SIMULTANEOUS METHOD DEVELOPMENT USING REVERSED-PHASE AND HYDROPHILIC INTERACTION CHROMATOGRAPHY

Waters
THE SCIENCE OF WHAT'S POSSIBLE.™

Kenneth J. Fountain, Hillary B. Hewitson, Eric S. Grumbach, Damian Morrison
Waters Corporation, 34 Maple Street, Milford, MA USA 01757

INTRODUCTION

Reversed-phase chromatography (RPLC) is still the most universally accepted and implemented technique for method development. Recently, there has been resurgence in the use of hydrophilic interaction chromatography (HILIC) to complement RPLC due to its ability to retain and separate highly polar compounds. Combining these two techniques during method development will ensure the broadest range of selectivity is investigated for mixtures containing compounds with a broad range of chemical properties. However, there are many challenges associated with the instrument setup and column conditioning/equilibration procedures that prevent simultaneous method development with both RPLC and HILIC.

The current work reviews the principles of a logical screening approach to method development for both RP and HILIC chromatography. The influence of various instrument parameters on the chromatographic performance of HILIC is also discussed. A protocol is presented for the sequential development of chromatographic methods using both RPLC and HILIC. New capabilities in instrument operation enable RP and HILIC separations to be performed in a single experiment, thus eliminating the need to switch mobile phases and/or wash solvents. Further, the columns used in this protocol provide a wide range of selectivity for mixtures containing polar and non-polar analytes. The combined RP/HILIC method development approach will be applied to the areas of food safety and monitoring of controlled substances.

CHROMATOGRAPHIC MODES

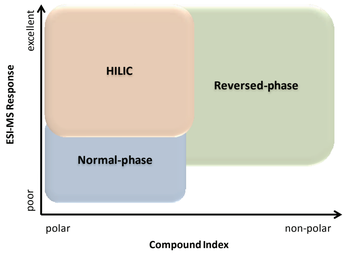

Figure 1. Diagram showing partial overlap in the compounds that can be analyzed with three different modes of chromatography. The volatile mobile phases used in both RP and HILIC make them ideal modes for modern applications.

METHOD DEVELOPMENT PARAMETERS FOR RP AND HILIC

Influence of Column Chemistry

Reversed-Phase

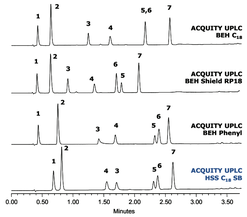

Figure 2. Analysis of basic drugs on different UPLC columns. ACQUITY UPLC system with PDA. Mobile phase A is 10 mM NH_4COOH, pH 3; mobile phase B is MeOH. Gradient from 35 to 80% B in 3 min. Flow rate is 0.4 mL/min. UV @ 260 nm, 1 µL injection, 30 °C. All columns 2.1 x 50 mm, 1.7 µm.

HILIC

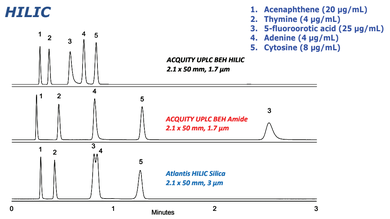

Figure 3. Comparison of different HILIC stationary phases. ACQUITY UPLC system with PDA. The isocratic mobile phase was 90/10 ACN/H_2O with 10 mM NH_4COOH, pH 3. The flow rate was 0.5 mL/min. The column temperature was 30 °C and the UV detector was set to 254 nm. Injection volume is 5 µL.

Influence of Mobile phase pH

Reversed-Phase

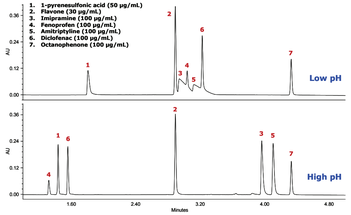

Figure 4. Influence of mobile phase pH on selectivity and retention in RP. ACQUITY UPLC system with PDA. The low pH mobile phase is 0.1% HCOOH in water. The high pH mobile phase is 0.1% NH_4OH in water. The organic solvent is ACN. Gradient from 5-95% ACN in 5 min. The flow rate was 0.5 mL/min. The column temperature was 30 °C and the UV detector was set to 254 nm. Injection volume is 2 µL. The sample diluent is 75/25 MeOH/water.

