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Selective comprehensive multi-dimensional separation for resolution enhancement in high performance liquid chromatography. Part I: Principles and instrumentation

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ABSTRACT

An approach to enhancing the resolution of select portions of conventional one-dimensional high performance liquid chromatography (HPLC) separations was developed, which we refer to as selective comprehensive two-dimensional HPLC (sLC × LC). In this first of a series of two papers we describe the principles of this approach, which breaks the long-standing link in on-line multi-dimensional chromatography between the timescales of sampling the first dimension (^{1}D) separation and the separation of fractions of ^{1}D effluent in the second dimension. This allows rapid, high-efficiency separations to be used in the first dimension, while still adequately sampling ^{1}D peaks. Transfer, transient storage, and subsequent second dimension (^{2}D) separations of multiple fractions of a particular ^{1}D peak produces a two-dimensional chromatogram that reveals the coordinates of the peak in both dimensions of the chromatographic space. Using existing valve technology we find that the approach is repeatable (%RSD of peak area <1.5%), even at very short first dimension sampling times – as low as 1 s. We have also systematically studied the critical influence of the volume and composition of fractions transferred from the first to the second dimension of the sLC × LC system with reversed-phase columns in both dimensions, and the second dimension operated isocratically. We find that dilution of the transferred fraction, so that it contains 10–20% less organic solvent than the ^{2}D eluent, generally mitigates the devastating effects of large transfer volumes on ^{2}D performance in this type of system. Several example applications of the sLC × LC approach are described in the second part of this two-part series. We anticipate that future advances in the valve technology used here will significantly widen the scope of possible applications of the sLC × LC approach.

1. Introduction

Advances in the applied sciences continue to challenge the state-of-the-art of separation science, demanding greater resolution of complex mixtures in less time and at lower cost. Analysis of compounds present at low concentrations in complex mixtures is especially challenging because the number of interfering compounds present at similar concentrations increases exponentially as the concentrations of target compounds decrease [1,2]. The analytical solution to these problems often involves the use of a separation method (e.g., gas or liquid chromatography, capillary electrophoresis) followed by a selective detection method (e.g., mass spectrometry or fluorescence). Recent work has placed significant emphasis on improvements in detector selectivity and sensitivity [3], as well as selective sample preparation procedures [4]. Increased chromatographic separation can reduce the need for advanced detectors or extensive sample preparation. However, in the case of conventional one-dimensional separations, increased resolution is achieved only through an increased analysis time [5]. In high performance liquid chromatography (HPLC), approaches to improve the speed of analysis include high temperature conditions, high pressure conditions, and the development of new stationary phase support technologies but they have real theoretical and practical limits [6]. In this work we describe an approach to efficiently increase the resolving power of conventional ^{1}D separations, without increasing analysis time, by selectively employing the principles of comprehensive two-dimensional chromatography at specific points during the analysis.

Multi-dimensional liquid chromatography (MDLC) has long been seen as a potential solution to increase resolution and improve the speed of analysis, particularly in the separation of complex mixtures. MDLC methods are typically divided into two main groups: comprehensive separations (denoted LC × LC for a

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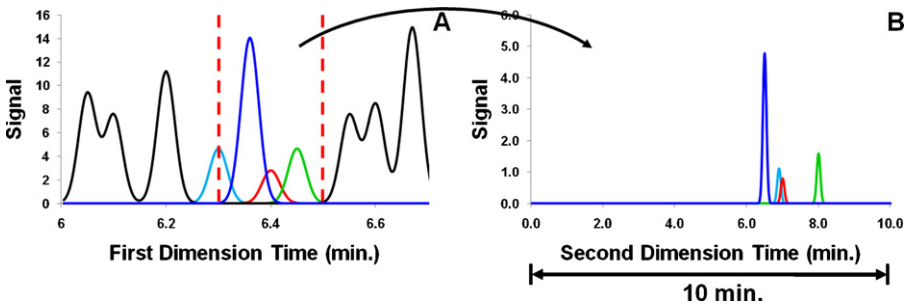

Fig. 1. Simulated heartcutting two-dimensional separation of a target analyte (red trace) from a complex sample matrix. Panel (A) shows the first dimension separation where the target analyte is clearly not well resolved. Panel (B) shows the simulated second dimension separation of the single fraction of ^{1}D effluent taken from 6.3 to 6.5 min (indicated by the dashed lines of panel A) where four constituents are transferred when the sampling window is wide enough to ensure that all of the red constituent is captured. The overlap of the teal and red peaks in panel (B) illustrates a fundamental problem with conventional heartcutting multidimensional separations – that when the target analyte is transferred in one large fraction much of the ^{1}D separation of that target from neighboring peaks is lost, placing increased burden on the ^{2}D separation. (For interpretation of the references to color in text, the reader is referred to the web version of the article.)

two-dimensional separation) concerned with the separation and quantitation of large numbers (ca. 10 s to 1000 s) of constituents of a sample [7], and targeted 'heartcutting' or 'coupled-column' methods (LC–LC for a two-dimensional separation) concerned with the analysis of a few (ca. 1–5) constituents of the sample matrix. In the past decade, research on the development of practically useful LC × LC has been particularly active [5,8–11].

In his foundational theoretical work on multi-dimensional separations, Giddings [12,13] clearly described what we now view as the two fundamental challenges that need to be overcome to realize the full potential of these separations. In this paper we describe a new methodology that addresses both of these problems. The first fundamental problem is that the separation mechanisms used in each dimension of a ^{2}D system must subject sample constituents to two separation steps involving mechanisms that are dependent upon different physico-chemical factors. In the current literature, this requirement is described as selecting 'orthogonal' separation modes. Although it is straightforward to conceive of combinations of separation modes that should satisfy this requirement, practical implementation of these combinations is often not so simple. Second, Giddings stated that separation gained in the first dimension of a multi-dimensional separation must not be lost in subsequent separation steps. The difficulties associated with each of these problems are described in turn in greater detail below and in Section 2.

The first problem, that of choosing orthogonal separation modes, becomes difficult because of issues related to solvent incompatibility [14]. The potentials of various mode combinations have been discussed extensively [8,12,15,16], and several combinations have been implemented in both LC × LC and LC–LC separations [14,17,18]. The most prominent problem associated with the coupling of different separation modes – solvent incompatibility – manifests either in a true physical sense (immiscibility), or in that a 'strong' solvent in one mode is a 'weak' solvent in the other mode. For example, if a NP separation involving hexane as the primary mobile phase is used in the first dimension, transfer of first dimension (^{1}D) effluent onto the second dimension (^{2}D) RP column can cause devastating peak broadening due to the solvent strength of hexane in a RP system (retention in hexane is much lower than in a typical RP eluent). We have strongly advocated for the use of LC × LC [11] and LC–LC [19] systems involving RP separations in both dimensions, minimizing – but not eliminating – the solvent compatibility issue. In this case, we inevitably face the situation where a sample constituent is highly retained on the ^{1}D column; it is transferred to the ^{2}D column in a sample that contains more organic solvent than the ^{2}D eluent required for reasonable retention, causing obvious losses in second dimension column performance. To minimize this problem, the more retentive stationary phase is generally used as the ^{2}D column, offering the possibility of on-column focusing at the head of the ^{2}D column [11,20]. This strategy eliminates solvent compatibility issues but limits the number of possible combinations of orthogonal columns.

