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Selective comprehensive multidimensional separation for resolution enhancement in high performance liquid chromatography. Part II: Applications

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ABSTRACT

In this second paper of a two-part series, we demonstrate the utility of 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 three quite different example applications. In the first paper of the series we described the principles of this approach, which breaks the long-standing link in online 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. In the first example, the power of the sLC × LC approach to significantly reduce the analysis time and method development effort is demonstrated by selectively enhancing the resolution of critical pairs of peaks that are unresolved by a one-dimensional separation (1D-LC) alone. 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 peaks in the 2D separation space. The added time dimension of sLC × LC chromatograms also facilitates the application of sophisticated chemometric curve resolution algorithms to further resolve peaks that are otherwise chromatographically unresolved. This is demonstrated in this work by the targeted analysis of phenytoin in urban wastewater effluent using UV diode array detection. Quantitation by both standard addition and external calibration methods yielded results that were not statistically different from 2D-LC/MS/MS analysis of the same samples. Next, we demonstrate the utility of sLC × LC for reducing ion suppression due to matrix effects in electrospray ionization mass spectrometry through the analysis of cocaine in urban wastewater effluent. Finally, we explore the flexibility of the approach in its application to two select regions of a single ^{1}D separation of triclosan and cocaine. The diversity of these applications demonstrates the power and versatility of the sLC × LC approach, which will benefit tremendously from further optimization and advances in valve technology that specifically address the needs of this new technique.

1. Introduction

In Part I of this two-part series we discussed the principles of a new approach to two-dimensional separation we refer to as 'Selective Comprehensive Two-Dimensional Liquid Chromatography' (sLC × LC). We assert that this approach fills a large void between the experimental extremes of two-dimensional separation: heartcutting, where a single fraction of first dimension (^{1}D) effluent containing compounds of interest is transferred to the second dimension; and fully comprehensive separation (LC × LC), where tens or hundreds of fractions of ^{1}D effluent are transferred to the second column for further separation. Our view is that the sLC × LC approach combines positive features of both the heartcutting and fully comprehensive approaches, while minimizing their weaknesses. The chief benefit of sLC × LC over the heartcutting approach is that it preserves the ^{1}D resolution of one or more target compounds from closely neighboring peaks, during the transfer and subsequent second dimension (^{2}D) separation of fractions of ^{1}D effluent. This is accomplished by capture, transient storage, and subsequent re-injection of multiple fractions (six in this work) of ^{1}D effluent, some of which contain compounds of interest that are not resolved in the first dimension, for further separation in the second dimension. This approach produces two-dimensional (in time) chromatograms for select portions of the overall separation; the multi-dimensional structure of these data lends itself to sophisticated chemometric analysis that is not applicable to data from heartcutting methods [1,2].

The chief advantage of sLC × LC over on-line fully comprehensive methods is that the approach breaks the long-standing link in fully comprehensive work between the timescales of sampling ^{1}D peaks, and the ^{2}D separation. In the fully comprehensive case this link forces serious compromises to be made between the rate

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at which the ^{1}D separation is sampled, and the performance of the ^{2}D separation [3–5]. The fundamental problem is that avoiding significant loss of ^{1}D resolution requires sampling of ^{1}D peaks at a rate of three to four fractions per peak width ($8^1\sigma$). With the exception of fully comprehensive approaches involving multiple ^{2}D columns operated in parallel [6], the ^{1}D sampling time must equal the ^{2}D analysis time. Adequate sampling of ^{1}D separations of even modest efficiency inevitably leads to extremely short ^{2}D analysis times (<5 s), compromising the contribution of the ^{2}D separation to the overall resolving power of the two-dimensional separation. In practice, both the sampling process and the performance of the ^{2}D separation are compromised [3,4,7,8]. The sLC × LC approach breaks this link, allowing adequate sampling of narrow peaks in highly efficient ^{1}D separations, while leveraging the resolving power of ^{2}D separations on the 10 to 30-s timescale.

In Part I we described the configuration of off-the-shelf valve technology for sLC × LC, evaluated the run-to-run repeatability of sLC × LC separations using short ^{1}D sampling times in the range of 1–3 s, and reported the results of calculations and experiments that showed the tremendous benefit of diluting ^{1}D effluent online with weak solvent during the fraction transfer step when reversed-phase columns are used in both dimensions of a sLC × LC system. In this paper we demonstrate the practical utility of the sLC × LC approach using three very different example applications. In the first example we use sLC × LC to selectively enhance the resolution of a pair of unresolved peaks in a rapid 5-min analysis of nine opiates. Systematic approaches to method development for 1D-LC focus effort on increasing the resolution of critical pairs of peaks until some satisfactory level of resolution is reached [9]. In our experience the rest of the resulting separation is often 'over-optimized' in the sense that the irregular spacing of other peaks in the chromatogram leads to wasted chromatographic space and analysis time. Here we show that sLC × LC can be used to reduce this wasted time by selectively shifting the burden of separation for the critical pair to the second dimension of the sLC × LC separation.

In the second example we demonstrate the targeted analysis of phenytoin in urban wastewater effluent using sLC × LC coupled with UV detection as the second and third dimensions of a 3D-LC separation. Subsequent chemometric data analysis was used to mathematically resolve the phenytoin peak from neighboring interferent peaks. In previous work we reported a heartcutting 3D-LC analysis of phenytoin in a sample matrix that was nominally identical to the one used here [10]. While the 3D-LC method was very effective for the analysis of phenytoin, even at the parts-per-trillion level, the slow speed of the terminal separation prohibited the analysis of more than one or two target compounds in a reasonable time. In the sLC × LC approach some of the resolving power of the terminal dimension is traded for shorter analysis times, which allows multiple fractions of the target peak to be captured and transferred to the terminal dimension. This adds a time dimension to the resulting chromatogram, which facilitates the application of multi-way curve resolution methods [1,2] with the aim of mathematically resolving peaks that would otherwise be chromatographically poorly resolved.

In a third example we demonstrate the use of sLC × LC to mitigate ionization suppression effects that are encountered in the analysis of complex matrices when electrospray ionization is used in conjunction with mass spectrometric detection. Previously, both heartcutting [11] and fully comprehensive [12] two-dimensional HPLC methods have been used to reduce ionization suppression effects. In principle, the sLC × LC approach offers advantages over both the heartcutting and fully comprehensive approaches for this type of application.

In each of these three examples just one selected region of the ^{1}D separation is targeted for resolution enhancement using the second dimension of the sLC × LC system. Thus, we close with a final example of a separation in which two separate regions of a ^{1}D separation are targeted to show that the technique can be extended to multiple sampling windows within a single analysis as needed. To demonstrate this ability, cocaine and triclosan are separated from a spiked sample of DI water. Cocaine and triclosan have significantly different ^{1}D retention times, thus making the transfer of both to the second dimension in a single sampling period impractical. Because sufficient retention differences exist between the two compounds in the first dimension, each can be independently collected and subjected to further separation and resolution enhancement.

2. Materials and methods

2.1. 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 and triclosan were from Sigma–Aldrich (St. Louis, MO); cocaine, d_3-cocaine, morphine-3-β-D-glucuronide, oxymorphone, oxycodone, morphine, codeine, hydrocodone, morphine-6-β-D-glucuronide, hydromorphone, and 6-acetylmorphine were from Cerilliant (Round Rock, TX). Acetonitrile was LC/MS grade and obtained from Fisher Scientific (Fair Lawn, NJ). Phosphoric acid was also from Fisher Scientific (HPLC grade, Fair Lawn, NJ), and ammonium acetate, formic acid, and trifluoroacetic acid were reagent grade or better, from Sigma–Aldrich. All mobile phases were degassed prior to use either by vacuum degassing or sparging with helium.

