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Recent innovations in UHPLC columns and instrumentation

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ABSTRACT

Ten years after the introduction of the first commercial ultra-high-performance liquid chromatography (UHPLC) system, the pace of progress has not slowed. We describe recent innovations in UHPLC columns and instruments, focusing on those intended primarily for reversed-phase separations of analytes with molecular weights less than about 5000 Daltons, using columns with internal diameters of 2.1 mm. New columns packed with sub-2-µm solid-core particles have produced efficiencies greater than 400,000 plates/m, more than 40% higher than those of columns packed with sub-2-µm fully porous particles. In addition, columns containing charged surface particles give higher peak capacities for separations of positively charged analytes when using the low ionic strength, acidic mobile phases preferred for electrospray mass spectrometric detection. The narrow peaks produced by these columns require instruments having extremely low dispersion. We review recent progress in measuring and reducing system dispersion.

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Contents

1. Introduction

Ultra-high-performance liquid chromatography (UHPLC) encompasses LC separations using columns containing particles smaller than the 2.5–5-µm sizes typically used in HPLC. The benefit of using columns containing smaller particles (typically sub-2 µm) is greater efficiency per unit time [1]. While UHPLC was initially demonstrated using home-built equipment [2], the introduction in 2004 of the first commercial UHPLC system enabled the broad adoption of this technique. To realize the benefits of columns containing sub-2-µm particles, the instrumentation needed to be optimized in concert with the columns. Not only did the system need to be capable of reliable operation at pressures up to 1000 bar, it also needed to have low extra-column dispersion and a column compartment designed to minimize radial temperature gradients [1]. In this article, we describe recent innovations in UHPLC columns and instrumentation, focusing on those intended primarily for reversed-phase (RP) separations of analytes with molecular weights less than about 5000 Da, using columns with internal diameters of 2.1 mm. This represents the largest segment of applications for UHPLC today.

Abbreviations: Da, Daltons; i.d, Internal diameter; TFA, Trifluoroacetic acid; ZDV, Zero dead volume; POISe, Performance-optimizing injection sequence; LEM, Linear extrapolation method.

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2. Recent innovations in UHPLC columns

The first commercial columns designed for UHPLC were packed with 1.7-µm particles, and had dimensions of 2.1 x 50 mm and 2.1 x 100 mm [1]. The particles were fully porous with an average pore size of 130 Å and were made of an ethylene-bridged organic/inorganic hybrid derivatized with C_{18} groups [3]. The columns were intended for RP separations of analytes with molecular weights less than about 5 kDa. Maximum efficiencies of 280,000 plates/m have been reported for these columns, corrected for system dispersion [4]. Over the past 10 years, a large number of additional surface chemistries have been introduced on both hybrid and silica sub-2-µm particles [5]. In addition to the RP mode, UHPLC columns have been commercialized for hydrophilic interaction [6], normal-phase [7], size-exclusion [8–10] and ion-exchange [9] chromatographies. UHPLC columns are now available for separations of analytes up to ~2 MDa, including biopolymers and industrial polymers [8–12]. UHPLC columns are commercially available in internal diameters (i.d.) of 0.075–4.6 mm. While the range of selectivity and application diversity have expanded dramatically, there has been less progress in increasing the efficiency per unit length of UHPLC columns, a fundamental measure of the ability of the column to produce narrow peaks. However, this has begun to change with the recent introduction of columns containing sub-2-µm solid-core particles (SCPs).

2.1. Columns packed with 2.6–2.7-µm SCPs

Unlike the fully porous particles (FPPs) traditionally used in HPLC columns, SCPs have a porous shell surrounding a non-porous core, as shown in Fig. 1. This particle morphology has also been named pellicular, superficially porous, fused-core, core-shell and shell. The history of the development of SCPs for chromatographic applications was recently reviewed [13,14]. After a long period of minimal activity, SCP technology gained prominence in 2007 with the introduction of columns packed with 2.7-µm solid-core silica particles [15]. The use of 4.6 mm i.d. columns packed with these particles on HPLC instruments was described as an alternative to UHPLC, offering high efficiency without the need for higher pressures [15]. A list of the currently available columns containing SCPs may be found in a recent review [16].