The utility of on-column focusing in MDLC has a long history in both LC × LC and LC–LC [21,22]. More recently, on-column focusing has been described as an advantageous means to counteract the dilution of analytes through the ^{1}D column prior to injection of transferred analytes into the ^{2}D column [23,24]. Possible solutions have been proposed to solve the solvent incompatibility problem in LC × LC systems: reverse osmosis [5], partial vaporization [25], trapping [26,27], and on-column focusing at the inlet of the ^{2}D column by dilution of ^{1}D effluent prior to transfer to the ^{2}D column. This has been implemented occasionally in LC–LC [28]; surprisingly, however, it has rarely been done in LC × LC separations [26,29]. In our own work, we have successfully used this dilution approach in the coupling of three different RP columns for targeted analysis by heartcutting three-dimensional HPLC [19]. A significant advantage of this approach is the freedom allotted during method development to choose columns for each dimension independent of their general retentivity level.

Giddings' second requirement – that the separation gained in one dimension should not be compromised as a result of the implementation of subsequent dimensions – has been difficult to satisfy, both in heartcutting and in comprehensive multi-dimensional separations. In the heartcutting case, it is standard practice to transfer a single portion of ^{1}D effluent containing one or more target compounds of interest to a ^{2}D column. As is shown in detail in Fig. 1, this invariably remixes previously separated constituents during the transfer of the heartcut portion [30]. In the comprehensive case, the ability to satisfy this second requirement is inhibited by the slow speed of ^{2}D separations relative to the inherent width of ^{1}D peaks prior to the transfer process. The impact of the loss of ^{1}D resolution on the performance of comprehensive two-dimensional separations, now referred to as the under-sampling problem, was first discussed by Opiteck et al. [31], followed by a detailed analysis by Murphy et al. [32], and has been studied extensively by several other groups since then [33–36]. With the exception of fully comprehensive approaches involving multiple ^{2}D columns operated in parallel [37], the ^{1}D sampling time must equal the ^{2}D analysis time. In comparisons of 1D-GC and GC × GC [33] and 1D-LC and LC × LC [38], Blumberg et al. and Stoll et al. found that the slow speed of the second dimensions of these comprehensive two-dimensional separations was a major factor limiting their performance. This becomes especially evident when comparing to ^{1}D separations with short ($\ll$1 h) analysis times.

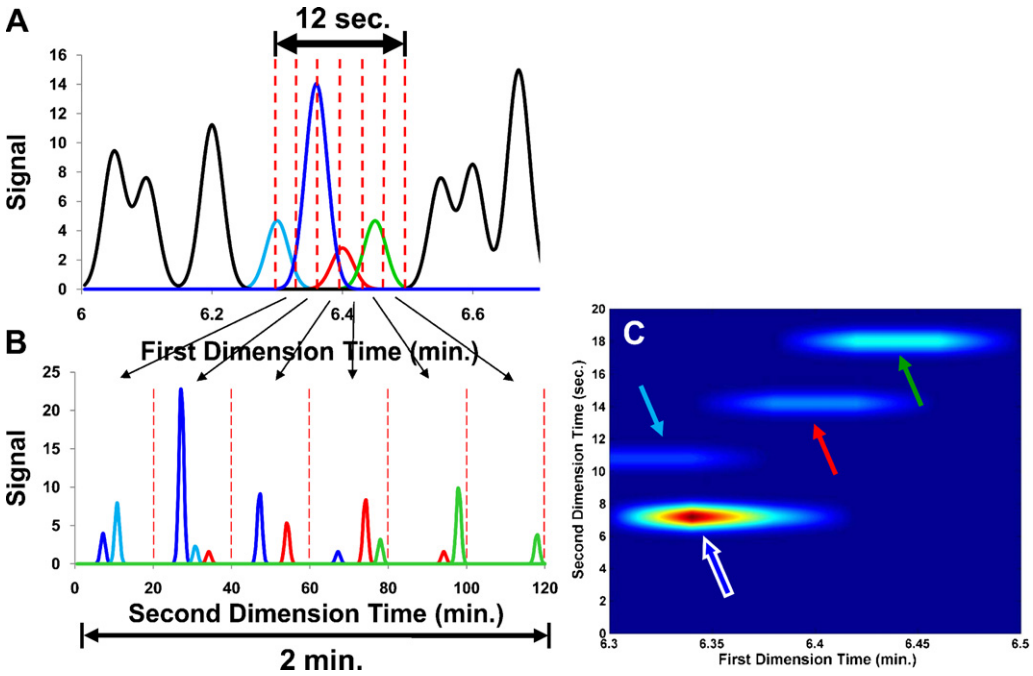

Fig. 2. Simulated sLC × LC separation of same complex matrix and target constituent as in Fig. 1. A series of six 2-s fractions of the first dimension effluent are captured and temporarily stored, followed by re-injection into the second dimension where each fraction is subjected to a 20-s separation, resulting in the series of six chromatograms separated by dashed lines of panel (B). When the chromatograms from panel (B) are reformatted into the contour plot in panel (C), we see that the target constituent indicated by the red arrow is well resolved from neighboring peaks in both the first and the second dimensions, because the resolution achieved in the ^{1}D separation has been maintained through adequate sampling of the first separation. (For interpretation of the references to color in text, the reader is referred to the web version of the article.)

In this paper, we describe an approach to multi-dimensional separations we refer to as 'selective comprehensive multi-dimensional liquid chromatography', and use the nomenclature sLC × LC. We believe the methodology provides effective means of addressing both of the practical challenges described above related to the under-sampling and solvent compatibility problems. The approach is selective in the sense that only selected regions of a ^{1}D separation are treated, or sampled in a comprehensive, multi-dimensional manner (see Fig. 2). Although the use of strategies involving multiple heartcuts has been described [19,39,40], we are not aware of any previous description of experimental work of the kind reported here. This approach has significant practical advantages over traditional heartcutting and fully comprehensive methods in a variety of applications which are described in Part II of this work. We view this approach as bridging the very large gap in experimental work between the extremes of online multi-dimensional chromatography represented by the heartcutting and fully comprehensive approaches. We are hesitant to refer to the approach as 'targeted' because, although it certainly is useful for highly targeted work, it is also very useful in analytical situations where the analysis of tens of compounds is required and much of the required resolution can be provided by a first dimension column alone. The key advantages of sLC × LC arise from the ability to break the long-standing link between the timescales of the first and second dimension separations, through novel implementation of existing valve technology. In some ways these advantages are similar to those derived from the offline approach to LC × LC, but without most of the major drawbacks of offline work [14]. In this paper we describe an instrument configuration that is useful for sLC × LC and characterize its performance. Because of the critical impact of the transfer of fractions of ^{1}D effluent to the ^{2}D column on the performance of the sLC × LC system, we also report the results of calculations and experiments that demonstrate the extraordinary effectiveness of simply diluting ^{1}D effluent prior to injection into the ^{2}D column.

