Journal of Chromatography A, 1217 (2010) 7648–7660

Contents lists available at ScienceDirect

Journal of Chromatography A

journal homepage: www.elsevier.com/locate/chroma

Targeted three-dimensional liquid chromatography: A versatile tool for quantitative trace analysis in complex matrices

Scott W. Simpkins, Jeremy W. Bedard, Stephen R. Groskreutz, Michael M. Swenson, Tomas E. Liskutin, Dwight R. Stoll*

Gustavus Adolphus College, 800 West College Avenue, Saint Peter, MN 56082, USA

ARTICLE INFO

Article history:
Received 13 July 2010
Received in revised form 5 September 2010
Accepted 8 September 2010
Available online 1 November 2010

Keywords:
Multidimensional
HPLC
LC/MS
Trace analysis
Three-dimensional

ABSTRACT

Targeted multidimensional liquid chromatography (MDLC), commonly referred to as 'coupled-column' or 'heartcutting', has been used extensively since the 1970s for analysis of low concentration constituents in complex biological and environmental samples. A primary benefit of adding additional dimensions of separation to conventional HPLC separations is that the additional resolving power provided by the added dimensions can greatly simplify method development for complex samples. Despite the long history of targeted MDLC, nearly all published reports involve two-dimensional methods, and very few have explored the benefits of adding a third dimension of separation. In this work we capitalize on recent advances in reversed-phase HPLC to construct a three-dimensional HPLC system for targeted analysis built on three very different reversed-phase columns. Using statistical peak overlap theory and one of the most recent models of reversed-phase selectivity we use simulations to show the potential benefit of adding a third dimension to a MDLC system. We then demonstrate this advantage experimentally by developing targeted methods for the analysis of a variety of broadly relevant molecules in different sample matrices including urban wastewater treatment effluent, human urine, and river water. We find in each case that excellent separations of the target compounds from the sample matrix are obtained using one set of very similar separation conditions for all of the target compound/sample matrix combinations, thereby significantly reducing the normally tedious method development process. A rigorous quantitative comparison of this approach to conventional 1DLC–MS/MS also shows that targeted 3DLC with UV detection is quantitatively accurate for the target compounds studied, with method detection limits in the low parts-per-trillion range of concentrations. We believe this work represents a first step toward the development of a targeted 3D analysis system that will be more effective than previous 2D separations as a tool for the rapid development of robust methods for quantitation of low concentration constituents in complex mixtures.

1. Introduction

As the fields at the interface of chemistry, biology, medicine, and environmental science continue to advance, so do the demands on the analytical aspects of work in these areas. Such fields often require the detection and quantitation of analytes found both at low concentrations and in complex mixtures, presenting a particularly difficult combination of two already challenging analytical problems. Many solutions to the analytical demands of these fields consist of a separation method (e.g., gas or liquid chromatography, capillary electrophoresis) followed by a sensitive and selective detection method (e.g., mass spectrometry, fluorescence). The selective detector is usually necessary because the chromatographic component of the analysis is unable to adequately separate chemically similar analytes of interest prior to detection [1]. For small organic molecules (less than 1000 Da), the current upper bound on the peak capacity of state-of-the-art liquid chromatography is about 500, for analysis times of a few hours or less [2]. Recent improvements in the robustness of electrospray ionization (ESI) mass spectrometry have accelerated the wide adoption of high performance liquid chromatography coupled with ESI–MS (referred to hereafter as LC/MS). Indeed, in many applications LC/MS is an indispensable tool that literally changes the way experiments are executed, making possible experiments that would otherwise not be conceivable. In particular, the ability to quantify small organic compounds (e.g., pesticides, pharmaceuticals) in complex matrices (e.g., river water, blood) at parts-per-billion (ppb) and parts-per-trillion (ppt) levels with little sample clean-up prior to analysis is now commonplace thanks to LC/MS technologies [3,4]. However, such detection technology is costly, not only in terms of capital

* Corresponding author. Tel.: +1 507 933 6304.
E-mail address: dstoll@gustavus.edu (D.R. Stoll).

0021-9673/$ – see front matter © 2010 Elsevier B.V. All rights reserved.
doi:10.1016/j.chroma.2010.09.023

investment in equipment, but also in maintenance costs that make per-sample analysis costs prohibitively expensive in some cases. In addition, sacrifices are made in terms of the quality of quantitative data as a result of non-linearity of detector response, matrix effects on the detector response, and instability of the detector over time [5,6]. These factors have been a significant motivator for the development of better chromatographic separation methods, including multidimensional chromatography [6–8].

Since the initial theoretical descriptions and experimental accounts of multidimensional chromatographic methods in the 1970s [9–13] the resolving power provided by these methods has steadily increased and the time required (i.e., analysis time) to achieve such performance has steadily decreased. Surprisingly, almost all of these improvements have taken place within the bounds of two-dimensional separations. Multidimensional chromatography methods can be divided into two main groups: comprehensive separations (denoted LC × LC for a two-dimensional separation) are concerned with the separation and quantitation of a large number (tens, hundreds, or thousands) of constituents of a sample [1], whereas 'heartcutting' or 'coupled-column' methods (LC–LC for a two-dimensional separation) are considered targeted methods, usually focused on the separation, and subsequent detection and quantitation, of a few constituents of particular interest from the sample matrix. Research in the area of LC × LC has been particularly active in the past five years or so [14–16] and has progressed to the point that separations of several hundred sample constituents on the 1-h timescale are no longer rare.

Early work on LC–LC highlighted the potential of these methods for targeted analysis in complex matrices. Indeed, Snyder initially discussed the main benefit of LC–LC separations as "their ability to provide sharply increased resolution at specific points in the chromatogram" [9]. In their pioneering work in online LC–LC, Majors and coworkers [11] alluded to the possible use of LC–LC as a "...'universal' LC separation system for aqueous samples." The feasibility of this notion was demonstrated through the quantitative analysis of a variety of target molecules in diverse matrices without prior sample preparation; these included caffeine in urine and blood plasma, with a minimum detectable concentration of 0.5 μg/mL in each matrix. These exciting initial results led to the development of LC–LC methods over the past three decades for the analysis of a variety of compound types including pesticides, proteins, biomarker compounds, and pharmaceuticals in diverse matrices [17–19].

From here on we will refer to 'coupled-column' and related targeted multidimensional methods simply as targeted multidimensional liquid chromatography (MDLC). These techniques have been successful in the analysis of a large variety of trace level targets in complex mixtures. Several review articles have described the versatility of targeted MDLC approaches, and reviewed different instrument configurations and application areas [17–20]. Recent manifestations of the technique have achieved notable selectivity, sensitivity, and quality of quantitation with and without the use of mass spectrometric detection [21]. In many cases the additional resolving power provided by the second dimension of the MDLC system is used as an alternative to extensive sample preparation prior to analysis using conventional HPLC methods [22–24]. In possibly the most extensive scheme to date, method development has been standardized for analyses of a variety of pesticides in water samples using two-column MDLC systems; methods can be developed for one or multiple pesticides chosen from a list of more than 75 compounds, and at least seven of these have been detected at or below 100 ppt [17,25].

Although targeted separations have been quite successful for a number of diverse applications, the methodology arguably has not lived up to the initial promise suggested by the notion of a 'universal analysis system' [11]. A number of factors offer an explanation as to why this 30-year-old idea has not been realized in practice. Published methods currently consist of multidimensional analyses utilizing conditions specific to only one compound or group of compounds. This suggests that these methods were not designed beyond the scope of the necessary analyses, although some have addressed universality within certain classes of compounds [17,25,26]. In addition, while mass spectrometric detection is often required for 2D analyses of the most complex samples [15], its widespread use in the analysis of less complex samples has diminished the perceived benefits of increased chromatographic selectivity in the analysis of more complex samples. The laboriousness of targeted multidimensional method development appears to have deterred many, and few have taken advantage of the ultimate analytical benefits of MDLC for targeted work.

In nearly all existing MDLC methods the potential resolving power of the system is compromised by the specific way in which the separation is executed. For example, the technique of backflushing from one column to the next is often used, which causes losses of potential resolving power due to remixing of previously separated compounds and does not result in a truly multidimensional separation [13,22,24,27]. Others may discourage the use of backflushing [8,25] but perform sample cleanup using a low efficiency first dimension separation, choosing not to resolve analyte groups of interest until the second dimension; this provides for the complete transfer of the analyte group from one dimension to the next but in no way maximizes chromatographic performance [25]. Furthermore, many targeted multidimensional methods have not significantly improved upon Snyder's original conception of coupled-column separations, in which the target analyte is subjected to an initial separation on a short analytical column followed by a final separation on a longer column packed with particles of the same chemistry [9,17,23,25].

As it was conceived 30 years ago by others, our definition of a 'universal analysis system' involves the complete separation of any target analyte from its matrix without the absolute requirement of highly selective detection [11]. Over three decades of work with various 2D separation systems have shown that two dimensions do not provide sufficient resolving power to be used in the "universal" way that we assume Apffel and coworkers must have imagined; this has motivated us to look at the potential benefit of the addition of a third dimension of separation in a system designed for targeted analysis. To our knowledge the most elaborate and powerful 3D separation to date was actually a comprehensive 3D separation, used to demonstrate the separation of hen ovalbumin peptide fragments using size exclusion and reversed-phase chromatography in the first and second dimensions, and capillary zone electrophoresis in the third dimension [28]. Unfortunately, the substantial decrease in peak capacity provided by each successive dimension and the extremely long analysis times (tens of hours) of comprehensive 3D separations have contributed greatly to their current lack of popularity despite the allure of their raw resolving power [29].

A successful three-dimensional MDLC system should also have benefits over one- and two-dimensional systems in situations where ESI-MS detection is desired and/or required. The increased separation power holds the potential to drastically reduce ion suppression effects of coeluting compounds in electrospray ionization [5,6]. In one recent study, Sancho and coworkers [30] demonstrated that a LC–LC separation prior to ESI-MS detection allowed the use of external calibration to analyze a pesticide and one of its metabolites in human serum and urine, with a very linear calibration curve ($r^2 > 0.9995$) from 1 to 100 ppb and recoveries over 87%. A subsequent study gave similar results for another pesticide and a metabolite, although an increase in separation power from the original method was required to achieve acceptable recoveries [31]. Rogatsky and coworkers have thoroughly investigated the effects of

chromatographic selectivity on MS detection, and they have shown both improvements in signal to noise ratio and reductions in matrix effects by replacing the selectivity of the fragmentation process (e.g., triple quadrupole MS) and subsequent multiple reaction monitoring with a second dimension of chromatographic separation in the analysis of polypeptide biomarkers [7]. In some cases, and especially in the analysis of certain classes of biological molecules, the power of a three-dimensional system may also provide the only means of separating isobaric compounds that are indistinguishable by mass spectrometry [32,33].

