

Direct Interface between Digital Microfluidics and High Performance Liquid Chromatography–Mass Spectrometry

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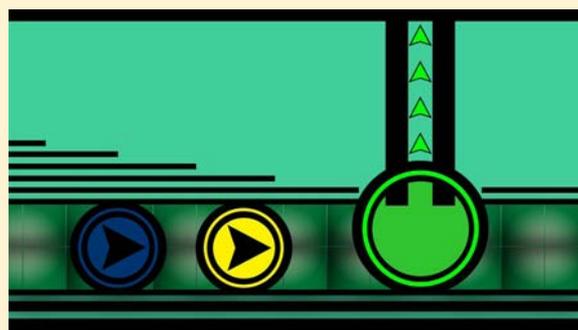
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S Supporting Information

ABSTRACT: We introduce an automated method to facilitate in-line coupling of digital microfluidics (DMF) with HPLC-MS, using a custom, 3D-printed manifold and a custom plugin to the popular open-source control system, DropBot. The method was designed to interface directly with commercial autosamplers (with no prior modification), suggesting that it will be widely accessible for end-users. The system was demonstrated to be compatible with samples dissolved in aqueous buffers and neat methanol and was validated by application to a common steroid-labeling derivatization reaction. We propose that the methods described here will be useful for a wide range of applications, combining the automated sample processing power of DMF with the resolving and analytical capacity of HPLC-MS.



Mass spectrometry (MS) is a widely used tool for the identification and quantification of diverse analytes in myriad applications.^{1,2} Unfortunately, many samples require laborious and time-consuming processing regimens prior to MS analysis. Thus, there is great interest in the development of new technologies that integrate sample processing with analysis by MS.^{3–5} Digital microfluidics (DMF), a liquid-handling technology that facilitates the translation of droplets on an array of electrodes,^{6,7} has been touted as a potential solution to this problem.⁸ Through the application of a series of potentials to the DMF electrodes, picoliter-to-microliter-sized droplets (each serving as an isolated vessel for chemical reactions and other processes) can be made to move, merge, split, and dispense from reservoirs. The absolute control over samples and reagents, and the convenient solvent exchange and product collection afforded by DMF makes it an attractive technique for automated processing of complex samples. For example, DMF methods have been developed for automated solid-phase extraction,^{9,10} liquid–liquid extraction,^{11,12} analyte extraction from dried blood spots,^{13,14} and proteomic sample reduction, alkylation, and digestion.^{15–17}

There has been great progress made toward coupling DMF-based sample processing directly to analytical MS through direct infusion⁸ (with no in-line separations). But for many applications, the quality and reproducibility of information collected by MS is limited without incorporation of a chemical

separation step.¹⁸ There are a few examples of coupling DMF directly to separations. For example, we developed a “hybrid microfluidic” technique that allows for an in-line connection between DMF sample preparation and microchannel electrophoresis.^{19,20} After processing (by DMF) and separation (by electrophoresis), samples can be delivered to microfluidic nanoelectrospray emitters for analysis by MS.¹³ Likewise, the Kaljurand group²¹ demonstrated the coupling of DMF with a (nonmicrofluidic) capillary electrophoresis system. Sample droplets and buffer droplets were actuated to the inlet of a separation capillary for electrophoretic separations, after which they could be analyzed either by MALDI-²² or ESI-MS.²³ These are useful advances, but there has been little progress in mating DMF directly to the much more common separation technique (for integrating with MS) of high-performance liquid chromatography (HPLC).

