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Dried Blood Spot Analysis by Digital Microfluidics Coupled to Nanoelectrospray Ionization Mass Spectrometry

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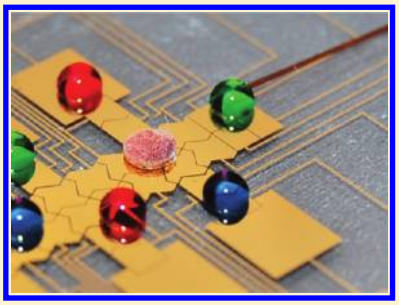
ABSTRACT: Dried blood spot (DBS) samples on filter paper are surging in popularity as a sampling and storage vehicle for a wide range of clinical and pharmaceutical applications. For example, a DBS sample is collected from every baby born in the province of Ontario, Canada, for quantification of approximately one hundred analytes that are used to screen for 28 conditions, including succinylacetone (SA), a marker for hepatorenal tyrosinemia. Unfortunately, the conventional methods used to evaluate DBS samples for newborn screening and other applications are tedious and slow, with limited options for automated analysis. In response to this challenge, we have developed a method to couple digital microfluidics (DMF) to nanoelectrospray ionization mass spectrometry (nESI-MS) for SA quantification in DBS samples. The new system is formed by sandwiching a pulled glass capillary emitter between the two DMF substrates such that the capillary emitter is immobilized without external seals or gaskets. Moreover, we introduce a new feedback control system that enables high-fidelity droplet manipulation across DBS samples without manual intervention. The system was validated by application to on-chip extraction, derivatization, and analysis of SA and other analytes from DBS samples, with comparable performance to gold-standard methods. We propose that the new methods described here can potentially contribute to a new generation of analytical techniques for quantifying analytes in DBS samples for a wide range of applications.



Dried blood spot (DBS) samples stored on filter paper are surging in popularity for applications ranging from screening for genetic disorders in newborn patients[1] to point-of-care testing for infectious diseases[2] to metabolic profiling in drug discovery and lead validation.[3] An example of the former application is the quantification of succinylacetone (SA) in DBS samples collected from newborn patients as a biochemical hallmark for tyrosinemia type 1, also known as hepatorenal tyrosinemia (HT).[4] In such tests, a punch from a DBS is pre-extracted in methanol and then the SA (which remains in the residual spot) is extracted and derivatized with hydrazine to form a hydrazone derivative that can be quantified using tandem mass spectrometry (MS/MS).[5,6] This method is particularly convenient because the analytes extracted into the methanol in the first step can be quantified to screen for other diseases such as phenylketonuria and homocystinuria.[7] This process (sequential extraction in methanol and hydrazine) is applied to more than 140 000 DBS samples each year at the Newborn Screening Ontario (NSO) facility at the Children's Hospital of Eastern Ontario.[8]

Unfortunately, analytical technology has not kept pace with the surging popularity of DBS analysis for newborn screening and other applications. Although there is great interest in developing new techniques for analyzing DBS samples,[9−13] there are few automated solutions available, and most methods used now are manual, tedious, and slow.[3] We report here a new method to analyze DBS samples for SA and other analytes. This new method is powered by digital microfluidics (DMF), a fluid-handling technique in which discrete droplets are manipulated on an open surface by applying a series of electric potentials to an array of electrodes.[14,15] Jebrail and Yang et al.[16] recently introduced the utility of digital microfluidics for in-line extraction and analysis of analytes in DBS samples. The digital microfluidic format is particularly well-suited for DBS analysis, as there are no channels that might otherwise become clogged by the particulates in the sample, and in addition DMF is compatible with mesoscale extraction/reagent volumes that are useful for extracting analytes from ∼millimeter-diameter punches from filter paper.

The work described here includes several advances relative to the methods described by Jebrail and Yang et al.,[16] including a new, straightforward interface between DMF and mass spectrometry for in-line extraction and analysis. In addition, the new method features automated control over droplet

