High throughput capillary liquid chromatography using a droplet injection and application to reaction screening

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Abstract

The cycle time of a standard liquid chromatography (LC) system is the sum of the time for the chromatographic run and the autosampler injection sequence. Although LC separation times in the 1-10 s range have been demonstrated, injection sequences are commonly >15 s, limiting throughput possible with LC separations. Further, such separations are performed on relatively large bore columns requiring flow rates of ≥5 mL/min, thus generating large volumes of mobile phase waste when used for large scale screening and increasing the difficulty in interfacing to mass spectrometry. Here, a droplet injector system was established that replaces the autosampler with a four-port, two-position valve equipped with a 20 nL internal loop interfaced to a syringe pump and a three-axis positioner to withdraw sample droplets from a well plate. In the system, sample and immiscible fluid are pulled alternately from a well plate into a capillary and then through the injection valve. The valve is actuated when sample fills the loop to allow sequential injection of samples at high throughput. Capillary LC columns with 300 µm inner diameter were used to reduce the consumption of mobile phase and sample. The system achieved 96 separations of 20 nL droplet samples containing 3 components in as little as 8.1 min with 5-s cycle time. This system was coupled to a mass spectrometer through an electrospray ionization source for high-throughput chemical reaction screening.

Keywords: Liquid Chromatography; High-Throughput; Reaction Screening; Segmented Flow Droplets

INTRODUCTION

Recent years have seen the development of increasingly rapid HPLC separations. Separation times of 1-15 s have been demonstrated for several sample types including chiral, achiral, and small biomolecules.[1-8] These separations have been achieved by using short HPLC columns (0.5-5.5 cm long), high flow rates (1-8 mL/min), and systems modified to have reduced dead volumes. Although peak capacity of these fast isocratic separations has been modest compared to longer analysis times, the use of peak deconvolution methods can increase information content.[2, 6, 9, 10] Up to 10 components were resolved in 0.9 s using such approaches.[2] One potential application demonstrated by fast separations was the increased throughput of standard pharmacopeial methods.[1, 4, 11] In principle, separation times of 1-15 s have appealing applications in a variety of high-throughput experiments (HTE) such as screening organic reactions, biocatalyst development, chemical "sensing", process monitoring, and quality testing of chemical libraries, among others. A bottle neck for throughput, however, has been injection methods. Typical autosamplers are not designed for throughputs of 1 samples/s and injection cycle times are generally in the range of 15-45 s. A second limitation has been use of larger bore columns, necessitating flow rates in the 5-8 mL/min range to achieve fast separations. Such flow rates would generate substantial amounts of mobile phase waste for large scale screens. Further, they hinder interface to mass spectrometry (MS) detection since optimal flow rates for LC-electrospray ionization (ESI)-MS experiments are typically 10 µL/min – 300 µL/min.[12, 13] Therefore, faster injection schemes and smaller scale columns would make high-throughput LC more feasible and useful. The potential for higher throughput injection has been demonstrated with a commercial HPLC-style injector that has been interfaced to solid-phase extraction-ESI-MS for analysis rates up to 10 s per sample.[14-16] Numerous applications have been demonstrated with this system.[14-24] While clearly powerful and filling a need, it does not provide chromatographic separation, relies on high flow rates and consumes 10 µL sample per assay. Limited attention has been devoted to the potential utilization of droplet microfluidics for injections in liquid chromatography.[25] In this work, a high-throughput droplet microfluidic injection for capillary LC and demonstrate its application to screening organic reactions is described.

Previously, we demonstrated that segmented flow could be used to enable rapid injections for HPLC. In this approach, a train of 4 µL plugs of sample separated by air bubbles is sequentially loaded into an external loop HPLC valve to achieve up to 1 s/sample analysis rates with a 5 mm long x 2.1 mm bore HPLC column operated at 5 mL/min. [26] Work with that system demonstrated some areas that could be improved. Sample loading was a two-step process of generating an array of segmented samples followed by pumping into the loop. It would be preferable to be able to continuously load samples to simplify and automate operation for larger scale screens. Also, that previous work did not address the large mobile phase and sample consumption of high-throughput HPLC with analytical-scale columns.

Here, we employ capillary LC columns (20 mm long x 0.3 mm inner diameter) coupled to a 20 nL internal loop injection valve for segmented injections. In this approach, a syringe pump operated in withdraw mode is connected to the waste side of the injection valve (Figure 1). The pump is operated in withdraw mode to continuously generate samples segmented by perfluorodecalin (PFD) from a well plate and pull them into the valve for injections at 4-s

intervals. 160 nL of sample is consumed per injection, and total mobile phase flow rates are 70 µL/min, an 70-fold reduction over standard HPLC systems. This approach was inspired in part by the report of a custom fabricated chip LC injector that used an internal loop design to inject droplet microfluidic samples.[27] That pioneering work demonstrated much lower sample consumption, with only 10 nL used for each injection. However, that work did not emphasize throughput, so LC separation times were a few minutes and the analysis of trains of droplets was not realized. Also, samples were pumped in and not withdrawn, precluding interface to a well plate or external source of sample. Another work has demonstrated sampling from a well plate of samples covered by an immiscible fluid; however, the injection process did not involve using segmented flow to the injection valve and there was no gain in throughput over a conventional autosampler.[28]

Among the possible applications of high-throughput HPLC, we demonstrate its use in reaction screening. An important emerging trend for organic reaction development has been to prepare arrays of reactions in well plates using automated fluid control borrowed from high-throughput bioassays.[29-32] By screening >1000 reactions at once, it is possible to quickly generate data sets that reveal new reactions, catalyst substrate scope, optimal reaction conditions, and new chemical insights.[33-36] Most analysis from these small batch reactions has been done using direct MS analysis such as ESI-MS or matrix assisted laser desorption ionization (MALDI) MS. [29, 37-39] Incorporation of fast capillary LC into such systems promises to add new capabilities for such studies. For example, it enables more information-rich analyses with less ionization suppression and potential for isomer separations compared to direct injection MS.

EXPERIMENTAL SECTION

Chemicals and Reagents

Thiourea, acetophenone, propiophenone, 2,5-dihydroxybenzoic acid, phenylacetic acid, trifluoroacetic acid (TFA), propylamine, and benzylamine were purchased from Sigma Aldrich (St. Louis, MO). Trans-beta-nitrostyrene and acetonitrile were purchased from Millipore Sigma (Burlington, MA). Methanol was purchased from Fisher Scientific (Hanover Park, IL). HPLC-grade water was purchased from ThermoFisher (Grand Island, NY). Perfluorodecalin was purchased from Oakwood Products (West Columbia, SC).

Instrumentation

Unless stated otherwise, all separation experiments were performed using a Luna C18(2) HPLC guard column (20 mm length x 0.3 mm i.d., 5 µm particles) from Phenomenex (Torrance, CA). For LC-UV experiments, a binary Waters Acquity Arc LC pump (Milford, MA) coupled to a LINEAR UVIS-205 absorbance detector (Auburn, CA) set to 214 nm. Data was collected at 20 Hz through a NI USB-6008 data acquisition card (National Instrument, Austin, TX, USA) controlled by a custom LabView program. Prism 10 (GraphPad Software, Boston, MA) was used for data processing. For all LC-MS experiments, a 1290 Infinity II LC pump (Agilent Technologies, Santa Clara, CA) coupled to an Agilent 6410 Triple Quadrupole mass spectrometer via a capillary electrophoresis sheath flow electrospray source (Agilent G1607B) was used. Electrospray ionization was achieved under the following conditions: temperature, 300 °C; gas flow, 10 L/min; nebulizer, 15 psi; capillary voltage: 3000 V; scan time: 10 ms; operating mode, positive.