In this work we describe the development of a system for heartcutting three-dimensional HPLC (h3DLC), and its application in the quantitation of different target compounds present at trace levels in different complex matrices. We have two primary goals in this first report on the h3DLC technique. First, we demonstrate both theoretically and experimentally the exceptional chromatographic resolving power provided by a 3D separation consisting of three very different reversed-phase columns, for a variety of target molecule and sample types, and show that excellent quantitative results are obtained in these different target/sample systems using very similar separation conditions in each case. Second, we demonstrate the benefits of such high resolving power in trace level quantitative analysis using both UV absorbance and mass spectrometric detection. In addition to these primary goals, we also describe a new approach to the 'heartcutting' process that makes the implementation of h3DLC substantially more flexible and simplifies the method development process for MDLC methods.

Substantial advances have been made in both the theory and practice of reversed-phase HPLC since the early development of targeted MDLC several decades ago. In this work we have implemented this knowledge to significantly advance the resolving power and applicability of targeted MDLC methods. The analytical power of h3DLC lies in the utilization of three very different, yet compatible dimensions of separation. In spite of our exclusive use of reversed-phase columns, which provides for three compatible dimensions of separation, the variety of column chemistries used ensures that the separation power of the MDLC system is not compromised. Solvent incompatibility has historically been a limitation in the development of targeted MDLC, as the pursuit of orthogonal multidimensional separations has often led to the pairing of normal- and reversed-phase columns. However, this is not a problem with our approach, as the variety of column chemistries used provides for three sufficiently different mechanisms of separation, all via reversed-phase columns. In the first, second, and third dimensions of our h3DLC system, we have utilized columns with weak cation exchange, carbon clad zirconia (C/ZrO_2), and conventional C18-type functionalities, respectively. Among these, we believe the C/ZrO_2 plays a particularly important role in contributing to this technique's separation power. Fundamental studies comparing the selectivity of carbon-based materials to conventional reversed-phases have clearly shown that the retention and separation mechanisms of carbon phases are different from those of conventional aliphatic bonded reversed-phase materials [34]. Despite these dramatic differences in reversed-phase selectivity, implementation of carbon-based phases has been extremely limited until recently, particularly in targeted MDLC systems. Aside from the work of Shalliker and coworkers incorporating these phases in MDLC analyses of organic oligomers [35], we are aware of only two other instances of targeted 2D methods involving a carbon-based phase [36,37].

To maximize the resolving power of our h3DLC system, all three separations are as independent as possible and operated as efficiently as possible within the constraints of a 3DLC system. In this work we have utilized generic gradient elution conditions in each dimension. When utilizing multiple gradient separations, mobile phase incompatibilities are an issue when transferring a highly retained analyte from one dimension to the next. However, aqueous dilution of the column effluent solves this problem and even allows for on-column focusing [28,38]. This focusing of analytes at the head of the column provides for powerful, high resolution, and independent separations, and it allows one to vary the transfer volume from one dimension to the next depending on the width of the analyte peak.

No initial demonstration of the power of h3DLC can adequately represent the performance of the technique in a wide range of analytical contexts. However, a few judiciously chosen examples begin to show the range of situations where the technique does perform well and give a sense for the ease with which the system is implemented to handle new target compounds in new sample matrices. Several experiments were performed using phenytoin, chlorophene, nicosulfuron, and hydrocortisone (see Fig. 1) as target compounds as a means of validating h3DLC as an analytical methodology. To evaluate its quantitative merits, the results of h3DLC analyses were compared with those of conventional 1DLC–MS/MS analyses of identical samples; we are aware of only one other such rigorous comparison involving a MDLC system [21]. With only minor modification to the separation conditions, we achieved complete or nearly complete separation of the target analytes from their complex matrices. This allowed us to achieve method detection limits in the low ppt range, even with UV absorbance detection. The compounds and respective sample matrices employed in this study represent relevant analysis problems in environmental, medical, and biological fields of research. The presence of pharmaceuticals and pesticides in surface water has raised concerns over potential effects on drinking water supplies and ecosystems [39]. Phenytoin has been a commonly prescribed anticonvulsant over the last half century; its excretion and improper disposal through sewer systems over an extended period of time renders it a relevant target to analyze in wastewater treatment plant effluent. The minor use of the bactericide chlorophene in personal care products results in very low concentrations in urban wastewater; we have chosen this target/sample combination to challenge the sensitivity of the h3DLC technique. The sulfonylurea herbicide nicosulfuron is an important target for analysis in surface water because of its current use (over 90,000 kg/year on corn) in local Minnesota agriculture [40], and because its high solubility in water presents a challenge to a MDLC system composed entirely of reversed-phase columns. Detection of hydrocortisone in urine is critical to the enforcement of anti-doping rules [41], and this compound can also be used as a biomarker for human stress response [42]. These analytes have been previously analyzed in the respective matrices utilized here. Specifically, method quantitation limits of 55 ppt [43], 5 ppt [44], and 5 ppb [45] have been reported for phenytoin, chlorophene, and hydrocortisone, respectively, and an instrument quantitation limit of 24 ppt has been reported for nicosulfuron [46].

There is much still to be explored within the rapidly growing area of multidimensional chromatography. We believe the implementation of a three-dimensional MDLC system composed of three efficient and functionally diverse reversed-phase separations represents a major step toward achieving a "universal analysis" for targeted analysis of organic compounds in aqueous samples. The use of carbon-based stationary phases with vastly different selectivities compared to other reversed-phase materials holds enormous potential for the development of reversed-phase multidimensional separations amenable to the analysis of polar, structurally similar, and other difficult to resolve compounds. We view this work as a first step in the demonstration of the utility of targeted 3DLC to simplify targeted analysis of a wide range of compounds relevant to current and future research in a variety of scientific fields.

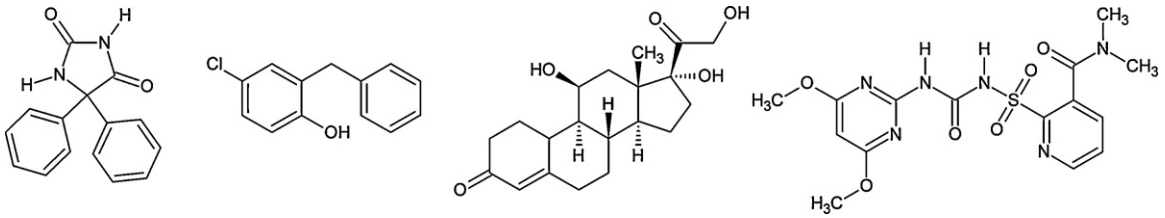

Fig. 1. Structures of the target compounds used in this work. From left to right: phenytoin, chlorophene, hydrocortisone, and nicosulfuron.

2. Theory

The theoretical advantages of comprehensive multidimensional separations have been articulated well for several decades. In this case the primary metric of separation performance is the peak capacity of the separation (n_c), where n_c represents the number of peaks that can be fit side by side (given some specified resolution, usually taken as unity) into the separation space. Here the great value of a two-dimensional (2D) separation lies in the fact that the peak capacity of a 2D separation is approximated by the product (not the sum) of the peak capacities of the individual one-dimensional (1D) separations that constitute the 2D separation [13,47]. Unfortunately the peak capacity concept is not very useful in targeted analysis, because the analyst really does not care what happens in the portions of the separation space that do not contain the analyte of interest. We propose that in this case a more useful metric is the probability (P_1) that a target analyte will be separated from the sample matrix and appear as a pure, single-constituent peak. We rely on the well developed Statistical Overlap Theory (SOT) of Davis and Giddings [48] to calculate the improvement in this probability of separation as additional dimensions of separation are added. Fig. 2 shows the chromatogram for a simulated 1D separation of 1000 detectable constituents from a complex sample, using a single channel detector (i.e., one wavelength in UV absorbance, or one m/z channel in MS detection). Here the retention times of individual constituents are randomly assigned (Poisson distribution), as are their peak heights (concentrations, from an exponential distribution). The peak widths are set such that the peak capacity of the simulated chromatogram in panel A is 100. SOT allows us to calculate P_1 for the 1D separation as a function of the number of detectable sample constituents, m, and the peak capacity of the separation, n_c, using Eq. (1) [48]:

$$P_1 = \exp\left(\frac{-2m}{n_c}\right) \quad (1)$$

The choice of parameters here is deliberate, in the sense that they are realistic estimates of what is encountered in practice in the analysis of biological mixtures, for example. We find that for a sample containing 1000 detectable constituents, the probability of observing a target analyte as a pure peak in a separation with a peak capacity of 100 is just 2×10^{-7}%; for analytical chemists and all users of chromatography this a devastating result, and shows just how limited conventional 1D separations are for the analysis of complicated samples [49]. Even if one supposes that a peak capacity of 400 can be achieved using state-of-the-art separations at high pressure (>1000 bar) and long analysis times, P_1 only improves to 0.7%. Surely this situation can be improved by adjusting the position of the target analyte through changes in the selectivity of the chromatographic separation; however, for very complex samples this merely results in a rearrangement of peak patterns and does not significantly improve P_1. We then continue the simulation by pulling out the retention coordinates of the constituents eluting between the two dashed vertical lines in Fig. 2A, and simulating the subsequent separation of these constituents using a second separation device whose retention characteristics are not at all related to the first dimension separation. We see in Fig. 2B that the separation is now greatly simplified (m is reduced to 69, because only a fraction of the sample is transferred to the second separation), however the target analyte is still not resolved from the simplified sample matrix. Assuming a peak capacity of 50 for this second dimension separation, we find that P_1 is improved dramatically to 6.3%. Because there are regions of the second dimension chromatogram that are not very crowded with other peaks, there is at least a chance, albeit small, that the target compound will be resolved as a pure, single-constituent peak. If we repeat the fraction transfer and separation process one last time, again assuming a peak capacity of 50 in the third dimension (m is further reduced to 5) we find that P_1 again improves dramatically to 82%, a result consistent with Fig. 2C where much of the baseline is actually unoccupied, leaving ample room for resolution of the target analyte from a relatively simple mixture.

