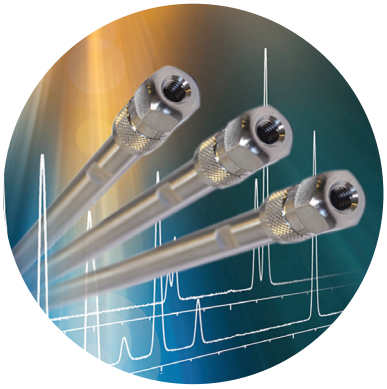
Computer aided method development using *in-silico* computer modelling and 6 columns with unique selectivities

An off-line version of ChromSword (ChromSword 2) without instrument connection can be used as an aid to HPLC method development.



INTRODUCTION

Reversed-phase method development can be a lengthy process which can tie up valuable resources in an analytical laboratory. The process followed and success obtained can vary depending upon experience, resources and available time. Often, a trial and error (or one factor at a time) approach is adopted, where parameters are adjusted and decisions made according to the analytical results obtained for each iterative step. This approach can produce acceptable separations, but it may fail to identify the most suitable method, i.e. the most robust or the most cost effective (e.g. fastest) method. The adoption of a structured approach to method development is helpful for many reasons: It can lead to the development of better, more robust methods, generate useful retention knowledge for analytes, and has the potential to provide significant savings in both development time and costs. A popular approach is to use screening protocols to systematically explore individual chromatographic parameters (such as column stationary phase, eluent composition, pH etc) and their effects upon retention/separation. Once screening is complete, the most promising combination of conditions can be further optimised, if needed, to produce the final method. This approach is useful, informed and highly recommended. Taking this process further (if required), the screening data can be input to LC retention modelling software to generate retention models and predict analyte retention behaviour at this final optimisation stage. This can be helpful for many industries to improve retention understanding and explore method robustness. Once models have been generated, further method changes can be predicted *in silico* (e.g. changing gradient ranges/slope) and then experimentally verified with a few injections. The need for further actual experimental work is therefore greatly reduced.

The ACE® ChromSword® Method Development Kit (MDK) is designed to be the perfect way to develop robust analytical methods and introduce a combined screening/modelling approach to the lab in an extremely cost effective manner.

SIX UNIQUE PHASES FOR INTELLIGENT COLUMN SCREENING

The key to developing a new LC method is to first optimise the selectivity of the separation. Table 1 ranks key chromatographic parameters according to how influential they are for affecting selectivity. Most chromatographic parameters affect selectivity to different degrees, so it is helpful to focus on those

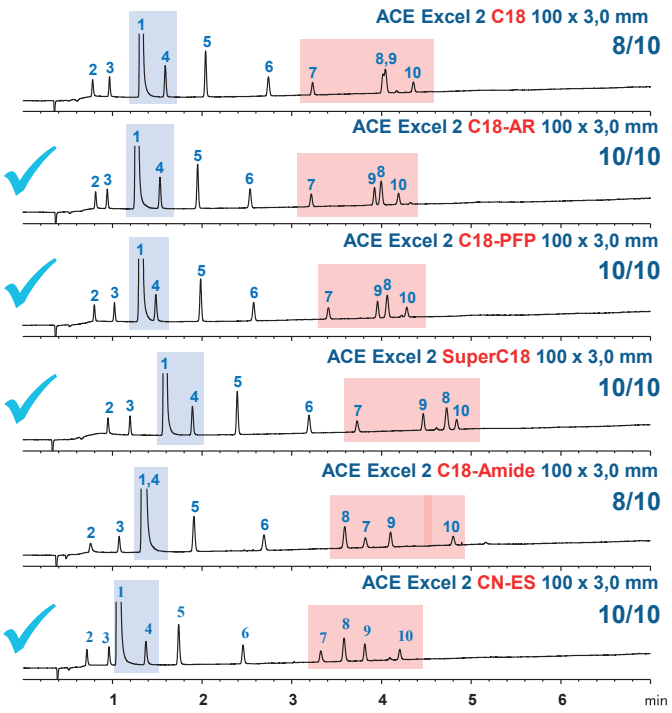
Table 1. Parameters affecting LC selectivity, ranked[1] according to their relative influence.