Synchronous Droplet Generation and Injection

Synchronous droplet generation and injection was achieved by using a four-port injection valve (C74MH, VICI AG-Valco Instruments, Houston, TX) equipped with a 20 nL internal injection channel and an electrical actuator (EHCA-CE, VICI AG-Valco Instruments, Houston, TX). (See Figures 1 and S1.) Two ports of the valve were connected to the LC binary sample pump and the column, respectively. On the other side of the valve, two pieces of perfluoroalkoxy alkane (PFA) tubing (one for sampling and one for waste, 40 and 25 cm in length, respectively) with 360 µm o.d. and 100 µm i.d. (IDEX Health & Science, Rochester, NY) were connected. A computerized numerical control (CNC) XYZ-positioner was used to position the inlet of the sampling tube for collecting samples from a well plate. The outlet of the waste tube was connected to a 25 µL Hamilton syringe (Reno, NV) mounted on a Fusion 400 syringe pump (Chemyx, Strafford, TX) operated in withdrawal mode. Droplet samples segmented by perfluorodecalin (PFD) were generated by using the CNC positioner to move the sampling tube inlet alternatingly into wells containing sample and PFD while the syringe pump was withdrawing at 1.5-2.6 µL/min. The droplets flowed through the sampling tube and into the internal sample groove of the valve for injection. Timing for actuating the valve was determined by visual observation (under microscope) of the droplet passing the base of the opaque PEEK ferrule (VICI), which is 1 cm away from the bore. Excess sample was collected in the waste tubing. A total volume of 160 nL for each droplet was used to guarantee overfilling the loop and maintain successful injection cycles.

For most tests, a standard mixture of 13.3 mM thiourea, 6.7 mM 2,5-dihydroxybenzoic acid, and 16.7 mM phenylacetic acid in 20:80 acetonitrile/water v/v and its 2-fold dilution were used as the samples. For standard carryover tests, a mixture of 16 mM thiourea, acetophenone, and propiophenone in 20% acetonitrile was used.

Organic Reaction Screen

48 variations of a Michael addition of amines to a nitrostyrene in different solvent systems were screened in duplicate (Scheme 1).[40] Stock solutions of trans-β-nitrostyrene in acetonitrile or methanol were filtered and dispensed in 48 Eppendorf tubes in a final concentration of 1 mg/mL and 1 mL volume. The 48 reactions were divided into four groups of 12 in terms of variables to screen: groups 1 and 3 each contained 2 and 1 equiv of benzylamine, respectively. Groups 2 and 4 each contained 2 and 1 equiv of propylamine, respectively. In each group, there were 12 solvent systems, comprising the following compositions: 0, 0.2, 2, 10, 20, and 30% water in acetonitrile, as well as 0.5, 5, 10, 25, 50, and 100% methanol in acetonitrile. After addition of amines to each tube, the reaction mixtures were incubated at room temperature overnight and subsequently pipetted into 48 different wells of a 384-well plate. Droplet samples of all reactions segmented by perfluorodecalin oil were generated and separated by the fast LC system described above. Effluent from the LC column was directly sent to the mass spectrometer through a 19.3 cm long, 360 µm o.d. and 40 µm i.d. fused silica capillary.

RESULTS AND DISCUSSION

Fast Capillary HPLC separations

Initial experiments focused on achieving fast separations of a test mixture that included thiourea, 2,5-dihydroxybenzoic acid, and phenylacetic acid. Figure 2A shows that when using the capillary column (20 mm length x 0.3 mm inner diameter, 5 µm), it is possible to increase the flow rate from 25 to 85 µL/min and maintain resolution of these test compounds while reducing the separation time to 4 s. Increasing the flow rate from 70 to 85 µL/min increased back pressure from 3,600 to 4,400 psi, close to the stated column pressure limit of 5,000 psi, with only a modest improvement in speed; therefore, we used 70 µL/min for our HTE trials. A key to maintaining resolution was minimizing the extra column band broadening.[41] The pre-column tube connecting the injector valve and the column was 25 µm i.d. x 5 cm in length, giving only 24.5 nL pre-column dead volume. The largest potential source of extra column band broadening was the connection between the column and detector. A 10 cm length was required to reach the column exit to the detector. Reducing the inner diameter of this tubing improves resolution but increases backpressure and decreases signal (Figure 2B). The latter effect is because the i.d. of the connecter tubing is also the pathlength in the flow through cell for the detector. Based on these trade-offs, we selected a 10.2 cm length of 40 µm i.d. tubing for connection.

To test the robustness and repeatability of this rapid separation, 96 injections of the test mixture were performed. For this test, the solution was continuously infused into the injection port via a syringe pump at 2 µL/min and injections made by alternating the valve between load and inject on a 4-s cycle with 2 s in the "load" position and 2 s in the "inject" position. Using this method, 96 injections were completed within 6.4 min. Relative standard deviation (RSD) for the peak heights of each analyte were 1.10%, 1.75%, 1.55%, respectively (Figure 3A). The separation performance was consistent and repeatable in terms of peak shape and retention time, which can be visualized by overlapping the chromatograms of the first, middle, and last separations. (Figure 3C) The average resolution of thiourea and 2,5-dihydroxybenzoic acid was 1.25 +/- 0.04, and the average resolution of 2,5-dihydroxybenzoic acid and phenylacetic acid was 1.50 +/- 0.03.

It was also observed that the same column could be used repeatedly under such conditions. These results indicate that the capillary LC column is sufficiently stable to high flow rates and multiple valve actuations to robustly separate consecutive samples.

Online Droplet Generation for Analysis

Results from Figure 3A,C show that the column is stable to repeated, fast separations. The test was then repeated using the system shown in Figure 1 to test stability with droplet injections. For this sequence of injections, the well plate was loaded with PFD and standard samples of two different concentrations. Droplets of the two samples were alternately drawn into the sample tube and segmented by PFD at 2.6 µL/min a total of 48 times each. The resulting chromatograms (Figure 3B,D) show the good stability of the system. The RSD for the peak heights of each analyte were 3.06, 2.62, and 2.46% for high concentration samples and 2.76, 2.67, and 2.23% for low concentration samples, respectively. Resolution was maintained throughout the entire sequence of injections as visualized by overlapping the first, middle, and last chromatograms from the sequence (Figure 3D). The average resolution of thiourea and 2,5-dihydroxybenzoic acid was 1.21+/- 0.05, and the average resolution of 2,5-dihydroxybenzoic acid and phenylacetic acid was 1.56 +/- 0.05. These data indicate the separations and stability from the droplet injector are similar to those obtained from continuous sample flow into the

injector (Figure 3A,C). It was further observed that performance was consistent throughout the entire time span of this project with thousands of injections of various analyte species, as long as the system remained clog-free and the system back pressure was below the column pressure limit of 5,000 psi.

Although the performance was similar to continuous flow injections, one difference observed was throughput. For the droplet injector test shown in Figure 3B, the droplet generator was programmed to dwell in sample wells for 3.7 s and in oil wells for 0.3 s, suggesting that the injection interval should be the same as the separation time which is 4 s (Figure 3B). However, the fluid flow in the PFA tubing was briefly blocked during each valve position switch because the two connecting ports are temporarily sealed by the rotor surface instead of opened to the rotor groove during the transition. As a result, a 0.4-0.6 s delay occurs after each valve actuation. Over the course of 96 injections, this effect increased the total analysis time 8.1 min compared to 6.4 min when infusing directly.

With normal, successful injections, column performance was maintained; however, if PFD entered the column e.g., from a mis-timed injection, the column performance was significantly degraded. Column performance could be restored by pumping 100% acetonitrile through the column for ~2 min at the separation flow rate. (Figure S2) In general, the current mode of observing the droplets entering the capillary to actuate the valve is not optimal and a method of detecting droplets entering the injection channel would be necessary to fully automate the injection process and prevent injection of carrier fluid.

An alternative to using an immiscible carrier fluid would be to pull samples with small miscible fluid plugs (e.g., mobile phase or sample solvent) between them into the injection valve. To test the feasibility of this idea, samples were pulled through the sampling tube in the continuous phase (without immiscible segmenting plugs) and directly through the UV detector to determine if the sample zones would be suitable for injection. As shown in Figure S3, the sample zone is diluted to about 60% of the initial concentration and broadened 5-fold to a Gaussian shaped peak by Taylor dispersion. As a result, it would be difficult to reproducibly inject a portion of this plug because small variations in timing would result in different concentrations injected. The breadth of the resulting zones would also constrain how close the zones could be and therefore how fast injections could be made in sequence. In contrast, when samples are compartmentalized with PFD (or air bubbles as in Figure S3), they do not broaden or dilute allowing representative portions of the sample to be injected regardless of which portion of the droplet is injected. Samples can also be placed closer together with droplets for faster injections because of the absence of broadening. Also, without the meniscus between immiscible phases, it would be complicated to observe the sample entering the valve to time the injection. These results highlight the importance of using the immiscible fluid for segmenting the sample for high-throughput injections. Interestingly, during these experiments we observed that small air bubbles could be formed on each side of the sample and create a compartmentalization effect that appeared comparable to the PFD in effectiveness. Therefore, air could likely also be used for segmentation in this system.

