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Capillary Fractionation of HPLC Substrates by a Microfluidic Droplet Generator for High Throughput Analysis

Shereef Hamed, Brian Shay, and Amar S. Basu, *Member, IEEE*

***Abstract* — Biochemical samples are complex mixtures containing 1000's of components which often must be fractionated prior to analysis. Conventional fraction collectors, which can only accommodate 10's of fractions, are not well suited for high throughput analysis. This paper describes microfractionation in droplets (µFD), a scalable microfluidic technique for generating thousands of fractions. A drop generator, placed downstream from a high performance liquid chromatography (HPLC) column, encapsulates the separated components into a serial array of monodisperse droplets. The droplets can be stored in a capillary or immediately used in subsequent assays. Using µFD, a mixture of 3 dyes separated in a C18 column was fractionated into 2,160 droplets in <6 min. The volume and frequency of the droplet fractions are governed by the capillary number (*Ca*), which depends on the viscosity of the carrier fluid, flow rate, and interfacial tension. With HPLC-compatible flow rates of 0.38-0.7 mL/min, in a 1.5 mm Teflon capillary, fractions contain volumes of 1-6 µL and are generated at 2-10 drops/s. Droplet fractions can be mixed with a subsequent reagent using a downstream tee junction. In theory, µFD can be coupled to a wide variety of separation processes, enabling high throughput fractionation and screening of complex mixtures in µL to sub-nL volumes.**

I. Introduction

Preparative chromatographic methods for isolating compounds commonly rely on fractionation techniques to keep each component separated in its own respective container. When this strategy is used for high throughput screening (HTS), it becomes limited as one physical container is needed per fraction. This limits the number of fractions which can be feasibly collected. As HTS becomes increasingly important in '-omics' research [1], a scalable and rapid fractionation technique would reduce the overall cost of screening [2-4]. We propose microfractionation in droplets (µFD), a 'containerless' fractionation technique where separated fractions are encapsulated into aqueous microdroplets within an immiscible carrier fluid [5] (Fig. 1). Microdroplet systems can store and screen compounds at nL and pL volumes without cross-contamination, while performing up to 10,000 assays a second [6-8]. Mixing and assaying can be accomplished by mixing individual droplets together. Due to small volumes, microdroplets can provide a 10-1000x reduction in reagent consumption per assay [9-11], greatly reducing the large recurring costs in HTS. The practical benefits of µFD over conventional fractionation include eliminating the use of robotic arms, eliminating the use of wells, and providing an overall cheaper and faster platform for completing assays. The high speed and low volumes (nL-pL) possible with µFD can greatly increase the temporal resolution of fraction analysis, and droplet fractions can be immediately coupled to a downstream screening assay [5-7] (enabling 'online screening').

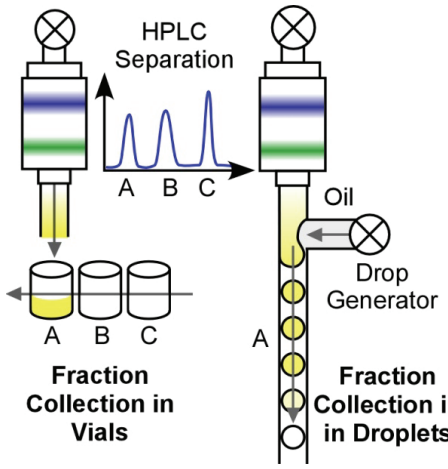

Figure 1: Concept of µFD coupled to a commercial HPLC (right) compared to conventional fraction collection (left).

µFD's simple design allows it to be coupled to virtually any separation scheme [5, 8]. Our previous studies have shown that it can be adapted to chromatographic methods [5] to form libraries of monodisperse droplets, each containing a separated compound. In this case, the separation technique (SPE cartridge) yields limited separation efficiency and relatively low purity of the resulting fractions. We now utilize high performance liquid chromatography (HPLC) as the separation process, yielding complete separations and high purity droplet fractions. A similar approach used ultra-performance liquid chromatography (UPLC) and a microfluidic chip, and the effect of eluent properties on droplet radii was presented [12]. We utilize a modular microfluidic approach where samples are fractionated within a conventional capillary and junction compatible with standard HPLC instruments. We demonstrate the tuning of fraction volumes and droplet frequency by changing the

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S. Hamed is with the department of Biomedical Engineering, Wayne State University, Detroit MI.

