Peilin Yang[1]
Terry McCabe[1]
Matthias Pursch[2]

[1]The Dow Chemical Company, Analytical Technologies, Midland, MI, USA
[2]Dow Deutschland GmbH, Analytical Technologies, Rheinmuenster, Germany

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Research Article

Practical comparison of LC columns packed with different superficially porous particles for the separation of small molecules and medium size natural products

Commercial C_{18} columns packed with superficially porous particles of different sizes and shell thicknesses (Ascentis Express, Kinetex, and Poroshell 120) or sub-2-μm totally porous particles (Acquity BEH) were systematically compared using a small molecule mixture and a complex natural product mixture as text probes. Significant efficiency loss was observed on 2.1-mm id columns even with a low dispersion ultra-high pressure liquid chromatography system. The Kinetex 4.6-mm id column packed with 2.6-μm particles exhibited the best overall efficiency for small molecule separations and the Poroshell 120 column showed better performance for mid-size natural product analytes. The Kinetex 2.1-mm id column packed with 1.7-μm particles did not deliver the expected performance and the possible reasons besides extra column effect have been proved to be frictional heating effect and poor column packing quality. Different column retentivities and selectivities have been observed on the four C_{18} columns of different brands for the natural product separation. Column batch-to-batch variability that has been previously observed on the Ascentis Express column was also observed on the Kinetex and Poroshell 120 column.

Keywords: Kinetex C_{18} / Natural product / Poroshell 120 EC-C_{18} / Sub-2-μm particles / Superficially porous particles
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1 Introduction

High-performance liquid chromatography (HPLC) is the predominant analytical technique in pharmaceutical, agricultural, and food industries. Modern quality control (QC) laboratories rely heavily on HPLC analysis for product release and process monitoring. Reliable and cost-effective instrumentation, rugged and reproducible analytical methods, user friendly and intuitive data systems are among the top criteria that someone working in a QC environment would desire. The emergence of ultra-high pressure liquid chromatography (UHPLC) instrumentation and the advent of columns packed with sub-2-μm particles have brought liquid chromatography development to a new era of high efficiency, high speed, and high throughput. Numerous advantages of UHPLC have been discussed extensively since the first commercialization of UHPLC instrument in 2004 [1–5]. Despite the outstanding features it can offer including high efficiency, short analysis time, and low solvent consumption, UHPLC has not become an industrial standard technique after many years of improvement in instrumentation and column performance. Practical issues such as method transferability, precision, sensitivity, ease of use, and column lifetime have outweighed the benefits of UHPLC for many users [6]. Initial capital investment in more expensive UHPLC systems, which are often required for UHPLC methods due to the higher backpressure resulting from smaller particle size, is another hurdle for the proliferation of this new technology [7–9]. From a practical point of view, a more cost-effective and straightforward way of improving the chromatographic performance of HPLC analysis would be more desired by QA/QC laboratories.

Another evolutionary event in the HPLC technology advancement was marked by the commercialization of a new generation HPLC column packed with superficially porous particles which are also known as core–shell or Halo™ or Fused-core™ particles. The concept of the superficially porous particles was not new and the early work can be traced back to 1960s [10]. But these particles did not draw broad attention until recently when the particle

Correspondence: Dr. Peilin Yang, Analytical Technologies, The Dow Chemical Company, 1897 Building, Midland, MI 48667, USA
E-mail: pyang@dow.com
Fax: +1-989-636-8450

Abbreviations: QC, quality control; **UHPLC**, ultra-high pressure liquid chromatography

size had been reduced to sub-3 μm. Initial sub-3-μm superficially porous particles from Advance Material Technology (AMT) were made of a 1.7-μm solid core surrounded by a 0.5-μm porous silica shell giving a total particle size of 2.7 μm. The Ascentis Express column from Supelco and the Halo column from MAC-MOD packed with the same 2.7-μm superficially porous particles have been observed to offer comparable separation efficiency to that of columns packed with sub-2-μm totally porous particles for various applications [11–14]. Extensive fundamental studies, column evaluation, and comparison work have been carried out over the past 3–4 years on the chromatographic performance of superficially porous particles and operational considerations [12, 15–21]. In spite of the improved performance of these particles resulting from the reduced mass transfer term and narrow particle size distribution [22], the advantages found in small molecule separations were not observed for larger molecules [23]. Also, significant column batch-to-batch variability has been observed for natural product separations, which raised another concern of column ruggedness for complex samples, especially for methods under regulatory compliances [14].