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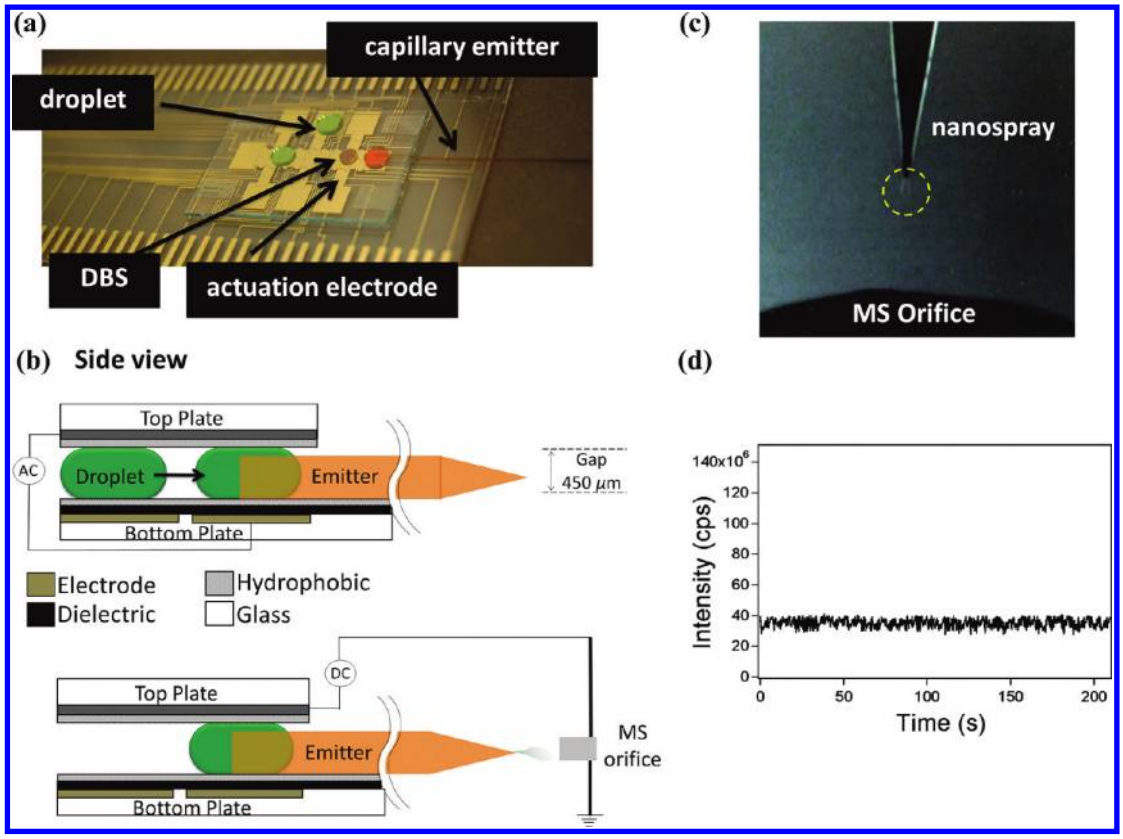

Figure 1. Digital Microfluidics–nanoElectrospray Ionization-Mass Spectrometry (DMF–nESI-MS) interface. (a) Image of a device (bearing colored droplets and a punched DBS sample) mated to a capillary emitter. The contact pads on the sides of the device mate with a 40-pin connector for automated droplet control. (b) Side-view schematics. (top) AC electric potentials are applied between the top and bottom substrates to actuate the droplets. (bottom) DC electric potentials are applied between the top plate and the MS orifice to generate a nanoelectrospray. (c) Image of spray generated at the tip of the capillary emitter. (d) Total ion count as a function of time from a 15 μL droplet of tyrosine (5 μM). The spray was stable for >200 s, with a RSD of 7.3%.

position, and multistep liquid extraction into multiple solvents. We propose that the new device format and method described here may be useful for myriad applications in which DBS samples must be processed prior to analysis with mass spectrometry.

EXPERIMENTAL SECTION

Reagents and Materials. Unless otherwise specified, reagents were purchased from Sigma Chemical (Oakville, ON). 3,4,5,6,7-^{13}C-succinylacetone ($^{13}C_5$-SA) was obtained from Cambridge Isotope Laboratories (Andover, MA). Whatman 903 filter paper was purchased from Fisher Scientific (Ottawa, ON). Parylene-C dimer was from Specialty Coating Systems (Indianapolis, IN), and Teflon-AF was purchased from DuPont (Wilmington, DE). All working solutions were prepared using deionized (DI) water that had a resistivity of 18 MΩ·cm at 25 °C, filtered with nylon syringe filters from Millipore (Billerica, MA, 0.2 μm pore diameter).

Dried blood spot (DBS) samples were formed in Toronto (University of Toronto) and in Ottawa (Newborn Screening Ontario, Children's Hospital of Eastern Ontario). For samples formed in Toronto, individual male units of blood in EDTA (K3) were purchased from Biochemed Services, Winchester, VA, and were fortified with SA (5, 10, 20, 50, or 80 μM) and fixed amounts of $^{13}C_5$-SA (15 μM). Aliquots (100 μL) were spotted on filter paper (thickness ~350 μm measured using a caliper) and dried at ambient temperature overnight. After drying, 3.2 mm diameter punches (thickness including blood ~430 μm measured using a caliper) were generated using a biopsy punch tool (Surgical Tools, Bedford, VA). These samples were stored at 4 °C in Petri dishes (Fisher Scientific, Ottawa, ON) when used within one week; otherwise, they were stored at −20 °C (to avoid overestimation of SA concentration[5]). Blood samples formed in Ottawa containing varying concentrations of SA were spotted, dried, and punched (3.2 mm diameter) by Newborn Screening Ontario (NSO) staff using standard techniques[17] and were stored at −20 °C. Before analysis, each such punch was fortified with 10 μL of $^{13}C_5$-SA (15 μM) and then air-dried at room temperature for 2 h.

DMF Device Fabrication. Digital microfluidic devices were fabricated in the University of Toronto Emerging Communications Technology Institute (ECTI) cleanroom facility, using a transparent photomask printed at Pacific Arts and Design (Markham, ON). The bottom plates of DMF devices were formed from glass substrates bearing patterned chromium electrodes and contact pads by photolithography and etching as described previously[18] and were coated with 7 μm of Parylene-C and 50 nm of Teflon-AF. Parylene-C was applied using a vapor deposition instrument (Specialty Coating Systems), and Teflon-AF was spin-coated (1% wt/wt in Fluorinert FC-40, 1000 rpm, 30 s) followed by postbaking on a hot-plate (160 °C, 10 min). Dicing tape was placed on the electrode contact pads prior to parylene coating and was removed after coating to facilitate electrical contact. In addition to patterned bottom-plate substrates, unpatterned indium tin oxide (ITO)-coated glass substrates (Delta Technologies Ltd., Stillwater, MN) were