2.2. Sample pre-concentration

Samples of urban wastewater treatment plant effluent (sampled October and November 2010) were pre-concentrated by Solid Phase Extraction (SPE) prior to analysis. SPE columns were prepared in-house by dry packing 2.0 g of carbon on silica (30–62 μm, 16%, w/w) (Part # 9000-1032, United Science, Minneapolis, MN) into empty 20 mL polypropylene SPE tubes fitted with 20 μm polyethylene frits (Supelco, Bellefonte, PA). A total of 16 L of wastewater treatment plant effluent was pre-concentrated to a 16 mL sample. No attempt was made to selectively pre-concentrate the samples; the goal was to provide a complex sample matrix to challenge the resolving power of the sLC × LC system. The effluent was first filtered through a 0.45 μm nylon membrane filter. Then, it was pulled through the SPE column at approximately 50 mL/min using house vacuum. After approximately 2 L of effluent was pulled through, the SPE column was dried for 5 min by drawing air through it. Retained compounds were eluted with five 1-mL aliquots of acetonitrile. The total volume eluted from extraction of 16 L of effluent was decreased to about 4 mL using vacuum centrifugation at 60 °C for 1 h. The remaining sample was reconstituted to 16 mL with DI water to give a final pre-concentration factor of 1000-fold. The reconstituted sample was stored at 4 °C until use. The pre-concentrated waste water treatment plant effluent sample will be referred to hereafter as WWTPE. Spiked WWTPE samples were prepared within 24 h of analysis.

2.3. sLC × LC instrumentation and chromatographic conditions

2.3.1. sLC × LC instrumentation

A complete schematic showing the instrument configuration used in sLC × LC separations is shown in Fig. 1. A detailed description of its setup and operation are included in Part 1 of this series of

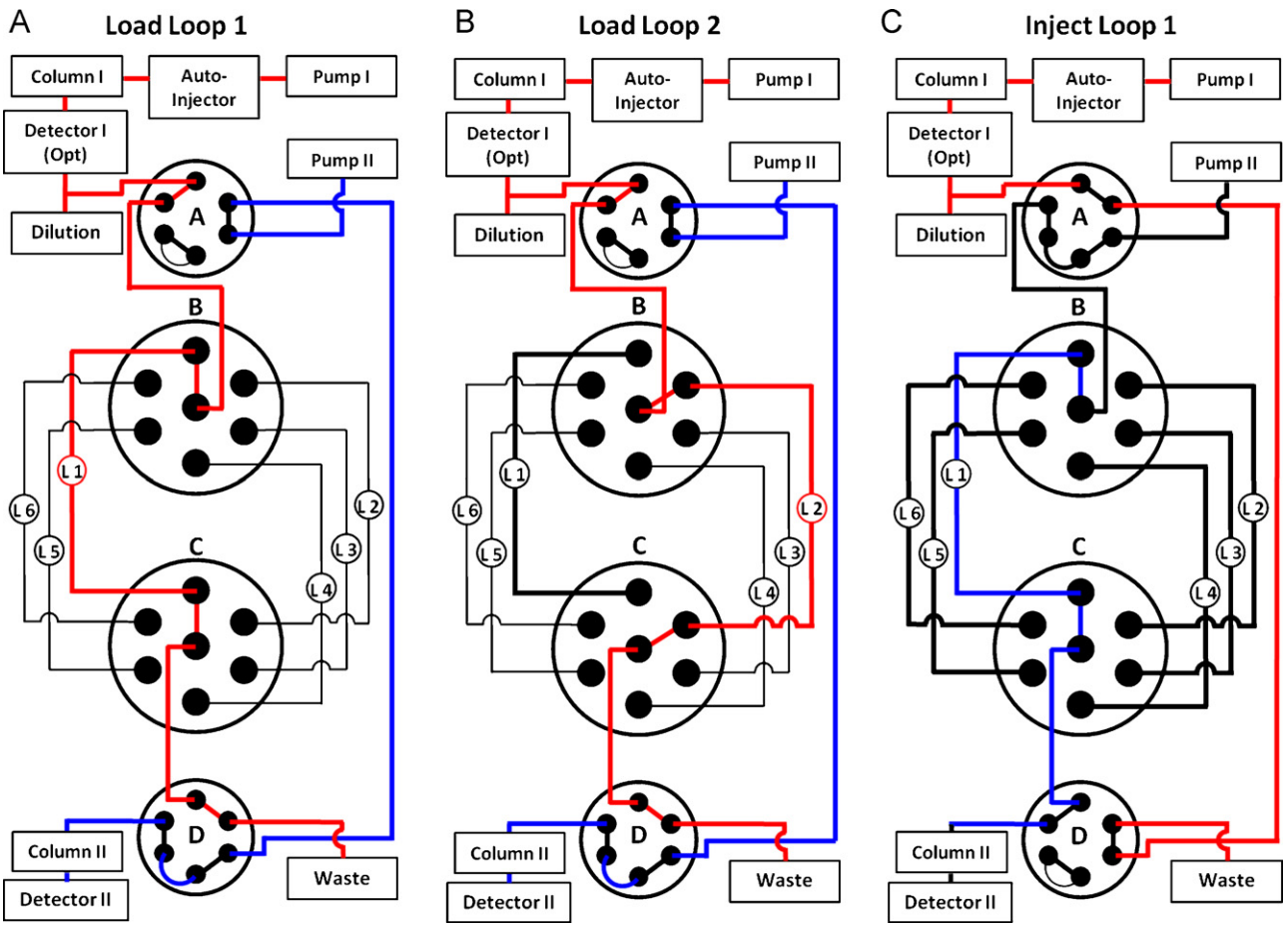

Fig. 1. Schematic of instrument configuration for sLC × LC. Pump I delivers eluent to Column I while Pump II pushes captured fractions of ^{1}D effluent out of sample loops L1 through L6 and delivers eluent to the ^{2}D column. Detector I is optional in the sense that it can be removed to reduce extra-column 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, store, and re-inject six 1–5 s portions 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).

papers; the salient features are included here for convenience. First and second dimension separations were carried out using separate HP1050 quaternary pumps. The ^{1}D system included a modified HP1050 auto-injector, equipped with a 900 μL syringe plunger and 400 μL sample loop. The ^{1}D detector was an "optional" G1312 variable wavelength UV absorbance detector (Agilent Technologies, Santa Clara, CA), which may be removed after determination of the location of the ^{1}D peak(s) of interest. A series of six fractions was captured from the ^{1}D target analyte peak(s) and subsequently re-injected into the ^{2}D column for further separation. In most cases (see Section 2.3.7 for MS/MS detection) the ^{2}D detector was a G1315 (Agilent) photodiode array UV absorbance detector. Individual instrument modules were controlled by Chemstation Software (Agilent, A.08.03).

Following the ^{1}D separation, the ^{1}D effluent was diluted with a stream of aqueous solvent at a flow rate between 0.5 and 2.1 mL/min using a Varian 212LC single channel pump (Agilent) prior to capture by the valve system. The four sampling valves at the heart of the sLC × LC system (labeled (A)–(D) of Fig. 1) were operated as related, but independent pairs. Valves A and D were a pair of six-port, two-position valves (Rheodyne Model 7010, Rohnert Park, CA) that controlled the loading/injecting of the sample loops L1–L6. Panels (A) and (B) of Fig. 1 depict the valve configuration for the loading of sample loops L1 and L2, the flow path is shown as a red trace. The blue trace in panel (C) depicts the flow path for the re-injection from loop L1. Coordinated rotation of Valves B and C determined which loop was being filled/injected within each cycle; both were six-position flow path selection valves from Cadence Fluidics (Model UBX-1701-0607-0001, Petaluma, CA). In all cases, loops L1–L6 were 75 μL, constructed from 240 μm I.D. PEEK tubing. The coordination of the timing of the different modules and fraction transfer between dimensions was controlled by simple LabView program written in-house.