More recently, both Phenomenex and Agilent introduced a new brand of columns packed with sub-3-μm superficially porous particles. It has been claimed by both companies that different processes such as sol–gel techniques for Kinetex columns [21, 24] and one-step polymerization techniques for Poroshell columns have been used in manufacturing these particles aiming to further improve the particle size distribution, surface roughness, and column batch-to-batch variability. The Kinetex columns from Phenomenex are available in two different particle sizes of 2.6 and 1.7 μm with 0.35 and 0.23 μm shell thickness, respectively. Improved performance has been observed with these new columns and some of the contributing factors include further reduced shell thickness thus even faster mass transfer, extremely smooth surface, and wider pore size distribution due to the stratified arrangement of the shells [19, 24–26]. The Agilent Poroshell 120 columns were packed with particles of the same dimension and shell thickness as the original Halo particles but with pore size of 120 Å instead of 90 Å. Little evaluation work on the Poroshell 120 column has been presented in the literature as of today.

The aim of our study was to make a practical comparison of a fully porous packing material with a particle size of 1.7 μm (Waters Acquity™) with three commercial superficially porous materials (Halo™, Kinetex™, and Poroshell 120™). Both 4.6 mm × 100 mm and 2.1 mm × 100 mm columns were evaluated with the aim to address the practical benefits and issues of different column dimensions such as extra-column effects and frictional heating effects. C_{18} stationary phase was chosen for all the tested columns in order to consistently compare the chromatographic performance of the columns and compare the phase selectivity for complex mixtures. Both small molecule test compounds and a complex mixture sample containing 16 medium size (Mw 700–800) naturally derived compounds with very similar structures, functional groups, and polarities were used as model analytes. Natural products are one of the most important sources for drug discovery, yet they are challenging in nature to separate and analyze due to their structural complexity [14, 27]. Column batch-to-batch variability was observed previously for natural product separation on the Ascentis Express column packed with Halo particles [14]. Minimal column batch-to-batch variability is one of the most critical factors to consider in method development and validation for extremely complex mixtures. It is our main goal to gain a better understanding of this new column technology to help develop reliable QC methods using these new generation columns packed with superficially porous particles.

2 Materials and methods

2.1 Materials and reagents

HPLC-grade water, HPLC-grade acetonitrile, HPLC-grade methanol, potassium phosphate monobasic, and potassium phosphate dibasic were purchased from Fisher Scientific (Pittsburgh, PA, USA). The Acquity BEH C_{18} column was purchased from Waters (Milford, MA, USA). The Ascentis Express C_{18} column was purchased from Supelco (Bellefonte, PA, USA). The three Kinetex C_{18} columns were purchased from Phenomenex (Torrance, CA, USA). The Poroshell 120 EC-C18 columns were purchased from Agilent Technologies (Palo Alto, CA, USA). Dimensions and properties of all six tested columns were summarized in Table 1.

Sample 1 was a mixture of uracil (2.5 μg/mL), acetophenone (10 μg/mL), toluene (0.5 mg/mL), ethyl benzene (0.5 mg/mL), and biphenyl (12.5 μg/mL) prepared in 50:50 acetonitrile/water. Sample 2 was a mixture of 16 amine-containing agricultural reference compounds (structures not disclosed here) derived from natural origin with molecular weights ranging from 700 to 800 Da. The sample was prepared by dissolving 2.5 mg of each compound in 100 mL of methanol to give a 25-ppm w/v solution.

2.2 Instrumentation

All the experiments were performed on an Agilent 1290 Infinity HPLC system (Agilent Technologies). The system included a 1290 Infinity binary pump, a thermal column compartment, and a programmable auto-sampler. The injection volume was set at 10 μL for 4.6-mm id columns and 2 μL for 2.1-mm id columns. The instrument was equipped with a 1290 Infinity Diode Array Detector with Max Light® cartridge flow cell (volume 1 μL and path length 10 mm). The in-line filter and column switching valve were intentionally removed to reduce system dwell volume. 0.12-mm id stainless steel capillaries (red tubing) were used for making all the connections.