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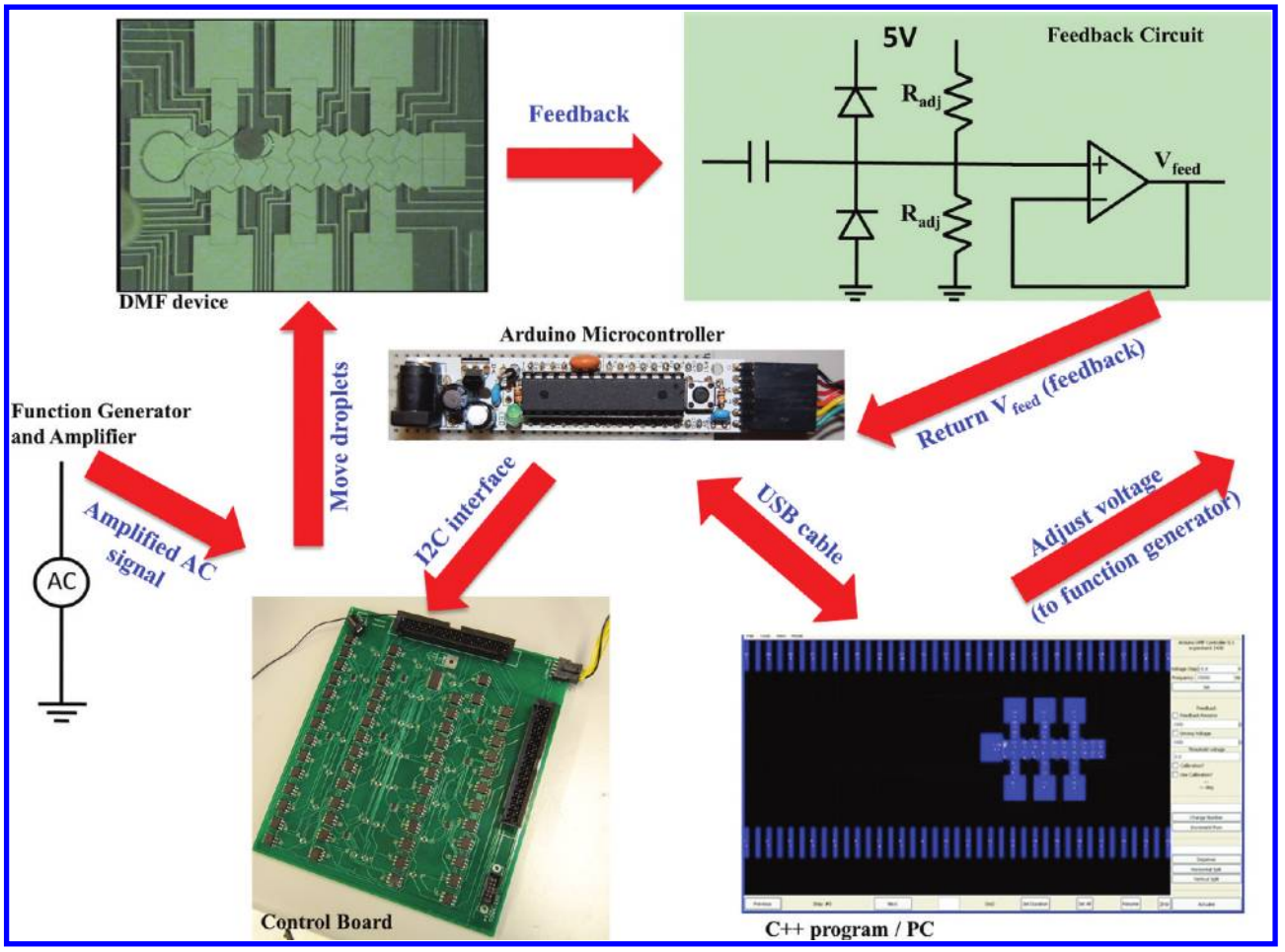

Figure 2. Impedance-based feedback control system. The schematic illustrates the relationships between the function generator and amplifier, the control board bearing high voltage relays, the RBBB Arduino microcontroller, the DMF device, the PC and C++ program, and the feedback circuit. To program a given droplet movement, the user clicks the appropriate position in the graphical C++ interface. After all movements are programmed, 5 V_{pp} signals are sent to the Arduino to activate the designated high voltage relays on the control board. In each droplet movement, the feedback circuit detects the impedance and compares it with a threshold to determine whether the droplet movement was successful. In any case in which an initial movement is not successful, a series of actuation potentials with higher magnitude are applied until droplet movement succeeds.

Table 1. Differences between the New Feedback Control System Used Here and the Feedback Control System Described Previously[19]

