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A digital microfluidic interface between solid-phase microextraction and liquid chromatography–mass spectrometry☆

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ABSTRACT

We introduce a method to couple solid-phase microextraction (SPME) with HPLC-MS using digital microfluidics (DMF). In the new system, SPME fibers are used to extract analytes from complex sample solutions, after which the analytes are desorbed into solvent droplets in a DMF device. The open geometry of DMF allows straightforward insertion of SPME fibers without requiring a complicated interface, and automated droplet manipulation enables multiplexed processing of the fibers. In contrast to other multiplexed SPME elution interfaces, the low volumes inherent to DMF allow for pre-concentration of analytes prior to analysis. The new SPME-DMF-HPLC-MS method was applied to the quantification of pg/mL-level free steroid hormones in urine. We propose that this new method will be useful for a wide range of applications requiring cleanup and pre-concentration with convenient coupling to high-performance analytical techniques.

1. Introduction

Solid-phase microextraction (SPME) is an analytical technique in which a fiber coated with a thin layer of extraction sorbent is exposed to a sample to collect measurable amounts of analyte for subsequent analysis [1,2]. SPME is widely used in biochemical [3–6] and environmental [7,8] analysis because it (a) allows for clean-up of complex sample matrices, and (b) is an effective solution to the "sample-to-analysis" problem—that is, in many real-world samples, analytes are present in dilute concentration in large volumes, while analysis techniques are often limited to small volumes. SPME solves the sample-to-analysis problem by enabling rapid, quantitative analyte extraction from large volumes into small volumes that are compatible with a wide range of analytical methods.

SPME is often coupled with gas chromatography (GC) via a simple, robust thermal desorption interface [9]. But there are many analytes with limited volatility that are better suited for high performance liquid chromatography (HPLC) than GC. Coupling SPME to HPLC is complicated, as analytes in SPME fibers must be desorbed in an appropriate solvent prior to analysis. For small numbers of experiments, it is straightforward to desorb analytes manually (e.g., in microcentrifuge tubes) for off-line analysis. But for larger numbers of experiments, it is useful to employ specialized interfaces that allow for hands-free integration of SPME with HPLC. These kinds of interfaces have been reported previously relying on (1) in-line injection into modified HPLC injection valves [10–15] or (2) off-line desorption into multiwell plates for subsequent analysis [16,17]. These are useful advances, but they suffer from a number of challenges including (1) sample carryover between analyses, fiber damage, and leaky seals, or (2) dilution into large elution volumes (limiting the capacity to pre-concentrate, which is often necessary detect analytes present in trace concentrations).

Here, we introduce a new solution to the SPME-HPLC interface problem, relying on digital microfluidics (DMF). DMF is a

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fluid-handing technique in which discrete droplets of sample and reagents are manipulated electrostatically on an array of electrodes [18–20]. In contrast to related techniques relying on enclosed microchannels, DMF is often operated open-to-atmosphere, which allows for straightforward interfacing with flexible probes from the outside world, including nanoelectrospray emitters [21–23], optical fibers [24], and capillary-based analysis systems [25–28]. Here we describe a new technique in which SPME fibers are interfaced directly with DMF. As far as we are aware, this represents the first integration of SPME sampling with any form of microfluidics.

The new technique is enabled by a simple interface in which analytes are first loaded onto SPME fibers and then are eluted in droplets actuated by DMF. The technique was used to quantify steroid hormones in urine, an important problem in tests for athletic doping. We propose that this method represents an attractive new tool in the analyst's toolbox, particularly for analytes that are present at dilute concentrations in complex sample matrices.

2. Experimental

2.1. Reagents and materials

4-Androsten-3-17β-ol-3-one (testosterone, TS), 4-androsten-3-17 β-ol-3-one-16,16,17-d3 (deuterotestosterone-d3, TSd3), 4-androsten-3,17-dione (androstenedione, AD), 4-androsten-3,17-dione-2,2,4,6,6,16,16-d7 (deuteroandrostenedione-d7, ADd7), 4-pregnen-3,20-dione, (progesterone, PG) and 4-pregnen-3,20-dione-2,2,4,6,6,17α,21,21,21-d9 (deuteroprogesterone-d9, PGd9) were obtained from Steraloids (Newport, RI). Human urine (pooled from male, non-pregnant female, and pregnant female subjects) was purchased from BioChemed Services (Winchester, VA). HPLC-grade methanol, acetonitrile, and all other reagents with no designated vendor were from Fisher Scientific (Ottawa, ON). Dulbecco's Phosphate-Buffered Saline (PBS) and 50% formic acid were from Sigma (Oakville, ON). Deionized (DI) water had a resistivity of 18 MΩ cm at 25 °C.

