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Performance optimizing injection sequence for minimizing injection band broadening contributions in high efficiency liquid chromatographic separations

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ABSTRACT

The observed performance when using high efficiency, low volume (U)HPLC columns has and continues to be limited by the extra column dispersion introduced by the chromatographic system. Even with the latest UHPLC instruments the injection and detection systems are still major contributors to peak broadening, especially when using 50 mm × 2.1 mm columns. A previously described injection technique now termed Performance Optimizing Injection Sequence (POISe) was shown to reduce or eliminate the impact of the injection system on the observed chromatographic performance. The POISe technique involves injecting a defined volume of weak solvent along with the sample in order to increase retention factors during sample loading. In the present study, a newly developed equation describing the phenomena involved during sample introduction is presented and shows that analyte bands are compressed at the head of the column in proportion to their retention factor independent of the elution mode (i.e. gradient or isocratic). This phenomenon is termed isocratic focusing and is shown to be most effective with analytes having retention factors in the range of 0.4–3. Additionally, it is shown that gradient compression plays a minor role in band compression when using this technique with analytes having retention factors of 1 or higher. The POISe technique is further investigated experimentally to determine its optimum configuration. It is also demonstrated to be effective with different HPLC and UHPLC instrument platforms and different high efficiency columns, such as those packed sub-2 μm and core–shell particles.

1. Introduction

Significant developments in recent years have further established HPLC as the primary technique in pharmaceutical, clinical, toxicological and environmental analyses. Developments in HPLC instruments and sorbents have enabled greatly improved chromatographic resolution in very short analysis times. This new generation of instruments, columns and practices belong to the technique collectively called ultrahigh pressure liquid chromatography (UHPLC). The first generation of very high efficiency columns and the instruments capable of operating them emerged in the last five years and are gradually penetrating many analytical laboratories. UHPLC is now becoming a major component of the family of "enabling technologies" used in the pharmaceutical industry.

The first generation of columns for use in UHPLC was based on traditional chromatographic sorbents made of porous silica particles less than 2 μm in size ("sub-2 μm particles"). Such columns provide plate heights on the order of 3–3.5 μm with only limited loss in performance when operated at flow rates beyond the optimum value observed on H/u curves. The practical implications are fast analyses with exceptional resolution. The major drawback of using columns packed with sub-2 μm particles is the very high operational pressure requiring the use of specialized instrumentation. Besides the high cost and limited availability of such instrumentation, UHPLC performed with columns packed with sub-2 μm particles is burdened by an undesirable side effect resulting from the high operating pressure: band spreading caused by frictional heating [1]. This phenomenon becomes significant at high mobile phase flow rates with longer columns and viscous mobile phases (e.g. methanol based mobile phases used at low temperature).

An effective and elegant solution to this problem is the use of columns made with partially porous chromatographic particles, the core–shell particles. Most of the recently developed such particles are made of a non-porous silica core 1.7–1.9 μm in diameter surrounded by a 0.3–0.5 μm thick porous shell. The morphology of this porous layer is similar to that of conventional, fully porous particles. The chromatographic performance of core–shell particle based columns is similar to that of columns made with sub-2 μm fully porous particles with the significant difference that their exceptional performance can be achieved at significantly lower (−30%) operational pressures [2–5]. Due to their larger

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average particle size, commercial core-shell columns such as Kinetex 2.6 μm, Halo 2.7 μm and Poroshell 2.7 μm (in any surface modification) generate back pressures of less than 400 bar under common operating conditions, including flow rates around their optimum levels. Hence, commonly used column dimensions such as 100 mm × 4.6 mm and 75 mm × 4.6 mm can provide UHPLC levels of performance even when operated on conventional HPLC instruments such as the Agilent 1100 liquid chromatograph.

HPLC instrumentation plays an equally important role in achieving top chromatographic performance (as in maximum plate count achieved within a given set of experimental conditions) as the column itself. Instruments not only have to be capable of delivering the desired flow rate against the resistance exerted by the column (i.e. the back pressure generated by the column) but also to add as little dispersion as possible to the analyte bands. During analysis a sample of finite volume is introduced into the mobile phase stream using an injection system. Various configurations of injection systems are used with the most common being the flow through needle and the fixed volume loop types, each having their respective strengths and weaknesses in terms of precision and dispersion. During the passage of the sample band through the various components of the injection system its volume increases (i.e. the analyte band widens) due to various dispersive effects. The consequence of all dispersive effects taking place outside the chromatographic column are collectively called extra-column band spreading. HPLC instruments operating columns of very high efficiency and small total volume (e.g. with dimensions of 50 mm × 2.1 mm) can preserve this exceptional column performance only if they are specifically designed to ensure minimal extra-column band spreading. This requirement becomes even more stringent with the most efficient chromatographic columns available today and certainly with the columns of the (near) future.

In our experience the injector is typically a significant contributor to extra column band spreading due to the numerous connections and flow passages. Historically, the primary consideration for an injector was precise delivery of sample volumes into the mobile phase stream [6]. As with any other instrument component [7], minute features of this device may have a major impact on its contribution to band spreading. A poorly designed and/or manufactured injector assembly or poorly maintained injector components (such as worn out seals) can dramatically affect its dispersion characteristics thus reducing the observed chromatographic performance. The injection step of chromatographic analysis has received relatively little attention over the many years of HPLC practice in spite of the fact that Guiochon et al. recognized very early the importance of what they suggestively called “the injection problem” [8].

Very small diameter columns require proportionally smaller sample volumes to avoid performance losses due to volume overload [9]. Vissers et al. investigated the role the sample-loop volume and diameter, as well as on-column focusing can play in maintaining low peak dispersion when making large volume injections onto micro-columns [10]. Foster et al. investigated various injector geometries in an attempt to improve injector performance [11]. Sandwich injection was proposed to prevent sample precipitation in connecting tubing [12], or to selectively elute water-soluble sample components [13]. At-column dilution is practiced in some applications (mainly in preparative chromatography) with the purpose of reducing the eluting strength of the sample solvent(s) [14].

According to Guiochon et al. the continuous improvement in HPLC column performance strides ahead of instrument design “sending engineers back to the drawing board” in an effort to catch up with the needs of the day [15]. Although significant efforts are directed to improving instrument performance, specifically to reducing instrument induced analyte band spreading, the most commonly used instrument models show great variability in this respect. As shown by Gritti et al. [16], some current models can add as much as 200% to the peak variance observed with small volume columns significantly reducing chromatographic resolution. Furthermore, significant variability is found between individual instruments of the same model [17]. In light of these observations and the fact that the IQ/OQ protocols practiced by most instrument manufacturers simply verify the operation of individual components, it is important to add chromatographic testing and performance specifications to new instrument installation and operational qualification acceptance tests.

In this work we refine the understanding of a novel injection technique capable of significantly improving chromatographic performance by preserving the efficiency of HPLC columns of very high efficiency [16]. This novel injection technique, hereafter referred to as the Performance Optimizing Injection Sequence (POISe) reduces analyte band widths by minimizing the extra column dispersion taking place ahead of the column. It can be performed with most HPLC instruments in current use, and has a dramatic positive effect on those that have not been carefully optimized to prevent excessive band spreading. The physical modifications required to reduce the extra column dispersion of an existing system are typically beyond what is practical for most users and therefore the use of this simple injection technique is proposed as a practical alternative. In contrast, POISe requires no physical modifications to the injector assembly and is non-disruptive to workflows.

