

Method Development in LC/MS analysis of polar compounds using a new HILIC stationary phase

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Overview

Purpose: To evaluate LC/MS method development parameters when using stationary phases specific for the retention of polar analytes: effect of mobile phase composition on retention and sensitivity, effect of injection solvent on chromatographic performance, use of gradient elution.

Introduction

Hydrophilic interaction liquid chromatography (HILIC) provides a viable and increasingly popular alternative technique to reversed-phase liquid chromatography (RP-LC) for the analysis of highly polar and hydrophilic compounds. The term HILIC was coined by Alpert in 1990 [1] to describe a LC technique which utilizes water as the stronger eluting component in a water / organic solvent mobile phase and a hydrophilic stationary phase. The water / polar solvent forms a polar layer semi-immobilized onto the surface of the stationary phase into which analytes partition. The retention mechanisms in HILIC are complex but are believed to be a combination of partitioning, adsorption and hydrogen bonding [2]. Using a highly organic mobile phase containing low levels of salts, HILIC is ideal for use with electrospray mass spectrometry.

HILIC is not generally suitable for the separation of samples containing a mixture of hydrophilic and hydrophobic compounds, as the latter are not retained. There are stationary phases that provide retention of both hydrophobic and hydrophilic compounds, through a combination of reversed and normal phase mechanisms. Retention is due to interactions that take place because of the aqueous nature of the mobile phase (RP) and those that provide retention that mimics a normal phase (NP) mechanism, where retention increases as the mobile phase organic content increases. This dual mechanism is better described as Aqueous Normal Phase (ANP) [3].

Thermo Scientific Hypercarb Porous Graphitic Carbon (PGC) has unique properties as a stationary phase in HPLC, providing unique retention and separation of highly polar compounds. PGC behaves in a similar manner to a strongly retentive alkyl-bonded phase for non-polar analytes, but it can also retain very polar analytes. The retention mechanisms involved are a combination of dispersive interactions between the analyte-mobile phase and analyte-graphitic surface, together with charge induced interactions of polar analytes with the polarizable surface of the graphite.

HILIC and ANP have the advantage of improved sensitivity with MS detection, since the mobile phases have high organic content (>70% aprotic solvent, typically acetonitrile) which assists nebulisation in electrospray ionisation (ESI). A second advantage is the possibility of using high flow rates for fast analysis, since the mobile phases have low viscosities.

The work presented in this poster compares chromatographic behaviour in HILIC mode, using an unmodified silica phase and a polar-bonded silica phase and also RP and ANP modes using PGC. The method parameters studied are mobile phase composition and its effect on the retention and sensitivity, choice of injection solvent and the use of gradient elution. Some examples of HILIC/ESI/MS applications are also shown.

Methods

Columns:

Hypercarb® 5 µm, 100 x 4.6 mm, Hypercarb 3 µm, 100 x 2.1 mm, Hypercarb 3 µm, 50 x 2.1 mm
Experimental Unmodified Silica, 5 µm, 150 x 4.6 mm (Thermo Scientific)
Experimental HILIC (polar-bonded phase), 150 x 4.6 mm 5 µm and 150 x 2.1 mm 5 µm (Thermo Scientific)
Thermo Scientific Hypersil GOLD™ 5 µm, 150 x 2.1 mm

Instrumentation:

Thermo Scientific Surveyor™ HPLC system, Thermo Scientific Accela™ UHPLC system, Thermo Scientific Surveyor MSQ™ and Thermo Scientific LCQ™ Deca XP.

Test Method I – Effect of organic on capacity factor (Figure 1)

Columns: 150 x 4.6 mm 5 µm (Experimental Unmodified Silica and HILIC); Hypercarb 5 µm, 100 x 4.6 mm
Mobile phase: 10 mM Ammonium Acetate, pH 5.0 / MeCN;
Flow rate: 0.6 mL/min; Detection: UV at 254 nm; Temperature: 30 °C;
Analytes: 1. 10 marker (acetone for Hypercarb, benzophenone for polar-bonded phase, toluene for unmodified silica

Test Method II – Effect of injection solvent (Figure 2)

Column: Experimental HILIC, 150 x 4.6 mm 5 µm
Mobile phase: 10 mM Ammonium Acetate, pH 5.0 / MeCN (10:90);
Flow rate: 0.6 mL/min; Detection: UV at 254 nm; Temperature: 30 °C;
Analytes: 1. Benzophenone, 2. Uracil, 3. Cytosine, 4. Acyclovir.

