Using Different HPLC Column Chemistries To Maximise Selectivity For Method Development

The purpose of HPLC method development is to determine conditions that provide adequate separation of analytes of interest in a reasonable time. Obtaining suitable selectivity should be the first step in this process, before final optimisation of the separation. Column chemistry (or more accurately the mechanisms of interaction between the stationary phase and the analyte that determine retention and separation) is one of the most powerful parameters that can be used to maximise selectivity. Sometimes phases other than C18 can be more helpful! In this Knowledgebase article, we discuss different column chemistries and their mechanisms of interaction to see how they can be a powerful ally for rapid LC method development.

Introduction

Selectivity (α) may be defined as the ratio of retention factors (k) for two adjacent eluting analyte peaks in a chromatogram as depicted in Figure 1.

The separation selectivity may be affected by many parameters that include mobile phase conditions, stationary phase chemistry and the instrument setup. Some parameters are known to have a larger impact on selectivity, whilst others can have a smaller influence. Column stationary phase chemistry is known to have a significant effect upon selectivity [1].

HPLC is an important technique across many different application areas for separating and quantifying analytes of interest within mixtures. This requires the analytes of interest to be sufficiently resolved from adjacent peaks in the chromatogram. From a practical point of view, resolution can be defined by Equation 1, where

R_s = resolution, N = number of theoretical plates, α = selectivity and k = retention factor.

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

Equation 1

Changing selectivity, efficiency or retention can affect resolution. However, if we represent the equation graphically (Figure 2), theory clearly indicates that selectivity is the most powerful term with respect to maximising resolution within this mathematical expression.

For method development purposes, exploring column chemistry and mechanisms of interaction to maximise selectivity is therefore helpful to resolve analytes of interest.

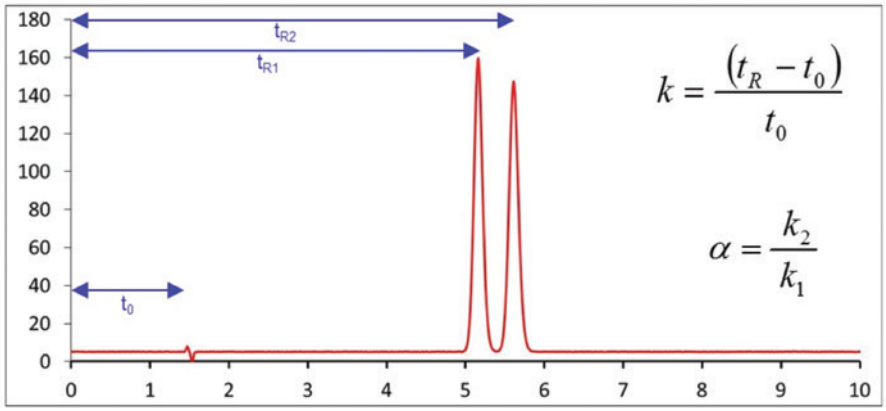

Figure 1: Retention factor and selectivity definition.

Reversed-Phase LC Mechanisms of Interactions

The most common column chemistry used across many application areas is C18. The C18 chemistry typically provides good performance and excellent column lifetimes / reproducibility under various conditions. It is also seen as broadly applicable for many analyte classes. The dominant retention and separation mechanism with alkyl chain based stationary phases is hydrophobic interactions. Depending upon the stationary phase chemistry, other mechanisms may be possible: should the stationary phase contain an aromatic ring or substituted aromatic ring (e.g. C18-AR or C18-pentaflurorophenyl (PFP)) and the analyte has regions of aromaticity, then pi-pi interactions may be possible. If polarisable functionality exists within the stationary phase chemistry (e.g. C18-PFP or CN-ES), then dipole-dipole interactions between the stationary phase and analyte may be achieved. Mechanisms such as hydrogen bonding (from the silica surface and polar moieties, e.g. polar embedded groups such as C18-Amide or carbamate) and shape selectivity (C18-PFP and C18) are also known. It is possible to determine the weighting of such mechanisms on each stationary phase using published defined protocols and tests [2]. This helps the analyst understand which mechanisms may operate with different stationary phases. Table 1 lists some different stationary phase chemistries and their main mechanisms of interaction. More stars indicate a higher weighting for that particular mechanism with the stationary phase.

Using complementary stationary phases during LC method development increases the potential interactions between analyte and stationary phase, therefore maximising selectivity and resolution. A simple and powerful way to optimise column selectivity is to screen a sample on a selection of different stationary phases using a generic set of gradient conditions. Figure 3 shows the chromatograms obtained using this approach for each of the stationary phases in Table 1, for the analysis of paracetamol (as the parent compound) and 9 related substances (spiked at 0.5% w/w).

The coloured boxes highlight retention and selectivity differences with the different column chemistries. The green box highlights how peak 4 may elute before, after, or completely coelute with the parent compound peak 1. Depending upon the mechanisms of interaction the selectivity is quite different. The red box highlights peak pair 7 & 8. Whilst generally well separated on all phases, the elution order is observed to reverse with the polar embedded ACE C18-Amide and ACE CN-ES stationary phases. Peaks 9 & 10 range from partial coelution to complete coelution and fully separated depending upon the stationary phase. Interestingly, it can be noted from these data that the ACE C18-Amide and ACE CN-ES provide complete separation of all 10 components compared to the other phases. The different mechanisms of interaction for these 2 phases provide suitably different selectivity to obtain complete resolution. One of the key points from these data is that the ACE C18 chemistry would not have separated the components using these gradient conditions. Further work would have been required, involving more time to determine a suitable separation. Using the power of stationary phase chemistry to maximise selectivity, there are 2 options where complete resolution of the 10 components is achieved, without the need for further method development.