To date, the vast majority of applications using both DMF and HPLC-MS have been implemented off-line,²⁴ which requires additional sample transfer and processing steps that can lead to nonspecific adsorption, contamination, and sample loss. The one report that we are aware of describing a direct interface between DMF and HPLC was presented at a

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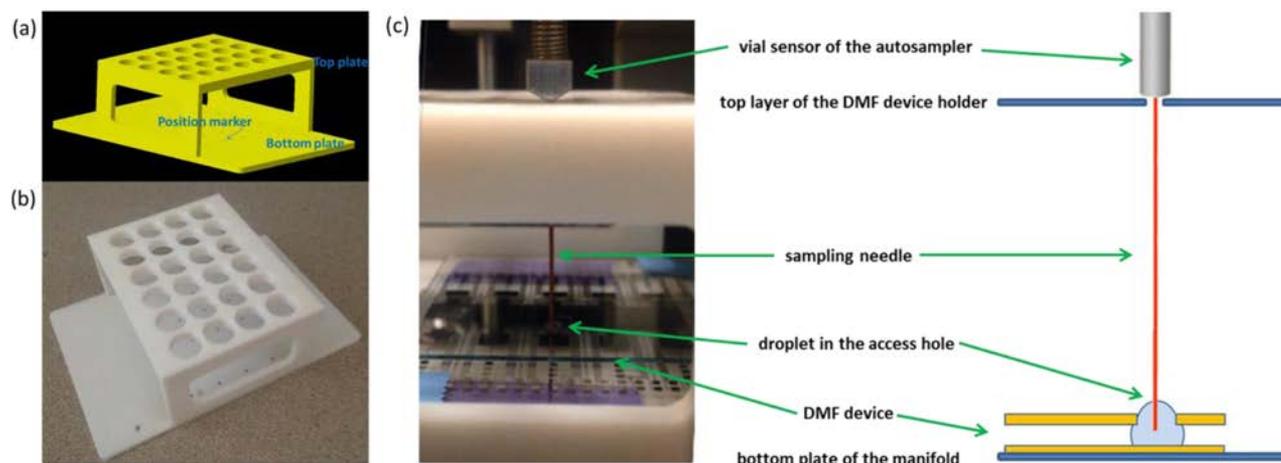


Figure 1. DMF-autosampler interface for DMF-HPLC-MS. (a) Three-dimensional rendering and (b) picture of the manifold designed to interface a DMF device with an HPLC autosampler. (c) Picture (left) and cartoon (right) depicting the process of sample aspiration from the access-hole in the top plate of a DMF device into an HPLC-MS via the sampling needle of the autosampler.

conference in 2012.²⁵ This method comprises an interface in which droplets of reaction products on a DMF device are transferred to a primary vial using a strong vacuum, followed by a second transfer to a secondary vial for a degassing step using a weak vacuum. In a final step, the degassed droplets are transferred a third time, injected onto an HPLC system for separations. This method represents an important step forward, but has a number of limitations, including the potential for sample loss in multiple transfers and vials, the necessity of extensive HPLC firmware modification for operability, and the requirement of large ($\sim 65 \mu\text{L}$) droplets for analysis. Here, we report a new interface in which droplets of “standard” DMF size ($1\text{--}5 \mu\text{L}$) are injected directly into an HPLC-MS for analysis that is compatible with a commercial autosampler system without any modifications. We propose that this interface will be useful for a wide range of applications, combining the automated sample processing power of DMF with the resolving and analytical capacity of HPLC-MS.

EXPERIMENTAL SECTION

Reagents and Materials. Testosterone was purchased from Cerillant (Round Rock, TX). Methanol and acetonitrile were from Caledon Laboratories Ltd. (Georgetown, Ontario, Canada), and acetic acid was obtained from Sigma-Aldrich (Oakville, Ontario, Canada). Tryptic digest of beta-galactosidase was purchased from SCIEX (Framingham, MA). The Amplifex Keto Reagent, also known as quaternary aminoxy (QAO) reagent²⁶ or (*O*-(3-trimethylammoniumpropyl) hydroxylamine) bromide, was also from SCIEX. Distilled deionized (DI) water ($18 \text{ M}\Omega$) was produced in-house using a Millipore Integral 10 water purification system (Millipore, Billerica, MA).

