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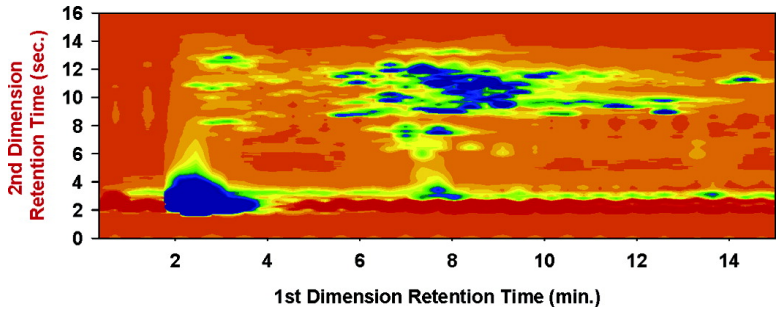

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Fast, Comprehensive Two-Dimensional HPLC Separation of Tryptic Peptides Based on High-Temperature HPLC

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Two-dimensional HPLC (2D-LC) has recently received considerable attention.[1] This interest is kindled by both the statistical model of overlap (SMO) theory, which makes it clear that the resolving power of one-dimensional HPLC is grossly insufficient for many purposes,[2] and by the demands of many bio-analytical applications.[1] Gradient elution 2D-LC is most important to peptide and protein separations, and is used as an alternative to 2D gel electrophoresis for use in proteomics.[3] The greatest impediment to the widespread use of 2D-LC is its long analysis time ranging up to days per analysis,[4,5] making the technique impractical for many jobs. Here we focus on improving the speed of gradient separations since these are typically used as the second dimension for peptide separations. Specifically we describe high-temperature,[6,7] ultrafast HPLC conditions,[8−10] along with the instrument modifications needed to reduce the analysis time of each complete second-dimension gradient separation to tens of seconds. Most importantly, this system is capable of generating the high peak capacity (>1000) characteristic of comprehensive 2D-LC in a relatively shorter analysis time (under 30 min) with simpler instrumentation than currently used.[11] For a complete 20-minute 2DLC analysis of tryptic peptides, the peak capacity of the system is about 1350, equivalent to one unit of peak capacity per second.[12]

Recognizing that sluggishness is its chief limitation, several approaches have been taken to improve the speed of 2D-LC. Tanaka[13] and others[14] have tested monolithic columns as the second dimension of a 2D-LC system. Unfortunately, pumping systems that give highly reproducible retention times, which are essential for 2D-LC, on capillary monolithic columns and monolithic columns with inner diameters in the range of 1−2 mm are not available,[15] and consequently very high flow rates exceeding 10 mL/min must be used to significantly improve the separation speed when monoliths are used.[13] Another approach is to use multiple second-dimension columns so that several second-dimension separations can be done simultaneously.[11,13,16,17] While this does improve the overall speed of the 2D-LC analysis, it requires more elaborate instrumentation[11,17] and introduces difficulty in data analysis, given that alternating separations must be performed on different columns which are rarely identical.[18]

Our approach focuses on greatly improving the speed of the second-dimension reversed-phase gradient separation. In a more detailed study of the factors controlling gradient cycle time (i.e. the gradient time plus the reequilibration time), we found that excellent repeatability of retention time (<±0.002 min) could be realized with narrow bore (2.1 mm), wide pore (300 Å) columns at higher temperature (100 °C) with only about one-column volume of flushing with the initial eluent.[19] We found that the foremost limitation to the reduction of the gradient cycle time when using short, narrow-bore columns for ultrafast separations (<30 s) at higher temperatures was the time required to flush the final eluent out of the HPLC hardware. High temperatures also facilitate a further reduction in gradient cycle time. The decrease of viscosity at high temperatures (a factor of 3.5 for a 20/80 acetonitrile/water mixture at 120 °C compared to 25 °C) allows a much higher eluent linear velocity (interstitial velocity, 3−10 cm/s) through the column to allow faster gradient development and also largely mitigates the loss in efficiency which occurs at high linear velocity.[7,10] While concerns for analyte instability under such high-temperature conditions have been raised repeatedly, our studies show that with residence times of 10−20 s very little detectable degradation of either small, labile organic analytes or proteins occurs.[6,20]

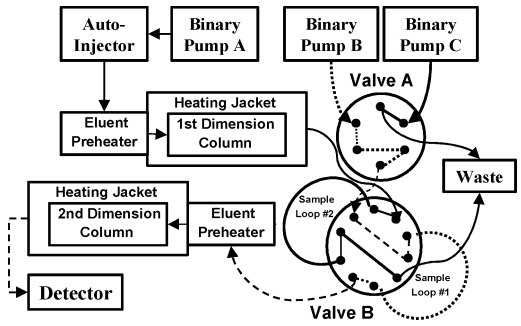

Figure 1. Schematic of an instrument for fast, comprehensive 2D-LC. Binary pumps C and valve A, necessary for the ultrafast second-dimension gradients in this work, are additions to the original setup of Bushey and Jorgenson.[21] The dashed and solid lines indicate independent continuous flow paths.

We modified the 2D-LC instrumentation described by Jorgenson[21] to form the instrument shown schematically in Figure 1. The salient features of this instrument are the addition of valve A to allow switching between two independent binary solvent streams, a second binary pump capable of developing fast binary gradients in the second dimension, and devices to properly heat both the column and the eluent to avoid thermal mismatch broadening (see Supporting Information). Under typical conditions the effective gradient time is 16 s, and the effective reequilibration time (including instrument flush-out) can be reduced to 5 s, giving a total cycle time of 21 s.