As mentioned above, the instrument contribution of the observed peak variance is most dramatic with small volume columns. For this reason this study focuses on the effectiveness of the POISe technique as a means of reducing the extra column contributions to band variance specifically when using small volume columns such as 50 mm × 2.1 mm packed with very high efficiency particles. Also, an alternative equation to that previously described is proposed which demonstrates that analyte focusing, not (step) gradient compression, is the dominant process giving the observed performance improvements when using the POISe technique [16]. Additionally, the optimum configuration when using the POISe technique is established and the general applicability of the technique with columns and instruments from different manufacturers are demonstrated.

2. Theory

2.1. Background

As described in our previous paper the band broadening introduced by the injector may be essentially eliminated by use of a sample focusing technique [16]. This sample focusing technique, now termed POISe, involves injecting a defined volume of weak solvent along with the sample. The sample and weak (focusing) solvent mix during transit through the injector, tubing and connections ahead of the column and as a result the elution strength of the sample band entering the column is reduced. The reduced elution strength of the sample band provides increased retention of most analytes during loading causing them to be focused at the head of the column. A related technique for reducing the contribution of very large injection volumes to the band variance was previous described as on-column focusing [18–20]. POISe is differentiated from on-column focusing in 2 important respects: (1) the primary objective with POISe is to improve chromatographic performance when using conventional injection volumes and (2) POISe does not require the sample to be prepared in a non-eluting solvent. The relevant factors involved in the POISe technique are briefly reviewed and then are elaborated further below.

POISe can be particularly useful, for example, in bionalaytical applications by allowing flexibility in choosing an adequate

reconstitution solvent for sample preparation. In these applications the analytes of interest along with the remaining sample matrix must be solvated by the reconstitution solvent which often requires a significant volume fraction of organic solvent. A strong sample solvent limits the volume of sample that can be injected as it reduces the retention and increases the band spreading of analytes having lower affinity for the sorbent in the injection solvent than in the mobile phase, thus reducing the limit of detection of the method [21].

All components in the chromatographic path contribute to the total variance of a chromatographic band (σ^2_{tot}) according to the well known equation,

$$\sigma^2_{tot} = \sigma^2_{inj} + \sigma^2_{col} + \sigma^2_{det} \quad (1)$$

where (σ^2_{inj}), (σ^2_{col}), (σ^2_{det}) are the variances caused by the injector, column and detector, respectively. As mentioned previously the injector is a major contributor to extra-column band dispersion. This is especially true when using short and small internal diameter columns (e.g. 50 mm × 2.1 mm) due to the exceptionally low dispersion and thus peak volumes produced. In our experience the current UHPLC detectors equipped with micro volume flow cells add significantly less band variance than the injection system and, therefore, methods to reduce the impact of the injector on the observed chromatographic performance are the focus of this investigation.

The linear solvent strength model (LSSM) is often used to approximate retention in isocratic elution and is typically described using the following equation,

$$\log k_a = \log k_w - S\varphi_b \quad (2)$$

where k_a is the retention factor in a particular mobile phase, k_w is the retention factor in pure water, S is a solvent/solute dependant factor and φ_b is the volume fraction of organic modifier in the mobile phase. Rearranging this equation and assuming $S = 3$ [22] shows that the retention factor (in isocratic elution) changes approximately 2-fold with a 10% change in organic modifier:

$$k_a = k_w \times 10^{\bigwedge - S\varphi_b} \quad (3)$$

2.2. Isocratic focusing factor

This relationship between k_a and the volume fraction of organic modifier along with consideration of mobile phase and analyte linear velocities provide insight into methods to facilitate analyte focusing. Analyte linear velocity (μ_a) in isocratic elution is typically described by the following equation:

$$\mu_a = \frac{\mu_0}{1 + k_a} \quad (4)$$

where μ_0 is the mobile phase linear velocity. Assuming a plug flow profile (i.e. no dispersion) between the injector and column inlet and insignificant extra column volume the time (t_{inj}) required to load the sample of volume V_{inj} onto the column is given by,

$$t_{inj} = \frac{V_{inj}}{F} \quad (5)$$

where F is the flow rate.

During sample loading, the sample band enters the column at the linear velocity of the mobile phase (μ_0). Assuming the sample is dissolved in mobile phase, when the front of the sample band first encounters the sorbent the analyte velocity (μ_a) is reduced relative to an unretained compound in proportion to its retention factor (k_a) in the given mobile phase. This reduction in linear velocity due to retention on the sorbent allows the rear of the analyte band to catch up with the front giving what will be referred to hereafter as isocratic focusing of the band. Equations describing this phenomenon are provided in the following discussion.

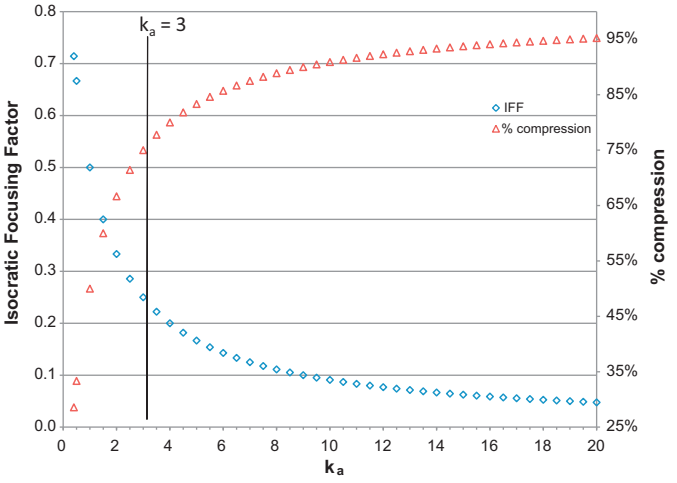

Fig. 1. Isocratic focusing factor and % analyte band compression vs. retention factor when the sample is dissolved in mobile phase.

The distance traveled by the front of an analyte band dissolved in mobile phase during loading (d_{inj}) is given by:

$$d_{inj,a} = t_{inj} \times \mu_a = \frac{V_{inj}}{F} \times \frac{\mu_0}{1 + k_a} \quad (6)$$

while the distance traveled by an unretained component ($k = 0$) during loading is given by:

$$d_{inj,0} = \frac{V_{inj}}{F} \times \mu_0 \quad (7)$$

The isocratic focusing factor then is defined as the ratio of the distance traveled by a retained compound relative to an unretained compound and is given by:

$$\text{Isocratic focusing factor(IFF)} = \frac{[(V_{inj}/F) \times (\mu_0/1 + k_a)]}{[(V_{inj}/F) \times \mu_0]} = \frac{1}{1 + k_a} \quad (8)$$

In order to avoid confusion with gradient theory (namely "gradient compression") the term isocratic focusing instead of isocratic compression has been adopted with the understanding that the physical manifestation of this phenomenon results in an effective compression of the volume occupied by the analyte at the head of the column. A plot of the isocratic focusing factor (Eq. (8)) and the degree of analyte band compression versus k_a is shown in Fig. 1 which assumes the sample is dissolved in mobile phase. This plot shows that isocratic focusing gives significant band compression (i.e. focusing) for analytes having sufficient affinity for the sorbent even when the sample is dissolved in mobile phase. For example, the IFF for an analyte with $k_a = 3$ is about 0.25 corresponding to an analyte band compression of approximately 75% when the sample is dissolved in mobile phase. In contrast, for an analyte with a retention factor of 0.4 the IFF is only 0.71 corresponding to a band compression of only 29% of the injected band width.

Fig. 1 also shows that a significant compression of the analyte band width takes place in the range of k_a 1–8 with less than ideal compression when $k_a < 3$. Additionally, the incremental improvement in compression gradually decreases, but is still significant, in the range k_a 3–8. From the plot in Fig. 1 it is obvious isocratic focusing will take place in almost any sample solvent, but will be effective only for the sample components that have a retention factor >3 in the given sample solvent. Along these lines, the stronger the eluting strength of the sample solvent, the more limited the

range of analytes that will have retention factors large enough for effective focusing (>3).