DMF Device Fabrication and Operation. Two-plate digital microfluidic devices were fabricated at the University of Toronto Nanofabrication Center (TNFC), as described elsewhere.^{27,28} Bottom plates feature an array of 80 square actuation electrodes ($2.2 \times 2.2 \text{ mm}$ each) and 12 reservoir electrodes ($16.4 \times 6.7 \text{ mm}$ each), coated with $\sim 7 \mu\text{m}$ Parylene-C (Specialty Coating Systems, Indianapolis, IN) and $\sim 200 \text{ nM}$ Teflon-AF (DuPont, Wilmington, DE). Top plates were formed from 0.7 mm thick indium tin oxide (ITO) coated glass substrates (Delta Technologies, Ltd., Loveland, CO). A

$1/16''$ diameter through-hole was drilled through each top plate, after which each substrate was coated with $\sim 200 \text{ nm}$ Teflon-AF. Devices were assembled with an ITO-glass top-plate and a patterned bottom-plate separated by a spacer formed from two pieces of double-sided tape (total spacer thickness was $180 \mu\text{m}$). The drilled hole on the top plate was aligned to cover an actuation electrode of the bottom plate. The open-source DropBot control system²⁹ (and attendant MicroDrop software) was used to manage the application of AC sine waves ($80\text{--}120 V_{\text{RMS}}$, 10 kHz) between the top plate (ground) and sequential electrodes on the bottom plate. A MicroDrop plugin (included here as [Supporting Information](https://github.com/wheeler-microfluidics/AnalystControl/releases/latest) and available online at <https://github.com/wheeler-microfluidics/AnalystControl/releases/latest>) was developed to allow DropBot to trigger sampling into the HPLC (details below). Droplet evaporation was not observed to be a problem for the applications described here; if it proves to be problematic in the future, potential solutions include enclosure in a vapor-saturated chamber,³⁰ or periodic replenishment with fresh solvent.³¹

QAO Derivatization. QAO was reacted with testosterone using methods similar to those described previously.²⁶ Briefly, one aliquot each of testosterone (20 ng/mL in methanol) and QAO solution (5 mg/mL in methanol premixed with acetic acid, $4:1, \text{ v/v}$) were loaded into reservoirs on the device. A unit droplet of each solution was dispensed onto the array of electrodes, where the two droplets were merged. The combined droplet was moved continuously in a square pattern^{32,33} for 5 min . After completing the reaction, an aqueous quench-step was implemented as part of the loading process onto the autosampler (described below).

DMF-HPLC Interface. A $128 \text{ mm} \times 86 \text{ mm} \times 40 \text{ mm}$ manifold was designed to interface DMF devices with a standard HPLC autosampler (Figure 1a,b). The .stl file for this design is included in the [Supporting Information](#), and the manifold was fabricated using Fortus 400mc 3D printer (Stratasys Ltd., Eden Prairie, MN). Devices were inserted such that the top-plate access hole was aligned with a position-marker on the bottom surface of the manifold. In a typical experiment, a droplet on the DMF device was driven to the top-plate access hole. In some experiments, the hole was dry, while in other experiments, a $5 \mu\text{L}$ aliquot of DI water or other

aqueous solution was pipetted into the access hole. After delivery of the sample droplet to the hole, the manifold was inserted into an Eksigent AS1 Autosampler (SCIEX, Dublin, CA) bearing a standard stainless steel sampling needle (150 μm i.d., 1/32" o.d.). With Eksigent control software needle position set to 1, the sampling needle was lowered into the access hole to aspirate 1 μL of the droplet into the HPLC-MS system (Figure 1c).

HPLC-MS/MS Analysis. Samples were separated using a nano-LC system (Tempo, SCIEX, Dublin, CA) equipped with a nano-LC capillary column (75 μm i.d. \times 15 cm Eksigent ChromXP column packed with C18 modified 3 μm diameter particles with 120 \AA diameter pores, SCIEX, Dublin, CA). Analytes were injected from the autosampler onto the column at a flow rate of 300 nL/min in direct-inject configuration. A linear gradient of acetonitrile/DI water with 0.1% formic acid was applied from 5%/95% to 60%/40% over 30 min for the analysis of steroids derivatives and from 5%/95% to 35%/65% over 15 min for β -galactosidase digests. Samples eluted from the nano-LC column were electrosprayed in positive mode using a NanoSpray interfaced to a QTRAP 5500 MS/MS system (SCIEX, Concord, Canada). For the analysis of QAO-testosterone, the multiple reaction monitoring (MRM) transition was set as 403.3 \rightarrow 164.2, and optimized peak intensities were observed at a declustering potential of 60 V, a collision energy of 62 eV, and source temperature of 150 $^{\circ}\text{C}$. For the analysis of digested β -galactosidase, MRM transitions and collision energies are listed in Table 1, with a declustering potential of 150 V.

