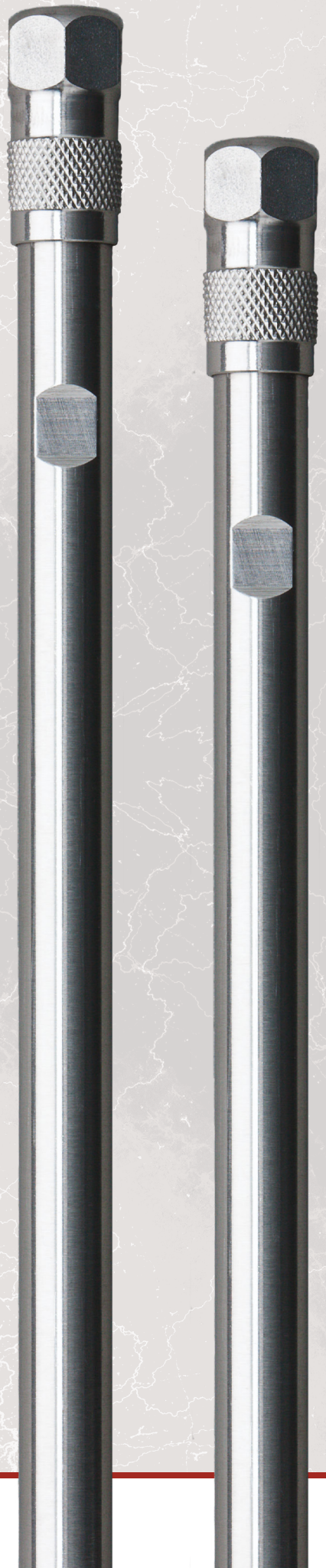
macmod
SMARTER CHROMATOGRAPHY

ACE®
HPLC and UHPLC Columns

Achieving Superior Separations with Innovative Chromatography Technologies



Achieving Superior Separations with Innovative Chromatography Technologies

Introduction

The ACE range of UHPLC and HPLC columns is designed to offer exceptional performance and reproducibility independent of which ACE column chemistry is being used for a particular application. In recent years, the ACE reversed-phase column chemistry portfolio has grown (through in-house organosilane applied expertise) with the introduction of a range of novel chemistry bonded phases that combine mechanisms of interaction to impact selectivity not available with traditional stationary phases. These rationally designed, next generation chemistries, offer the opportunity to explore the selectivity space and accelerate method development whilst not compromising robustness and reproducibility. This article discusses the technology and data behind these developments, including aspects such as scientifically led rational phase design, characterisation, performance, and reproducibility (column-to-column and batch-to-batch) that can be expected from all ACE UHPLC and HPLC columns for even the most demanding applications.

The Need for Reproducibility in LC Analysis

One of the most important aspects of a chromatographic method is that it should give reproducible performance. From injection-to-injection, system-to-system, and day-to-day, it is vital that the method provides predictable and consistent separation of the particular analytes of interest. Numerous factors can affect the reproducibility of a method, such as temperature fluctuations, mobile phase preparation variations, and instrument-to-instrument performance variability. Many of these factors are suitably controlled by the analyst, often by the adoption of good housekeeping practices and procedures. For example, using documented and well-defined mobile phase preparation procedures, controlling column temperature with column ovens, and regular maintenance of the LC system to meet system suitability criteria can all help to minimize method variability. One fundamental factor of LC methods that the analyst cannot directly control is the manufacturing of the column being used for the separation.

To provide a highly consistent and reproducible column (and therefore separation), all elements of the manufacturing process of the LC column must be subjected to quality control processes. It is therefore important for the chromatographer to work with high-quality columns from a well-established and trusted column manufacturer. ACE UHPLC and HPLC columns are designed and manufactured in laboratories accredited to the ISO9001 standard.

ACE columns are subjected to extensive controls at each stage of the manufacturing process, to ensure the products deliver reliable, predictable performance from batch to batch, lot to lot, and column to column. **Figure 1** demonstrates the excellent silica and silane batch-to-batch reproducibility provided by the ACE C18 (novel chemistries data provided later), achieved by using finely controlled protocols for all manufacturing elements involving silica quality and purity, chemistry bonding, and column packing. ACE columns are designed to deliver industry-leading performance and peak shape (**Figure 2**) and to be ultra robust and stable under challenging conditions as well as to provide excellent column lifetime and reproducibility (**Figure 3**).