From Eq. (8) it is obvious that the degree of band compression can be manipulated by adjusting the retention factors of the analyte bands during sample loading. This focusing step plays an important role in reducing the impact of injection volume and injector variance in both isocratic and gradient elution. The POISe technique is a special case of isocratic focusing where the retention factor of the analyte in the injected sample band (in which case k_a becomes k_i) is temporarily increased:

$$\mathrm{IFF_{POISe}} = \frac{1}{1+k_i} \tag{9}$$

The following equation was proposed in our previous paper [16] to describe sample focusing:

$$\text{sample focusing factor} = \frac{1+k_a}{1+k_i} \tag{10}$$

An apparent shortcoming of calculating the sample focusing factor using Eq. (10) is the lack of focusing when analytes are dissolved in mobile phase, i.e. $k_a = k_I$. From the above description of isocratic focusing it is clear that analytes with adequate retention factors will be focused at the head of the column during sample loading as a function of their k_a. Therefore Eq. (9) is preferred over Eq. (10) when describing analyte focusing. It should be emphasized that isocratic focusing occurs only during sample loading which takes place essentially before the separation has begun.

This isocratic focusing is a likely explanation for the improved performance generally observed in isocratic separations with analytes having retention factors >3. Simple experiments varying the retention of analytes by adjusting the volume fraction of organic modifier in the mobile phase clearly demonstrate that chromatographic performance improves significantly when the mobile phase is adjusted to give a retention factor greater than 3.

It is common practice to inject small sample volumes onto columns of low volume (such as the 50 mm × 2.1 mm) in order to reduce performance losses due to sample-volume overload. Generally, a 10% loss in efficiency caused by the injection step is considered practically reasonable and acceptable [23]. However, methods to reduce the impact of the injector on the observed performance have received little attention in the recent literature. As demonstrated in the next section, the POISe technique can reduce the impact of the injector to near zero with the proper choice of conditions.

2.3. Compressed band variance

The volume occupied by the analyte band, V_{analyte}, immediately after completion of the loading step is given by,

$$V_{\mathrm{analyte}} = d_{\mathrm{inj},a} \times \frac{\pi d_c^2 \varepsilon_t}{4} = \frac{V_{\mathrm{inj}}}{F} \times \frac{\mu_0}{1+k_a} \times \frac{\pi d_c^2 \varepsilon_t}{4}$$

$$= \mu_0 \times \frac{1}{F} \times \frac{\pi d_c^2 \varepsilon_t}{4} \times \frac{V_{\mathrm{inj}}}{1+k_a} = \frac{V_{\mathrm{inj}}}{1+k_a} \tag{11}$$

where ε_t is the total porosity of the column (~0.53 for core–shell, ~0.7 for fully porous [24]) and d_c is the column inner diameter (in mm). The right side of Eq. (11) is similar to the expression proposed for displaced volume when using on-column focusing in microbore LC, in which case the injection volume was significantly increased with the purpose of improving sensitivity [25].

Conditions for reducing the injector contribution to the observed chromatographic performance are most easily identified using peak variances. Since the peak width at base is defined as 4σ the volume occupied by the analyte band at the head of the column can be assumed to be equal to 4σ. Eq. (11) can then be used to calculate the variance of the compressed analyte band ($\sigma^2_{\mathrm{analyte}}$) from the variance of the injected band (σ^2_{inj}) as shown below:

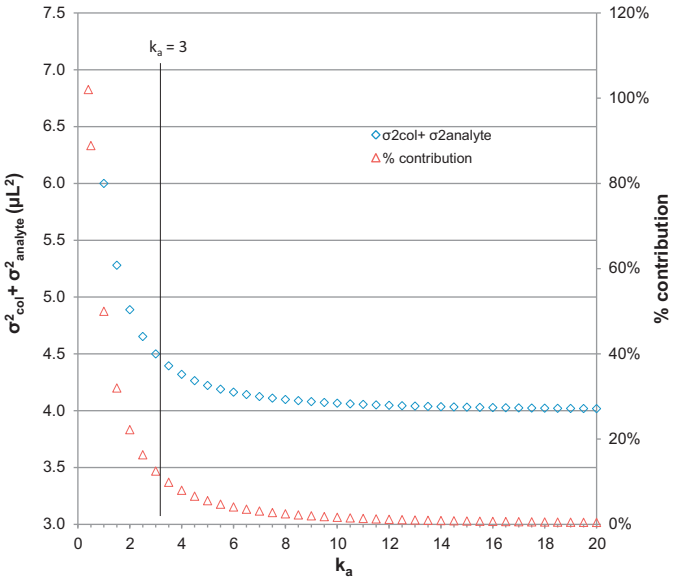

Fig. 2. Variance contribution of the compressed analyte band, ($\sigma^2_{\mathrm{analyte}}$), to the column peak variance, (σ^2_{col}), as a function of retention factor of the analyte in the sample band (k_i), assuming $V_{\mathrm{inj}} = 4\,\mu\mathrm{L}$, ($\sigma^2_{\mathrm{col}}$) = 4 µL², ($\sigma^2_{\mathrm{injdisp}}$) = 4 µL².

$$\left(\frac{V_{\mathrm{analyte}}}{4}\right)^2 = \sigma^2_{\mathrm{analyte}} = \left[\frac{(V_{\mathrm{inj}}/4)}{1+k_i}\right]^2 = \frac{\sigma^2_{\mathrm{inj}}}{(1+k_i)^2} \tag{12}$$

The variance introduced by the injector is the combination of the variance induced by the volume of sample loaded (V_{inj}) and the inherent dispersion of the injector used, ($\sigma^2_{\mathrm{injdisp}}$). The total injector variance can be calculated using [26]:

$$\sigma^2_{\mathrm{inj}} = (0.4 \times V_{\mathrm{inj}})^2 + \sigma^2_{\mathrm{injdisp}} \tag{13}$$

Inserting the above expression for the injector variance into Eq. (12) allows the contribution from the sample volume and the inherent injector variance to be treated independently:

$$\sigma^2_{\mathrm{analyte}} = \frac{(0.4 \times V_{\mathrm{inj}})^2 + \sigma^2_{\mathrm{injdisp}}}{(1+k_i)^2} \tag{14}$$

The contribution of the compressed analyte band variance is largest (i.e. causes the largest decrease in performance) for analytes having $k_a < 3$, since limited isocratic focusing of the injected band takes place (only 25% or less as discussed above in relation to Fig. 1). For example, assuming the sample is dissolved in mobile phase, a 4 µL injection volume and an inherent injector variance of 4 µL², the variance of the injected analyte band, ($\sigma^2_{\mathrm{analyte}}$), in the absence of focusing (i.e. $k_a = 0$) is 6.5 µL². In the case of an analyte having a retention factor of $k_a = 1$ the variance of the injected analyte band after isocratic focusing is reduced to 1.6 µL². Further, assuming a column variance, (σ^2_{col}), of 4 µL² (i.e. 8 µL peak volume eluting from a 50 mm × 2.1 mm column, with $N \sim 10{,}000$ at $k_a = 1$, and $V_0 \sim 100\,\mu\mathrm{L}$) the variance contribution of the focused analyte band with $k_a = 1$, ($\sigma^2_{\mathrm{analyte}}$), to the column variance ($\sigma^2_{\mathrm{analyte}}/\sigma^2_{\mathrm{col}} \times 100$) is a very significant 40%.