B. Shay is with the Biomedical Mass Spectrometry Facility in the Department of Pharmacology at the University of Michigan Ann Arbor.

A. Basu is with the department of Electrical and Computer Engineering and the department of Biomedical Engineering at Wayne State University (phone: 313-577-3990; email: abasu@eng.wayne.edu).

HPLC elution speed, eluent properties, and oil/water flow rates. Because the droplet fractions are generally larger in a capillary, they can also be potentially subjected to a second dimension of separation. Compatibility with HPLC instruments at standard flow rates makes it suitable for a wide range of analytical and preparative analyses.

II. Concept

Microfractionation in droplets is accomplished by coupling a droplet generator to the exit of an HPLC column (Fig. 1). As the separated components exit the column, they are encapsulated into droplets which permanently isolate the components, and prevent dispersion. The concentration of each separation is measured by absorbance, and retention times are determined by interactions between the substrate and column matrix. Using a C-18 column, more hydrophobic dyes retain in the column the longest. HPLC elutions are isocratic, applying an ethanol/water mobile phase. Chromatograms generated by a UV absorbance detector also catalog the purity, location, and retention time of each droplet fraction. Chromatograms are generated with different HPLC elution speeds, and different ratios of the binary mixture, which effects the formation of droplets.

The drop generator consists of a tee junction, where one input contains the HPLC eluent, and the second contains an immiscible carrier fluid. The T junction breaks up the eluent stream into monodisperse drops by orthogonal shear forces [13]. This can also be done using flow focusing, which requires a cross junction [14]. At a low capillary number (Ca) and small geometries, the train of droplets exiting the junction is monodisperse.

It is important to note that the carrier fluid will form a thin lubricating film which separates the droplets from the channel walls, preventing cross contamination between fractions. The wetting effect occurs so long as the surface tension between the droplet and wall material is greater than that of the carrier fluid and the wall [15]. The thickness of the wetting film can be approximated by Bretherton's law [16] $h = 1.34rCa^{2/3}$, where h is the thickness of the film, and r is the capillary radius. Ca is given by $\eta v/\sigma$, where η is the viscosity of the carrier fluid, v is the velocity of the carrier fluid, and σ is the interfacial tension.

Ca also determines the size of the droplets [17]. At $Ca<10^{-2}$ (squeezing regime), drop diameters scale with the relative flow rate of aqueous phase and the carrier, Q_A/Q_C; and are relatively independent of interfacial tension and viscosity. At $Ca>10^{-2}$, (dripping regime), droplet sizes depend on relative flow rate, and also scale inversely with Ca. In our experiments, with $10^{-3}<Ca<10^{-1}$, we expect to see characteristics of both regimes. In all cases, droplet volume v_d and frequency F_d are related by the conservation of mass formula $Q_A = F_d\, v_d$,.

III. Experimental Setup

A. *HPLC*

The HPLC utilized in this experiment is a Waters LC Module I (Waters Corp, Milford MA) fitted with an Atlantis dC-18 hydrophobic column (Waters Corp, Milford MA). 1 mm glass vials are used with the built in autosampler (Waters Corp, Milford MA), from which the dye mixtures are inserted into the machine. The dyes being used in this experiment are FD&C Red 40, FD&C Blue 1, and FD&C Yellow 5 (McCormick, Sparks MD). Eluents include 99% HPLC grade methanol (EMD, Gibbstown NJ), and 100% deionized water contained within 500 mL pyrex flasks. Chromatograms are recorded with the absorbance reader calibrated at 254 nm and 1.0 AUFS.