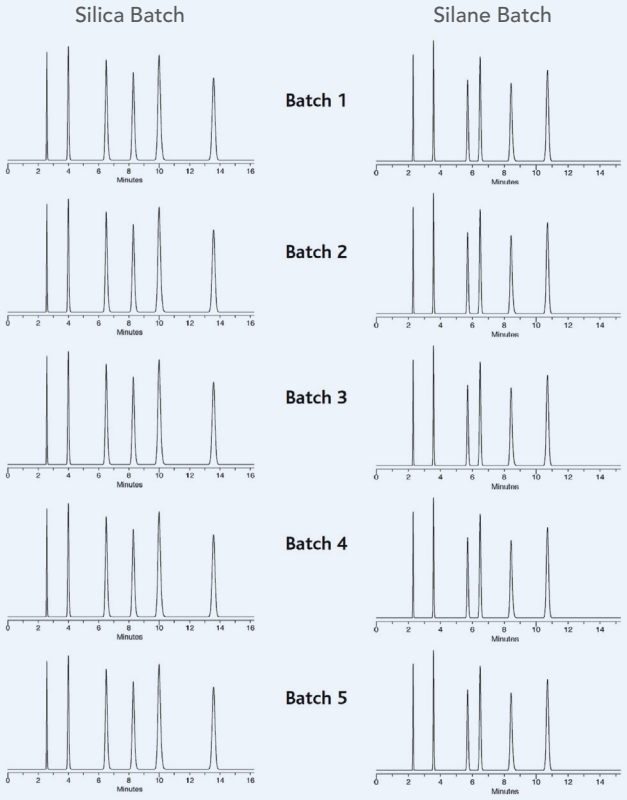
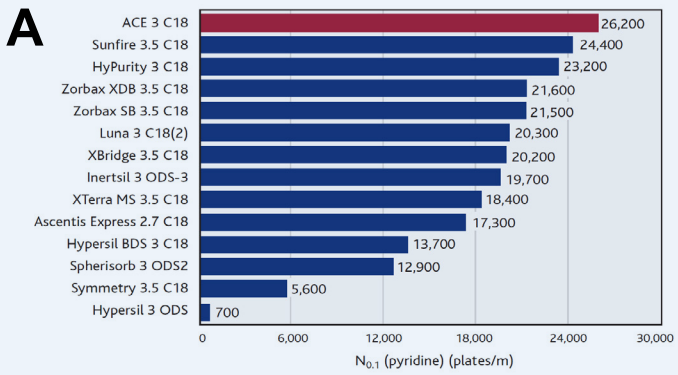


Figure 1: Historical test data demonstrating the batch-to-batch reproducibility of the ACE C18.

Conditions for left-hand chromatograms:
Column: ACE 5 µm C18, 250 x 4.6 mm
Mobile Phase: 80:20 (v/v) MeOH/0.025 KH_2PO_4 (pH 6.0)
Flow Rate: 1.0 mL/min
Sample: 1) Uracil, 2) Desipramine, 3) Doxepin, 4) Imipramine, 5) Amitriptyline, 6) Phenanthrene

Conditions for right-hand chromatograms:
Column: ACE 5 µm C18, 250 x 4.6 mm
Mobile Phase: 35:65 (v/v) MeCN/0.1% TFA in H_2O
Flow Rate: 1.0 mL/min
Sample: 1) Uracil, 2) 4-Hydroxybenzoic acid, 3) Acetylsalicylic acid, 4) Benzoic acid, 5) 2-Hydroxybenzoic acid, 6) Ethyl paraben

The comparative data presented here may not be representative of all applications. Please see References section for acknowledgement of trademarks



Conditions:
Column Dimensions: 50 x 2.1 mm
Mobile Phase: 40:60 (v/v) MeOH:H_2O
Flow Rate: 0.20 mL/min
Temperature: 22 °C
Detection: 254 nm
Sample: 1) Uracil, 2) Pyridine, 3) Phenol

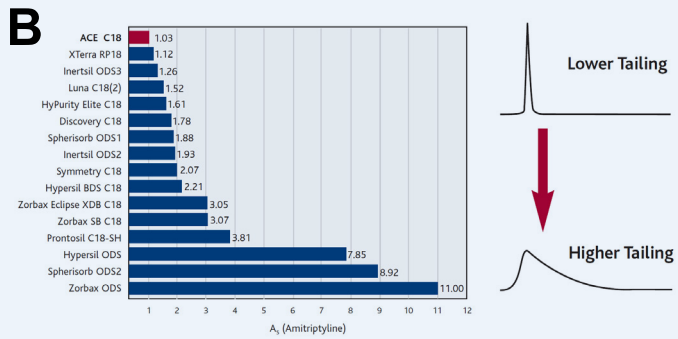


Figure 2: ACE columns deliver industry-leading peak shape and efficiency.*
A) Peak efficiency comparison
B) Peak asymmetry comparison for the ACE 3 µm C18 and various competitor C18 phases currently available on the market

Conditions:
Column Dimensions: 150 x 4.6 mm, 5 µm
Mobile Phase: 80:20 (v/v) MeOH:5 mM potassium phosphate buffer (pH 7.0)
Flow Rate: 2.0 mL/min
Temperature: 24 °C
Sample: Amitriptyline

The comparative data presented here may not be representative of all applications. Please see References section for acknowledgement of trademarks

*The data was obtained from the National Institute of Standards and Technology (NIST), Certificate of Analysis for Standard Reference Material 870 – "Column Performance Test Mixture for Liquid Chromatography" at the NIST website http:/ois.nist.gov/srmcatalogue/certifictates/870.pdf in September 2002. The NIST test miture, which is designed to characterize general aspects of HPLC was revised in December 2002. Comparative data may not be representative of all applications. Please see References section for acknowledgement of trademarks.