2. Theory

In the following discussion a two-dimensional separation is assumed, however the concepts described here are not necessarily restricted to two dimensions of separation. One of the principle advantages of sLC × LC over conventional heartcutting multi-dimensional separations for targeted analysis is illustrated in Fig. 1. Panel A shows that when a heartcut is wide enough to ensure that the target constituent is quantitatively transferred to the second dimension (considering potential retention time shifts), resolution of the target constituent from neighboring ^{1}D peaks is lost. Along with neighboring ^{1}D peaks, the separation burden is also transferred to the second dimension shown in panel B. If the second dimension does not have good selectivity for the peaks that are adjacent in the first dimension (e.g., the red and teal peaks are not separated in the second dimension, whereas they were in the first), then the net result of the multi-dimensional separation is poor. This poor result is not because the selectivity of the separation is inadequate *per se*, but because of the way in which the separation is executed.

The solution to this problem afforded by the sLC × LC approach is illustrated in Fig. 2, where the same ^{1}D separation shown in Fig. 1 is assumed. In this case the ^{1}D effluent containing the target constituent is transferred to the subsequent dimension in several small fractions, and a complete ^{2}D separation of each of these fractions is executed in the second dimension as shown in panel B. As will be discussed below, our approach to this process allows transient storage of these fractions such that *the sampling time in the first dimension need not be the same as the analysis time in the second dimension*. In this example, samples of the ^{1}D effluent are captured every 2 s, but each ^{2}D separation is allotted 20 s. Herein lies the important distinction of this work – the sLC × LC approach breaks the long-standing link between the timescales of the ^{1}D and ^{2}D separations of conventional online LC × LC. When the six ^{2}D chromatograms from panel B are reformatted into the contour plot shown in panel C, the target constituent is clearly well resolved

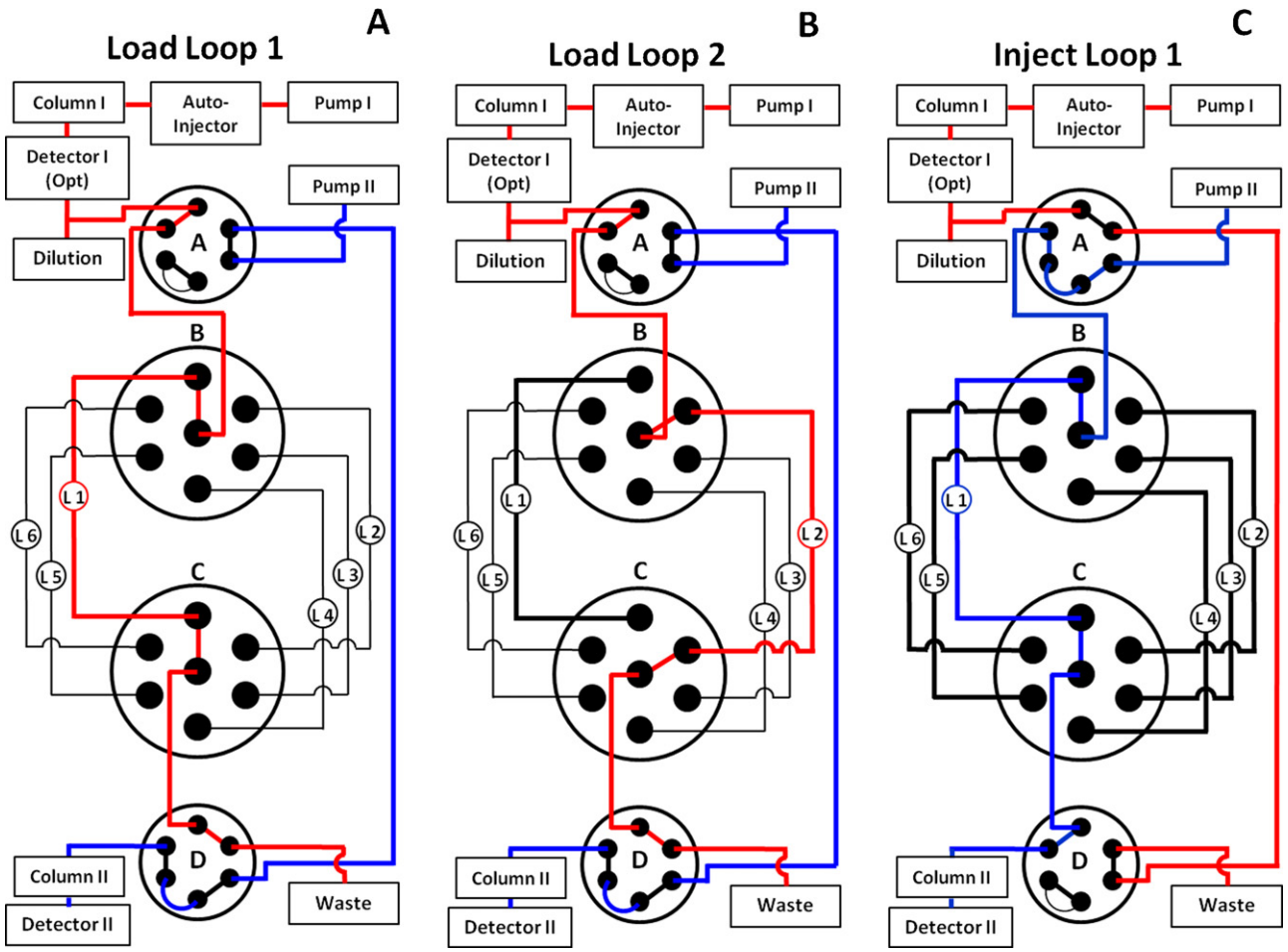

Fig. 3. Schematic of instrument configuration for sLC × LC. Pump I delivers eluent to Column I while Pump II pushes captured fractions of ^{1}D effluent out of sample loops L1 through L6 and delivers eluent to the ^{2}D column. Detector I is optional in the sense that it can be removed to reduce extra-column broadening after ^{1}D elution times of target compounds have been determined. Dilution of ^{1}D effluent was achieved by an external, low-pressure pump T-ed into the ^{1}D flow path to achieve effective on-column focusing in the second dimension. A series of four valves labeled A–D were used to capture and store six fractions of ^{1}D effluent. Example valve configurations required for fraction capture are shown in panels (A and B). Loop L1 is loaded by the ^{1}D flow marked in red in panel (A), then valves (B and C) rotate to load L2 shown in panel (B) while the flow from Pump II shown in blue remains unchanged. Panel (C) shows an example of a flow path required to re-inject captured fractions into the ^{2}D column. Subsequent injection of all six fractions is achieved by simultaneous rotation of valves B and C. (For interpretation of the references to color in text, the reader is referred to the web version of the article.)

from all of the neighboring peaks. Here, the good resolution is due in part to the selectivity of the second dimension, but more importantly, it is due to the integrity of the sampling process which preserves the resolution achieved in the first dimension.

