Knowledge note #0018

Step-by-Step Protocol for Streamlined Reversed-Phase Method Development using Avantor® ACE® MDKs

INTRODUCTION

The use of a systematic screening strategy to explore the stationary phase selectivity for new samples is a well established approach to method development and allows chromatographers to rapidly identify a suitable stationary phase and analytical conditions. Avantor® ACE® Method Development Kits (MDKs) contain three LC columns, each providing substantially different selectivity and are therefore ideally suited to this approach. This Knowledge Note outlines a simple and systematic protocol for screening new samples using reversed-phase conditions, that can help rationalise and streamline the development of new LC methods.

WHY USE COLUMN SCREENING?

Reversed-phase LC columns offering different selectivity to a standard C18 phase are widely available (e.g. PFP, phenyl, polar embedded phases etc.). Changing the column stationary phase can have a dramatic impact on selectivity (Figure 1). As part of any method development strategy, it is therefore useful to assess the stationary phase chemistry to obtain a successful separation. However, it is often difficult to predict which stationary phase will be the most suitable for a new separation. Screening columns at the beginning of method development, using identical mobile phase conditions, is an efficient way to assess the impact of stationary phase selectivity and can help achieve your desired separation quicker with better resolution.

ACE MDKs group columns with different stationary phase chemistries (i.e. different mechanisms of analyte-stationary phase interaction) to maximise selectivity and increase the likelihood of separating challenging mixtures. These MDKs offer a highly cost effective solution for method development. The two most popular ACE reversed-phase (RP) MDKs (see Table 1) include unique phases engineered to exploit different retention mechanisms and maximise selectivity. All six phases can be used with standard RP conditions and are as robust as a C18 phase. Other ACE MDK's including HILIC, Bioanalytical 300 Å, UltraCore and Microbore are also available.
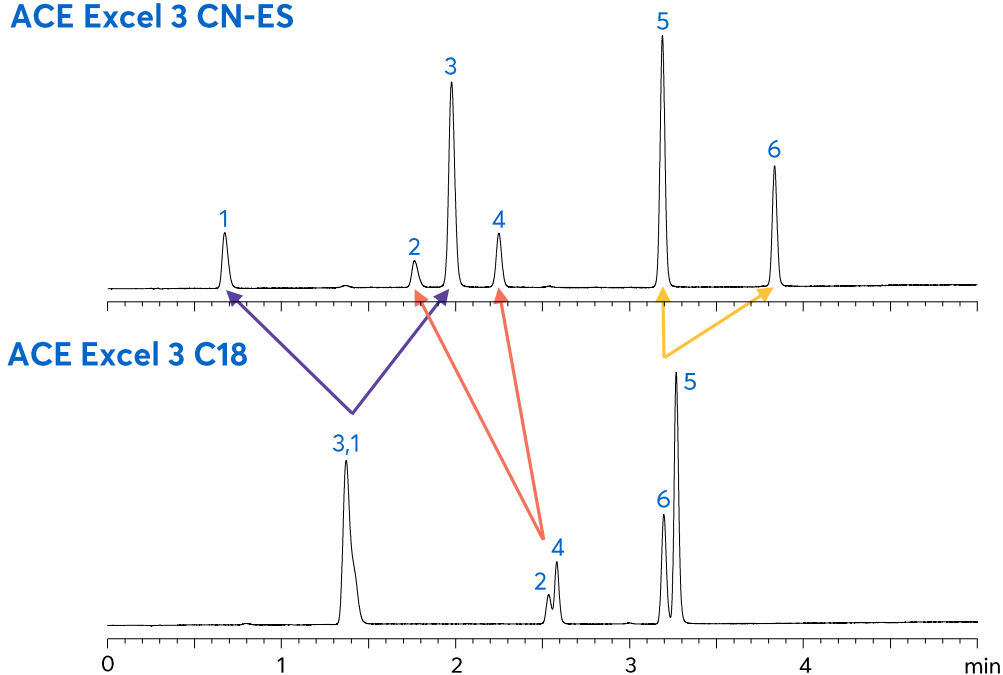
Avantor® ACE®

Figure 1: The effect of changing column stationary phase chemistry.

