A Review of Two-Dimensional Liquid Chromatography Approaches Using Parallel Column Arrays in the Second Dimension

Samuel W. Foster[1], Deklin Parker[1], Sangeeta Kurre[1], John Boughton[1], Dwight R. Stoll[2], James P. Grinias[1,*]

[1] Department of Chemistry & Biochemistry, Rowan University, 201 Mullica Hill Rd., Glassboro, NJ 08028 USA

[2] Department of Chemistry, Gustavus Adolphus College, 800 West College Avenue, Saint Peter, MN 56082 USA

*Corresponding Author: James P. Grinias, grinias@rowan.edu

Abstract

Multi-dimensional liquid chromatography techniques play an important role in the analysis of complex mixtures. The keys to maximizing peak capacity in these methods are fast sampling rates and sufficient complementarity between the first- (^{1}D) and second- (^{2}D) dimension separations. One way that these criteria have been met is by using ^{2}D parallel column arrays. This review covers demonstrations of this approach in the literature that have been published over the past three decades. Two or more identical ^{2}D columns can be operated in a sequential order to permit increased separation times and higher peak capacities in the second dimension without the concomitant decrease in sampling rate. The parallel column arrays can also be operated simultaneously to reduce total analysis time. Columns with different stationary phase chemistries can be used in the ^{2}D column array to increase complementarity by utilizing specific stationary phases for various first dimension fractions. More recently, this type of platform has been used to automate the development of two-dimensional (2D) achiral-chiral LC methods. These strategies, as well as recent efforts towards the development of integrated, spatial multi-dimensional LC devices that include parallel column arrays, are discussed here.

Keywords: two-dimensional liquid chromatography, 2D-LC, multi-dimensional liquid chromatography, parallel column arrays, method development

1. Introduction

Maximizing separation peak capacity is a critical aspect of the analysis of complex mixtures by liquid chromatography (LC). However, there are practical limits to the peak capacity that can be obtained in one-dimensional separations given constraints based on the maximum achievable pressure (based on both system and column) and the time needed to perform the separation [1]. Because of these limits, multi-dimensional separation techniques have become an increasingly popular approach to the separation and analysis of complex mixtures.

Multi-dimensional liquid chromatography relies upon the use of two or more complementary separations (*i.e.* the separation mechanisms are completely independent from each other, also referred to as "orthogonal") to enhance the resolution of analytes that cannot be isolated using a single separation mode. It is a powerful tool because the total peak capacity of the comprehensive multi-dimensional method is the product of the peak capacity of each individual separation. However, this relationship only holds true if (1) the dimensions are completely complementary (complete usage of the two-dimensional separation space), and (2) there is sufficient sampling of the preceding dimension's separation into the subsequent dimensions (*i.e*. at least four samples across a peak bandwidth of eight standard deviations [2]). The first condition can be somewhat difficult to achieve because separation modes that are compatible for coupling often have some overlap between retention/elution behavior based on molecular size, polarity, and/or charge. The second requires very fast separations in the latter dimensions so that resolution obtained in a given separation is not lost in the transfer of fractions to the subsequent dimension. A number of review articles have focused on various aspects of column selection, method development, and hardware design in multi-dimensional LC approaches designed to achieve higher peak capacity [3–10].

The central theme of this review article is the use of multiple second-dimension (^{2}D) columns to increase complementarity and/or sampling rate compared to the use of a single ^{2}D column. These columns are typically arranged in parallel arrays, and such arrays previously been used in one-dimensional LC separations to either improve analytical throughput [11–20][21–23], increase method selectivity by using multiple stationary phases [24–33][34], or increase sample loadability by splitting the injected volume onto multiple columns [35]. Although the term "parallel" has been used to describe both instrument features and gradient method design in the multi-dimensional LC literature to date, the term is specifically used in this article to designate system designs in which more than one column is connected to the same incoming sample/eluent stream. In these arrays, the columns are operated in either an (1) alternating, (2) sequential, or (3) simultaneous way. To improve sampling rates while also potentially increasing the ^{2}D

peak capacity (n_c), multiple columns with the same stationary phase can be operated in a ^{2}D column array. To increase complementarity, multiple columns with different stationary phases can be employed to utilize more of the two-dimensional (2D) separation space and achieve higher peak capacities. In this review article, we will discuss recent demonstrations of both strategies, as well as describe how they have been applied to improve the capabilities of 2D-LC separations.

2. Second-Dimension Column Arrays Using Identical Stationary Phases

2.1 Parallel Column Arrays Operated in an Alternating or Sequential Order

Early experimental demonstrations of ^{2}D dual-column arrays that were operated alternately to separate each eluted first-dimension (^{1}D) fraction focused on the analysis of chiral compounds. Originally, a serial arrangement of achiral and chiral columns was applied for the analysis of the cancer drug leucovorin (administered as a diastereomeric mixture) and its metabolite, 5-methyltetrahydrofolate [36]. However, poor chromatographic efficiency on the chiral column led to broad peaks, which prevented extending the validated quantitation range of the drug in plasma below 1 μg/mL. To improve the technique, they switched the order of the columns, and sent two heart-cut fractions from a ^{1}D chiral separation to two independent ^{2}D achiral C18 columns (**Figure 1**) [37]. Peaks were refocused at the head of the ^{2}D columns and further separated from co-eluting compounds (full method described in **Figure 1** caption), building upon a similar approach that used two C18 trapping columns for modulation between the chiral and achiral separations [38]. The peak refocusing on the ^{2}D columns extended the lower end of the validated range to 25 ng/mL, permitting extended pharmacokinetic studies to measure drug clearance over longer time periods [37]. This foundational work demonstrated how this type of ^{2}D column arrangement could be used to facilitate improved 2D-LC separations by analyzing each heart-cut fraction on a separate column.

The next significant advances in this dual column approach came several years later, focusing on the development of comprehensive techniques for biomolecular analysis. For size-exclusion chromatography (SEC) × reversed phase (RP) LC analysis of peptides from trypsin-digested proteins, several SEC columns were connected in series, with the outlet of the final column connected to a ^{2}D dual-column array controlled by two four-port valves (**Figure 2**) [39]. A higher flow rate of 1 mL/min was used for the first 40 min of the separation to enable compounds to elute through the large volume SEC columns, followed by a drop to 0.1 mL/min for 140 min to perform comprehensive analysis on 35 fractions (cycle time on the ^{2}D columns was 4 min, including a 3 min gradient and 1 min equilibration). This

approach achieved n_c of 495, which was a more than ten-fold improvement from approximate one-dimensional n_c values of 45 that were common at the time. To further improve the sensitivity of the technique, the internal diameter of the ^{2}D RP columns was reduced from 4.6 mm to 1.0 mm [40]. Finally, to extend the system to the analysis of whole proteins from a cell lysate, the ^{2}D columns were replaced with polymeric perfusion-style particles and directed to a UV absorbance detector followed by a fraction collector [41]. Fractions of interest were further characterized with off-line MS analysis performed through both ESI and MALDI ionization modes.

In the early 2000s, a series of reports were published focusing on the combination of ^{1}D ion-exchange (IEX) separations with ^{2}D RP column arrays for protein analysis. With a 10-port single valve modulator, both weak-anion exchange (WAX) [42] and strong-cation exchange (SCX) [43] ^{1}D separations were coupled to a dual-column array of short 4.6 mm x 14 mm columns packed with 1.5 µm non-porous silica particles bonded with C18 for rapid ^{2}D RP separations. Twenty fractions (60 s each) were collected during a 20 min separation of a mixture of 11 intact proteins, with an estimated maximum n_c of 600. System capabilities were later increased through the addition of an in-line, size-based sample preparation step using a restricted access material (RAM) column prior to the first dimension IEX column, and an increase in the number of RP columns in the ^{2}D array from two to four (**Figure 3**) [44]. Here, a 96 min ^{1}D separation was fractionated into twenty-four 4 min fractions. Each ^{2}D RP column went through a 16 min cycle of sample loading (4 min), elution (8 min), and column regeneration (4 min), with the entire process controlled using three 10-port valves. This approach was an early demonstration increasing the ^{2}D n_c, and thus the overall n_c, while still maintaining sufficient fractionation sampling rates by utilizing a multiple ^{2}D column array. For the analysis of a human hemofiltrate sample primarily consisting of peptides and small proteins, the increased ^{2}D n_c value of 130 provided a total calculated n_c of 3000 (based on 24 fractions) for the 2D method. This platform was later applied for the identification of peptides in a similar sample matrix obtained from patients with chronic renal failure [45].

Various other reports using two or more identical ^{2}D columns have also focused on protein and/or peptide separations. Using capillary-scale monolithic columns, IEX was coupled to a ^{2}D array of three RP columns for the separation of intact proteins [46]. In this array, one column was being loaded with effluent from the ^{1}D IEX column, another was being equilibrated with a starting condition solution (or washed to remove salts if sample was already loaded), and the third had the RP gradient method running for separation and elution of proteins. The full cycle time for a single ^{2}D column to go through these sequential steps was 45 min. Fractions eluted from the ^{2}D column were collected following the separation of an *E.*

coli lysate, and although most still contained multiple proteins, each fraction was found to contain 10 or fewer proteins (as shown by SDS-PAGE), thus greatly reducing the complexity of the original sample. To increase the frequency at which fractions could be collected while not reducing the ^{2}D analysis time to avoid loss of n_c, an additional switching valve was added and the number of ^{2}D columns was increased to twelve (**Figure 4**) [47]. Over 500 differentiated peaks were identified using the system with three ^{2}D columns and the number exceeded 900 when twelve ^{2}D columns were used. Absorbance detection was employed in both studies, with three individual detectors used with the smaller column array and a novel capillary-scale detector array [48] implemented for the twelve column set. In another approach, a ^{1}D WAX column was connected to two 4.6 mm x 50 mm C18 RP columns for alternate modulation of ^{1}D fractions [49]. To increase the overall n_c, the ^{2}D column connected to the ^{2}D pump for elution was also connected to a second, identical column in series for a combined ^{2}D column length of 100 mm. For the analysis of a tryptic digest of a four protein mixture, n_c of 650 was obtained. When the second 50 mm column in the ^{2}D serial arrangement was heated to 80°C to decrease the ^{2}D separation time, the number of analyzed fractions increased from 25 to 37, reducing undersampling and increasing n_c to 890.

To expand the technique to the use of normal phase (NP) LC × RPLC with a ^{2}D dual column array, a modulation approaching using two 10-port valves was developed [50]. The first valve operated similarly to a typical 2D-LC modulation valve with two sample loops between dimensions, while the second valve enabled selection between the two ^{2}D columns (each connected to their own independent pumping system) for alternating operation. When coupling a 1.0 mm x 250 mm NP diol column to two parallel 4.6 mm x 50 mm RP columns, a 1 min sampling time was achieved with the two ^{2}D columns each having a 2 min full cycle time. For the total 25 min analysis time of a small molecule test mixture, n_c of 487 was obtained (**Figure 5**), which was close to the theoretical maximum product value of each dimension based on the high complementarity between dimensions. When the ^{1}D separation time was extended to 90 min for the analysis of a lemon oil sample, the observed n_c was 1,095, which was more than double the value achieved with a traditional modulation approach using a single ^{2}D column. Lower effective n_c of 82 was observed for the analysis of a steroid sample using RPLC (cyanopropyl) × RPLC (C18) with the same system because of the lower complementarity compared to NPLC × RPLC. A follow-up study for the analysis of fish oil samples using the same columns showed similar losses due to poorer complementarity, with a theoretical n_c of 290 dropping to a calculated value of 159 when accounting for use of the 2D separation space [51]. For the analysis of tryptic peptide mixtures with a similar instrument setup, RPLC (low pH) × RPLC (high pH) was employed to increase complementarity even though RP columns were used in both

dimensions [52]. To achieve higher ^{1}D n_c, four 2.1 mm x 150 mm columns packed with sub-3 µm superficially porous C18 particles were coupled in series, while the second dimension employed 4.6 mm x 50 mm columns packed with 3.5 µm fully porous C18 particles as in the previous work. With a 6 hr analysis time for the ^{1}D separation, an effective n_c of 4,677 was observed, with the theoretical maximum n_c diminished due to some undersampling of the narrow ^{1}D peaks.