Table 1. Dimensions and material characterization of the tested columns from manufacturers' literature

Column	Type	id (mm)	Length (mm)	Particle size (μm)	Shell thickness (μm)	Pore size (Å)	Surface area (m^2/g)	Carbon load (%)	Bonding density (μmol/m^2)
Supelco Ascentis Express C_{18}	Superficially porous	4.6	100	2.7	0.5	90	150	–	3.5
Agilent Poroshell 120 EC-C_{18}	Superficially porous	4.6	100	2.7	0.5	120	130	8	3.3
Phenomenex Kinetex C_{18}	Superficially porous	4.6	100	2.6	0.35	100	200	12	2.8
Phenomenex Kinetex C_{18}	Superficially porous	2.1	100	2.6	0.35	100	200	12	2.6
Phenomenex Kinetex C_{18}	Superficially porous	2.1	100	1.7	0.23	100	200	12	2.8
Waters Acquity BEH C_{18}	Totally porous	2.1	100	1.7	–	130	185	18	3.1

2.3 Chromatographic conditions

The performance of the Waters Acquity BEH C_{18}, the Supelco Ascentis Express C_{18}, Phenomenex Kinetex C_{18}, and Agilent Poroshell 120 EC-C_{18} was compared using sample 1 and sample 2. Brand new columns (from different lots) and the same preparation of mobile phase were used for the evaluation of column lot-to-lot variability to eliminate any variation caused by previous column use histories.

2.3.1 Conditions for small molecule analytes (sample 1)

Ten microliters (4.6-mm id columns) or 2 μL (2.1-mm id columns) of test mixture (sample 1) was injected and separated under isocratic conditions using 50:50 acetonitrile/water v/v at different flow rates for the efficiency study. The column ovens were maintained at 50°C and UV detection was at 250 nm.

2.3.2 Conditions for natural product mixtures (sample 2)

Ten microliters (4.6-mm id columns) or 2 μL of test mixture (sample 2) was injected and separated under isocratic conditions 58.8:14:27.2 v/v/v acetonitrile/methanol/25 mM pH 6.2 potassium phosphate buffer as the eluent at different flow rates for the efficiency study. The column ovens were maintained at 35°C and UV detection was at 250 nm.

2.4 Data acquisition and analysis

The system was controlled by ChemStation software and the signal was acquired with a sampling rate of 80 Hz. The separation efficiency was calculated using the peak width at half-height formula.

2.5 Method of calculation

In the column efficiency comparison study, superficial linear velocities were used instead of the chromatographic linear velocity to take into account the difference between the porosities of the superficially porous and fully porous particles [28]. The superficial linear velocity (u_s) is given by

$$u_s = \frac{F_v}{\pi R^2}$$

where F_v is the flow rate and R is the inner column radius.

Because the van Deemter equation $H = A+(B/u)+cu$ does not account for the fact that A and C terms are coupled through particle diameters, the Knox equation $h = Av^{1/3}+(B/v)+cv$ was used for the efficiency evaluation, where h is the reduced plate height and v is the reduced linear velocity. The reduced plate height is given by

$$h = \frac{H}{d_p}$$

where H is the HETP and d_p is the average particle size of column packing material. The reduced linear velocity was calculated according to the formula

$$v = \frac{u_s d_p}{D_M}$$

where u_s is the superficial linear velocity and D_M is the analyte diffusion coefficient.

Analyte diffusion coefficients were roughly estimated using the Wilke–Chang equation [29]

$$D_M = 7.4 \times 10^{-8} \frac{(\Psi M_s)^{0.5} T}{\eta V_A^{0.6}}$$

where Ψ is the solvent association factor, M_s is the molecular weight of the mobile phase, η is the viscosity, T is the temperature, and V_A is the solute molecular volume.

3 Results and discussion

Columns packed with superficially porous particles have attracted attention and gained popularity in recent years as they are lower pressure alternatives to the columns packed with sub-2-μm particles for achieving high separation efficiency. These columns are available in different dimensions, particles sizes, and shell thicknesses from different vendors. This manuscript systematically compares the performance of five columns packed with superficially

porous particles and one column packed with fully porous sub-2-μm particles (Table 1) using both a small molecule test mixture and a mid-size natural product mixture as model analytes.