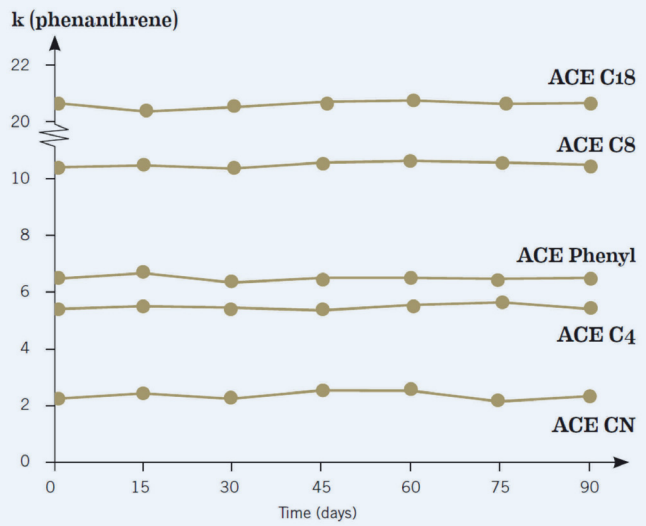


Figure 3: Long-term acid stability of ACE phases at pH 1.8.

Conditions:
Column Dimensions: 150 x 4.6 mm
Mobile Phase: 50:50 (v/v) $CH_3CN:H_2O$
Flow Rate: 1.0 mL/min
Temperature: 22 °C
Sample: Phenanthrene

Acidic Exposure Conditions:
Mobile Phase: 50:50 (v/v) MeCN:0.1% TFA in H_2O pH 1.8
Flow Rate: 1.0 mL/min
Temperature: 22 °C

ACE Phase Design and Novel Selectivity Developments

The reliability, reproducibility, selectivity, and stability of C18 phases have led to them being the most widely used column chemistry for a broad range of LC applications. The mechanism of separation for C18 columns is dominated by hydrophobic interactions between the analyte and stationary phase. Many analytes will benefit from other modes of interaction, depending upon their chemical structure, chemical conformation, and constituent functional groups, to enable separation from other species in the sample. It is therefore desirable to have other bonded phase chemistries available to provide additional mechanisms of interaction. Currently, popular RPLC non-C18 chemistry options include alkyl aromatic phases (e.g., propyl phenyl, hexyl phenyl, or propyl pentafluorophenyl (PFP)) and polar embedded phases (e.g., embedded amide, carbamates). However, these phases can display properties such as shorter column lifetimes, phase bleed, or reproducibility issues which will be undesirable for developing robust methods.

To combat this shortcoming, novel ACE chemistries have been designed that combine the robustness of traditional C18 bonded phases with the alternative selectivity provided by other functional groups. This concept and approach has led to the development of the ACE range of novel phase chemistries, specifically designed to maximize chromatographic selectivity by bringing together different mechanisms of interaction within a single stationary phase. Applying this phase design approach has resulted in the development of five novel chemistries that include the ACE C18-AR, ACE C18-PFP, ACE C18-Amide, ACE CN-ES, and ACE SuperC18. (See addendum for structures.) These novel phases incorporate added functional groups within the C18 bonded phase ligand to provide substantially different selectivity to traditional C18 phases which will maximize selectivity for a particular separation.

For example, the ACE C18-AR incorporates aromatic functionality into a C18 ligand to provide enhanced interactions with an electron deficient aromatic analyte functional group. Conversely, the C18-PFP incorporates the pentafluorophenyl functional group which provides enhanced interactions with an electron-rich aromatic group. The ACE C18-Amide was designed with a longer spacer between the silica particle and the embedded amide group, to provide the unique selectivity of embedded amide phases with added stationary phase stability. The ACE CN-ES was designed to provide interesting CN selectivity but specifically address the low stability of traditional CN columns. By extending the bonded ligand alkyl spacer, the ACE CN-ES stability is significantly improved over traditional CN phases. Finally, the ACE SuperC18 utilizes novel encapsulated bonding technology (EBT) to extend the usable pH range of this phase and allows chromatographers the option of operating their methods at extremes of pH (pH 1.5-11.5).

Chromatographic selectivity is the most influential term in the resolution equation (see **Figure 4** for equation and a graph of resolution vs. selectivity, efficiency, and retention factor). Whilst retention factor (*k*) and column efficiency (*N*) can be utilized to increase resolution, optimizing the selectivity of the separation (*α*) provides the most effective option for maximizing resolution.

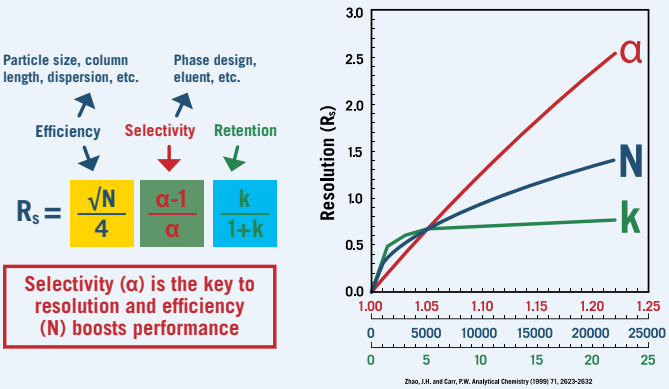


Figure 4: Resolution equation describing relationship with efficiency, selectivity, and retention.

Providing different mechanisms of interaction through different column chemistries maximizes selectivity and therefore resolution of analytes from each other. Whilst C18 phases can provide good separations in many cases, there are many examples where other mechanisms of interaction, provided by a different stationary phase, are required to deliver a separation that is not possible with a C18 phase. For example, **Figure 5** shows the separation of a mixture of sulphur-containing analytes under identical conditions using ACE C18 and ACE C18-AR columns. While the C18 phase alone (hydrophobic interactions) cannot separate the analytes, in this case, the C18-AR (similar hydrophobicity to C18 with additional pi-pi and weak dipole-dipole interactions) is able to resolve all four analytes.