Table 1. Mass Spectrometry Parameters for the Analysis of Digested β -Galactosidase

peptide	Q1 (<i>m/z</i>)	Q3 (<i>m/z</i>)	collision energy (eV)
FNDDFSR	450.7	524.2	28
YSQQQLMETSHR	503.2	760.3	27
GDFQFNISR	542.3	636.4	26
IDPNAWVER	550.3	871.4	27
VDEDQFPAPVK	671.3	755.5	33
DWENPGVTQLNR	714.8	884.5	32
APLNDIGVSEATR	729.4	832.5	48

RESULTS AND DISCUSSION

DMF–HPLC Interface. The overall goal for this work was to develop an interface to allow for automated transition of droplets on DMF to analysis by HPLC-MS. A custom manifold (.stl file included as Supporting Information) was designed to house DMF devices and match the dimensions (128 mm \times 86 mm) of a standard 48-well HPLC vial holder (Figure 1a,b). Thus, the manifold serves as a “virtual sample tray” and can be inserted directly into a standard autosampler. The experiments described here were implemented using an Eksigent AS1 autosampler, but we propose that manifold should be compatible with most commercial systems.

The DMF-HPLC interface was designed such that the autosampler needle can be lowered to penetrate an access hole in the top-plate of a two-plate DMF device (Figure 1c). This can be controlled manually (via the autosampler control software) or can be triggered using a custom plugin (included here as Supporting Information) developed for the open-source DMF control system, DropBot. Once lowered, the needle contacts the droplet(s) sandwiched within the device, where

some portion (or all) of the droplet(s) can be aspirated and then automatically injected into the HPLC-MS. Ease of alignment was a critical design goal, which led us to include an array of markers on the bottom surface of the manifold. These markers can be recognized as “sample vial” surrogates by the autosampler control software. While the present study describes injections from only a single access-hole, we propose that it will be straightforward to use DMF devices with an array of access holes (positioned to match the pitch of the manifold markers) to allow for flexibility to use DMF manipulations, the X-Y manipulator, or both to choose which samples to load into the instrument. In this implementation, DMF droplet manipulation allows for the queuing and delivery of different samples to the HPLC (in place of using the autosampler’s X-Y manipulator to sample from different vials in the tray).

Another element considered in the manifold design was a capacity to trigger the autosampler’s vial sensor. In conventional autosampler operation, as the needle descends, the vial sensor makes physical contact with the top of an HPLC vial, which limits the vertical travel-distance of the needle (to avoid crashing into the bottom of the vial). An analogous feature was enabled here by designing the manifold to be 40 mm high. As depicted in Figure 1c, the top of the manifold triggers the vial sensor such that the needle (when engaged in setting “1” in the software) penetrates to an appropriate position to sample the droplet without crashing into the bottom plate of the device. This feature allows the new system to operate “out of the box” with no requirement for modification of the autosampler in any way.

Finally, the most important design consideration for the new system was dictated by sample volume. The devices used here operate with unit volumes (i.e., the volume of a droplet that covers a single 2.2 \times 2.2 mm driving electrode) of \sim 0.85 μL ; by dispensing, mixing and merging unit droplets, it is common to work with droplets as large as \sim 5 μL . This range of volumes is a perfect match for standard HPLC autosamplers, which are designed to aspirate 1–10 μL of sample to inject onto the column. In contrast, the one DMF-HPLC interface described previously²⁵ required the use \sim 65 μL samples, which would (a) occupy nearly the entire array of electrodes on the devices used here, and (b) is nearly an order of magnitude larger than typical samples injected onto HPLC (a characteristic that would be particularly undesirable for applications involving precious samples such as core-needle biopsies²⁴). In summary, we propose that the system described here represents a useful combination of existing techniques, forming an integrated microfluidic sample processing module connected to a high-performance analyzer.