It is worth noting here that Fig. 2 emphasizes the fact that when non-selective detection is used for targeted analysis in a complex matrix, detection limits are dictated more by our inability to separate the target compound from the sample matrix (background signal) than the inability of the detector to provide a response to the target of interest that is significantly higher than the detector noise level.

The impact of adding second and third dimension separations to a separation system for targeted analysis as discussed above and depicted in Fig. 2 is striking, however this assumes that we have at our disposal three sufficiently different separation devices so that retentions of the sample constituents on one device are not related to the retentions of those constituents on the second and third devices. In practice, differences of this degree are not found, however some systems do exhibit differences that are large enough to be useful. This especially occurs when very different separation modes are combined, for example in the early work of Apffel and coworkers [11] combining size exclusion chromatography (SEC) and reversed-phase HPLC (RPLC), or in later work combining RPLC and normal-phase HPLC (NPLC) [14]. In our work we have focused on the development of a 3D separation system composed of separation media whose separation mechanisms are at least characterized as reversed-phase, even though all three materials exhibit at least some mixed-mode retention behavior. This type of system, if successful, avoids the major solvent incompatibility issues encountered in other systems (e.g., NPLC × RPLC), and the low separation efficiencies characteristic of SEC, for example. Given our extensive experience with carbon-based phases for RPLC, we naturally turned to one phase of this kind (ZirChrom-CARB) for inclusion in the 3D system because these phases are known to exhibit extreme differences in selectivity compared to other RPLC phases under RPLC conditions [34]. To the best of our knowledge, carbon-based media have not been employed in MDLC separations, with the exception of own work, the 2D separation by Thiebaut and coworkers, and that of Shalliker and coworkers [16,35,36,48,50,51], as discussed above. A natural second choice of materials was a typical aliphatic bonded phase based on silica (Zorbax Eclipse XDB C8 here), because of the excellent peak shapes and separation efficiencies that can be obtained with these mate-

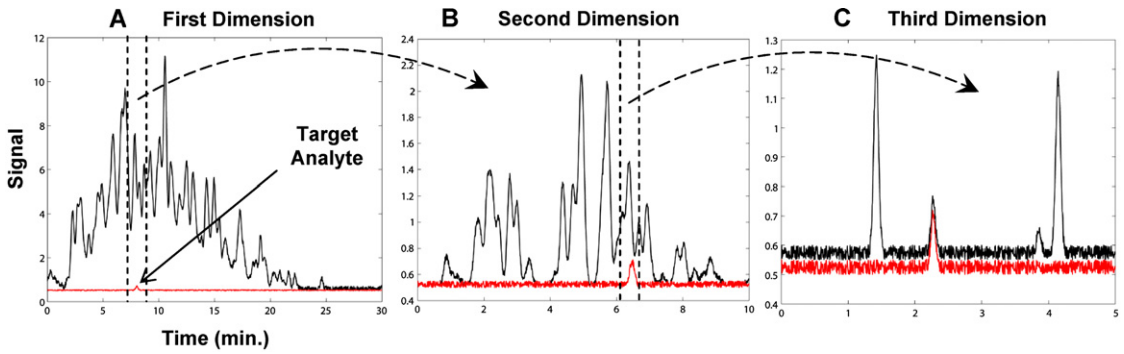

Fig. 2. Simulated h3DLC separation of a target analyte (pure peak (—-), chosen at random) from a matrix of 1000 constituents, with non-selective detection. Constituent retention times and intensities are randomly assigned from Poisson and exponential distributions, respectively. Peak capacities of first, second, and third dimension separations are 100, 50, and 50, respectively, which are easily achieved in the times shown using modern equipment.

rials. Finally, our choice of the third material was guided by the notion that many biological samples (e.g., river water, urine) contain ionizable compounds, and that a reversed-phase material that exhibited selectivity for ionizable compounds would be useful. In this case we chose a silica-based material [52] functionalized first with a highly aromatic polymer network, followed by modification with C8 aliphatic groups and carboxylic acid groups (referred to hereafter as HC–COOH). When used above ca. pH 5 this material exhibits both reversed-phase and cation-exchange characteristics due to the presence of the deprotonated carboxylate functional group.

Experimental separations of real mixtures using a 3D system built around these three columns are shown below and are the ultimate measure of the usefulness of this combination of columns, but it is instructive to examine differences between these columns using existing methods of characterizing RPLC selectivity. One widely used approach is the so-called Hydrophobic Subtraction Model (HSM) of reversed-phase selectivity developed by Snyder and Dolan and coworkers [53]. In this approach RPLC columns are characterized by fitting experimental retention data for 16 carefully chosen solutes to a 5-parameter linear model, where the five parameters (H, S, A, B, and C, below) represent contributions to retention arising from interactions between the solute and stationary phase and are related to hydrophobic interactions, steric effects, hydrogen bond acidity and basicity, and Coulombic interactions. One particularly useful outcome of this model in the context of our work is that comparisons of the selectivities of two RPLC columns can be made if the five column parameters described above are known for a pair of phases of interest. The so-called F_s factor (see Eq. (2)) is a measure of the 'distance' between the two columns in question in a five dimensional selectivity space:

$$F_s = ([12.5(H_2 - H_1)]^2 + [100(S_2 - S_1)]^2 + [30(A_2 - A_1)]^2 + [143(B_2 - B_1)]^2 + [83(C_2 - C_1)]^2)^{1/2} \quad (2)$$

Snyder and coworkers state that a F_s less than 3 indicates that a pair of columns is sufficiently similar to justify an attempt to use one column in place of the other for a particular separation, with the expectation that small differences in relative peak spacing will be observed when using the alternate column. There is no theoretical upper bound (the lower bound is zero) to F_s, but given Eq. (2) it is evident that a larger F_s value indicates a larger difference between the selectivities of the pair of columns in question. Column parameters for 476 RPLC columns, including three zirconia-based phases are publicly available [54], however ZirChrom-CARB and the HC–COOH phase described above are not among them. Using parameters for the HC–COOH phase measured by Zhang and Carr [52], and parameters for the ZirChrom-CARB phase measured by us (unpublished results) using the protocol established by Snyder and coworkers [53], we have calculated the F_s values shown in Table 1 for the three columns used in this work. It is important to note that we have used the C-term (for cation-exchange contributions to retention) determined at pH 7 for the HC–COOH phase since we have used it at pH 6 where the carboxylate functional groups are nearly fully deprotonated.

Table 1
Selectivity comparisons for the three columns used in this work.

Column pair	F_s value
HC–COOH/ZirChrom-CARB	178
HC–COOH/XDB C8	143
ZirChrom-CARB/XDB-C8	261

Calculated using Eq. (2), solute parameters published by Snyder and coworkers (XDB-C8 [51]) and Zhang and Carr (HC–COOH [50]), and us (unpublished, ZirChrom-CARB).

These F_s values clearly indicate that the three columns used here are very different, however a brief discussion of the parameter space is useful here to get a sense for how different these columns may be. First, it is important to qualify this discussion by saying that the fit of the experimental ZirChrom-CARB data to the HSM model is not good, and thus we limit our discussion of differences between these phases to qualitative interpretation and in no way suggest that good quantitative estimates of retention can be obtained for the ZirChrom-CARB phase using this model. When F_s values are calculated for all possible pairs of 476 silica-based phases in the dataset, we find that the maximum F_s value is 437; thus, the values in Table 1 in the range of ca. 150–250 correspond to some of the most different column pairings we see. We also note that the smallest F_s value observed for the ZirChrom-CARB paired with any other column in the expanded dataset including zirconia-based phases and the HC–COOH phase is 178, which happens to correspond to one of the pairs of columns used in this work.

Another useful aspect of the HSM is that parameters have also been tabulated for 90 low molecular weight compounds [55,56] which allows the prediction of retention for these 90 solutes on 476 different RPLC phases. Here we are concerned with the ability of the three columns described above to distribute the low molecular weight constituents of the samples we have chosen over the three-dimensional separation space such that the target analytes in these samples are well separated from the sample matrix. Fig. 3 shows the predicted distribution of 89 of compounds in the Snyder–Dolan solute set (picric acid was excluded due to its exceptionally high k' that skews the entire plot) over a three-dimensional separation space, using the three columns chosen for this work. We see that there is extensive dispersion of the compounds along all three axes of the plot, which is required for the enhancement of separation due to additional dimensions of separation. This plot suggests that

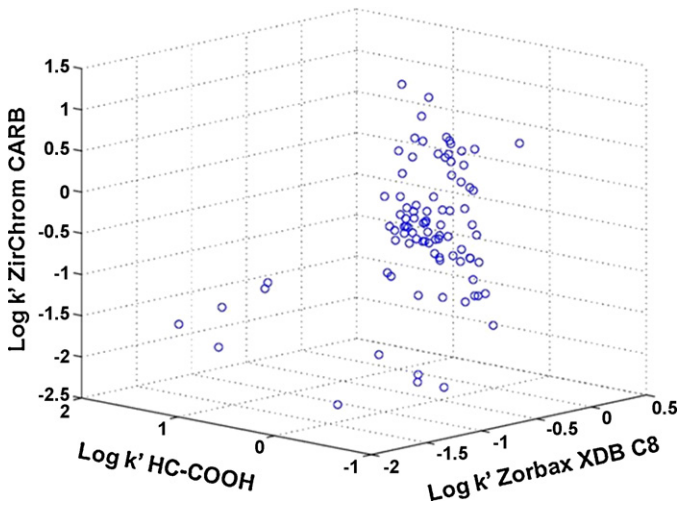

Fig. 3. Three-dimensional selectivity plot showing the predicted retention factors of the 89 (picric acid is excluded because its large k' skews the entire figure) functionally diverse small molecules initially used in the development of the Hydrophobic Subtraction Model of reversed-phase selectivity by Snyder and Dolan [53]. Although there is some clustering of the data along the diagonal in the x–y plane in particular, the overall dispersion of the data suggests that these three reversed-phases are sufficiently different to be useful in a three-dimensional separation system.

separations based on these columns will not fully utlilize the three-dimensional space, however we believe this is due more to the limitations of the HSM in representing the chemical diversity of real samples than it is due to the similarities of the selectivities of these columns. The experimental data below will show that these three columns are certainly different enough to be useful in this application.