property	system reported here	system used previously[19]
feedback voltage measurement circuit	buffered system, shown in Figure 2, managed by an RBBB Arduino microcontroller (Modern Device, Providence, RI)	passive system relying on resistors and capacitors
circuit tuning mechanism	digital potentiometer (R_{adj} in Figure 2 = 1−10 kΩ) managed by Arduino capable of automatic thresholding for new liquids during a given experiment	manual potentiometer (1−20 kΩ) which must be adjusted for each new liquid prior to each experiment
high voltage source and degree of control	sine-wave from function generator (managed by software) and amplifier which can be adjusted during each experiment	sine-wave from function generator and amplifier which must be adjusted prior to each experiment
length of droplet actuation pulse	505 ms (500 ms driving pulse + 5 ms measurement)	215 ms (200 ms driving pulse + 15 ms measurement)
control software and interface	custom C^{++} program interfaced to Arduino microcontroller via USB cable	Custom LabView (National Instruments, Austin, TX) program interfaced to a DAQPAD 6507 (National Instruments)
number, type, and configuration of relays/switches	80 AQV259H (Panasonic) solid-state relays, 2 per output (total 40 outputs), with each output capable of two states: high-voltage, ground	96 RT424012F (Tyco Electronics) mechanical relays, 2 per output (total 48 outputs) with each output capable of three states: high-voltage, ground, and float
feedback response to droplet movement failure	repeated pulses *with a 50 V_{rms} step-up in voltage* (up to a maximum of 350 V_{rms}) until droplet movement is achieved	repeated pulses of the *same actuation voltage* until droplet movement is achieved

coated with Teflon-AF (50 nm, as above) for use as top-plate substrates.

The device design (Figure 1a) features an array of 22 roughly square actuation electrodes (2.2 × 2.2 mm each) connected to 7 reservoir electrodes (5 × 5 mm each), with interelectrode gaps of 30 μm. Each electrode is connected to an array of contact pads on the side of the device spaced appropriately to interface with a 40-pin connector (Compar Inc., Burlington ON). Devices were assembled with an unpatterned ITO−glass top plate and a patterned bottom plate such that the 37.5 mm

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× 25 mm top plate was roughly aligned with the outer edges of the reservoir electrodes on the bottom plate. The two plates were separated by a spacer formed from five pieces of double-sided tape (total spacer thickness ~450 μm) to ensure a larger gap than the thickness of the DBS punches (see above). With these dimensions, 15 μL droplets were used for most experiments and covered approximately two 2.2 × 2.2 mm electrodes.

DMF Device Operation. Droplet driving potentials (150−350 V_{rms}) were generated by amplifying the sine wave output of a function generator (Agilent Technologies, Santa Clara, CA) operating at 15 kHz. Aliquots (~15 μL) of reagents were loaded onto a DMF device by pipetting a droplet onto the bottom plate at the edge of the top plate, and simultaneously applying driving potential to the appropriate reservoir electrode (relative to the top-plate electrode) to draw the fluid into the reservoir. Thereafter, droplets were manipulated by applying driving potential to sequential actuation electrodes on the bottom plate relative to the top-plate electrode.

Droplet driving potentials were managed using an automated feedback control system, in which droplet movement is monitored by impedance sensing such that if a movement failure is observed, additional voltage pulses are applied until the droplet completes the desired operation. The feedback control system is built around an RBBB Arduino microcontroller (Modern Device, Providence, RI) and is depicted in Figure 2. The system used here is a new generation of an older system that was described previously.[19] The differences between the two systems are listed in Table 1.

DMF−nanoESI-MS Interface. To form the DMF−MS interface, one or more ~5 cm long, 360 μm o.d., 50 μm i.d., 30 μm tip i.d. pulled glass capillary nanoelectrospray ionization (nanoESI) emitters (New Objective Inc., Woburn, MA) were inserted between the two plates of the DMF device. The capillaries were positioned such that they penetrated ~2.7 cm into the device, an arrangement that was found to secure the tip against unwanted movements. The device was then positioned such that the tapered tip of one of the capillaries was ~3 mm away from the orifice of the mass spectrometer. To initiate analysis by mass spectrometry, a droplet was driven to the edge of the entrance orifice of a pulled-glass emitter, and after filling by capillary action, 1.7−2.2 kV (DC) was applied to the ITO-coated top plate of the DMF device to generate a nanoelectrospray into an LTQ Mass Spectrometer (Thermo Scientific). The fluid consumption was observed to be ~800 nL/min, such that a single droplet was sufficient for several minutes of data collection. Fifteen microliter droplets of tyrosine (5 μM in 50:50 methanol:DI H_2O) were actuated to the capillary to evaluate the flow rate (by determining the average duration of measurable signal that could be generated from individual droplets) and the stability of the spray as a function of total ion count (TIC).

DMF-Driven DBS Processing and Analysis. In typical experiments, a 3.2 mm diameter DBS punch was positioned on the bottom plate of a device, the top plate was positioned, and a nine-step procedure was implemented. In step 1, a 15 μL droplet of pre-extraction solvent (neat methanol, MeOH) was loaded and actuated to the DBS punch and incubated for 15 min at room temperature. In step 2, the MeOH was actuated away from the DBS and collected in a waste reservoir. Steps 3−4 and 5−6 were repeats of steps 1−2. In step 7, a 15 μL droplet of SA extraction/derivatization/MS solvent (6.5 mM hydrazine and 15 μM $^{13}C_5$-SA in 80:20% acetonitrile:DI water with 0.1% v/v formic acid) was loaded and actuated to the residual blood spot and incubated for 15 min at 37 °C on a hot-plate until evaporation. In step 8, a fresh 15 μL droplet of SA extraction/derivatization/MS solvent was loaded and delivered to the residual blood spot and was incubated at room temperature for 1 min. During that minute, the droplet was actuated around the five electrodes adjacent to the DBS in a circular fashion. In step 9, the droplet was actuated to the inlet of the capillary emitter for analysis.