Tanaka Characterization of ACE UHPLC and HPLC Columns

All ACE column chemistries have been designed and characterized using data from the Tanaka protocol[1] – a well-established and accepted approach to understanding stationary phase retention mechanisms. Using a series of small probes under specific conditions, values are derived for specific mechanisms present (or absent) for the stationary phase tested (five key tests are performed and collected, representing different mechanisms and column characteristics). The data from these five tests can help the analyst understand the weightings of different mechanisms that contribute to analyte retention on each column used for a separation. **(Note: Tanaka tests are described later.)** Stationary phases can be designed to optimize specific retention mechanisms, providing complementary stationary phases with different selectivity to one another. The data for five key mechanisms of interaction determined for ACE columns have been converted to stars for simplicity in **Table 1** (where more stars means the mechanism has a higher weighting with that chemistry).

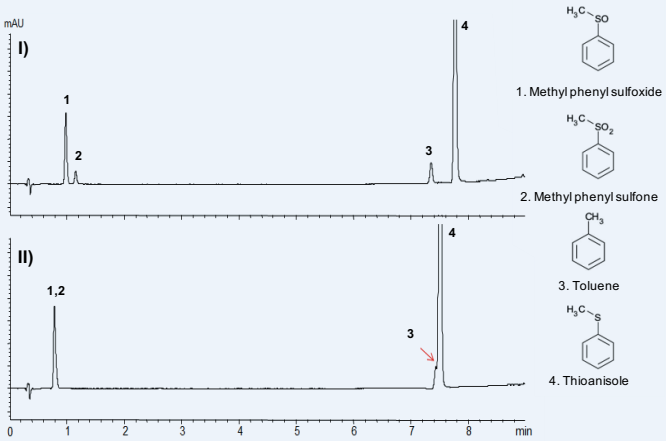


Figure 5: Separation of sulphur-containing analytes on the ACE C18-AR and ACE C18. The novel ACE C18-AR has multiple modes of interaction to provide separation where a standard C18 chemistry cannot achieve full resolution of the analytes.

Conditions:

Columns: ACE 3 µm, C18-AR, 50 x 2.1 mm
ACE 3 µm, C18, 50 x 2.1 mm

Part Numbers: ACE-119-0502
ACE-111-0502

Mobile Phase: A: H_2O
B: MeOH

Time (mins)	%B
0.0	30
5.0	30
9.0	95
9.5	30

Post Time 4 mins

Flow Rate: 0.5 mL/min
Injection: 1 µL
Temperature: 22 °C
Detection: UV, 254 nm

Bonded Phases	Separation Mechanism and Relative Strength[2]				
	Hydrophobic Binding	π-π Interaction	Dipole-Dipole	Hydrogen Bonding	Shape Selectivity
ACE C 18	****	-	-	*	**
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.
[2]Separation mechanisms provided by the ACE C18 and five novel phases.

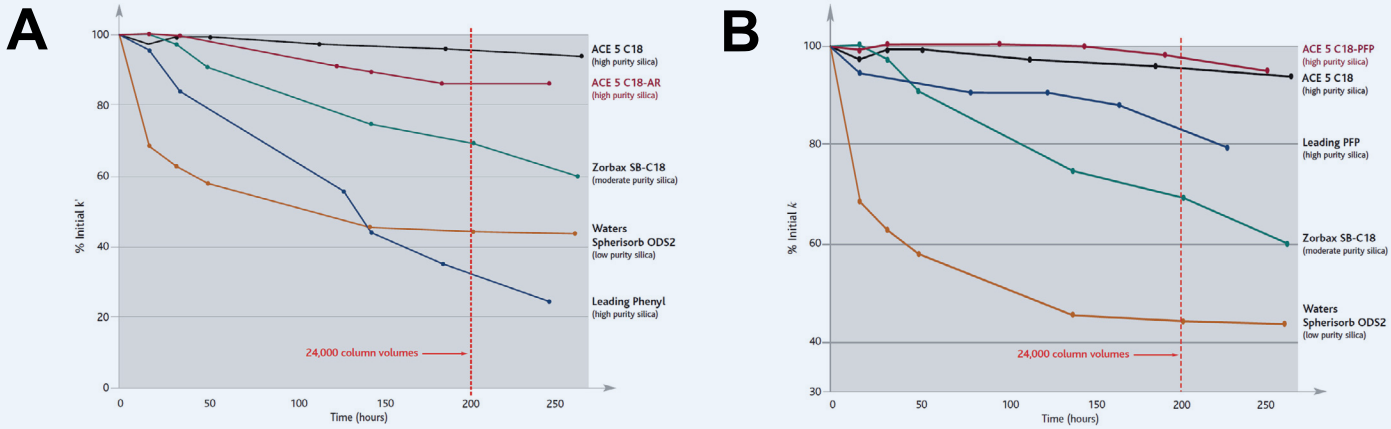


Figure 6: Accelerated low pH stability studies at high temperature for A) the ACE C18-AR and B) the ACE C18-PFP. Both phases show similar stability to the ACE C18, whilst outperforming leading phenyl, PFP, and C18 phases.