3. Materials and methods

3.1. Reagents

Standard solutions of the target analytes were prepared by dissolving first in acetonitrile, then diluting to the desired concentration with deionized (DI) water. Phenytoin was from Sigma–Aldrich (St. Louis, MO); Chlorophene was from SPEX Certiprep Group (Metuchen, NJ); Hydrocortisone was from Sigma–Aldrich; Nicosulfuron was from Chem Service (West Chester, PA). Acetonitrile was LC/MS grade, obtained from Fisher Scientific (Fair Lawn, NJ). Phosphoric acid was from Fischer Scientific, and ammonium acetate and formic acid were from Sigma–Aldrich. DI water was obtained from an in-house Millipore (Billerica, MA) water purification system, and was used without further treatment. All mobile phases were degassed prior to use either by vacuum degassing or sparging with helium.

3.2. Sample preconcentration

Samples of wastewater treatment plant effluent (St. Peter, MN), human urine, and Minnesota River water (St. Peter, MN) were all preconcentrated by Solid Phase Extraction (SPE) prior to analysis. SPE columns were prepared in house by slurrying 0.25 g of HLB SPE particles (40–60 μm, Supelco, Bellefonte, PA) in about 5 mL of isopropanol, and transferring to an empty 6 mL polypropylene SPE tube fritted with a 20 μm polyethylene frit. The details of the sample loading, elution, and post-SPE treatment processes varied slightly and are given below. No attempt was made in any case to selectively preconcentrate the samples; the goal of this step was simply to prepare a very complex sample matrix to challenge the separation power of the h3DLC system.

3.2.1. Wastewater treatment plant effluent (St. Peter, MN, sampled September 2009)

A total of 16 L of effluent was preconcentrated in 1 L portions, first filtering through a 0.45 μm nylon membrane filter, then pulling it through the SPE cartridge at approximately 20 mL/min. using house vacuum. The SPE bed was then dried by pulling air through for 15 min, followed by elution with a three 1-mL portions of 98/2 MeOH/ammonium hydroxide. The volume of the eluted sample was decreased to about 200 μL by vacuum centrifugation at 50 °C for 20 min. The remaining sample was diluted to 1 mL using deionized water to give a final preconcentration factor of 1000-fold. The reconstituted sample was stored at 4 °C until analysis. Spiked extract samples (phenytoin and chlorophene) were prepared immediately prior to analysis.

3.2.2. Human urine

A 100 mL sample of urine from a healthy male volunteer was collected over approximately a 36-h period. This sample was processed in 10 mL portions, using the approach described above. The final sample was brought to a total volume of 10 mL using deionized water to give a final preconcentration factor of 10-fold. The reconstituted sample was stored and spiked (hydrocortisone) prior to analysis as described above.

3.2.3. Minnesota River water (St. Peter, MN, sampled April 2010)

A total of 16 L of river water was processed using the same approach as described above for the wastewater treatment plant effluent. Here again the final preconcentration factor was 1000-fold, and the extract sample was stored and spiked prior to analysis (nicosulfuron) as described above.

3.3. Instrumentation

3.3.1. Conventional 1DLC separations

Conventional 1DLC separations were carried out using standard equipment involving one of two systems. In each case all instrument modules were from Hewlett Packard (Palo Alto, CA) or Agilent Technologies (Santa Clara, CA), with the exception of the triple quadrupole mass spectrometer (Model 320) from Varian Inc. (Palo Alto, CA). The first system was composed of a HP1050 quaternary pump, HP1050 autosampler equipped with a 900 μL syringe plunger and 400 μL sample loop, and a G1312 variable wavelength UV absorbance detector. The second system was composed of a G1314 binary pumping system, G1313 autosampler (100 μL sample loop), and the same G1312 variable wavelength UV detector as above. There was no preference for either system in this part of the work, except that the first system allowed large injection volumes to be used. The 1DLC–UV separations were carried out using a serially coupled pair of 150 mm × 4.6 mm i.d. Zorbax XDB-C8 (3.5 μm) columns. The 1DLC–MS/MS analyses of HC in urine were carried out using just one of the 15 cm XDB columns. Detailed chromatographic conditions accompany the discussion of results below, as they varied slightly from sample to sample.

3.3.2. Targeted 3DLC separations

A simplified schematic of the instrument configuration used for the h3DLC separations in this work is shown in Fig. 4. All three pumps (I, II, and III below) were HP1050 quaternary pumps (Hewlett Packard), the auto-injector was the HP1050 module described above with the 900 μL syringe plunger, Detector I was the G1312 (Agilent) variable wavelength UV detector described above, and Detectors II and III were G1315 (Agilent) photodiode array UV detectors. A G1316 thermostated column compartment (Agilent) was used to preheat the first dimension eluent and column at 40 °C. The eluents in the second and third dimensions were preheated by passing the eluent through a short (ca. 5 cm) length of stainless

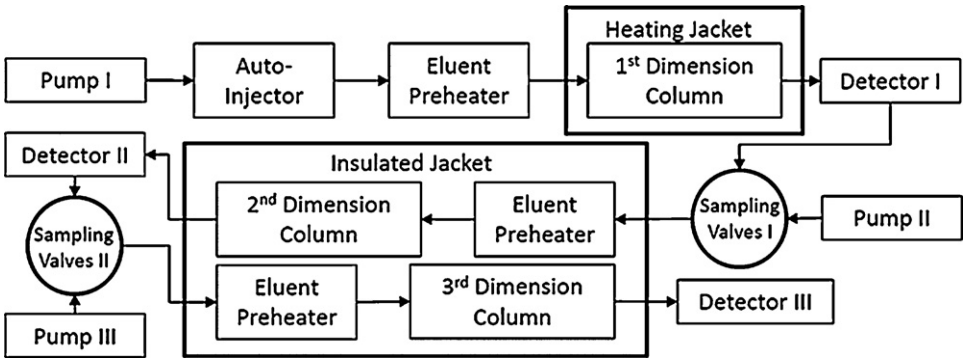

Fig. 4. Schematic of instrument configuration for targeted three-dimensional HPLC. Pumps I, II, and III are all capable of delivering solvent gradients to the three HPLC columns. Detectors I, II, and III are all UV absorbance detectors; in the case of the hydrocortisone analysis, a triple quadrupole mass spectrometer was connected to the outlet from detector III for h3DLC–MS/MS. Two pairs of six-port valves, indicated by 'sampling valves' I and II are used to capture portions of target analyte peaks from the first and second dimension column effluent streams and transfer those fractions to the second and third dimension columns for further separation.

steel connecting tubing immersed in a water jacket thermostated at 40 °C. The second and third dimension columns themselves were not actively heated but were insulated using 1 in. thick polystrene foam to maintain a consistent column temperature of approximately 40 °C. The sampling valves (I and II) positioned between dimensions I and II, and II and III were comprised of pairs of six-port valves and two 75 μL sampling loops made of 0.010″ i.d. PEEK tubing. The configuration of these pairs of valves for the purpose of sampling in MDLC was described in detail previously [35,57]. The total volume of first or second dimension effluent transferred to the second or third dimension columns was controlled by the number of 75 μL fractions transferred, in six-second intervals. This approach is very flexible, allowing the total transfer volume to be changed using the instrument control software rather than having to change the instrument hardware, while keeping the effective gradient delay volume of the fluid path between pump and column reasonable. Finally, the effluent leaving detectors I and II was diluted with water at a flow rate between 400 and 700 μL/min (depending on the particular analysis) to allow effective focusing of target analytes at the inlets of the second and third dimension columns [28,38]. These water dilution streams were delivered using two Varian 212LC single channel pumps (Varian, Inc., Palo Alto, CA).

Individual instrument modules were either controlled by Chemstation Software (Agilent, A.08.03) or Varian MS Workstation (Varian, Inc., rev 6.9.3). The coordination of the timing of the different modules and fraction transfer between dimensions was controlled by simple LabView (National Instruments, Austin, TX, rev. 8.5) code written in-house and a USB PC interface (USB-6009).

The chemistries of the HPLC columns used in the h3DLC system were described above, but their dimensions are given here. In each 3D separation a 50 mm × 4.6 mm i.d. (5 μm) HC–COOH column was used (see Section 2 for details, this is a mixed-mode RPLC/weak cation exchange phase). All second dimension separations involved a carbon-clad zirconia phase (1.3%, w/w carbon, 50 mm × 4.6 mm i.d., 3.0 μm) from ZirChrom Separations, Inc. (Anoka, MN). All third dimension separations involved a C18 type RPLC column; in the case of the separation of nicosulfuron from river water extract a 150 mm × 4.6 mm i.d. (2.7 μm) Ascentis Express C18 column (Supleco, Bellefonte, PA) was used, whereas all other separations involved a 150 mm × 4.6 mm i.d. (3.5 μm) Zorbax XDB-C8 column. The Ascentis Express column was used in the third dimension for the nicosulfuron analysis (the most recent work) in anticipation of using these highly efficient columns based on fused-core particle technology [58] for the C18 type phases in our future work.

3.3.3. Chromatographic conditions

Detailed chromatographic conditions are given below accompanying the discussion of results since small changes were made depending on the target analyte and sample matrix. The general scheme in the case of the h3DLC separations involved the use of an acetonitrile gradient in all three dimensions, beginning with a weak enough eluent to allow focusing of analyte from the large injection volumes used (100–360 μL), and ending with an organic-rich eluent to flush strongly retained sample constituents from the column prior to subsequent analyses (typically a 10–100% gradient). A pH 6 ammonium acetate buffer was used in the first dimension to enhance the cation exchange component of the selectivity of the HC–COOH column. A pH 2 phosphoric acid buffer was used in the second dimension with the exception of the nicosulfuron analysis in which a significant cation concentration was needed to elute the target analyte from the ZirChrom-CARB phase. Finally, either a pH 2 phosphoric acid buffer (UV detection) or a pH 3 formic acid buffer (MS detection) was used in the third dimension. Here we stress the fact that although small changes were made to these general conditions depending on the analysis at hand, the same basic set of columns and conditions were used for all analyses, producing excellent separations without extensive effort devoted to method development.