2.3.2. *Rapid sLC × LC separation of nine opiates*

A mixture of nine opiates was separated using the sLC × LC system with a total analysis time of about 5 min. Second dimension separations were used only to separate oxycodone and 6-acetylmorphine, which were unresolved in the ^{1}D separation. An Ascentis Express F5 perfluorophenyl stationary phase was used for ^{1}D separations (75 mm × 2.1 mm I.D.; 2.7 μm; Supelco, Bellefonte, PA). The flow rate was 0.70 mL/min and a 2.8-min linear gradient from 7 to 43% B solvent (50/50 acetonitrile/20 mM ammonium phosphate, pH 4) was used, with a hold at 43% B until 3 min; the A solvent was 10 mM H_3PO_4. The column temperature was 30 °C, the injection volume was 10 μL, and peaks were detected by absorbance of UV light at 210 and 220 nm. First dimension effluent containing the oxycodone and 6-acetylmorphine peaks was captured in the time window from 2.50 to 2.65 min in six 1.5-s fractions. Effluent fractions were diluted with 0.1% trifluoroacetic

Table 1
Example of the sequence of timed events for the operation of the sLC × LC system for the separation of nine opiate compounds (see Section 2.3.2 for other conditions). ^{1}D sampling time was 1.5 s, and each ^{2}D separation was 25 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	^{1}D effluent through loops/to waste (Fig. 1, panel (A))	L1/L1
120 (2 min)	0	Pump II start		L1/L1
150	30	Load loops		L1/L1
151.5	31.5			L2/L2
153	33			L3/L3
154.5	34.5			L4/L4
156	36			L5/L5
157.5	37.5			L6/L6
159	39	Loading finished	Switch Positions	
160	40.2	Inject from loops	Pump II eluent through loops/to Column II (Fig. 1, panel (C))	L1/L1
185	65			L2/L2
210	90			L3/L3
235	115			L4/L4
260	140			L5/L5
285	165			L6/L6
300 (5 min)	180	^{1}D stop time		L6/L6
310	190	^{2}D stop time		

[a] See Fig. 1.

acid at 2.1 mL/min prior to storage in loops L1–L6 shown in Fig. 1 (70 μL/fraction). These fractions were then injected into the ^{2}D column at 25-s intervals beginning 2.67 min from the start of the ^{1}D separation. Details of the sequence of timed events for the rapid sLC × LC separation of nine opiates shown in Fig. 2 are given in Table 1.

The ^{2}D column in this case (50 mm × 4.6 mm I.D.) was based on a novel carbon-modified silica-based stationary phase referred to hereafter as 'COS'. This material 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 [13,14]. 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 [10,15,16]. The COS material (15% carbon, w/w) was obtained from United Science (Minneapolis, MN) and packed into stainless steel columns in-house. The ^{2}D eluent was 15/85 acetonitrile/0.1% trifluoroacetic acid, pumped at a flow rate of 3.0 mL/min. The column was thermostated at 40 °C, and peaks were detected by absorption of UV light at 220, 254, and 280 nm.

2.3.3. 3D-LC/UV analysis of phenytoin in WWTPE

Quantitation of phenytoin in WWTPE using UV absorbance detection required a three-dimensional separation with an additional dimension of separation preceding the sLC × LC separation because of the complexity of the sample matrix. The first dimension was used to reduce the number of compounds from the WWTPE transferred to the highly retentive COS columns used in the second dimension. The first dimension was sampled by simply diverting the effluent stream of the ^{1}D column directly into the ^{2}D column using the approach described by Rogatsky and Stein [17].

An Ascentis Express F5 perfluorophenyl stationary phase was used for ^{1}D separations (50 mm × 2.1 mm I.D.; 2.7 μm; Supelco, Bellefonte, PA). The flow rate was 0.50 mL/min and a 5-min linear gradient from 15 to 100% B solvent (acetonitrile) was used; the A solvent was 10 mM ammonium acetate, pH 5.0. The column temperature was 40 °C, and the injection volume was 300 μL. The phenytoin peak was heartcut from the ^{1}D separation between 2.3 and 3.5 min and diluted with DI water at a flow rate of 0.5 mL/min during transfer to the ^{2}D column. The ^{2}D column was a serially coupled pair of 50 mm × 2.1 mm I.D. columns (total length of 10 cm) prepared in-house with COS (15% carbon, w/w, United Science, LLC), and the ^{2}D separation was isocratic using 40/60 acetonitrile/10 mM H_3PO_4. The column was heated to 40 °C and the flow rate was maintained at 0.5 mL/min. The ^{2}D effluent was diluted with DI water at a flow rate of 0.50 mL/min and six 2-s fractions were captured in the time window from 7.82 to 8.02 min. These fractions were then injected into the ^{3}D column beginning at 8.04 min and following at 20-s intervals. Third dimension separations used an Ascentis Express C18 column (30 mm × 2.1 mm I.D., 2.7 μm; Supelco, Bellefonte, PA) operated at a flow rate of 2.0 mL/min and 50 °C. The ^{3}D eluent was 25/75 acetonitrile/10 mF H_3PO_4, and peaks were detected by absorbance of UV light at 210, 220, and 254 nm.UV/Vis spectra were also collected in the range of 200–800 nm at a rate of 25 scans/s to facilitate four-way chemometric analysis of the data [18,19].

2.3.4. Heartcutting 2D-LC/MS/MS analysis of phenytoin in WWTPE

The concentration of phenytoin in WWTPE was determined using heartcutting 2D-LC/MS–MS and the method of standard addition. This experiment utilized the first single quaternary pump and large injection system described above (Section 2.3.1). The first dimension was sampled by simply diverting the effluent stream of the ^{1}D column directly into the ^{2}D column using the approach described by Rogatsky and Stein [17]. In our experience, the added resolving power of the second dimension was very beneficial for reducing matrix effects of the WWTPE sample on the phenytoin signal normally observed in 1D-LC/MS/MS analyses.

The first dimension separation utilized the two serially coupled 50 mm × 2.1 mm I.D. COS columns described in Section 2.3.3. A 7.5-min gradient from 10 to 100% B solvent (acetonitrile) was used, followed by a hold at 100% B for an additional 2.5 min; the A solvent was 10 mM ammonium acetate, pH 5.0. The flow rate was 0.50 mL/min, the column was thermostated at 40 °C, and the injection volume was 100 μL. A variable wavelength UV absorbance detector set at 210 nm was used initially to determine the elution time of the phenytoin peak in the first dimension. Effluent from the ^{1}D column was transferred to the ^{2}D column from 6.1 to 7.0 min.

Second dimension separations were carried out with a 50 mm × 4.6 mm I.D. (2.7 μm) Ascentis Express C18 column (Supelco). A 2-min gradient from 10 to 100% acetonitrile (with 0.1% formic acid as the aqueous solvent) was used with a hold for 2 min at 100% acetonitrile. The gradient began at the 7-min mark of the ^{1}D separation and ran until 11 min after the ^{1}D injection. The flow rate remained constant throughout the separation at 0.5 mL/min. Phenytoin was detected by MS/MS in the negative mode; detailed conditions are given below (Section 2.3.6).