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

[1] *Adapted from Snyder L, Kirkland J, Dolan J, 2010, Introduction to Modern Liquid Chromatography, 3rd ed., New Jersey, Wiley & sons.*

parameters that maximise selectivity. Column selectivity is one of the most powerful parameters and, therefore, column screening using a range of stationary phase chemistries (with different mechanisms of interaction) is a widely adopted approach. This can then be expanded by also screening different organic modifiers in the eluent (e.g. methanol or acetonitrile) to explore the selectivity of protic and aprotic solvents. Screening combinations of column chemistries and solvents provides a useful, systematic approach to exploring analyte retention changes to achieve the desired separation. The ACE® reversed-phase method development poster (available free on request, see below) provides step-by-step guidance on how to design and perform column screening experiments.

The ACE® ChromSword® MDK contains six columns (including five novel chemistries with different mechanisms of interaction) specifically designed to provide alternative selectivity. Through rational design of the stationary phase chemistries, they each retain sample analytes by a unique combination of retention mechanisms, as summarised in Table 2. Screening a sample on these six chemistries, therefore, increases the likelihood of separating complex mixtures through multiple modes of interaction. Figure 1 shows the screening data on the six ACE® columns for paracetamol and related impurities. The most common starting point for method development, the C18 phase, fails to separate all the sample components. By screening the sample



SCOUTING CONDITIONS

Columns: 100x3,0 mm, 2 µm
A.: 20 mM ammonium acetate pH 6,0
B.: 20 mM ammonium acetate pH 6,0 in MeCN:H_2O (9:1 v/v)
Gradient: 5 to 95%B in 10 min
Temperature: 40 °C, injection: 2 µl, Flow rate: 1,2 ml/min
Sample: Acetaminophen with rel. subs. at 0,5% w/w

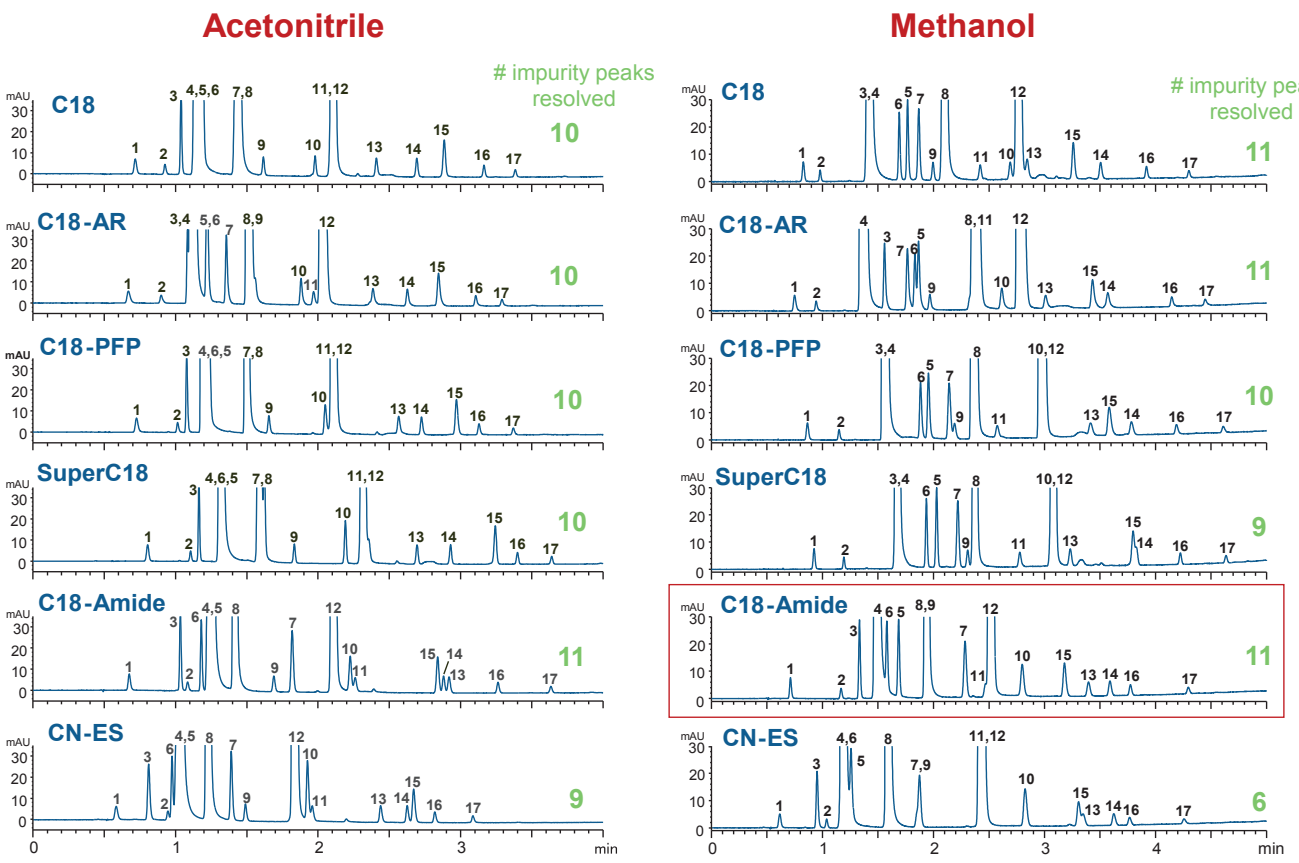
1. Paracetamol
2. 4-Aminophenol
3. Hydroquinone
4. 2-Aminophenol
5. 2-Acetamidophenol
6. Phenol
7. 4-Nitrophenol
8. 2-Nitrophenol
9. 4-Chloroacetanilide
10. 4-Chlorophenol