To demonstrate the resolving power of this fast, comprehensive 2D-LC system, we separated a tryptic digest of bovine serum albumin (BSA) using a strong cation-exchange column (SCX, PO_4-ZrO_2 prepared as described previously,[22] 5 μm, 250 Å pore size, 50 mm × 2.1 mm i.d.) in the first dimension, and a reversed-phase column judiciously chosen for its high stability in hot, acidic mobile phases in the second dimension (SB-C_{18}, 3.5 μm, 300 Å pore size, 50 mm × 2.1 mm i.d.). Details of the chromatographic conditions used in the first and second dimensions are provided as Supporting Information; briefly, a buffered (pH = 2.9) gradient of sodium phosphate was used in the first dimension, and a reversed phase gradient of acetonitrile−water with 0.1% trifluoroacetic acid was used in the second dimension on the relatively thermally stable reversed-phase column. It is particularly important to note that both the first- and second-dimension columns are carefully thermostated

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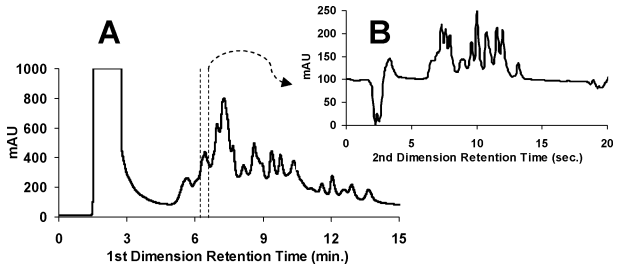

Figure 2. Example of a single second-dimension chromatogram resulting from the injection of the first-dimension peak captured from 6.65 to 7.00 min.

at 35 and 100 °C, respectively. The flow rate through the second-dimension column was 3.0 mL/min. (27.5 MPa), and each injection of effluent from the first-dimension column onto the second-dimension was 35 μL. Figure 2A shows the chromatogram resulting from the separation of the tryptic peptide sample on the first-dimension SCX column alone, along with a representative chromatogram (Figure 2B) generated by the injection of a 35 μL fraction of effluent captured from the first-dimension column during the 6.65−7.00 min interval. Peptides were detected by their absorbance at 214 nm. The complete, 2D chromatogram (Figure S1) shows several peaks in each second-dimension separation. Most components eluting from the first-dimension column appear in at least two second-dimension chromatograms, thus indicating that we are sampling at a rate sufficient to nearly maximize the two-dimensional information content.[23] By counting the number of peaks appearing in individual second-dimension chromatograms, and assuming that each component is present in an average of three second-dimension separations, we estimate that more than 100 components were resolved in this 2D-LC separation.

To evaluate the precision in peak height and area, and retention time of the 2D-LC system, we used a mixture of four cationic undecapeptides (Alberta Peptide Institute, Catalog no. CES-00P50) and completed three full 2D-LC separations of this mixture; we chose not to evaluate the precision of peaks in the tryptic BSA digest separation because of the low probability of finding completely pure peaks (using UV detection), thus invalidating the precision calculations. The average precision of the retention time (peak maximum), peak height, and peak area for the four components are quite satisfactory at 0.32, 2.9, and 3.3% RSD, respectively (complete data in Table S2) and considerably superior to that achieved by the multicolumn approach. To estimate the peak capacity of the second-dimension gradient separation, we measured the peak width at half-height ($w_{1/2}$), and converted these widths to full (4σ) peak widths. A plot of full second-dimension peak width vs gradient retention time (Figure S3) shows that the peak width does not correlate with retention time, and the average 4σ peak width is about 0.5 s. Using the gradient elution peak capacity equation of Neue,[24] we estimated that the peak capacity of each second-dimension gradient was 32; based on this the probability of resolving a four-component mixture into four singlet peaks, assuming that the retention times are distributed in a Poisson fashion, is about 0.3−0.5.[2] During a full 20-min 2D-LC separation (including 5 min for SCX column reequilibration), 42 individual 35-μL fractions were captured from the first-dimension column and injected onto the second-dimension column. We further assumed, as has been done by others,[17] that the total peak capacity is the product of the second-dimension peak capacity (32) and the number of first-dimension fractions taken (42). We have followed the rule of Murphy[23] regarding the rate of sampling of the first-dimension eluent; however, we use the above approximation, despite its shortcomings, to facilitate comparison to previous studies. The total peak capacity calculated using this method is 1350 for the 20-min analysis, or approximately 1 peak/s (4000/h). This compares quite favorably to a recent report of 2000/h using a far more elaborate 2D-LC system.[17]

The dramatic improvement in the speed of reversed-phase gradient elution through the use of high-temperature HPLC and modest instrument modifications has led to a significant improvement in the performance of 2D-LC. Further optimization of this system will lead to improvements in the peak capacity productivity and make 2D high-temperature HPLC a practical approach for the analysis of complex biological mixtures.

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Supporting Information Available: Complete ref 13, detailed description of the operation of the instrumentation used, chromatographic conditions, data analysis information, tryptic digest conditions, a full 2D-LC chromatogram (Figure S2), peak width (Figure S3) and precision data (Table S2) (PDF). This material is available free of charge via the Internet at http://pubs.acs.org.

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