After the nine-step sample processing procedure was complete, a nanoelectrospray was generated from the emitter. MS/MS analysis of derivatized SA extracted from blood spots was carried out by introducing 25% collision energy to the parent ions, and fragments over the m/z range of 100−300 were scanned. Derivatized SA product ions, which exhibit a loss of H_2O (18 m/z), were observed in the second mass selection, and the peak intensities were used for quantification. Spectra were collected as an average of 50 acquisitions, and at least three samples were evaluated for every condition recorded. Calibration curves were generated by plotting the intensity ratios of the derivatized SA product ions relative to those of the derivatized internal standard ($^{13}C_5$-SA) as a function of SA concentration. These data were fit with a linear regression to form the DBS-calibration curve, and the detection limit was calculated as the amount of SA corresponding to the average signal measured on blank samples (DBS punches with no SA) plus three times the standard deviation (SD). Total recovery (including matrix suppression effects) was determined by comparing the amount of SA from a DBS punch from the DBS-calibration curve with a calibration curve generated from SA solution standards. All data in the calibration curves were generated from at least four replicate DBS punch samples formed in Toronto, and the calibration curves were used to evaluate DBS punches formed in Ottawa.

In some experiments, a shortened protocol was used (not involving a DBS punch) to evaluate tip washing efficiency. A 15 μL droplet of 80 μM SA standard in SA extraction/derivatization/MS solvent (see above) was dispensed from a reservoir and actuated to the capillary emitter and sprayed as above. After 5 min of collecting MS/MS data, a 15 μL droplet of SA extraction/derivatization/MS solvent (not containing SA standard) was dispensed and actuated to the tip and sprayed (with MS/MS data collection) to rinse the tip for 5 min. This rinse process was repeated five times, and the SA concentration during each rinse was calculated by evaluating the ratio of intensities of product ions (derivatized SA/derivatized $^{13}C_5$-SA) with respect to the calibration curves described above.

In some experiments, an extended protocol was used to extract and analyze amino acids in a first procedure, followed by SA in a second. In these experiments, devices with two capillary emitters (one at each end of the device) were used. In these experiments, the initial methanol extraction step (step 1) was similar to what is described above but with a 5 min (instead of 15 min) incubation. In step 2, the MeOH droplet was actuated away from the DBS punch to a nonreservoir electrode and evaporated to dryness (~15 min, room temperature). Two additional steps were then implemented (steps 2a and 2b). In step 2a, a 15 μL droplet of AA derivatization reagent (3 N HCl in n-butanol) was loaded into the reservoir, actuated to the dried extract, and incubated at 75 °C on a hot-plate until dryness. In step 2b, a 15 μL droplet of AA MS solvent (acetonitrile/water 4:1 v/v) was loaded into the reservoir, driven to the dried extract to collect the derivatized amino

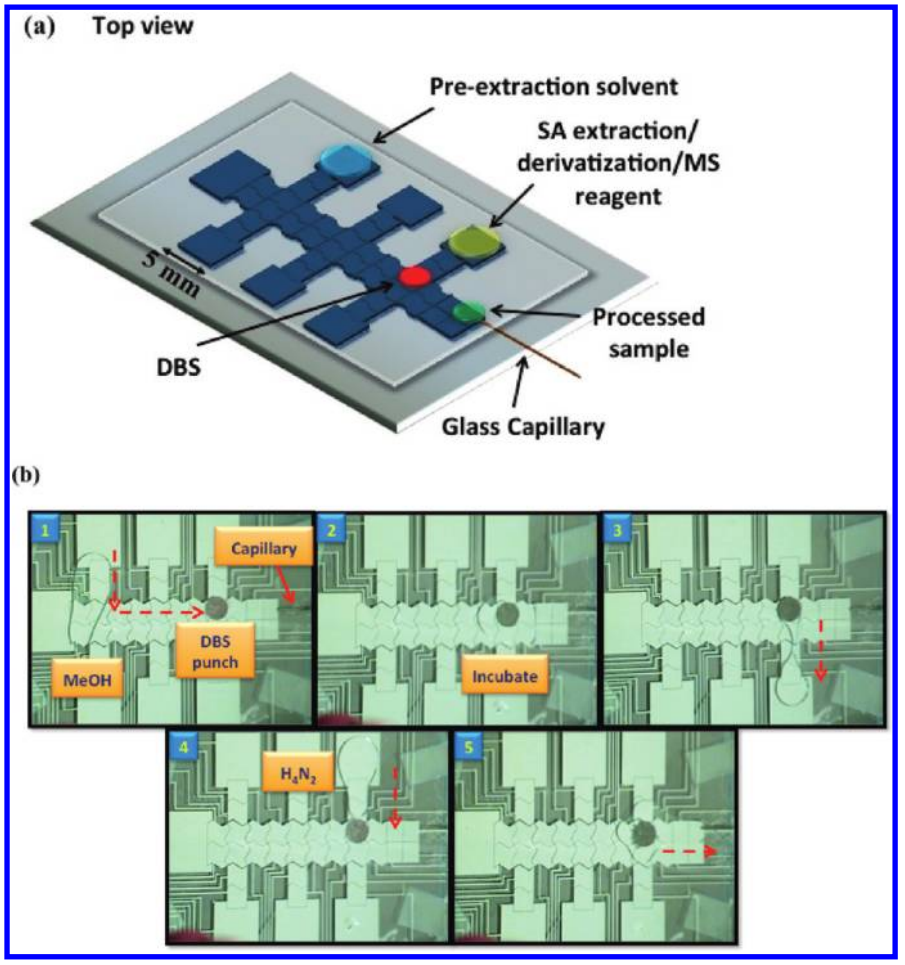

Figure 3. SA analysis method. (a) Schematic of the DMF device with capillary emitter. (b) Sequence of frames from a movie depicting SA extraction from a DBS punch, including pre-extraction in methanol (frames 1–3), and extraction and derivatization of SA with hydrazine (frames 4 and 5).

acids, and delivered to one of the capillary emitters. A nanoelectrospray was generated, and MS data were collected using the same parameters as SA analysis. Steps 3–6 (with 15 min incubations and moving MeOH droplets to waste) and 7–9 (described above) were then implemented as above.