Several other applications have been demonstrated using 2D-LC techniques with parallel ^{2}D column arrays. To separate phenolic antioxidants and flavonoids, a method using complementary PEG-silica and Zr-Carbon phases in each dimension was developed [53]. A 10-port valve was used to alternate between the two 2.1 mm x 50 mm Zr-Carbon ^{2}D columns. No assessment of peak capacity was reported, although high retention time repeatability was shown for the method (%RSD < 5%). Later, the same ^{2}D Zr-Carbon column array was coupled to a ^{1}D C18 column for the analysis of beer and wine samples [54]. The capability to operate the zirconia-based phases at higher temperatures up to 120ºC allowed for higher throughput in the ^{2}D separation that could be used in increase the overall sampling rate of the technique. For the analysis of ribosomal proteins from a yeast sample, a ^{1}D SCX was performed using a step salt gradient followed by 18 min gradient RP separations on two alternating ^{2}D C4 columns [55]. Other efforts relied upon monolithic columns with reduced flow resistance to achieve higher throughput ^{2}D separations. An IEX × RPLC separation of human urine metabolites utilized two ^{2}D C18 monolith columns [56]. Monolith columns have also been used for RPLC × RPLC separations, with a ^{1}D 4.6 mm x 150 mm column packed with fully porous pentabromobenzyl-phase particles connected to two ^{2}D 4.6 mm x 50 mm C18 silica monoliths [57]. The ^{2}D columns were operated at an exceptionally high 16 mL/min flow rate to achieve a 12 s ^{2}D separation time, which was then applied to the analysis of polyaromatic hydrocarbons in gasoline extract samples. Other aromatic and polyaromatic mixtures were separated with a modified column arrangement of a 4.6 mm x150 mm fluoro-alkyl column coupled to 4.6 mm x 30 mm C18 silica monoliths [58]. Here, additional valves containing sample loops were added to the modulator platform, permitting 15 s fractionation and a 30 s overall ^{2}D separation time, with an observed n_c of 1190 for a 65 min analysis. A summary of all of the experimental demonstrations of sequential ^{2}D column operation in 2D-LC is provided in **Table 1**.

The theoretical foundation of the ability for ^{2}D multi-column arrays to enhance the overall n_c through this type of sequential operation relative to the use of a single column has been previously detailed [59]. Based on a series of calculations and simulations primarily dependent on the ^{2}D cycle time, it was shown that increasing the number of columns in a ^{2}D parallel array does increase n_c, albeit not linearly.

Because the increase in performance levels off as the number of columns increases, a practical limit of five columns in a ^{2}D array was proposed. The more important advantage provided by these arrays was a reduction in the need for separations that meet minimum sampling time rates, easing the need for incredibly fast ^{2}D cycle times. This is somewhat akin to current commercial instrumentation containing multiple stored injection loops that can be injected sequentially onto a ^{2}D column [60] or previously reported research-based set-ups using 18 solid-phase extraction (SPE) cartridges instead of loops [61,62]. The theoretical study does bring up a number of experimental factors that must be considered when setting up a parallel column array for 2D-LC, specifically in terms of column, tubing, and pump reproducibility to ensure that the exact same separation is being performed in each ^{2}D column. This can be especially difficult to achieve in terms of identical columns, as even the best column manufacturers have slight column-to-column repeatability differences when using traditional slurry packing techniques. In practice as a research tool, especially when combined with MS detection, slight differences in the separations may not be as critical as they would be in a QA/QC environment. For these more quantitative applications, the complexity of instrumentation (especially in terms of the additional valves and control schemes that were required for most of the methods described in this section) and issues with exactly matching the ^{2}D separations may preclude wide adoption of the technique.

2.2 Parallel Column Arrays Operated Simultaneously

To reduce total analysis time, individual columns in ^{2}D column arrays can be operated at the same time rather than sequentially. One approach to coupling a ^{1}D SCX separation to 18 ^{2}D RP capillary LC columns was achieved through the combination of dual selector valves for fractionation and a micro-flow splitter to achieve simultaneous elution in the ^{2}D column array (**Figure 6**) [63]. Each step change in the ^{1}D SCX salt gradient was fractionated onto a different ^{2}D column by varying the position of the selector valves. Once all the fractions were loaded, an in-house fabricated 18-channel flow splitter was then used to send the mobile phase flow from a single pump to all 18 ^{2}D RP columns for concurrent 90 min gradient separations (180 min total separation time). The effluents from the ^{2}D columns were deposited onto MALDI plates at 15 s intervals, providing 360 spots per column and a total of 6,480 spots for MALDI-TOF-TOF-MS off-line detection, which added an additional day to the total analysis time. In this original study, the MALDI matrix was added to each individual spot following the parallel LC elution, while a similar follow-up study using a 10-capillary ^{2}D array included online mixing of a 1:1 addition of matrix to each effluent channel [64]. A modified set-up with a ^{1}D SAX separation followed by an array of eight

^{2}D analytical-scale RP columns that eluted into 96-well plates was applied for the identification of proteins from human plasma samples [65]. Following fractionation into the well plates (1 min fractions per channel, 0.2 mL total volume based on 0.2 mL/min flow rate, 110 total ^{2}D separation time), a bicinchoninic acid assay was performed to identify which fractions contained high abundance proteins. The high abundance proteins were then removed from these fractions with an immunoaffinity protein depletion kit to improve detection of low abundance proteins when fractions were subsequently analyzed by nano-LC-MS/MS. Over 1,300 unique proteins were identified, with concentrations ranging from 0.01 ng/mL up to 41 g/L in plasma [65]. A similar instrumental design and workflow that included an automated fraction collector [66] was more recently used to identify 4,436 proteins from HeLa cell extracts [67]. A summary of these methods is provided in **Table 2**. A limitation to most of these approaches was detection throughput, especially since many involved the use of off-line detectors. Multiplexed detection, such as the UV absorbance array described in [48] could reduce the total analysis time of simultaneous column operation. The disadvantage of this approach is that different detectors operating simultaneously may have slightly different signal response and would need to be calibrated individually, making effective quantitation more difficult to achieve.

Looking toward the future, the need for increasingly higher n_c to effectively analyze complex mixtures suggests adoption of higher-order multi-dimensional separation techniques, including three-dimensional (3D)-LC [68,69]. In spatial 3D-LC approaches that utilize integrated microfluidic devices to perform the separation, a core design component is the use of column arrays in the second and third dimensions that are operated simultaneously [70]. A foundational demonstration of this approach was an integrated microfluidic device that connected a ^{1}D separation channel to a perpendicular spatial array of 21 ^{2}D columns (**Figure 7**) [71]. This was later expanded to a 3D device that contained 16 ^{2}D channels and 256 third dimension (^{3}D) channels [72]. A key consideration in these devices is flow distribution to ensure that mobile phase is effectively delivered to all channels in the column arrays. To optimize this process, several computational and experimental studies have focused on flow distributor designs in spatial multi-dimensional chromatographic devices [73–77]. Active control of these flow distributors to improve performance can be achieved through rotational confinement devices that can be used to quickly shift flow into perpendicular directions to modulate between dimensions [78,79] or with freeze-thaw valve actuation [80]. Further details on the design of these devices and potential approaches to detection for spatial 3D-LC separations were recently described by the Eeltink group [70]; interested readers are referred to this publication for more information.

3. Second-Dimension Column Arrays Using Different Stationary Phases

The concept of using multiple columns in a 2D-LC separation to expand the complementarity of the technique dates back to some of the earliest published discussions of the technique [81]. One of the key impediments to fully utilizing the 2D separation space is lack of complementarity, as coupling very distinct separation mechanisms can often be difficult due to incompatible mobile phase conditions [8]. With a parallel array of ^{2}D columns each containing different stationary phases, the potential for expanding the complementarity between the dimensions increases. Experimental demonstrations of such an approach are summarized in **Table 3**. In the early 2000s, this strategy was demonstrated experimentally through the coupling of a single ^{1}D RP ODS-AQ column to parallel ^{2}D amino- and cyano-phase columns [82]. Modulation to two different columns was controlled through a 12-port valve, with a variety of flow splitters and unions used to enable system operation with a single pumping system and a single in-line detector combination (PDA and MS). For the analysis of a mixture of various aromatic compounds, the ^{1}D fractions that contained co-eluting analytes were both able to be separated on the ^{2}D columns. The elution order was different on each column due to differences in stationary phase interactions for amine-containing compounds (**Figure 8**). The n_c of this approach was calculated at 450 for a 30 min total analysis time. For the separation of a mixture of barbiturates and other drug compounds, this same platform was used with a ^{1}D C18 column and two identical ^{2}D phenyl columns, allowing for a RPLC × RPLC separation with some complementarity based on π-π interactions with the phenyl phase [83]. The system was modified to utilize a separate pump for each dimension and a separate detector for each of two parallel ^{2}D columns [84]. In this case, two 4.6 mm x 20 mm mixed mode RP columns were used in the ^{2}D array. By using two different pumps, mobile phase conditions were selected that promoted separations based on ^{1}D hydrophobic interactions and ^{2}D ionogenic interactions in the mixed mode columns, providing complementarity for non-neutral compounds in the mixture. A different group later adopted this same instrument arrangement with two individual ^{2}D detectors, but implemented two identical 3.0 mm x 50 mm columns packed with sub-3 µm core-shell materials for the ^{2}D array in a RPLC (low pH) × RPLC (high pH) method [85]. The full cycle time for the ^{2}D gradient separation was 30 s, allowing for a sampling time of 15 s through the use of two columns. The method was successfully applied for the analysis of co-eluting pharmaceutical degradants and other minor components in an active pharmaceutical ingredient sample.

Multiple reports have described the use of complementary ^{2}D column arrays to aid in the separation of charged species. For the analysis of surfactant mixtures, a ^{1}D NP separation using a 1.0 mm x 250 mm

diol column was coupled to a ^{2}D array that contained a 4.0 mm x 125 mm C4 column and a 4.0 mm x 250 mm C2 column [86]. Four heart-cut fractions were collected, with valve timing selected to elute the first fraction containing cationic and amphoteric species and the third fraction containing anionic sulfate species onto the C4 column. The second fraction containing nonionic species and the fourth fraction anionic sulfonate species were sent to the C2 column. The total analysis time was 54 min and detection limits as low as 10 ng on-column were observed using evaporative light scattering detection (ELSD). A 2D-ion chromatography (IC) method has also been reported in which two ^{2}D columns (cation- and anion-exchange, respectively) are used to separate both cations and anions following fractionation on a ^{1}D trap column [87]. The combined method was used to separate and detect seven inorganic anions and six inorganic cations in a single injection, with detection limits in the 1 – 300 ppb range achieved using conductivity detection.

Systems using different phases within the ^{2}D column array have been used for a number of other specific applications requiring 2D separations. An aforementioned dual C18 silica monolith column array was modified to increase dimensional complementarity by replacing one of the ^{2}D columns with a pentabromobenzyl silica monolith [58]. Because the ^{2}D columns were operated in an alternating fashion, two complete analyses were needed to obtain a separation on each ^{2}D column (*e.g.* odd ^{1}D fractions would elute onto the C18 monolith in run one and even ^{1}D fractions would elute onto the C18 monolith in run two), doubling the total analysis time. A hydrophilic interaction (HILIC)-RPLC method for the identification of 12 small molecules in tartary buckwheat samples used a ^{1}D mixed-mode HILIC column and two different ^{2}D RP columns (phenyl and polar embedded phases) [88]. Although not comprehensive, the high selectivity of the method did permit quantitation of the similar analytes of interest, which are difficult to separate with 1D or less complementary 2D separations. For combined metabolomic and lipidomic methods in a single analysis, a short ^{2}D trap column separated polar metabolites from more hydrophobic metabolites and lipids, with each fraction diverted to a different ^{2}D RP column for sequential analysis [89]. More than 3200 features were observed in the analysis of a pooled human plasma sample, which is nearly identical to the summed features of independent metabolomic and lipidomic analyses; 98.8% of the features identified in the combined method were the same as the two individual runs. This methodology was later applied to the analysis of acyl-CoAs in mutated glioma cells [90] and serum samples from esophageal squamous cell carcinoma patients [91]. A multi-detector approach for the lipidomic analysis of triacylglycerols in adult/infant formula has also been reported, with non-aqueous RP methods being used in both dimensions (^{1}D C18 column and two C30 columns with different lengths in

the ^{2}D array) [92,93]. The eluent from the ^{1}D column first passed through in-line UV absorbance and FLD detector flow cells, then to a flow splitter that separated flow five ways: to a CAD detector, APPI-MS, ESI-MS, and each of the ^{2}D columns. The 50 mm length C30 column was connected to in-line UV and ESI-MS detectors, while the 100 mm length C30 column had a post-column split to ELSD and ESI-MS detectors. Sixteen analytes fell within the certified reference value ranges of a NIST standard for adult/infant formula out of nineteen targeted compounds, demonstrating a potential alternative to the standard GC-FID method. The need for multiple MS instruments in this platform may make it difficult for many to adopt based on cost and laboratory space requirements. Although different ionization sources were used for these studies, multi-emitter tip designs [94] or motorized stages [95] that can be used to connect different columns to the same MS inlet may be a feasible alternative.