3.1 Column efficiency

3.1.1 Extra column effect

The six columns evaluated in this study were divided into two groups for the efficiency comparison according to their column inner diameter (4.6 mm versus 2.1 mm). As our main goal was to provide a practical guide to column selection for method development rather than to find the theoretically highest efficiency possible for these columns, the measured peak widths were not corrected for the extra-column peak dispersion as it is impossible to completely eliminate the extra-column effect in real-life situations albeit there are approaches to minimize it [30, 31]. The Agilent 1290 Infinity LC instrument used in our study has an extra-column variance of 7 μL^2 measured in house, smaller than that of most of the HPLC systems on the market. However, as compared in Table 2, significant efficiency loss was observed on the 2.1-mm id Kinetex column compared with the 4.6-mm id column packed with exactly the same particles, especially for less retained analytes. On a traditional HPLC system with larger extra-column variance, the efficiency loss on the 2.1-mm id column would be more significant. Therefore, in spite of the solvent saving benefit from using 2.1-mm id column, one has to consider the compromise in separation efficiency, which could be crucial for complex samples.

3.1.2 Column efficiency for small molecules

To compare the three 4.6-mm id columns packed with different superficially porous particles for small molecule separation, a Knox plot for well-retained biphenyl peak (retention factor k' of 7–8) was constructed and shown in Fig. 1A. All three columns maintained low reduced plate height at high reduced velocities, enabling fast analysis without compromising efficiency. The Kinetex 4.6-mm id column packed with 2.6 μm particles with 0.35 μm shell thickness offered the highest efficiency with reduced plate height of 1.6. Part of the reason was the reduced mass transfer resistance (C-term) due to the reduced shell thickness and slightly smaller overall particle size. Other factors found previously such as particle smoothness and pore structures may have also contributed to the outstanding performance of the Kinetex column [19, 32].

A similar plot was constructed for the three 2.1-mm id columns as shown in Fig. 1B. The two Kinetex columns packed with superficially porous particles outperformed the Acquity column packed with totally porous particles at high flow velocities as expected, primarily due to the reduced mass transfer. The Kinetex 2.1-mm id column packed with 1.7-μm particles with 0.23-μm shell surprisingly showed lower efficiency than the same dimension column packed with 2.6-μm particles with 0.35-μm shell. This lower efficiency of the 1.7-μm particles was likely caused by multiple contributing factors. It has been reported that the 1.7-μm Kinetex particles have wider particle size distribution than their 2.6-μm counterparts, and some irregular shape particles have been observed with the 1.7 μm format [24]. We did not observe any difference in peak shapes or tailing factors for the two columns. Another contributing factor we believe

Table 2. Plate number calculated by ChemStation™ software for the four analyte peaks separated on the Kinetex 4.6-mm or 2.1-mm id C_{18} columns packed with 2.6-μm superficially porous particles

Column id	Acetophenone ($k = 1$)	Toluene ($k = 3$)	Ethyl benzene ($k = 5$)	Biphenyl ($k = 7$)
4.6 mm	22 649	24 009	23 215	22 681
2.1 mm	6236	14 186	16 345	17 659
Efficiency loss (%)	72	41	30	22

The efficiency loss was calculated from the difference in plate numbers.

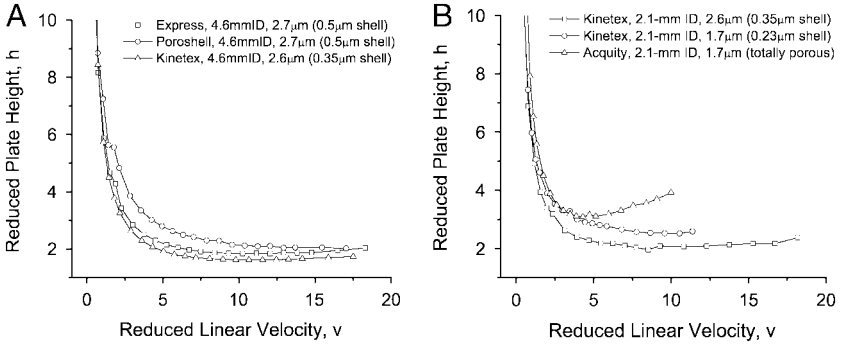

Figure 1. Knox plots for the 4.6-mm id (A) and 2.1-mm id (B) columns tested. The reduced plate height was calculated from the experimentally obtained HETP data for the biphenyl peak, and the reduced superficial linear velocity was calculated from the superficial linear velocity. The mobile phase was 50:50 acetonitrile/water. More experimental details are found in Section 2.

is the frictional heating effect. Column packed with smaller particles will generate higher backpressure thus more heat at the same linear velocity. Frictional heating has been known to affect the performance of 2.1-mm id columns packed with sub-2-μm particles [33, 34]. One simple way of measuring the frictional heating effect was to plot the retention factor with pressure. Figure 2 illustrates the change in retention factor of biphenyl with increasing pressure. A decrease in the retention factor by 7% was observed with a pressure increase from 10 to 1000 bar for both the 2.1-mm id Acquity and Kinetex columns packed with 1.7-μm particles. This result confirmed that frictional heating can have a significant impact on the efficiency of columns packed with sub-2-μm particles.