RESULTS AND DISCUSSION

DMF–nESI-MS Interface. Jebrail and Yang et al.[16] recently reported the first microfluidic method for direct analysis of DBS samples, in which amino acids were extracted, derivatized, and analyzed by tandem mass spectrometry. The authors of that work described a method relying on multilayer "hybrid microfluidics"[20] for in-line analysis, in which samples were transferred from a digital microfluidic module (on the top of the device) to a microchannel (on the bottom of the device) with an integrated nanoelectrospray ionization (nESI) emitter for mass spectrometry. This method is an important step forward for the field, as the enthusiasm for automated processing techniques for analyzing DBS samples is well documented.[9−13] But the Jebrail and Yang et al.[16] technique suffers from a key limitation: devices formed in this manner require a complex series of fabrication and alignment steps prior to thermal bonding of the two substrates; this complexity may limit widespread application of the technique.

Here, we report a new DMF–nESI interface that is much more straightforward to fabricate. As shown in Figure 1, the interface is constructed by inserting a conventional pulled-glass capillary nESI emitter between the two substrates of an assembled DMF device. In developing this method, we were inspired by recent studies in which capillaries are interfaced with digital microfluidic devices for separations,[21,22] bead-based DNA extractions,[23] and mass spectrometry.[22,24] We propose that this configuration is more flexible than the "hybrid microfluidics" interface, as it allows for the droplet manipulation to be decoupled from the nESI interface.

As shown in Figure 1, the new method was realized by using a spacer between the DMF top- and bottom-plates that was slightly larger than the outer diameter of the emitter-capillary. With this configuration, emitters were easily introduced and removed in seconds, without requiring that the device be disassembled. In practice, droplets are manipulated on the DMF device by applying AC fields between electrodes on the top and bottom plates as illustrated in Figure 1b (top). When a droplet is moved such that it touches the inlet of the capillary, it spontaneously fills by capillary action in seconds (the emitters used here hold ~100 nL). To generate a spray, a DC potential is applied between the top-plate DMF electrode and the mass spectrometer, as illustrated in Figure 1b (bottom), leading to a stable spray (Figure 1c) for analysis (Figure 1d). The droplet consumption rate (presumed to originate from a combination of flow through the capillary and evaporation) was estimated to be ~800 nL/min, and the spray was stable for ~200 s with a relative standard deviation of 7.3% in total ion count (Figure 1d). This method is similar to a DMF–ESI interface recently

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reported by Baker and Roper,[24] with a key difference being that the nESI interface reported here does not require an external pressure source for sampling into the MS.

Automated Droplet Control. A second drawback of the proof-of-concept system described in Jebrail and Yang et al.[16] is lack of full automation (i.e., actuation potentials in the previous study[16] were applied to contact pads manually). Automating droplet movement on digital microfluidic devices is challenging, as droplets are sometimes observed to have imperfect actuation fidelity (i.e., a given applied voltage pulse may not move a droplet onto the energized electrode).[19] This phenomenon is caused by droplet sticking to device heterogeneities such as scratches or dust. This is exacerbated for the application reported here, as DBS punches serve as three-dimensional, high surface-area obstacles that impede droplet movement. Thus, a primary goal for the work reported here was the development of a robust automation system that is capable of high-fidelity actuation of various liquids across DBS punches.

Shih et al.[19] recently reported an impedance sensing and feedback control system for high-fidelity droplet actuation in digital microfluidics. The system was demonstrated to be particularly useful for manipulating sluggish fluids that are susceptible to sticking to device surfaces.[19] Here, we report an improved feedback control circuit suitable for DBS sample analysis by digital microfluidics, depicted in Figure 2. The many improvements relative to the system reported previously[19] are listed in Table 1; the most important improvement is an active control system that facilitates automated application of driving potentials with increasing magnitude to droplets that resist movement. This is a useful advance, as in practice, it is preferable to actuate droplets at a low driving voltage, because the lower electrical fields are less likely to cause dielectric breakdown of the device insulating layer. But for a droplet that resists movement at low voltage, it is useful to increase the voltage temporarily to generate higher forces; after the droplet has moved, the voltage can be reduced again for subsequent movements. As far as we are aware, the system reported here is the first to facilitate automated, feedback-controlled variation of actuation potentials.