HILIC

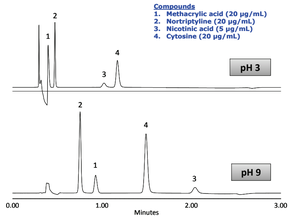

Figure 5. Influence of mobile phase pH on selectivity and retention in HILIC. ACQUITY UPLC system with PDA. The isocratic mobile phase was 90/10 ACN/H_2O with either 10 mM NH_4COOH, pH 3 or 10 mM NH_4CH_3COO, pH 9. The flow rate was 0.5 mL/min. The column temperature was 30 °C and the UV detector was set to 220 nm. Injection volume is 5 µL. The sample diluent is 75/25 ACN/MeOH.

Influence of Organic Solvent

Reversed-Phase

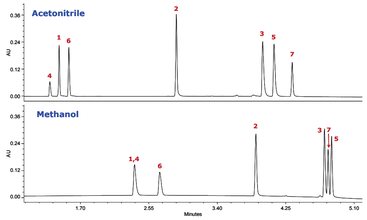

Figure 6. Influence of elution solvent on selectivity and retention in RP. ACQUITY UPLC system with PDA. Mobile phase A is 0.1% NH_4OH in water. Mobile phase B is ACN or MeOH. All other conditions as in Figure 4. Peak ID is in Figure 4.

HILIC

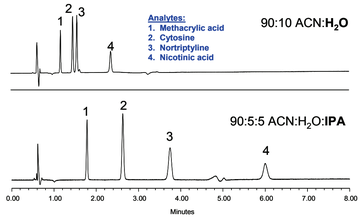

Figure 7. Influence of organic solvent on selectivity and retention in HILIC. ACQUITY UPLC system with PDA. The isocratic mobile phase is 10 mM NH_4CH_3COO with 0.02% acetic acid.

SELECTIVITY DIFFERENCES BETWEEN RP AND HILIC

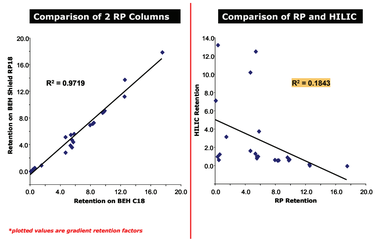

Figure 8. Comparison of the selectivity differences between RP and HILIC. The orthogonality (scattered distribution of data in the plot) of RP and HILIC makes them ideal modes of chromatography to be combined into one automated method development scheme.

Automating RP/HILIC Method Development

Performing both RP and HILIC method development using a single system configuration is difficult. First, the mobile phases need to be premixed for HILIC on a binary pumping system. Second, the presence of a weak and strong needle wash on a fixed loop-style injector can influence the chromatography since the needle wash or washes may come into contact with the sample plug (see Figure 9 below).

The design of the ACQUITY UPLC H-Class sample manager allows both HILIC and RP to be run without needle wash modification. Thus, method screening using both RP and HILIC can be automated. This is due to the fact that the purge and strong wash solvents do not contact the sample. In addition, the ability to pump four mobile phases at once eliminates the need for separate buffer preparations for RP and HILIC. The technique only requires 2 dilutions of the same sample. In order to maximize selectivity differences, 3 RP columns and 1 HILIC column are proposed for routine screening.

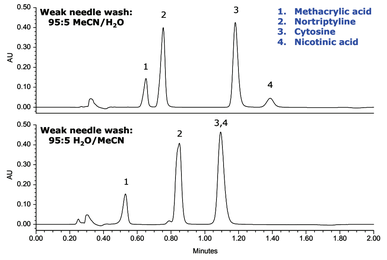

Figure 9. Influence of the weak needle wash on peak shape and retention for a fixed loop-style injector. The mobile phase is 90/10 MeCN/H_2O with 10 mM NH_4CH_3COO pH 9. The flow rate is 0.5 mL/min, 30 °C, UV 220 nm. Injection volume and sample concentrations as in Figure 5.