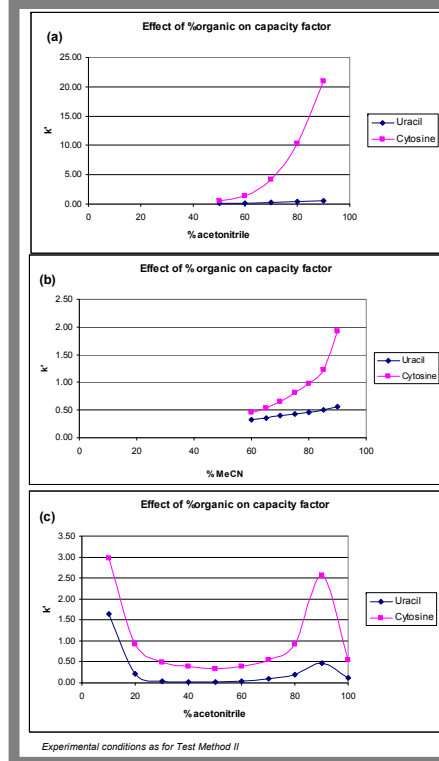
Test Method III – MS sensitivity (Figure 4)

Column: Hypercarb 3 µm, 100 x 2.1 mm
Mobile phase: 50 mM Ammonium Acetate, pH 5.0 / MeCN
Flow rate: 0.2 mL/min; Detection: ESI (+ve for cytosine, -ve for uracil); Temperature: 25 °C

Results

1) Retention

FIGURE 1. Analyte Retention as a Function of Percentage Organic in the Mobile Phase. (a) Silica phase; (b) Polar bonded silica phase; (c) PGC.



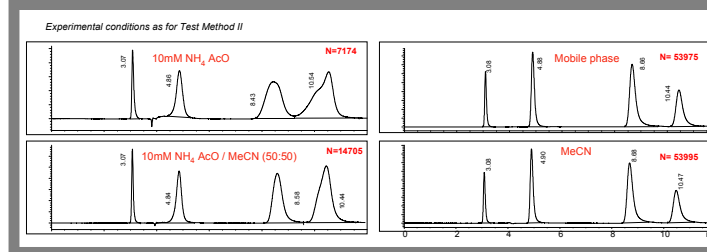
2) Injection solvent

Figure 2 shows the effect of injection solvent on the quality of the separation: peaks get sharper as the percentage of MeCN is increased, and therefore the strength of the injection solvent is decreased

In HILIC mode, retention of polar analytes increases as the quantity of non-polar solvent in the mobile phase (generally acetonitrile) increases. This behaviour is demonstrated in Figure 1 (a) and (b) for unmodified silica and polar-bonded silica phases.

PGC, which has a hydrophobic surface, displays both typical reverse and normal phase mode retention characteristics as shown in Figure 1 (c). Analyte retention first decreases as the percentage of organic increases (typical reverse phase behavior) and then increases as the concentration of acetonitrile becomes greater (normal phase behavior). Between 90 – 100% organic concentration analyte retention reverts to reverse phase trends. This dual behavior allows PGC to be used for the separation of polar analytes either under reversed or aqueous normal phase conditions.

FIGURE 2. Effect of Injection Solvent on Quality of Separation: (a) ammonium acetate 10 mM; (b) Ammonium acetate 10 mM / MeCN (50:50); (c) mobile phase (d) MeCN.



3) Sensitivity

The use of HILIC is often highlighted as advantageous for use with ESI MS detection by virtue of the fact that the highly organic mobile phases ensure efficient desolvation, which in turn leads to improved sensitivity. This concept is shown in Figure 3, where the S/N for nicotine is 15 times higher in HILIC than in RPLC; the S/N for cotinine is 5 times higher in HILIC than in RPLC.

FIGURE 3. RPLC (on the left) and HILIC (on the right) experiments.