The sum of the variance of the focused analyte band and the column variance as a function of analyte retention factor is plotted in Fig. 2. The plot shows that the variance contribution of the focused analyte band is reduced to <10% of the column variance when the retention factor of the analyte in the sample band is at least 3. Hence, increasing the retention factors of most analytes in the

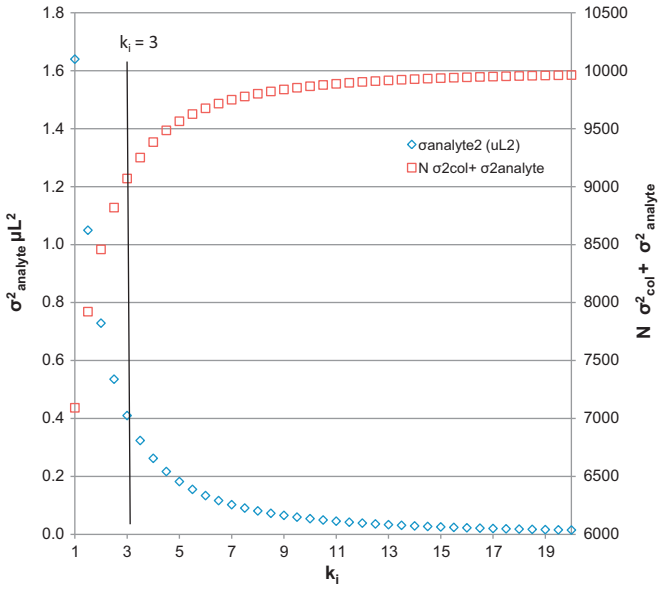

Fig. 3. Impact of the compressed analyte band variance, ($\sigma^2_{analyte}$), on the efficiency calculated from the sum of the compressed analyte band variance, ($\sigma^2_{analyte}$), (as a function of retention factor of the analyte in the injected band, k_i) and the column variance, (σ^2_{col}), assumed to be constant 4 μL^2.

injected sample band to a minimum value of 3 during sample loading is the primary objective of the POISe technique. Furthermore, Fig. 2 shows that the ideal sample loading condition is when $k_a > 8$ where the contribution of the injection step to total band spreading is practically insignificant. To further illustrate the impact of reducing the injection variance on the observed chromatographic performance the efficiency calculated using the sum of the analyte band variance (as a function of k_i in the injected sample band) and a constant column variance, (σ^2_{col}), of 4 μL^2 is plotted in Fig. 3. This plot demonstrates the importance of reducing the injection system contribution to band variance. This plot also shows the relatively insignificant improvement to efficiency beyond a k_i of ~8 indicating the variance introduced by the injection system has essentially been eliminated.

As stated earlier a 10% reduction in the organic modifier content of the mobile phase generally produces a 2-fold increase in retention factor. As an example, consider a sample dissolved in acetonitrile:water 7:3 (v/v) as in protein precipitated plasma samples with no further sample work-up. For an injected sample volume of 1 µl an equal amount of a "weak solvent" having a composition of 1:1 acetonitrile:water reduces the acetonitrile content of the mixed sample plug when reaching the head of the column to 60% (assuming complete mixing). In this case, the retention factor of all analytes in the sample band will be increased by ~2×. Sample components having a k_a of 1.5 in 7:3 acetonitrile:water will have a k_i of ~3 in the injected sample band (acetonitrile:water 6:4) and would be focused into bands having less than 10% impact on the column variance. Using a larger volume of weak solvent with even lower eluting strength than the above example (e.g. 4 µl of water) would provide higher k_i values, in many cases above 8 (see Section 3). These examples reinforce the observation that POISe has the largest impact on sample components having retention factors (k_a) of <3.

2.4. Step gradient compression

In our previous paper it was assumed that POISe induces not only what we now call isocratic compression but also step gradient compression arising from the early elution of the rear of the analyte band by the mobile phase (of higher eluting strength than that of the mixture sample solvent + weak solvent) moving behind the sample plug. It was also assumed that the relative impact of the two processes (isocratic compression and step gradient compression) could not be distinguished. Step gradient compression was introduced by Snyder et al. [27] and later revised by Gritti et al. [16] as:

$$\text{step gradient compression factor}(G_{12}) = \frac{(1+k_i)}{k_i} \times \frac{k_a}{(1+k_a)} \quad (15)$$

While it is acknowledged that isocratic focusing and step gradient compression are 2 distinct phenomena, in the literature to date only gradient compression has been proposed as the singular phenomenon responsible for the narrow elution bands observed in gradient chromatography. As pointed out above, isocratic focusing is very effective in reducing the impact of the injection step on the observed chromatographic performance and is also present in gradient analysis (albeit only during the loading step). Therefore, a comparison of the relative impact of isocratic focusing and gradient compression is relevant to this discussion. The gradient compression and isocratic focusing factors based on Eqs. (9) and (15) are plotted in Fig. 4. The plots show that isocratic focusing during sample loading has a significantly larger impact than step gradient compression taking place immediately after loading regardless of k_a. For example, the bandwidth of an analyte having $k_a = 1$ (in the mobile phase), corresponding to a $k_i = 8$ (in the diluted sample solvent) is compressed to less than 11% of its original volume by isocratic focusing, while to only 56% by step gradient compression. Therefore, the impact of step gradient compression is limited when analyte bands are effectively focused during sample loading (i.e. $k_a > 1$). It should be mentioned that gradient compression during elution is a separate and effective phenomenon minimizing the impact of the column variance on the observed chromatographic performance.

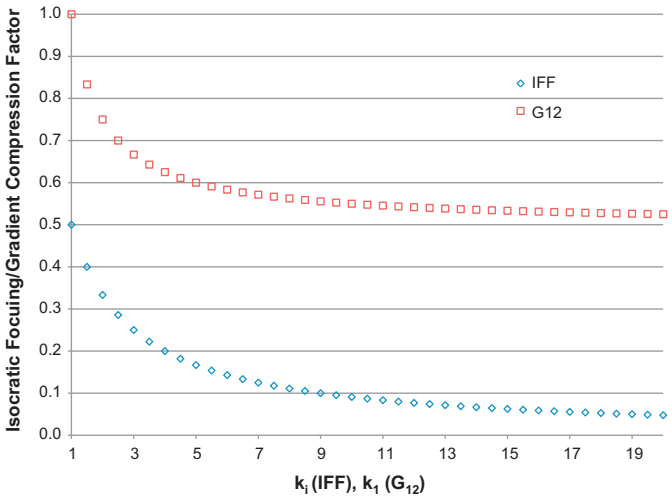

Fig. 4. The gradient compression and isocratic focusing factors calculated based on Eqs. (9) and (15) as a function of the analyte retention factor in the diluted sample plug; for the gradient compression factor (G_{12}), the retention factor in mobile phase, k_2, was assumed to be 1.

3. Experimental

3.1. Materials

The test standard, containing uracil (0.01 mg/mL), acetophenone (0.22 mg/mL), toluene (9.42 mg/mL), and naphthalene (9.42 mg/mL) was obtained from Phenomenex (Torrance, CA, USA), and was diluted 5× so the sample solvent matched the mobile

phase, i.e. 50% water and 50% acetonitrile by volume. HPLC grade water was obtained by using a Milli Q water purification system (Millipore, Billerica, MA, USA). All organic solvents were HPLC grade and purchased from VWR (Westchester, PA, USA). The columns used, Kinetex 2.6 μm XB-C18 (Phenomenex, Torrance, CA), Kinetex 1.7 μm XB-C18, Zorbax Eclipse XDB-C18 1.8 μm (Agilent Technologies, Santa Clara, CA), and Acquity UPLC® BEH C18 1.7 μm (Waters Corporation, Milford, MA), were purchased from their respective manufacturers and used as received. All columns were 50 mm × 2.1 mm in dimension unless otherwise noted.

