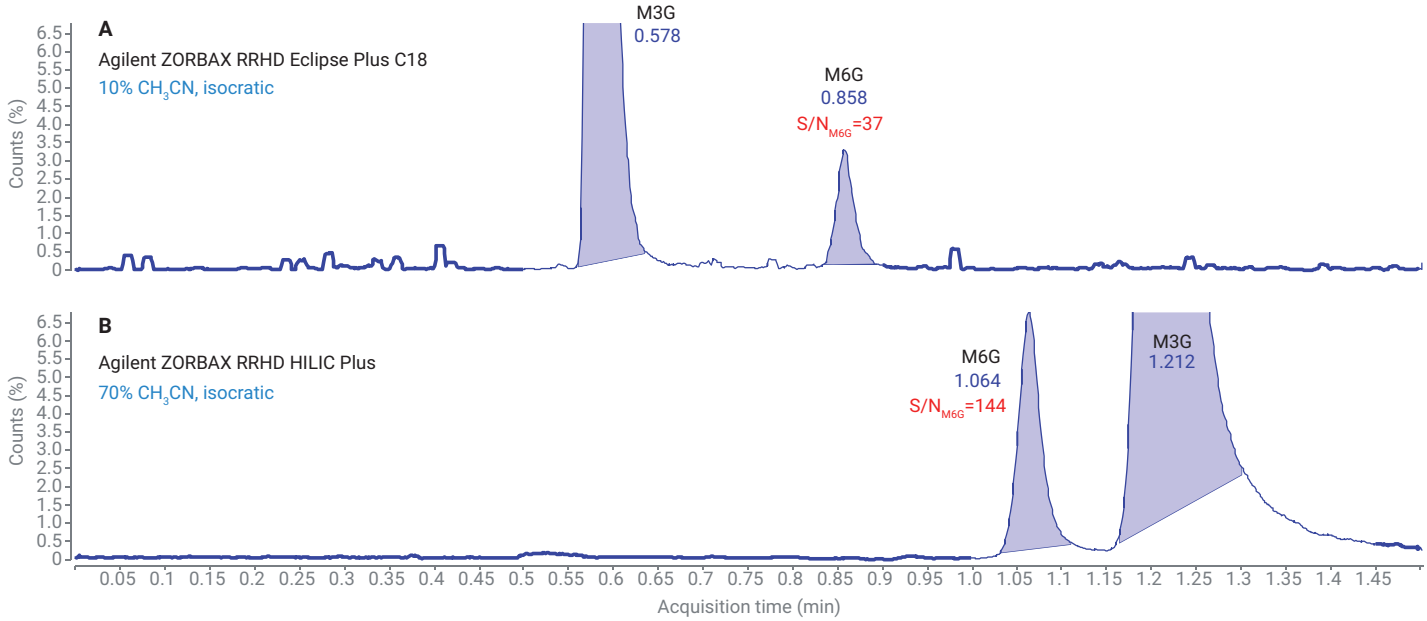


Figure 8. HILIC pairs well with LC/MS, and can improve sensitivity compared to RPLC for opioid metabolites.

If method development is difficult, and more mobile phase flexibility is needed, then RPLC with UV detection may be desired. All the common buffer options listed in Table 4 are compatible with RPLC and UV detection. Conversely, the lack of compatibility of most of the low pH options and the phosphates is shown for HILIC and MS analyses. If very low pH is needed for an analysis, such as for acidic compounds, then RPLC may be the preferred methodology.

Table 4. Reversed-phase LC and UV detection are compatible with a wider range of mobile phases, especially at Low pH.

Mobile Phase	Useable pH/Range	Recommended for HILIC?	Recommended for MS?	Recommended for RPLC and UV?
TFA	<1.5	No	No	Yes
Phosphate	1.1–3.1	No	No	Yes
Formic Acid	<2.8	No	Yes	Yes
Acetic Acid	<3.8	No	Yes	Yes
Formate	2.8–4.8	Yes	Yes	Yes
Acetate	3.8–5.8	Yes	Yes	Yes
Carbonate	5.4–7.4	Yes	Yes	Yes
Phosphate	6.2–8.2	No	No	Yes
Bicarbonate	6.6–8.6	Yes	Yes	Yes
Ammonia	8.2-10.2	Yes	Yes	Yes
Phosphate	11.3-13.3	No	No	Yes

Conclusion

Find the best column to retain and separate all analytes.

- RPLC cannot retain all polar/ionized analytes; HILIC may work for these.
- Some analytes can be retained and separated equally well in both modes of LC.

Consider the sample: analyte solubility and sample solvent.

- Injecting strong solvent in both RPLC and HILIC will negatively affect chromatographic quality.
- Strong solvent effects get worse with larger injection volumes.
- Polar compounds are generally more soluble in water than acetonitrile, which is good for RPLC.

What type of detection is available?

- Ensure that analytes are compatible with detector choice, and that sensitivity needs are met.
- HILIC can improve LC/MS analyses due to more volatile mobile phases.
- UV and RPLC are compatible with a wider variety of mobile phases, which may improve analyte retention and separation.

Choosing between HILIC and RPLC represents a balancing act that benefits from careful consideration of chromatographic separation requirements, analyte polarity and solubility, the nature of the sample solvent, and available detection modalities.

Reference

1. Long, W. Automated Amino Acid Analysis Using an Agilent Poroshell HPH-C18 Column. *Agilent Technologies Application Note*, publication number 5991-5571EN, **2017**.

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