An example of the flow and solvent profiles used in the three dimensions of the 3D system is shown below (Fig. 5) for the case of the analysis of phenytoin and chlorophene in wastewater treatment plant effluent extract. This figure effectively conveys two important points about the execution of the 3D separations. First, it is evident that there is some amount of overlap in time (that is, they are operated partially in parallel) between the execution of the three separations. In other words, the second dimension separation is started as soon as the first target analyte is transferred from the first to the second dimension column, and likewise for the third separation. This means that the effective analysis time of the entire 3D method is considerably shorter than it would be if the three separations were executed in a strictly serial fashion. The effective analysis times for the three different h3DLC separations discussed below are between 8 and 15 min. Second, it is important to point out that the flow rate is changed in all three dimensions during the method. Typically the flow rate is high initially to ensure efficient flushing of the sample transfer loops that capture analyte fractions and deliver them to the next column, and to quickly flush the gradient delay volume of the pumping systems (ca. 1 mL for the HP1050 pumps). During elution of the target analyte from the first and second dimension columns the flow rate is dropped to 0.35 mL/min so that the total flow (including dilution) of effluent entering the sample transfer loops does not exceed their volume

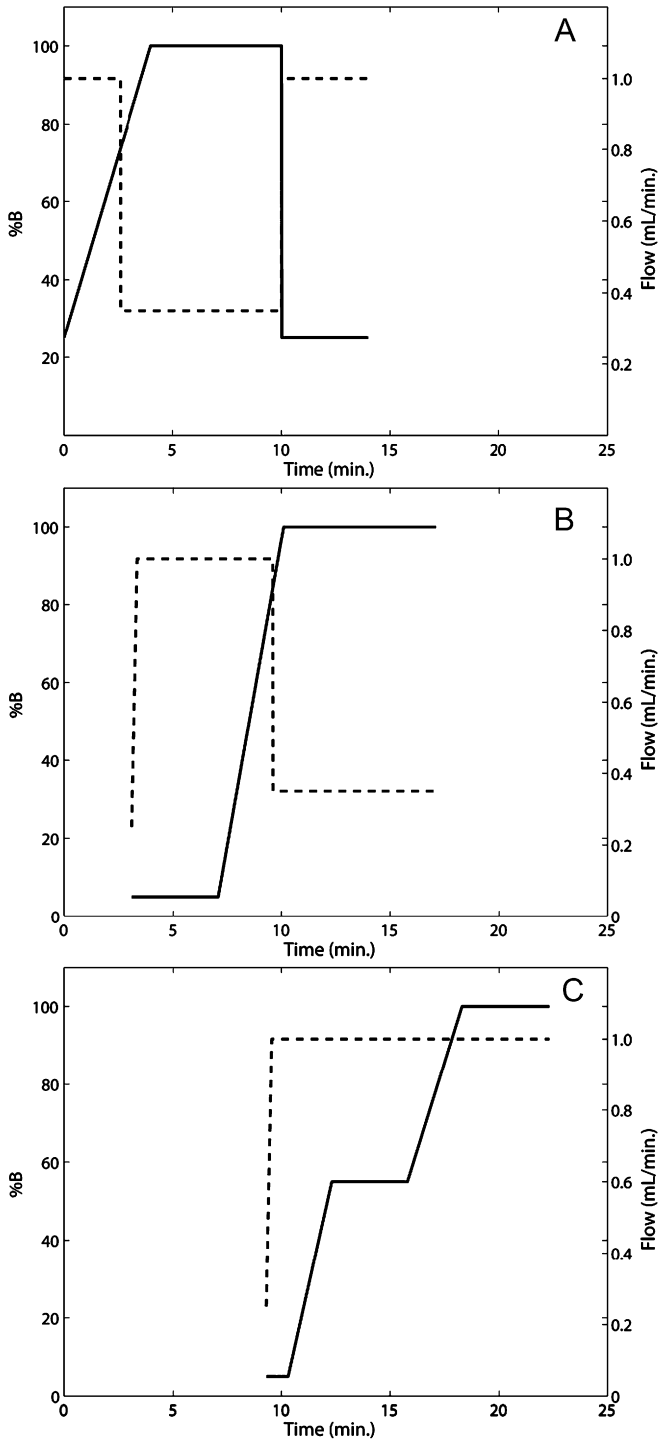

Fig. 5. Flow rate (. . .. . .) and mobile phase composition (—) profiles used in the three dimensions (A, B, C for first, second, and third dimensions) of the h3DLC system for the analysis of phenytoin and chlorophene in WWTP effluent (see Fig. 7). The time axis represents time relative to an injection at time zero in the first dimension; here it is evident that the three dimensions of separation are not strictly serial, which improves the total analysis time. Flow rates in the first and second dimensions were dropped to 0.35 mL/min. during transfer of the target analyte peak to the next dimension to minimize peak elution volume, whereas the flow rate during the rest of the analysis was increased to 1 mL/min. to maintain reasonable gradient delay and system volume flush out times.

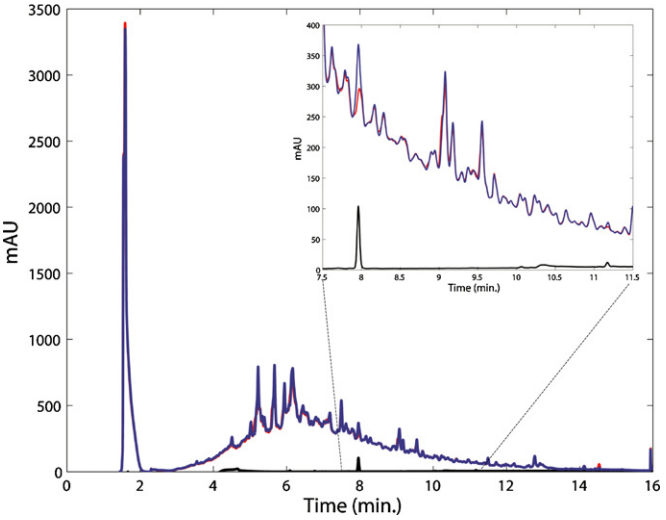

Fig. 6. 1DLC–UV separation of 1000-fold concentrated wastewater treatment plant effluent. The elution times of phenytoin and chlorophene are shown in the black trace (ca. 8.0 and 11.2 min.), for a sample of DI water spiked at 500 and 50 ppb, respectively. The red trace is the raw extract sample, and the blue trace shows the separation of the extract with phenytoin and chlorophene standards spiked in at 500 and 50 ppb after the sample pre-concentration step. *Chromatographic conditions*: 300 mm × 4.6 mm i.d. Zorbax Eclipse XDB-C8, 5 μm; flow rate, 2.25 mL/min.; injection volume, 360 μL; gradient elution from 5 to 100% acetonitrile from 0 to 14 min, with 10 mM phosphoric acid buffer as the aqueous component of the eluent; 210 nm absorbance detection; column temperature, 40 °C.

during the six-second transfer time interval. This evidently complex aspect of the methods would nearly be eliminated if pumping systems with lower gradient delay volumes could be used; indeed, this is a high priority in our ongoing work.

3.3.4. Mass spectrometry

Mass spectrometry analyses were conducted on the Varian Model 320 electrospray ionization triple quadrupole mass spectrometer described above. Phenytoin was detected in the negative mode at (−) 4500 V. Nitrogen was used as the nebulizing gas and was set to 55 psi; the drying gas was nitrogen at 200 °C. Quantitation of phenytoin was based on single reaction monitoring (SRM) and the transition from m/z 251.0 → 101.7. Hydrocortisone was detected in the positive mode with the electrospray needle set to (+) 5000 V. The remaining mass spectrometer settings were identical to the phenytoin analyses. Quantitation of hydrocortisone was based on SRM and the transition from m/z 363.5 → 120.7.

4. Results and discussion

4.1. Qualitative comparisons of 1D and 3D separations

4.1.1. Separation of phenytoin and chlorophene from wastewater effluent extract—UV detection

A 1DLC–UV separation of a 1000-fold concentrated sample of wastewater treatment plant (WWTP) effluent containing the target compounds phenytoin and chlorophene is shown in Fig. 6. The black trace shows the separation of phenytoin and chlorophene spiked in deionized (DI) water at 500 and 50 ppb (equivalent to original concentrations of 500 and 50 ppt in a sample that is concentrated 1000-fold). The red trace shows the neat WWTP extract, and the blue trace shows the WWTP sample spiked with phenytoin and chlorophene at the same levels as in the DI water sample. This overlay shows that while there is ample signal for both the phenytoin and chlorophene peaks above the background in the DI water sample, there is no chance for accurate quantitation in the WWTP sample because the peaks for the target compounds are severely overlapped with peaks for other constituents of the

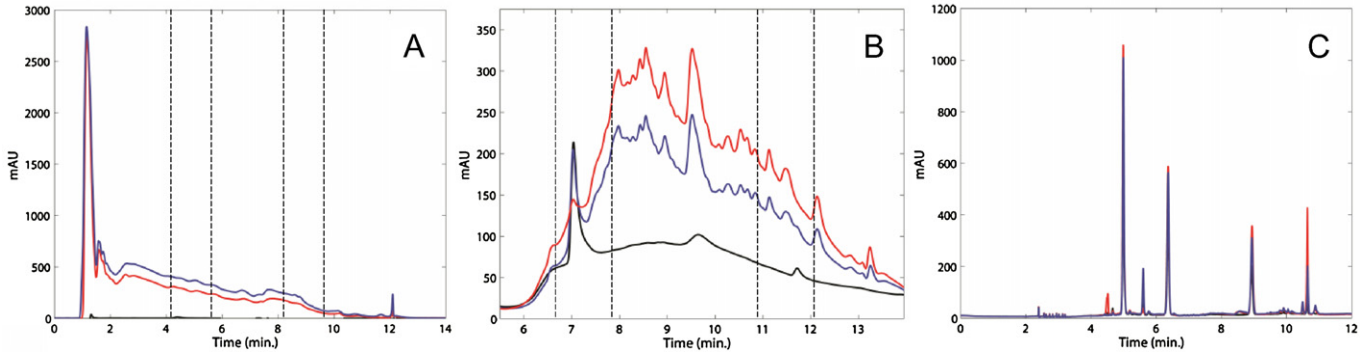

Fig. 7. Chromatograms observed at the outlet of each dimension of separation in the 3DLC/UV system for the separation of the 1000-fold concentrated WWTP effluent sample. Detailed flow rate and solvent gradient profiles for the three dimensions are shown in Fig. 4. The HC–COOH, ZirChrom-CARB, and Zorbax XDB-C8 columns are used in the first, second, and third dimensions, respectively, and acetonitrile/buffer gradients are used in each dimension. The buffers used in the first, second, and third dimensions were 10 mM ammonium acetate at pH6, 10 mM phosphoric acid, and 10 mM phosphoric acid. The injection volume was 360 μL, and all column temperatures were 40 °C. Absorbance detection at 210 nm is used in each case; the gradient background is subtracted from each first dimension chromatogram to minimize the visual effect of the strong absorption of the acetate buffer. Chromatograms are color coded in the same manner as in Fig. 6.