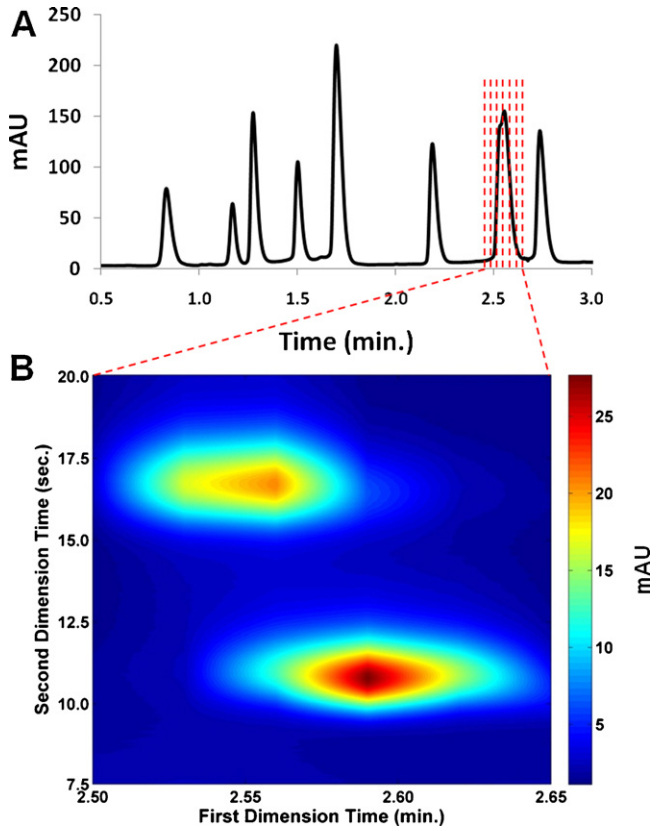

Fig. 2. Chromatograms observed at the outlet of ^{1}D (A) and ^{2}D (B) separations of a sLC × LC separation of a mixture of nine opiate compounds in DI water at 10 μg/mL. Panel (A) shows the ^{1}D chromatogram where seven of the nine compounds are well-resolved. Peaks correspond to morphine 3-β-D glucuronide, morphine 6-β-D-glucuronide, morphine, oxymorphone, hydromorphone, codeine, oxycodone, 6-acetylmorphine, and hydrocodone (in order of ^{1}D elution). Sampling of ^{1}D effluent occurred from 2.50 to 2.65 min corresponding to the elution window of oxycodone and 6-acetylmorphine. Panel (B) shows the result of the ^{2}D separation where the six ^{2}D chromatograms have been reformatted into a contour plot where the oxycodone and 6-acetylmorphine peaks are well resolved. Beginning at 2.67 min the ^{1}D and ^{2}D separations are carried out in parallel using two independent UV detectors. For detailed chromatographic conditions, see Section 2.3.2.

2.3.5. Reduction of matrix effects in the analysis of cocaine in WWTPE by sLC × LC/MS/MS

d_3-Cocaine was analyzed in WWTPE by both 1D-LC/MS/MS and by sLC × LC/MS–MS to quantify the reduction of matrix effects using the sLC × LC approach. For this comparison, the chromatographic conditions of the 1D-LC/MS/MS and the first dimension of the sLC × LC system were identical. Both utilized the same serially coupled 50 mm × 2.1 mm I.D. COS columns described in Section 2.3.3. Linear 5-min gradients from 10 to 100% B solvent (acetonitrile) were used followed by a hold at 100% B solvent for an additional minute; the A solvent was 0.1% formic acid in water. The flow rate was 0.25 mL/min, and in both cases 100 μL samples of WWTPE were injected containing 100 ppb d_3-cocaine. The first dimension effluent was diluted with DI water at 0.75 mL/min. The sLC × LC separations used a 30 mm × 2.1 mm I.D. Ascentis Express C18 (2.7 μm) column, in the second dimension, operated isocratically at 50 °C and a flow rate of 2.0 mL/min of 15/85 acetonitrile/0.1% formic acid in water. A UV detector was used to determine the ^{1}D elution time; however, once this was established it was taken out of the flow path to minimize extra-column peak broadening. Six 4-s fractions were captured between 4.32 and 4.72 min, and 20-s ^{2}D separations were performed on each fraction beginning at 4.75 min. MS/MS conditions were again identical in the 1D-LC and the sLC × LC experiments; detailed MS/MS conditions are given in Section 2.3.7.

2.3.6. Analysis of multiple compounds or groups of compounds by sLC × LC

To demonstrate the ability of a sLC × LC method to include multiple sampling windows within a single analysis, cocaine and triclosan were simply separated from a spiked sample of DI water. Cocaine and triclosan had significantly ^{1}D retention time differences, thus making the transfer of both to the second dimension in a single sampling period impractical. The chromatographic conditions were the same as in Section 2.3.4 with a few small changes. The ^{1}D gradient was extended to 10 to 100% acetonitrile in 8 min and held at 100% acetonitrile until 9 min. In the second dimension, a stepped isocratic solvent program was used (15% acetonitrile from 2 to 6 min for the analysis of lower retention cocaine, 65% acetonitrile from 6 to 11 min for the analysis of higher retention triclosan, and 90% acetonitrile from 11 to 12 min). The stepwise isocratic program allowed both cocaine and triclosan to have a reasonable retention time in the second dimension. Two sets of six 4-s fractions of ^{1}D effluent were captured from the first dimension, one beginning at 3.35 min to capture the cocaine peak, and the other at 8.35 min to capture the triclosan peak. These fractions were injected into the ^{2}D column at 20-s intervals beginning at 3.77 min for cocaine and 8.77 min for triclosan.

2.3.7. Mass spectrometric detection

A Varian Model 320 electrospray ionization triple quadrupole mass spectrometer was used for mass spectrometric detection. Nitrogen was used as the nebulizing (55 psi) and drying gas (21 psi, 300 °C). All chromatograms were obtained using selected reaction monitoring (SRM) following fragmentation of parent compounds using argon as the CID gas at 1.5 mTorr. Phenytoin was detected in the negative mode with the electrospray needle set to (−)4500 V, and quantitation was based on the transition from *m/z* 251.0 → 101.7. Cocaine and d_3-cocaine were detected in the positive mode at (+)4500 V, and quantitation of cocaine and d_3-cocaine was based on the transitions from *m/z* 304.2 → 182.0 and *m/z* 307.2 → 185.0, respectively. Triclosan was detected in the negative mode at (−)4500 V, using the transition *m/z* 287.0 → 35.0.

3. Results and discussion

3.1. Rapid sLC × LC separation of nine opiates

Of the applications of sLC × LC described in this work, the one that most clearly demonstrates the advantages of the approach is the rapid separation of nine opiate compounds. Because of the unordered nature of retention of compounds in most mixtures, the development of methods for separations of compounds by conventional 1D-LC is typically driven by the resolution of one or a few critical pairs. Attempts to resolve one particularly critical pair through changes in operating conditions often can resolve that pair, but the changes frequently create another critical pair through concurrent changes in selectivity [9]. The names of the nine opiate compounds studied in this work are given in the caption of Fig. 2. Seven of the nine compounds are easily separated in less than 3 min using an Ascentis Express F5 column under conventional 1D-LC conditions, as shown in the top trace of Fig. 2. However, 6-acetylmorphine and oxycodone co-elute at about 2.6 min, and their retention factors essentially co-vary with mobile phase composition, as shown in Fig. 3, to the extent that it is not possible to resolve them by changing gradient steepness, for example. It is quite likely that this pair of compounds, and probably all nine compounds, could be resolved on some other RP column, or with some other buffer on the F5 column. However, finding these conditions is time-consuming and expensive. The sLC × LC approach allows us to use the existing 1D-LC separation to separate seven of the nine

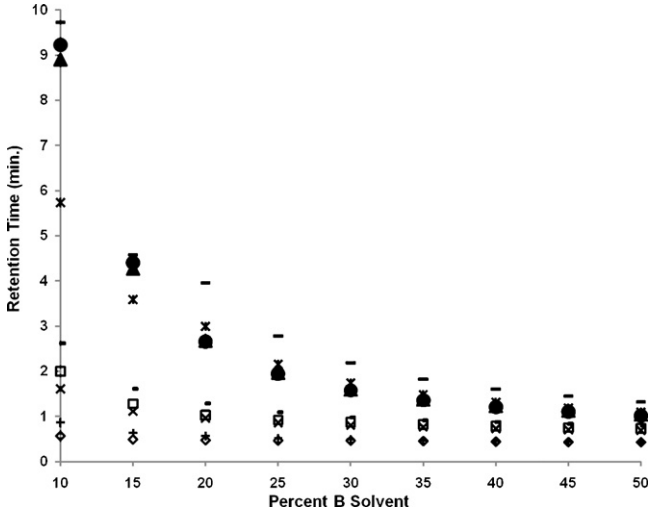

Fig. 3. Retention times of nine opiates under isocratic RP conditions with an Ascentis Express F5 column (75 mm × 2.1 mm I.D., 2.7 μm); Morphine 3-β-D-glucuronide (◊), morphine 6-β-d-glucuronide (+), morphine (×), oxymorphone (□), hydromorphone (−), codeine (*), oxycodone (▲), 6-acetylmorphine (●), and hydrocodone (-). The B solvent was 90:10 acetonitrile/100 mM ammonium acetate (pH 4.0), and the A solvent was 0.1% (w/w) formic acid in water. The flow rate was 0.5 mL/min., and the column was thermostatted at 40 °C. Retention times of 6-acetylmorphine and oxycodone (▲, ●) are highly overlapped and co-vary with eluent composition, requiring the use of a ^{2}D separation to resolve these two peaks.