The pharmaceutical industry has demonstrated the use of parallel ^{2}D column arrays using column selection valves to empirically identify the most complementary column combinations as part of automated method development processes. To help improve the detection and identification of impurities and degradation products in pharmaceutical formulations, a ^{1}D RP separation was coupled to a six-column ^{2}D array [96]. Some of the ^{2}D columns were also RP but had slightly different selectivity, enabling the separation of a small impurity from the tail of the large active pharmaceutical ingredient peak observed in the first dimension. One of the columns in the ^{2}D array was actually identical to the ^{1}D column and was used for mobile phase additive exchange from phosphate buffer to formic acid to enhance MS detection. Finally, an inorganic salt impurity was separated and detected by using a ^{2}D HILIC column coupled to a charged aerosol detector. A similar instrument setup was later utilized for 2D achiral-chiral separations with a chiral column array in the second dimension (**Figure 9**) [97]. For method screening, each relevant ^{1}D heart-cut peak could be held in a sample loop and then a small aliquot of each loop could be sent to six different ^{2}D columns to identify which chiral phase provided the highest resolution. Once the best chiral column from the array was identified for each peak, single methods could then be designed that sent each compound to a different column to ensure chiral separation, a feat that is very difficult to achieve when only a single ^{2}D chiral column is used in a 2D achiral-chiral method. This is especially valuable for compounds with multiple chiral centers. To further automate method development for achiral-chiral peak purity analysis, column selection valves have been used for both ^{1}D RP and ^{2}D chiral column arrays, along with a mobile phase selector to screen additives for the achiral separation [98]. For the analysis of complex biopharmaceutical mixtures, automated method screening using the dual array platform employed ^{1}D SEC, IEX, and RP columns and a variety of ^{2}D RP columns was demonstrated on a standard protein

mixture [99]. More recently, ^{2}D column arrays have been used for the automated characterization of multiple monoclonal antibody structural attributes [100]. These platforms utilizing parallel column arrays in the second dimension, and sometimes the first dimension as well, provide an automated approach to method development that can simplify the integration of 2D-LC into pharmaceutical purity and biopharmaceutical characterization analyses. Many of the examples in the preceding paragraphs of Section 3 focused on methods developed for single applications, which may have less broad applicability. Automated pharmaceutical method development provides a much more likely area in which ^{2}D column arrays using columns with different stationary phases will be adopted.

4. Conclusions

The collection of articles reviewed here highlight the various ways in which parallel column arrays can enhance multi-dimensional LC methods. The ^{2}D separation time can be extended while minimizing the effects on sampling rate and total analysis time when multiple identical column arrays are used. Simultaneous operation of multiple ^{2}D columns, especially at the capillary-scale or in integrated microfluidic devices, can also be performed to maximize ^{2}D throughput. Detection can be challenging for these approaches, but MALDI-MS detection of spatial arrays of eluted fractions can be utilized. When different stationary phases are used in ^{2}D column arrays, complementarity can be increased to better utilize the 2D separation space. This strategy is also becoming increasingly important for automated method screening in industry, especially when developing 2D achiral-chiral methods for pharmaceutical compounds. More traditional comprehensive 2D-LC techniques have increased in popularity with the wider availability of commercial solutions, both in terms of hardware and software. Further research building upon the multi-column approaches to improve 2D-LC performance described here, especially through higher complementarity based on the use of multiple stationary phases, may eventually increase the ubiquity of these methods as well. However, the complexity of instrumentation remains a barrier to wider adoption of this technique, and commercially available solutions may increase usage as has been observed with more traditional 2D-LC techniques.

Acknowledgements

This work was supported by the Chemical Measurement and Imaging Program in the National Science Foundation Division of Chemistry under Grants CHE-2045023 (to JPG) and CHE-2003734 (to DRS).

References

[1] G. Guiochon, The limits of the separation power of unidimensional column liquid chromatography, J. Chromatogr. A. 1126 (2006) 6–49. https://doi.org/10.1016/j.chroma.2006.07.032.

[2] R.E. Murphy, M.R. Schure, J.P. Foley, Effect of Sampling Rate on Resolution in Comprehensive Two-Dimensional Liquid Chromatography, Anal. Chem. 70 (1998) 1585–1594. https://doi.org/10.1021/ac971184b.

[3] D.R. Stoll, X. Li, X. Wang, P.W. Carr, S.E.G. Porter, S.C. Rutan, Fast, comprehensive two-dimensional liquid chromatography, J. Chromatogr. A. 1168 (2007) 3–43. https://doi.org/10.1016/J.CHROMA.2007.08.054.

[4] P. Dugo, F. Cacciola, T. Kumm, G. Dugo, L. Mondello, Comprehensive multidimensional liquid chromatography: Theory and applications, J. Chromatogr. A. 1184 (2008) 353–368. https://doi.org/10.1016/j.chroma.2007.06.074.

[5] I. François, K. Sandra, P. Sandra, Comprehensive liquid chromatography: Fundamental aspects and practical considerations-A review, Anal. Chim. Acta. 641 (2009) 14–31. https://doi.org/10.1016/j.aca.2009.03.041.

[6] M. Iguiniz, S. Heinisch, Two-dimensional liquid chromatography in pharmaceutical analysis. Instrumental aspects, trends and applications, J. Pharm. Biomed. Anal. 145 (2017) 482–503. https://doi.org/10.1016/j.jpba.2017.07.009.

[7] D.R. Stoll, P.W. Carr, Two-Dimensional Liquid Chromatography: A State of the Art Tutorial, Anal. Chem. 89 (2017) 519–531. https://doi.org/10.1021/acs.analchem.6b03506.

[8] B.W.J. Pirok, A.F.G. Gargano, P.J. Schoenmakers, Optimizing separations in online comprehensive two-dimensional liquid chromatography, J. Sep. Sci. 41 (2018) 68–98. https://doi.org/10.1002/jssc.201700863.

[9] B.W.J.J. Pirok, D.R. Stoll, P.J. Schoenmakers, Recent Developments in Two-Dimensional Liquid Chromatography: Fundamental Improvements for Practical Applications, Anal. Chem. 91 (2019) 240–263. https://doi.org/10.1021/acs.analchem.8b04841.

[10] J. De Vos, J.P. Grinias, D. Stoll, S. Buckenmaier, S. Eeltink, Advances in ultra-high-pressure and multi-dimensional liquid chromatography instrumentation and workflows, Anal. Sci. Adv. 2 (2021) 171–192. https://doi.org/10.1002/ansa.202100007.

[11] L. Zeng, D.B. Kassel, Developments of a Fully Automated Parallel HPLC/ Mass Spectrometry

System for the Analytical Characterization and Preparative Purification of Combinatorial Libraries, Anal. Chem. 70 (1998) 4380–4388. https://doi.org/10.1021/ac9805448.

[12] V. De Biasi, N. Haskins, A. Organ, R. Bateman, K. Giles, S. Jarvis, High throughput liquid chromatography/mass spectrometric analyses using a novel multiplexed electrospray interface, Rapid Commun. Mass Spectrom. 13 (1999) 1165–1168. https://doi.org/10.1002/(SICI)1097-0231(19990630)13:12<1165::AID-RCM638>3.0.CO;2-4.

[13] W.A. Korfmacher, J. Veals, K. Dunn-Meynell, X. Zhang, G. Tucker, K.A. Cox, C. Lin, Demonstration of the capabilities of a parallel high performance liquid chromatography tandem mass spectrometry system for use in the analysis of drug discovery plasma samples, Rapid Commun. Mass Spectrom. 13 (1999) 1991–1998. https://doi.org/10.1002/(sici)1097-0231(19991030)13:20<1991::aid-rcm743>3.3.co;2-u.

[14] D.L. Hiller, A.H. Brockman, L. Goulet, S. Ahmed, R.O. Cole, T. Covey, Application of a non-indexed dual sprayer pneumatically assisted electrospray source to the high throughput quantitation of target compounds in biological fluids, Rapid Commun. Mass Spectrom. 14 (2000) 2034–2038. https://doi.org/10.1002/1097-0231(20001115)14:21<2034::AID-RCM124>3.0.CO;2-6.

[15] C.K. Van Pelt, T.N. Corso, G.A. Schultz, S. Lowes, J. Henion, A four-column parallel chromatography system for isocratic or gradient LC/MS analyses, Anal. Chem. 73 (2001) 582–588. https://doi.org/10.1021/ac0006876.

[16] Y. Shen, N. Tolić, R. Zhao, L. Paša-Tolić, L. Li, S.J. Berger, R. Harkewicz, G.A. Anderson, M.E. Belov, R.D. Smith, High-throughput proteomics using high-efficiency multiple-capillary liquid chromatography with on-line high-performance ESI FTICR mass spectrometry, Anal. Chem. 73 (2001) 3011–3021. https://doi.org/10.1021/ac001393n.

[17] M. Jemal, M. Huang, Y. Mao, D. Whigan, M.L. Powell, Increased throughput in quantitative bioanalysis using parallel-column liquid chromatography with mass spectrometric detection, Rapid Commun. Mass Spectrom. 15 (2001) 994–999. https://doi.org/10.1002/rcm.330.

[18] R.C. King, C. Miller-Stein, D.J. Magiera, J. Brann, Description and validation of a staggered parallel high performance liquid chromatography system for good laboratory practice level quantitative analysis by liquid chromatography/tandem mass spectrometry, Rapid Commun. Mass Spectrom. 16 (2002) 43–52. https://doi.org/10.1002/rcm.539.

[19] H. Wang, S.M. Hanash, Increased throughput and reduced carryover of mass spectrometry-based

proteomics using a high-efficiency nonsplit nanoflow parallel dual-column capillary HPLC system, J. Proteome Res. 7 (2008) 2743–2755. https://doi.org/10.1021/pr700876g.

[20] P. Diederich, S.K. Hansen, S.A. Oelmeier, B. Stolzenberger, J. Hubbuch, A sub-two minutes method for monoclonal antibody-aggregate quantification using parallel interlaced size exclusion high performance liquid chromatography, J. Chromatogr. A. 1218 (2011) 9010–9018. https://doi.org/10.1016/j.chroma.2011.09.086.

[21] A. Nagy, A. Gaspar, Packed multi-channels for parallel chromatographic separations in microchips, J. Chromatogr. A. 1304 (2013) 251–256. https://doi.org/10.1016/j.chroma.2013.06.065.

[22] J. Huft, C.A. Haynes, C.L. Hansen, Fabrication of high-quality microfluidic solid-phase chromatography columns, Anal. Chem. 85 (2013) 1797–1802. https://doi.org/10.1021/ac303153a.

[23] J. Huft, C.A. Haynes, C.L. Hansen, Microfluidic integration of parallel solid-phase liquid chromatography, Anal. Chem. 85 (2013) 2999–3005. https://doi.org/10.1021/ac400163u.

[24] G.M. Gross, B.J. Prazen, R.E. Synovec, Parallel column liquid chromatography with a single multi-wavelength absorbance detector for enhanced selectivity using chemometric analysis, Anal. Chim. Acta. 490 (2003) 197–210. https://doi.org/10.1016/S0003-2670(03)00669-X.

[25] Y. Wang, R. Lehmann, X. Lu, X. Zhao, G. Xu, Novel, fully automatic hydrophilic interaction/reversed-phase column-switching high-performance liquid chromatographic system for the complementary analysis of polar and apolar compounds in complex samples, J. Chromatogr. A. 1204 (2008) 28–34. https://doi.org/10.1016/j.chroma.2008.07.010.

[26] K. Klavins, H. Drexler, S. Hann, G. Koellensperger, Quantitative metabolite profiling utilizing parallel column analysis for simultaneous reversed-phase and hydrophilic interaction liquid chromatography separations combined with tandem mass spectrometry, Anal. Chem. 86 (2014) 4145–4150. https://doi.org/10.1021/ac5003454.

[27] K. Ortmayr, S. Hann, G. Koellensperger, Complementing reversed-phase selectivity with porous graphitized carbon to increase the metabolome coverage in an on-line two-dimensional LC-MS setup for metabolomics., Analyst. 140 (2015) 3465–3473. https://doi.org/10.1039/c5an00206k.