DMF-HPLC-MS/MS for Aqueous Sample Analysis. The new system reported here was designed such that droplets manipulated on two-plate DMF device (sandwiched between a top and bottom plate) can be sampled via an access-hole drilled in the top plate. We hypothesized that aqueous droplets manipulated in the hydrophobic (i.e., Teflon-AF-coated) devices would spontaneously “wet” the access-hole, the sides of which feature exposed hydrophilic glass, in a manner similar to how droplets were made to interface with drilled holes in multilayer “hybrid” microfluidic devices.²⁰ This hypothesis was correct—as shown in Figure 2a and Movie S1, when a water droplet is driven to an access hole, it instantaneously wets the empty hole. In the course of hundreds of trials with droplet volumes ranging from 0.85 to 5 μL , the droplet always wetted the hole, where it was accessible for analysis.

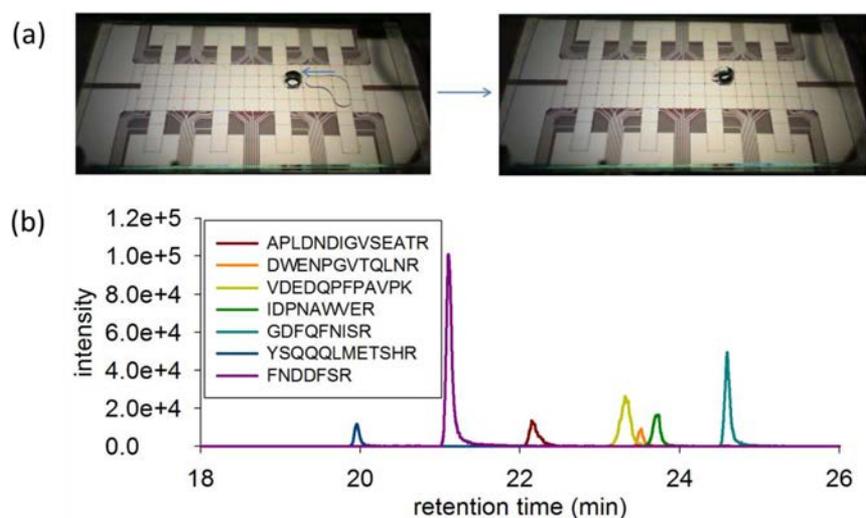


Figure 2. Aqueous sample analysis by DMF-HPLC-MS/MS. (a) Frames from a movie ([Movie S1](#) in the [Supporting Information](#)) demonstrating the loading of a 5 μL droplet of water into an access hole. (b) Representative extracted ion chromatogram (XIC) generated from injection of a 1 μL portion of a droplet manipulated on DMF containing digested β -galactosidase. The peaks (shown in different colors) match the MRM transitions assigned to the seven tryptic peptides predicted for this analyte.