Columns: 50 x 2.1 mm; Mobile phase A: 0.1% Formic acid in H_2O, B: 0.1% Formic acid in MeOH:H_2O (9:1 v/v); Gradient: 3 to 100% B in 5 minutes; Flow rate: 0.21 mL/min; Temperature: 40 °C; Detection: UV, 254 nm; Sample: 1) Metronidazole, 2) Benzyl alcohol, 3) Hydrochlorothiazide, 4) Vanillin, 5) Methyl Paraben, 6) 1,2-Dinitrobenzene.

Table1: Phase characteristics of columns included in Avantor® ACE® reversed-phase MDKs.

	Bonded phase	Separation Mechanism and Relative Strength[1]				
		Hydrophobic Binding	π-π Interaction	Dipole-Dipole	Hydrogen Bonding	Shape Selectivity
ACE Advanced Method Development Kit	ACE C18	****	-	-	*	**
	ACE C18-AR	****	*** (donor)	*	**	***
	ACE C18-PFP	****	*** (acceptor)	****	***	****
ACE Extended Method Development Kit	ACE SuperC18	****	-	-	-	**
	ACE C18-Amide	****	-	**	****	**/***
	ACE CN-ES	***	*	***	**	*

[1]weightings and/or by reference to other ACE phases using >100 characterising analytes.

COLUMN SCREENING WITH AVANTOR® ACE® MDKS - FOUR STEPS FOR RATIONAL METHOD DEVELOPMENT

Column screening is a simple yet powerful approach, allowing a suitable column to be quickly identified. The approach can also be made more comprehensive by screening two different mobile phase organic modifiers (e.g. methanol and acetonitrile) to further asses selectivity. The flow chart in Figure 2 summarises how a method development screen can be carried out in four simple steps.
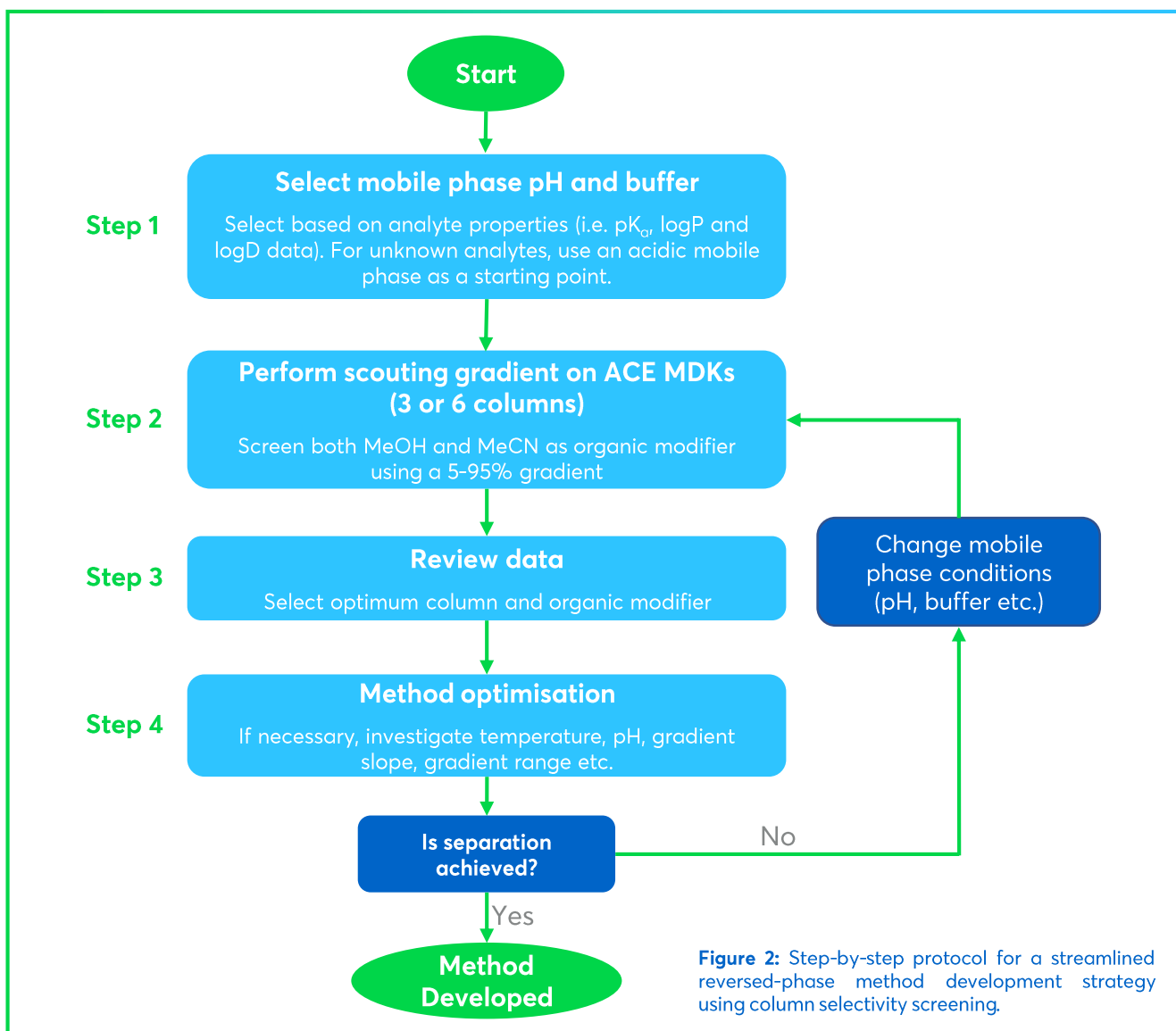
Start