FIGURE 1: ACE® column screening for paracetamol and related substances.

Table 2. The six ACE®-bonded phases and relative contributions to their phase character.

Bonded phase	Separation mechanism and relative strength[1]				
	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	***	*	***	**	*

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



SCOUTING CONDITIONS

Columns: ACE® Excel 2 µm, 100x3,0
A.: 20 mM ammonium formate pH 3,0
B.: 20 mM ammonium formate pH 3,0 in MeOH:H_2O (9:1 v/v)
Gradient: 5 to 95%B in 5 min
Temperature: 40 °C, injection: 2 µl, Flow rate: 1,2 ml/min
Sample:

1. 2-Aminophenol
2. Hydroquinone
3. Theobromine
4. Paracetamol
5. Theophylline
6. Paraxanthine,
7. 4-Hydroxybenzoic acid
8. Caffeine
9. 2-Acetamidophenol
10. 2-Hydroxybenzoic acid
11. Phenol
12. Aspirin
13. 4-Nitrophenol
14. 4-Chloroacetanilide
15. 2-Nitrophenol
16. Acetylsalicylsalicyclic acid
17. Salsalate

FIGURE 2: ACE® column screening for a triple API pharmaceutical sample on the 6 ACE® column chemistries, using both methanol and acetonitrile as the mobile phase organic modifier.

on multiple columns, four separations are immediately identified that require no further method development. Closer examination of the data (highlighted boxes) shows that the six phases demonstrate different selectivity to one another, with co-elution occurring on some phases and complete reversals in the elution order of some peak pairs also observed (e.g. peaks 7 and 8 on the ACE® C18-Amide). This example clearly demonstrates the power of the column screening approach.

CHROMSWORD® 2 SOFTWARE FOR METHOD OPTIMISATION

Often, the optimum result from the column screening approach doesn't provide a full separation of all the sample analytes. In this case, further method development is required through varying other chromatographic parameters, such as temperature and gradient time. The ChromSword® 2 software, included in the ACE® ChromSword® MDK, provides a streamlined solution to this optimisation process. By entering retention data from as few as two experimental runs into the software, a retention model can be established. From the model, it is then possible to simulate thousands of potential separations without the need to perform additional experiments. Figure 2 shows the results from