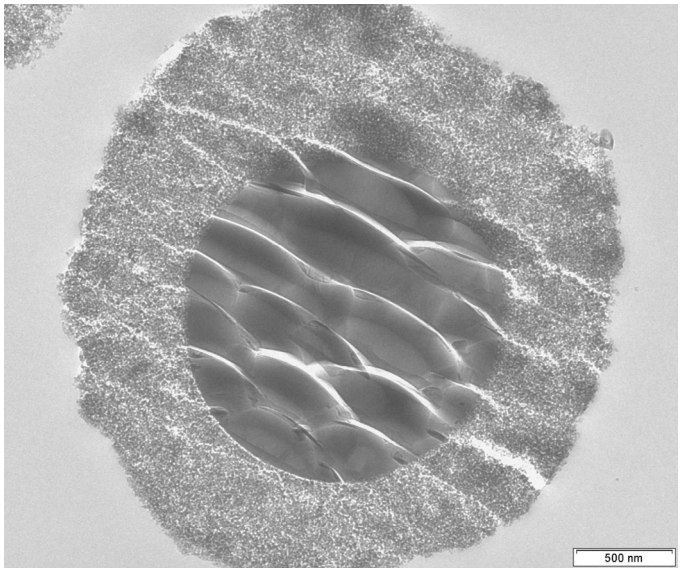

Fig. 1. Transmission electron microscope image of a cut 2.7-µm Halo particle showing the solid core and the porous shell. {Reprinted with permission from [13], ©2011 Elsevier}.

Compared to similar size FPPs, SCPs have been shown to give higher column efficiencies as well as slower decreases in efficiency above the optimum flow rate [13,14]. The reasons for these benefits have been investigated. Contrary to popular belief, the reduction of the diffusion path in SCPs is responsible for only a very small portion of the efficiency increase for low-molecular-weight analytes [13]. The largest contributions to the higher efficiency come from reductions in longitudinal diffusion and eddy dispersion. The former is a result of the lower pore volumes of SCPs, while the latter is due to more uniformly packed beds. Efficiencies of 250,000–300,000 plates/m have been achieved for 2.6–2.7-µm SCPs in 4.6-mm i.d. columns [13]. However, significantly lower efficiencies (160,000–220,000 plates/m) have been achieved for these particles in 2.1-mm diameter columns, even after correction for extra-column dispersion [13]. This is believed to be due to non-optimal column packing [17]. It is important to note that there is significant variability in column efficiency within a brand of columns. Studies of this variability for three brands of columns packed with 2.6-µm or 2.7-µm SCPs were recently reported [18–20]. The relative standard deviations for the efficiencies of 2.1 x 100 mm columns (n = 6) were in the range 4.6–6.8%.

2.2. Columns packed with sub-2-µm SCPs

Instead of viewing columns packed with 2.6–2.7-µm SCPs as an alternative to UHPLC, an important recent trend is the development of columns packed with sub-2-µm SCPs expressly for use on UHPLC systems. This provides the possibility of efficiencies and speeds significantly greater than those achievable using columns packed with sub-2-µm FPPs or 2.6–2.7-µm SCPs. However, achieving higher efficiencies requires optimized packing of these particles. The first commercially available columns packed with 1.7-µm SCPs did not consistently have higher efficiencies than the best 1.7-µm FPP columns, due to a high eddy-dispersion contribution [21]. But, the columns packed with SCPs did show a slower decrease in efficiency at high flow rates when 100% acetonitrile was used as the mobile phase. This was attributed to smaller radial temperature gradients as a result of the greater thermal conductivity for the bed of SCPs [21]. An efficiency of 350,000 plates/m (corrected for system dispersion) was reported for a column packed with 1.7-µm SCPs having a thinner shell [22,23]. The thickness of the shell was demonstrated to have a significant effect on efficiency and retention [23]. While particles with thinner shells offer higher efficiencies, they also result in reduced retention.