B. *Microfluidic Setup*

1.5785 mm diameter Teflon tubing (Small Parts, Miramar FL) is attached between the exit of the HPLC column and one port of a T-junction connector (Value Plastics, Fort Collins CO). The second port of the T-junction is connected by a Teflon capillary to a 10 mL syringe (for oil). The syringe is mounted on a syringe pump (model KDS230, KD Scientific, Holliston MA), by which drop generation speeds are controlled. The oil phases being used include 28 cSt oleic acid and 1000 cSt silicone oil (Dow Corning, Midland MI). Teflon is chosen as the capillary material because it is chemically inert, gas permeable, and hydrophobic.

C. *Experimental Procedure(s)*

The sample mixture contains 1 part yellow dye for 100 parts deionized (DI) water, and 2 parts blue dye for 30 parts DI water. Each HPLC injection contains 10 µl of this mixed sample. The first separation protocol utilizes a linear isocratic gradient, where the mobile phase contains a 40% to 60% methanol water mixture. The procedure was conducted at HPLC elution speeds of 0.7 ml/min. The second separation protocol is also isocratic, utilizing a 30% to 70% mixture of methanol and water. This mixture is optimal for separating the organic dyes with the equipment used. The protocol is repeated at HPLC flow rates of 0.7 ml/min, 0.5 ml/min, and 0.38 ml/min. All chromatograms are digitally recorded using Labview 2010 (National Instruments, Austin TX).

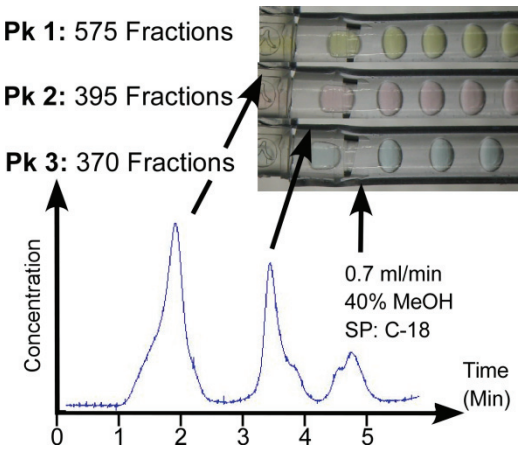

Figure 2: Chromatogram and fraction pictures of yellow, red, and blue food dyes separated at 0.7 mL/min HPLC elution speed, 0.49 mL/min carrier speed and 40/60 MeOH-Water elution profile. Droplets are generated at 6 drops/s with volumes of 1.94 uL.

In both protocols, the eluents are fractionated into microdroplets using oleic acid as the carrier fluid. Separations involving the 30/70 eluent mixture are additionally fractionated with silicone oil as the carrier. The volume and frequency of droplet fractions are tuned using carrier fluid flow rates of 0.15 ml/min, 0.35 ml/min, 0.49 ml/min, and 0.75 ml/min, and HPLC flow rates of 0.37 ml/min, 0.5 ml/min, and 0.75 ml/min. Droplet radii and frequencies are determined through frame-by-frame analysis of videos taken with a CCD camera.

IV. Results And Discussion

A. *HPLC Separations and Fractionation*

To demonstrate μFD with HPLC, we separated a mixture of 3 dyes with different hydrophobicities. The ratio of solvent in the binary eluent alters its interfacial properties with the oil phase, and effects capillary fractionation. When the concentration of MeOH is set to 40%, the separations complete by 4 min from the first peak (Fig. 2). Higher concentration of MeOH reduces the number of blank fractions between each separation, but it decreases resolution. Oleic acid is used as the carrier fluid at 0.49 ml/min, with the HPLC flow rate set at 0.7 ml/min. The surface tension of the 40/60 binary liquid is lower than the 30/70 mixture, and thus allows droplet generation to perform faster with smaller fractions. Fractions are generated at 6 drops/s, with volumes of 1.94 μL (Fig. 2). The overall reduction of interfacial tension increases the total number of fractions containing substrates, each with smaller diameters. The contact angle also decreases as a result of increasing the MeOH concentration, and therefore wetting of the capillary wall increases slightly [16]. Using the conservation of mass formula, the total number of drop fractions amounts to 575 for yellow (1.12 mL of eluent), 395 for red (0.77 mL of eluent), and 370 for blue (0.72 mL of eluent).