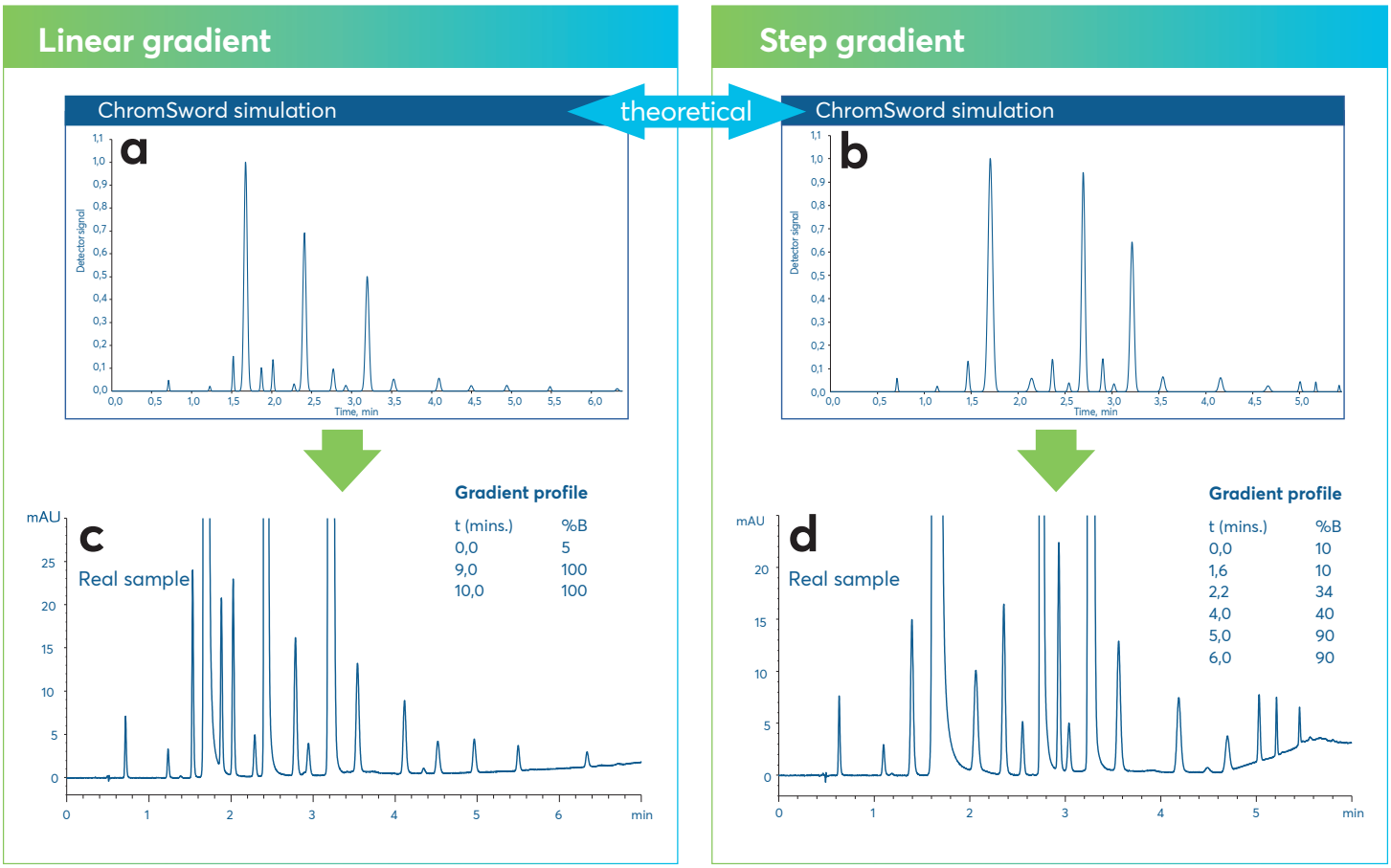
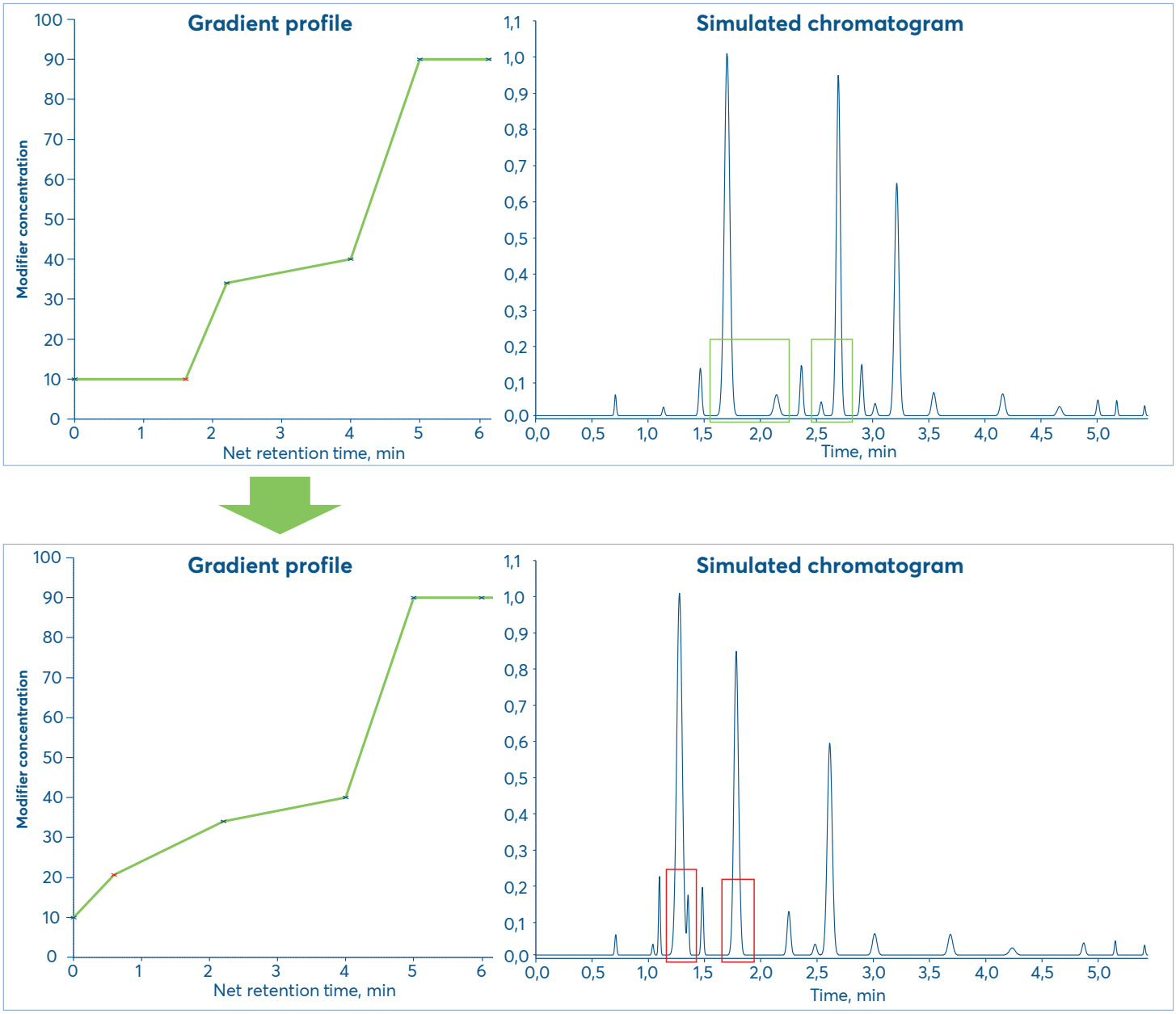