[28] J. Robles-Molina, B. Gilbert-López, J.F. García-Reyes, A. Molina-Díaz, Simultaneous liquid chromatography/mass spectrometry determination of both polar and “multiresidue” pesticides in food using parallel hydrophilic interaction/reversed-phase liquid chromatography and a hybrid sample preparation approach, J. Chromatogr. A. 1517 (2017) 108–116.

https://doi.org/10.1016/j.chroma.2017.08.041.
[29] E. Rampler, H. Schoeny, B.M. Mitic, Y. El Abiead, M. Schwaiger, G. Koellensperger, Simultaneous non-polar and polar lipid analysis by on-line combination of HILIC, RP and high resolution MS, Analyst. 143 (2018) 1250–1258. https://doi.org/10.1039/c7an01984j.
[30] M. Schwaiger, H. Schoeny, Y. El Abiead, G. Hermann, E. Rampler, G. Koellensperger, Merging metabolomics and lipidomics into one analytical run, Analyst. 144 (2019) 220–229. https://doi.org/10.1039/c8an01219a.
[31] M.A.I. Prodhan, B. Shi, M. Song, L. He, F. Yuan, X. Yin, P. Bohman, C.J. McClain, X. Zhang, Integrating comprehensive two-dimensional gas chromatography mass spectrometry and parallel two-dimensional liquid chromatography mass spectrometry for untargeted metabolomics, Analyst. 144 (2019) 4331–4341. https://doi.org/10.1039/c9an00560a.
[32] F. Yuan, S. Kim, X. Yin, X. Zhang, I. Kato, Integrating two-dimensional gas and liquid chromatography-mass spectrometry for untargeted colorectal cancer metabolomics: A proof-of-principle study, Metabolites. 10 (2020) 343. https://doi.org/10.3390/metabo10090343.
[33] F. Yuan, J. Harder, J. Ma, X. Yin, X. Zhang, M.M. Kosiewicz, Using Multiple Analytical Platforms to Investigate the Androgen Depletion Effects on Fecal Metabolites in a Mouse Model of Systemic Lupus Erythematosus, J. Proteome Res. 19 (2020) 667–676. https://doi.org/10.1021/acs.jproteome.9b00558.
[34] M. Komendová, S. Nawada, R. Metelka, P.J. Schoenmakers, J. Urban, Multichannel separation device with parallel electrochemical detection, J. Chromatogr. A. 1610 (2020) 460537. https://doi.org/10.1016/j.chroma.2019.460537.
[35] M.J. Gray, P.J. Slonecker, G. Dennis, R.A. Shalliker, A column capacity study of single, serial, and parallel linked rod monolithic high performance liquid chromatography columns, J. Chromatogr. A. 1096 (2005) 92–100. https://doi.org/10.1016/j.chroma.2005.06.091.
[36] I.W. Wainer, R.M. Stiffin, Direct resolution of the stereoisomers of leucovorin and 5-methyltetrahydrofolate using a bovine serum albumin high-performance liquid chromatographic chiral stationary phase coupled to an achiral phenyl column, J. Chromatogr. B Biomed. Sci. Appl. 424 (1988) 158–162. https://doi.org/10.1016/S0378-4347(00)81088-9.
[37] L. Silan, P. Jadaud, L.R. Whitfield, I.W. Wainer, Determination of low levels of the stereoisomers of leucovorin and 5-methyltetrahydrofolate in plasma using a coupled chiral-achiral high-performance liquid chromatographic system with post-chiral column peak compression, J.

Chromatogr. B Biomed. Sci. Appl. 532 (1990) 227–236. https://doi.org/10.1016/S0378-4347(00)83774-3.

[38] A. Walhagen, L.-E. Edholm, Coupled-column chromatography on immobilized protein phases for direct separation and determination of drug qenantiomers in plasma, J. Chromatogr. A. 473 (1989) 371–379. https://doi.org/10.1016/S0021-9673(00)91321-9.

[39] G.J. Opiteck, J.W. Jorgenson, R.J. Anderegg, Two-Dimensional SEC/RPLC Coupled to Mass Spectrometry for the Analysis of Peptides, Anal. Chem. 69 (1997) 2283–2291. https://doi.org/10.1021/ac961156d.

[40] G.J. Opiteck, J.W. Jorgenson, M.A. Moseley, R.J. Anderegg, Two-Dimensional Microcolumn HPLC Coupled to a Single-Quadrupole Mass Spectrometer for the Elucidation of Sequence Tags and Peptide Mapping, J. Microcolumn Sep. 10 (1998) 365–375. https://doi.org/10.1002/(SICI)1520-667X(1998)10:4<365::AID-MCS6>3.0.CO;2-E.

[41] G.J. Opiteck, S.M. Ramirez, J.W. Jorgenson, M.A. Moseley, Comprehensive two-dimensional high-performance liquid chromatography for the isolation of overexpressed proteins and proteome mapping, Anal. Biochem. 258 (1998) 349–361. https://doi.org/10.1006/abio.1998.2588.

[42] K.K. Unger, K. Racaityte, K. Wagner, T. Miliotis, L.E. Edholm, R. Bischoff, G. Marko-Varga, Is multidimensional High Performance Liquid Chromatography (HPLC) an alternative in protein analysis to 2D gel electrophoresis?, HRC J. High Resolut. Chromatogr. 23 (2000) 259–265. https://doi.org/10.1002/(SICI)1521-4168(20000301)23:3<259::AID-JHRC259>3.0.CO;2-V.

[43] K. Wagner, K. Racaityte, K.K. Unger, T. Miliotis, L.E. Edholm, R. Bischoff, G. Marko-Varga, Protein mapping by two-dimensional high performance liquid chromatography, J. Chromatogr. A. 893 (2000) 293–305. https://doi.org/10.1016/S0021-9673(00)00736-6.

[44] K. Wagner, T. Miliotis, G. Marko-Varga, R. Bischoff, K.K. Unger, An automated on-line multidimensional HPLC system for protein and peptide mapping with integrated sample preparation, Anal. Chem. 74 (2002) 809–820. https://doi.org/10.1021/ac010627f.

[45] E. Machtejevas, H. John, K. Wagner, L. Ständker, G. Marko-Varga, W.G. Forssmann, R. Bischoff, K.K. Unger, Automated multi-dimensional liquid chromatography: Sample preparation and identification of peptides from human blood filtrate, J. Chromatogr. B Anal. Technol. Biomed. Life Sci. 803 (2004) 121–130. https://doi.org/10.1016/j.jchromb.2003.07.015.

[46] Z. Zhu, H. Chen, J. Ren, J.J. Lu, C. Gu, K.B. Lynch, S. Wu, Z. Wang, C. Cao, S. Liu, Two-dimensional chromatographic analysis using three second-dimension columns for continuous

comprehensive analysis of intact proteins, Talanta. 179 (2018) 588–593. https://doi.org/10.1016/j.talanta.2017.11.060.

[47] J. Ren, M.A. Beckner, K.B. Lynch, H. Chen, Z. Zhu, Y. Yang, A. Chen, Z. Qiao, S. Liu, J.J. Lu, Two-dimensional liquid chromatography consisting of twelve second-dimension columns for comprehensive analysis of intact proteins, Talanta. 182 (2018) 225–229. https://doi.org/10.1016/j.talanta.2018.01.072.

[48] K.B. Lynch, Y. Yang, J. Ren, S. Liu, Multiple-channel ultra-violet absorbance detector for two-dimensional chromatographic separations, Talanta. 181 (2018) 416–421. https://doi.org/10.1016/j.talanta.2018.01.045.

[49] D. Li, L. Zhang, Z. Wang, A. Intisar, W. Zhang, Development of a parallel-tandem column interface in a two-dimensional liquid chromatography system, Chromatographia. 73 (2011) 871–877. https://doi.org/10.1007/s10337-011-1974-x.

[50] I. François, A. de Villiers, B. Tienpont, F. David, P. Sandra, Comprehensive two-dimensional liquid chromatography applying two parallel columns in the second dimension, J. Chromatogr. A. 1178 (2008) 33–42. https://doi.org/10.1016/j.chroma.2007.11.032.

[51] I. François, P. Sandra, Comprehensive supercritical fluid chromatography × reversed phase liquid chromatography for the analysis of the fatty acids in fish oil, J. Chromatogr. A. 1216 (2009) 4005–4012. https://doi.org/10.1016/j.chroma.2009.02.078.

[52] I. François, D. Cabooter, K. Sandra, F. Lynen, G. Desmet, P. Sandra, Tryptic digest analysis by comprehensive reversed phase x two reversed phase liquid chromatography (RP-LC x RP-LC) at different pH's, J. Sep. Sci. 32 (2009) 1137–1144. https://doi.org/10.1002/jssc.200800578.

[53] F. Cacciola, P. Jandera, E. Blahová, L. Mondello, Development of different comprehensive two dimensional systems for the separation of phenolic antioxidants, J. Sep. Sci. 29 (2006) 2500–2513. https://doi.org/10.1002/jssc.200600213.

[54] F. Cacciola, P. Jandera, L. Mondello, Temperature effects on separation on zirconia columns: Applications to one- and two-dimensional LC separations of phenolic antioxidants, J. Sep. Sci. 30 (2007) 462–474. https://doi.org/10.1002/jssc.200600387.

[55] H. Liu, S.J. Berger, A.B. Chakraborty, R.S. Plumb, S.A. Cohen, Multidimensional chromatography coupled to electrospray ionization time-of-flight mass spectrometry as an alternative to two-dimensional gels for the identification and analysis of complex mixtures of intact proteins, J. Chromatogr. B Anal. Technol. Biomed. Life Sci. 782 (2002) 267–289.

https://doi.org/10.1016/S1570-0232(02)00554-8.
[56] S.P. Dixon, I.D. Pitfield, D. Perrett, Comprehensive multi-dimensional liquid chromatographic separation in biomedical and pharmaceutical analysis: A review, Biomed. Chromatogr. 20 (2006) 508–529. https://doi.org/10.1002/bmc.672.
[57] T. Murahashi, Comprehensive two-dimensional high-performance liquid chromatography for the separation of polycyclic aromatic hydrocarbons, Analyst. 128 (2003) 611–615. https://doi.org/10.1039/b212643e.
[58] N. Tanaka, H. Kimura, D. Tokuda, K. Hosoya, T. Ikegami, N. Ishizuka, H. Minakuchi, K. Nakanishi, Y. Shintani, M. Furuno, K. Cabrera, Simple and Comprehensive Two Dimensional Reversed-Phase HPLC Using Monolithic Silica Column, Anal. Chem. 76 (2004) 1273–1281. https://doi.org/10.1021/ac034925j.
[59] J.N. Fairchild, K. Horváth, G. Guiochon, Theoretical advantages and drawbacks of on-line, multidimensional liquid chromatography using multiple columns operated in parallel, J. Chromatogr. A. 1216 (2009) 6210–6217. https://doi.org/10.1016/j.chroma.2009.06.085.
[60] Y. Chen, L. Montero, O.J. Schmitz, Advance in on-line two-dimensional liquid chromatography modulation technology, TrAC - Trends Anal. Chem. 120 (2019) 115647. https://doi.org/10.1016/j.trac.2019.115647.
[61] S.R. Wilson, M. Jankowski, M. Pepaj, A. Mihailova, F. Boix, G. Vivo Truyols, E. Lundanes, T. Greibrokk, 2D LC separation and determination of bradykinin in rat muscle tissue dialysate with on-line SPE-HILIC-SPE-RP-MS, Chromatographia. 66 (2007) 469–474. https://doi.org/10.1365/s10337-007-0341-4.
[62] A. Mihailova, H. Malerød, S.R. Wilson, B. Karaszewskl, R. Hauser, E. Lundanes, T. Greibrokk, Improving the resolution of neuropeptides in rat brain with on-line HILIC-RP compared to on-line SCX-RP, J. Sep. Sci. 31 (2008) 459–467. https://doi.org/10.1002/jssc.200700257.
[63] X. Gu, C. Deng, G. Yan, X. Zhang, Capillary array reversed-phase liquid chromatography-based multidimensional separation system coupled with MALDI-TOF-TOF-MS detection for high-throughput proteome analysis, J. Proteome Res. 5 (2006) 3186–3196. https://doi.org/10.1021/pr0602592.
[64] C. Liu, X. Zhang, Multidimensional capillary array liquid chromatography and matrix-assisted laser desorption/ionization tandem mass spectrometry for high-throughput proteomic analysis, J. Chromatogr. A. 1139 (2007) 191–198. https://doi.org/10.1016/j.chroma.2006.11.019.