sample. The results of h3DLC–UV separations of the same samples described above in Fig. 6 are shown in Fig. 7. The elutions of the phenytoin and chlorophene peaks from the first dimension column (panel A, 4.4 and 9.0 min) are completely obscured by the elution of many other compounds during the same times. Portions of first dimension effluent were transferred to the second dimension column during the times bracketed by the pairs of dashed lines shown in panel A. Panel B shows the chromatograms obtained at the outlet of the second dimension column, where we see that the maximum absorbance signal is significantly reduced from 2000 to 300 mAU. It is difficult to say that fewer peaks are observed in panel B compared to panel A because there is so much overlap in A. However, the decrease in the overall magnitude of the absorbance signal is strong evidence that the sample initially injected into the first dimension has been simplified considerably upon transfer of select portions of effluent into the second dimension column. Here the elution times for phenytoin and chlorophene from the second dimension column are 7.0 and 11.7 min, respectively. Although the sample was cleaned up by the first dimension column and further separated by the second dimension column, there still is not enough separation at this point to achieve reasonable quantitation of the target compounds using a non-selective detector. Fractions of second dimension effluent were again transferred to the third dimension column during the times bracketed by the pairs of dashed lines shown in panel B. Finally, panel C shows the chromatogram obtained at the outlet of the third dimension column. This chromatogram is significantly less crowded than either of the chromatograms from the first or second dimension separations, and is dominated by the presence of the four retention alignment marker peaks (benzyl alcohol, m-dinitrobenzene, toluene, and tetramethylbenzene) at approximately 5.0, 6.3, 9.0, and 10.7 min. With the exception of these marker peaks, the dominant feature of the chromatogram is actually the phenytoin peak at 5.6 min. This is an indication of the extent to which the complicated sample is cleaned up after separation by three very different dimensions of the 3D system.

Fig. 8 provides a different view of Fig. 7C with the scales expanded to focus on the separation of the phenytoin and chlorophene peaks from the sample matrix. The baseline resolution of the phenytoin peak from the sample matrix is especially impressive. Despite the transfer and injection of about 2 mL of second dimension effluent to the third dimension column, symmetrical narrow peaks are observed for both phenytoin and chlorophene which speaks to the significance of the effluent dilution step between dimensions and the analyte focusing that takes place early in the gradient elution program in the third dimension.

Although the phenytoin peak in particular appears to be well resolved from sample matrix as shown in Fig. 8, we realize that there is no way, using UV detection, to prove that the phenytoin peak is pure (although the spectral consistency across the peak looks good as well; data not shown). To assess the quantitative accuracy of this h3DLC–UV method for phenytoin in the WWTP effluent matrix we have carefully compared the quantitative results to those obtained from a conventional 1DLC–MS/MS method which we perceive to be the 'gold standard' technology for this type of determination. At this point we refer the reader to the section on quantitation below for this comparison, and continue in this section with more qualitative comparisons of 1D and 3D separations for other target analytes in other sample matrices.

4.1.2. Separation of hydrocortisone from human urine—UV detection

One of the significant potential advantages of the 3D separation approach described here over conventional 1D separations is that the extreme separation power afforded by the 3D approach should minimize the need for extensive method development to resolve target compounds from the sample matrix of interest. As a first step in assessing the practical utility of this notion, we attempted to separate hydrocortisone from a 10-fold concentrated sample of human urine. As with the analysis of phenytoin in WWTP extract, we made no attempt to selectively pre-concentrate the sample, only to concentrate it to the point where the target compound was readily detected above the detector background noise using UV absorbance detection. A detailed description of the chromatographic conditions used for these separations is given above in the experimental section; the conditions used in this case were nearly identical to those used for phenytoin. The buffers, organic solvents, and gradient elution profiles used in all three dimensions were similar to those used for phenytoin (Fig. 7), with adjustments made only to position the hydrocortisone peak during the gradient elution to minimize the total analysis time of the 3D separation process. Fig. 9 shows a comparison of conventional 1DLC–UV, and h3DLC–UV analyses of the 10-fold concentrated urine, along with a 100 ppb hydrocortisone standard sample, and the urine sample spiked with hydrocortisone at the same level.

Again good separation of hydrocortisone from the sample matrix is obtained, as with phenytoin above, however in this case the target peak is slightly overlapped with a smaller peak. No attempt was made to resolve these two peaks through adjustments of the operating conditions. As is discussed below, this slight overlap did not affect the accuracy of quantitative analysis, as compared to 1DLC–MS/MS analysis.

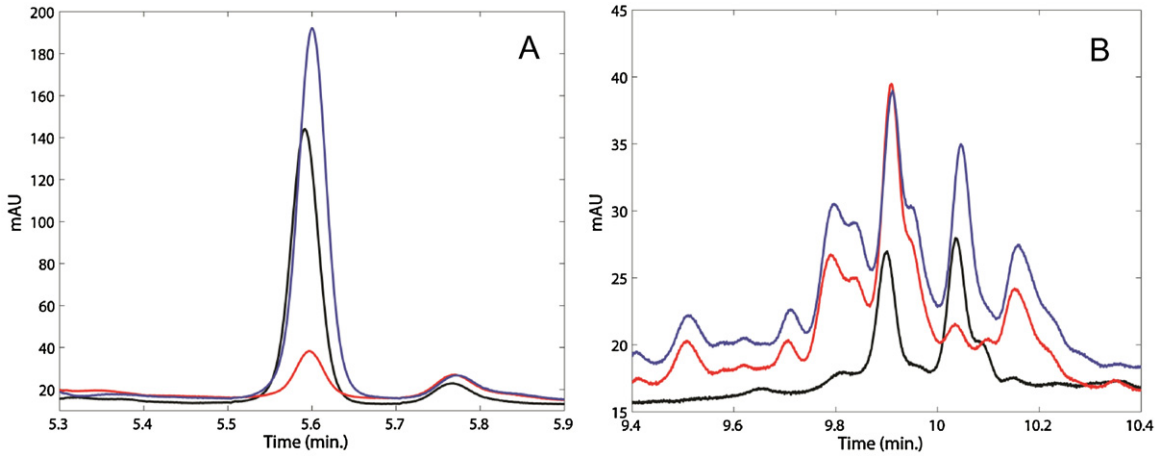

Fig. 8. Expanded view of the chromatogram obtained at the outlet of the third dimension of the h3DLC–UV system highlighting the regions near the elution of phenytoin and chlorophene. Chromatograms are color coded in the same way as in Fig. 6. Baseline resolution is achieved between the phenytoin peak and the sample matrix. The chlorophene peak is not as well resolved, but comparison of the black and red traces suggests that several of the peaks in this region of the chromatogram are due to impurities in the analytes used as retention alignment markers, and we have eliminated this problem in subsequent work.

4.1.3. Separation of hydrocortisone from human urine—MS/MS detection

It is clearly evident from the previous two examples that the resolving power of a 3D separation is very beneficial when UV absorbance detection is used to detect target compounds in complex matrices. In the case of the separation of hydrocortisone in urine we also find that the resolving power of the 3D separation is also very beneficial even when tandem mass spectrometry (MS/MS) is used for detection. The limitations of conventional HPLC are known in the area of steroid analysis because of the structural similarity of many endogenous compounds of this type, thus the crowded 1DLC–MS/MS chromatogram for the analysis of hydrocortisone in urine shown in Fig. 10A is not entirely surprising. Fig. 10A shows that there are many other compounds in the urine sample that share the same parent mass to charge ratio (m/z, $[M+H^+]^+$, 363.5) and fragment mass (m/z, 120.7), some of which have ion intensities similar to that of hydrocortisone. Fig. 10B shows the MS/MS chromatogram for the same 363.5 → 120.7 transition, but at the outlet of the third dimension column of the 3DLC system. The elimination of other compounds from the background is really striking; there is only one other detectable peak in the baseline at ca. 4.85 min in addition to the hydrocortisone peak at 5.05 min.

4.1.4. Separation of nicosulfuron from Minnesota River water extract—UV detection

The final example of a different sample matrix/target analyte combination discussed here is the analysis of the herbicide nicosulfuron (see Fig. 1) in Minnesota River water. Nicosulfuron is the active ingredient in a herbicide formulation that is widely used in the southern Minnesota watershed that empties into the Minnesota River. Fig. 11 shows a comparison of 1DLC–UV and h3DLC–UV separations of a 1000-fold concentrated extract of Minnesota River water for the analysis of nicosulfuron. As with hydrocortisone in urine, only small changes were made to the operating conditions initially used for the phenytoin analysis described above. The most significant change was the use of a 100 mM ammonium acetate buffer (pH 6) in the second dimension, which was required to elute nicosulfuron from the ZirChrom-CARB column. Aside from this change no others were made to enhance the selectivity of the separation. As with the previous two cases, there is excellent separation of the target compound from the sample matrix, even when

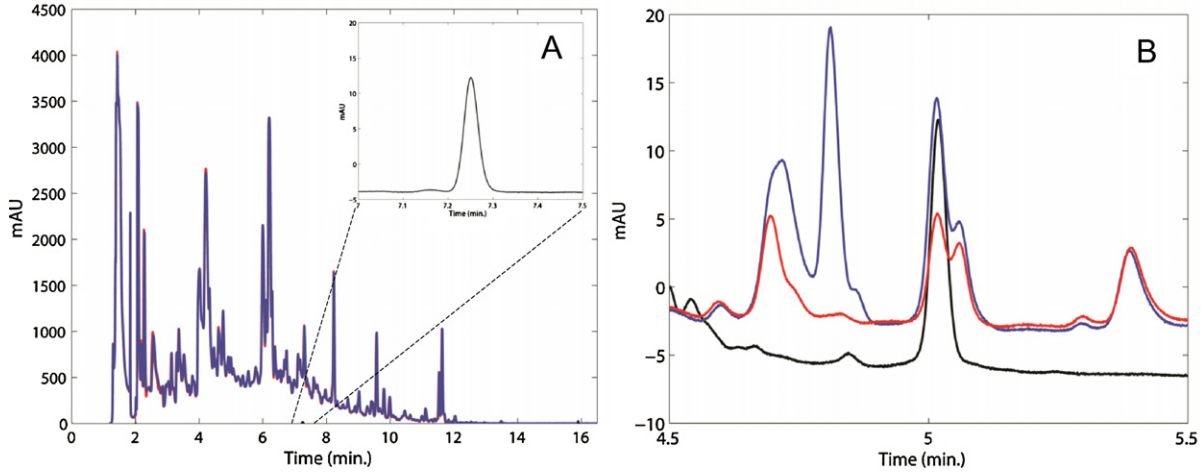

Fig. 9. Comparison of 1DLC–UV and h3DLC–UV separations of a 10-fold concentrated human urine sample for the target analyte hydrocortisone. Color coding of the chromatograms is the same as in Fig. 6. 1D and 3D Chromatographic conditions were the same as those described in Figs. 6 and 7, except that in the 3D system the organic solvent in the second dimension was 10/90 1-octanol/acetonitrile; minor adjustments were made to the gradient profiles to position the target peak in the low flow rate windows shown in Fig. 5. The hydrocortisone peak is slightly overlapped with one other measurable peak, but in general the resolution of hydrocortisone from the complex matrix is very good. No attempt was made to resolve these two peaks through adjustments of chromatographic conditions.