In 2013, 2.1 x 50 mm columns packed with 1.3-µm SCPs were introduced [24]. Corrected for system dispersion, maximum efficiencies of 435,000–510,000 plates/m were measured for these columns [25,26]. However, they have been reported to have much higher pressures than columns packed with 1.7-µm SCPs [24,26]. The specific permeability of a column containing the 1.3-µm particles was found to be 45% lower than that of a column containing 1.7-µm SCPs [26]. This results in pressures that are more than double those of columns containing 1.7-µm SCPs, for constant flow rate and column length. Consequently, only a 50-mm-long column could be operated at the optimum flow rate using a UHPLC system with a maximum pressure of 1200 bar [24]. In contrast, lengths up to 150 mm may be used for columns packed with 1.7-µm particles.

Also in 2013, columns packed with 1.6-µm SCPs were introduced. Corrected for system dispersion, maximum efficiencies of 330,000–420,000 plates/m have been reported for these columns

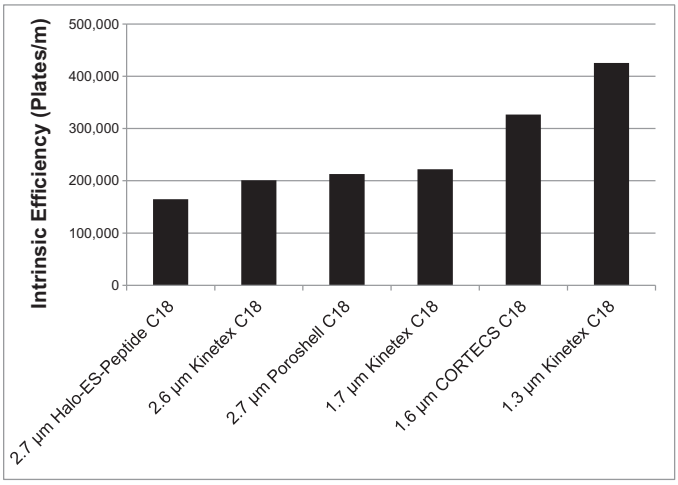

Fig. 2. Comparison of the maximum efficiency per unit length (corrected for system dispersion) for six types of column packed with solid-core particles (calculated from the intrinsic plate height values in [25]). All columns were 2.1 x 100 mm except for the 1.3-µm Kinetex C_{18} column, which was 2.1 x 50 mm.

[25,27–29]. Despite the smaller particle size, they have pressures similar to columns packed with 1.7-µm FPPs. This is due to the higher interstitial porosity of columns packed with SCPs, as well as the narrower particle-size distribution [13]. The higher interstitial porosities generally found for columns packed with SCPs are believed to be due to the rough surface morphology of most SCPs [13,14]. Shown in Fig. 2 is a summary of the efficiencies per unit length (corrected for system dispersion) for six brands of columns packed with SCPs [25]. All were 2.1-mm i.d. columns. The large increase in efficiency per unit length for the columns packed with 1.6-µm and 1.3-µm particles may clearly be seen.

2.3. Charged surface-particle technology

The discussion above focused on column efficiency measurements employing primarily neutral analytes. While such compounds best reveal the maximum efficiency of a column, most applications of UHPLC involve the separation of molecules that are much more complex. It has been shown that analyte properties and mobile-phase composition may affect the kinetic performance of columns [30]. Positively-charged analytes, such as protonated bases, often give broad, tailing peaks [31]. One way to mitigate this effect, particularly when using low ionic strength acidic mobile phases, such as 0.1% formic acid, is to incorporate a low level of positive charge into the particle surface [32,33]. Columns with this technology were initially introduced using 1.7-µm totally porous hybrid particles. The theoretical basis for the behavior of cations on positively-charged RP porous particles has been elaborated [34]. Columns containing these particles have been shown to be particularly useful for separation of peptides [35]. An example is shown in Fig. 3, where a comparison is made of Lys-C peptide maps of trastuzumab obtained using two different 2.1 x 150 mm columns packed with 1.7-µm FPPs. The mobile phase contained 0.02% TFA and 0.08% formic acid. The column employing charged surface hybrid (CSH) technology shows narrower peaks, with a 90% increase in the peak capacity ($P_{C,4\sigma}$). Recently, this technology became available on 1.6-µm SCPs. Columns containing these particles have been shown to be useful for the quantification of human insulin and five recombinant analogs in plasma using multidimensional LC/MS/MS [36].