compounds, but then selectively use LC × LC conditions to resolve the remaining pair. Fig. 4 shows the gradient elution retention times of all nine opiates on two RP columns: the Ascentis Express F5, and a prototype Carbon on Silica phase (COS). We see that the COS phase easily separates the 6-acetylmorphine and oxycodone, which makes it a good complement in a sLC × LC separation; however, it would not separate all nine compounds by itself in a conventional 1D-LC separation (e.g., the glucuronides co-elute). The contour plot in Fig. 2 shows the LC × LC separation of the critical pair which co-elute on the F5 column. For this separation, six 1.5-s fractions of ^{1}D effluent were collected and subsequently analyzed in six 25-s isocratic ^{2}D analyses in parallel with the ongoing first dimension

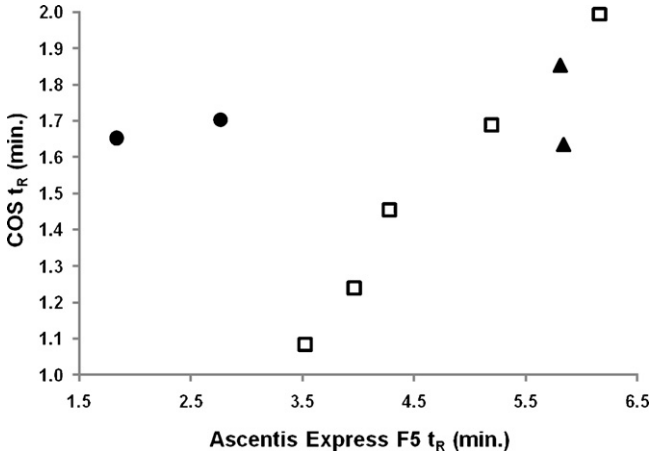

Fig. 4. Retention times of nine opiates under RP gradient elution conditions on Ascentis Express F5, and Carbon on Silica (COS). 6-acetylmorphine and oxycodone (▲) are unresolved by the Ascentis Express F5 column, but are well resolved by the COS column, whereas the two glucuronides (●) are well resolved by the F5 column but not by the COS column. Chromatographic conditions: Ascentis Express F5: (75 mm × 2.1 mm I.D., 2.7 μm), 20 μL injection, 5–40% acetonitrile in 6 min; A solvent was 0.1% formic acid; flow rate, 0.25 mL/min; COS: (50 mm × 4.6 mm I.D.), 20 μL injection, 5–40% acetonitrile over 4 min; A solvent was 10 mF H_3PO_4; flow rate, 1.0 mL/min.

separation (i.e., two UV detectors were used in parallel). The average relative precision of total peak areas for compounds 7 and 8 over four replicate injections were 0.87 and 1.86%, respectively. It is very clear from this plot that the measurable but incomplete separation of this pair of peaks along the ^{1}D axis is preserved by adequately sampling the ^{1}D effluent in this window. In this case, the resolution of the pair in the second dimension is so good that the ^{1}D separation is not needed. However, one can certainly imagine situations where the resolution in both dimensions is needed to fully resolve the pair, and sLC × LC makes this possible.

The advantage of the sLC × LC approach with respect to analysis time cannot be overstated in this case. The conventional approach to LC × LC separations is not ideal in this type of application, because the slow speed of second dimension separations forces one of two negative outcomes: either ^{1}D peaks are inadequately sampled across their width causing severe loss of ^{1}D resolution (in this case only one or two samples could be taken during the elution of the ^{1}D peak pair), or the first dimension must be slowed down to make the peaks sufficiently wide that they can be adequately sampled, resulting in a significantly slower overall analysis [20]. It is our view that the sLC × LC approach is a very powerful tool to dramatically reduce the analysis time for moderately complex samples where the resolution of one or more critical pairs limits throughput and dominates method development efforts.

In this separation, the nine opiates were detected by a pair of diode array UV detectors operated in parallel. Using two detectors allowed the ^{2}D analysis to begin immediately after all six fractions were captured. A similar separation of the nine opiates was also done using a tandem mass spectrometer as the detector (data and chromatographic conditions not shown). The sLC × LC fractions were stored in the sample loops until all nine opiates eluted from the first dimension. This demonstrates the ability of the sLC × LC system to perform online comprehensive separations which do not need to be done in real time.

3.2. Separation and quantitation of phenytoin in wastewater treatment plant effluent

The results of a sLC × LC separation of 1000× concentrated WWTPE are shown in Fig. 5. In this case, the separation dimensions referred to as 'second' and 'third' are actually the second and third dimensions of a three-dimensional separation where the transfer of ^{1}D effluent to the second dimension is a simple heartcut process. Since the focus of this paper is on the sLC × LC process we focus here on the second and third dimensions of separation. The actual first dimension separation of the WWTPE extract was very similar to those shown in our previous work on targeted 3D-LC (see Fig. 7A in Ref. [10]). The overlay shown in panel (A) of Fig. 5 shows the consecutive third dimension separations of a 50 ppb phenytoin standard in DI water (black trace), and WWTPE extract spiked with 50 ppb phenytoin (blue trace). This comparison shows that, even with this three-dimensional separation method, the fractions transferred from the second to the third dimension contained several detectable constituents in addition to phenytoin. The most abundant of these interferents eluted just prior to phenytoin in the third dimension. Panels (B) and (C) of Fig. 5 show the interferent more clearly after the reformation of the ^{3}D chromatograms into a contour plot. The maxima of the phenytoin and the interferent peaks are located at approximately 7.92 min in the ^{2}D time axis and 11 s on the ^{3}D axis. Fig. 5C provides a different view of Fig. 5B with the ^{3}D timescale expanded from 7.5 to 15 s to focus on the phenytoin peak, marked with an arrow, and the neighboring interferent peak. In this figure phenytoin and the interferent are the two major features; based on the shift in retention time between the third and fourth ^{3}D separations, one could conclude that there are actually two chemically distinct compounds eluting