In initial studies, we observed that the most common failure point in SA analysis in DBS samples was the movement of a droplet of hydrazine-containing solvent after it contacted a DBS punch. For example, in a series of experiments using a constant driving voltage of 150 V_{rms} (as in the system reported previously[19]), successful movement of 15 μL hydrazine-containing droplets away from DBS punches required an average of 25.6 ± 4.8 voltage pulses (N = 5). Moreover, in 40% of cases, the droplet failed to move even after application of this large number of pulses. The new system shown in Figure 2 solves this problem. In a series of experiments using the new system, a 100% droplet movement success rate was achieved for the same operation with an average of 4.8 ± 1.3 voltage pulses and an average increase in voltage (relative to the starting potential of 150 V_{rms}) of 80 ± 27 V_{rms} (N = 13). The new system was capable of implementing complex droplet manipulation protocols (as described below) with perfect droplet movement fidelity. We speculate that this approach will be useful for a wide range of automated applications, especially those involving the manipulation of aqueous droplets onto and off of three-dimensional solids such as filter paper punches (as reported here), porous polymer monoliths[25] for solid-phase extraction, and hydrogel discs[26,27] for forming enzyme microreactors or for three-dimensional cell culture.

SA Analysis. An automated nine-step digital microfluidic method was developed to analyze SA in DBS samples. A portion of an experiment is depicted in Figure 3. Briefly, the DBS punch is inserted into the device and then is pre-extracted in droplets of methanol. The residual DBS punch is then extracted and derivatized in droplets containing hydrazine (for reaction mechanism see Sander et al.[6]), after which the derivatized SA is delivered to the capillary for analysis by nESI-MS/MS. The extraction steps in this method require 60 min to complete (similar to 65 min required using conventional techniques[5]). A representative secondary mass spectrum generated using the DMF method is shown in Figure 4a.

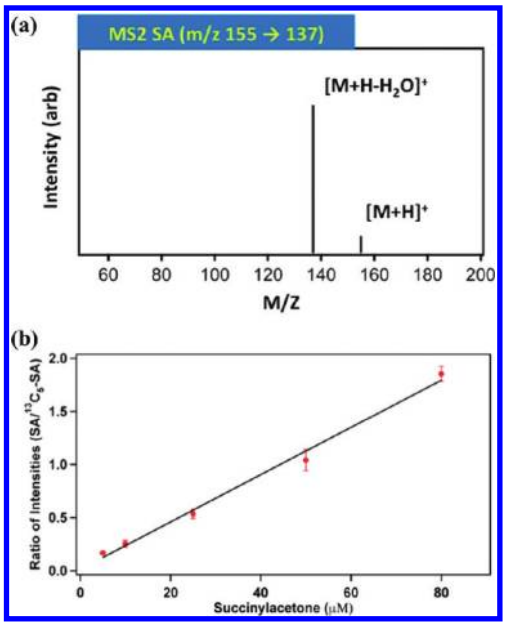

Figure 4. SA analysis data. (a) Representative secondary MS (MS2) spectrum of derivatized SA after collision induced dissociation (CID). (b) Calibration curve of spiked SA in dried blood spot punches. Each data point represents at least four replicate measurements, and error bars represent ±1 SD.

When the product ions were subjected to collision-induced dissociation (CID), a characteristic fragmentation (neutral loss of 18) was observed (i.e., m/z 155→137 for SA and 160 → 142 for $^{13}C_5$-SA), which was used to quantify SA. Figure 4b is a calibration curve with R^2 = 0.9923, generated by analyzing DBS punch standards from the abundance ratio of the SA peak intensity relative to the internal standard ($^{13}C_5$-SA) peak intensity in the secondary (MS2) spectra. The extraction efficiency was found to be 85.2% with a CV of 6.85%, and a limit of detection of 4.95 μM (2.43 ng). Patients suffering from HT typically have SA blood concentrations in the range of 16–150 μM,[28] making the microfluidic method a good fit for screening for this condition. Future improvements in sensitivity (for example, using a triple quadrupole mass spectrometer in place of the linear ion trap used here) will likely make the method useful for monitoring and follow-up of patients as they recover (and SA concentrations decrease to low levels).

We evaluated the new digital microfluidic method relative to gold standard techniques, which comprise a series of manual or robotic steps including serial extractions in multiple solvents, mixing extractates with derivatization reagents and internal standards, pooling extractates for analysis, solvent exchange for mass spectrometry, and off-line MS/MS.[5,6,28−32] A series of

punches from blood samples containing various concentrations of SA were processed by the digital microfluidic method, and punches from the same samples were evaluated using the conventional technique at the NSO facility in Ottawa, ON. The measured values originating from the tests are listed in Table 2.

Table 2. Measured Succinylacetone (SA) Concentration in 3.2 mm Diameter Punches from Filter Paper Bearing Dried Blood Spots Using Digital Microfluidics (left) and Standard Techniques at Newborn Screening Ontario (right)[a]

sample	measured SA concentration (μM) using DMF method	measured SA concentration (μM) using standard NSO technique
1	<4.95	6.4
2	<4.95	1.5
3	57.2	60
4	58.3	60
5	<4.95	<1.5
6	<4.95	6.4
7	40	35.2
8	37.6	40

[a]A paired *t*-test revealed no statistical difference between the two data sets at a 95% confidence interval.

A paired t test revealed no significant statistical difference between the two data sets at a 95% confidence level ($P = 0.05$ and $t = 0.8177$). This result is notable, as the two data sets were generated using different extraction volumes and techniques as well as different mass spectrometers (from different manufacturers) in different locations run by different operators. Furthermore, the digital microfluidic method facilitates significant reduction in reagent use (75 μL vs 200−310 μL[5,32]). Finally, we propose that the in-line analysis afforded by this technique would be useful for automated SA quantification in laboratories that lack robotic instrumentation.

