

Fast Method Development in U-HPLC with 1.9 µm Particle Packed Columns

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Abstract

Purpose: show a strategy for fast method development in U-HPLC, applied to a test mixture comprising basic, acidic and neutral analytes.

Method: the analysis was based on the preliminary screening of six column chemistries packed with 1.9 µm particles. Mobile phase composition and column temperature were also investigated. Method optimization was then conducted on the best column candidate.

Results: a method was developed in a few hours, which lead to the separation of the analytes, without compromising their resolution.

Introduction

The reduction in particle size of packing materials used in HPLC has allowed for the use of shorter columns, whilst maintaining efficiency and thus leading to quicker run times. The use of sub-2 µm particle packed short columns has also shown the great advantage of not compromising resolution [1].

Separation selectivity is the main contributor to resolution. In the equation below resolution (R_s) is expressed as a function of capacity factor (k), selectivity (α), and efficiency (N):

$$R_s = \frac{1}{4} \frac{(\alpha-1)}{\alpha} \sqrt{N} \frac{k}{1+k}$$

Variations of selectivity in reversed phase HPLC (RP-HPLC) are generally obtained by changing the mobile phase composition or column chemistry. Since generic mobile phases, such as aqueous acetonitrile or aqueous methanol are generally chosen first, the analyst is often left with column chemistry, to adjust the selectivity of the RP-HPLC method. It is therefore very important that a range of column chemistries is available, when developing new methods with sub-2 µm materials.

Because of the speed of analysis, method development time in U-HPLC amounts to hours rather than days. However, it can be reduced further by screening different column chemistries with a generic mobile phase, at different temperatures, and then optimizing on the column that gives the best performance.

The current work evaluates selectivity changes obtained when employing alkyl chain, polar endcapped, cyano, phenyl and perfluorinated chemistries (Table 1). A range of acidic, neutral and basic analytes (listed on Figure 1) were run under generic gradient conditions, on all six column chemistries with UV detection. The mobile phases selected were 0.1% formic acid in water/ acetonitrile or 0.1% formic acid in water/methanol. Column temperature was screened at 25, 40 and 60 °C.

Column chemistries

Following the success of the Hypersil GOLD™ stationary phase for providing extremely symmetrical peaks even for very basic analytes, additional chemistries have been developed using the same highly pure silica support and similar robust bonding process. These include C8, a polar endcapped C18, cyano, phenyl and perfluorinated chemistries (Table 1). The polar functional group used to endcap Hypersil GOLD aQ™ provides an additional interaction mechanism by which polar compounds can be retained and resolved. Additionally, the polar endcapping allows for the use in 100% aqueous mobile phases without the risk of loss of performance or poor stability. Hypersil GOLD C8 offers similar selectivity to the original Hypersil GOLD but is less retentive, whereas Hypersil GOLD CN offers a cyano chemistry with alternative selectivity for reversed-phase and can also be used for normal phase separations. Hypersil GOLD Phenyl provides alternative selectivity and is particularly suitable for aromatic or moderately polar compounds. The phenyl bonded phase contains a butyl linker which allows for alignment of the phenyl ring with aromatic molecules, enhancing π-π interactions and therefore retention. Furthermore, the C4 linker provides the stationary phase with moderate hydrophobicity, making it ideal for the separation of analyte mixtures with varying degrees of polarity and aromaticity. The sixth chemistry, Hypersil GOLD PFP, has a pentafluorophenyl ligand that provides extra selectivity of halogenated compounds and also molecules which contain several nitro, hydroxyl, carboxyl or other polar groups that may not be well retained or resolved on alkyl chain phases. The introduction of a fluorine group in the alkyl stationary phase causes significant changes in hydrophobic phase interactions. The carbon-fluorine bond is more polar than the carbon-hydrogen bond, which explains the extra selectivity and retention observed for compounds containing halogens and other polar groups. The extra rigidity of the perfluorinated ring may explain why a high degree of shape selectivity for isomers can be observed.

TABLE 1: Specifications of Hypersil GOLD phases.

	Chemistry	Pore size (Å)	% Carbon	Particle size (µm)
Hypersil GOLD	C18 Selectivity	175	10	1.9, 3, 5, 8, 12
Hypersil GOLD C8	C8	175	8	1.9, 3, 5, 8
Hypersil GOLD aQ	C18 polar endcapped	175	12	1.9, 3, 5, 8, 12
Hypersil GOLD CN	Cyano	175	4	1.9, 3, 5
Hypersil GOLD Phenyl	Phenyl	175	8	1.9, 3, 5
Hypersil GOLD PFP	Pentafluorophenyl	175	8	1.9, 3, 5, 8, 12

Materials & Methods

Columns:

Hypersil GOLD 1.9 µm, 50 x 2.1 mm; Hypersil GOLD C8 1.9 µm, 50 x 2.1 mm; Hypersil GOLD aQ 1.9 µm, 50 x 2.1 mm; Hypersil GOLD PFP 1.9 µm, 50 x 2.1 mm; Hypersil GOLD Phenyl 1.9 µm, 50 x 2.1 mm and 100 x 2.1 mm; Hypersil GOLD CN 1.9 µm, 50 x 2.1 mm; (Thermo Scientific, Bellefonte, PA). Column names are abbreviated to GOLD, C8, aQ, PFP, Phe and CN respectively.