3.1.3 Column efficiency for mid-size natural products

To evaluate the column performance for mid-size natural products, Knox plots were again constructed for the 4.6 and 2.1-mm id column groups. As shown in Fig. 3A for the 4.6-mm id column group, minimal reduced plate height was achieved at reduced linear velocity of around 7 and increased with increasing linear velocity. The Poroshell 120 column showed higher efficiency at higher linear velocity compared with the Kinetex and Ascentis Express column, making it more suitable for the separation of higher molecular weight analytes.

The Acquity 2.1-mm id column continued to show lower efficiency at higher linear velocity compared with the two Kinetex columns as illustrated in Fig. 3B. The 2.1-mm id Kinetex column packed with 1.7-μm particles again exhibited significant lower performance than the same dimension Kinetex column packed with 2.6-μm particles, consistent with previous observations with the small molecule analytes.

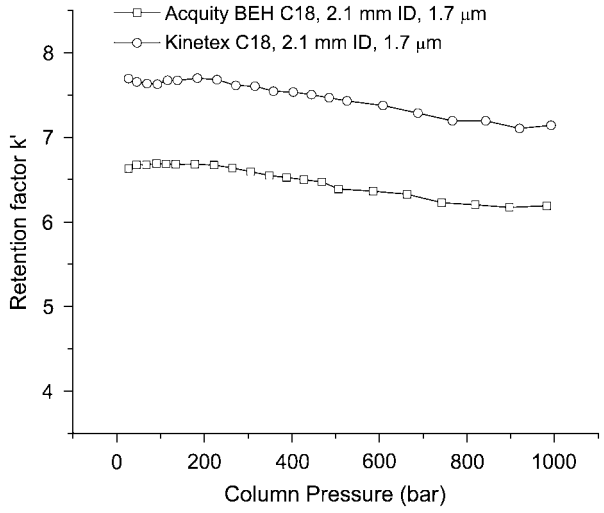

Figure 2. Plots of retention factor (k') for biphenyl on the Acquity BEH C_{18} and Kinetex C_{18} column as a function of column pressure. The mobile phase was 50:50 acetonitrile/water. The flow rate was varied and the pressure was recorded from the instrument pressure reading.

3.2 Column backpressure

The column backpressure was measured at varying linear velocities as illustrated in Fig. 4. According to $\Delta P = (\varphi \eta L u_0 / d_p^2)$, where ΔP is the pressure drop and ϕ is the flow resistance, column backpressure is inversely proportional to particle size (d_p). As expected, higher backpressure was observed for the two columns packed with sub-2-μm particles. The 4.6-mm id Kinetex column packed with 2.6-μm particles showed higher backpressure than the 2.1-mm id Kinetex column packed with the same size particles at same linear velocities, reflecting higher flow resistance. The higher flow resistance indicates denser packing and better packing quality [26]. The poorer packing quality of the 2.1-mm id Kinetex column could also explain the performance difference between the 4.6 and 2.1-mm id columns. The Poroshell column packed with 2.7-μm particles also generated higher backpressure than the Ascentis Express and Kinetex column packed with the same or smaller particles, indicating again a different packing density or potential differences in particle size distribution of the Poroshell column.