Fig. 2 demonstrates in qualitative terms (although physically realistic retention times and peak widths were used) that the sLC × LC approach effectively maintains the resolution achieved in the first dimension of a MDLC system, in the context of targeted analyses. The loss of first dimension resolution due to slow sampling of the target ^{1}D peak has also been studied quantitatively by several groups, beginning with Murphy et al. [32], continuing with the work of Seeley [36] and Horie et al. [35], and most recently by Davis et al. [34] and Blumberg [33]. The effective first dimension resolution ($^1R'_S$) of a target constituent from a neighboring peak contributing to the total 2D resolution is a function of the relationship between the sampling time, t_S, and a measure of the ^{1}D peak width prior to the sampling process [34] ($^1\sigma$):

$$^1R'_S = \frac{^1R_S}{\sqrt{1 + 0.21(t_S/^1\sigma)^2}} \quad (1)$$

The rule-of-thumb that a ^{1}D peak should be sampled three to four times across its 8σ width to avoid significant loss of ^{1}D resolution [32] follows from this type of equation. In the experimental work described here we have generally adhered to a sampling rate of about four samples per 8σ width, both because of the limitations of current valve hardware (the number of loops) and because four samples per peak gives a good estimate of the target peak location within the sampling window in the ^{1}D time axis. Although some recent optimization studies in online LC × LC have shown that lower sampling rates are optimal (2–3 samples per 8$^1\sigma$) [24,35,37], we assert that this is unique to online LC × LC and does not apply here because the timescales of the sampling process and ^{2}D separation are not linked in sLC × LC as they are in LC × LC.

Fig. 3 shows a series of schematic flow diagrams that describe the application of existing valve technologies to achieve sLC × LC separations. This setup allows the capture and transient storage of up to six fractions of one or more ^{1}D peaks, followed by serial injection of those fractions into a ^{2}D column for further separation. Very narrow ^{1}D peaks (ca. 5-s 4σ width) can be sampled adequately, thus maintaining highly efficient ^{1}D separations while preserving adequate separation in the second dimension at longer analysis times (ca. 10–30 s). A specific example of the timing of the entire sLC × LC process is shown below in Table 1.

The scheme for sLC × LC described here is not void of the practical difficulties faced in LC × LC separations. Foremost among these

Table 1
Example of the sequence of timed events for the operation of the sLC × LC system (see Section 3.3.2 for other conditions); ^{1}D sampling time was 1.0 s and each ^{2}D separation was 15 s.

Time from ^{1}D start (s)	Time from ^{2}D start (s)	Process	Valves A/D[a] position	Valves B/C[a] position
0		^{1}D inject load loops		L1/L1
163 (2.69 min)				L1/L1
164			^{1}D effluent through loops/to waste (Fig. 3C, panel A)	L2/L2
165				L3/L3
166				L4/L4
167				L5/L5
168		Loading finished		L6/L6
169	0	Pump II start	Switch positions	
184	15	Inject from loops		L1/L1
199	30			L2/L2
214	45		Pump II eluent through loops/to Column II (Fig. 3C, panel C)	L3/L3
229	60			L4/L4
244	75			L5/L5
259	90			L6/L6
275	120	^{2}D stop time		L6/L6
300 (5 min)	155	^{1}D stop time		

[a] See Fig. 3.

are the challenges of interfacing the two dimensions of separation, particularly the large injection volumes normally associated with the fraction transfer process [24]. An example of typical conditions in a LC × LC experiment makes this problem clear. Suppose that we wish to use a 2.1 mm I.D. column in our ^{1}D separation. It follows from this initial choice that a reasonable (though not absolutely required) choice of ^{2}D column diameters would be 2.1 mm to avoid significant dilution of the analyte band prior to detection, and thus poorer detection limits [23,24,41]. We would also like to use a flow rate through the first column that is near the optimum velocity (in a van Deemter sense) to maintain good separation efficiency and peak capacity in the first dimension; for a 2.1 mm I.D. column this optimal flow rate is on the order of 0.5 mL/min (8.3 μL/s). A column of reasonable efficiency operated at this flow rate under gradient elution conditions will produce peaks for low molecular weight compounds with widths on the order of 5 s (8σ width). If a first dimension peak is quantitatively transferred to the second dimension as a single heartcut fraction, the injection volume into the second dimension is on the order of 50 μL. As will be discussed below, this situation is ameliorated somewhat if we sample the peak multiple times and transfer several smaller fractions to the second dimension (e.g., 4 fractions of 12.5 μL each). However, this scenario is particularly problematic when the composition of the effluent containing the compound of interest in the first dimension is 'strong' (i.e., high fraction of organic solvent in a RP system) relative to the eluent into which the fraction is injected in the second dimension [42]. Given that the 50 μL fraction described is on the same order as the entire dead volume of short 2.1 mm I.D. column needed for fast ^{2}D separations, this kind of situation can lead to devastating peak broadening (see Fig. 5) and complete loss of useful separation efficiency in the second dimension of an LC × LC system.

The concept of mitigating the kind of 'injection broadening' described above by taking advantage of on-column focusing effects has a long history [23,24,43–45]. This has been shown to be particularly useful in the case of gradient elution separations where analytes injected in a large volume of weak solvent are focused at the column inlet due to their high initial retention in the gradient elution scheme [42,46]. One way of achieving these conditions is to dilute the sample with weak solvent prior to injection, either offline or online through a dilution line [28]. In our own work [19], we have found this online dilution approach to be so effective that we could use reversed-phase columns in all three dimensions of a three-dimensional HPLC system for targeted analysis, and still maintain excellent separation performance (i.e., avoid significant injection broadening) in the third dimension. While the utility of this dilution approach in LC × LC systems has been alluded to frequently, we are not aware of any systematic experimental studies of the potential benefit when isocratic elution is the intended mode in the terminal dimension of a multi-dimensional system.

Recently the potential benefit of on-column focusing in online LC × LC has been discussed by three different groups [23,24,42]. Their theoretical framework is useful both for assessing the potential benefit of online dilution of the ^{1}D effluent in LC × LC, and predicting the dilution factors that are required for success with this approach. The salient features of the theory are repeated here for convenience. We begin with the premise that we would like to know how much the effluent of a ^{1}D separation must be diluted prior to injection into the ^{2}D column (under isocratic conditions) to avoid seriously compromising the performance of the ^{2}D column. We assert that while the following example calculation is somewhat specific to our system, it is also very firmly rooted in reality. That is, the assumptions we make about typical transfer volumes are based on typical flow rates, column efficiencies, and peak widths encountered in experimental separations conducted in our laboratory. We begin with the goal of maintaining 90% of the native efficiency of the ^{2}D column (10% loss due to injection broadening). For the purpose of this calculation we ignore post-separation broadening processes (e.g., tubing and detector contributions) such that the effective peak variance in the second dimension, $^2\sigma^2_{eff}$ is due only to broadening inside the column, $^2\sigma^2_{col}$, and injection broadening, $^2\sigma^2_{inj}$:

$$^2\sigma^2_{eff} = {}^2\sigma^2_{inj} + {}^2\sigma^2_{col} \quad (2)$$

The effective isocratic plate count, $^2N_{eff}$, of the ^{2}D column is a function of the effective variance (in time units) and the retention time of the peak, ^2t_r:

$$^2N_{eff} = \left(\frac{^2t_r}{^2\sigma_{tot}}\right)^2 \quad (3)$$

In their work, Horvath et al. [23] assumed a linear relationship between the natural logarithm of the analyte retention factor and the volume fraction of organic solvent in the mobile phase (ϕ). While this is convenient, in our experience the significant curvature in the dependence of $\ln k'$ on ϕ can lead to overly optimistic errors in $2\sigma^2_{inj}$ when using their form of Eq. (5). Therefore we use