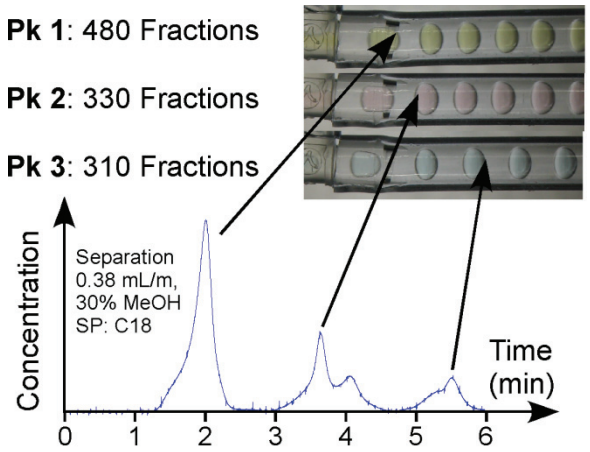

Figure 3: Chromatogram and fraction pictures of yellow, red, and blue food dyes separated at 0.7 mL/min HPLC elution speed, 0.49 mL/min carrier speed and 30/70 MeOH-Water elution profile. Droplets are generated at 5 drops/s with volumes of 2.33 μL.

Using a 30/70 MeOH-water mixture and 0.7 ml/min HPLC flow rate, dye mixtures separate within 5 minutes from the beginning of the first peak (Fig. 3). As in Figure 2, the carrier fluid speed is 0.49 ml/min. The resulting fractions are produced at 5 drops/s with each drop measuring 2.33 μL. The volumes of combined fractions are similar to those in Figure 2.

Keeping the parameters the same as in Figure 3, but reducing the HPLC flow rate to 0.38 mL/min, the colors are separated over a period of 10 min (Fig. 3). The number of fractions collected is less than in Figure 1, and fraction spacing is greater. This is because droplets are generated at 4 drops/s, with droplet volumes equaling around 1.58 μL. This HPLC protocol provides better fraction resolution for samples that may elute at very similar times. Even better resolution can be achieved by increasing the speed of the carrier, which increases *Ca* and creates drops of yet smaller volume and frequency. The total number of drop fractions amounts to 385 for yellow (0.62 mL of eluent), 265 for red (0.42 mL of eluent), and 245 for blue (0.39 mL of eluent).

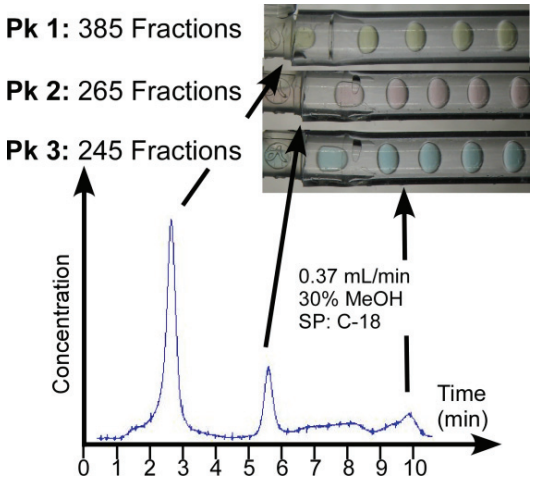

Figure 4: Chromatogram and fraction pictures of yellow, red, and blue food dyes separated at 0.38 mL/min HPLC elution speed, 0.49 mL/min carrier speed and 30/70 MeOH-Water elution profile. Droplets are generated at 4 drops/s with volumes of 1.6 uL.