3.3 Retentivity and selectivity

Gaining sufficient resolution for critical pairs is usually the most challenging task in method development for complex

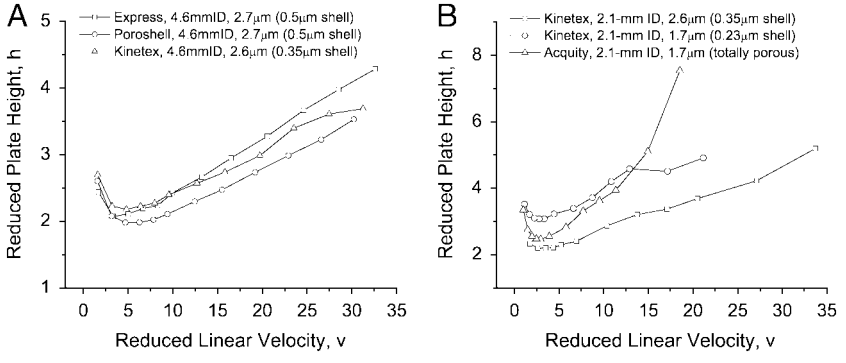

Figure 3. Knox plot for the 4.6-mm id (A) and 2.1-mm id (B) columns tested. The reduced plate height was calculated from the experimentally obtained HETP data for a peak with k' around 8–9, and the reduced superficial linear velocity was calculated from the superficial linear velocity. The mobile phase was 58.8:14:27.2 v/v/v acetonitrile/methanol/25 mM pH 6.2 potassium phosphate buffer. More experimental details are found in Section 2.

mixtures. In principle, increasing the retention factor, maximizing the column efficiency, and improving the selectivity are the three ways to enhance resolution, but improving the separation selectivity is by far the most powerful option. Therefore, besides column efficiency, a comparison of column retentivity and selectivity would also be very helpful for guiding the method development strategies on these new generation columns.

Figure 5 compares the separation of the small molecule test mixture on the four different brands of columns. The retention factor of the biphenyl peak was calculated. Surprisingly, the Poroshell 120 column was significantly more retentive than the other three columns; however, the bonding density of the Poroshell 120 column was similar to that of the other columns and the surface area and carbon loading were less than that of the other column as listed in Table 1. The reason for this abnormal retention behavior was unknown.

All the columns evaluated in this study have C_{18} stationary phase, but subtle difference in stationary phase chemistry and bonding process in some cases can significantly impact the selectivity of complex mixtures. The natural product mixture used in a previous study [14] served as good test probes for comparing the column selectivity due to the complexity and structural similarity of the analytes. Figure 6 shows the chromatograms of the same natural product test mixture on four different columns. The flow rate for the Acquity column was adjusted to achieve similar linear velocity. Significant difference in selectivity was observed for several critical pairs or groups, such as peak 4/5, 7/8, 9–12, 13/14. This result suggests that for method development it is important to be aware of the selectivity difference of the columns packed with superficially porous particles with C_{18} chemistry from different vendors.

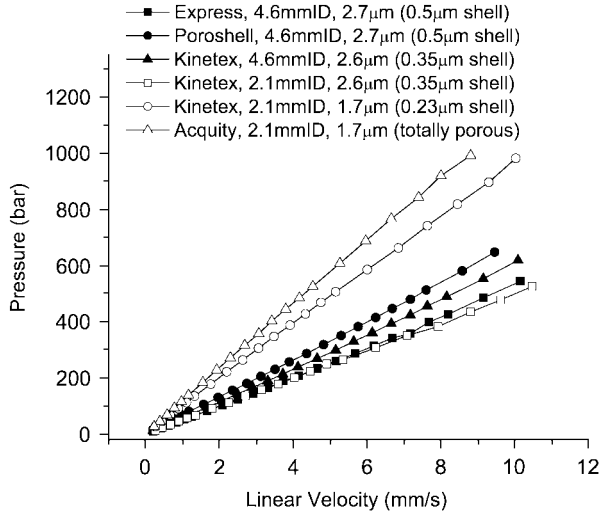

Figure 4. Plot of pressure drop as a function of linear velocity for six columns tested. Mobile phase composition and other conditions are the same as in Fig. 1.

3.4 Column batch-to-batch variability

A rugged QC method should be able to tolerate small variations in method parameters, such as small changes in temperature, detector wavelength, mobile phase compositions, etc. Column batch-to-batch variability is one of the critical measures in evaluating the ruggedness of a QC method. Once a regulatory enforcement method is implemented in a manufacturing QC lab, the same method is required to be used for years or even decades and any changes to the method need to be submitted to regulatory authorities with explanation and correlation data. Replacing an aging column is a common practice in QC labs, but when columns are purchased at different times, the particles used to pack the column are likely to be from a different batch, which sometimes can cause problems in the reproducibility of the method especially for impurity methods. Significant batch-to-batch variability of the Ascentis Express column was observed in our previous study for the separation of the same natural product test mixture [14]. As both Phenomenex and Agilent had claimed a different particle manufacturing process for the Kinetex and Poroshell 120 column, it was worthwhile to evaluate the batch-to-batch variability for these new columns.