Step 1

Select mobile phase pH and buffer

Select based on analyte properties (i.e. pK_a, logP and logD data). For unknown analytes, use an acidic mobile phase as a starting point.

Step 2

Perform scouting gradient on ACE MDKs (3 or 6 columns)

Screen both MeOH and MeCN as organic modifier using a 5-95% gradient

Step 3

Review data

Select optimum column and organic modifier

Change mobile phase conditions (pH, buffer etc.)

Step 4

Method optimisation

If necessary, investigate temperature, pH, gradient slope, gradient range etc.

Is separation achieved?

No

Yes

Method Developed

Figure 2: Step-by-step protocol for a streamlined reversed-phase method development strategy using column selectivity screening.

SELECTING COLUMN DIMENSIONS AND PARTICLE SIZE

The column dimensions can be defined by the LC system and user preference. In general, for 400 bar HPLC systems, 5 µm 150 x 4.6 mm is a good choice. For 600 bar optimised HPLC systems, 2 and 3 µm particles in shorter columns (e.g. 100 mm) can be used. 1.7 µm UHPLC particles in short column lengths (e.g. 50 mm) are suitable for UHPLC systems.

HOW TO DETERMINE AN APPROPRIATE SCREENING GRADIENT TIME.

A suitable gradient time for the screening experiments can be selected using equation 1. The column volume (V_M) can be estimated using equation 2. It is important to always include a post-gradient isocratic re-equilibration of at least 10 x V_M before the next injection.

$$t_G = \frac{k^* \times \Delta\varphi \times V_M \times S}{F} \quad (1)$$

$$V_M = \frac{0.5 \times L \times d_c^2}{1000} \quad (2)$$

t_G = Gradient time (mins.)
k^* = Gradient retention factor (typically set to approx. 5)
$\Delta\Phi$ = Gradient range (for a 5-95%B gradient, $\Delta\Phi$ = 0.9)
V_M = Column internal volume (mL)
S = 4 for small molecules
F = Flow rate (mL/min)
L = Column length (mm)
d_c = Column internal diameter (mm)

WORKED EXAMPLE

Figure 3 shows an example of the application of the column screening protocol to a pharmaceutical sample containing acetaminophen (paracetamol) and related substances. As per Figure 2, the mobile phase pH was selected based on logD and pK_a data for the 10 analytes. The sample was screened on the ACE Advanced and Extended MDKs (six ACE stationary phase chemistries) detailed in Table 1.