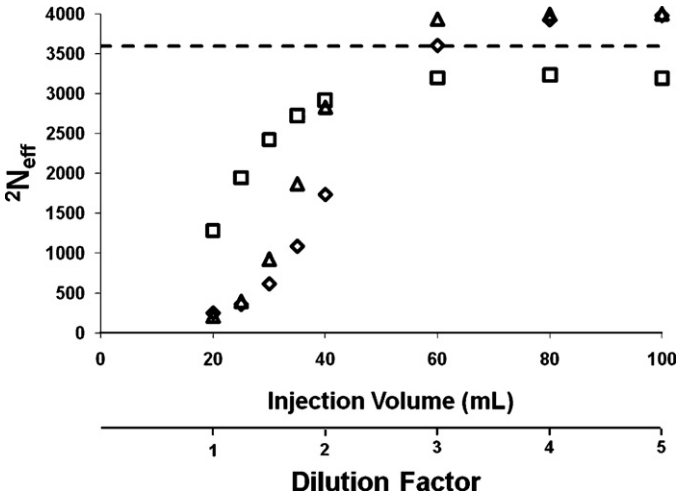

Fig. 4. Calculated effect of the dilution of ^{1}D effluent on the performance of ^{2}D separations in sLC × LC. Effective plate counts ($^2N_{eff}$) that account for band broadening due to injection and intra-column processes were calculated using Eqs. (2)–(5) for serotonin (□), phenytoin (◊), and triclosan (△), assuming a native column efficiency of 4000 plates. Other parameters are as follows: flow rate, 1.5 mL/min; 30 mm × 2.1 mm I.D. Ascentis Express C18 (2.7 μm). The volume of undiluted ^{1}D effluent was assumed to be 20 μL in all cases. Values of $^1\phi$ were 0.10, 0.45, and 0.90. Experimentally determined fitting parameters (k_w, a, and B) from Eq. (4) were 152, 1.03, and 42.9 for serotonin, 90,600, 4.45, and 92.4 for phenytoin, and 271,000, 1.72, and 41.9 for triclosan.

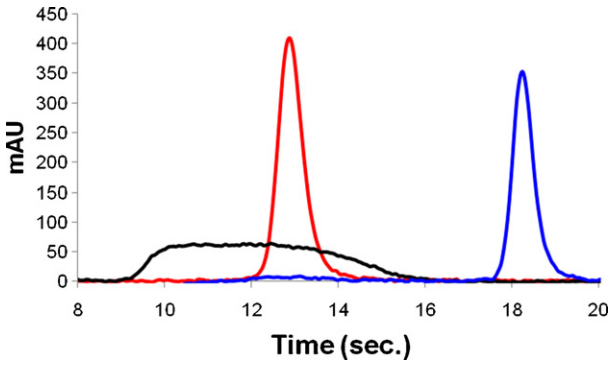

Fig. 5. Demonstration of the potential benefit of on-column focusing achieved by dilution of ^{1}D effluent prior to ^{2}D separation. All chromatograms were obtained using conventional ^{1}D instrumentation and a single 30 mm × 2.1 mm I.D. Ascentis Express C18 column with mobile phase of 35/65 acetonitrile/10 mM H_3PO_4 in water at 1.0 mL/min. and ambient temperature. The red trace shows 'typical' performance of the column under these conditions; a 1 μL injection of a 20 μg/mL solution of phenytoin in eluent was made. The black trace shows the devastating effect a 20 μL injection containing 45% acetonitrile (1.0 μg/mL phenytoin); this would be typical in a sLC × LC experiment without dilution of ^{1}D effluent. The blue trace shows the result of the injection of 75 μL of the same effluent fraction, but diluted three-fold such that the organic content is just 15% acetonitrile (0.30 μg/mL phenytoin). All of the performance lost as a result of injecting a large volume of organic-rich sample is re-gained by simply diluting the sample, despite that the sample volume is actually larger than the dead volume of the column itself. (For interpretation of the references to color in text, the reader is referred to the web version of the article.)

the dependence of $\ln k'$ on ϕ described recently by Neue and Kuss [47].

$$\ln k' = \ln k'_w + 2\ln(1 + a\phi) - \frac{B\phi}{1 + a\phi} \quad (4)$$

where k'_w is the retention factor of the analyte in pure weak solvent (pure aqueous phase in a RP separation), and a and B are fitting coefficients.

The injection broadening is then given by Eq. (5), where $^2k'$ is the retention factor of the analyte in the ^{2}D eluent, and k'_s is the retention factor of the analyte in the fraction of ^{1}D effluent that is transferred to the ^{2}D column, both calculated using Eq. (4). We refer readers to previous publications [20,23,24,44] for details of the derivation of this expression (Eq. (5)). Note that as the degree of dilution of a fixed fraction volume of ^{1}D effluent is changed, both $^2t_{inj}$ and k'_s change as well.

$$^2\sigma^2_{t,inj} = \frac{^2t^2_{inj}}{12}\left(\frac{1 + {}^2k'}{1 + k'_S}\right)^2 \quad (5)$$

We then choose an initial column efficiency of 4000 plates which corresponds to the typical efficiency of the 30 mm × 2.1 mm I.D. Ascentis Express C18 column used under the conditions of our work (ca. 1.5 mL/min and 40 °C), and calculate the effective column efficiency for three compounds with quite different retention characteristics (serotonin, phenytoin, and triclosan) as a function of the extent to which the fraction of ^{1}D effluent is diluted prior to injection into the ^{2}D column; Fig. 4 shows the results for all three compounds. In each case we assume that the volume of the ^{1}D effluent fraction prior to dilution is 20 μL. We assume (based on experience) that the $^1\phi$ values prior to dilution are 10, 45, and 90% acetonitrile for serotonin, phenytoin, and triclosan, respectively. The values of k_w, a, and B for each compound were determined experimentally and are reported in the caption of Fig. 4. The dashed horizontal line in Fig. 4 represents 90% of the native column efficiency. In the case of triclosan, the results show that the performance of the ^{2}D column can benefit tremendously from rather minor dilution of the ^{1}D effluent with weak solvent during the transfer process. For example, the effective column efficiency increases from 200 to 3900 plates by simply diluting the effluent by a factor of three, in spite of the fact that the injection volume triples from 20 to 60 μL. A similar trend is observed for phenytoin, except that slightly more dilution is needed to obtain the same degree of benefit. This is consistent with the long-held understanding that the more retained a compound is in weak solvent, the greater the potential impact of the analyte-focusing effect [48]. Nevertheless, the practical consequences of diluting the ^{1}D effluent during the transfer process are striking. Even in the case of phenytoin, which is only moderately retained under RP conditions ($\Delta^2k'/\Delta\phi$ is 26 at $^2k'$ of 2), we only need to dilute the ^{1}D effluent by a factor of 3 or 4 to reach 90% of the native column efficiency. The curve for serotonin illustrates that the outcome of this calculation is very highly dependent on the setup of the sLC × LC conditions. Despite the very low k_w value of just 152, the use of similar ^{1}D and ^{2}D eluent compositions and the very strong curvature of the dependence of $\ln k'$ on ϕ lead to quite reasonable values of N_{eff}, though the 90% target is difficult to reach. It is interesting that Eq. (5) is independent of the ^{2}D retention factor (i.e., the curve for a given compound does not change as $^2k'$ is changed by varying $^2\phi$). As $^2k'$ increases, $^2\sigma_{col}$ increases such that the relative contribution to $^2\sigma_{tot}$ by $^2\sigma_{inj}$ decreases, but at the same time $^2\phi$ becomes closer to ϕ_S as $^2k'$ is increased, thus diminishing the focusing effect; these two effects exactly oppose each other in these calculations. To some the beneficial effects of sample dilution may seem obvious based on their previous experience, yet the notion that injecting significantly more sample (resulting from diluting the fraction during transfer) can lead to significantly better results is indeed counterintuitive. Perhaps this is why such a simple approach to solving the interfacing problem in LC × LC has not been used, with one exception of which we are aware [29]. In addition to using this dilution approach very successfully in all of our sLC × LC work – all ^{1}D effluent is diluted continuously online prior to capture, storage, and re-injection of selected fractions from the first dimension (see 'Dilution' in Fig. 3) – we have also verified the results of these calculations, at least qualitatively, for phenytoin and triclosan. To keep the length of this paper reasonable we only report the experimental data for phenytoin here (see Section 4 and Figs. 5 and 6). We note here that none of the equations discussed