Carryover and Elimination

A potential limitation of the system was the possibility of carryover from sample to sample. A carryover test for the mixture of thiourea, acetophenone, and propiophenone was performed by injecting five acetonitrile (blank) droplets following each standard sample droplet

(Figure 4A). Analytes were detected in all first blank droplets, indicating that carryover exists in this system but can be eliminated by one wash droplet. Subsequently, a range of wash droplet volumes were evaluated by introducing a specific volume of wash droplet between the sample and the first blank in the droplet array, without injecting it (Figures 4B and S4). We found that just a 10 nL wash droplet (100% acetonitrile) was sufficient to eliminate >96% carryover, and the impact of 10 nL and 100 nL wash droplets was remarkably similar. This finding suggests that a wash droplet as small as 10 nL could be adequate for mitigating the carryover. Carryover was found to be analyte-specific [26, 42] and tailoring the cleaning approach might also be necessary for distinct analytes.

Application to a Michael Addition Reaction Screen

To test the capability of this system for a potential application, we used it to analyze 48 different Michael addition reaction mixtures in duplicate. To facilitate the analysis, the capillary LC was interfaced to a mass spectrometer, which allowed measurements even if analytes were not sufficiently resolved from other reaction components or did not have sufficient absorbance. The screened reactions are shown in Scheme 1 and all variants are shown in Table S1. Prior research into Michael addition of amines to nitrostyrenes has indicated the creation of N-butyl or benzyl imines as side products via a retro-aza-Henry-type transformation in tandem with the Michael-adducts. [40, 43-45] A mechanistic study of the nitroalkane elimination has concluded that this process is dual-catalyzed by the presence of protic solvent and an excess of amines. Experimentally, using 1 equiv of amine yielded only 58% imine from the initial methyl-trans-styrene substrate, but 1.5 and 2 equiv yield 95 and 99%, respectively. Adding small amount of protic solvents such as 0.2% (v/v) water or 0.5% (v/v) MeOH into acetonitrile drove the synthesis of imines from 12% to 82% and 85%, respectively. [40] This ability to vary output of the reaction provides a convenient test of the high-throughput capillary LC. We therefore created an array of 48 reactions that used two different substrates at different equivalents and protic solvent content on a well plate for screening. (See Table S1 for layout of reactions).

Figure 5A shows the trace for the entire capillary LC-MS screen of the reaction array in duplicate. The inset of Figure 5A shows a zoomed-in view of selected sections. The chromatograms show that it was possible to partially resolve the reactants and products for the different reactions in 7 s. This resolution was stable throughout the screen. The use of MS further allowed improved detection specificity of targeted analytes.

Using the peak heights of the imines compared to the summed total peak heights for both products from the MS traces, it was possible to semi-quantify the reaction results. Using this approach, we found the expected qualitative trends in product formation as a function of protic solvent content and equivalent of amines (Figure 5B). Specifically, as the percentage of water or methanol is increased in the reaction mixture, the fraction of the imine product was increased. The minor influence of the equivalent amine relative to prior work can be attributed to our substitution of trans-styrene for methyl-trans-styrene as originally utilized in the precedent study, which was used to improve solubility. Furthermore, variations in critical reaction parameters such as temperature, types of amines, and concentration between the two studies necessitate further exploration for a comprehensive conclusion. Reproducibility was good as illustrated by the overlap of duplicate assays in Figure 5B, in which the dots are individual data points, and the lines are the averaged values of the duplicates. Accuracy of results was marginally affected by carryover. Studies of the reaction mixture showed that carryover only exists in the first blank

injections. (Figure 5C) The peak heights for the carryover signals averaged 6.8% of the samples (range of 0.4% to 11.6%). As discussed above, wash droplets could be used to minimize this effect.

The screening experiment illustrates the potential for rapid capillary LC to be used for high-throughput chemical experimentation with reduced time and material consumption. The duty cycle for each injection was 8.3 s including 7 s for analysis and an added 1.3 s due to the flow pause during the valve actuation. Therefore, the full run of 96 samples was completed within 13.5 min. The screen was achieved at 50 µL/min mobile phase flow rate so that each sample consumed < 7 µL solvent to analyze. In principle, the system is also flexible allowing different columns or detectors depending upon the application.

Potential for High-Throughput Experiments (HTE)

The advantage of capillary LC over HPLC in terms of resource utilization is more pronounced in the context of HTE. Assuming a mobile phase flow rate of 70 µL/min and a 4-s separation time, the total consumption of mobile phase would be 47 mL and analysis time would be just 11 h for a 10,000-sample experiment. In comparison, the same experiment performed using a typical LC system with an autosampler and a relatively large bore column would require 21,000 mL of mobile phase and 44 h, assuming a 15 s autosampler sequence and 1 s separation time at a flow rate of 8 mL/min. According to this calculation, our system could reduce mobile phase consumption by a factor of 446 and complete the process in a quarter of the time compared to a fast HPLC separation using a conventional autosampler. Using the AMGS greenness calculator (see the Supporting Information (SI) for information) the HPLC method for 10,000 samples would have a greenness of 12,666 compared to 301 for the segmented flow, capillary LC method. This improvement in greenness is driven by both instrument electricity usage (shorter time) and less solvent consumption. Therefore, the droplet capillary LC system has potential to achieve HTE in a greener and more time efficient way than HPLC. The system also reduces sample consumption. The current mode consumed 160 nL of sample (20 nL injected) for each assay, a 25-fold reduction over our previous fast LC system. [26] The sample consumption of just 160 nL compared to 1 µL is also valuable for reducing waste and making the screen compatible with small scale reactions or other sample limited experiments. We believe sample consumption can be further reduced by automation of the injection process so that extensive overfilling of the loop is not necessary.

The use of a capillary LC also facilitates the interface to MS. The typical operational flow rate of the fast capillary LC system is 20-90 µL/min, making it compatible with common ESI sources without modifications such as split flow. In a prior fast HPLC-MS experiment, the flow rate was reduced from 5 to 1.5 mL/min, thus decreasing throughput to make the system more compatible with MS. [26]

The use of a capillary LC also facilitated the ability to continuously inject from a well plate rather than using a two-step process of loading samples into a capillary tube, which is then pumped into the injector. Such capability not only simplifies automation of the injector but also makes true high throughput experiments more feasible (e.g., by coupling the system to a plate handling robot for multiple plates). In previous experiments with a segmented flow injector for HPLC, it was not possible to stably withdraw samples fast enough with a syringe pump to fill the injection loop. The lower injection volumes needed with capillary LC allowed lower sample

withdraw flow rates, which were stable and readily attained using the simple syringe pump system.

The fast separations demonstrated here are limited to analysis of relatively simple mixtures, although coupling to MS or use of peak deconvolution [2, 6, 9, 10] can further expand the scope. Even with this limitation, numerous applications can be envisioned besides the rapid organic reaction screen demonstrated here. Such applications include enzyme engineering or synthetic biology, where many enzyme variants are screened for product formation, screening of chemical libraries for quality control, process monitoring, or drug development screens.

Enabling these applications and expanding them to more complex mixtures will require further development. As mentioned above, automating injection is a priority. It may be possible to improve the resolution with different columns. We tested a Waters nanoEase™ M/Z column (50 mm length x 0.3 mm i.d., 1.8 μm) and a Phenomenex Kinetex core-shell column (30 mm length x 0.3 mm i.d., 2.6 μm) for fast separations (Figure S5) and both produced good separations in less than 21 s. In principle, the smaller particles used in these columns should allow higher performance at fast times; however, pressure limits of the columns or system used here prevented exploring this possibility. Importantly, these columns are produced as guard columns and are not necessarily optimized for high-flow rate LC separation. Therefore, further column development and perhaps the use of smaller particles for higher resolution and higher pressure systems are of interest. Finally, the development of rapid gradient systems for capillary LC will widen the scope of applications.

CONCLUSION

This work has demonstrated the feasibility of achieving throughput of 0.2 samples/s by capillary LC through sample introduction utilizing droplet microfluidics and an internal loop injector. Discrete samples can be automatically and continuously pulled into the injector without detrimental effects on the separation. Separation times of 4 s were possible at 70 μL/min for a 3-component mixture. Resolution enhancements by further reduction of extra column effects and improvements in columns may allow for higher resolution and shorter times. The relatively low flow rate of capillary LC reduced mobile phase consumption 70-fold over HPLC for greener separations and facilitated interface to MS. The system was demonstrated for screening a reaction. This work suggests motivation for continued development of capillary LC and expanding the potential applications of high-throughput LC.