[65] Z. Huang, G. Yan, M. Gao, X. Zhang, Array-Based Online Two Dimensional Liquid Chromatography System Applied to Effective Depletion of High-Abundance Proteins in Human Plasma, Anal. Chem. 88 (2016) 2440–2445. https://doi.org/10.1021/acs.analchem.5b04553.
[66] X. Wang, M. Gao, X. Zhang, Microliter-level multi-channel fraction collector for high-throughput separation system, J. Chromatogr. A. 1656 (2021) 462535. https://doi.org/10.1016/j.chroma.2021.462535.
[67] X. Wang, G. Yan, H. Zheng, M. Gao, X. Zhang, Strategy for high-throughput identification of protein complexes by array-based multi-dimensional liquid chromatography-mass spectrometry, J. Chromatogr. A. 1652 (2021) 462351. https://doi.org/10.1016/j.chroma.2021.462351.
[68] E. Davydova, P.J. Schoenmakers, G. Vivó-Truyols, Study on the performance of different types of three-dimensional chromatographic systems, J. Chromatogr. A. 1271 (2013) 137–143. https://doi.org/10.1016/j.chroma.2012.11.043.
[69] N. Abdulhussain, S. Nawada, P. Schoenmakers, Latest Trends on the Future of Three-Dimensional Separations in Chromatography, Chem. Rev. 121 (2021) 12016–12034. https://doi.org/10.1021/acs.chemrev.0c01244.
[70] T. Themelis, A. Amini, J. De Vos, S. Eeltink, Towards spatial comprehensive three-dimensional liquid chromatography: A tutorial review, Anal. Chim. Acta. 1148 (2021) 238157. https://doi.org/10.1016/j.aca.2020.12.032.
[71] B. Wouters, J. De Vos, G. Desmet, H. Terryn, P.J. Schoenmakers, S. Eeltink, Design of a microfluidic device for comprehensive spatial two-dimensional liquid chromatography, J. Sep. Sci. 38 (2015) 1123–1129. https://doi.org/10.1002/jssc.201401192.
[72] B. Wouters, E. Davydova, S. Wouters, G. Vivo-Truyols, P.J. Schoenmakers, S. Eeltink, Towards ultra-high peak capacities and peak-production rates using spatial three-dimensional liquid chromatography, Lab Chip. 15 (2015) 4415–4422. https://doi.org/10.1039/c5lc01169h.
[73] E. Davydova, S. Wouters, S. Deridder, G. Desmet, S. Eeltink, P.J. Schoenmakers, Design and evaluation of microfluidic devices for two-dimensional spatial separations, J. Chromatogr. A. 1434 (2016) 127–135. https://doi.org/10.1016/j.chroma.2016.01.003.
[74] S. Jespers, S. Deridder, G. Desmet, A microfluidic distributor combining minimal volume, minimal dispersion and minimal sensitivity to clogging, J. Chromatogr. A. 1537 (2018) 75–82. https://doi.org/10.1016/j.chroma.2018.01.029.
[75] T. Adamopoulou, S. Nawada, S. Deridder, B. Wouters, G. Desmet, P.J. Schoenmakers,

Experimental and numerical study of band-broadening effects associated with analyte transfer in microfluidic devices for spatial two-dimensional liquid chromatography created by additive manufacturing, J. Chromatogr. A. 1598 (2019) 77–84. https://doi.org/10.1016/j.chroma.2019.03.041.

[76] T. Adamopoulou, S. Deridder, T.S. Bos, S. Nawada, G. Desmet, P.J. Schoenmakers, Optimizing design and employing permeability differences to achieve flow confinement in devices for spatial multidimensional liquid chromatography, J. Chromatogr. A. 1612 (2020) 460665. https://doi.org/10.1016/j.chroma.2019.460665.

[77] R. Ghosh, G. Chen, U. Umatheva, P. Gatt, A flow distribution and collection feature for ensuring scalable uniform flow in a chromatography device, J. Chromatogr. A. 1618 (2020) 460892. https://doi.org/10.1016/j.chroma.2020.460892.

[78] T. Adamopoulou, S. Deridder, G. Desmet, P.J. Schoenmakers, Two-dimensional insertable separation tool (TWIST) for flow confinement in spatial separations, J. Chromatogr. A. 1577 (2018) 120–123. https://doi.org/10.1016/j.chroma.2018.09.054.

[79] T. Themelis, J. De Vos, J.L. Dores-Sousa, T. van Assche, S. Eeltink, Engineering solutions for flow control in microfluidic devices for spatial multi-dimensional liquid chromatography, Sensors Actuators, B Chem. 320 (2020) 128388. https://doi.org/10.1016/j.snb.2020.128388.

[80] S.H. Nawada, T. Aalbers, P.J. Schoenmakers, Freeze-thaw valves as a flow control mechanism in spatially complex 3D-printed fluidic devices, Chem. Eng. Sci. 207 (2019) 1040–1048. https://doi.org/10.1016/j.ces.2019.07.036.

[81] F. Erni, R.W. Frei, Two-dimensional column liquid chromatographic technique for resolution of complex mixtures, J. Chromatogr. A. 149 (1978) 561–569. https://doi.org/10.1016/S0021-9673(00)81011-0.

[82] C.J. Venkatramani, Y. Zelechonok, An automated orthogonal two-dimensional liquid chromatograph, Anal. Chem. 75 (2003) 3484–3494. https://doi.org/10.1021/ac030075w.

[83] C.J. Venkatramani, A. Patel, Towards a comprehensive 2-D-LC-MS separation, J. Sep. Sci. 29 (2006) 510–518. https://doi.org/10.1002/jssc.200500341.

[84] C.J. Venkatramani, Y. Zelechonok, Two-dimensional liquid chromatography with mixed mode stationary phases, J. Chromatogr. A. 1066 (2005) 47–53. https://doi.org/10.1016/j.chroma.2004.11.103.

[85] A.J. Alexander, L. Ma, Comprehensive two-dimensional liquid chromatography separations of

pharmaceutical samples using dual Fused-Core columns in the 2nd dimension, J. Chromatogr. A. 1216 (2009) 1338–1345. https://doi.org/10.1016/j.chroma.2008.12.063.
[86] O.P. Haefliger, Universal two-dimensional HPLC technique for the chemical analysis of complex surfactant mixtures, Anal. Chem. 75 (2003) 371–378. https://doi.org/10.1021/ac020534d.
[87] Y. Fa, Y. Yu, F. Li, F. Du, X. Liang, H. Liu, Simultaneous detection of anions and cations in mineral water by two dimensional ion chromatography, J. Chromatogr. A. 1554 (2018) 123–127. https://doi.org/10.1016/j.chroma.2018.04.017.
[88] Q. Ren, C. Wu, J. Zhang, Use of on-line stop-flow heart-cutting two-dimensional high performance liquid chromatography for simultaneous determination of 12 major constituents in tartary buckwheat (Fagopyrum tataricum Gaertn), J. Chromatogr. A. 1304 (2013) 257–262. https://doi.org/10.1016/j.chroma.2013.07.008.
[89] S. Wang, L. Zhou, Z. Wang, X. Shi, G. Xu, Simultaneous metabolomics and lipidomics analysis based on novel heart-cutting two-dimensional liquid chromatography-mass spectrometry, Anal. Chim. Acta. 966 (2017) 34–40. https://doi.org/10.1016/j.aca.2017.03.004.
[90] S. Wang, Z. Wang, L. Zhou, X. Shi, G. Xu, Comprehensive Analysis of Short-, Medium-, and Long-Chain Acyl-Coenzyme A by Online Two-Dimensional Liquid Chromatography/Mass Spectrometry, Anal. Chem. 89 (2017) 12902–12908. https://doi.org/10.1021/acs.analchem.7b03659.
[91] W. Ma, S. Wang, T. Zhang, E.Y. Zhang, L. Zhou, C. Hu, J.J. Yu, G. Xu, Activation of choline kinase drives aberrant choline metabolism in esophageal squamous cell carcinomas, J. Pharm. Biomed. Anal. 155 (2018) 148–156. https://doi.org/10.1016/j.jpba.2018.03.062.
[92] W.C. Byrdwell, Breaking the rules: Two- and three-dimensional liquid chromatography with four dimensions of mass spectrometry, Curr. Trends Mass Spectrom. 19 (2021) 6–11.
[93] W.C. Byrdwell, H.K. Kotapati, R. Goldschmidt, P. Jakubec, L. Nováková, Three-dimensional liquid chromatography with parallel second dimensions and quadruple parallel mass spectrometry for adult/infant formula analysis, J. Chromatogr. A. 1661 (2022) 462682. https://doi.org/10.1016/j.chroma.2021.462682.
[94] C.J. Chen, F.A. Li, G.R. Her, Development of a low-flow multiplexed interface for capillary electrophoresis/electrospray ion trap mass spectrometry using sequential spray, Electrophoresis. 29 (2008) 1997–2003. https://doi.org/10.1002/elps.200700445.
[95] E.A. Livesay, K. Tang, B.K. Taylor, M.A. Buschbach, D.F. Hopkins, B.L. LaMarche, R. Zhao, Y.

Shen, D.J. Orton, R.J. Moore, R.T. Kelly, H.R. Udseth, R.D. Smith, Fully automated four-column capillary LC-MS system for maximizing throughput in proteomic analyses, Anal. Chem. 80 (2008) 294–302. https://doi.org/10.1021/ac701727r.

[96] K. Zhang, Y. Li, M. Tsang, N.P. Chetwyn, Analysis of pharmaceutical impurities using multi-heartcutting 2D LC coupled with UV-charged aerosol MS detection, J. Sep. Sci. 36 (2013) 2986–2992. https://doi.org/10.1002/jssc.201300493.

[97] J. Lin, C. Tsang, R. Lieu, K. Zhang, Fast chiral and achiral profiling of compounds with multiple chiral centers by a versatile two-dimensional multicolumn liquid chromatography (LC–mLC) approach, J. Chromatogr. A. 1620 (2020) 460897. https://doi.org/10.1016/j.chroma.2020.460987.

[98] H. Wang, H.R. Herderschee, R. Bennett, M. Potapenko, C.J. Pickens, B.F. Mann, I.A. Haidar Ahmad, E.L. Regalado, Introducing online multicolumn two-dimensional liquid chromatography screening for facile selection of stationary and mobile phase conditions in both dimensions, J. Chromatogr. A. 1622 (2020) 460895. https://doi.org/10.1016/j.chroma.2020.460895.

[99] C.J. Pickens, I.A. Haidar Ahmad, A.A. Makarov, R. Bennett, B.F. Mann, E.L. Regalado, Comprehensive online multicolumn two-dimensional liquid chromatography-diode array detection-mass spectrometry workflow as a framework for chromatographic screening and analysis of new drug substances, Anal. Bioanal. Chem. 412 (2020) 2655–2663. https://doi.org/10.1007/s00216-020-02498-8.

[100] L. Verscheure, G. Vanhoenacker, S. Schneider, T. Merchiers, J. Storms, P. Sandra, F. Lynen, K. Sandra, 3D-LC-MS with 2D Multimethod Option for Fully Automated Assessment of Multiple Attributes of Monoclonal Antibodies Directly from Cell Culture Supernatants, Anal. Chem. 94 (2022) 6502–6511. https://doi.org/10.1021/acs.analchem.1c05461.

Figure Captions

Figure 1. Early demonstration of dual column array in the second dimension for the multiple heart-cut chiral-achiral 2D-LC analysis of stereoisomers of leucovorin (LV) and 5-methyltetrahydrofolate (5-METHF) in plasma. For the first 16.5 min after injection (panel A), eluent from the first dimension Resolvosil BSA-7 column was directed to waste (red trace) while ^{2}D column B is equilibrated (blue trace). Then, the *S* diastereomer of LV was loaded onto ^{2}D column A over the next six minutes (red trace in panel B). From 22.5 min to 46.5 min (panel C), an elution gradient was performed on ^{2}D column A (blue trace) while the *R* diastereomer of LV and both diastereomers of 5-METHF were loaded onto ^{2}D column B (red trace). In the final 24 min of the method, an elution gradient was performed on ^{2}D column B (same position as panel A). Adapted from reference [37] with permission from Elsevier (Copyright 1990).

Figure 2. Schematic of a SEC-RPLC-UV-MS technique using a dual ^{2}D column array with two switching valves. Reproduced from reference [39] with permission from the American Chemical Society (Copyright 1997).

Figure 3. Instrument diagram for the IEX-RPLC-UV separation of peptides and small proteins with fraction collection for offline MALDI-MS analysis. This system included four identical RP columns in the ^{2}D array. Reproduced from reference [44] with permission from the American Chemical Society (Copyright 2002).

Figure 4. Schematic of a 2D IEX-RPLC separation for the analysis of intact proteins using a ^{2}D array of twelve columns connected to two selector valves. Reproduced from reference [47] with permission from Elsevier (Copyright 2018).