As described above, a major advantage of the new method reported here relative to that described by Jebrail and Yang et al.[16] is the decoupling of the nESI-MS interface to the sample preparation system. Thus, when needed (e.g., when there is a clog in the tip), a new tip can be exchanged for an old one for continued operation. But there may be cases in which it is useful to reuse the same tip for multiple analyses. To investigate the compatibility of the new technique with tip reuse, we performed an experiment to determine the number of droplets required to effectively rinse the SA from a tip after an analysis. Figure 5 summarizes the data; as shown, four to five rinse droplets is sufficient to reduce the signal originating from SA to being below the detection limit of the technique. For the data shown in Figure 5, we used the extraction/derivatization/MS solvent (including internal standard) used for the DBS analysis procedure as a rinse solution; this was required to quantify the SA in each step. But in future work, other rinse solutions (e.g., aqueous solutions of NaOH or acetic acid[33]) may prove to be useful for tip washing in fewer steps. Regardless, the data in Figure 5 demonstrate that DMF can be used for automated tip washing, which may be useful for a variety of applications.

A final goal for this work was to demonstrate that the new method is compatible with complex, multistep protocols enabling quantification of both SA (as in Figure 4 and Table 2), and amino acids that are routinely evaluated in newborn screening. Figure 6a depicts the setup used for these experiments. As described in the Experimental Section, in these experiments, the methanolic pre-extractate was isolated

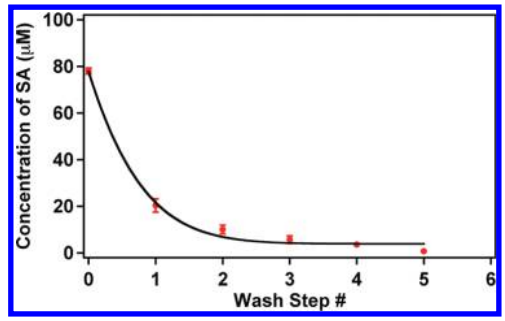

Figure 5. Graph of SA concentration (red dots) as a function of wash step (the black line is included to guide the eye). SA was measured first in a droplet containing a standard solution of 80 μM SA and 15 μM SA internal standard (wash step 0) and then in a series of droplets containing only the internal standard (wash steps 1−5). Each data point represents three replicate measurements, and error bars represent ±1 SD.

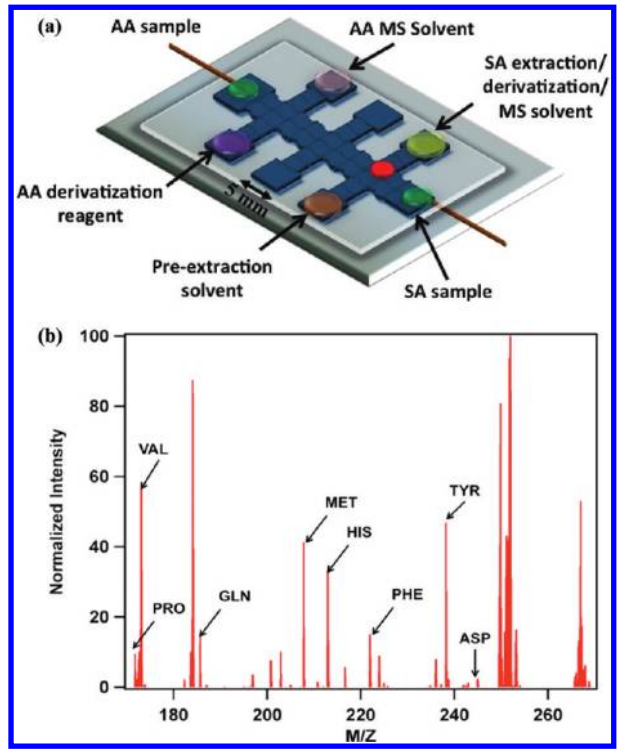

Figure 6. (a) Schematic of device setup used to analyze amino acids (AAs) and then succinylacetone. (b) Representative primary MS spectrum showing several amino acids identified in a DBS using DMF. The data in part b were generated using the nESI tip at the top left of (a), and data used to quantify SA (not shown, similar to Figure 4) were generated using the nESI tip at the bottom right of part a.

and derivatized with n-butanol for analysis in one nESI tip and the hydrazine extractate was directed to a second nESI tip. A representative primary mass spectrum of derivatized amino acids is shown in Figure 6b. These proof-of-concept (and nonquantitative) results suggest the possibility of future techniques in which a group of markers that are typically evaluated in newborn screening (including amino acids, acylcarnitines, and others) are simultaneously analyzed on an integrated microfluidic platform.

The use of two nESI tips in these experiments is interesting; as far as we are aware, this is the first example of a digital microfluidic device with multiple interfaces to a mass

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spectrometer. In this proof-of-concept work, the device was repositioned manually when switching between tips, but we can envision future devices with multiple tips positioned on an automated translation stage. An alternative would be to use a single tip with programmed rinse steps (as in Figure 5) between the analyses.

CONCLUSION

We have developed an automated microfluidic device with on-chip coupling with nanoelectrospray ionization for in-line analysis of dried blood spot samples by mass spectrometry. The new method incorporates a feedback control system enabling facile, high-fidelity droplet movement without manual intervention. As proof of principle, the method was used to perform on-chip extraction and quantification of succinylacetone, a specific marker of tyrosinemia type 1, in dried blood spot samples. We propose that the new system represents a prototype for fast and inexpensive screening of dried blood spots and other complex samples for a wide range of applications.

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Notes

The authors declare no competing financial interest.

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