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Supporting Information. Description of greenness calculation, details on experimental setup and reaction conditions for screen, and data on: oil effect on column, broadening of sample zones without segmentation, use of wash droplets for carryover, and comparison of different capillary LC columns.

Figures

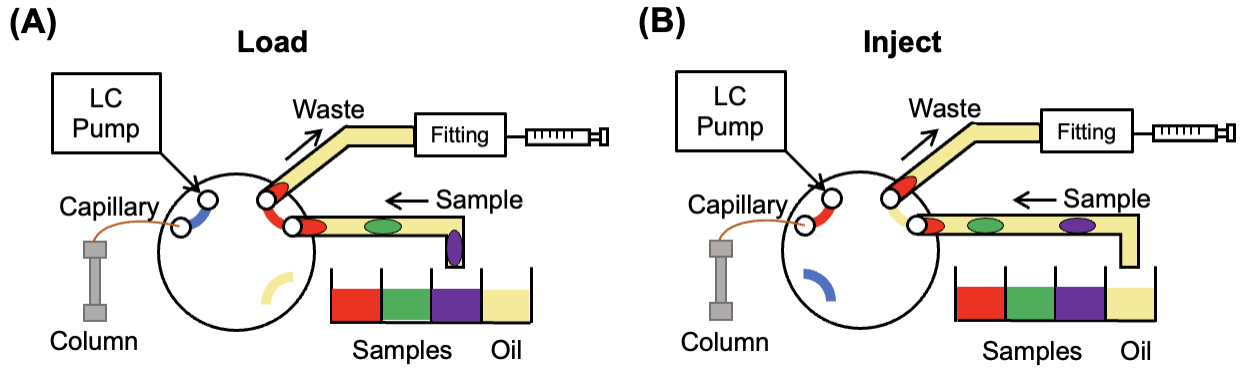


Figure 1. Illustration of online sample generation and separation. (A) The valve is at the loading position, the droplet samples are drawn from the well plate and segmented by perfluorodecalin oil (yellow) in the perfluoroalkoxy alkane (PFA) tubing by sampling alternately from PDF and sample wells and sent to the internal sample groove in the valve. (B) The valve is at the injection mode. The internal sample groove loaded with a sample is sent to the left side for separation.

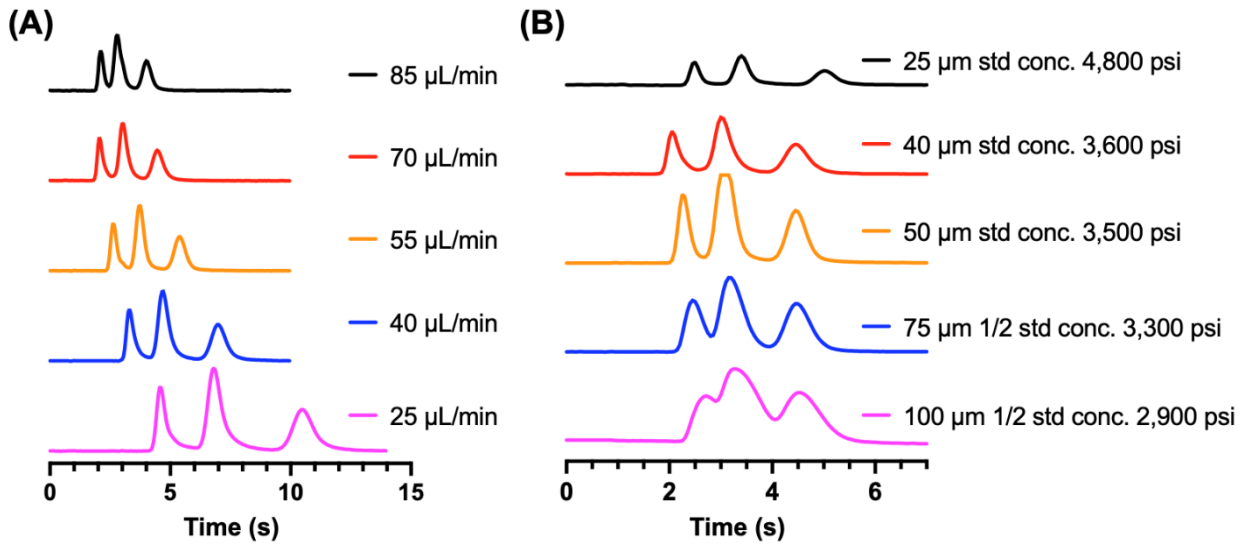


Figure 2. Effect of flow rate and column to detector tubing dimensions on LC separation of 3-component test mixture. (A) Different flow rates tested on the same column (Luna) with the same mobile phase (35/65 v/v acetonitrile/water + 0.1% TFA) for standard sample mixture of 13.3 mM thiourea, 6.7 mM 2,5-dihydroxybenzoic acid, and 16.7 mM phenylacetic acid. (B) Effect of changing inner diameter of column to detector tubing. 10.2 cm length fused silica capillaries with varying of inner diameters were installed on the same column and tested with the mobile phase as in (A). The standard concentration (std conc.) samples indicated above were used for 25 µm, 40 µm, and 50 µm i.d. capillaries, and half concentrated standard samples (1/2 std conc.) were used for 75 µm and 100 µm i.d. capillaries. Flow rates for all assays were 70 µL/min except for the 25 µm i.d. capillary, which operated at 60 µL/min and a resulting back pressure of 4,800 psi, which was just below the 5,000 psi pressure limit of the column.