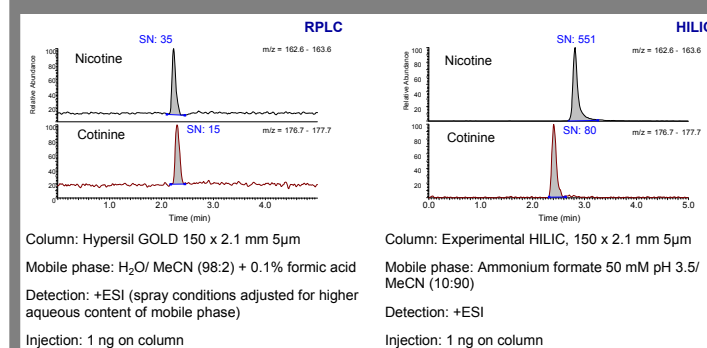
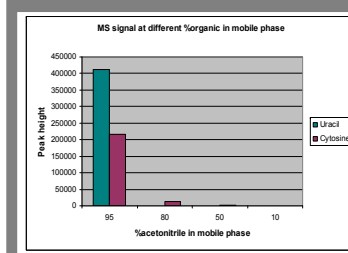


FIGURE 4. ESI-MS signal in ANP and RP on PGC for cytosine and uracil.



As demonstrated in Figure 1, PGC can be used for the retention of polar analytes in RP with a mobile phase rich in the aqueous component, and also in ANP with a mobile phase rich in the organic component.

The sensitivity of ESI detection of uracil and cytosine was measured for mobile phase containing low, intermediate and high organic content. The data displayed in Figure 4 shows that the best signal (measured as peak height) is obtained at high organic content, under ANP conditions. Figure 5 illustrates the separation of cyclic monophosphate nucleotides under RP and ANP conditions, using a gradient of ammonium acetate and acetonitrile.

4) Applications

FIGURE 5. Comparison of the gradient separation of cyclic nucleotides on PGC in RP and ANP modes.

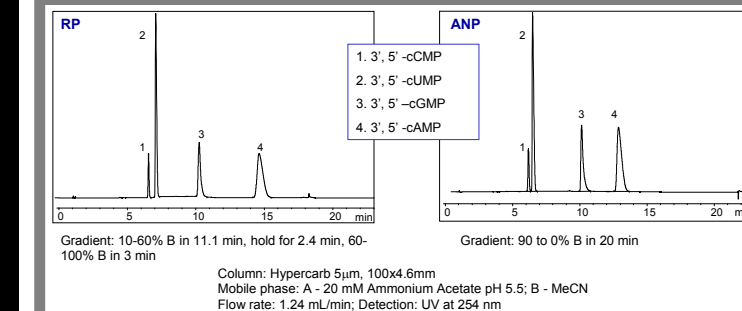
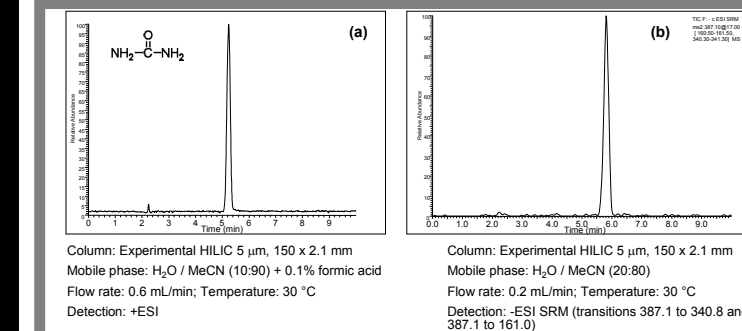


FIGURE 6. HILIC / MS analysis of urea (a) and lactulose (b).



Conclusions

- The unmodified silica and the polar-bonded silica phases exhibit typical HILIC behaviour: increased concentration of apolar solvent (acetonitrile) in the mobile phase results in increased retention of polar analytes.
- PGC exhibits typical reversed and normal phase behaviors for polar analytes, which can be retained and separated in either mode.
- It is shown that sample eluent composition is critical to peak shape.
- The high percentage organic mobile phases used in HILIC and ANP offers improved mass spectrometric sensitivity.
- PGC can be used for the separation of polar analytes in both reversed and aqueous normal phase modes.

References

- [1] A.J. Alpert, J. Chromatogr. 499, 177-196 (1990)
- [2] P. Hemström and K. Irgum, J. Sep. Sci. 29, 1784-1821 (2006)
- [3] J. Pesek, M. T. Matyska, LCGC, May 2007

Additional Information

For additional information, please browse our Chromatography Resource Centre which can be accessed from: www.thermo.com/columns.

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