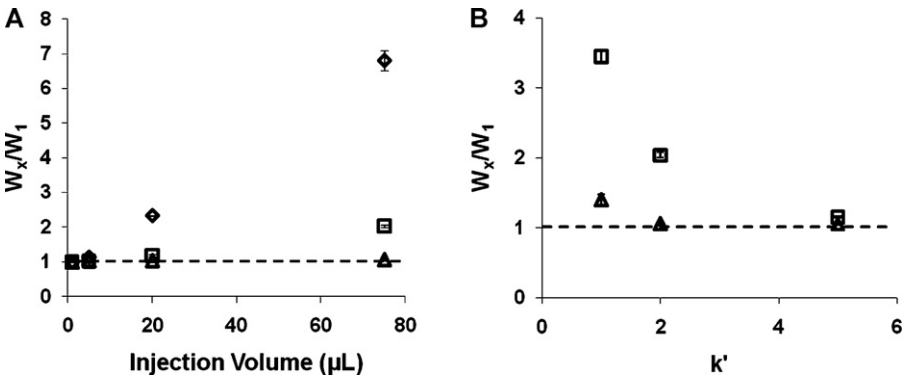

Fig. 6. Results of a systematic study of the on-column focusing effect under conditions typical of those used in the second dimension of a sLC × LC system. Panel (A) shows the experimentally determined peak width of phenytoin at a given injection volume (w_x) relative to the width measured for a 1 μL injection, where the sample solvent contained 0 (◊), 10 (□), and 20% (△) less acetonitrile than the eluent. Panel (B) shows the quantitative dependence of the benefit derived from reducing the organic content of the sample on the analyte retention factor; the effect of dilution decreases as the analyte retention factor increases. *X*-axis *k*′ values are approximate, corresponding to mobile phase compositions of 35, 25, and 17.5% acetonitrile, respectively. Under the reference condition (1 μL injection) plate counts of about 3000 were obtained for phenytoin (plate counts of about 5000 were obtained for toluene under the same conditions). Error bars represent one standard deviation of the measured peak widths.

here predict the effect of large injection volumes on retention time, which can be substantial as shown in Fig. 5. This portion of the work is ongoing and a more extensive analysis of the results will be reported elsewhere.

3. Materials and methods

3.1. Reagents

Standard solutions of target analytes were prepared by first dissolving the analyte in acetonitrile, then diluting to the desired concentration and solvent composition with deionized (DI) water. DI water was from an in-house Millipore water purification system (Billerica, MA), and was used without further treatment. Phenytoin was from Sigma–Aldrich (St. Louis, MO); phosphoric acid was from Fisher Scientific (HPLC grade, Fair Lawn, NJ), and formic acid was reagent grade or better, from Sigma–Aldrich. All mobile phases were degassed prior to use either by vacuum degassing or sparging with helium.

3.2. Injection volume studies

3.2.1. Instrumentation

Studies of the effect of injection volume and sample composition on the ^{2}D column performance were conducted using a dedicated free-standing injection valve such that the injector could be placed very close to the column and detector to minimize connecting tubing lengths. A HP1050 quaternary pump from Agilent Technologies (Santa Clara, CA) was used with a six-port two-position valve from Rheodyne (Model 7010, Rohnert Park, CA) equipped with injection loops of 1, 5, 20, or 75 μL made of 240 μm I.D. PEEK tubing, corresponding to the injection volumes used in this experiment. The injector valve was actuated pneumatically with air at 70 psi. An Agilent G1315 photodiode array UV detector was used with a 500 nL flow cell, again to minimize extra-column peak broadening. The extra-column variance was measured by replacing the column with a zero dead volume union and found to be $(2\ \mu L)^2$ at a flow rate of 1.0 mL/min. A 30 mm × 2.1 mm I.D. (2.7 μm) Ascentis Express C18 column (Supelco, Bellefonte, PA) was connected directly to the detector using a 10 cm length of 63 μm I.D. PEEK tubing. The column was used at ambient temperature and selected for this study because of the high efficiency offered by core–shell particles at moderate pressures. A simple LabView (National Instruments, Austin, TX, rev. 8.5) program written in-house, and a USB PC interface (USB-6009) were used to control the synchronization of the injector and detector data acquisition.

3.2.2. Chromatographic conditions

Standard solutions of phenytoin were made and diluted to specific analyte concentrations which contained specified levels of organic solvent. Samples of three different compositions were injected into mobile phases containing 45, 35, and 27.5% acetonitrile (10 mM phosphoric acid as the aqueous component), corresponding to phenytoin retention factors of 1, 2, and 5. The sample compositions were chosen such that the injected sample contained 0, 10, or 20% less acetonitrile than the mobile phase into which it was injected. This resulted in a series of nine different mobile phase/sample composition combinations. For each of these combinations, injection volumes of 1, 5, 20, and 75 μL were made. To achieve consistent mass loading of analyte across this wide range of volumes, analyte concentrations were adjusted accordingly, with a target of 20 ng injected on column. All separations were carried out at a flow rate of 1.25 mL/min. Analytes were detected by UV absorption spectroscopy.

3.3. sLC × LC instrumentation and chromatographic conditions

3.3.1. sLC × LC instrumentation

A complete schematic showing the instrument configuration used in sLC × LC separations is shown in Fig. 3. First dimension separations were carried out on a system, comprising Pump I, Auto-Injector, and Column I of Fig. 3. This system was composed of an HP1050 quaternary pump and modified HP1050 auto-injector, equipped with a 900 μL syringe plunger and 400 μL sample loop. Each first dimension separation utilized a thermostated column compartment (G1316, Agilent) to preheat the first dimension eluent and column and was equipped with a G1312 variable wavelength UV absorbance detector (Agilent). This detector is described in the schematic as 'Optional'; once the location of the first dimension peak is determined it can be removed from the flow path to decrease extra-column peak broadening during fraction transfer between dimensions. Gradient elution was used in the first dimension of all sLC × LC separations, followed by isocratic ^{2}D separations. This allowed for fast ^{2}D separations because the need for ^{2}D column re-equilibration was eliminated.