Stock solutions (10 μg/mL) of each hormone standard (TS, AD, and PG) were prepared in methanol. Two stock mixtures in methanol were also prepared: mixture A contained the three standard hormones (100 ng/mL ea.), and mixture B (internal standard, I.S.) contained the three deuterated hormones (TSd3, ADd7, and PGd9, 100 ng/mL 100 ea.) in methanol. For SPME experiments, hormone stock solutions were diluted (individually) into working solutions in (a) PBS to final concentrations of 0, 20, 100, or 500 pg/mL, and (b) in human urine diluate (supernatant collected from 36 mL of human urine mixed with 4 mL 2 M phosphate buffer, pH 6.8, centrifuged at 300*g*, 5 min) to final concentrations of 0, 100, 500, or 1000 pg/mL. For SPME experiments, mixture B was diluted into desorption solvent (acetonitrile:isopropyl alcohol:50 mM ammonium acetate, 40:40:20, v/v, with 0.1% formic acid) to a final concentration (of each I.S. in the mixture) of 1000 pg/mL. For the generation of calibration curves without SPME, working solutions were formed by diluting mixtures A and B into methanol:water 1:1 v/v, to a final concentration of 20, 50, 100, 500, 1000, 2000 and 5000 pg/mL of each standard in mixture A, as well as1000 pg/mL of each I.S. in mixture B.

2.2. Digital microfluidic device fabrication and operation

Digital microfluidic devices were fabricated at the University of Toronto Nanofabrication Center (TNFC) as described elsewhere [29]. Briefly, device bottom plates bearing patterned electrodes and contact pads were prepared by photolithography and wet etching of chromium and photoresist (AZ1500) coated glass slides obtained from Telic Co (Santa Clarita, CA). These substrates were then coated with 7 μm Parylene-C and 50 nm Teflon-AF. The bottom-plate design features an array of 18 actuation electrodes (6 × 6 mm ea.) with inter-electrode gaps of 30 μm as shown in Fig. 1. Unpatterned DMF device top plates were formed from ITO-coated glass substrates (Delta Technologies Ltd., Loveland, CO) coated with 50 nm Teflon-AF (as above). Devices were assembled with an ITO–glass top-plate and a patterned bottom-plate separated by a spacer formed from five pieces of double-sided tape (total spacer thickness 450 μm). Droplet movement (driven by applying an 80 V_{RMS}, 10 kHz sine wave between the top-plate electrode and sequential electrodes on the bottom plate) was programmed and managed via the open-source DropBot droplet manipulation system [30].

2.3. SPME-DMF

All experiments were performed at room temperature (25 °C). C18 coated SPME fibers were from Supelco (Bellfonte, PA). In each experiment, a SPME fiber was conditioned by immersing in 4 mL of 50:50 methanol:DI water for at least 30 min. The fiber was then inserted into 4 mL of sample solution and incubated for 60 min while stirring with a stir bar at 500 rpm. This process was determined to be non-equilibrium for the analytes evaluated here using methods reported elsewhere [5] (Supplementary Fig. S1). After extraction, the fiber was rinsed with DI water and inserted into a DMF device. Analytes were then desorbed into a 35 μL droplet of desorption solvent. In some experiments, the desorption droplet was driven onto the fiber and incubated without moving for one hour (in these experiments, an extra 15 μL droplet of desorption solvent without I.S. was added after 30 min to compensate for evaporation). In most experiments, the desorption droplet was agitated during incubation (for 5–20 min) by cycling between electrodes while maintaining contact with the fiber. In all cases, after incubation, the droplet was driven away from the SPME fiber and the fiber was retracted into the needle. Then the droplet was actuated onto the collection zone, where it was stored at room temperature until the top plate was removed to allow the droplet to evaporate to dryness (~30 min). Then, the analytes were reconstituted in 35 μL of 50:50 methanol:DI water prior to injecting into an LC–MS system. In some experiments samples were injected onto the HPLC manually; in others, analytes were directly loaded from the DMF device onto the HPLC using a custom DMF-HPLC-MS interface [31].