Figure 5. 2D contour plot for NPLC x RPLC separation using dual ^{2}D column. Peak capacity of 487 was obtained in this mixture of 28 standard compounds. Reproduced from reference [50] with permission from Elsevier (Copyright 2008).

Figure 6. Schematic of a 2D SCX-RPLC system with a ^{2}D array of 18 capillary. These columns elute onto a MALDI plate for offline MALDI-TOF-TOF-MS detection. Adapted from reference [63] with permission from the American Chemical Society (Copyright 2006).

737

738 **Figure 7.** Microfluidic device with a single ^{1}D column channel, a ^{2}D flow distributor, 21 parallel ^{2}D
739 column channels, and an outlet flow collector. Reproduced from reference [71] with permission from John
740 Wiley and Sons (Copyright 2015).

741

742 **Figure 8.** 2D contour plots for the 2D-LC separation of a test mixture of aromatic compounds. The ^{1}D
743 ODS-AQ column eluent was sampled onto cyano and amino ^{2}D columns in an alternating sequence.
744 Reproduced from reference [82] with permission from the American Chemical Society (Copyright 2003).

745

746 **Figure 9.** Schematic of an achiral-chiral 2D-LC separation with six ^{2}D chiral columns for automated
747 column screening during method development. Reproduced from reference [97] with permission from
748 Elsevier (Copyright 2020).

749

Figures

Figure 1.

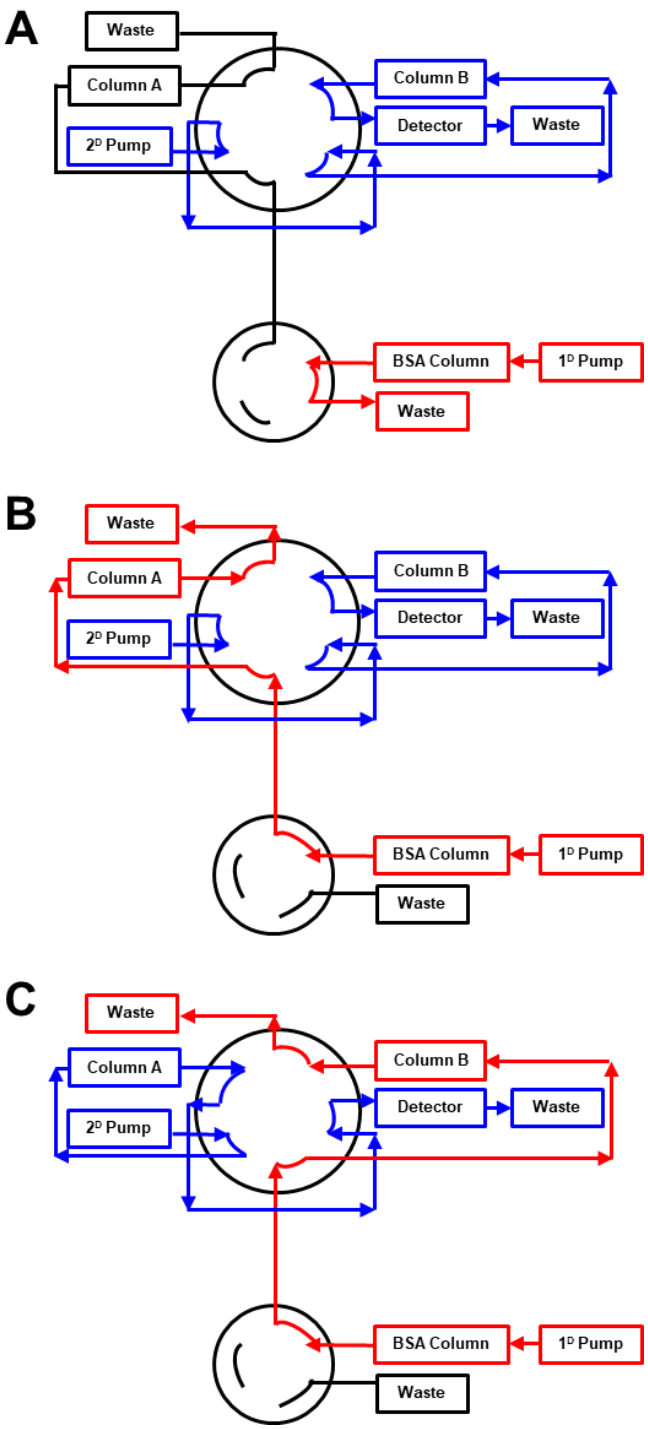


Figure 2.

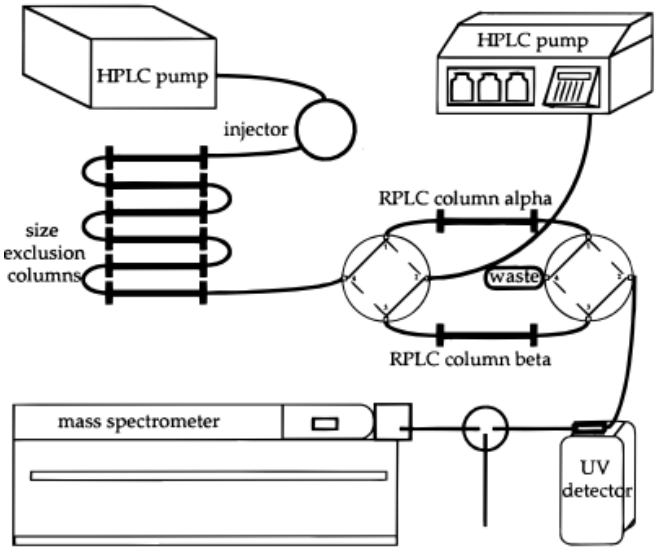
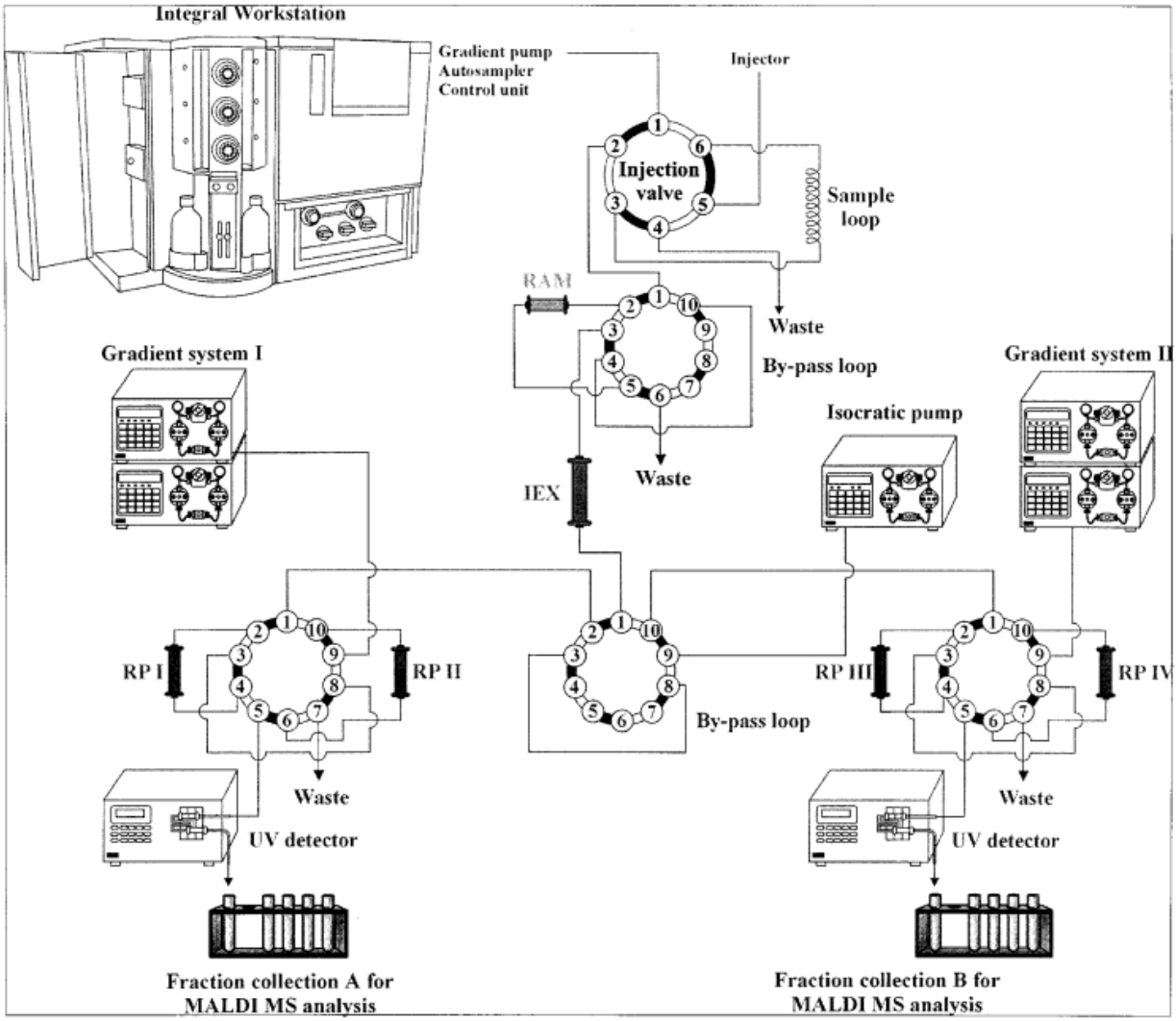
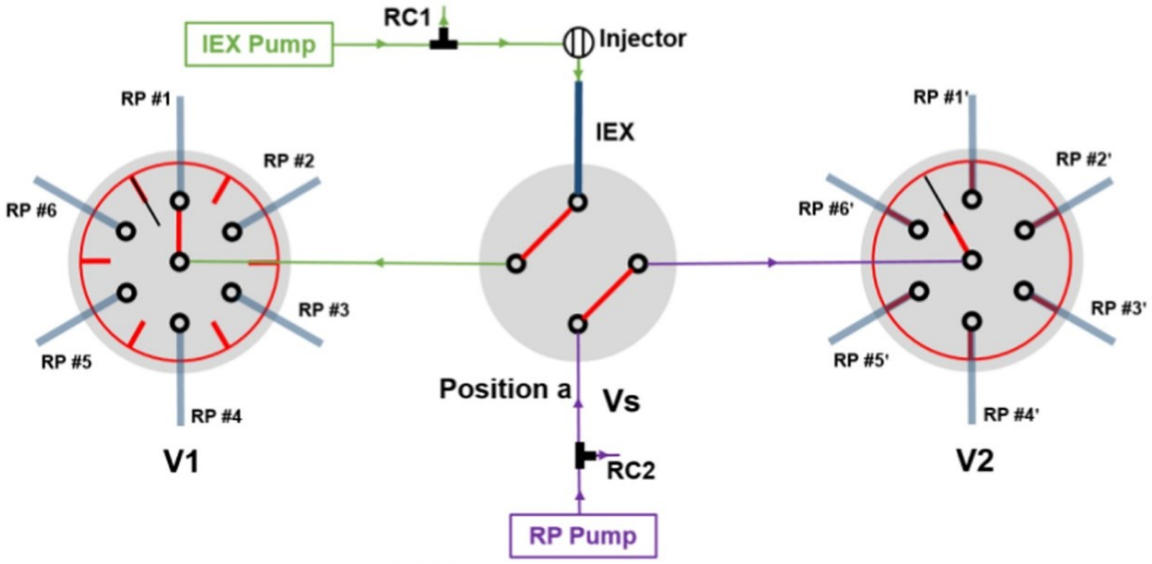

HPLC pump
HPLC pump
injector
size
exclusion
columns
RPLC column alpha
waste
RPLC column beta
mass spectrometer
UV
detector

Figure 3.