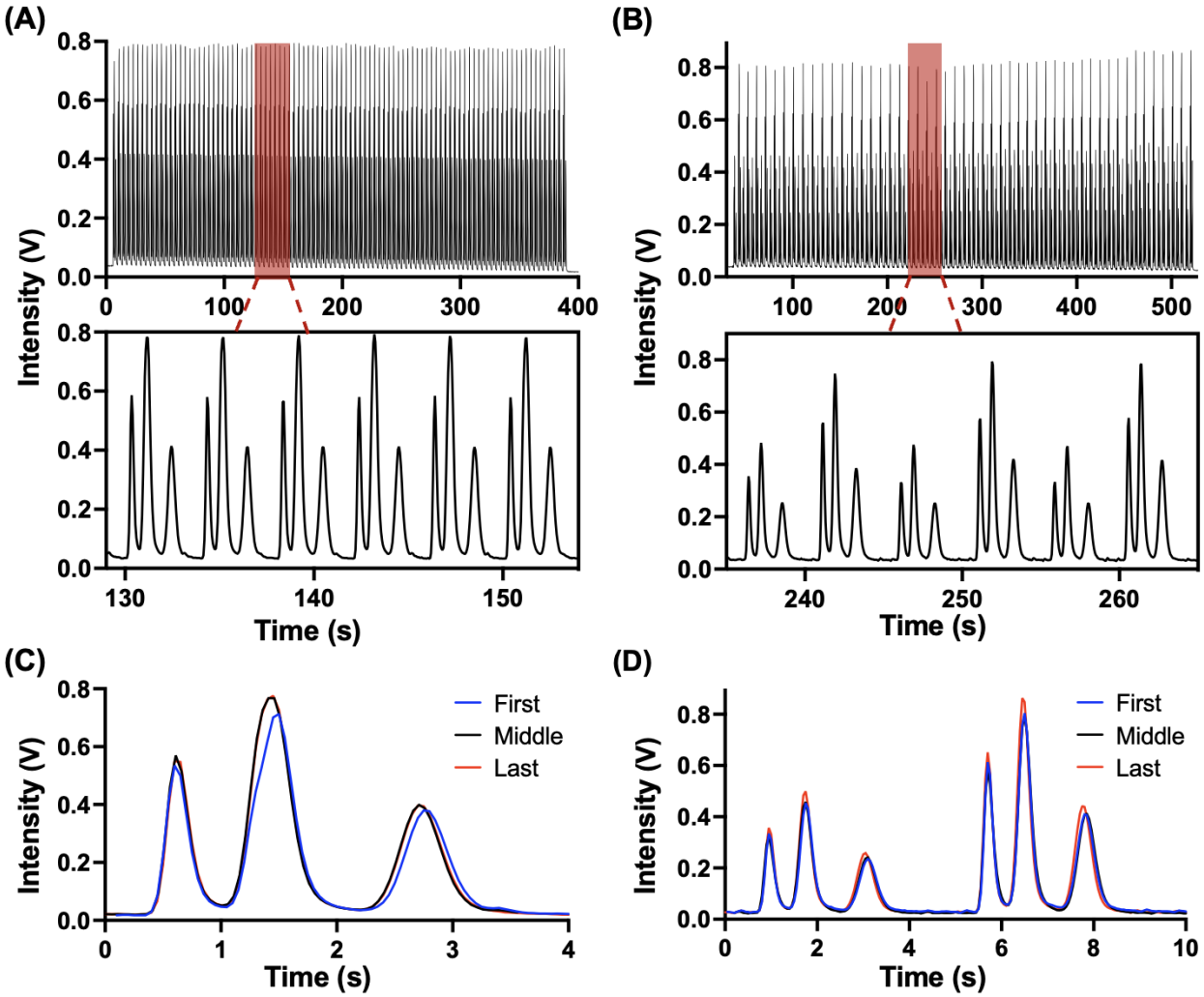
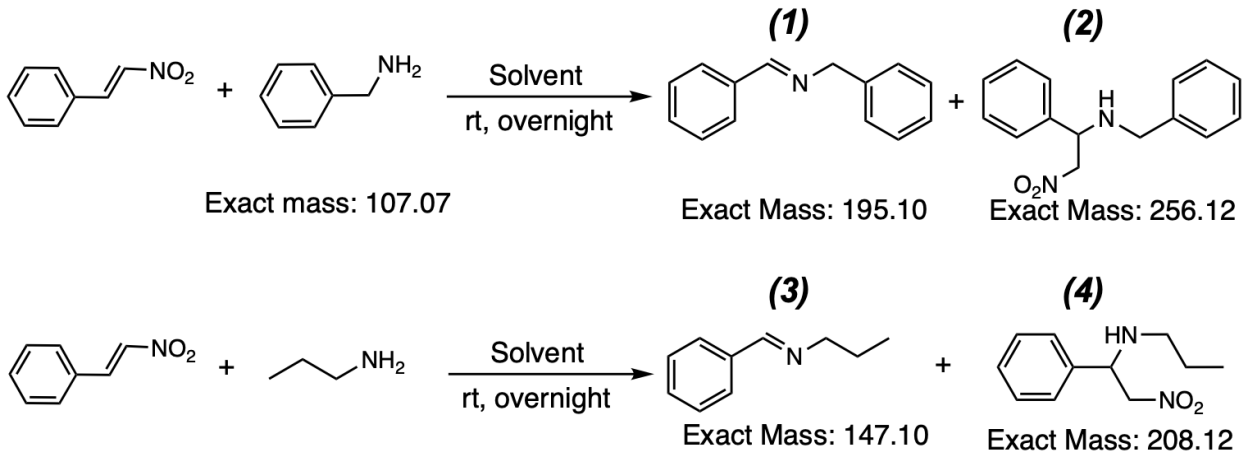


Figure 3. High-throughput fast separation tests with standard samples. (A) Top: 96 injections of samples infused in a continuous flow from the syringe. Bottom: zoomed-in of six separations from 129 s to 154 s. (B) Top: 96 injections of samples drawn from the well plate as segmented droplets of alternating concentrations. Bottom: zoomed-in view of 6 separations from 235 s to 265 s. (C) First, middle, and last separations in A overlaid. (D) First, middle, and last separations in B overlaid. For both experiments, the mobile phase was 35/65 acetonitrile and water + 0.1% TFA. Flow rate was 70 µL/min. The analytes were 13.3 mM thiourea, 6.7 mM 2,5-dihydroxybenzoic acid, and 16.7 mM phenylacetic acid dissolved in aqueous solutions with 20% acetonitrile. The wavelength for UV detection was 214 nm.



Scheme 1. Reaction schemes between trans-nitrostyrene and benzylamine (top) or propylamine (bottom), with (***1***), (**2**), and (***3***), (***4***) as products, respectively. Both reactions were run by incubating at room temperature overnight within solvent systems containing varying portions of protic solvents (methanol or water) in aprotic solvent (acetonitrile).

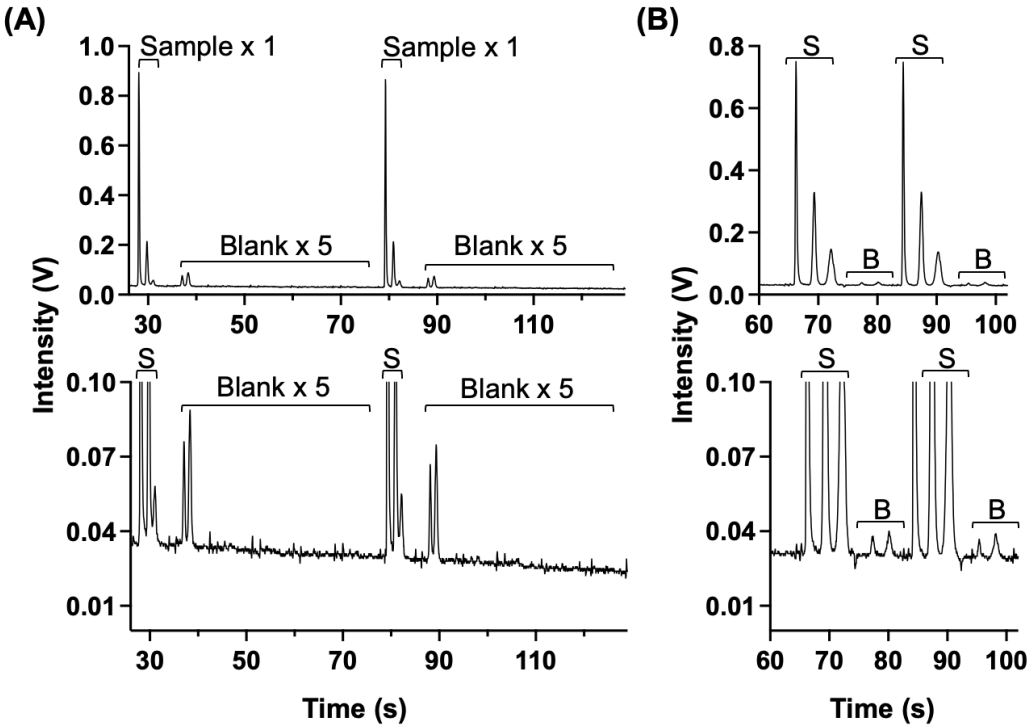


Figure 4. Evaluation of carryover and its elimination. (A) Top: duplicate injections of one sample containing 16 mM thiourea, acetophenone, and propiophenone, followed by 5 blank droplets. Only the first blank has carryover signals. Bottom: zoomed-in view of intensity between 0 to 0.1 V. (B) Top: duplicate injections of one sample and one blank droplet with a 10 nL uninjected wash droplet (100% acetonitrile) in between. The carryover of thiourea, acetophenone, and propiophenone was reduced to 0, 2.3%, and 7.8%, respectively, calculated from the peak heights. Bottom: zoomed-in view of intensity between 0 to 0.1 V.