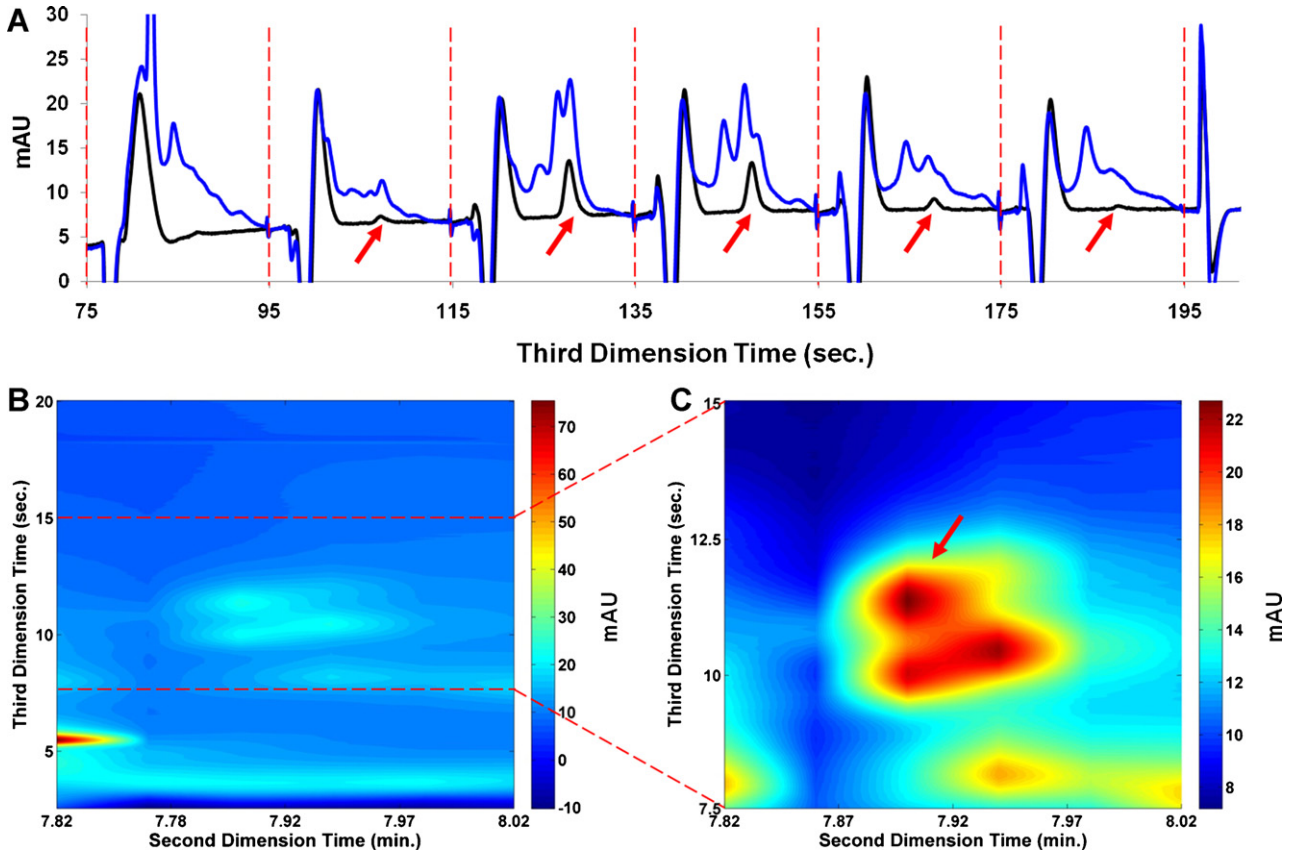

Fig. 5. Chromatograms showing the results of a 3D-LC separation of phenytoin from WWTPE where the sLC × LC approach is used in the second and third dimensions of the 3D system. Panel (A) shows the sequence of ^{3}D chromatograms observed at the outlet of the ^{3}D column using a UV absorbance detector. The black and blue traces show the chromatograms for samples of DI water and WWTPE, each spiked with 50 ppb phenytoin. Arrows are used to indicate the location of the phenytoin peak within each ^{3}D separation (each separation is bound by vertical dashed lines). Panel (B) is a contour plot created from the reformation of the data shown in panel (A). Two peaks are observable at 7.92 min in the second dimension and 10–11 s in the third dimension; panel (C) focuses in on this region to display the phenytoin peak (arrow) and a large interferent peak that elutes at about the same time in the second dimension, but is slightly resolved in the third dimension. There is a slight retention shift (ca. 0.2 s) in the third dimension which leads to the slight offset appearance of the peaks. This is the very worst deviation observed in the entire experiment (most separations were far better), and did not affect the results of subsequent chemometric processing of the data for quantitative purposes. For detailed chromatographic conditions, see Section 2.3.3. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

with slightly different ^{3}D retention times. However, we are confident that the interferent peak is composed of just one compound because replicate experiments performed on the same WWTPE sample at different phenytoin spike levels did not show this shift in retention. We show this particular example to demonstrate that the chemometric data analysis method applied (discussed below) to resolve the phenytoin peak from the rest of the sample was effective in spite of retention shifts on the order of 0.25 s in adjacent ^{3}D chromatograms.

Although the phenytoin peak is not nearly as well-resolved from the sample matrix as it was in our previous work on a similar WWTPE sample (see Fig. 8A in Ref. [10]), this result was not entirely unexpected. The application of the sLC × LC methodology compromises the amount of separation efficiency achievable in the third dimension in the interest of using short analysis times in the terminal dimension to analyze several fractions of ^{2}D effluent, which preserves the ^{2}D separation. Each 2-s ^{2}D fraction was subjected to a 20-s separation (indicated by the dashed lines of Fig. 5A), as opposed to a 10-min gradient used in our previous heartcutting work. Despite the loss of resolving power in the third dimension, we believe that two major advantages of the sLC × LC approach outweigh this disadvantage. First, taking multiple fractions of ^{2}D effluent containing the target compound clearly identifies where the peak maximum is in the second dimension, and this allows us to verify that the target compound has been quantitatively transferred to the third dimension. As will be shown below, this is not a trivial matter; complex sample matrices can have a significant effect on the retention of specific compounds. Thus, the target peak may shift slightly from analysis to analysis depending on what exactly is injected into the ^{1}D column. In the case of traditional heartcutting work, this problem is circumvented by using a sufficiently wide sampling window to ensure that the target compound is quantitatively transferred even if retention shifts do occur. Indeed, in our own work we have used sampling windows on the order of two to three times the width of the actual target peak. This obviously places greater demands on subsequent separation dimensions. The second advantage of sLC × LC is that the separation done in a comprehensive manner for a select portion of the analysis adds a time dimension to the data structure that was not present in our previous heartcut data. This is significant because this higher order data structure is amenable to sophisticated chemometric data analysis strategies including PARAFAC and related algorithms [21], which can mathematically resolve chromatographically unresolved peaks.

Fig. 6 shows results from the analysis of a dataset comprised of a set of phenytoin standards in DI water and WWTPE extract samples both spiked with the same levels of phenytoin standards (ten total samples, each analyzed in duplicate). The data were analyzed using a previously published method [18] referred

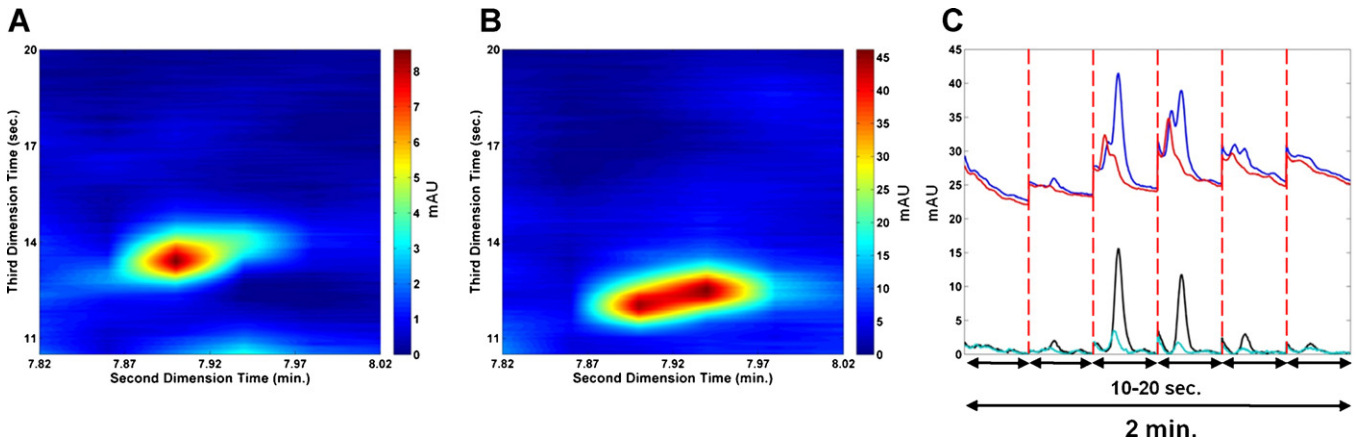

Fig. 6. Results of the application of the IKSFA-ALS algorithm to the dataset containing both DI water and WWTPE samples spiked with phenytoin in the range of 0–150 ppb. Panel (A) shows the phenytoin peak (50 ppb spike) that has been mathematically resolved from the large neighboring interferent peak in the WWTPE sample, shown in panel (B). The top two traces in panel (C) show the raw sequential ^{3}D chromatograms (10-s segments of each 20-s ^{3}D separation) as obtained directly from the UV detector at 216 nm for samples spiked with either 0 (red trace) or 150 (blue) ppb phenytoin. The bottom two traces show the IKSFA-ALS-resolved chromatograms for the component corresponding to the phenytoin peak in the same two WWTPE samples; the 0 and 150 ppb spiked samples are shown by the teal and black traces, respectively. The two sets of traces were offset by 15 mAU to facilitate visualization. In the case of the unspiked sample the phenytoin peak is barely recognizable as a small shoulder on the right side of the larger interferent peak (red trace), whereas the phenytoin peak is clearly evident in the IKSFA-ALS-resolved chromatogram (teal trace). This example shows the value of the added time dimension of the data obtained from sLC × LC separations in subsequent chemometric analysis of the data. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

