Chromatography Solutions

Knowledge note #0013

The Power of Stationary Phase Selectivity

INTRODUCTION

During LC method development, a range of method parameters are typically assessed to achieve the desired separation. One of the most powerful parameters that can be varied is stationary phase selectivity. By varying stationary phase chemistry, differing mechanisms of interaction with analyte molecules can be exploited to optimise the separation selectivity. This Knowledge Note will briefly describe the key parameters that affect separation selectivity and how novel stationary phase chemistries from the Avantor® ACE® portfolio can be utilised during method development to achieve better separations.

SELECTIVITY

Analyte retention time (t_R) is described as the time taken for an analyte to elute from the column. Retention factor (k) describes the analyte elution from the column taking into account the column void volume (t_0). Selectivity (α) is the ratio of retention factors of two adjacent analytes and is described by Equation 1.

$$\propto = \frac{k_2}{k_1} \qquad (1)$$

An α-value of 1 indicates coelution of the two analytes. The combination of the column and elution conditions prohibits the separation of those peaks, regardless of the column efficiency. This therefore indicates further work must be performed to improve separation. Many method and instrument parameters can affect the separation as described in Table 1. Column stationary phase (and the various mechanisms of interaction) is a powerful parameter to explore when developing methods.

Selectivity is important, but other parameters must also be considered when developing methods, to provide suitable resolution between analytes. Resolution can be described using Equation 2, where *W* corresponds to the peak width of the adjacent peaks.

$$R_s = \frac{2(t_{R2} - t_{R1})}{W_1 + W_2} \qquad (2)$$

Table 1: Parameters affecting LC selectivity, ranked[1] according to their relative influence.

Isocratic Separations		Gradient Separations
- Column stationary phase - pH (ionisable analytes only) - Organic modifier type - % Organic modifier - Buffer selection - Column temperature - Buffer concentration	MOST influential LEAST influential	All parameters for isocratic separations PLUS: - Gradient steepness - Dwell volume - Column dimensions

[1] Adapted from reference 1.

A minimum resolution value of 1.5 for the critical pair (i.e. closest eluting pair in the chromatogram) is recommended for most chromatographic separations. The resolution equation can also be expressed according to Equation 3. This new equation can be used to understand the influence of efficiency, selectivity and retention on resolution.

Efficiency, Selectivity, Retention

$$R_s = \frac{\sqrt{N}}{4} \frac{(\alpha-1)}{\alpha} \frac{k}{(1+k)} \qquad (3)$$

From this equation, resolution can be improved by varying either k, α or N. However, as can be seen from the graph in Figure 1, the selectivity parameter is the most powerful parameter that affects resolution. It is therefore highly recommended that column chemistry is explored during method development to optimise the separation selectivity.

COMMON REVERSED-PHASE INTERACTIONS

Avantor ACE columns are manufactured using modern ultra-inert, base-deactivated type-B silica, which increases batch-to-batch reproducibility. The tightly controlled bonding procedure ensures that residual silanols are minimised, which improves peak shape by reducing potential secondary interactions between functionalised analytes and the silica surface.

The ACE reversed-phase portfolio (Table 2), was designed and engineered with the aim of producing a broad array of functionalities offering orthogonal selectivity. The complementary phases cover a range of retention mechanisms, namely hydrophobic, π-π interactions, dipole-dipole, hydrogen bonding and shape selectivity.

Hydrophobic retention is the dominant retention mechanism for alkyl ligand stationary phases, such as C18, C8 and C4. Whilst the C18 phase remains the most commonly utilised in reversed-phase LC, other mechanisms of interaction, provided by different stationary phases, are often advantageous.