Mobile phases:

A - H₂O + 0.1% formic acid; B - MeOH + 0.1% formic acid or MeCN + 0.1% formic acid; Generic gradient - 5 to 100 %B in 2.9 min.

Optimized gradient¹ - 10 to 100 %B in 3.4 min; 60 to 90% B from 3.4 to 3.6 min.

¹ Flow rate - 0.50 mL/min; Inj Vol - 0.7 µl on 50 x 2.1 mm column.

² Flow rate - 0.55 mL/min; Inj Vol - 1.4 µl on 100 x 2.1 mm column.

³ Column temperature - 25, 40 and 60 °C.

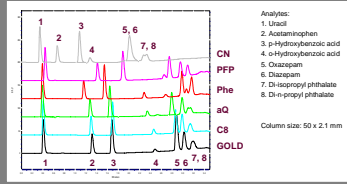
⁴ Detection - UV at 225 and 254 nm.

⁵ U-HPLC Instrumentation: Accela™ (Thermo Scientific, San Jose, CA).

Results & Discussions

Step 1: column chemistry and mobile phase evaluation. The approach for screening different column chemistries when developing U-HPLC methods was taken to analyze a mixture of eight compounds (Figure 1).

FIGURE 1: Selectivity changes with column chemistry. Mobile phase: acidified aqueous MeOH, delivered in a generic gradient. Temperature: 25 °C.

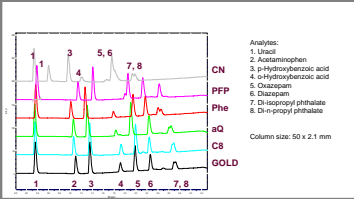


In relation to the alkyl chain phases, selectivity differences observed are:

- Higher resolution of 5 + 6 on aQ
- 5 + 6 co-elute on CN
- Lower resolution of 4 + 5 on PFP.

Where there is co-elution or poor resolution, no attempts were made to resolve co-eluting analytes. A change in mobile phase composition was applied, as shown in Figure 2.

FIGURE 2: Selectivity changes with column chemistry. Mobile phase: acidified aqueous MeCN, delivered in a generic gradient. Temperature: 25 °C.



In relation to the alkyl chain phases, selectivity differences observed are:

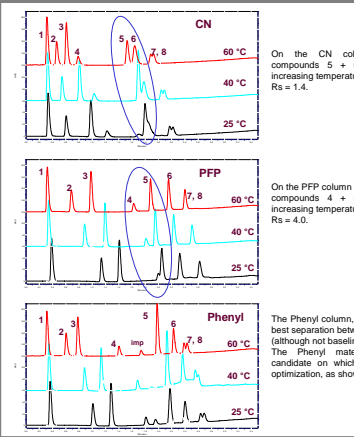
- Higher resolution of 5 + 6 on aQ
- 5 + 6 co-elute on CN
- Lower resolution of 4 + 5 on PFP.

Best separation of 7 + 8 on Phenyl.

Step 2: column temperature evaluation.

Where there is co-elution or poor resolution, attempts were made to resolve the co-eluting analytes by increasing the temperature, as shown in Figure 3.

FIGURE 3: Selectivity changes with column temperature. Mobile phase: acidified aqueous MeCN, delivered in a generic gradient. Column size: 50 x 2.1 mm.

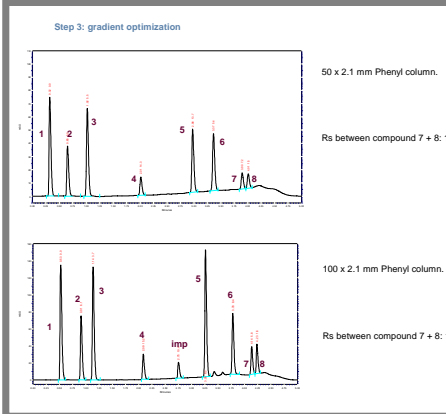


On the CN column separation of compounds 5 + 6 was achieved by increasing temperature from 25 to 60 °C. $R_s = 1.4$.

On the PFP column separation of compounds 4 + 5 was achieved by increasing temperature from 25 to 60 °C. $R_s = 4.0$.

The Phenyl column, at 60 °C provides the best separation between compounds 7 + 8 (although not baseline resolved). The Phenyl material was the best candidate on which to perform method optimization, as shown in Figure 4.

FIGURE 4: Separation of the eight components present in test mixture on Phenyl columns. Mobile phase: acidified aqueous MeCN, delivered in the 'optimized' gradient. Temperature: 60 °C.



Step 3: gradient optimization

50 x 2.1 mm Phenyl column.

R_s between compound 7 + 8: 1.5.

100 x 2.1 mm Phenyl column.

R_s between compound 7 + 8: 1.6.

Conclusions

- Selectivity differences between alkyl chain, polar endcapped C18, cyano, phenyl and perfluorinated column chemistries under generic mobile phase conditions are demonstrated, when either MeOH or MeCN are employed as organic modifiers.
- A column temperature increase, using generic mobile phase conditions, allows for selectivity variations on cyano, phenyl and perfluorinated phases.
- Following a preliminary selectivity screening, a quick method optimization (gradient 'optimization') on phenyl material leads to the separation of a mixture of basic, acidic and neutral analytes. This is achieved in 5 hours, rather than days.

References

- [1] L. Pereira, C. Blythe, H. Ritchie, Poster presented at Pitcon 2006, Orlando, Florida, USA.

Additional Information

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

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