At the outlet of the ^{1}D column, or after the ^{1}D detector, effluent was diluted with water to reduce the percentage of organic solvent in the fractions transferred to the second dimension. This dilution stream was delivered by a Varian 212LC single channel

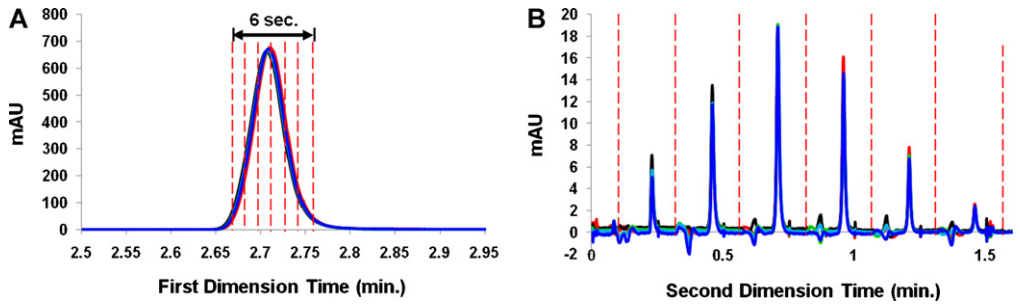

Fig. 7. Five replicate chromatograms observed at the outlet of the ^{1}D (A) and ^{2}D (B) detectors of a sLC × LC separation of phenytoin in DI water. One-second fractions of the ^{1}D peak (A) were transferred to the second dimension where 15-s isocratic separations of each fraction were performed. The repeatability of the sLC × LC system is evident by the consistent size and retention time of second dimension peaks. The small differences in the size of the peaks in adjacent ^{2}D separations in panel (B) were due to the slight drift in retention time of the ^{1}D peak. For detailed chromatographic conditions, see Section 3.3.2.

pump (Agilent). Following dilution, flow was directed to a series of four sampling valves at the heart of the sLC × LC system (labeled A, B, C, D of Fig. 3). Valves A and D were a pair of six-port, two-position valves (Rheodyne Model 7010, Rohnert Park, CA) and valves B and C were six-position flow path selection valves from Cadence Fluidics (Model UBX-1701-0607-0001, Petaluma, CA). Both the six-port valves (A and D) and six-position valves (B and C) were operated as related, but independent pairs. Valves A and D controlled the loading/injecting of the sample loops L1–L6, whereas valves B and C controlled which loop was being filled/injected within the loading/injection cycle. In all cases, loops L1–L6 were 75 μL, constructed from 240 μm I.D. PEEK tubing. This valve configuration allowed a series of six fractions, from ca. 1 to 5 s in length, to be captured from the ^{1}D target analyte peak(s) and subsequently re-injected into the ^{2}D column for further separation. The detailed flow paths in panels A and B of Fig. 3 show how effluent from the ^{1}D column (red path) first filled loop L1, followed by rotation of valves B and C to load L2 while maintaining the positions of valves A and D. Rotation of B and C continued until all six loops were loaded. In the next step, all four valves rotate to achieve the flow path depicted in panel 3 and begin injections of the stored fractions into the ^{2}D column. In this configuration L1 is injected into Column II at a high flow rate, typically 1–2 mL/min, using isocratic eluent delivered by a second HP1050 quaternary pump (Pump II) shown by the flow path outlined in blue. Valves B and C were rotated to select for all six loops at specified intervals corresponding to the ^{2}D analysis time for each fraction. Finally, all four valves were returned to their original positions shown in panel A. The coordination of the timing of the different modules and fraction transfer between dimensions was controlled by simple LabView code written in-house and controlled as above. The details of the sequence of timed events for the sLC × LC separation of phenytoin in DI water shown in Fig. 7 are given in Table 1. The ^{2}D detector used in this study was a G1315 (Agilent) photodiode array UV absorbance detector. Individual instrument modules were controlled by Chemstation Software (Agilent, A.08.03).

3.3.2. Repeatability of sLC × LC at short sampling times

To test the repeatability of the sLC × LC system at short sampling times, sets of five replicate separations of a sample of phenytoin in DI water were carried out with ^{1}D sampling times of 1 and 3 s. A 50 mm × 4.6 mm I.D. Carbon on Silica (COS) column (17% carbon, w/w) packed in-house was used for each ^{1}D separation. This column was based on a novel carbon-modified silica-based stationary phase that exhibits selectivity characteristics similar to other commercially available carbon-based phases including porous graphitic carbon and carbon-clad zirconia, including enhanced retention of polarizable compounds and geometric isomer selectivity [20,49]. Preparation and characterization of this material will be described elsewhere. Previously we discussed and demonstrated the utility of these carbon-based phases in multidimensional separations [11,19,50]. The COS material (15% carbon, w/w) was obtained from United Science (Minneapolis, MN). The flow rate was 1.25 mL/min and a 3.3-min linear gradient from 10 to 100% B solvent (acetonitrile) was used with a hold at 100% B from 3.3 min until 5 min; the A solvent was 0.1% formic acid. The column was heated to 40 °C, the injection volume was 45 μL, and detection was achieved by UV absorption spectroscopy. Six 1-s ^{1}D effluent fractions containing the phenytoin peak (2.7–2.8 min) or six 3-s fractions (2.6–2.9 min) were diluted with DI water at 1.25 mL/min prior to storage and subsequent transfer to the ^{2}D column at 15-s intervals starting at 2.8 and 2.9 min from the start of the first ^{1}D dimension separation for the 1-s and 3-s sampling times, respectively.

Second dimension separations were performed using an Ascentis Express C18 column (30 mm × 2.1 mm I.D., 2.7 μm; Supelco, Bellefonte, PA). The ^{2}D eluent consisted of 35/65 acetonitrile/10 mM H_3PO_4 at a flow rate of 1.5 mL/min. The column was heated to 40 °C, and peaks were detected by UV absorption spectroscopy.

4. Results and discussion

4.1. Impact of fraction volume and composition on ^{2}D performance

Before discussing the results of our systematic study of the impact of the volume and composition of the fraction transferred from the first to the second dimension of a LC × LC system, it is useful to consider the results of the following scenario. Suppose that we perform a sLC × LC separation focused on the analysis of phenytoin which elutes from our ^{1}D column during a gradient elution separation when the composition of the effluent exiting the column is 45% acetonitrile. Further suppose that the corresponding fraction volume is 25 μL, and that this fraction is injected into a 30 mm × 2.1 mm I.D. ^{2}D column, which is suitable for rapid ^{2}D separations. At room temperature the ^{2}D eluent composition needed to obtain a retention factor of about 3 is 35% acetonitrile. The devastating impact of such a scenario on column performance (simulated using a simple 1D-LC experiment) is shown in the black trace in Fig. 5, where we see that the peak becomes very wide and short. For reference the red trace shows the column performance under more ideal circumstances, where the same conditions were used except the injection volume was 1 μL and the sample composition was 35% acetonitrile. The phenytoin concentration was varied to obtain an injection of 20 ng analyte independent of the injection volume. The combination of the large injection volume (relative to the ~50 μL column volume) and the high concentration of acetonitrile in the sample relative to the eluent severely distorts the peak. The benefit of diluting the sample with water to lower the fraction of organic solvent relative to the eluent composition is shown by the blue trace. Here we see that despite the fact that the

sample volume has been increased three-fold to 75 μL, the peak shape improves dramatically because of the low acetonitrile content of the sample (15%) relative to the eluent. The peak shape is very similar to that obtained under favorable conditions of a 1 μL injection volume. This result very clearly shows the counter-intuitive, yet very powerful, principle in sLC × LC that diluting the ^{1}D effluent and injecting a larger sample into the ^{2}D column can actually be very advantageous, so long as the diluent is the weak component of the ^{2}D eluent.