Figure 7 shows the chromatograms of the same sample (sample 2) on the three Kinetex columns with different batch numbers. Differences in resolution for the critical

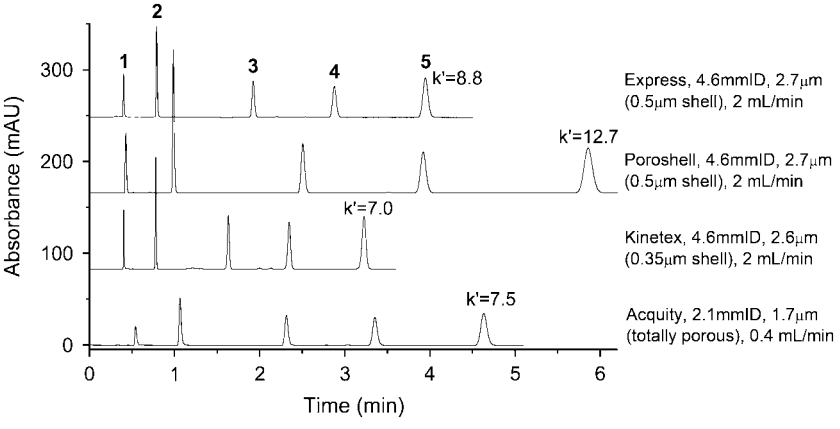

Figure 5. Comparison of column retentivity using sample 1 as test mixture. Retention factor (k') of biphenyl was calculated for the four columns tested. Experimental conditions were the same as described in Fig. 1.

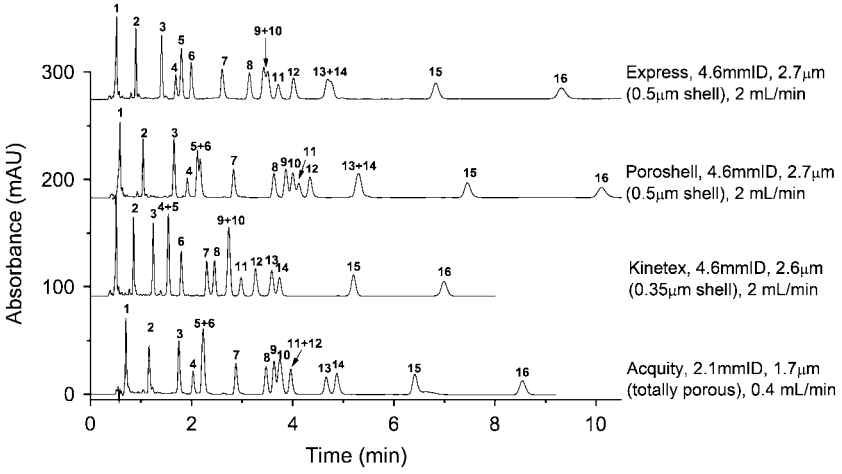

Figure 6. Comparison of column selectivity using sample 2 as the test mixture. Column dimensions and flow rate are shown in the figure. Other experimental conditions were the same as described in Fig. 3. More experimental details are found in Section 2.

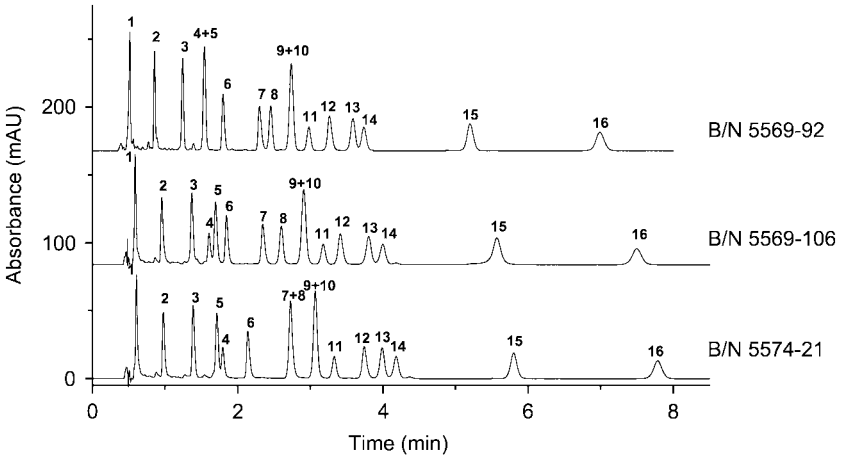

Figure 7. Batch-to-batch variability of the Kinetex C_{18} column (4.6 mm × 100 mm, 2.6 μm particles). Sample 2 was separated on column from three different batches using the same conditions as described in Fig. 3. Resolution of critical pairs 4/5 and 7/8 was different with the different batches.