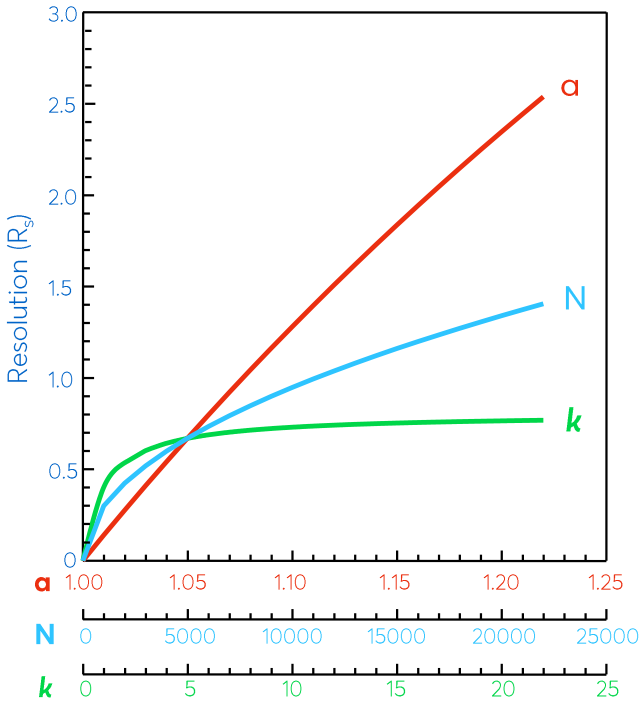

Figure 1: Effect of k, N and α on resolution.[2]

Table 2: Six ACE bonded phases and relative contributions to their phase character.

Bonded phase	Separation Mechanism and Relative Strength[2]				
	Hydrophobic Binding	π-π Interaction	Dipole-Dipole	Hydrogen Bonding	Shape Selectivity
ACE C18	★★★★	–	-	★	★★
ACE C18-AR	★★★★	★★★ (donor)	★	★★	★★★
ACE C18-PFP	★★★★	★★★ (acceptor)	★★★★	★★★	★★★★
ACE SuperC18	★★★★	–	-	-	★★
ACE C18-Amide	★★★★	–	★★	★★★★	★★/★★★
ACE CN-ES	★★★	★	★★★	★★	★

[2] Approximate value – determined by semi-quantitative mechanism weightings and/or by reference to other ACE phases using >100 characterising analytes.

The ACE C18-AR contains an electron-rich ring attached to an extended alkyl chain, combining the hydrophobicity of a C18 phase with additional aromatic selectivity. The chromatograms in Figure 2 compare the selectivity of the ACE C18, ACE Phenyl and ACE C18-AR with toluene, trinitrobenzene, dinitrobenzene and nitrobenzene. The ACE C18-AR and ACE C18 provide similar retention of the hydrophobic marker, toluene. However, the ACE C18-AR shows stronger retention of the nitro-substituted aromatic analytes, along with a different elution order and successfully provides full resolution of the analytes.

The ACE C18-PFP combines hydrophobicity from the alkyl chain with shape selectivity, dipole-dipole and π-π interactions, provided by the electron deficient pentafluorophenyl ring. The ACE C18-Amide contains an extended alkyl tail to increase hydrophobic retention, whilst the embedded amide moiety is ideal for analytes able to hydrogen bond, such as acidic, phenolic and amino analytes.

The terminal polar CN group on the ACE CN-ES utilises a polar and dipole mechanism whilst, again, the alkyl chain enhances hydrophobicity. Figure 3 demonstrates how the

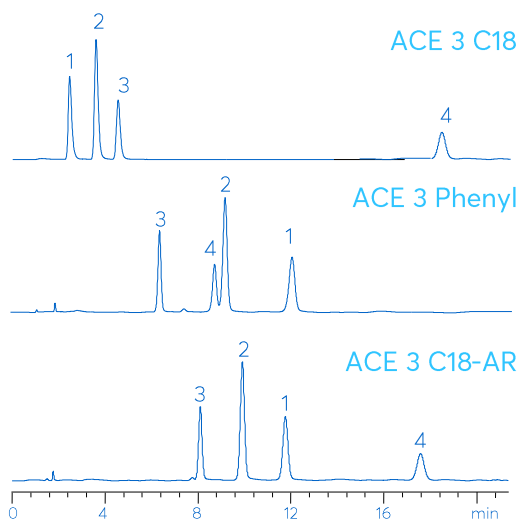

Figure 2: Separation of substituted aromatic analytes. Column dimensions: 150 x 4.6 mm; Mobile phase: MeOH/H_2O 1:1 v/v; Flow rate: 1 mL/min; Temperature: 40 °C; Detection: UV, 210 nm. Sample: 1. 1,3,5-trinitrobenzene, 2. 1,3-dinitrobenzene, 3. nitrobenzene, 4. toluene.