to as Iterative Key Set Factor Analysis with Alternating Least Squares and spectral selectivity constraints (IKSFA-ALS-ssel). A six-component model was used to fit a portion of the data shown in Fig. 5B containing the data from 10.5 to 20 s in all six ^{3}D separations, and UV–vis absorbance spectra from 200 to 800 nm in 4 nm increments (resulting in a 191 × 6 × 101 × 20 matrix, including sample-to-sample concentration variation as a dimension). In addition to four unique chromatographic background components, phenytoin and the major interferent eluting prior to phenytoin in the third dimension (Fig. 5C) were resolved as separate components in the IKSFA-ALS-ssel model. Contour plots for the resulting resolved components for phenytoin and the interferent are shown in Fig. 6A and B, respectively. The effectiveness of the multiway IKSFA-ALS-ssel algorithm in resolving the phenytoin from chromatographically unresolved peaks is demonstrated most convincingly in the series of ^{3}D chromatograms shown in Fig. 6C. The red and blue traces show the series of raw ^{3}D chromatograms for an unspiked WWTPE sample and one spiked with 50 ppb phenytoin. The teal and black traces show the phenytoin peaks resolved from these same samples using the IKSFA-ALS-ssel approach. In the red trace the phenytoin peak is barely evident as a shoulder on the larger interferent peak eluting earlier in the ^{3}D axis, whereas this peak is clearly evident as an easily integrated peak in the resolved profile (teal).

The inclusion of spiked WWTPE samples and standards in DI water allowed the quantitation of phenytoin in the unknown WWTPE sample by both the standard addition and external calibration methods. The same set of samples was also analyzed by heartcutting 2D-LC/MS/MS [10] as a means of evaluating the quantitative accuracy of the sLC × LC approach with UV absorbance detection and IKSFA-ALS-ssel analysis. The results of this comparison are shown in Table 2. The results of the 3D-LC method with sLC × LC in ^{2}D and ^{3}D are not statistically different from the 2D-LC/MS/MS results, showing that the 3D-LC method is quantitatively accurate. Further, quantitation by external calibration is also accurate in the 3D case because the UV detection signal is not significantly altered by matrix effects. These results are qualitatively comparable to the findings of our previous work with a similar sample and heartcutting 3D-LC [10].

3.3. Ion suppression of deuterated cocaine in WWTPE samples

The increased separation power of multi-dimensional separations has advantages even when coupled with detection by tandem mass spectrometry. As shown previously by Pascoe et al. [12] and others [10,11], the added resolving power of multi-dimensional separations can help mitigate the effects of coeluting compounds that can either enhance or suppress the ionization of target compounds when electrospray ionization is used for LC/MS. This is particularly useful in cases where stable isotope-labeled internal standards (SILIS) are not commercially available, or are prohibitively expensive, as the use of SILIS is the most commonly used way to overcome matrix effects for quantitative purposes [22].

In principle, the sLC × LC approach described here should provide these benefits, but in a more time-effective manner compared to previously used approaches. To illustrate this benefit, 1DLC/MS–MS and sLC × LC/MS–MS separations of a 1000-fold concentrated sample of WWTPE spiked with a deuterated cocaine (d_3-cocaine) standard are shown in Fig. 7. The analysis of cocaine in wastewater is an established method for estimating drug use in a community [23,24] and measuring the efficacy of wastewater treatment plants [25]. In each panel the black trace shows the signal for a sample of 100 ppb d_3-cocaine in DI water, and the blue trace shows

Table 2
Comparison of phenytoin quantitation results for 3D-LC/UV[a] and 2D-LC/MS/MS.

Method	Phenytoin concentration (ppb)[b,c]	
	Standard addition	External calibration
3D-LC/UV	39.9 ± 9.9 (R^2 = 0.988)	33.7 ± 3.4 (R^2 = 0.998)
Heartcutting 2D-LC/MS/MS	42 ± 19 (R^2 = 0.96)	

[a] The sLC × LC approach was used in the second and third dimensions of the 3D-LC separation.
[b] Concentration in analytical sample; concentration in original WWTPE sample is 1000-fold lower, in units of ppt.
[c] Uncertainties are expressed as confidence intervals calculated at 95% confidence level, and standard deviations calculated by accounting for the standard deviation of the regression in both the case of external calibration [22] and the case of standard addition [23].

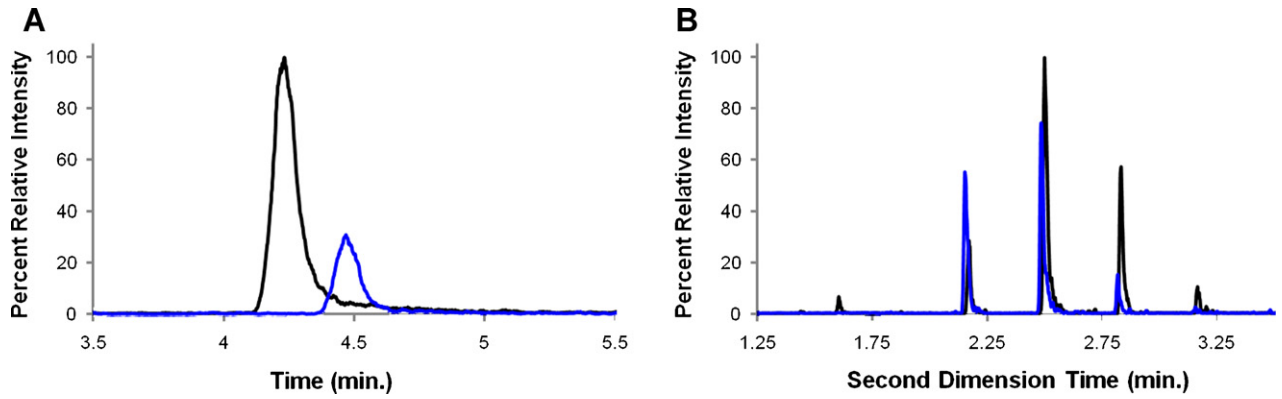

Fig. 7. Comparison of 1D and sLC × LC separations of d_3-cocaine in 1000× concentrated WWTPE using MS/MS detection. Panel (A) shows the SRM chromatogram obtained from a conventional 1D-LC separation, and panel (B) shows the SRM chromatogram obtained at the outlet of the second dimension of the sLC × LC system. The black trace on both panels shows the separation of a 100 ppb spike of d_3-cocaine into DI water and the blue traces are for WWTPE spiked with 100 ppb d_3-cocaine. In the sLC × LC case the sampling window was adjusted to accommodate the ^{1}D retention shift observed in panel (A) such that the two profiles overlap nicely in panel (B) (18 s shift). For detailed chromatographic conditions, see Section 2.3.5.

the signal in the WWTPE sample spiked at the same level. Panel (A) clearly shows the suppression of signal in the WWTPE sample, presumably due to matrix effects on ionization. Qualitatively, panel (B) shows a significant improvement in the relative signal intensity for d_3-cocaine in the WWTPE sample. A quantitative comparison of the two methods was made by calculating the ratio of the d_3-cocaine peak area in the WWTPE sample relative to the DI water sample in each case. For the sLC × LC data, the total peak area was obtained by simply summing the areas of the peaks observed in five consecutive ^{2}D analyses. In the 1D-LC/MS–MS separation, the detector response for d_3-cocaine in WWTPE was just 28% of that in the DI water sample (i.e., ca. 4-fold suppression). With the sLC × LC system, the detector response for d_3-cocaine in WWTPE was 70% of that obtained for the DI water sample, which constitutes a modest improvement. This comparison shows that ion suppression effects can be decreased with the use of sLC × LC, in this case by a factor of 2.5. Clearly the magnitude of this improvement will be sample- and matrix-dependent, and will depend on the time allotted to each ^{2}D separation for resolution of the target compound from the portion of sample matrix that is transferred from the first to the second dimension with the target compound.