2.4. HPLC-MS/MS

Chromatographic separations were performed using an Agilent Technologies 1200 series HPLC system (Santa Clara, California) with an injection volume of 20 μL. An Agilent Zorbax Eclipse Plus C18 column (2.1 mm i.d. × 100 mm long, 1.8 μm particle dia.) was used for separation. A 15.5-min elution was programmed featuring gradients of (A) DI water and (B) methanol: from 0 to 1 min (isocratic step at 50% B), 1–1.5 min (linear increase from 50 to 80% B), 1.5–2.5 min (linear increase from 80 to 100% B), 2.5–8 min (isocratic step at 100% B), 8–8.1 min (linear decrease from 100 to 50% B), 8.1–15.5 min (isocratic step at 50% B). The HPLC was operated at ambient temperature in gradient elution mode at a flow rate of 0.1 mL/min with post-column addition of 2% ammonia in methanol at 3 μL/min by a syringe pump (Harvard Apparatus, Holliston, MA). HPLC eluent was interfaced to a QuattroMicro triple quadrupole mass spectrometer (Waters, Milford, MA) via positive-mode electrospray ionization (ESI). The settings included capillary voltage 3.0 kV, extractor cone voltage 3 V, detector voltage 600 V, cone gas flow 50 L/h, desolvation gas flow 600 L/h, source temperature 100 °C, and desolvation temperature 400 °C. The system was operated in multiple reaction monitoring (MRM) mode, and the MRM transitions, cone voltages, and collision energies were varied for each analyte as a function of retention time (Table S1 in

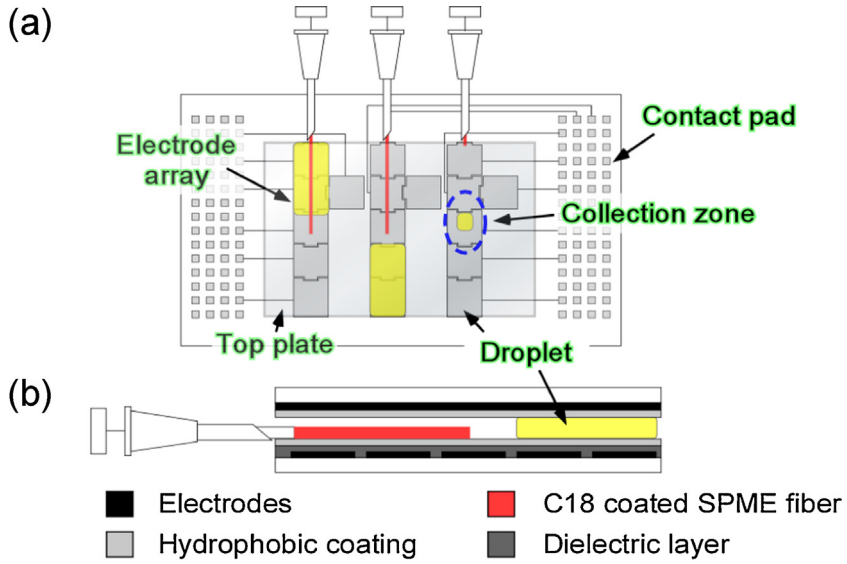

Fig. 1. SPME-DMF. Schematics of (a) top-view and (b) side-view of the new SPME-DMF interface.

the supplementary information). For quantitative analysis, calibration curves were constructed by plotting the peak area ratio of the analytes to I.S. as a function of analyte concentration. Regression lines were fit to the raw data (i.e., not the averages of replicates), and limits of detection (LODs) and limits of quantitation (LOQs) were determined as the analyte concentrations corresponding to the signal of the sample blank plus three or ten times the standard deviation of the average blank signal.