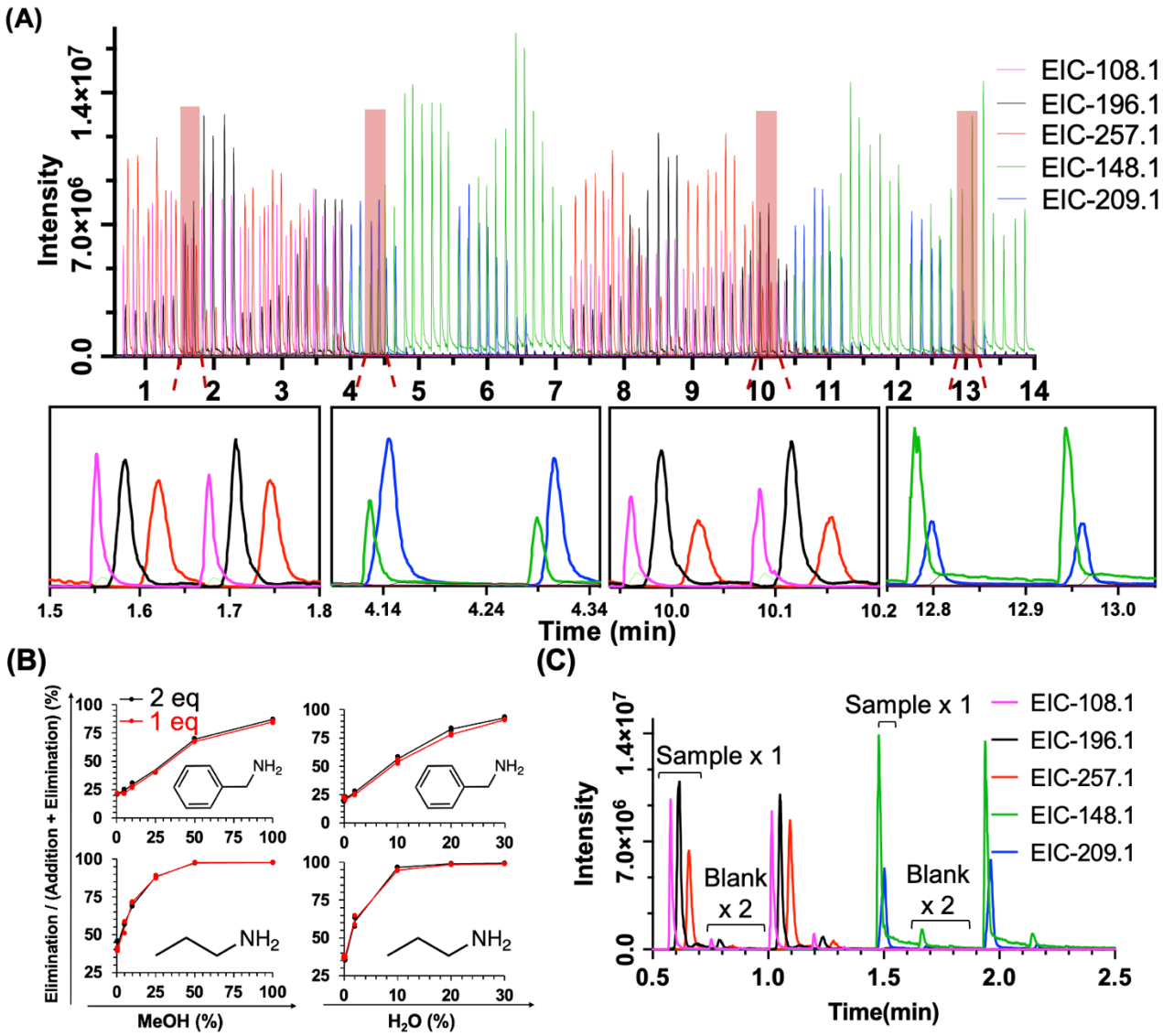


Figure 5. (A) Extracted ion chromatograms from a screen of 96 reaction mixtures with the fast capillary LC-MS and droplet injector. EIC traces are color-coded by detected m/z as shown. Scheme 1 shows the analytes with exact masses. Insets show sections with two sample reactions analyzed. (B) The percentage of elimination product, measured using EIC peak heights of both products, as a function of the percentage of MeOH and H_2O in reaction mixture for two different equivalents of amine. The dots are individual data points, and the lines are the averaged value of the duplicates for each condition. (C) Carryover test of selected reaction screening samples. Duplicate injections of 2 blank droplets of acetonitrile following one sample droplet for reaction mixtures of trans-nitrostyrene and either 2 equiv of benzylamine in 10/90 water/acetonitrile solvent (magenta, black, and red traces) or 2 equiv of propylamine in 10/90 methanol/acetonitrile solvent (green and blue traces), respectively. For both (A) and (C), magenta, black, red, green, and blue traces correspond to benzylamine, (***1***), (***2***), (***3***), and (***4***) in scheme 1, respectively. The mobile phase was 30/70 acetonitrile/water + 0.1% TFA, and the flow rate was 50 µL/min.

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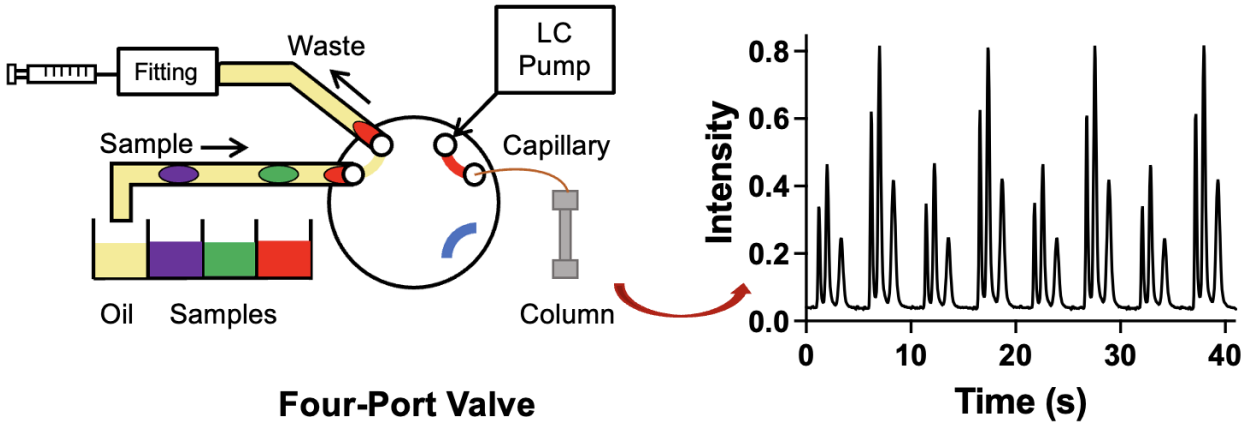
TOC Graphic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0.4
0.2
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10
20
30
40
Time (s)

Supporting Information

High throughput capillary liquid chromatography using a droplet injection and application to reaction screening

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Methods

Greenness score of a hypothetical 10,000 sample screen was calculated using the ACS Green Chemistry Institute calculator at https://www.acsgcipr.org/amgs/. The calculations used an isocratic method with 30% acetonitrile / 70% water (v/v) as mobile phase. Run time was 4 s at 50 µL/min for capillary LC with droplet injection and 16 s at 8 mL/min for HPLC with autosampler. Both screens assumed 150 mL to create the 10,000 samples, same solvent as mobile phase, and 10 standards for calibration. The calculator returned the following values:

	HPLC	**Capillary LC**
Greenness	12,666	301
Instrument Energy	1147	287
Solvent Energy	7470	64
Solvent EHS	4048	35

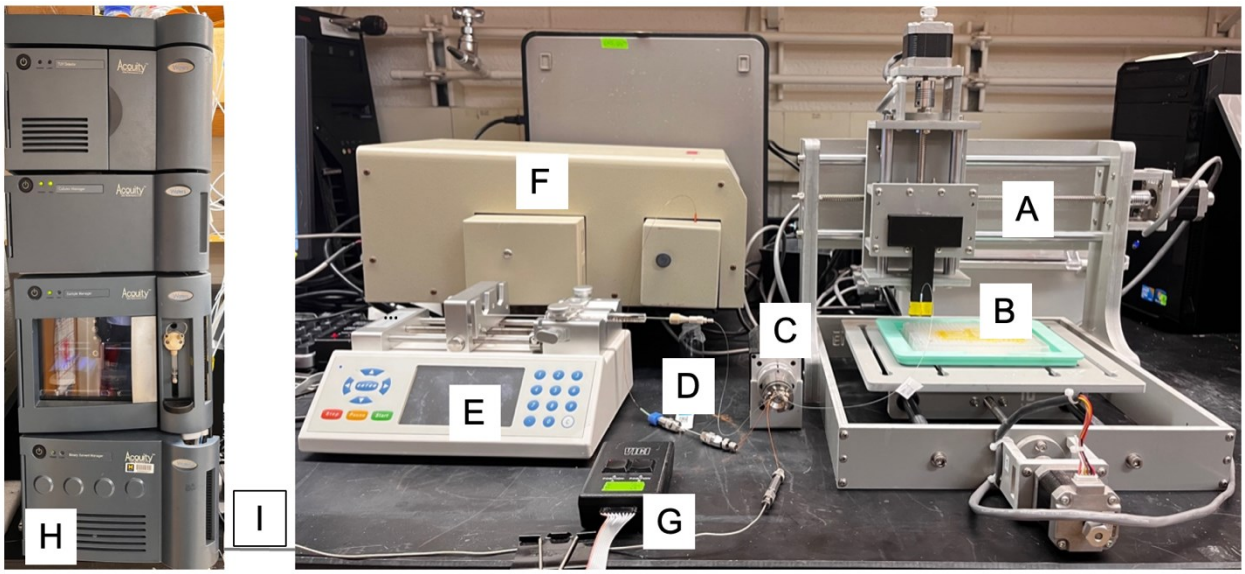

Figure S1. Experimental setup for fast capillary LC-UV. (A) Computerized numerical control (CNC) XYZ-positioner. (B) Well plate. (C) Four-port nano valve. (D) Capillary column. (E) Syringe pump. (F) UV detector. (G) Valve actuation remote. (H) Waters Acquity binary solvent manager. (I) Stainless steel tubing connecting the LC pump to the injector valve.