RP/HILIC SCREENING PROTOCOL

Instrument

ACQUITY UPLC H-Class with ACQUITY PDA and column manager

Columns

ACQUITY UPLC BEH C18, 2.1 x 50 mm, 1.7 µm
ACQUITY UPLC BEH Shield RP18, 2.1 x 50 mm, 1.7 µm
ACQUITY UPLC HSS C18 SB, 2.1 x 50 mm, 1.7 µm
ACQUITY UPLC BEH Amide, 2.1 x 50 mm, 1.7 µm

Mobile phases and gradient conditions

Mobile phase A:	H_2O
Mobile phase B:	ACN
Mobile phase C:	200 mM NH_4COOH, pH 3
Mobile phase D:	200 mM NH_4CH_3COO, pH 10
Gradient (RP):	5-95 % B in 5 min with 5 % C or D
Gradient (HILIC):	90-50 % B in 5 min with 5 % C or D
Flow rate:	0.6 mL/min
Column Temp.:	30°C
Needle wash:	50/50 ACN/H_2O
Sampling Rate:	20 points/sec
Time Constant:	0.1

RP/HILIC FOR MORPHINE-RELATED COMPOUNDS

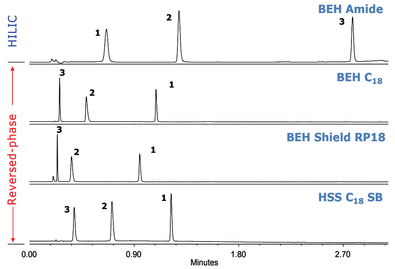

Figure 10. RP/HILIC screening of morphine-related compounds using one instrumental setup. The chromatograms shown are acquired using the low pH conditions (ammonium formate, pH 3) specified above. The sample diluent for RP is 81/12/7 water/MeOH/ACN w/0.2% HCOOH. The sample diluent for HILIC is 75/25 ACN/MeOH. Each compound is present at a concentration of 25 µg/mL. UV detection is at 280 nm. Peaks: [1] 6-acetylmorphine, [2] morphine, [3] morphine-3β-D-glucuronide.

RP/HILIC FOR WATER-SOLUBLE VITAMINS

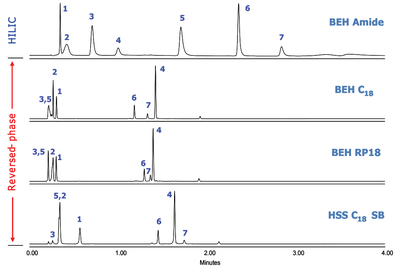

Figure 11. RP/HILIC screening of water-soluble vitamins using one instrumental setup. The chromatograms shown are acquired using the low pH conditions (ammonium formate, pH 3) specified in Figure 10. The sample diluent for RP is water with 75 mM KH_2PO_4. The sample diluent for HILIC is 68/32 ACN/water. Each compound is present at a concentration of 50 µg/mL. UV detection is at 265 nm. Peaks: [1] Nicotinamide, [2] Pyridoxal, [3] Nicotinic acid, [4] Riboflavin, [5] Thiamine, [6] Folic acid, [7] B12

CONCLUSIONS

- **The same experimental parameters (mobile phase pH, column chemistry, and organic solvent) affect retention and selectivity in both RP and HILIC modes of chromatography.**
- **RP and HILIC provide truly orthogonal modes of separation that benefit the method development process for complex mixtures.**
- **Instrument parameters can significantly affect the quality of the chromatography in HILIC, thus making automation of a combined RP/HILIC method development protocol difficult.**
- **The ACQUITY UPLC H-Class system allows simultaneous method development using both RP and HILIC.**
- **A method development protocol using both RP and HILIC was proposed for complex mixtures containing polar molecules.**

Suggested Reading

1. Neue, U. D., *J. Sep. Sci.* **2007**, *30*, 1611-1627.
2. Grumbach, E. S., Diehl, D. M., Neue, U. D., *J. Sep. Sci.* **2008**, *31*, 1511-1518.
3. Fountain, K.J., Xu, J., Diehl, D.M., Morrison, D., *J. Sep. Sci.* **2010**, *33*, 740-751.

TO DOWNLOAD A COPY OF THIS POSTER, VISIT WWW.WATERS.COM/POSTERS