3. Results and discussion

3.1. SPME-DMF-HPLC-MS

The overall goal of this work was to develop a robust, automated interface between SPME and HPLC-MS. Digital microfluidics (DMF) proved useful for this purpose, given that its open geometry facilitates direct interaction between droplets manipulated in the devices and capillaries/fibers inserted at the open edges [21–28]. As shown in Fig. 1, in the new system reported here, SPME fibers bearing analytes can be inserted into the open space between top- and bottom-plates on the DMF device. One or more solvent droplets can then be actuated to the fiber to remove analytes from the coating, after which they can be analyzed by HPLC-MS. Use of a recently developed manifold integrating a DMF device with an autosampler [31] makes the entire process automated.

In initial tests, different desorption solvents, volumes, times, and schemes were evaluated. It was determined that a 35 μL droplet of acetonitrile:isopropyl alcohol:50 mM ammonium acetate (40:40:20, v/v with 0.1% formic acid) was appropriate to elute steroid hormone analytes from the SPME sorbent phase (C18) used here. Further, it was determined that an "active" desorption scheme, in which the desorption droplet is continually agitated around the fiber (depicted in Fig. 2a), is more efficient than a "static" desorption scheme in which the desorption droplet is simply moved onto the fiber and then incubated without moving until collection. As shown in Fig. 2b, testosterone collected onto a fiber from a 100 pg/mL solution in PBS is completely eluted in a single 20 min active desorption, while appreciable testosterone was still detected in the third consecutive static desorption. Further, the same performance was observed for agitation in elution solvent for only 5 min; thus, the active desorption scheme with 5 min incubation was used for all experiments described below.

The new method introduced here joins techniques described previously for interfacing SPME with HPLC: in-line methods relying on modified injection valves [10–15], and off-line methods relying on multiwell plates and robotics [16,17]. The new SPME-DMF-HPLC method has a number of potential advantages, as summarized in Table 1. First, the previously reported in-line (serial) techniques often allow for mechanical agitation of the fiber during desorption to improve the desorption rate. The new system reported here has a similar advantage of "active" elution (as illustrated in Fig. 2), but without the propensity for fiber damage (which has been reported previously [14]). Second, the previously reported in-line techniques are susceptible to sample carryover between injections, while DMF devices can be rinsed and reused many times with no cross-contamination [32] or can formed (by ink-jet printing on paper) at extremely low cost [33,34] for single use. Third, the previously reported in-line techniques are inherently serial, making multiplexing impossible. In contrast, the DMF system can be multiplexed (3-plex system described here, but higher levels of multiplexing would be straightforward), analogous to the off-line methods described for multi-well plates.

A fourth advantage of the new system is the capacity to pre-concentrate analytes prior to analysis using small elution volumes. In a typical SPME process, n moles of analyte are loaded onto the fiber, and then assuming complete elution (as in the active case in Fig. 2b), the measured concentration c_m in the eluate is simply

$$c_m = \frac{n}{V_e}$$

where V_e is the elution volume. The low elution volume for the system reported here (V_e = 35 μL) results in significant advantages for pre-concentration relative to the multiplexed/off-line SPME techniques (for example, Mirnaghi et al. [17] reports V_e = 1 mL).

Finally, we note that as a fifth advantage, DMF is capable of automating complex, multi-step, heterogeneous sample processing regimens, such as in-line reduction, alkylation, and digestion in hydrogel microreactors [35], or magnetic bead-based immunodepletion [36]. In contrast, the in-line/injection valve [10–15] technique is unifunctional (i.e., desorption from SPME fibers), and the off-line/multiplexed [16,17] techniques have limited processing functionality (e.g., sample extraction and elution). Although multi-step processing was not featured in the work described here, we propose that they make the new technique a particularly