Conditions:
Column Dimensions: 50 x 2.1 mm
Mobile Phase: 5:95 (v/v) MeOH:0.1% TFA in H_2O pH 1.9
Flow Rate: 0.20 mL/min
Temperature: 80 °C

As can be seen, each ACE novel chemistry provides a complementary mixture of retention mechanisms to offer unique selectivity toward target analytes.

Building Stability and Reproducibility Into Novel Selectivity Columns

Often, columns which provide alternate selectivity to a C18 are perceived as being less robust. This is partly due to them being bonded with shorter chain ligands (e.g., phenyl, PFP, and cyano phases that frequently incorporate a propyl or hexyl linker within the ligand), which makes the bonded phase more prone to acid hydrolysis at low pH. These shorter chain chemistries are chemically labile and do not offer much to impede acid hydrolysis. Additionally, these phases offer less hydrophobic retention (a key mechanism in reversed-phase LC) than a C18 phase.

The ACE novel chemistries avoid these issues through rational phase design, and as such, can be considered as robust and reproducible as standard C18 phases. Additionally, these novel chemistries offer low phase bleed compared to shorter chain phenyl or PFP-based columns. The built-in stability of the ACE novel chemistries is demonstrated in **Figure 6**. **Figure 6A** shows an accelerated column stability study, performed under aggressive acidic conditions, comparing the ACE C18-AR to C18 and phenyl phases. **Figure 6B** shows an equivalent experiment for the ACE C18-PFP. Both the ACE C18-AR and ACE C18-PFP phases are shown to be as stable as the ACE C18. The unique chemistry of the ACE novel phases means that the chromatographer can expect the same run-to-run and sample-to-sample stability offered by C18 phases under even the most challenging conditions.

Batch-To-Batch Reproducibility

Variations between different batches of stationary phase are historically one of the most common causes of method development concern and was a feature of older Type A silica-based columns many years ago. Type A silicas were much less pure than Type B silicas that are commercially available today. The ACE novel phases, however, are manufactured using the same strict bonding protocols and ultra-inert base deactivated 'Type B' silica as the standard ACE C18. Stringent control of the entire manufacturing process, along with tight specifications for purity, selectivity, retention, asymmetry, and efficiency, means excellent batch-to-batch reproducibility is obtained for all ACE chemistries. As shown in **Figure 7** for ACE C18-PFP, thorough control of all manufacturing processes means that batch-to-batch variability of the parent silica (same particle size plus different particle sizes) and silane batches is very low. As a result, separations can be seamlessly and predictably scaled between different ACE particle sizes and column dimensions. Batch-to-batch and column-to-column reproducibility are guaranteed for all ACE columns.

As a further demonstration of the excellent batch-to-batch reproducibility offered by all phases, **Figure 8** shows an overlay of Tanaka characterization data (all five tests plus the additional two ion-exchange tests for mechanisms and column characteristics) for 9 batches of ACE C18-AR. Column characterization protocols, such as the Tanaka approach, use specially selected analytes chromatographed under specific conditions to characterize the properties of a bonded phase. The overlaid batch data shows that the unique selectivity of the ACE C18-AR is highly reproducible across multiple batches, therefore providing the chromatographer with assurance of the batch-to-batch reproducibility of ACE columns.