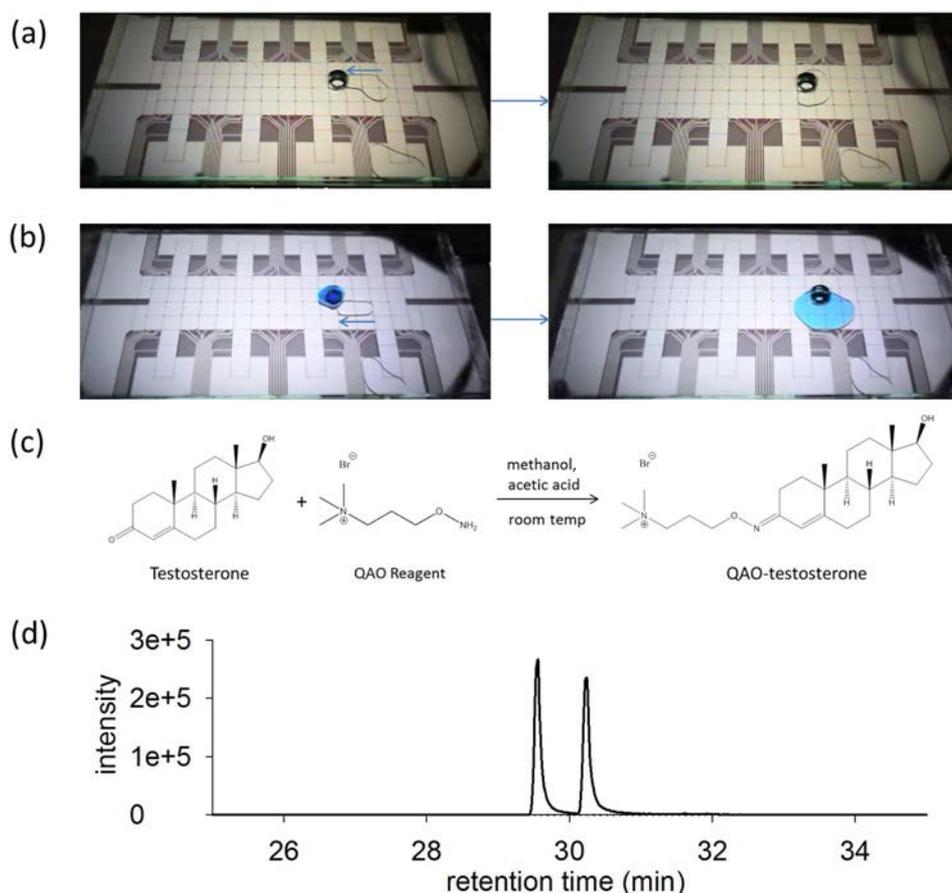


Figure 3. Methanolic sample analysis by DMF-HPLC-MS/MS. (a) Frames from a movie ([Movie S2](#) in the [Supporting Information](#)) demonstrating that a 3 μL methanol droplet does not spontaneously wet an empty access hole (instead forming a “doughnut” shape). (b) Frames from a movie ([Movie S3](#) in the [Supporting Information](#)) demonstrating that a 2 μL methanol droplet automatically merges with a “pre-wetted” 5 μL aqueous droplet (containing blue dye for visibility) in the access hole. (c) QAO-testosterone derivatization scheme. (d) Representative XIC generated from injection of a 1 μL portion of a droplet containing QAO-testosterone formed on-chip.

A model system (β -galactosidase digest in DI water with 0.1% formic acid) was chosen to evaluate the capacity of the new system for in-line aqueous droplet manipulation and

HPLC-MS analysis. In these experiments, 2 μL droplets of the digest were dispensed and driven to the access hole (as in [Figure 2a](#)), and then the autosampler needle was lowered into

the hole to aspirate 1 μL of the solution into the HPLC-MS system. A representative extracted ion chromatogram (XIC) generated from these experiments is shown in Figure 2b. In the proof-of-concept work shown here, the digestion was carried out off-chip, but in the future, a full proteomic sample preparation process might be implemented on-chip, including reduction, alkylation, and tryptic digestion,^{15–17} perhaps coupled with magnetic particle-based predepletion of abundant protein species.³⁴ Note that the ability to automate multistep sample processing without a robotic plate-handling/dispensing/aspiration system represents a significant advantage relative to methods relying on multiwell plates. We propose that these methods will be useful for proteomic sample processing and analysis, as well as a wide range of other applications that rely on samples dissolved in aqueous solvents.

DMF-HPLC-MS/MS for Methanolic Sample Analysis.