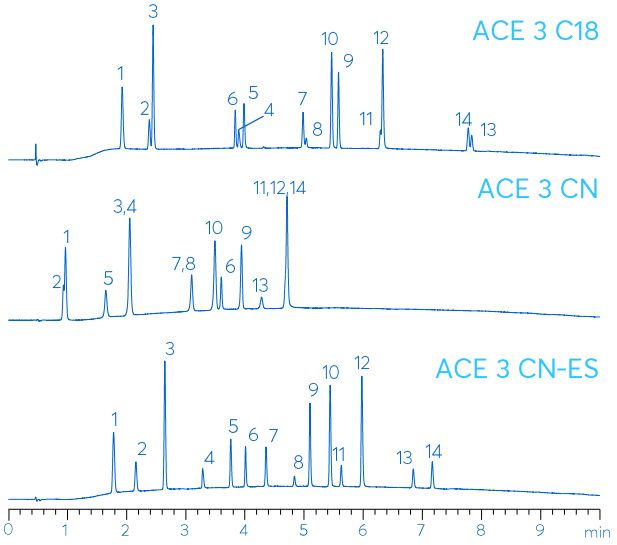

Figure 3: Advantages of multi-mode interactions on separations. Column dimensions: 100 x 2.1 mm; Mobile phase: A 0.1% formic acid in H_2O, B 0.1% formic acid in MeCN; Gradient: 3-100% B in 10 mins; Flow rate: 0.6 mL/min; Temperature: 40 °C; Detection: UV, 210 nm.

combined hydrophobic and cyano functionality provide different selectivity to standard C18 and cyano phases. Finally, the ACE SuperC18 is a hydrophobic C18 phase utilising unique encapsulated bonding that is stable using low, mid and high pH eluents.

Figure 4 shows a selection of acidic, basic and neutral analytes, chromatographed on a reversed-phase gradient, using five of the Avantor ACE reversed-phase columns detailed in Table 2. The data clearly shows that the different stationary phase chemistries provide substantially different selectivity to one another. The elution order of the seven peaks is different for each of the columns. This example clearly demonstrates how powerful stationary phase chemistry can be for exploring separation selectivity. If one stationary phase chemistry fails to resolve a critical peak pair, assessing the separation on a variety of phase chemistries offering complimentary selectivity can help rapidly identify a more suitable column for the separation.

UNDERSTANDING SELECTIVITY DIFFERENCES BETWEEN COLUMNS

Established column characterisation protocols, such as those devised by Tanaka or Synder and Dolan, can be used to understand the relative contributions of different retention mechanisms to analyte retention, such as hydrogen bonding capacity, ion-exchange capacity and hydrophobicity. Various databases are available which contain characterisation data for columns from different vendors. These can prove invaluable for identifying orthogonal stationary phase selectivity.