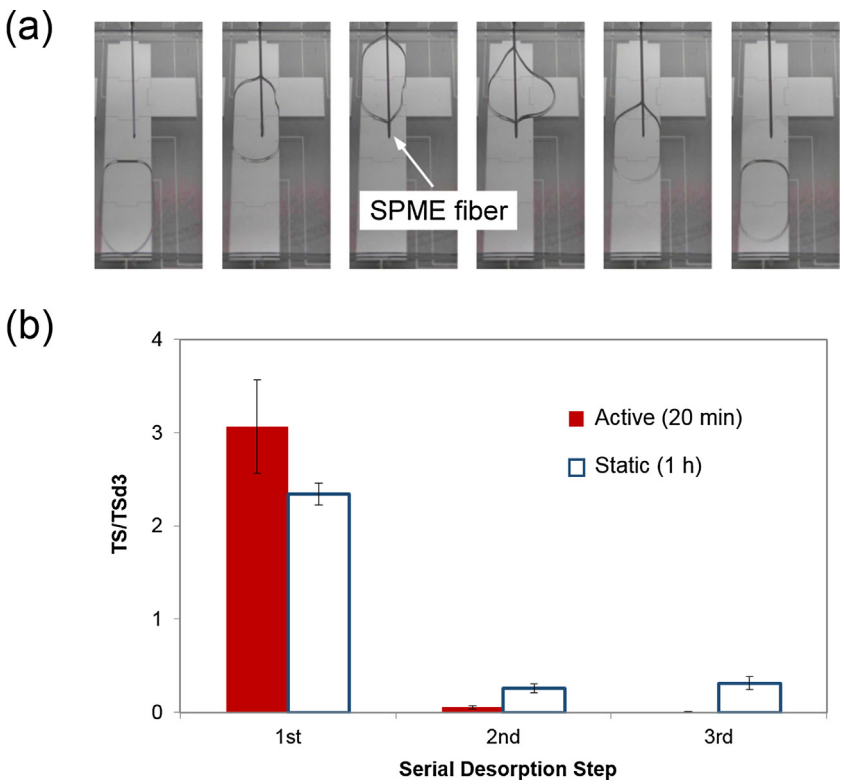

Fig. 2. SPME-DMF elution. Frames from a movie (a) (left-to-right) illustrating the "active" mode of incubation with elution solvent droplet. (b) Comparison of average signals [reported as a ratio of HPLC-MS/MS MRM peak area for testosterone (TS) relative to that for deuterated testosterone (TSd3)] observed for desorption of samples in the active [red filled boxes, as in panel (a) for 20 min] or static (empty blue boxes, for droplets with no movement for 1 h) modes. The sample was 100 pg/mL testosterone in PBS, and each SPME fiber was eluted three times in series (represented as 1st, 2nd, 3rd) on separate DMF devices. Error bars represent ±1 standard deviation for 3 fibers evaluated for each condition. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Table 1
Comparison between in-line SPME-HPLC [10–15] (relying on modified injection valves) and off-line SPME-HPLC [16,17] (relying on multiwell plates and robotics), and the new SPME-DMF-HPLC interface reported here.

Method	Active desorption without fiber damage	Sample carryover	Multi-plexing	Pre-concentration	Multistep, heterogeneous processing
In-line SPME-HPLC [10–15]	✗	✗	✗	✓	✗
Off-line SPME-HPLC [16,17]	✓	✓	✓	✗[a]	✗
SPME-DMF-HPLC (reported here)	✓	✓	✓	✓	✓

[a] Pre-concentration is possible using multiwell plate methods by evaporating the elution solvent and reconstitution, but this feature is rarely used because of the extremely long times (~5–6 h) required for evaporation of the desorption solvent volumes used.

appealing system to integrate with samples that require complex workup prior to analysis.

3.2. Quantitative analysis of steroids

As an initial application, a SPME-DMF-HPLC-MS method was developed to quantify anabolic-androgenic steroids (AAS, i.e., testosterone and its derivatives) in urine. AAS abuse is a huge problem – worldwide, it is estimated [37] that up to 6.4% of all men have engaged in non-medical AAS consumption. This problem is particularly pervasive in athletics – according to the World Anti-Doping Agency (WADA), 63% of 5271 positive tests for doping reported in 2014 (across a wide range of Olympic and non-Olympic sports) were caused by AAS abuse [38]. While AAS testing has historically relied on measuring so-called "total" hormone analysis [39,40] (requiring hydrolysis to release steroids from the glucuronated and sulfated forms that are dominant in urine), there is a recent trend to measure only the "free" hormones, which is faster and also a more reliable indicator of androgenic status [41,42] than "total" hormone levels. The drawback for this technique is concentration – many "free" hormones are present at low levels in urine (~pg/mL), which makes detection a challenge. This problem is exacerbated by matrix effects – urine contains a high concentration of salts that can interfere with analysis by MS. We hypothesized that SPME-DMF-HPLC-MS might be able to address these challenges.