FIGURE 3: Simulated and experimental results from optimisation of the separation in Figure 2 by ChromSword® 2; a) predicted linear gradient separation, b) predicted step-gradient separation, c) real life chromatogram for the gradient in a and d) real life chromatogram for the step gradient in b.

an ACE® 6-column, 2-solvent screening experiment performed on a triple API pharmaceutical sample containing 14 impurities and 3 active ingredients (17 components in total). The separation on the ACE® C18-Amide with a methanol containing mobile phase was selected as the most promising combination for further development (highlighted). This screening result shows insufficient resolution of peak pair 4 and 6 and co-elution of 8, 9 and 11, 12. To optimise the separation, data from the 5 minute screening run, along with data from additional 10 and 15 minute gradients, were entered into ChromSword® 2 to generate a simple retention model based on gradient time. The software was then used to automatically optimise the separation and provided both linear and step gradient solutions for complete separation of all analytes (Figures 3a and b). The software has effectively removed the trial and error based steps (e.g. change gradient slope and/or add gradient steps, then perform injections) from the method development process. The ChromSword® 2 software uses an advanced Monte Carlo optimisation algorithm to simulate thousands of potential separations and return an optimised gradient profile. The software predicted that all 14 impurities could be fully resolved using both linear and step-gradients. The computer-generated gradients were then transferred to the LC system and run experimentally to validate the simulations (Figures 3c and d). Excellent agreement between the simulated and experimental results was obtained. The gradient separations shown in Figure 3 were generated by ChromSword® 2 in just a couple of minutes, thereby saving valuable development time compared to following an experimental approach to optimisation. In addition to fully automatic optimisation, the user may manually



edit the gradient profile in any way desired and the corresponding chromatogram is automatically simulated (Figure 4).

FIGURE 4: Manual editing of the gradient profile. The gradient profile can be displayed alongside a simulated chromatogram and manually editing the gradient results in real time changes to the simulated chromatogram. In this example, altering the initial isocratic hold at the start of the gradient was found to result in co-elution of two peak pairs.

CONCLUSIONS

This short article has introduced a streamlined approach to reversed-phase method development that combines column screening with advanced computer simulation software. By screening a new sample on each of the six ACE® columns, a suitable column selectivity for separation can be quickly identified. From as little as two runs, the ChromSword® 2 software can then be used to simulate and rapidly optimise the final separation.

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