The plots in Fig. 6 show the results of a more systematic study, intended to yield an experimental rule-of-thumb for the degree to which the ^{1}D effluent should be diluted in a sLC × LC experiment to maintain good performance of the ^{2}D column in spite of large transfer volumes and the elution of target compounds from ^{1}D columns in organic-rich effluent. Panel A of Fig. 6 shows the experimentally measured peak width at a given injection volume (w_x) relative to the peak width measured in the case of a 1 μL injection (w_1), for situations where the sample solvent contained 0, 10, or 20% less acetonitrile than the eluent, and the eluent was adjusted to obtain a retention factor of 2 for phenytoin. We see that when the sample composition is matched to that of the eluent, severe peak broadening is observed, especially when injection volumes typical of those used in our sLC × LC work (20–75 μL) are used. On the other hand, reducing the organic content of the sample just 10% relative to the eluent composition has a significant positive effect, and if the sample contains 20% less acetonitrile, no measurable effect on the peak width is observed, even at injection volumes up to 75 μL. Panel B shows the apparent quantitative dependence of the benefit of reducing the organic content of the sample on the analyte retention factor. In this case, the eluent was adjusted to obtain retention factors for phenytoin of 1, 2, or 5. The injection volume is constant at 75 μL, and the relative peak width is plotted for situations where the sample composition contains 10 or 20% less acetonitrile than the eluent at each retention factor. Here we see that a 20% difference between sample and eluent compositions is particularly beneficial in situations where the analyte retention factor is less than 2, but that beyond a retention factor of about 4 even a 10% difference will produce peaks that are very close to the best possible performance. Based on these experimental results, we use the rule-of-thumb that we need to adjust the ^{2}D eluent to give analyte retention factors in the range of two to four, and then dilute the ^{1}D effluent to such that the sample injected into the ^{2}D column contains at least 10% less acetonitrile than the ^{2}D eluent. Obviously this rule-of-thumb is somewhat compound-specific and must be adjusted depending on the application, particularly when working with highly hydro- or lipophilic compounds. In our view, these experimental results are entirely consistent with the results of the calculations presented in Section 2. Quantitative comparison of the experimental and calculation results is difficult because retention times change in addition to peak widths, as shown in Fig. 5. The dependence of retention time on injection volume and the sample/eluent composition mismatch is important and will be discussed elsewhere; it is not incorporated into the model used above and is beyond the scope of this paper.

4.2. Repeatability of sLC × LC at short sampling times

As part of our assessment of the capabilities of the instrument setup for sLC × LC described, we evaluated the repeatability of the sLC × LC approach, with a focus on the repeatability of peak area at short ^{1}D sampling times. State-of-the-art column technologies make it possible to achieve sub-five second peak widths (8σ) in RP separations under gradient elution conditions. If we are to take full advantage of the sLC × LC approach, it will ultimately be important to sample ^{1}D peaks with very short intervals, on the order of 1 s or less. Given the complexity of the fluid path at the heart of the sLC × LC approach, one might reasonably question the overall repeatability of the sLC × LC separation process. To evaluate this we performed replicate separations of a standard solution of phenytoin in DI water, at sampling times of 1 and 3 s. Fig. 7 shows overlays of the ^{1}D (panel A) and ^{2}D (panel B) chromatograms for five replicate injections of the phenytoin standard, where the sampling time was set to 1 s. There is excellent alignment of both the ^{1}D and ^{2}D peak profiles, indicating excellent repeatability of the sLC × LC process, in spite of the very short sampling time. There are measurable shifts in ^{1}D retention time, which translate into small shifts in the envelope of consecutive ^{2}D peaks. The use of 1-s sampling intervals would not result in the quantitative transfer of the target analyte in six fractions in this case because the ^{1}D peak is too wide. We realize that this situation is not ideal, but we anticipate situations where much narrower ^{1}D peaks will be obtained, and wanted to test the capabilities of the setup shown in Fig. 3. The most important observation here is that the total peak area is very consistent. The relative precision of ^{1}D peak area was 1.1%, whereas the relative precision of the total ^{2}D peak area was 0.6% for both 1-s and 3-s sampling intervals. The precision of total ^{2}D area is not significantly different from that of the ^{1}D peak area, and both values are within the manufacturer specification of injection volume precision for the HP1050 autosampler used in the experiment. This excellent repeatability will allow us to preserve the narrow peak widths of ^{1}D peaks in sLC × LC separations involving very efficient ^{1}D separations, in contrast to existing heartcutting and LC × LC methodologies.

5. Conclusions

In this work we have described an approach to enhancing the resolution of select portions of conventional 1D-LC separations, which we refer to as selective comprehensive two-dimensional HPLC (sLC × LC). In our view, the primary advantages of this approach over conventional heartcutting and fully comprehensive approaches (LC × LC) are as follows.

(1) The sLC × LC approach breaks the long-standing link between the timescales of the sampling of the ^{1}D separation and the subsequent separation of fractions of ^{1}D effluent in the second dimension. This allows rapid, high-efficiency separations to be used in the first dimension of sLC × LC separations, while still adequately sampling first dimension peaks (to avoid undersampling-induced broadening) and capitalizing on the resolving power of moderately efficient ^{2}D separations.
(2) The added time dimension of sLC × LC datasets that comes about by sampling ^{1}D peaks multiple times across their widths will enable the use of sophisticated chemometric algorithms to mathematically resolve chromatographically unresolved peaks. Historically, the application of these methods to data from conventional heartcutting experiments has been very limited.
(3) Transfer and subsequent ^{2}D separations of multiple fractions of a particular ^{1}D peak produces a two-dimensional chromatogram that reveals the coordinates of the peak in both dimensions of the chromatographic space. This provides confidence that a particular peak has been quantitatively transferred from the first to the second dimension separation, or can reveal that conditions (e.g., ^{1}D sampling window) must be altered to achieve quantitative transfer.

Using existing valve technology we find that the approach is very repeatable, even at very short ^{1}D sampling times – as low as 1 s. We have also systematically studied the influence of the volume and composition of fractions transferred from the first to the second dimension, when the ^{2}D is operated isocratically, and quantified

the benefit to the ^{2}D separation of diluting the ^{1}D effluent online during the transfer process. In spite of the larger injection volumes associated with this dilution approach, we find that the effects of these large volumes can largely be eliminated if the ^{1}D effluent is diluted to the point where it contains 10–20% less acetonitrile than the ^{2}D eluent.

We anticipate that advances in valve technology that specifically address the needs of the sLC × LC approach will have an enormous impact and make the approach an even more powerful tool for selectively enhancing the resolution of existing 1D-LC methods to address the need for higher throughput in a variety of application areas, some of which are demonstrated in the second paper in this two-part series.

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