3. Recent innovations in UHPLC instrumentation

The use of columns packed with sub-2-µm particles necessarily increases the backpressure at which UHPLC systems operate [1]. UHPLC systems are now available from multiple vendors, with pressure limits up to 1300 bar [5,37]. Both binary high-pressure mixing and low-pressure mixing pumping technologies are available [5,37]. In general, the binary high-pressure mixing pumps have lower dwell volumes and are more suitable for very fast gradients at lower flow rates, while the low-pressure mixing pumps provide greater flexibility in method development and support gradients using more than two solvents/buffers [37]. Sample injectors are tuned to the lower injection-volume requirements of UHPLC columns [5]. Column ovens should avoid active circulation of the heating fluid (air) to minimize radial temperature gradients within the UHPLC column [38]. A wide variety of detectors are now available, including absorbance (tunable and photodiode array) [5], fluorescence [39], refractive index [10], multi-angle light scattering [10], evaporative light scattering [40], charged aerosol [40], electrochemical (amperometric and coulometric) [41], circular dichroism [42], and a variety of mass spectrometers [43]. In general, these detectors feature lower volume flow cells and higher data-sampling rates than their HPLC analogs [5,37].

3.1. Extra-column dispersion requirements

The small peak volumes associated with UHPLC make the reduction of extra-column dispersion a critical aspect of UHPLC instruments [4,5,44–46]. Under isocratic conditions, the dependence of the measured volume variance of a peak ($\sigma^2_{measured}$) on the extra-column variance (σ^2_{ex}) may be expressed as:

$$\sigma^2_{measured} = \sigma^2_{ex} + \frac{V_0^2}{N_{intrinsic}}(1+k')^2 \quad (1)$$

where V_0 is the column void volume, $N_{intrinsic}$ is the intrinsic column efficiency (the column efficiency in the absence of extra-column dispersion) and k' is the retention factor [29]. By definition, the measured efficiency ($N_{measured}$) is:

$$N_{measured} = \frac{V_R^2}{\sigma^2_{measured}} \quad (2)$$

where V_R is the retention volume, and

$$k' = \frac{V_R - V_0}{V_0} \quad (3)$$

By substituting Equation (1) into Equation (2) and rearranging, the following equation is obtained, expressing the extra-column variance as a function of the measured and intrinsic efficiencies:

$$\sigma^2_{ex} = V_0^2(1+k')^2\left(\frac{1}{N_{measured}} - \frac{1}{N_{intrinsic}}\right) \quad (4)$$

Fig. 4 shows plots of σ^2_{ex} *vs.* $N_{intrinsic}$, where $N_{measured}$ has been set to 90% of $N_{intrinsic}$, for three different values of k'. The $N_{intrinsic}$ and V_0 values were chosen to be appropriate for 2.1 x 100 mm columns packed with sub-2-µm SCPs. The results show that the extra-column variance must be less than 7.6 µL^2 for $k'=8$ and less than 0.85 µL^2 for $k'=2$ to achieve 90% of the intrinsic efficiency for a column with 40,000 plates (400,000 plates/m). Clearly, as the intrinsic column efficiency increases, the dispersion of UHPLC systems must be decreased to allow the higher efficiency to be realized.