The upper six chromatograms show the sample screened on the six ACE reversed-phase columns using methanol (MeOH) as the organic modifier in line B. In the lower six chromatograms, the experiment was repeated using
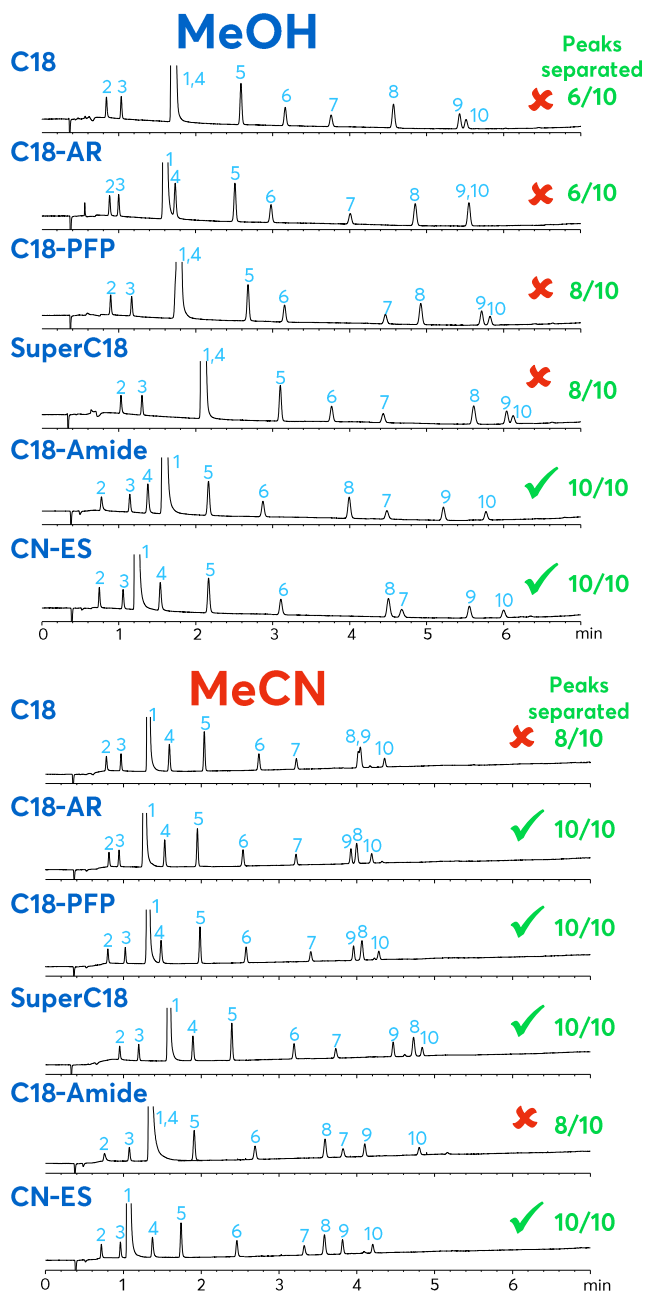

Figure 3: Worked example of a six column screen using the protocol outlined in Figure 2.
Columns: ACE Excel 2 µm, 100 x 3.0 mm; Mobile Phase A: 20 mM NH_4OAc pH 6.0 (aq); B: 20 mM NH_4OAc pH 6.0 in Organic:H_2O (9:1 v/v); Gradient: 5 to 95% B in 10 minutes; Flow rate: 1.2 mL/min; Temperature: 40 °C; Detection: UV, 210 nm; Sample:
1) Acetaminophen (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.

acetonitrile (MeCN) as the organic modifier. As can be seen, clear differences in analyte selectivity are apparent on the six columns. Additionally, methanol and acetonitrile also provide different analyte spacing. The most common starting point for method development (C18) did not separate all the analytes using either methanol or acetonitrile. Further method development would be required. On the novel phases however, this six column/two mobile phase screening strategy immediately provided six solutions for the separation of all of the sample components, meaning that no further method development was required.

CONCLUSION

This Knowledge Note has outlined a simple and universal protocol for reversed-phase method development using Avantor® ACE® Method Development Kits. Screening a new sample on multiple stationary phase chemistries and multiple organic modifiers allows chromatographers to quickly identify a suitable stationary phase/mobile phase combination for the separation. This helps to streamline the method development process. In the worked example shown, the screening protocol produced six possible options for the full separation of all sample components, with no further method development required.