3.2. HPLC equipment

Four different HPLC/UHPLC systems were used in this work. System one was an Agilent 1200 SL (Agilent Technologies, Santa Clara, CA, USA) consisting of a binary pumping system, well plate autosampler, column oven, and multiple wavelength detector with a micro flow cell (1.7 μL, 6 mm path). System two was an Agilent 1100 with a binary pumping system, a G1329A autosampler, and a diode array detector outfitted with a micro flow cell (1.7 μL, 6 mm path length). System three was an Agilent 1290 system with a 1290 Infinity binary pumping system, Infinity autosampler, column oven, and a 1200 SL variable wavelength detector outfitted with a micro flow cell (2 μL, 3 mm path length). The fourth system was a Waters Acquity UPLC with a binary solvent manager, sample manager, column oven and TUV detector outfitted with an analytical flow cell (500 nL, 10 mm path length). On all systems the column was connected directly to the injector and detector bypassing the column oven using a 20 cm piece of 75 μm i.d. PEEKSil tubing (SGE, Austin, TX, USA). Detectors on each system were set to their respective fastest collection rates. For all the experiments presented the mobile phase was 50% water and 50% acetonitrile (v/v) at a flow rate of 0.5 mL/min. The detection wavelength was 254 nm. For the Agilent 1100 and Agilent 1200SL instruments the acquisition software was Agilent Chemstation Rev B.04.01, while for the Agilent 1290 the acquisition software was Agilent Chemstation Rev B.04.03. For the Acquity UPLC system the acquisition software was Empower Pro 2 version 6.20.00.00. Regardless of the acquisition software, all data was processed using Agilent Chemstation Rev B.02.01. The peak widths reported are the 4σ peak widths and the theoretical plates were calculated using the half height method.

4. Results

4.1. Effect of weak solvent volume and configuration and reproducibility with POISe

The initial work performed in this study centered on determining the optimum volume of weak solvent required to realize the maximum gain in performance since this was not covered in the previous work [16]. Separations were performed using a mobile phase containing 50% water and 50% acetonitrile with a detection wavelength of 254 nm and a flow rate of 0.5 mL/min. While in this work we used 100% water as the weak solvent, we have used water containing 5–10% methanol in other experiments and seen similar results (data not included). Fig. 5 shows column efficiencies obtained for the various test analytes when using 1 μl (1× the injection volume) to 10 μL (10× the injection volume) of weak solvent with a Kinetex 2.6 μm C18 50 mm × 2.1 mm column on system 2 (Agilent 1100). As shown in Fig. 5, plate counts increase with increasing the volume of weak solvent. This performance benefit plateaus at 3–4 μL (3–4× the injection volume) indicating there is no significant benefit to weak solvent volumes greater than 3–4× the sample volume. In practical terms, the optimal ratio of weak solvent volume to sample volume should be 4:1. The use of larger

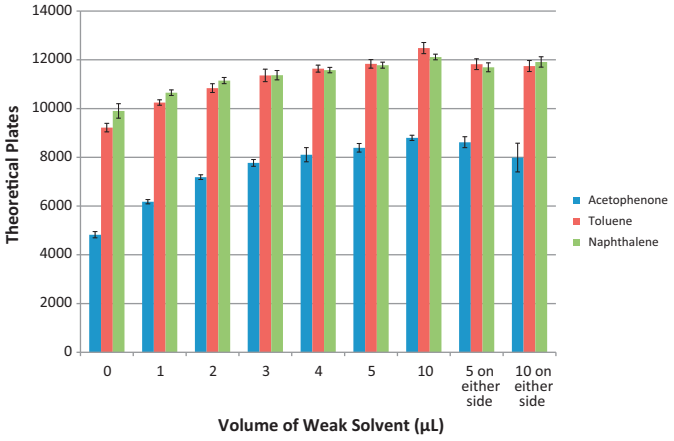

Fig. 5. Chromatographic performance obtained on a Kinetex 2.6 μm XB-C18 50 mm × 2.1 mm column with varying volumes of weak solvent and 1 μL of sample using the POISe injection technique on an Agilent 1100 HPLC system.

volumes of weak solvent does not provide additional benefit in terms of peak width; any observed change in efficiency is due to increased retention time. If a weak solvent other than water is used the volume should be adjusted to ensure an ~8× increase in retention factor in the diluted sample to realize the maximum benefit, whenever possible. Chromatograms obtained on a Kinetex 2.6 μm XB-C18 column on the Acquity UPLC system with and without POISe are shown in Fig. 6 which is the first reported example of the effect of POISe when using a UHPLC system.

The chromatographic performance data for the experiments varying the weak solvent volume are shown in Table 1. As expected the performance benefits realized were highest for acetophenone which is a weakly retained analyte ($k_a \sim 1$). This is consistent with isocratic focusing as described in Section 2 since the k_a for this analyte in mobile phase is $\ll 3$. The low retention factor for acetophenone in mobile phase does not allow significant isocratic focusing. With the 1:4 ratio of sample solvent to weak solvent (water), the percent organic in the injected sample plug is reduced

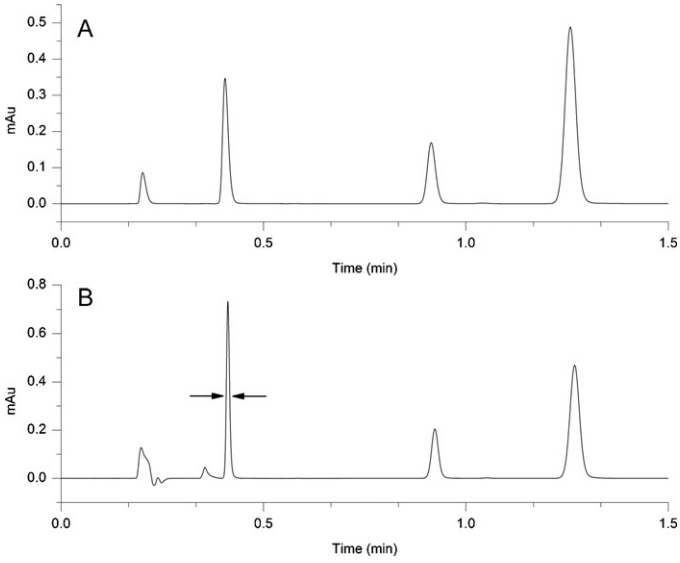

Fig. 6. Chromatograms obtained on a Kinetex 2.6 μm XB-C18 column (A) without and (B) with the use of the POISe injection technique on an Acquity UPLC system. Peaks A: (1) uracil, (2) acetophenone, (3) toluene and (4) naphthalene; Peaks B: (1) uracil, (2) unknown contaminant in weak wash solvent, (3) acetophenone, (4) toluene and (5) naphthalene. Conditions: mobile phase was 50% water and 50% acetonitrile (v/v) at a flow rate of 0.5 mL/min; detection wavelength at 254 nm.

Table 1
Number of theoretical plates and peak widths at base obtained with increasing volumes of weak solvent and fixed injection volume with a Kinetex 2.6 μm XB-C18 column on an Agilent 1100 HPLC instrument.