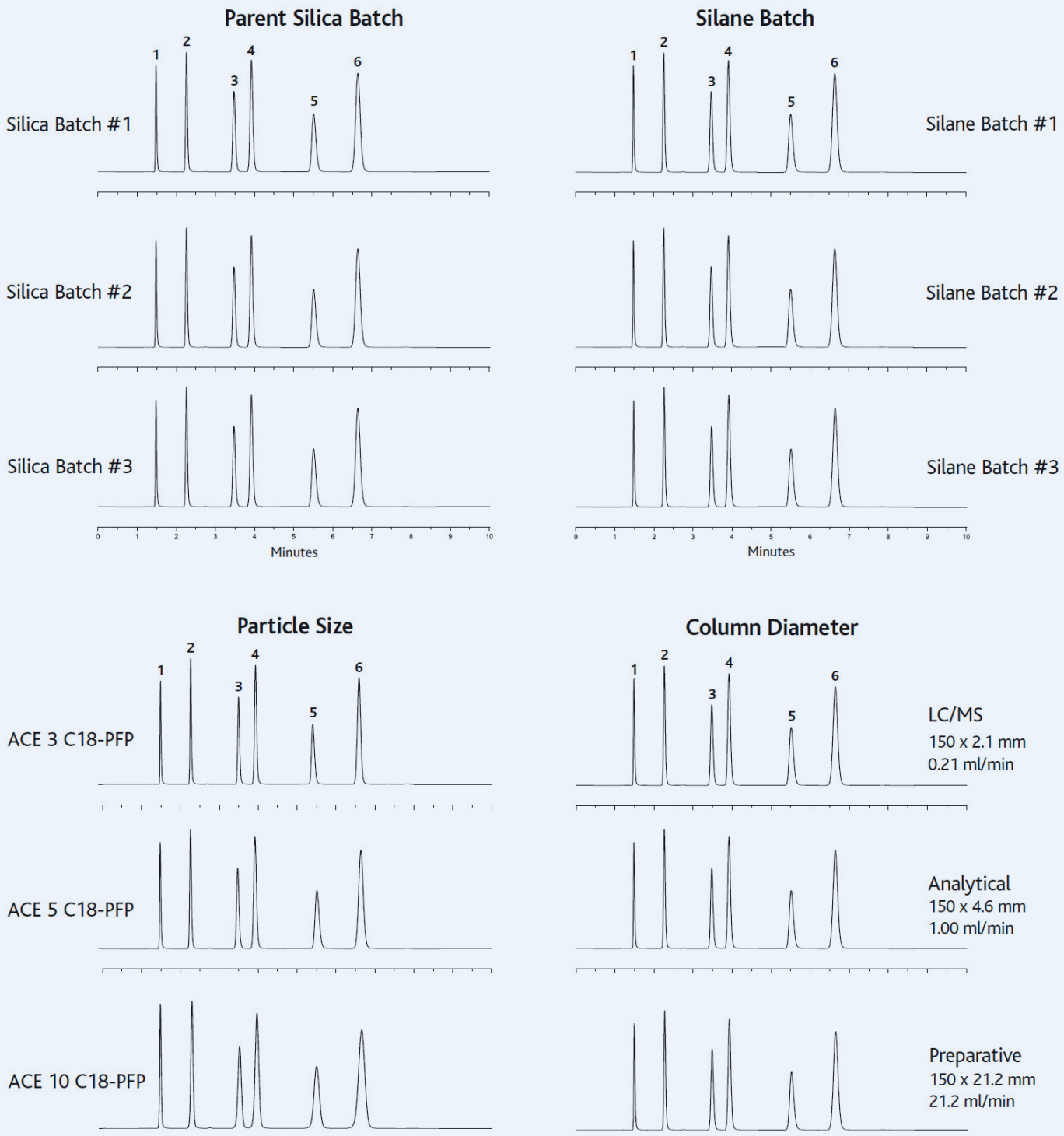


Figure 7: Chromatograms demonstrating the excellent reproducibility achieved with the ACE C18-PFP when silica and silane batches are changed. Separations can be reproducibly transferred between particle sizes and scaled between different column dimensions.

Conditions:
Column: ACE 5 µm C18-PFP, 150 x 4.6 mm (unless specified otherwise)
Mobile Phase: 35:65 (v/v) MeCN:0.1% TFA in H_2O
Flow Rate: 1.00 mL/min (unless specified otherwise)
Temperature: 22 °C
Detection: 254 nm
Sample: 1) Uracil, 2) 4-Hydroybenzoic acid, 3) Acetylsalicylic acid, 4) Benzoic acid, 5) 2-Hydroxybenzoic acid, 6) Ethyl paraben

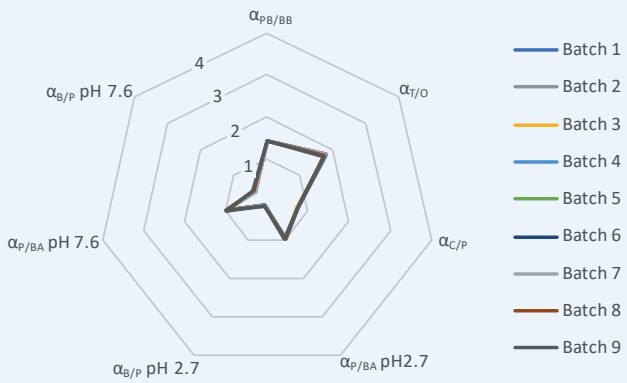


Figure 8: Tanaka characterization data for 9 batches of the ACE C18-AR.

Tanaka Characterization Parameters:

$\alpha_{PB/BB}$ – Hydrophobic selectivity
$\alpha_{T/O}$ – Shape selectivity
$\alpha_{C/P}$ – Hydrogen bonding capacity
$\alpha_{P/BA}$ pH 2.7 – Acidic cation exchange capacity
$\alpha_{B/P}$ pH 2.7– Phenolic selectivity
$\alpha_{P/BA}$ pH 7.6 – Total ion exchange capacity
$\alpha_{B/P}$ pH 7.6 – Phenolic selectivity

ACE Excel UHPLC Columns: Engineered for UHPLC Robustness

The adoption of Ultra-High-Pressure LC (UHPLC) separations using sub-2 micron particles has accelerated in recent years. The use of these smaller particle sizes is accompanied with a large increase in operating back pressure, placing increased demands on the analytical column. ACE Excel UHPLC columns are designed to take full advantage of low dispersion, UHPLC instruments (up to 1,000 bar, 15,000 psi), and are fully compatible with all commercially available UHPLC systems. ACE Excel UHPLC columns (available with 1.7 and 2 µm particles) are packed using proprietary HSC™ (High Stability Column) technology, which results in ultra-robust columns. This packing technology was implemented to ensure the stability of the stationary phase packed bed under the demanding high pressure and high mobile phase linear velocity conditions typically used with UHPLC applications. **Figure 9** shows a comparison of an ACE Excel column packed with HSC™ technology and a prototype column packed without the technology.

Both columns were subjected to a gradient cycle with a maximum pressure of 1,000 bar (15,000 psi). After 2,000 gradient cycles, the column efficiency, retention, and peak shape of the ACE Excel column was essentially unchanged. In contrast, the prototype column, packed without HSC™ technology, showed a rapid deterioration in performance. The ACE novel selectivity phases are all available in ACE Excel UHPLC column formats with 1.7 and 2 µm particles packed using HSC™ technology.