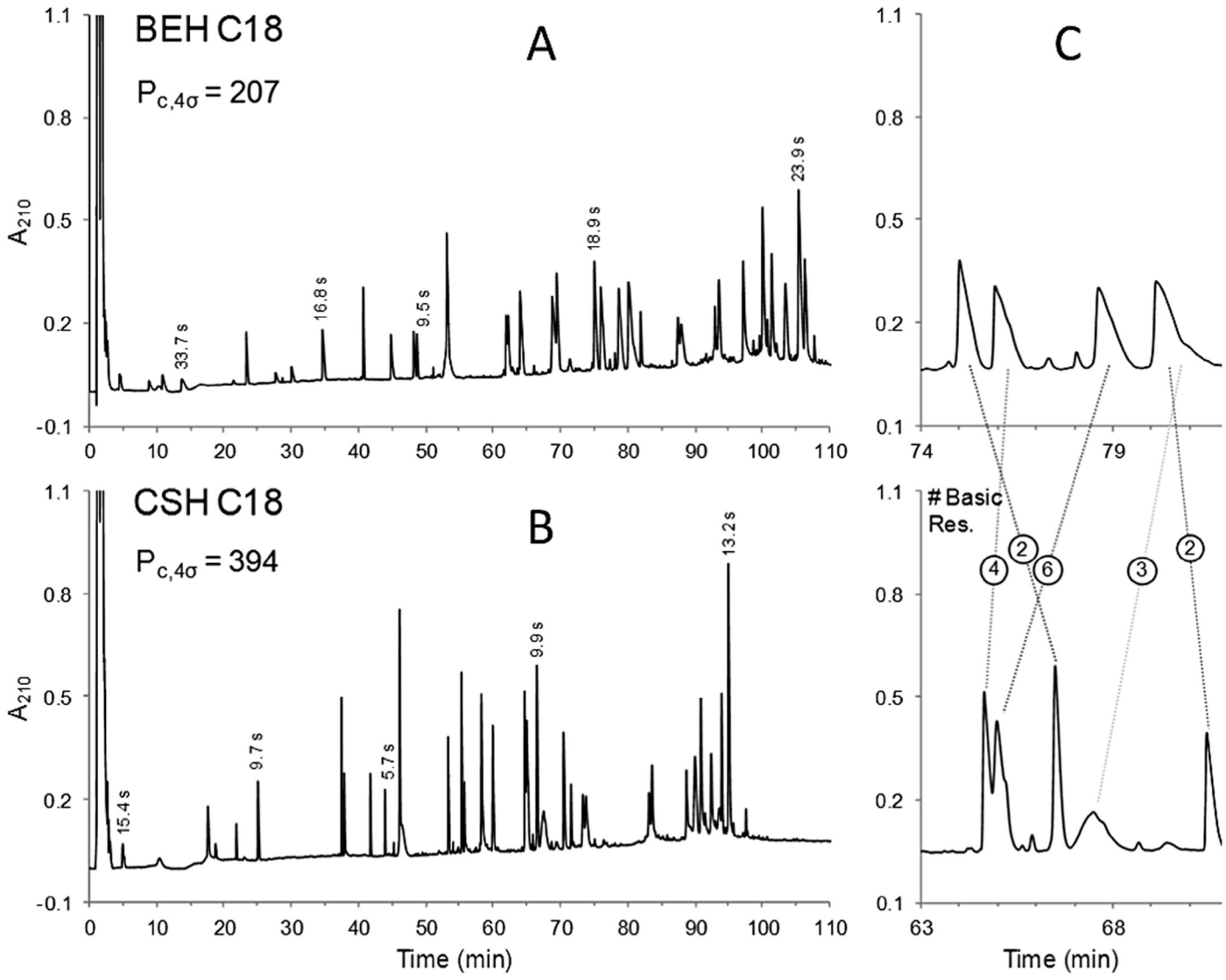

Fig. 3. Lys-C peptide maps of trastuzumab obtained with: (A) a 2.1 x 150 mm Acquity BEH C_{18}, 1.7-μm column; and, (B) a 2.1 x 150 mm Acquity CSH C_{18}, 1.7-μm column. Peak widths at half height ($w_{0.5}$) are shown for five peptides spread across the separations. Peak capacities (4σ) were calculated from the averages of these values. (C) Corresponding retention windows from each peptide map. Dotted lines indicate the change in elution order of the different peptide species, as detected by ESI-MS. The number of basic residues for each identified species is provided. {Reprinted with permission from [35], ©2013 American Chemical Society}.