Weak solvent volume (μL)	Peak	Plates	Peak width	% Decrease in width
0	Acetophenone	4726	0.024	0.00%
	Toluene	9393	0.039	0.00%
	Naphthalene	10024	0.051	0.00%
1	Acetophenone	6086	0.021	11.67%
	Toluene	10161	0.037	5.89%
	Naphthalene	10565	0.049	5.19%
2	Acetophenone	7255	0.020	18.10%
	Toluene	10861	0.036	9.11%
	Naphthalene	11260	0.048	7.07%
3	Acetophenone	7778	0.019	20.00%
	Toluene	11654	0.035	10.01%
	Naphthalene	11563	0.047	8.82%
4	Acetophenone	8304	0.019	22.72%
	Toluene	11727	0.035	10.94%
	Naphthalene	11615	0.047	8.86%
5	Acetophenone	8442	0.019	22.20%
	Toluene	11788	0.035	11.31%
	Naphthalene	11872	0.047	9.16%
10	Acetophenone	8855	0.019	20.91%
	Toluene	12444	0.034	13.22%
	Naphthalene	12191	0.047	9.58%
5 on either side	Acetophenone	7639	0.021	14.38%
	Toluene	11684	0.035	10.07%
	Naphthalene	11696	0.047	8.50%

from 50 v/v% to 10 v/v%. Based on Eq. (3), the expected increase in k is 2-fold with every 10 v/v% change in organic modifier, therefore, the k of acetophenone ($k_a = 1$) is expected to be increased to 8, i.e. an 8× increase in k. The predicted impact of the compressed analyte band variance on the column + detector band variance was calculated assuming a variance value of 1 μL^2 for the detector and 4 μL^2 for the column. The value for the detector variance is an average for several different detector types investigated in our laboratory (data not included). In contrast, the "experimental" percent contribution of the compressed analyte band variance to the column + detector band variance was calculated using:

$$\frac{\text{Peak variance without POISe} - \text{Peak variance with POISE}}{\text{Peak variance with POISE}} \times 100 \quad (16)$$

In this context, the experimental measurements with POISe are considered to give the closest approximation of the true column + detector variance. From Eq. (14), the expected decrease in the injection system contribution to the column + detector band variance (assuming a detector variance of 1 μL^2 and a column variance of 4 μL^2) when the k of an analyte is increased from 1 to 8 is 38% (see Fig. 2). As shown in Table 2, a 42% change in the contribution of the injection step to the column and detector variance was observed for acetophenone which is in good agreement with the 38% predicted by Eq. (14). For toluene, the predicted change in the contribution is 10% (see Fig. 2) while the observed change in contribution was 16% which again validates the proposed model (Eq. (14)).

Therefore, the lower limit for POISe to be effective is $k_a \sim 0.4$ which is within the range of practical interest in most cases. As discussed in Section 2.2, the upper limit of effectiveness is $k \sim 8$, beyond which further improvement is negligible. Therefore, the range of primary usefulness of the POISe technique is $0.4 < k_a < 8$ with the most benefit realized in the range of $0.4 < k_a < 3$. Hence, arguably, the range of practical interest of most applications can benefit from use of the POISe technique.

The optimal configuration in regard to the position of the plug of weak solvent in the POISe technique was established by comparing results for the following sample and weak solvent combinations: sample preceded by a plug of weak solvent, sample plug sandwiched between two plugs of weak solvent and sample followed by a plug of weak solvent. The data with the sample sandwiched between two plugs of weak solvent can be found in Table 1. When we compared the effects of a 10 μL weak solvent plug following the sample to the sample plug sandwiched between two 5 μL plugs of weak solvent the naphthalene peak was 9.6% narrower with the weak solvent following the sample versus only 8.5% narrower when the sample is sandwiched. Further, the acetophenone peak was 21% narrower with the 10 μL plug following the sample versus 14.4% when the sample was sandwiched. This demonstrates that having the weak solvent follow the sample into the column provides the best performance. The same conclusion was drawn when the weak solvent plug preceded the sample. These findings indicate that mixing is most effective when the weak solvent follows the sample band thru the injection system and this observation is consistent across different instrument platforms (data not shown).

Using the optimized POISe conditions, the reproducibility of the technique was evaluated. Six replicate injections were performed both with and without POISe. Table 3 lists the average values and relative standard deviations for each of the following chromatographic parameters: retention time, peak area, height and width at half height (width @ 50%), tailing factor and column efficiency, for an early eluting ($k_a = 1$) and also an intermediate eluting ($k_a = 3.5$) sample component. The values listed in Table 3 demonstrate that the reproducibility of the POISe technique is good and comparable to standard injection (no POISe). Furthermore, the data in Table 3 shows that peak height, width, tailing factor and efficiency are all improved with the POISe technique.

4.2. POISe versus sample pre-dilution

In order to determine the extent of mixing when using the POISE technique, experiments were performed where the

Table 2
Reduction in peak variance and decrease in the contribution of the injection step to the column variance when using the POISe technique with an Agilent 1290 UHPLC instrument.

Analyte	Inj tech	4σ Peak width (min)	Peak volume (μL)	Peak variance ($\mu L\hat{2}$)	Variance reduction ($\mu L\hat{2}$)	Contribution %
Acetophenone	NoPOISe	0.0197	9.86	6.07	N/A	N/A
	POISe	0.0165	8.26	4.26	1.80	42%
Toluene	NoPOISe	0.0356	17.8	19.8	N/A	N/A
	POISe	0.0331	16.5	17.1	2.75	16%

Table 3
Comparison of the reproducibility of chromatographic parameters with and without the POISe injection technique ($n = 6$) using an Agilent 1290 UHPLC instrument.

Analyte	Injection technique	RT, min (%RSD)	Peak area, mAU*s (%RSD)	Peak height, mAU (%RSD)	4σ Peak width, min (%RSD)	Tailing factor, (%RSD)	Efficiency, (%RSD)	4σ Resolution, (%RSD)
Acetophenone	NoPOISe	0.381 (0.37%)	90.7 (0.55%)	112 (1.4%)	0.0197 (0.49%)	1.54 (1.1%)	6400 (1.6%)	N/A
	POISe	0.394 (0.30%)	97.7 (0.31%)	147 (0.75%)	0.0165 (0.36%)	1.35 (0.59%)	9700 (1.5%)	N/A
Toluene	NoPOISe	0.879 (0.49%)	79.7 (2.9%)	57.8 (2.5%)	0.0356 (0.43%)	1.19 (0.97%)	10300 (1.5%)	18.0 (0.65%)
	POISe	0.893 (0.40%)	79.4 (1.8%)	62.6 (1.8%)	0.0331 (0.59%)	1.12 (0.23%)	12300 (1.3%)	20.1 (0.081%)

Table 4
Performance comparison when using the POISe injection technique versus offline sample dilution at the same ratio (1 μL of sample diluted with 4 μL of water) using an Agilent 1100 HPLC instrument.

Compound	Injection style	Retention time	Plates	Peak width
Uracil	POISe	0.191	380	0.0385
Acetophenone		0.387	8200	0.0179
Toluene		0.865	10100	0.0350
Naphthalene		1.17	9800	0.0481
Uracil	Conventional with	0.181	950	0.0223
Acetophenone	Diluted Sample	0.386	8400	0.0181
Toluene		0.859	10000	0.0351
Naphthalene		1.16	9700	0.0481

sample was pre-diluted to the optimum ratio (4:1) determined for the POISE technique. By comparison of the performance obtained with the pre-diluted sample to that obtained with the POISe technique the extent of mixing can be assessed. Table 4 lists the results for these experiments. As shown, the peak widths were identical whether the sample dilution was a result of using the POISe technique or if off line dilution was used. These experiments confirm the effectiveness of mixing in the pre-column space. This finding is in agreement with the practice called "at-column dilution" successfully used in preparative chromatography whenever sample solubility is the limiting factor to increasing productivity. In such cases, instead of loading very large volumes of dilute sample onto the preparative column, the crude sample is dissolved in a strong solvent (e.g. dimethylsulfoxide; which can be disruptive in nature to the chromatographic process) and the injection plug is mixed with mobile phase right before the column inlet. This technique allows for large loads of crude sample and does not seem to be burdened by sample precipitation at the column head. While sample solubility concerns remain when diluting samples with weak solvent, given the significantly lower sample loads practiced in chromatographic analysis compared to chromatographic purification, it should come as no surprise that sample precipitation is not a concern with POISe. This result also reinforces the postulate in the above theory that the benefits observed in POISe are mostly due to isocratic focusing as opposed to (step) gradient compression. If there was a significant contribution in POISe due to gradient compression, the POISe assisted separation should have significantly narrower peaks versus the off line dilution, which is in fact not the case.