Not only did the sample composition have an effect on the MS/MS signal but also on the retention time in the 1D-LC separations, and the ^{1}D elution time in sLC × LC separations. This effect is evident in panel (A) of Fig. 7 where the d_3-cocaine retention times are different for the WWTPE and DI water samples despite the nominally identical separation conditions. The cocaine in the WWTPE samples typically eluted 15 s later than the samples in DI water. If the sampling window in a sLC × LC experiment remained unchanged, a portion of the analyte in the WWTPE sample could be lost to waste, affecting the quantitative accuracy of the analysis. The solution to this problem in the conventional heartcutting 2D-LC approach is to make the sampling window wider which places greater burden on the ^{2}D separation because more sample constituents are transferred to the second dimension along with the target analyte. In the sLC × LC approach, ^{1}D retention shifts are clearly observed by changes in the ^{2}D chromatogram containing the largest peak for the target analyte. Based on this observation

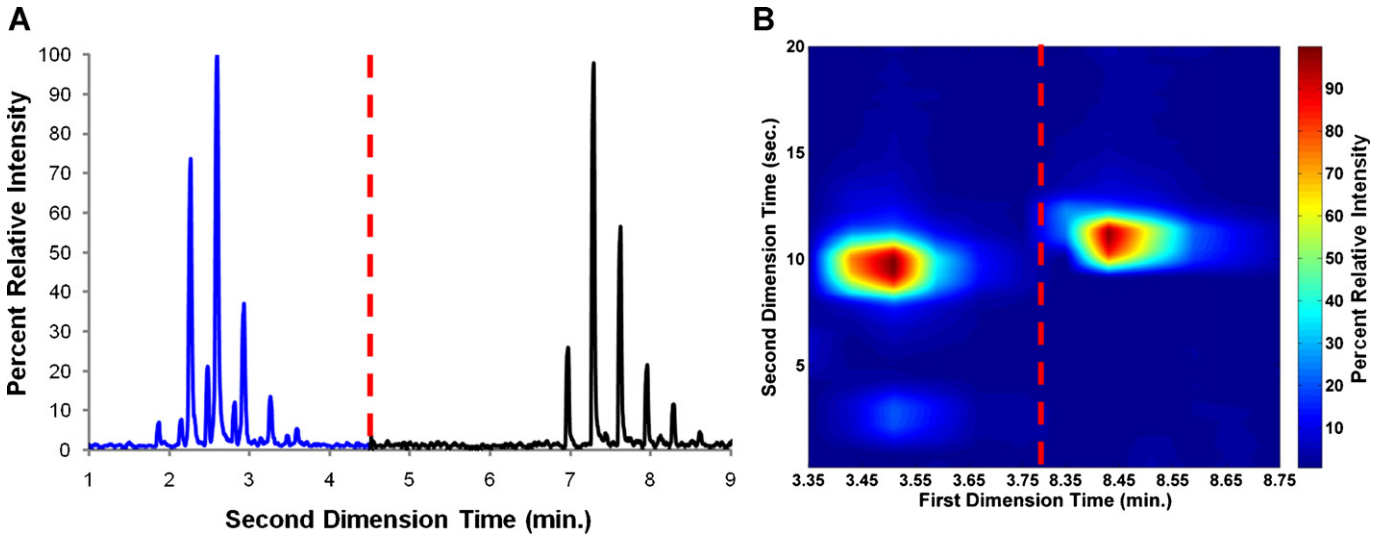

Fig. 8. sLC × LC/MS/MS chromatograms for the separation of cocaine and triclosan in a single analysis. Panel (A) shows sequential 2D chromatograms for cocaine (blue) and triclosan (black). The color change shows the point in time when the mass spectrometric detection conditions were changed. Panel (B) shows the same data as in panel (A), but reformatted into a contour plot to show the peak profiles in two-dimensional coordinates. Here we actually see an impurity resolved from the cocaine peak in the second dimension. The chromatographic conditions are the same as those in Fig. 7 for the analysis of d_3-cocaine in WWTPE, with a few exceptions as described in Section 2.3.6. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

the sampling window can easily be changed in subsequent analyses to ensure quantitative transfer of the analyte to the second dimension. The effectiveness of this change is observed in panel (B) of Fig. 7, where the sampling window in the sLC × LC experiment was changed (18 s delay in the start of the second dimension program) to accommodate the ^{1}D retention shift, resulting in improved alignment of the peak profiles in the DI water and WWTPE samples.

3.4. Analysis of multiple compounds or groups of compounds by sLC × LC

The sLC × LC separation and analysis of cocaine and triclosan is illustrated in Fig. 8. This example demonstrates the utility of the method by allowing multiple regions of overlapped ^{1}D peaks to be resolved by the ^{2}D column. Current limitations to this method still include the finite number (six) of ^{1}D effluent fractions that can be collected and stored. In this case this meant that cocaine had to be collected from the first dimension and separated in the second dimension prior to the elution of triclosan from the ^{1}D column. As a result, with existing valve technology regions of the ^{1}D separation containing poorly resolved compounds must be separated by ca. 2 min OR multiple target compounds must be transferred to the second dimension during one sampling window. Advances in valve technology will undoubtedly increase the flexibility of the sLC × LC approach which is currently limited more by available hardware than by conceptual limitations.

4. Conclusions

In the first paper of this series we demonstrated the ability to capture, store, and re-inject select fractions of ^{1}D effluent with sampling times as short as 1 s in an approach we refer to as 'Selective Comprehensive Two-Dimensional Liquid Chromatography' (sLC × LC). In this paper we set out to demonstrate the practical utility of the sLC × LC approach via a set of three distinct applications:

(1) Selective enhancement of the resolution of a critical pair of opiate compounds previously unresolved by a rapid 1D-LC analysis of a total of nine opiates. The resulting 2D chromatogram shows that both the first and second dimensions of the sLC × LC contribute to the total resolution of the critical pair, and most of the ^{1}D resolution is maintained by rapidly sampling the ^{1}D effluent at 1.5-s intervals.
(2) Targeted analysis of phenytoin in a wastewater treatment plant effluent (WWTPE) extract using sLC × LC with UV absorbance detection and subsequent chemometric data analysis using the previously developed IKSFA-ALS-ssel algorithm. We find that the IKSFA-ALS-ssel approach is very effective for resolving the target analyte peak from closely neighboring interferent peaks, resulting in quantitative results (ca. 35 ppt phenytoin) that are not statistically different from those obtained by 2D-LC/MS/MS in a head-to-head comparison.
(3) Mitigation of ionization suppression effects during the analysis of cocaine in a wastewater extract using electrospray ionization and tandem mass spectrometric detection. The apparent recovery of d_3-cocaine spiked into a 1000-fold concentrated WWTPE extract sample increased from 25 to 70% upon addition of the second dimension of the sLC × LC to an existing 1D-LC separation. A significant matrix effect was observed on the retention time of the target d_3-cocaine peak in both 1D and 2D separations. In the sLC × LC case this shift was revealed by a change in the ^{2}D chromatogram containing the largest d_3-cocaine peak, and a change in the sampling window was made to ensure quantitative transfer of the target peak from the first to the second dimension.

Finally, we show that the resolution of multiple regions of a 1D-LC separation can be enhanced, so long as the time between regions is sufficient to allow analysis of fractions of ^{1}D effluent collected in the first sampling period. The methodology is flexible and allows the use of different ^{1}D sampling times and ^{2}D analysis times in different regions of the ^{1}D separation. Nevertheless, currently the sLC × LC approach is limited far more by the restrictions of existing valve technology than by conceptual limitations. We anticipate that this initial work will drive the development of new valve technologies, which will significantly expand the scope of applications of sLC × LC.

Acknowledgements

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