3.2. Measurement of extra-column dispersion

A simple, accurate means of determining extra-column dispersion is needed to characterize UHPLC systems. The most common approach is conceptually simple: replace the column with a zero dead volume (ZDV) union, inject a small volume of a suitable sample, and determine the volume variance of the peak. The method can be precise and rapid and may be used to rank the relative extra-column dispersion of different UHPLC systems [5,37,46]. Values reported for several systems are given in Table 1. Note that extra-column dispersion depends on the specific configuration of a system, including the sample-loop volume, the injection mode, the detector flow-cell volume and the dimensions of the connecting tubing [4,44,45]. Extra-column dispersion also varies with flow rate, mobile-phase composition and temperature. The values in Table 1 were measured for the standard configuration of each system.

Recent studies by Gritti and Guiochon pointed out significant issues with this direct measurement [27,29]. When the results of measurements of extra-column dispersion employing a ZDV union were used to correct the reduced plate heights of naphthalene for the same column operated on three different UHPLC systems, the resulting van Deemter plots did not overlay, indicating that the estimate of extra-column dispersion was inaccurate [29]. The problem arises because the elevated backpressure of UHPLC columns is not emulated. When a column is installed the pressure in the pre-column tubing is elevated, which reduces diffusion coefficients. According to Aris dispersion theory, this will tend to increase the pre-column dispersion. Using an adjustable flow restrictor to vary the pressure, system dispersion was shown to increase 20–25% when the pressure was increased from < 100 bar to 528–582 bar [27].

The most common alternative to the so-called ZDV measurement is the linear extrapolation method (LEM) [47]. In the LEM, the observed peak variance is plotted *versus* $(1+k')^2$ and the y-intercept of the plot is taken to be the extra-column variance. The best estimates of the peak variances observed in these measurements are based on moment analysis [46,48]. Gritti and Guiochon proposed an alternate plot of the apparent plate height *versus* $1/(1+k')^2$ [29]. The slope of that plot contains the extra-column dispersion and the void volume of the column. The y-intercept is the intrinsic plate height. It is assumed that the sample is dissolved in the mobile phase,

Table 1
Extra-column variances for UHPLC instruments measured using a zero dead volume union

Instrument	Extra-column variance (μL^2) from [5][a]	Extra-column variance (μL^2) from [46][b]
Waters Acquity UPLC I-Class	1.0	
Waters Acquity UPLC H-Class	3.2	
Waters Acquity UPLC	5.8	6–7
Thermo Ultimate 3000 RSLC	11.6	
Shimadzu Nexera		13–20
Agilent 1200		13–20
Agilent 1290 Infinity LC	16.0	
Accela High Speed LC	17.6	
Perkin-Elmer Flexar		18–26

[a] Measured using caffeine with acetonitrile/water (50:50 v/v) at 0.5 mL/min.
[b] Measured using estradiol with acetonitrile/water (48:52 v/v) at 0.01–1.2 mL/min.