Conclusions

Selectivity is the key to analyte resolution in reversed-phase small molecule chromatography. Using different column chemistries (or mechanisms of interaction) is helpful as it affects analyte retention behaviour. Exploring the different column chemistries in a screen to maximise selectivity can be a useful approach to systematic method development.

[1.] Adapted from 'Introduction to Modern Liquid Chromatography, 3rd Edition, Snyder, Kirkland, Dolan, 2010, p29, Wiley and Sons

[2] . Kimata, K., Iwaguchi, K., Onishi, S., Jinno, K., Eksteen, R., Hosoya, K., Araki, M., Tanaka, N., J. Chromatogr. Sci. 1989 27, 721-728

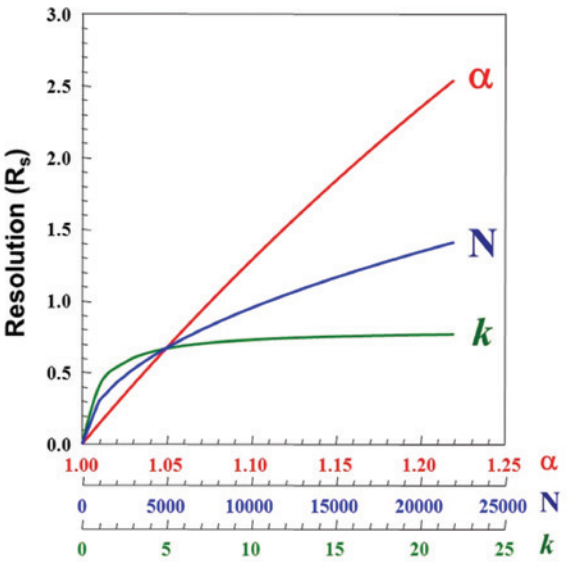

Figure 2: Selectivity: the key to chromatographic resolution.

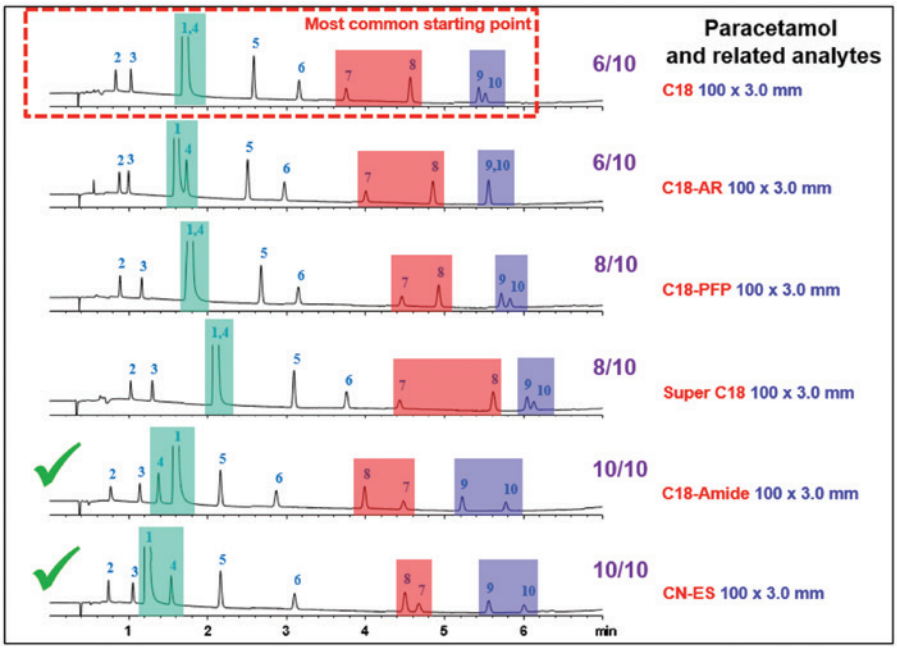

Figure 3: 100 x 3.0 mm columns, A= 20 mM CH_3COONH_4, pH 6.0 B= 20 mM CH_3COONH_4, pH 6.0 in 9:1 v/v MeOH:H_2O. 5-95 %B in 10 mins, 1.2 mL/min, 40 °C, 2 µL injection. Peak identities: 1. paracetamol, 2. 4-aminophenol, 3. hydroquinone, 4. 2-aminophenol, 5. 2-acetamidophenol, 6. phenol, 7. 4-nitrophenol, 8. 2-nitrophenol, 9. 4-chloroacetanilide, 10. 4-chlorophenol.

Table 1: Different stationary phase chemistries and the weighting of interaction mechanisms.

Chemistry	Example	Separation Mechanism and Relative Strength of Interaction[1]				
		Hydrophobicity	π-π interaction	Dipole-Dipole	Hydrogen Bonding	Shape Selectivity
C18	ACE C18	****	-	-	*	**
Extended alkyl chain aromatic	ACE C18-AR	****	*** (donor)	*	**	***
Extended alkyl chain PFP	ACE C18-PFP	****	*** (acceptor)	****	***	****
Wide pH range stable C18	ACE SuperC18	****	-	-	-	**
Polar embedded amide	ACE C18-Amide	****	-	**	****	** / ***
Extended alkyl chain CN	ACE CN-ES	***	*	***	**	*

[1] Approximate value - determined by semi-quantitative mechanism weightings with reference to other ACE products using >100 analytes