4.3. Effectiveness of the POISe technique in HPLC and UHPLC

It stands to reason that older generation instrumentation designed more than a decade ago would have notable extra column contributions to band spreading when challenged with the narrow peaks eluting from UHPLC columns [28]. In conjunction with the advent of UHPLC columns a new generation of instruments have been developed which are stated to have significantly reduced band dispersion. We investigated the effect of using the POISe injection technique with the new generation instruments as well as with a conventional Agilent 1100 instrument. The results obtained with and without the POISe technique on an Agilent 1100, Agilent 1200 SL, Agilent 1290, and Acquity UPLC instruments are shown in Table 5 when using a Kinetex 2.6 μm XB-C18 column in 50 mm $\times$ 2.1 mm format. It is important discussing here the significant differences in the design of the injection systems used in the Agilent and the Acquity UHPLC systems. The Agilent systems use a 40 or 100 μL loop which, under standard injection conditions, is partially filled with the desired sample volume while the rest of the loop is filled with mobile phase.

With the Acquity UPLC instrument 2 different wash solvents (strong and weak) are used for cleaning the injection needle after each injection. The Acquity system used for the experiments described here was outfitted with a 5 μL fixed loop and allowed for various injection modes. In the partial loop injection mode and when using the weak needle-wash option, the sample plug displaces an equivalent volume of weak wash solvent in the needle. Therefore, upon injection, the sample plug is followed by a plug of weak solvent. For example, when a 1 μL injection volume is

Table 5
Chromatographic performance obtained on a Kinetex 2.6 μm XB-C18 50 × 2.1 mm column with and without the POISe injection technique on different HPLC and UHPLC instruments.

Instrument	POISe	Peak	Plates	Width	% Decrease in width
Agilent 1100	None	Acetophenone	4700	0.0242	0.00%
		Toluene	9400	0.0391	0.00%
		Naphthalene	10000	0.0515	0.00%
	4 μL	Acetophenone	8300	0.0187	23%
		Toluene	11800	0.0348	11%
		Naphthalene	11600	0.0469	8.9%
Agilent 1200 SL	None	Acetophenone	4900	0.0229	0.00%
		Toluene	9300	0.0372	0.00%
		Naphthalene	10100	0.0484	0.00%
	4 μL	Acetophenone	7800	0.0189	18%
		Toluene	11600	0.0339	9.0%
		Naphthalene	11700	0.0457	6.0%
Agilent 1290	None	Acetophenone	6600	0.0196	0.00%
		Toluene	10400	0.0354	0.00%
		Naphthalene	10600	0.0478	0.00%
	4 μL	Acetophenone	9500	0.0165	16%
		Toluene	12300	0.0330	6.8%
		Naphthalene	12100	0.0454	4.9%
Waters Acquity	None	Acetophenone	3900	0.0270	0.00%
		Toluene	9200	0.0390	0.00%
		Naphthalene	10100	0.0510	0.00%
	4 μL	Acetophenone	13500	0.0150	44%
		Toluene	13700	0.0320	18%
		Naphthalene	12700	0.0460	9.8%

Table 6
Chromatographic performance obtained with and without the POISe injection technique with a variety of columns packed with totally porous or core-shell particles.

Column	Instrument	POISe Volume (μL)	Peak	Retention Factor	Plates	Width	% Decrease in Width
Kinetex 2.6 μm XB-C18	Agilent 1200 SL	0	Acetophenone	1.01	4700	0.0242	N/A
			Toluene	3.49	9400	0.0391	*N/A*
			Naphthalene	5.09	10000	0.0515	*N/A*
		4	Acetophenone	1.05	8300	0.0187	23%
			Toluene	3.50	11700	0.0348	11%
			Naphthalene	5.08	11600	0.0469	8.9%
Kinetex 1.7 μm XB-C18	Agilent 1200 SL	0	Acetophenone	1.09	5300	0.0240	*N/A*
			Toluene	3.83	10600	0.0395	*N/A*
			Naphthalene	5.73	11400	0.0532	*N/A*
		4	Acetophenone	1.12	8300	0.0197	18%
			Toluene	3.86	12700	0.0369	6.7%
			Naphthalene	5.77	12700	0.0511	3.9%
Acquity UPLC 1.7 μm BEH C18	Agilent 1200 SL	0	Acetophenone	1.17	7000	0.0250	*N/A*
			Toluene	4.25	10300	0.0498	*N/A*
			Naphthalene	6.34	10700	0.0680	*N/A*
		4	Acetophenone	1.17	9900	0.0219	12%
			Toluene	4.25	11000	0.0484	2.9%
			Naphthalene	6.34	11100	0.0665	2.1%
Zorbax 1.8 μm SB-C18	Agilent 1200 SL	0	Acetophenone	1.42	6600	0.0239	*N/A*
			Toluene	5.77	11200	0.0521	*N/A*
			Naphthalene	8.62	11600	0.0726	*N/A*
		4	Acetophenone	1.45	8300	0.0226	5.6%
			Toluene	5.77	12000	0.0505	2.9%
			Naphthalene	8.61	12000	0.0720	0.78%
Gemini NX 3 μm C18	Agilent 1100	0	Acetophenone	1.65	4400	0.0334	*N/A*
			Toluene	5.79	6600	0.0704	*N/A*
			Naphthalene	8.48	6500	0.0980	*N/A*
		4	Acetophenone	1.65	5900	0.0305	8.8%
			Toluene	5.67	7000	0.0687	2.3%
			Naphthalene	8.27	6900	0.0966	1.5%