Integral Workstation
Gradient pump
Autosampler
Control unit
Injector
Injection valve
Sample loop
Waste
RAM
By-pass loop
Waste
Gradient system I
IEX
Isocratic pump
Gradient system II
RP I
RP II
By-pass loop
RP III
RP IV
Waste
UV detector
Waste
UV detector
Fraction collection A for MALDI MS analysis
Fraction collection B for MALDI MS analysis

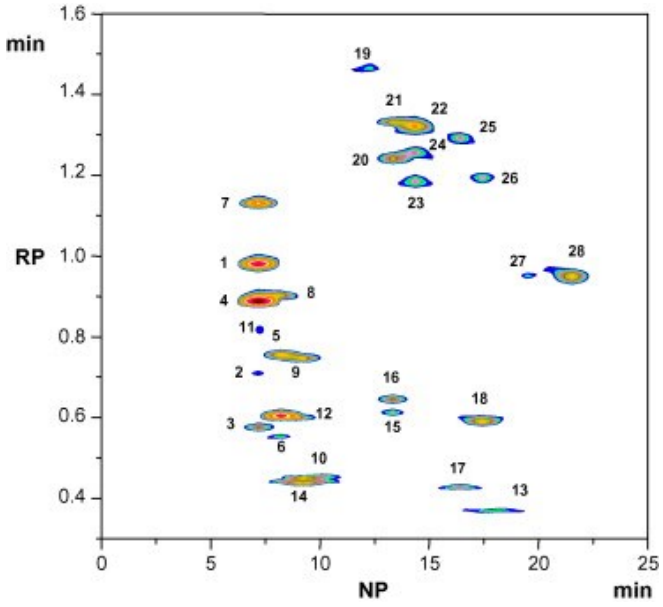
761 **Figure 4.**



762

763

764 **Figure 5.**



765

766

Figure 6.

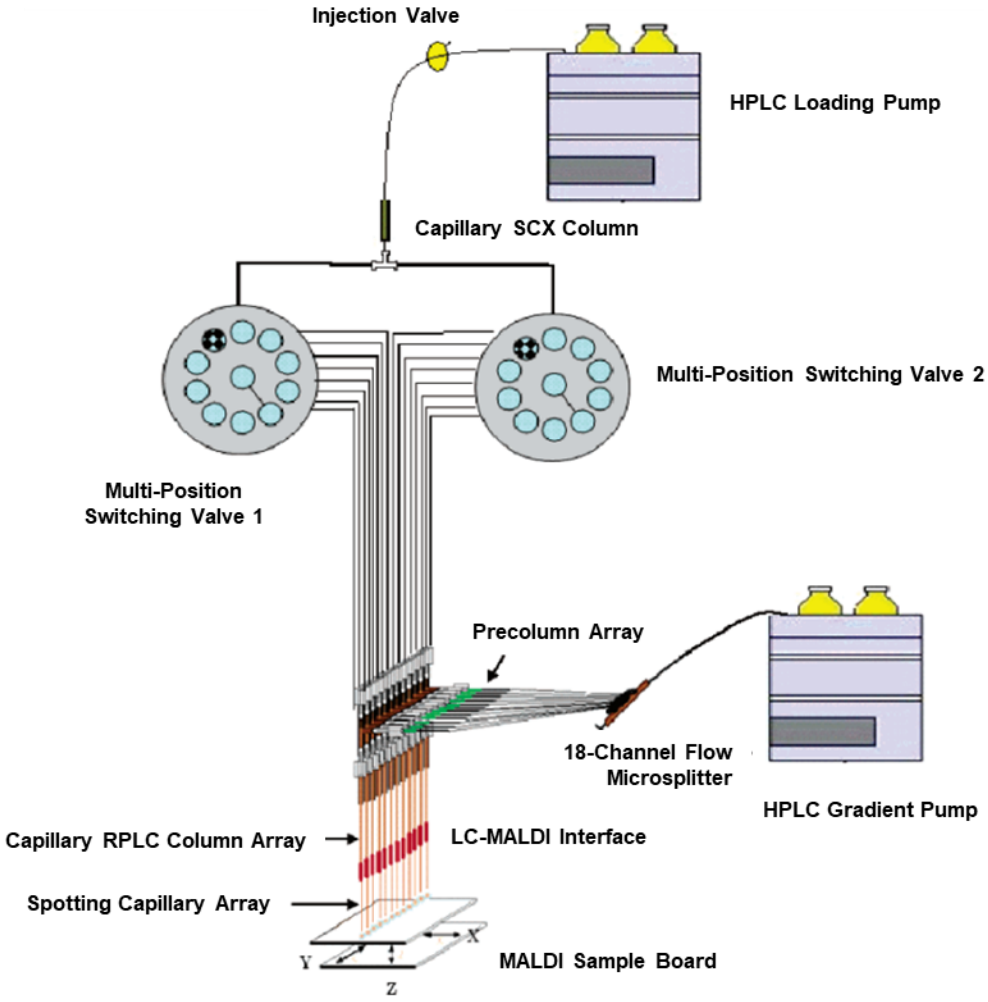
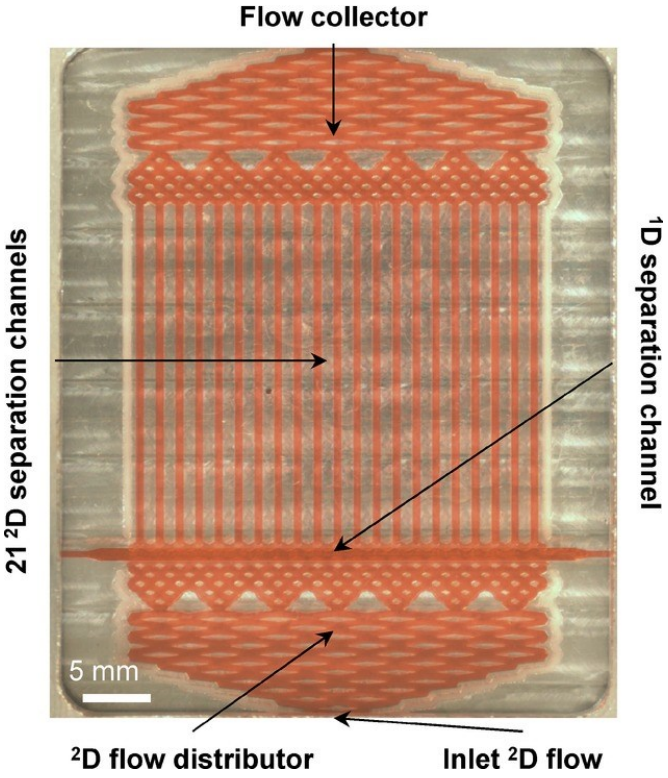
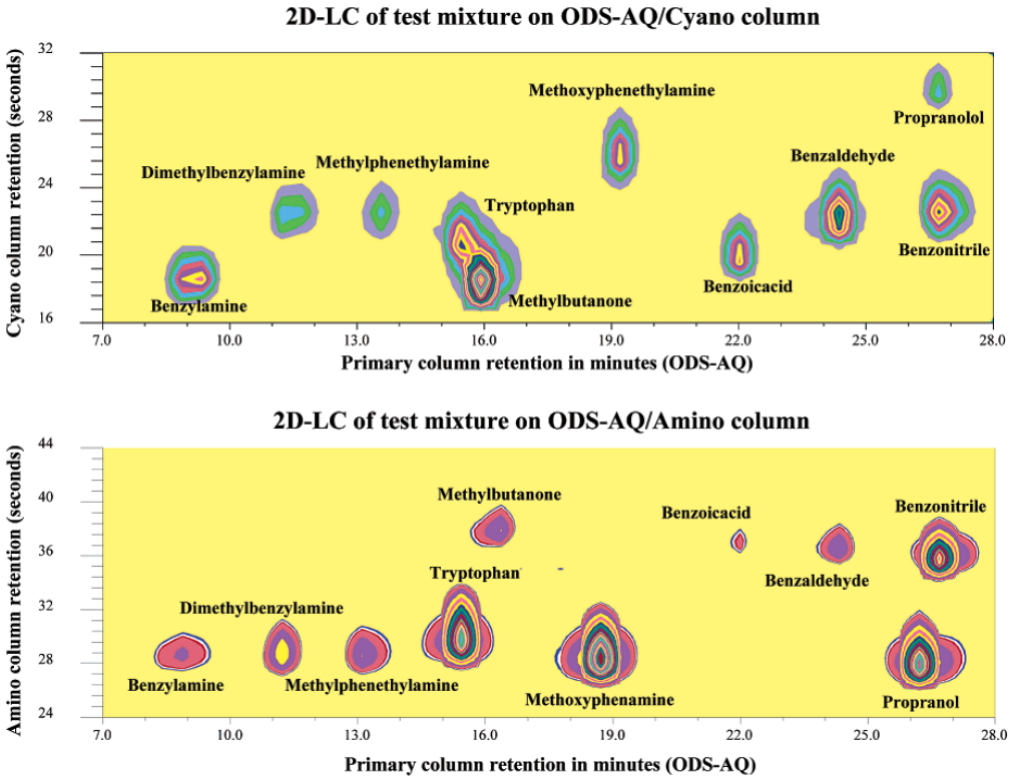

Injection Valve
HPLC Loading Pump
Capillary SCX Column
Multi-Position Switching Valve 2
Multi-Position Switching Valve 1
Precolumn Array
18-Channel Flow Microsplitter
HPLC Gradient Pump
Capillary RPLC Column Array
LC-MALDI Interface
Spotting Capillary Array
MALDI Sample Board
X
Y
Z

Figure 7.


Flow collector
21 ²D separation channels
¹D separation channel
5 mm
²D flow distributor
Inlet ²D flow

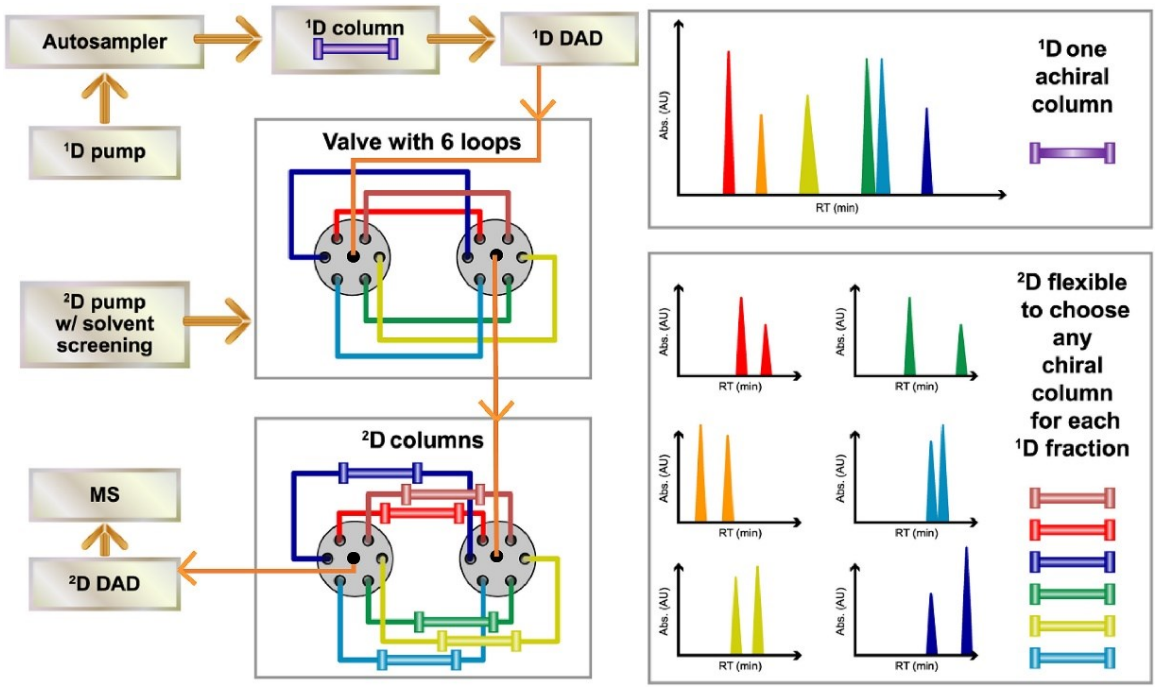
774 **Figure 8.**



775

776

Figure 9.