As an initial test for quantitation and precision of the new method, a dilution series of two model AAS analytes, testosterone (TS) and androstenedione (AD), as well as a third (related) steroid hormone, progesterone (PG), were prepared in phosphate buffered saline (PBS, a solution that, like urine contains a high concentration of salts). Samples were extracted, eluted, and analyzed using the new method to form calibration curves, plotting the peak area ratios of analytes relative to those of deuterated internal standards (included in the elution buffer) as a function of concentration. SPME-DMF-HPLC-MS data and calibration curves are shown in red filled circles in Fig. 3, and Table 2 summarizes the analytical performance of the method. To highlight the pre-concentration capacity of the new method, the same dilution series were evaluated with-

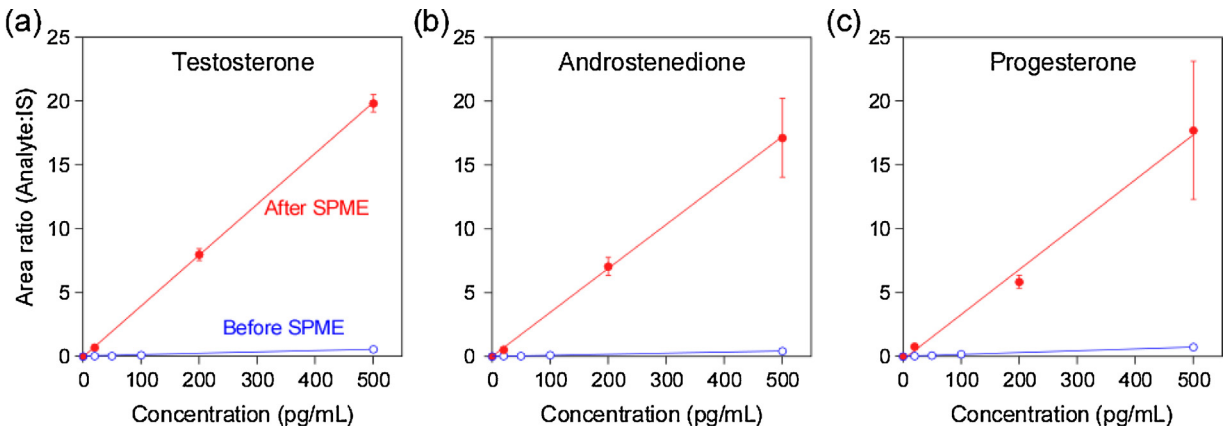

Fig. 3. Calibration data for model analytes extracted from PBS by SPME-DMF. HPLC-MS/MS MRM peak area ratios for (a) TS:TSd3 (b) AD:ADd7, and (c) PG:PGd9 as a function of initial concentration generated before (blue ○) or after (red ●) extraction. Error bars represent ±1 standard deviation for 3 replicate extractions of each analyte. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Table 2
Analytical performance of SPME-DMF-HPLC-MS.

Steroid hormone	Calibration curve (coefficients ± 1 S.D.)	R^2	LOD (pg/mL)	LOQ (pg/mL)	Linear range (pg/mL)
Testosterone	y = (0.0398 ± 0.0005) x − 0.0265 ± 0.1471	0.9981	1.3	2.1	2.1–500[a]
Androstendione	y = (0.0344 ± 0.0021) x − 0.0116 ± 0.5534	0.9655	1.5	3.0	3.0–500[a]
Progesterone	y = (0.0351 ± 0.0036) x − 0.2320 ± 0.9753	0.9041	9.9	13.7	13.7–500[a]

[a] 500 pg/mL is the highest concentration evaluated for these data, collected for analyte dissolved in PBS (reported here). For analysis of analytes dissolved in urine, calibration curves were generated from higher concentrations of spiked analyte (see Fig. S2 in the online supplementary information) for analysis by standard addition.