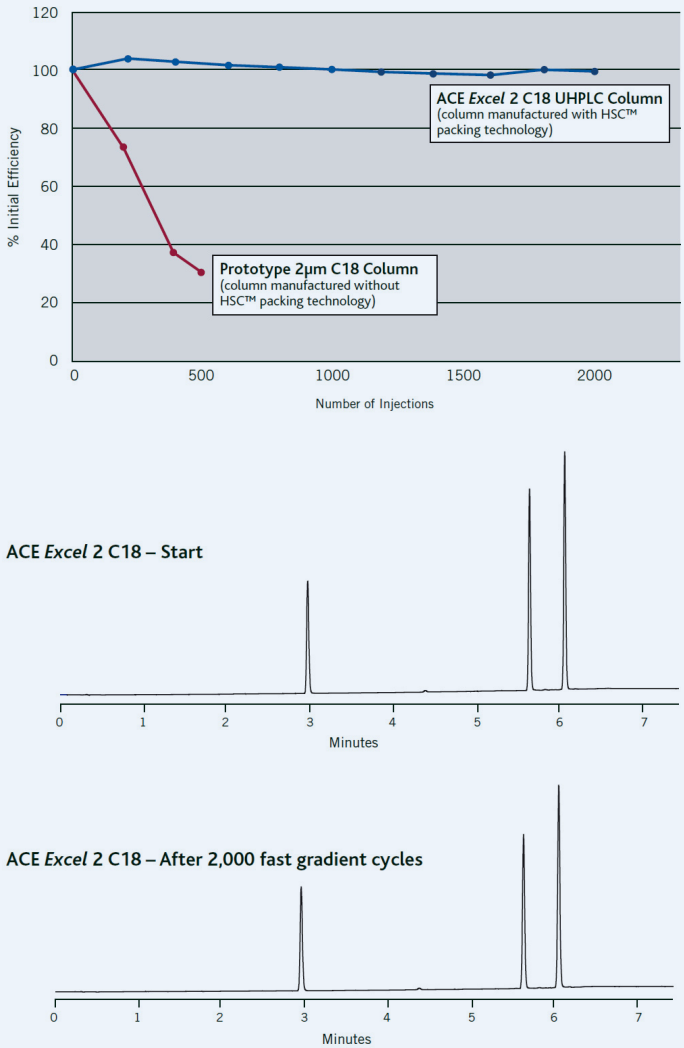


Figure 9: Comparison of the efficiency of an ACE Excel column packed with HSC™ technology and a prototype column packed without. Both columns were subjected to up to 2,000 injections on a high-pressure (1,000 bar maximum) gradient.

Conditions:

Column Dimensions: 100 x 2.1 mm
Mobile Phase: A = 0.1% formic acid in H_2O
Mobile Phase B = 0.1% formic acid in MeOH
Gradient A: 20 – 90% B in 5 minutes, 1 minute hold
Gradient B: 90% to 20% B in 1 minute, 2 minutes re-equilibration hold
Flow Rate: 0.73 mL/min
Pmax = 1,000 bar (15,000 psi)

Encapsulated Bonding Technology for Extended pH Stability

For ionisable analytes, pH is a powerful parameter for controlling the selectivity of a separation. For example, many basic pharmaceutical compounds can be chromatographed in their more polar ionised forms at low pH (e.g., pH 2), which can often result in low retention and poor peak shape. At high pH (>9) however, they are predominantly in their neutral, more hydrophobic form, making analysis at high pH potentially highly desirable. Most silica-based LC columns have an optimum lifetime when used at a pH approximately between 2 and 8, which precludes their use at high pH for basic analytes. The ACE SuperC18 (porous and solid core particles) and SuperPhenylHexyl (solid core particle) are ultra-inert phases with extended pH stability and have a recommended pH range of 1.5-11.5. These ACE column options have been developed to allow the analyst to fully exploit both low and high pH mobile phases.

This extended pH stability is achieved through novel organo-silane bonding termed Encapsulated Bonding Technology (EBT™). This approach dramatically increases ligand coverage at the silica surface and essentially eliminates the effect of unbonded silanol groups on analytes from separations. The use of EBT™ means that the ACE SuperC18 and SuperPhenylHexyl phases are ultra-resistant to ligand cleavage under aggressive acidic conditions and are protected from silica dissolution under basic conditions, which can lead to premature column deterioration. **Figure 10** demonstrates the prolonged use of the ACE SuperC18 under aggressive acidic and basic conditions. Under both sets of conditions, retention and column performance remain unaffected after >2,000 injections, confirming the stability and robustness of the stationary phase material.