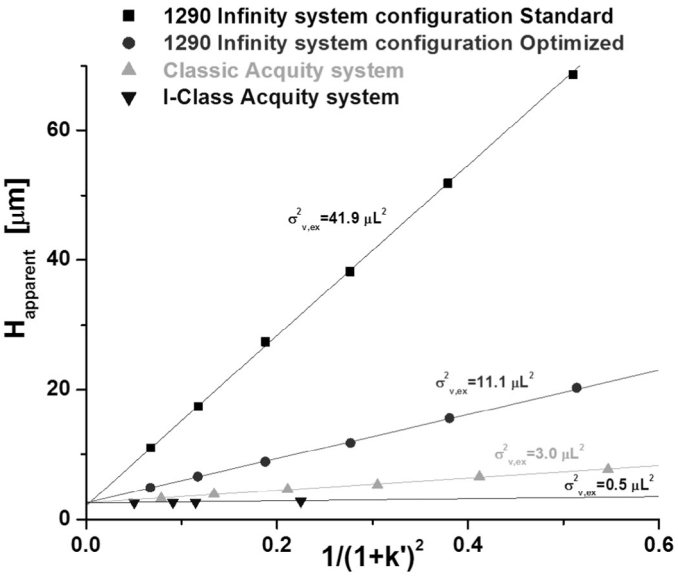

Fig. 5. Plots of the apparent (measured) plate height for several alkyl phenones *versus* $1/(1+k')^2$ for the same column used on four different UHPLC instruments. The 2.1 x 100 mm column was packed with 1.6-µm solid-core C_{18} particles, the mobile phase was acetonitrile/water (75/25 v/v) and the temperature was 24°C. Despite the large differences in extra-column volume variances (σ^2_{ex}), the y-intercept is nearly the same for all four instruments. {Reprinted with permission from [29], ©2014 Elsevier}.

that the retention factors are modest (<3) and that the flow rate is high enough to minimize axial diffusion within the column. As shown in Fig. 5, measurements of the intrinsic plate height based on this extrapolation method show agreement within 5% for four different UHPLC systems having extra-column dispersion variances in the range 0.5–41.9 μL^2 [29].

3.3. Reduction of extra-column dispersion

Examination of the Taylor-Aris equation describing dispersion in tubes [49] and the coupling equation of Fountain et al. [4] indicated that reducing the inner diameter and length of the tubing used to connect various post-injector components is the first step in decreasing extra-column dispersion. This necessarily increases the system backpressure as the instrumental cost of reducing extra-column dispersion. Active inlet preheating as a second temperature-control zone within adiabatic column ovens is another opportunity to reduce extra-column dispersion while preserving effective temperature control [5].

Pre-column dispersion can be reduced by co-injection of a solvent that is weaker than the mobile phase in the performance-optimizing injection sequence (POISe), which focuses the sample by reducing its initial retention factor [50]. Injectors that transfer a sample to a small loop (≤1 µL) can demonstrate low dispersion, but further reduction of the inner diameter of the sample loop can result in increased dispersion, resulting from changes in i.d. as the sample passes from the loop through the passages of the injection valve and into the connecting tubing [51]. It has been reported that, for injection volumes > 3 µL, the extra-column dispersion of a direct injector with First In, Last Out flow direction is superior to a fixed loop injector in which the sample is transferred via a sampling needle to the sample loop [50]. This is a consequence of the number of changes in diameter experienced by the sample volume in the fixed loop injector in which the sample is transferred from the sample vial to

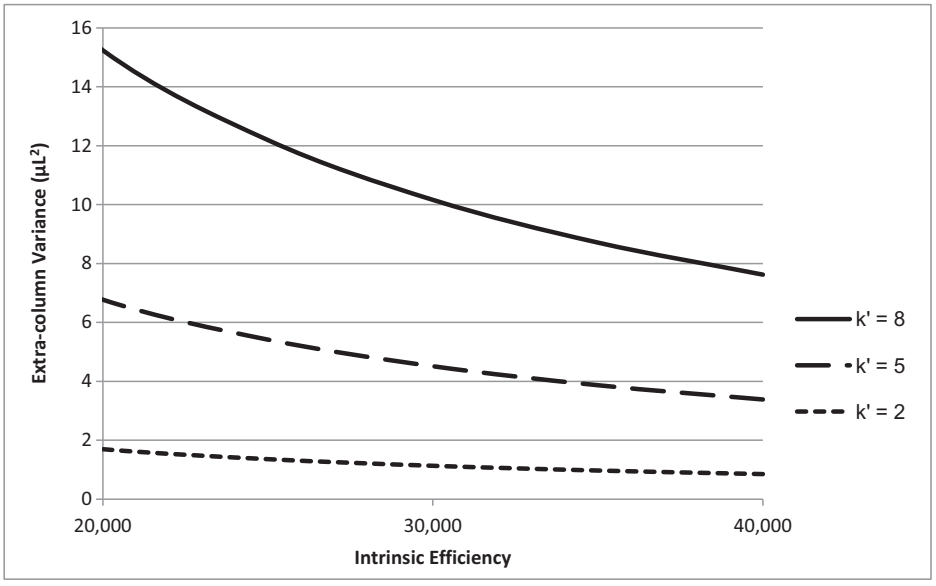

Fig. 4. Calculated extra-column variance required to maintain 90% of the intrinsic column efficiency as a function of intrinsic column efficiency for 2.1 x 100 mm columns, for three different values of the retention factor (k'). A V_0 value of 184 µL was used.

the sample loop via the sample needle and the associated transfer tubing.