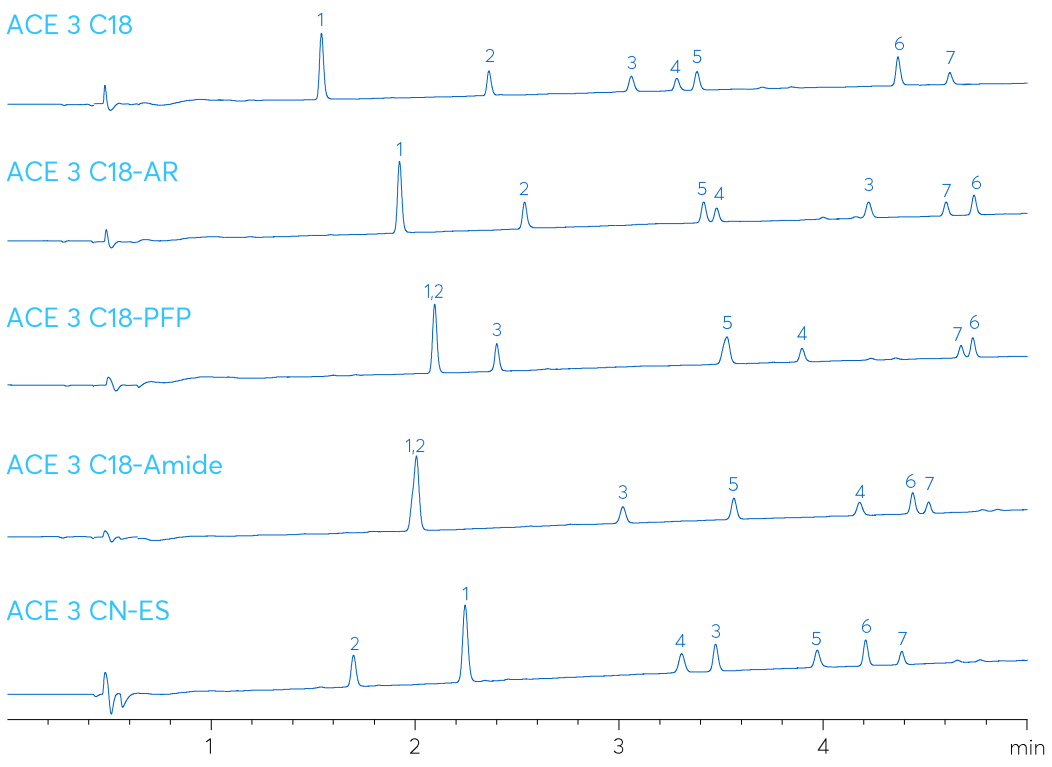

Figure 4: Retention of acidic, neutral and basic compounds with different elution orders, using the power of phase selectivity.
Column dimensions: 100 x 2.1 mm; Mobile phase: A 20 mM ammonium formate (aq), B 20 mM ammonium formate in MeOH/H_2O 90:10 v/v; Gradient: 3-100% B in 5 mins; Flow rate: 0.6 mL/min; Temperature: 40 °C; Detection: UV, 214 nm.
Sample: 1. hydrochlorothiazide, 2. methylphenylsulfoxide, 3. 1,3,5-trinitrobenzene, 4. myricetin, 5. *p*-cresol, 6. sulindac, 7. toluene.

Alternatively, differences in selectivity can be practically demonstrated using a selectivity screen and the Neue selectivity approach.[3] Using this approach, gradient retention times of a diverse set of 41 analytes, with different physico-chemical properties, are determined on each stationary phase.

A linear regression analysis of the plotted retention times for any column pair combination provides an R^2 value from which a Selectivity value (S) is determined, according to equation 4. The S-values quantify the difference in selectivity offered by the column pair. An S-value close to 0 indicates similar retention for the 41 analytes and therefore identical selectivity. S-values between 6 and 8 indicate that the two columns are similar to one another, with small observable differences in selectivity. Higher S-values indicate that the two columns are orthogonal and possess substantially different selectivity.

$$S = 100 \times \sqrt{(1 - R^2)} \qquad (4)$$

Table 3 shows the S-values determined for the six ACE phases using mobile phases containing both acetonitrile and methanol. Significant S-values were obtained between the majority of ACE column combinations, indicating meaningful selectivity differences between all six phases. Importantly, the data also revealed significant selectivity differences between methanol and acetonitrile for all six phases (data not shown, see reference 4 for further details). This indicates that the use of the six ACE phases with both organic modifiers provides a powerful tool for fully exploring selectivity during method development. Avantor ACE Knowledge Note #0018 provides further details of how stationary phase chemistry can be utilised in column screening strategies to help streamline method development.

CONCLUSION

This Knowledge Note has demonstrated that stationary phase chemistry is a powerful parameter to explore, during reversed-phase method development. By varying the stationary phase, substantially different selectivity can be obtained for a given separation. The Avantor ACE reversed-phase portfolio has been specifically designed to offer a range of complimentary stationary phases. Quantitative data has been presented which demonstrates that these phases provide valuable selectivity differences which can be exploited to help develop optimised LC separations.

Avantor® ACE®

A	C18	C18-AR	C18-PFP	CN-ES	C18-Amide	SuperC18
C18	0	19	15	25	18	7
C18-AR		0	15	27	28	18
C18-PFP			0	20	19	15
CN-ES				0	19	23
C18-Amide					0	20
SuperC18						0

B	C18	C18-AR	C18-PFP	CN-ES	C18-Amide	SuperC18
C18	0	13	11	15	20	9
C18-AR		0	8	17	26	11
C18-PFP			0	13	23	9
CN-ES				0	17	16
C18-Amide					0	24
SuperC18						0

Table 3: Experimentally determined S-values for the six ACE stationary phases, in mobile phases containing either methanol (A) or acetonitrile (B). Adapted from reference 4.

REFERENCES

1. Adapted from "Introduction to Modern Liquid Chromatography", 3rd Edition, Snyder, Kirkland, Dolan, 2010, p.29, Wiley & sons
2. J. H. Zhao, and P. W. Carr. *Analytical Chemistry*, 71 (1999) 2623-2632
3. U. D. Neue, J. E. O'Gara, A. Méndez, *J. Chromatogr. A* 1127 (2006) 161-174
4. M. R. Euerby, M. Fever, J. Hulse, M. James, P. Petersson, C. Pipe "Maximization of Selectivity in Reversed-Phase Liquid Chromatographic Method Development Strategies"; *LCGC Europe*, January (2016) 8–21.