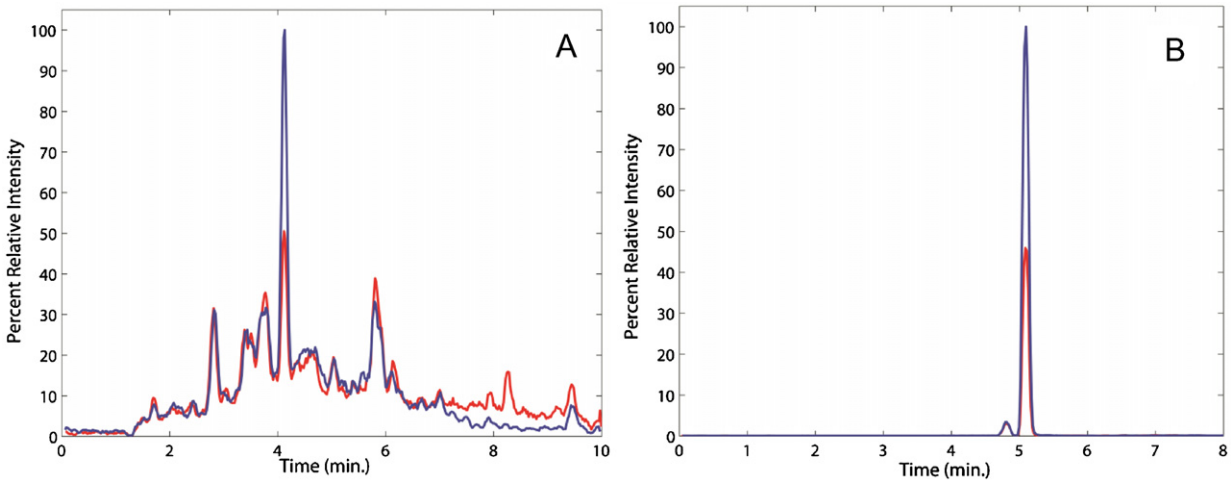

Fig. 10. Comparison of 1D and 3D separations of hydrocortisone from 10-fold concentrated human urine, using MS/MS detection. Panel A shows the SRM chromatogram obtained for a conventional 1DLC separation (target peak at 4.05 min.), and panel B shows the SRM chromatogram obtained at the outlet of the third dimension of our h3DLC system (target peak at 5.05 min.). Chromatographic conditions for the 1D separation were similar to those described in Fig. 6 except that a single 15 cm column was used at the analysis time was 10 min. Chromatographic conditions for the 3D separation were the same as those described in Figs. 7 and 9 except that 0.1% formic acid was used in the third dimension for LC/MS compatibility. Here the red traces are the chromatograms for the unspiked urine samples, and the blue traces are for urine spiked with 100 ppb hydrocortisone. The small peak eluting before hydrocortisone in panel B is the same size in both traces, and its identity is unknown to us.

considering nicosulfuron concentrations were 100 ppt in the original water sample. In this case we did not detect any nicosulfuron in the unspiked sample; however, this is quite reasonable as the water sample was collected in the spring of 2010 before herbicide application had begun for the year.

4.2. Comparison of quantitative results from 1D and 3D separations

In addition to the qualitative comparisons of 1D and 3D separations described above, we have also conducted rigorous quantitative comparisons of 3DLC separations with UV detection to conventional 1DLC separations with MS/MS detection for phenytoin in the wastewater effluent sample and hydrocortisone in urine. To the extent that 1DLC–MS/MS is perceived as the method of choice for trace level quantitation in complex matrices, we feel this comparison is the most appropriate and analytically demanding for this study. The key quantitative analyses described here use the strategy of standard addition as a means of dealing with matrix effects. We could also have used stable isotope labelled internal standards, but since this approach is not viable for UV absorbance detection, we elected to base the comparison primarily on quantitation by the standard addition method (Std. Add. below). This allowed us to analyze the exact same physical samples by both methods, thereby eliminating sample preparation as a variable in the comparison. We also made estimates of the phenytoin and hydrocortisone concentrations based on the use of external standards (Ext. Std. below) prepared in deionized water. While this is certainly inappropriate in the case of LC/MS because of susceptibility to alteration of ionization by the sample matrix, UV absorbance detection does not suffer from this particular matrix effect. Table 2 is a summary of the quantitative data

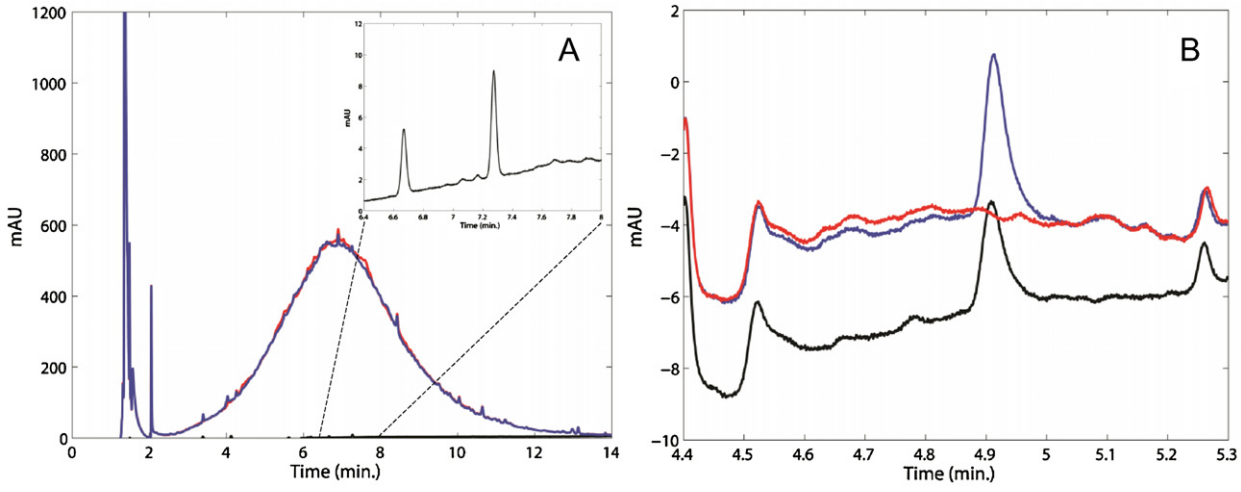

Fig. 11. Comparison of 1DLC–UV (A) and h3DLC–UV (B, third dimension only) separations of a 1000-fold concentrated Minnesota River water sample for the target analytenicosulfuron. Color coding of the chromatograms is the same as in Fig. 6, and the detection wavelength was 210 nm. The target peak was at 7.25 and 2.92 min in the 1D separation and third dimension of the 3D separation, respectively. 1D and 3D chromatographic conditions were the same as those described in Figs. 6 and 7, except that the buffer in the second dimension of the 3D system was 100 mM ammonium acetate at pH 6, and the column used in the 3rd dimension was the Ascentis Express C18. The nicosulfuron concentration in the standard sample and the spiked extract sample was 100 ppb. Here again it is remarkable that there are no significant interfering peaks from the river water matrix, as compared to the target compound at the 100 ppb level (100 ppt equivalent in the original sample).

Table 2
Estimates of phenytoin and hydrocortisone concentrations determined by 1DLC–MS/MS and h3DLC–UV and standard addition or external calibration approaches.[a].

	Phenytoin (ppb[b,c])		Hydrocortisone (ppb[d])	
Method	Std. Add.[e]	Ext. Cal.[f]	Std. Add.[d]	Ext. Cal.[e]
1DLC–MS/MS	176 ± 12	65 ± 28	84.8 ± 8.2	7.8 ± 10.8
h3DLC–UV	184 ± 15	172 ± 6	84 ± 11	85 ± 11

[a] Error estimates are 95% confidence intervals calculated using the standard deviations of the individual regressions, and the numbers of replicates given below.
[b] $n = 10$ or 12 for quantitation by external standards or standard addition, respectively.
[c] Concentration in analytical sample; concentrations in collected water samples are 1000-fold less.
[d] Concentration in analytical sample; concentrations in collected water samples are 10-fold less.
[e] Five standard additions were made in the range of 100–500% of the estimated concentration of the target compound in the analytical sample.
[f] Five external calibrants were prepared in DI water at concentrations bracketing the estimated concentration of the target compound in the analytical sample.

for phenytoin and hydrocortisone resulting from both methods of quantitation.

The most important aspect of the data in Table 2 is that for both phenytoin and hydrocortisone, there is no statistically significant difference between the concentrations determined by h3DLC–UV and 1DLC–MS/MS, when the standard addition method is used. Not only do we observe excellent separations of the target analytes from the sample matrices in a qualitative sense as shown above, but the h3DLC–UV method is also quantitatively accurate in these cases, as compared to 1DLC–MS/MS. We see that the precision of the two methods is quite similar, as measured by the size of the confidence intervals relative to the mean concentrations. Finally, a very interesting aspect of this part of the study is that the concentration estimates from the h3DLC–UV method using external calibration are not statistically different from the standard addition data. This is exciting because it suggests that actual 3DLC separation process is not prone to sample matrix effects. On the other hand, we see that the 1DLC–MS/MS method severely underestimates the analyte concentrations when external standards are used, presumably due to severe suppression of the target analyte signal by co-eluting constituents of the sample matrix.

We estimate that the method detection limit for phenytoin in WWTP effluent is 9 ppt for the h3DLC–UV method, which compares favorably to 0.4 ppt for the 1DLC–MS/MS method (assuming 400 μL injection in each case, and criterion of detection limit corresponding to a signal-to-noise ratio of three).