Autosampler
¹D column
¹D DAD
¹D pump
Valve with 6 loops
²D pump w/ solvent screening
²D columns
MS
²D DAD
Abs. (AU)
RT (min)
¹D one achiral column
²D flexible to choose any chiral column for each ¹D fraction

781 **Table 1.** Summary of Parallel Column Arrays Operated in an Alternating or Sequential Order

Method Designation	**^{1}D Column(s)**	**^{2}D Columns**	**Modulation Scheme**	**Sample Description**	**Total Separation Time**	**Peak Capacity**	**Reference**
Multiple heart-cut	Resolvosil BSA-7 (4 mm x 150 mm)	Two LiChrocart RP-18 (4 mm x 125 mm)	10-port valve	Pharmaceutical drugs in extracted plasma	71.5 min	N.R.	[37]
Comprehensive	G2000SW$_{XL}$ (7.8 mm x 300 mm, six coupled serially)	Two Micra RP-18 (4.6 mm x 33 mm)	Two 4-port valves	Tryptic digest of ovalbumin	180 min	495	[39]
Comprehensive	G2000SW$_{XL}$ (7.8 mm x 300 mm, six coupled serially)	Two Hypersil BDS C-18 (1.0 mm x 20 mm)	Two 4-port valves	Tryptic digest of bovine serum albumin	320 min	520	[40]
Comprehensive	G3000SW$_{XL}$ (7.8 mm x 300 mm, eight coupled serially)	Two PerSeptive R2/H (2.1 mm x 33 mm)	Two 4-port valves	Protein mixture (*E. coli* lysate)	390 min	N.R.	[41]
Comprehensive	G3000SW$_{XL}$ & G4000SW$_{XL}$ (7.8 mm x 300 mm, six of each coupled serially)	Two PerSeptive R2/H (2.1 mm x 100 mm)	Two 4-port valves	Protein mixture (*E. coli* lysate)	1050 min	1500	[41]
Comprehensive	TSK-gel DEAE-NPR or TSK-gel SP-NPR (4.6 mm x 35 mm)	Two Micra NPS ODS I (4.6 mm x 14 mm)	10-port valve	Standard protein mixture and human fibroblast cell extract	20 min	600	[42],[43]

Comprehensive	TSK-gel DEAE-NPR or TSK-gel SP-NPR (4.6 mm x 35 mm)	Four Micra NPS ODS I (4.6 mm x 14 mm)	Three 10-port valves	Human hemofiltrate (including from chronic renal patients) and human fibroblast cell lysate (20 kDa cutoff)	96 min	3000	[44],[45]
Comprehensive	Co-polymer monolith functionalized with tertiary amine groups (0.250 mm x 260 mm)	Three PS-DVB monolith (0.250 mm x 260 mm)	Two 7-port selector valves	Protein mixtures (Standards & *E. coli* lysate)	630 min	N.R.	[46]
Comprehensive	Co-polymer monolith functionalized with tertiary amine groups (0.250 mm x 300 mm)	Twelve PS-DVB monolith (0.250 mm x 300 mm)	Two 7-port selector valves & one 4-port valve	Protein mixtures (Standards & *E. coli* lysate)	252 min	N.R.	[47]
Comprehensive	BioBasic AX (4.6 mm x 250 mm)	Two (plus one additional in series) SinoChrom ODS-BP (4.6 mm x 50 mm)	10-port valve	Tryptic digest of standard protein mixture	100 min	650 (890 at high temp. conditions)	[49]
Comprehensive	Betasil Diol (1.0 mm x 250 mm)	Two Zorbax SB C18 (4.6 mm x 50 mm)	Two 10-port valves	Small molecule test mixture	25 min	516 (487 corrected for complementarity)	[50]
Comprehensive	Betasil Diol	Two	Two	Lemon oil extract	90 min	1840	[50]

	(1.0 mm x 250 mm)	Zorbax SB C18 (4.6 mm x 50 mm)	10-port valves			(1095 corrected for complementarity)	
Comprehensive	Zorbax SB CN (2.1 mm x 150 mm)	Two Zorbax SB C18 (4.6 mm x 50 mm)	Two 10-port valves	Steroid mixture	25 min	400 (82 corrected for complementarity)	[50]
Comprehensive	Zorbax SB CN (2.1 mm x 150 mm)	Two Zorbax SB C18 (4.6 mm x 50 mm)	Two 10-port valves	Sulfonamide mixture	75 min	N.R.	[50]
Comprehensive	Zorbax SB CN (2.1 mm x 150 mm)	Two Zorbax SB C18 (4.6 mm x 50 mm)	Two 10-port valves	Phenacyl esters of triglycerides in fish oil	60 min	290 (159 corrected for complementarity and 90 corrected for complementarity and undersampling)	[51]
Comprehensive	Halo C18 (2.1 mm x 150 mm, four coupled serially, low pH)	Two Zorbax 300 Extend ODS (4.6 mm x 50 mm, high pH)	Two 10-port valves	Tryptic digests of bovine serum albumin and human serum	360 min	4677 (corrected for complementarity and undersampling)	[52]
Comprehensive	Discovery HS PEG (4.6 mm x 150 mm)	Two Discovery ZR-CARBON (2.1 mm x 50 mm)	10-port valve	Standard mixture of phenolic compounds and flavonoids, beer	135 min	N.R.	[53]

Comprehensive	Discovery HS PEG (2.1 mm x 50 mm) & Purospher Star RP-18e (4.6 mm x 150 mm) connected in series	Two Discovery ZR-CARBON (2.1 mm x 50 mm)	10-port valve	Standard mixture of phenolic compounds and flavonoids, beer	160 min	N.R.	[53]
Comprehensive	Zorbax SB C18 (0.5 mm x 150 mm)	Two Discovery ZR-CARBON (2.1 mm x 50 mm)	10-port valve	Standard mixture of phenolic compounds and alkylbenzenes, beer, wine	130 min	N.R.	[54]
Comprehensive	Shodex SP-420N (4.6 mm x 35 mm)	Two Eichrom NPS C18 (4.6 mm x 33 mm)	10-port valve	Standard protein mixture	84 min	N.R.	[55]
Comprehensive	Shodex SP-420N (4.6 mm x 35 mm)	Two Symmetry300 C4 (2.1 mm x 50 mm)	10-port valve	Ribosomal proteins (yeast cell lysate)	200 min	~700	[55]
Comprehensive	SCX-SAX columns coupled in series	Two ODS monolith	6-port valve	Human urinary metabolites	160 min	N.R.	[56]
Comprehensive	Cosmosil 5PBB (4.6 mm x 150 mm)	Two Chromolith SpeedROD (4.6 mm x 50 mm)	10-port valve	Polycyclic aromatic hydrocarbons (standards and from gasoline extract)	30 min	N.R.	[57]
Comprehensive	Fluofix (4.6 mm x 150 mm)	Two ODS monolith	Flow switch and two	Standard mixture of hydrocarbons	65 min	1190	[58]

		(4.6 mm x 30 mm)	6-port valves	and benzene derivatives			
Comprehensive	Waters ODS-AQ (4.6 mm x 150 mm)	Two Exsil Amino (4.6 mm x 50 mm)	12-port valve	Small molecule standard test mixture	30 min	450	[82]
Comprehensive	X-Terra C18 (4.6 mm x 150 mm)	Two Zorbax SB-Phenyl (4.6 mm x 50 mm)	12-port valve	Small molecule drug mixture	35 min	N.R.	[83]
Comprehensive	Zorbax Extended C18 (2.1 mm x 150 mm)	Ascentis Express C18 (3.0 mm x 50 mm)	12-port valve	Mixture of drug degradants	17 min	N.R.	[85]

782

783

784 **Table 2.** Summary of Parallel Column Arrays Operated Simultaneously

Method Designation	^{1}D Column	^{2}D Columns	Modulation Scheme	Sample Description	Total Separation Time[a]	Number of Protein Identifications	Reference
Comprehensive	POROS SCX (0.53 mm x 150 mm)	18 Hypersil C18 (0.25 mm x 250 mm)	Two 11-port selector valves	Tryptic digest of proteins from human liver tissue	180 min	462	[63]
Comprehensive	POROS SCX (0.32 mm x 70 mm)	10 Zorbax 300 SB C8 (0.25 mm x 250 mm)	In-house fabricated multiple-channel interface	Tryptic digest of proteins from liver cancer tissue (from mouse model)	150 min	1202	[64]
Multiple heart-cut	ProPac SAX-10 (4.0 mm x 250 mm)	Eight Xtimate C8 (2.1 mm x 250 mm)	11-port selector valve	Proteins in human plasma	240 min	1332	[65]
Multiple heart-cut	PolyCATWAX50/50 (4.6 mm x 200 mm)	Eight UniPS 5-1000 SS (2.1 mm x 150 mm)	11-port selector valve	HeLa cell lysate	300 min	4436	[67]

785 [a] Times listed for primary 2D-LC separation and do not include additional off-line detection time by MS or LC-MS.

786

787 **Table 3.** Summary of Parallel Column Arrays with Different ^{2}D Stationary Phases

Method Designation	**^{1}D Column(s)**	**^{2}D Columns**	**Modulation Scheme**	**Sample Description**	**Total Separation Time**	**Reference**
Comprehensive	Waters ODS-AQ (4.6 mm x 150 mm)	Exsil Amino (4.6 mm x 50 mm) & Platinum Cyano (7.0 mm x 33 mm)	12-port valve	Standard mixture of small molecule aromatic compounds	30 min	[82]
Comprehensive	Primesep-100 (4.6 mm x 150 mm)	Primesep-100 (4.6 mm x 20 mm) & Primesep-B (4.6 mm x 20 mm)	12-port valve	Small molecule standard test mixture	30 min	[84]
Comprehensive	Primesep-B (4.6 mm x 150 mm)	Primesep-100 (4.6 mm x 20 mm) & Primesep-B (4.6 mm x 20 mm)	12-port valve	Small molecule standard test mixture	30 min	[84]
Multiple heart-cut	Nucleosil 100-5 OH (1.0 mm x 250 mm)	Nucleosil 120-5 C4 (4.0 mm x 125 mm) & Nucleosil 100-7 C2 (4.0 mm x 250 mm)	10-port valve	Complex surfactant mixture	54 min	[86]
Multiple heart-cut	Thermo Carbohydrate Removal Cartridge (2.0 mm x 150 mm) or IonPac CG12A (2.0 mm x 50 mm)	IonPac CS12A (2.0 mm x 250 mm) & IonPac AS11-HC (2.0 mm x 250 mm)	10-port valve	Standard mixture of inorganic ions and mineral water samples	25 min	[87]

Comprehensive	Fluofix (4.6 mm x 150 mm)	One ODS monolith and one PBB monolith (both 4.6 mm x 30 mm)	Flow switch and two 6-port valves	Standard mixture of hydrocarbons and benzene derivatives	65 min (130 min if run twice for full coverage)	[58]
Multiple heart-cut (with stop flow)	Acclaim Mixed-Mode HILIC-10 (2.1 mm x 10 mm)	Acclaim RSLC Polar Advantage II (2.1 mm x 150 mm) & Acclaim RSLC Phenyl-1 (2.1 mm x 150 mm)	10-port valve & 6-port valve	Small molecules from tartary buckwheat plants	75 min	[88]
Multiple heart-cut	Acquity BEH C8 (2.1 mm x 5 mm)	Acquity BEH C18 (2.1 mm x 50 mm or 2.1 mm x 100 mm) & Acquity HSS T3 (2.1 mm x 50 mm)	6-port valve, 8-port valve, & 10-port valve	Pooled plasma, mouse tissue liver extract, prostate cancer cells, serum from esophageal squamous cell carcinoma patients	30 min	[89], [90], [91]
Comprehensive (split-flow, multicycle)	Two Inertsil ODS-2 (4.6 mm x 250 mm) connected in series	Two Accucore C30 (2.1 mm x 50 mm & 3.0 mm x 100 mm)	Prototype valve switching system	Infant/adult nutritional formula	75 min	[92], [93]
Multiple heart-cut	XTerra RP18 (4.6 mm x 150 mm) or	Six assorted RP and HILIC columns (varying dimensions)	Two 6-port valves &	Pharmaceutical compound (peak purity profiling)	10 – 25 min	[96]

(with peak parking)	Zorbax Eclipse XDB C18 (4.6 mm x 150 mm)		four 7-port selector valves			
Multiple heart-cut	Ascentis Express C18 (4.6 mm x 150 mm) Or Poroshell EC-C18 (3.0 mm x 150 mm)	Six polysaccharide chiral phases (all 3.0 mm x 50 mm)	Four 7-port selector valves	Pharmaceutical compound (chiral purity profiling)	24 min	[97]
Multiple heart-cut	Six RP columns (all 2.1 mm x 50 mm)	Four chiral phase columns (all 4.6 mm x 50 mm)	Two 14-port, 6-position column selection valves & 8-port duo head valve	Pharmaceutical compound (chiral purity profiling)	<20 min	[98]
Comprehensive	C4, SEC, SAX, and SCX columns (various dimensions)	Three RP columns (all 3.0 mm x 50 mm)	Two 14-port, 6-position column selection valves & 8-port duo head valve	Standard protein mixture (both intact and chymotryptic digest)	<35 min	[99]
Multiple heart-cut	Bio-Monolith Protein A column (5.2 mm x 5 mm)	AdvanceBio SEC 300A (7.8 mm x 300 mm), Bio Mab NP5 PK	10-port active solvent modulation valve & two 8-port duo head valves	Monoclonal antibodies	<70 min (*includes ^{3}D desalting SEC column*)	[100]

		(2.1 mm x 250 mm, or alternate MabPac SCX-10), AdvanceBio HIC (4.6 mm x 100 mm)				

788