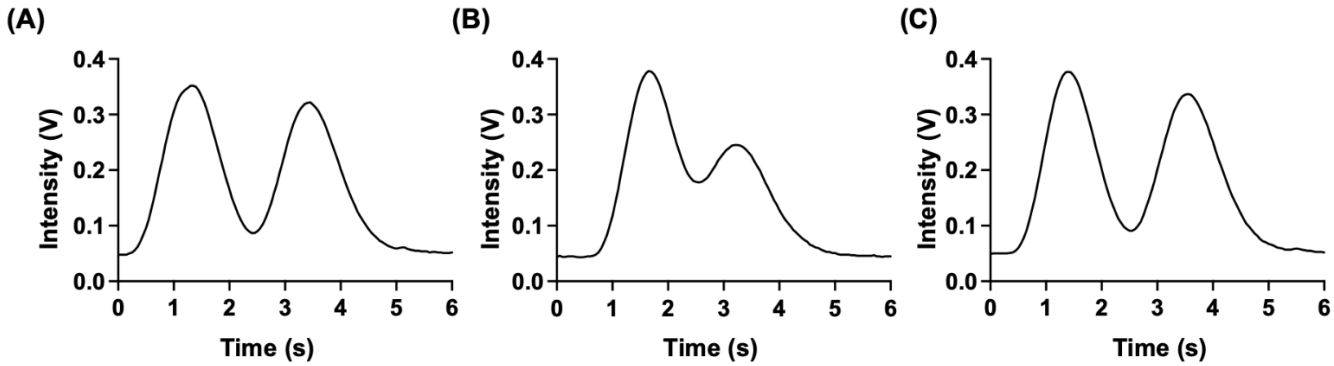

Figure S2. The effect of perfluorodecalin oil on column separation efficiency and recovery. From left to right: a) separation of thiourea and acetophenone before any oil ever entered the column; b) after accidently injected oil onto the column during droplet experiment; c) after running 100% acetonitrile at 80 µL/min for ~ 2 min to clean the oil out of the column. Mobile phase was premixed 45/55 acetonitrile/water + 0.1% TFA, flow rate was 80 µL/min.

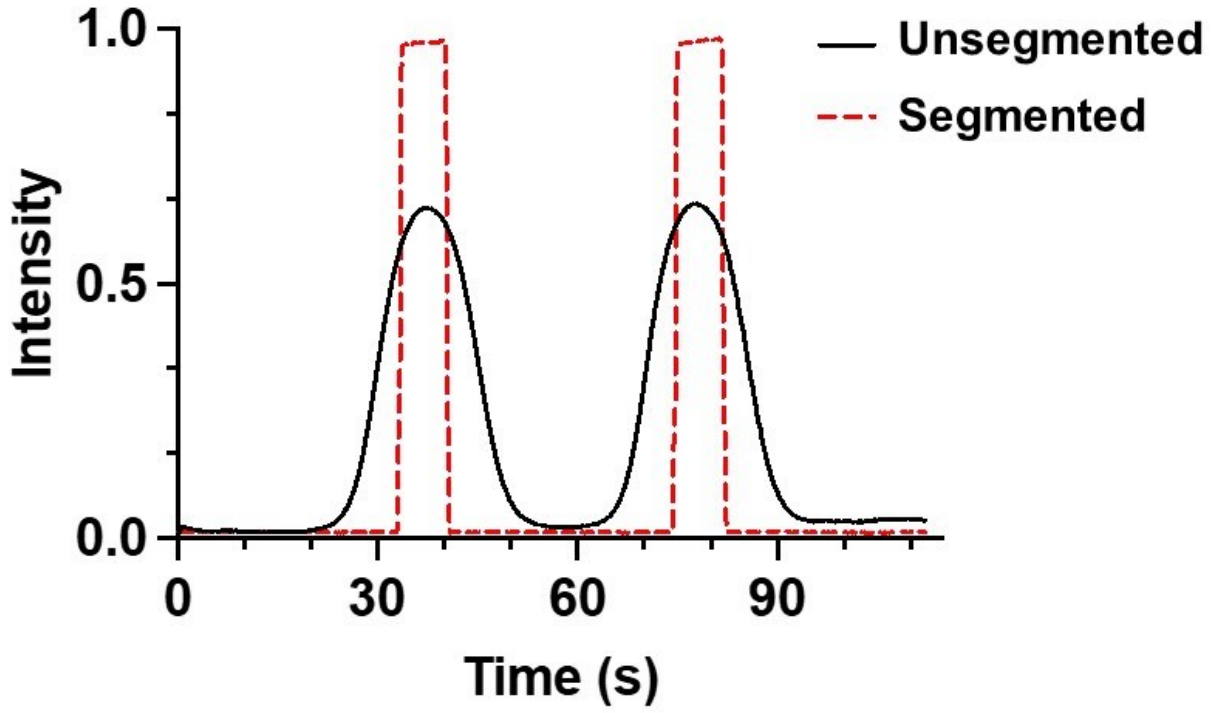

Figure S3. UV absorbance trace from pulling of sample through UV detector without segmenting and with air bubbles segmenting the sample. Sample was "sipped" twice using system in Figure 1 at 1.5 µL/min for 6.6 s (165 nL sample) with 33 s (825 nL) of blank in between. Sample was pulled through perfluoroalkoxy alkane (PFA) tubing (40 long x 360 µm o.d. x 100 µm i.d.) directly to the UV detector. The zone is broadened due to Taylor dispersion as shown. The segmented samples had air bubbles introduced between the sample plugs. Detection is at 214 nm. Sample was 6.65 mM thiourea, 3.35 mM 2,5-dihydroxybenzoic acid, and 8.35 mM phenylacetic acid in 20/80 ACN/H_2O v/v.