5. Conclusions

We have described the construction of a three-dimensional HPLC system geared toward targeted analysis in complex matrices, based on the use of three functionally different reversed-phase HPLC columns. This system has been applied to the analysis of four different target analytes in three very different sample matrices, using a set of nominally identical operating conditions. Following are the primary conclusions of the study.

1. Excellent separation of the target analyte from the sample matrix is observed in all four cases, even when relatively non-selective UV absorbance detection is used. We attribute this excellent separation to the power of the three-dimensional approach, and show that in the case of these very complicated matrices, two dimensions of separation is not adequate to achieve accurate quantitation for low-level sample constituents. In the case of the analysis of hydrocortisone in human urine, the additional separation power of the 3D method improved the signal to noise ratio for hydrocortisone, even when MS/MS detection was used.
2. Excellent separations of the target compounds and matrices were achieved with relatively little method development. Very similar organic solvents, buffers, and gradient elution programs were used for each separation problem. Here again we attribute this success to the high resolving power predicted for the combination of the three columns used in this work. With an overwhelming degree of resolving power in hand from the very beginning of the method development process, the probability of an initially successful separation of the target compound from the sample matrix is practically quite high.
3. Careful quantitative comparisons of the h3DLC–UV method and conventional 1DLC–MS/MS show that the 3D method not only appears to provide excellent separation, but is also quantitatively accurate, compared to the conventional methodology (LC/MS/MS) for trace analysis in complex matrices. Good linearity of standard addition and external calibration curves is observed with the h3DLC–UV system, leading to precise estimates of the concentrations of the target analytes, in spite of the apparent complexity of the 3DLC instrument. In our experience the system has been quite robust and reliable over the period of approximately 1 year.

We believe this work affirms the predicted value of a 3DLC system based on three very different reversed-phase stationary phases for the analysis of target compounds present at very low concentrations in complex matrices. At this point in time we believe this methodology has the greatest value as a research tool for rapid method development of targeted analyses in complex matrices. Our highest priority in ongoing work is to extend this methodology to target several compounds in a single analysis.

Acknowledgments

The authors acknowledge financial support from several organizations including: Gustavus Adolphus College, Sigma Xi (SWS), Camille and Henry Dreyfus Faculty Start-Up Program (DRS, MMS, TEL), Howard Hughes Medical Institute (SRG), the Minnesota River Board (SRG), and the Minnesota Chromatography Forum (SWS). Materials and expertise were provided by: Sigma–Aldrich (Ascentis Express column and SPE materials), ZirChrom Separations, Inc. (ZirChrom-CARB columns), Prof. Peter Carr (HC–COOH column, and several stimulating discussions over this work), Amanda Nienow (nicosulfuron sample), and Jeff Knutson, Director of the St. Peter Wastewater Treatment Plant.

References

[1] V.G. van Mispelaar, H.-G. Janssen, A.C. Tas, P.J. Schoenmakers, J. Chromatogr. A 1071 (2005) 229.
[2] X. Wang, W.E. Barber, P.W. Carr, J. Chromatogr. A 1107 (2006) 139.
[3] A.C. Chiaia, C. Banta-Green, J. Field, Environ. Sci. Technol. 42 (2008) 8841.
[4] R.N. Xu, L. Fan, M.J. Rieser, T.A. El-Shourbagy, J. Pharm. Biomed. Anal. 44 (2007) 342.
[5] F. Gosetti, E. Mazzucco, D. Zampieri, M.C. Gennaro, J. Chromatogr. A 1217 (2010) 3929.
[6] R. Pascoe, J.P. Foley, A.I. Gusev, Anal. Chem. 73 (2001) 6014.
[7] E. Rogatsky, V. Tomuta, H. Jayatillake, G. Cruikshank, L. Vele, D.T. Stein, J. Sep. Sci. 30 (2007) 226.
[8] E. Rogatsky, D.T. Stein, J. Sep. Sci. 29 (2006) 538.
[9] L.R. Snyder, J. Chromatogr. Sci. 8 (1970) 692.
[10] R.E. Majors, J. Chromatogr. Sci. 18 (1980) 571.

[11] J.A. Apffel, T.V. Alfredson, R.E. Majors, J. Chromatogr. 206 (1981) 43.
[12] E.L. Johnson, R. Gloor, R.E. Majors, J. Chromatogr. 149 (1978) 571.
[13] J.C. Giddings, Anal. Chem. 56 (1984) 1258A.
[14] I. François, K. Sandra, P. Sandra, Anal. Chim. Acta 641 (2009) 14.
[15] G. Guiochon, N. Marchetti, K. Mriziq, R.A. Shalliker, J. Chromatogr. A 1189 (2008) 109.
[16] D.R. Stoll, X. Li, X. Wang, S.E.G. Porter, S.C. Rutan, P.W. Carr, J. Chromatogr. A 1168 (2007) 3.
[17] E. Hogendoorn, E. Dijkman, Methods Biotechnol., 2006.
[18] R.E. Majors, LCGC North America 23 (2005) 1074.
[19] E. Hogendoorn, P. Van Zoonen, F. Hernandez, LC–GC Europe 16 (2003) 44.
[20] P. Dugo, T. Kumm, F. Cacciola, G. Dugo, L. Mondello, J. Liquid Chromatogr. Relat. Technol. 31 (2008) 1758.
[21] E. van der Heeft, E. Dijkman, R.A. Baumann, E.A. Hogendoorn, J. Chromatogr. A 879 (2000) 39.
[22] M.A.J. Bayliss, R.F. Venn, A.M. Edgington, R. Webster, D.K. Walker, J. Chromatogr. B 877 (2009) 121.
[23] A.R. Mughari, M.M. Galera, P.P. Vazquez, R.S. Valverde, J. Sep. Sci. 30 (2007) 673.
[24] R. Preuss, J. Angerer, J. Chromatogr. B 801 (2004) 307.
[25] E.A. Hogendoorn, E. Dijkman, B. Baumann, C. Hidalgo, J.-V. Sancho, F. Hernandez, Anal. Chem. 71 (1999) 1111.
[26] O.P. Haefliger, Anal. Chem. 75 (2003) 371.
[27] L. Blumberg, M.S. Klee, J. Chromatogr. A 1217 (2010) 99.
[28] A.W. Moore Jr., J.W. Jorgenson, Anal. Chem. 67 (1995) 3456.
[29] P.J. Schoenmakers, G. Vivo-Truyols, W.M.C. Decrop, J. Chromatogr. A 1120 (2006) 282.
[30] J.V. Sancho, O.J. Pozo, F. Hernandez, Rapid Commun. Mass Spectrom. 14 (2000) 1485.
[31] F. Hernández, J.V. Sancho, O.J. Pozo, J. Chromatogr. B 808 (2004) 229.
[32] M. Jayamanne, I. Granelli, A. Tjernberg, P.O. Edlund, J. Pharm. Biomed. Anal. 51 (2010) 649.
[33] M. Kiffe, D. Graf, M. Trunzer, Rapid Commun. Mass Spectrom. 21 (2007) 961.
[34] P.T. Jackson, M.R. Schure, T.P. Weber, P.W. Carr, Anal. Chem. 69 (1997) 416.
[35] M.J. Gray, G.R. Dennis, P.J. Slonecker, R.A. Shalliker, J. Chromatogr. A 1041 (2004) 101.
[36] D. Thiebaut, J. Vial, M. Michel, M.-C. Hennion, T. Greibrokk, J. Chromatogr. A 1122 (2006) 97.
[37] E.a. Hogendoorn, C. Verschraagen, U.A.Th. Brinkman, P. van Zoonen, Anal. Chim. Acta 268 (1992) 205.
[38] Y. Oda, N. Asakawa, T. Kajima, Y. Yoshida, T. Sato, J. Chromatogr. 541 (1991) 411.
[39] D.W. Kolpin, E.T. Furlong, M.T. Meyer, E.M. Thurman, S.D. Zaugg, L.B. Barber, H.T. Buxton, Environ. Sci. Technol. 36 (2002) 1202.
[40] D. Edwards, U.S. EPA, Report on the Food Quality Protection Act Tolerance Reassessment Progress and Risk Management Decision for Nicosulfuron, 2004.
[41] M. Duclos, M. Guinot, Y. Le Bouc, Appl. Physiol. Nutr. Metab. 32 (2007) 895.
[42] D.H. Hellhammer, S. Wust, B.M. Kudielka, Psychoneuroendocrinology 34 (2009) 163.
[43] F. Busetti, K. Linge, A. Heitz, J. Chromatogr. A 1216 (2009) 5807.
[44] A. Wick, G. Fink, T. Ternes, J. Chromatogr. A 1217 (2010) 2088.
[45] A.-E.F. Nassar, N. Varshney, T. Getek, L. Cheng, J. Chromatogr. Sci. 39 (2001) 59.
[46] G. Gervais, S. Brosillon, A. Laplanche, C. Helen, J. Chromatogr. A 1202 (2008) 163.
[47] B.L. Karger, L.R. Snyder, C. Horvath, An Introduction to Separation Science, Wiley & Sons, New York, 1973.
[48] J.M. Davis, J.C. Giddings, Anal. Chem. 55 (1983) 418.
[49] M. Martin, D.P. Herman, G. Guiochon, Anal. Chem. 58 (1986) 2200.
[50] M.J. Gray, G.R. Dennis, P.J. Slonecker, R.A. Shalliker, J. Chromatogr. A 1028 (2004) 247.
[51] M.J. Gray, A.P. Sweeney, G.R. Dennis, P.J. Slonecker, R.A. Shalliker, Analyst 128 (2003) 598.
[52] Y. Zhang, P.W. Carr, J. Chromatogr. A, submitted for publication.
[53] L.R. Snyder, J.W. Dolan, P.W. Carr, Anal. Chem. 79 (2007) 3254.
[54] U.S. Pharmacopeia, July 5, 2010, http://www.usp.org/USPNF/columnsPQRIapproach.html.
[55] N.S. Wilson, J.W. Dolan, L.R. Snyder, P.W. Carr, L.C. Sander, J. Chromatogr. A 961 (2002) 217.
[56] N.S. Wilson, M.D. Nelson, J.W. Dolan, L.R. Snyder, R.G. Wolcott, P.W. Carr, J. Chromatogr. A 961 (2002) 171.
[57] D.R. Stoll, J.D. Cohen, P.W. Carr, J. Chromatogr. A 1122 (2006) 123.
[58] J.J. DeStefano, T.J. Langlois, J.J. Kirkland, J. Chromatogr. Sci. 46 (2008) 254.