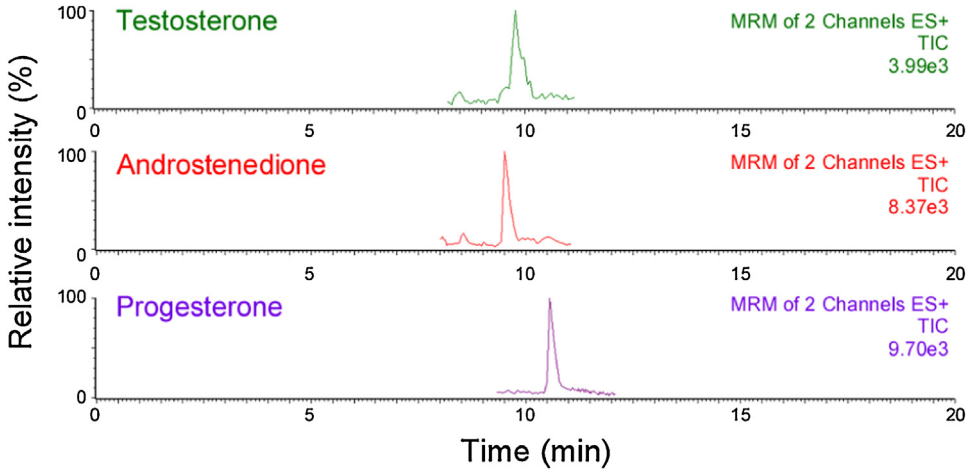

Fig. 4. Representative HPLC-MS/MS chromatograms (generated in MRM mode) for TS TS (green, top), AD (red, middle), and PG (purple, bottom) extracted from urine (spiked at 100 pg/mL for each analyte) with SPME-DMF. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

out SPME extraction and elution; these data are represented in blue open circles in Fig. 3. As shown, the SPME-extracted samples were concentrated ~25-fold relative to the original solutions, allowing for low detection limits (i.e., the SPME-DMF-HPLC-MS method has LOQ = 2.1, 3.0, and 13.7 pg/mL for TS, AD, and PG, respectively).

The SPME-DMF-LC/MS method was then applied to quantify hormones in human urine samples (building from related methods using SPME [5]). After minimal pretreatment, urine samples were extracted, eluted, and analyzed using the new technique. Representative chromatograms for each analyte are shown in Fig. 4. For quantitative analysis, the method of standard additions [43] was used (to correct for any matrix effects); calibration curves are shown in Fig. S2 in the online supplementary information. The calibration data was used to measure the "free" endogenous concentrations of each analyte in pooled male, non-pregnant female and pregnant female urine samples (Table 3). The measured levels in each sample agree with those reported previously [44,45] (noting that the PG measured in samples from pregnant females is higher than expected).

The results described here demonstrate that the new method is useful targeted analysis of free AAS in urine. In this work, basal levels were evaluated in pooled urine samples, but in the future, we propose that this technique will be useful for screening for AAS abuse in athletics and other settings. Given that SPME has also been shown to be useful for extracting many different conjugated steroids in urine [5], the technique described here is likely extensible to metabolomic profiling and discovery. More generally, given the rising popularity of SPME for *in-vivo* sampling, we propose that the new interface described here may find utility for non-destructive sampling in blood and interstitial fluids, position-sensitive analysis in precious tissue samples, longitudinal studies of drug fate, and beyond [6]. Finally, it may be possible to make such

Table 3
Concentrations of free steroid hormones measured in pooled human urine samples derived from males, non-pregnant females and pregnant females using SPME-DMF-HPLC–MS.

Steroid hormone	Concentrations ± 1 S.D. measured in urine (pg/mL)		
	Males	Non-Pregnant Females	Pregnant Females
Testosterone	780 ± 86	260 ± 7	200 ± 77
Androstendione	430 ± 130	150 ± 79	680 ± 50
Progesterone	200 ± 39	49 ± 61	1500 ± 550

systems portable for analysis in the field, using systems combining SPME-DMF and miniature mass spectrometry [23].

4. Conclusion

We have introduced a technique for coupling solid-phase microextraction (SPME) with HPLC-MS relying on digital microfluidics. In this technique, analytes in sample solution are extracted into a SPME fiber coating off-line, and then eluted into a droplet of desorption solvent on a digital microfluidic device, prior to analysis by HPLC-MS. We demonstrated that the SPME-DMF-HPLC-MS is useful for quantifying pg/mL concentrations of free steroid hormones in urine, and propose that the new technique will be useful for a wide range of applications which suffer from the sample-to-analysis problem of dilute analytes in large volumes.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.chroma.2016.03.029.