As described above, the new DMF-HPLC interface is particularly well-suited for analysis for analyzing aqueous droplets (which are observed to wet empty access holes spontaneously). But, in many applications, organic solvents, like methanol and acetonitrile, are also important for sample processing and cannot be replaced with aqueous solvents. Here, we used methanol as a representative solvent to evaluate compatibility with such applications.

As shown in Figure 3a and Movie S2, droplets containing 100% methanol do not wet an empty access hole; rather, the droplet forms a “doughnut” shape around the hole, where it cannot be accessed by the autosampler needle. This phenomenon is consistent with expectations; unlike aqueous samples, droplets of methanol should not experience a strong attraction to hydrophilic exposed glass relative to hydrophobic Teflon-AF. More generally, it was found that a droplet containing a mixture of methanol and water on a DMF device does not wet the access hole if the methanol content of droplet is >33%.

One solution to the problem described above would be to coat the walls of the access hole with a low-surface energy material (to allow spontaneous wetting of organic solvent-containing droplets). Another would be to use DMF to dispense, merge, and mix methanol and water droplets on the DMF device at an appropriate ratio prior to interfacing with HPLC. But desiring a more general solution with minimal impact on device fabrication and recognizing that many autosamplers have the capacity to both dispense and aspirate, we chose to evaluate a third strategy in which the access hole is prewetted with water prior to running an experiment. As shown in Figure 3b and Movie S3, a water droplet (5 μL) that is pipetted into the access hole remains in place, without spreading onto the hydrophobic DMF device. When a 2 μL methanol droplet is driven by DMF to the access hole, it merges with the preloaded water droplet, such that the merged droplet is accessible for loading into the autosampler. As expected (from the methanol/water mixture tests described above), the volume ratio of the aqueous and methanolic aliquots in this technique is critical. If the volume of the two solvents is the same (e.g., both 2 μL), upon merging, the combined droplet is pulled out of the hole and is not accessible for loading onto the HPLC (Movie S4). In general, it was found that the volume ratio of the preloaded water and DMF-actuated methanol should be greater than 2:1 to ensure successful loading of the methanolic droplet into the access hole. A volume ratio of 2.5:1 was used for the experiments reported here. As described in the supplementary methods and

results, this method was found to not interfere with the ability to mix samples and reagents prior to analysis.

Finally, armed with a method for using the new manifold to inject organic solvent-containing droplets into the HPLC-MS/MS, we used this strategy for on-chip steroid derivatization. Steroid hormones often have poor ESI-MS signal intensities because they are difficult to ionize; this has led to the development of reagents such as QAO that form derivatives that ionize easily. As shown in Figure 3c, when testosterone is combined with QAO reagent in the presence of acid (free from water), a derivative is formed that yields high signal when analyzed by ESI-MS.²⁶ In this work, two methanolic droplets, one containing testosterone and another containing QAO with acetic acid were dispensed, merged and mixed on a DMF device. After 5 min, this droplet was moved to the prewetted access hole to merge with the prefilled water droplet (which also served the function of quenching the reaction). The sampling needle was then lowered into the access-hole to aspirate the solution into the HPLC-MS system for analysis. As shown in Figure 3d, the reaction product of QAO-testosterone was successfully identified. QAO-derivatization of testosterone often leads to the formation of structural isomers that are separable via HPLC-MS/MS,²⁶ which were observed here as two separable chromatographic peaks. The peak intensities ($n = 3$, CV = 5%) and retention times ($n = 3$, CV = 0.06%) for multiple injections were reproducible run-to-run, suggesting that DMF-HPLC-MS/MS will be useful for in-line processing and analysis.

CONCLUSIONS

We introduced a technique for coupling digital microfluidics (DMF) with HPLC-MS. We propose that this technique will form the basis for an automated bioanalysis system that uses DMF for sample processing and pretreatment and HPLC-MS/MS for target-characterization.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.analchem.5b03616.

Details of the fluorescence experiments and the mixing in the “pre-wetting” strategy (PDF).

AnalystControl file (ZIP).

Manifold design (ZIP).

Supporting movies S1–S4 (ZIP).

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Notes

The authors declare no competing financial interest.

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