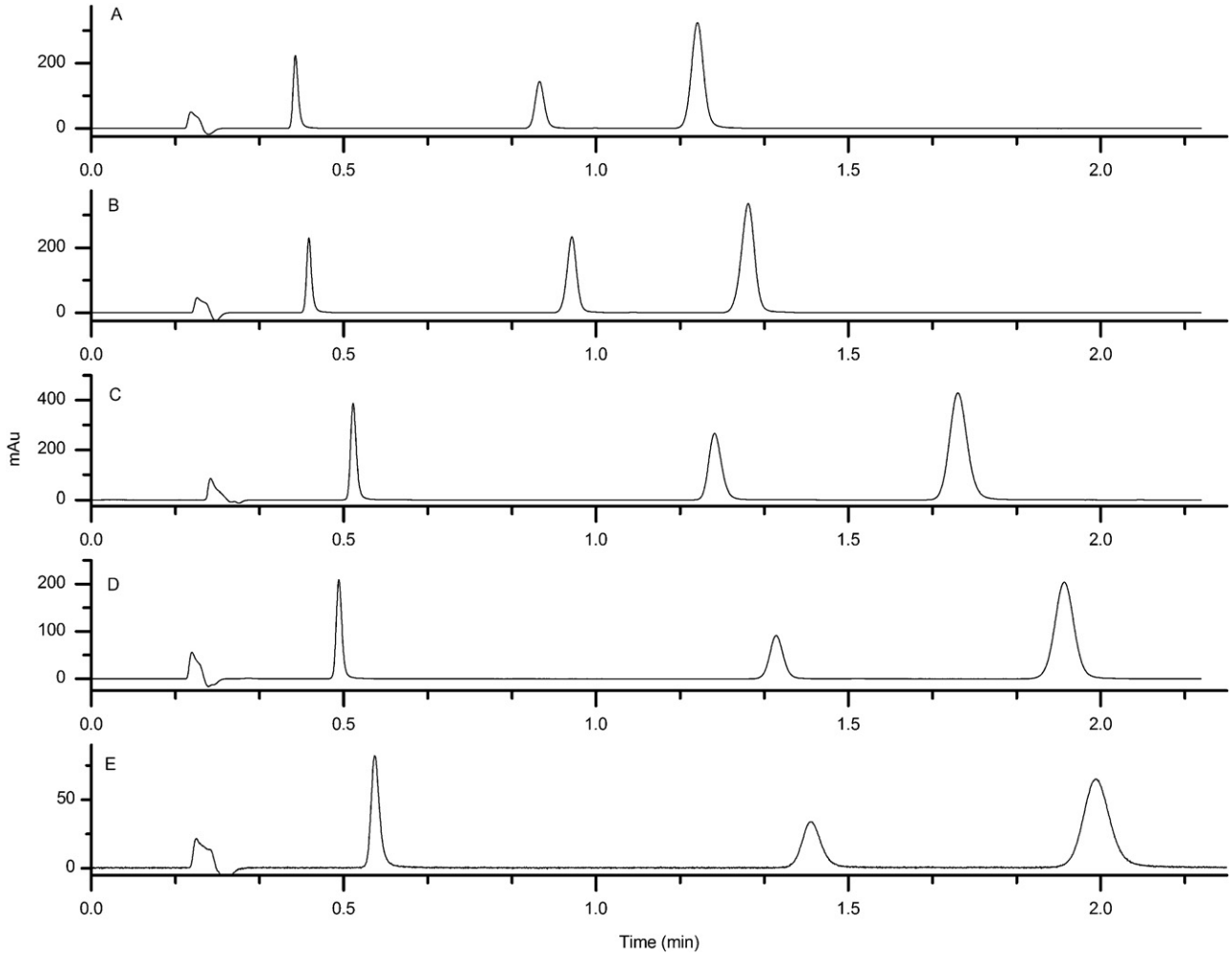

Fig. 7. Chromatograms obtained when using the POISe injection technique in conjunction with (A) Kinetex 2.6 μm XB-C18, (B) Kinetex 1.7 μm XB-C18, (C) Acquity 1.7 μm BEH C18, (D) Zorbax 1.8 μm XDB-C18, and (E) Gemini NX 3 μm C18 on an Agilent 1200SL UHPLC system. Peaks (1) uracil, (2) acetophenone, (3) toluene and (4) naphthalene.

specified the 5 μL sample loop will retain 4 μL of weak wash solvent. When operated in this configuration, the Acquity UPLC performs a POISe injection. This was confirmed by replacing the weak wash solvent with mobile phase and performing several analyses. When using mobile phase as the weak wash solvent the chromatographic performance severely degraded on the Acquity UPLC. While other injection systems have been proposed and tested for UHPLC separations, all of the injection systems on the instruments used for this study still employ a rotary injection valve [29].

For consistency 4 μL of weak solvent along with 1 μL of sample were used with all other HPLC systems. In Table 5 the plate counts and peak widths obtained on 3 different UHPLC instruments are shown with and without the POISe injection technique. For all instruments tested there was a 5–44% decrease in peak width when using the POISe injection technique depending on the k_i of the analyte. The largest improvement was observed on the Acquity UPLC system for the weakly retained solute, acetophenone. In this case there was a 44% decrease in peak width, where under the same conditions the Agilent UHPLC instruments showed a 16–18% decrease. The Agilent 1100 system showed a 23% decrease for the peak width of acetophenone in comparison to the 16–18% on the Agilent UHPLC instruments. This minor difference between the Agilent HPLC and the two UHPLC instruments shows that there has not been a significant improvement in the design of the Agilent UHPLC instruments. The Acquity UPLC result shows the large extra column variance introduced by its injection system and pre-column mobile phase heater. It also shows that the POISe technique effectively eliminates even large contributions from the injection step to the observed peak variance. Additionally, POISe is shown to be a useful diagnostic tool for assessing extra column contributions ahead of the column.

In spite of the excessive extra column volume identified for the Acquity UPLC it still showed the best overall performance when using the POISe technique in comparison to the other UHPLC instruments. With a well compressed analyte band at the head of the column and the same column in place, any difference in peak characteristics may be attributed to the dispersion caused by the detector. The differences in detector volumes for the different instruments (500 nL for the Acquity UPLC, while 1.7–2 μL for the Agilent HPLC instruments) is the likely explanation for the better overall performance of the Acquity UPLC when using the POISe technique. Regardless of whether an HPLC or UHPLC instrument was used the POISe injection technique consistently provided a significant improvement in chromatographic performance while only marginally affecting the cycle time (maximum of 3 second increase with Agilent 1200/1290; none with Acquity UPLC). In addition, even the newer generation UHPLC instruments have not significantly improved in regards of their sample delivery system and fortunately the POISe technique effectively minimizes this shortcoming.

4.4. Applicability of the POISe technique with different columns

To show the general applicability of the POISe technique four additional HPLC columns were tested, two packed with totally porous sub-2 μm particles (Acquity UPLC 1.7 μm BEH C18 and Zorbax 1.8 μm SB-C18) as well as a sub-2 μm core–shell column (Kinetex 1.7 μm XB-C18) and a conventional HPLC column (Gemini NX 3 μm C18). The traditional HPLC column was tested on an Agilent 1100 instrument while the sub-2 μm columns were tested on an Agilent 1200SL UHPLC instrument due to the high backpressure generated by these columns. The results from these experiments are presented in Table 6. For all columns, whether packed with core–shell or fully porous particles, there was a significant decrease in peak width when using the POISe technique versus standard injection. Chromatograms obtained on the UHPLC columns are shown in Fig. 7. On all tested columns, as was observed with the 2.6 μm core–shell column, the most significant improvement was observed with the early eluting analytes; however, all analyte peaks in the chromatogram show some improvement. It is interesting to note that for the most retained compound, naphthalene, the largest decrease in peak width (3.9%) was observed on the 1.7 μm core–shell column, while the smallest (0.8%) on the Zorbax column. This result can be explained by the difference in retention factors for naphthalene on the different columns: Kinetex had the lowest k_a of 5.5 while Zorbax had the highest k_a of 8.6.

5. Conclusions

The performance capabilities of high efficiency, small volume, (U)HPLC columns have and continue to be limited by the extra column dispersion of the (U)HPLC system. Conventional HPLC systems limit the observed performance of 50 mm × 2.1 mm columns packed with 3 μm fully porous particles. Unfortunately, even the latest UHPLC systems continue to limit the observed performance of the newest ultra high efficiency columns, such as core–shell and sub 2-μm fully porous, despite being designed specifically for such columns. As shown in this work the injection system is a major contributor to extra-column dispersion. The POISe injection technique provides a simple and effective procedure to reduce or eliminate the variance introduced by the injection system. An improved model was developed to reflect the dominant role isocratic focusing plays in both isocratic and gradient separations. Additionally, it was verified experimentally that the model provides reasonable predictions for the performance improvements achieved with the POISe technique. It was shown that the range of primary usefulness of the POISe technique is $0.4 < k_a < 8$ with the most benefit realized in the range of $0.4 < k_a < 3$. Hence, arguably, the range of practical interest of most applications can benefit from use of the POISe technique. Gradient compression from the step gradient following sample loading plays little, if any, role in the performance improvements observed using the POISe technique. However it may provide additional benefit for analytes whose retention factors cannot be increased significantly upon dilution with weak solvent. Band compression with the POISe technique is also effective in gradient elution mode and can also reduce the impact of instrument components intentionally added ahead of the column, such as heat exchangers and switching valves. These topics as well as the effect of larger sample injection volumes with higher organic solvent content will be covered in subsequent studies.

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