B. *Tuning the Size of Droplet Fractions*

One of the main governing principles for fraction morphologies, the capillary number, can be tuned by droplet generation speeds and the viscosity of the carrier fluid. When the carrier fluid is oleic acid, the droplet frequencies increase linearly with increasing HPLC elution speeds and with higher carrier flow rates (Fig. 5a). When compared with silicone oil (Fig. 6a), the frequency of droplet formation using oleic acid is much lower at a Q_C of 0.35 ml/min and higher. There is a ~37 times increase in viscosity that lowers *Ca,* and contributes significantly to the increase in frequency observed with silicone oil. Utilizing oleic acid, the droplet volumes increase linearly with increasing HPLC elution speeds and carrier speeds of 0.35 and 0.75 ml/min. At a Q_C of 0.15 ml/min, droplet volumes are highest with HPLC speeds of 0.5 ml/min, with no linearity. When the droplet volumes in oleic acid are compared to silicone oil, the volumes are generally lower at the same respective Q_A and

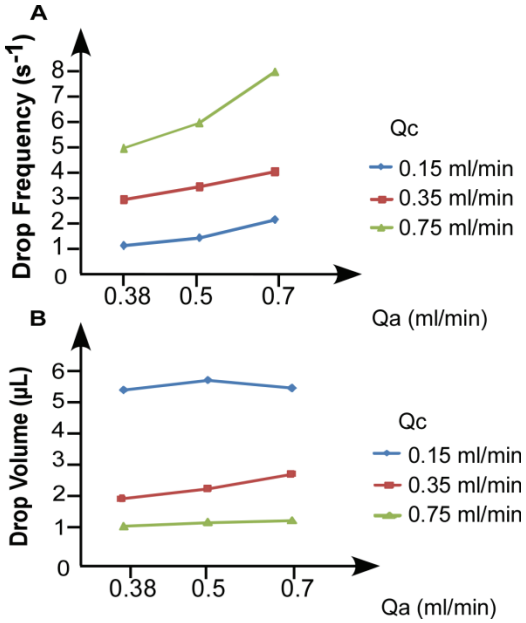

Figure 5: Tuning fraction frequency (A) and volume (B) using oleic acid as the carrier fluid. Q_C and Q_A are the flow rates of the carrier fluid and HPLC eluent, respectively.

Q_C (Fig. 5b, 6b). However, lower carrier speeds significantly increase the fraction volumes in both figures. Modulating the fraction volumes and concentrations with conventional fractionation techniques would require robotic programming with sophisticated machinery. The microfluidic setup utilized in this experiment costs a small fraction of conventional technologies, and has capabilities of generating heterogeneous libraries of isolated substrates for HTS. By utilizing microfluidics, fractions are modulated by fluid

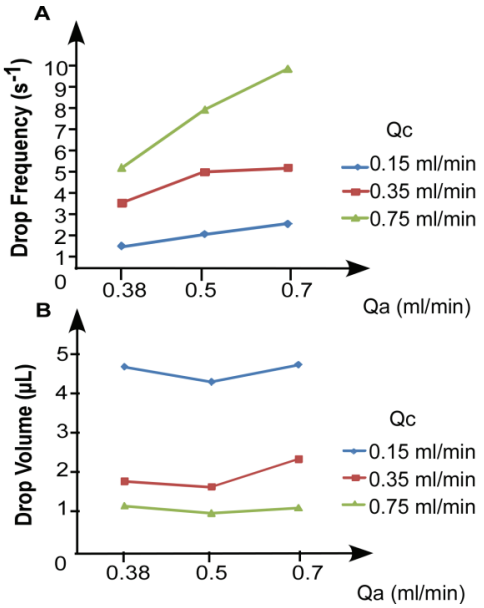

Figure 6: Tuning the frequency (A) and volume (B) of droplet fractions using silicone oil as the carrier fluid. Q_C and Q_A are the flow rates of the carrier fluid and HPLC eluent, respectively.

properties, flow rates, and interfacial tension. Although still in its infancy, microfluidic fractionation technologies show prospects in linking biological analytical techniques to online screening.

V. Conclusions

Microfractionation of HPLC separated mixtures in a standard Teflon capillary is an economical and versatile way to prepare biomolecules for screening assays. Fraction volume and frequency can be modulated to resolve substrates with similar retention times, or limit the amount of fractions in storage. The fractions can then be coupled to downstream screening assays.

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