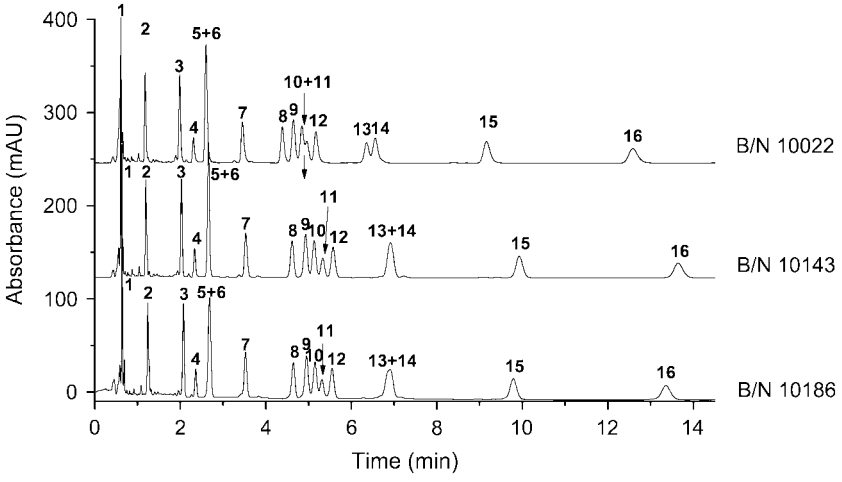

Figure 8. Batch-to-batch variability of the Poroshell 120 C_{18} column (4.6 mm × 100 mm, 2.7 μm particles). Sample 2 was separated on columns from three different batches using the same conditions as described in Fig. 3. Resolution of critical pairs 10/11 and 13/14 was different with the different batches.

pairs 4/5 and 7/8 were observed for the three column lots. Similarly, as shown in Fig. 8, slight variation in selectivity was also seen on the Poroshell columns from three different lots as reflected by the resolution change of critical pairs 10/11 and 13/14. Although a little disappointing to see similar column batch-to-batch variability for the new Kinetex and Poroshell columns, chances are low that the same degree of variability would be observed for other applications because this specific test mixture is known to be extremely sensitive to the subtle change of column chemistry, and the mobile phase conditions were unique to this test mixture. Nevertheless, future improvement in column batch-to-batch variability is still highly desired for these new generation columns since users are more likely to apply these columns for complex samples, which are more susceptible to batch variability.

4 Concluding remarks

The performance of six commercial C_{18} columns packed with superficially porous particles of different sizes and shell thicknesses or totally porous sub-2-μm particles was systematically compared using a small molecule mixture and a mid-size complex natural product mixture as test probes. All the five columns packed with superficially porous particles have shown very flat C-terms for small molecule analytes, allowing high-speed separation with high efficiency. The efficiency of 2.1-mm id columns was significantly affected by the system dwell volume. Even on a low dispersion UHPLC system, over 20% efficiency loss was observed for well-retained analyte in comparison with 4.6-mm id columns. The Kinetex 4.6-mm id column packed with 2.6-μm particles exhibited the best overall efficiency for small molecule separations and the Poroshell 120 column showed better performance for mid-size natural product analytes. The Kinetex 2.1-mm id column packed with 1.7-μm particles did not deliver the performance to our expectation and the possible reasons besides extra column effect have been suggested to be frictional heating effect and poor column packing quality. Using the 16-component natural product mixture as test sample, different column retentivities and selectivities have been observed for the four C18 columns of different brands. Column batch-to-batch variability that has been previously observed on the Ascentis Express column was also observed on the Kinetex and Poroshell 120 columns. This common issue should not be neglected when developing a QC method for complex samples on these new generation columns.

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The authors have declared no conflict of interest.

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