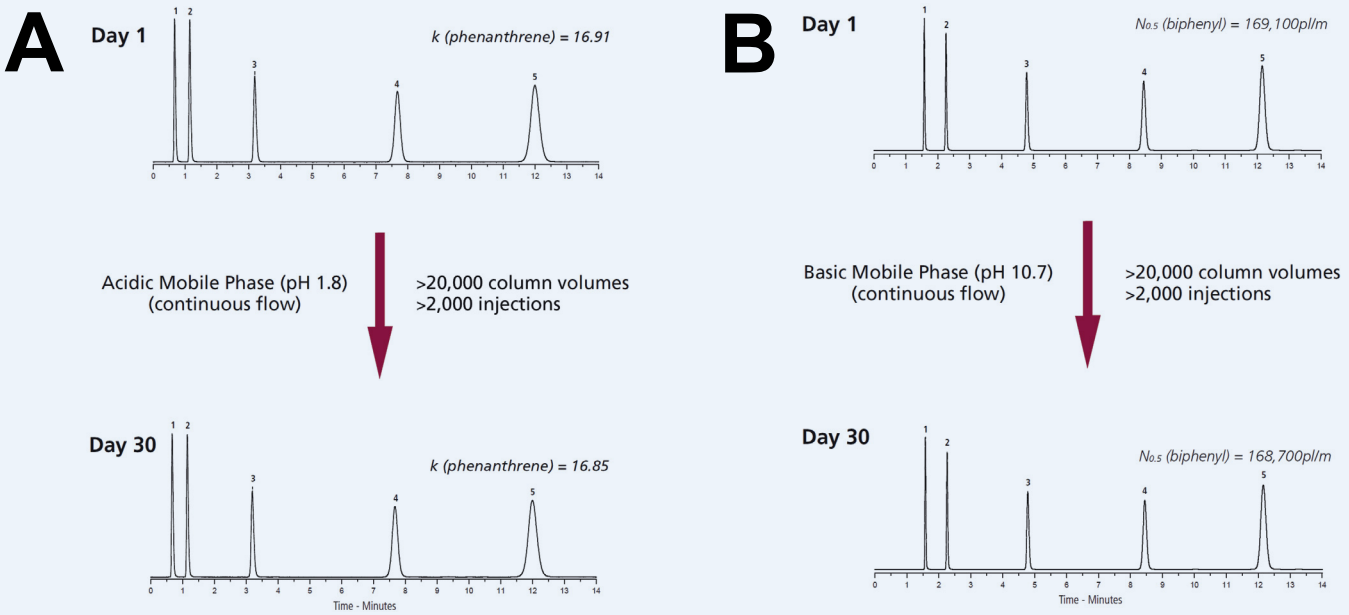


Figure 10: QC testing of the ACE Excel SuperC18 before and after >20,000 column volumes continuous flow exposure to A) acidic (pH 1.8) and B) basic (pH 10.7) mobile phase. The ACE SuperC18 shows no reduction in retention or performance after exposure to either set of conditions. The SuperC18 and SuperPhenylHexyl columns provide excellent separations for a range of small molecules with low and high mobile phase pH values. The wider pH range stability of the columns offers the analyst the option to explore pH without concerns about stability or poor column lifetime.

Acidic Flushing Conditions
Column: ACE Excel 2 µm SuperC18, 50 x 2.1 mm
Mobile Phase: 50:50 (v/v) MeOH:0.1% TFA in H_2O (pH 1.8)
Flow Rate: 0.20 mL/min
Temperature: 40 °C

Basic Flushing Conditions:
Column: ACE Excel 3 µm SuperC18, 150 x 4.6 mm
Mobile Phase: 50:50 (v/v) MeCN:0.1% NH_3 in H_2O (pH 10.7)
Flow Rate: 1.00 mL/min
Temperature: 40 °C

Conditions for Acidic Flow Evaluation:
Mobile Phase: 70:30 (v/v) MeOH:H_2O
Flow Rate: 0.20 mL/min
Temperature: 22 °C
Sample: 1) Uracil, 2) Eimethyl phthalate, 3) Toluene, 4) Biphenyl, 5) Phenanthrene

Conditions for Basic Flow Evaluation
Mobile Phase: 80:20 (v/v) MeOH:H_2O
Flow Rate: 1.00 mL/min
Temperature: 22 °C
Sample: 1) Uracil, 2) Dimethyl phthalate, 3) Toluene, 4) Biphenyl, 5) Phenanthrene

Conclusions

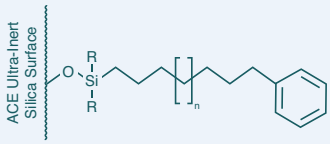
ACE UHPLC and HPLC phases have a well-deserved reputation for delivering exceptional column performance, peak shape, and reproducibility. Columns are manufactured within ISO 9001 accredited facilities, giving assurance to all users that a fully traceable Quality System is in place that records, monitors, and drives consistency and reproducibility of all ACE products. Based upon these solid manufacturing foundations with highly experienced chromatographers, novel ACE chemistries have been designed with unique organosilane technologies to provide new solutions for separation science. The novel ACE chemistries provide the expected robustness and reproducibility to deliver separation options for even the most difficult chromatography challenges in a diverse range of application areas including pharmaceutical, industrial, clinical, forensic, and environmental analyses.

References

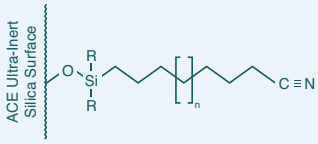
1. Kimata, K., Iwaguchi, K., Onishi, S., Jinno, K., Eksteen, R., Hosoya, K., Araki, M., Tanaka, N., J. Chromatogr. Sci. 1989 27, 721-728

Addendum

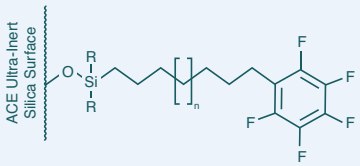
ACE C18-AR



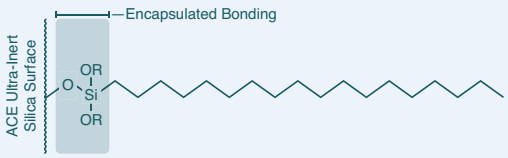
ACE CN-ES



ACE C18-PFP



ACE SuperC18



ACE C18-Amide