3.4. Detection requirements for UHPLC

Detectors contribute to the extra-column dispersion of a UHPLC system in two principal ways. First, the dispersion associated with the volume of the detector can be estimated from $\sigma_v^2 = V^2/12$, where V is the flow-cell volume [4]. The use of light guiding resulting from total internal reflection has allowed the reduction of flow-cell volumes to as little as 250 nL in commercial instruments while preserving sufficient light throughput to ensure acceptable noise performance for absorbance detection [52]. In addition to reducing the volume of the flow cell, it is necessary to increase the sampling rate and reduce the degree of electronic filtering to ensure peak fidelity. Fountain et al. treated the sampling interval as the principal contributor to temporal peak broadening [4].

Commercially available detectors provide sampling rates as high as 200 Hz [53]. It must be noted that sampling rates greater than 40 Hz are rarely required and that detector noise increases as the sampling rate is increased. This increase in noise is a consequence of reduced signal averaging at higher sampling rates and an increase in the relative contribution of the so-called "read noise" associated with measuring the photocurrent at the photo diode(s). In general, the sampling rate should be chosen to ensure that 20 points are collected across a single peak and 100 points are collected for merged peaks [54].

Fluorescence detection for UHPLC requires a reduction of flow-cell volume from the typical HPLC flow cells, but there is a loss in detector response associated with the smaller volume in which the emitted photons are observed. One fluorescence detector for UHPLC compensates for the reduced sensitivity from flow-cell volume reduction with the use of a mercury-doped xenon emission lamp, which increases the excitation energy at the mercury lines [55].

The extra-column dispersion of UHPLC/MS systems was investigated by Spaggiari et al. for several UHPLC instruments coupled to triple quadrupole, time-of-flight, quadrupole time-of-flight, and quadrupole ion-trap instruments [53]. The presence or the absence of an absorbance detector and/or a divert valve, as well as the length and the i.d. of the tubing used post-column, all had significant effects on band broadening observed at the mass spectrometer. It was noted that these dispersive elements all occur post-column and are not mitigated by sample focusing occurring with gradient separations. For flow rates greater than 200 µL/min, the extra-column variance measured from direct injection without a column increases as the sampling rate decreases. However capillary voltage, desolvation and cone-gas flow rates showed minimal impact on band broadening. The extra-column variances for the standard configurations of four systems were in the range ~25–95 μL^2 at 600 µL/min, much larger than the variances for the UHPLC/UV systems shown in Table 1. However, by reducing the length and the i.d. of the connecting tubing, the extra-column variances could be decreased to ~17–19 μL^2 at 600 µL/min for all the systems studied.

4. Conclusions

As UHPLC has continued to evolve over the past 10 years, the drive for higher column efficiency has emerged as a key theme. The development of columns containing sub-2-µm SCPs has enabled large efficiency increases (greater than 40%) relative to columns containing sub-2-µm FPPs. For columns containing 1.6-µm or 1.7-µm SCPs, this efficiency increase may be obtained with pressures similar to columns containing 1.7-µm FPPs. In contrast, columns containing 1.3-µm SCPs have ~100% higher pressures, limiting their utility on current UHPLC instruments. In addition, charged surface particle technology offers the ability to obtain high efficiencies for not only neutral molecules, but also positively-charged analytes when using the low ionic strength acidic mobile phases preferred for electrospray mass-spectrometric detection.

To obtain the full benefit of these higher efficiency columns, the extra-column dispersion of UHPLC instruments has needed to be reduced. While significant progress has been made in this endeavor, this remains a fruitful area for further improvements.

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