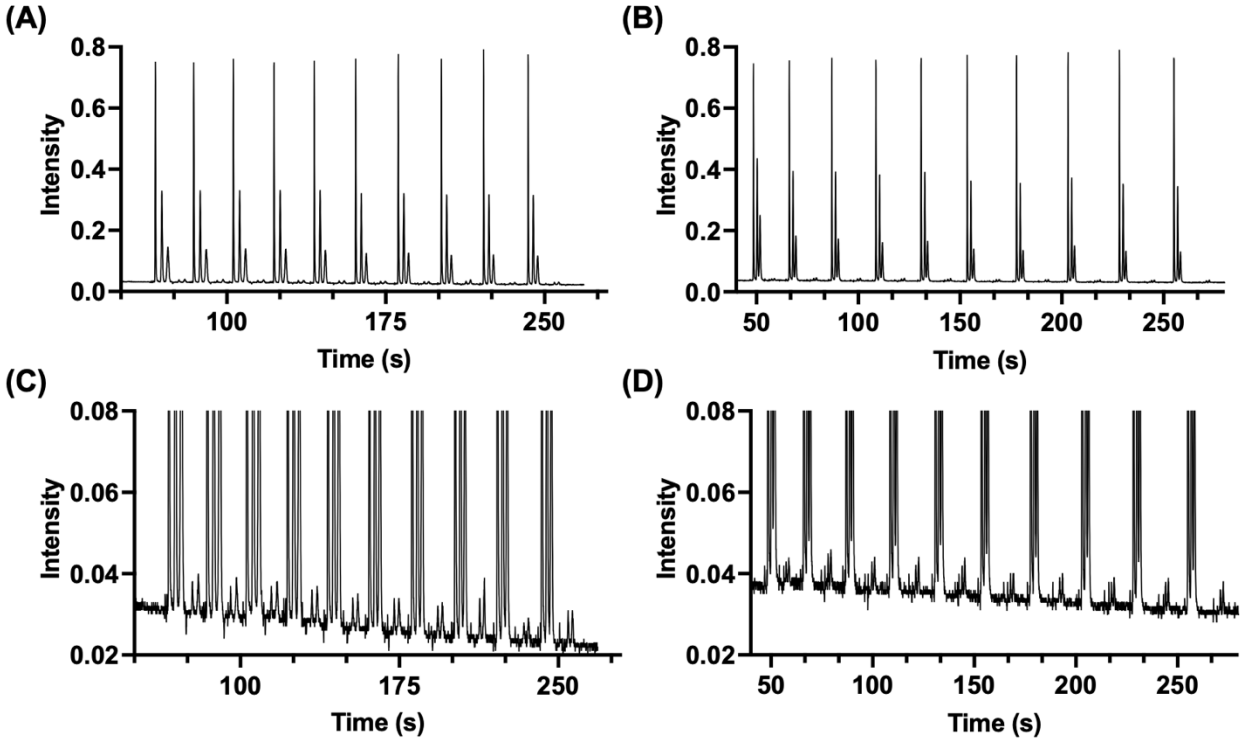

Figure S4. Effect of using different volumes of an uninjected wash droplet on carryover. Droplets were generated in a pattern of sample, wash droplet and a blank droplet. Only the sample and the blank droplets were injected to the column. (A) From left to right: duplicates of 10 nL, 20 nL, 30 nL, 40 nL, and 50 nL wash droplet tests. (B) From left to right: duplicates of 60 nL, 70 nL, 80 nL, 90 nL, and 100 nL wash droplet tests. (C) Zoom-in from 0.02 – 0.08 in intensity of A. (D) Zoom-in from 0.02 – 0.08 in intensity of B. Samples were mixture of 13.3 mM thiourea, 6.7 mM 2,5-dihydroxybenzoic acid, and 16.7 mM phenylacetic acid in aqueous solution with 20% acetonitrile. Wash and blank droplets were both 100% acetonitrile.

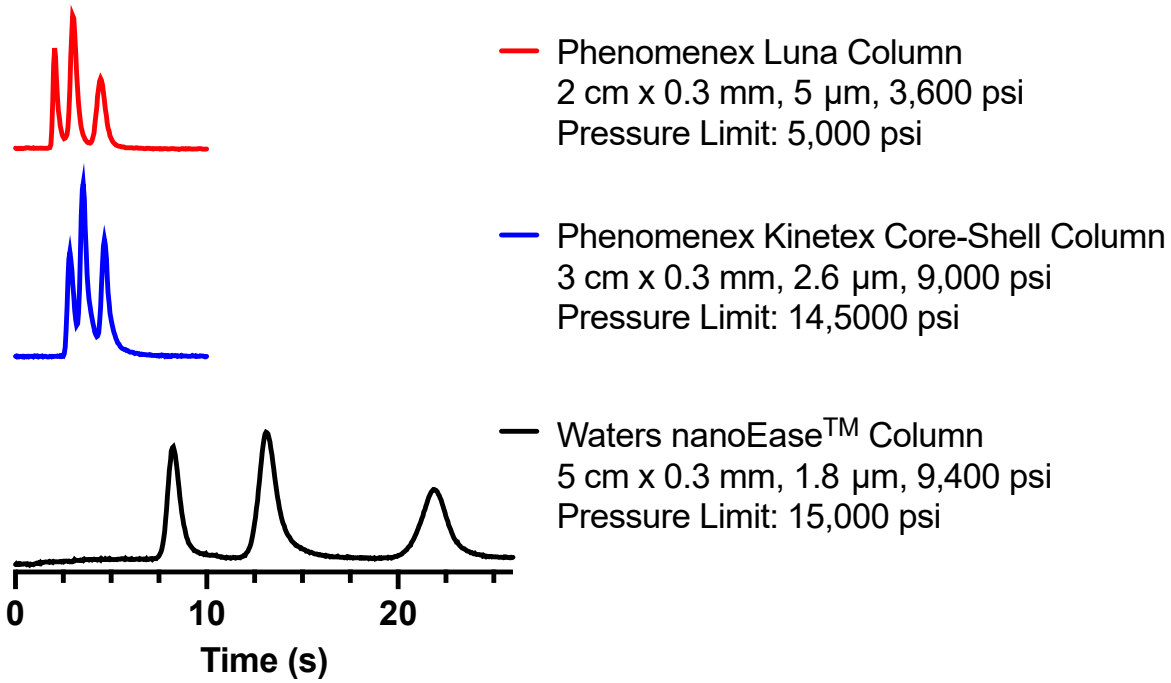

Figure S5. Comparison of 3 columns. Mobile phase: premixed 35/65 acetonitrile/water + 0.1% TFA. For the 2 cm, 3 cm, and 5 cm columns, the flow rates were 70 µL/min, 60 µL/min, and 35 µL/min, respectively; and their back pressures were 3,600 psi, 9,000 psi, and 9,400 psi, respectively. Chromatograms are normalized to the y-axis.

entry	Amine	Solvent	entry	Amine	Solvent
1	2 eq Benzylamine	MeCN	25	1 eq Benzylamine	MeCN
2	2 eq Benzylamine	MeCN+0.1% H2O	26	1 eq Benzylamine	MeCN+0.1% H2O
3	2 eq Benzylamine	MeCN+0.2% H2O	27	1 eq Benzylamine	MeCN+0.2% H2O
4	2 eq Benzylamine	MeCN+2% H2O	28	1 eq Benzylamine	MeCN+2% H2O
5	2 eq Benzylamine	MeCN+10% H2O	29	1 eq Benzylamine	MeCN+10% H2O
6	2 eq Benzylamine	MeCN+0.2% MeOH	30	1 eq Benzylamine	MeCN+0.2% MeOH
7	2 eq Benzylamine	MeCN+0.5% MeOH	31	1 eq Benzylamine	MeCN+0.5% MeOH
8	2 eq Benzylamine	MeCN+5% MeOH	32	1 eq Benzylamine	MeCN+5% MeOH
9	2 eq Benzylamine	MeCN+10% MeOH	33	1 eq Benzylamine	MeCN+10% MeOH
10	2 eq Benzylamine	MeCN+25% MeOH	34	1 eq Benzylamine	MeCN+25% MeOH
11	2 eq Benzylamine	MeCN+50% MeOH	35	1 eq Benzylamine	MeCN+50% MeOH
12	2 eq Benzylamine	100% MeOH	36	1 eq Benzylamine	100% MeOH
13	2 eq Propylamine	MeCN	37	1 eq Propylamine	MeCN
14	2 eq Propylamine	MeCN+0.1% H2O	38	1 eq Propylamine	MeCN+0.1% H2O
15	2 eq Propylamine	MeCN+0.2% H2O	39	1 eq Propylamine	MeCN+0.2% H2O
16	2 eq Propylamine	MeCN+2% H2O	40	1 eq Propylamine	MeCN+2% H2O
17	2 eq Propylamine	MeCN+10% H2O	41	1 eq Propylamine	MeCN+10% H2O
18	2 eq Propylamine	MeCN+0.2% MeOH	42	1 eq Propylamine	MeCN+0.2% MeOH
19	2 eq Propylamine	MeCN+0.5% MeOH	43	1 eq Propylamine	MeCN+0.5% MeOH
20	2 eq Propylamine	MeCN+5% MeOH	44	1 eq Propylamine	MeCN+5% MeOH
21	2 eq Propylamine	MeCN+10% MeOH	45	1 eq Propylamine	MeCN+10% MeOH
22	2 eq Propylamine	MeCN+25% MeOH	46	1 eq Propylamine	MeCN+25% MeOH
23	2 eq Propylamine	MeCN+50% MeOH	47	1 eq Propylamine	MeCN+50% MeOH
24	2 eq Propylamine	100% MeOH	48	1 eq Propylamine	100% MeOH

Table S1. Conditions of 48 Michael addition reactions for high-throughput screen test. All reactions were run in Eppendorf tubes and incubated at room temperature overnight before transferring to a well plate for analysis. Each reaction was analyzed in duplicate.