References

[1] C.L. Arthur, J. Pawliszyn, Solid-Phase microextraction with thermal-desorption using fused-silica optical fibers, Anal. Chem. 62 (1990) 2145–2148.
[2] A. Spietelun, A. Kloskowski, W. Chrzanowski, J. Namiesnik, Understanding solid-phase microextraction: key factors influencing the extraction process and trends in improving the technique, Chem. Rev. 113 (2013) 1667–1685.
[3] H.L. Lord, X. Zhang, F.M. Musteata, D. Vuckovic, J. Pawliszyn, In vivo solid-phase microextraction for monitoring intravenous concentrations of drugs and metabolites, Nat. Protoc. 6 (2011) 896–924.
[4] E. Cudjoe, B. Bojko, I. de Lannoy, V. Saldivia, J. Pawliszyn, Solid-phase microextraction: a complementary in vivo sampling method to microdialysis, Angew. Chem. Int. Ed. 52 (2013) 12124–12126.
[5] E. Boyaci, K. Gorynski, A. Rodriguez-Lafuente, B. Bojko, J. Pawliszyn, Introduction of solid-phase microextraction as a high-throughput sample preparation tool in laboratory analysis of prohibited substances, Anal. Chim. Acta 809 (2014) 69–81.
[6] G. Ouyang, D. Vuckovic, J. Pawliszyn, Nondestructive sampling of living systems using in vivo solid-phase microextraction, Chem. Rev. 111 (2011) 2784–2814.
[7] R. Xu, H.K. Lee, Application of electro-enhanced solid phase microextraction combined with gas chromatography–mass spectrometry for the determination of tricyclic antidepressants in environmental water samples, J. Chromatogr. A 1350 (2014) 15–22.
[8] E. Boyaci, J. Pawliszyn, Micelle assisted thin-film solid phase microextraction: a new approach for determination of quaternary ammonium compounds in environmental samples, Anal. Chem. 86 (2014) 8916–8921.
[9] J. O'Reilly, Q. Wang, L. Setkova, J.P. Hutchinson, Y. Chen, H.L. Lord, C.M. Linton, J. Pawliszyn, Automation of solid-phase microextraction, J. Sep. Sci. 28 (2005) 2010–2022.
[10] Y. Chen, L.M. Sidisky, A new interface for coupling solid phase microextraction with liquid chromatography, Anal. Chim. Acta 817 (2014) 23–27.
[11] J. Chen, J.B. Pawliszyn, Solid phase microextraction coupled to high-performance liquid chromatography, Anal. Chem. 67 (1995) 2530–2533.
[12] A.A. Boyd-Boland, J.B. Pawliszyn, Solid-Phase microextraction coupled with high-performance liquid chromatography for the determination of alkylphenol ethoxylate surfactants in water, Anal. Chem. 68 (1996) 1521–1529.
[13] J.C. Rodrigues, A.J.S. Neto, C. Fernandes, C. Alves, A.S. Contadori, F.M. Lancas, Development of an improved heated interface for coupling solid-phase microextraction to high-performance liquid chromatography, J. Chromatogr. A 1105 (2006) 208–212.
[14] H.L. Lord, Strategies for interfacing solid-phase microextraction with liquid chromatography, J. Chromatogr. A 1152 (2007) 2–13.
[15] Y. Chen, L.M. Sidisky, Improvement of solid phase microextraction fiber assembly and interface for liquid chromatography, Anal. Chim. Acta 743 (2012) 61–68.
[16] E. Cudjoe, D. Vuckovic, D. Hein, J. Pawliszyn, Investigation of the effect of the extraction phase geometry on the performance of automated solid-phase microextraction, Anal. Chem. 81 (2009) 4226–4232.
[17] F.S. Mirnaghi, Y. Chen, L.M. Sidisky, J. Pawliszyn, Optimization of the coating procedure for a high-throughput 96-blade solid phase microextraction system coupled with LC–MS/MS for analysis of complex samples, Anal. Chem. 83 (2011) 6018–6025.
[18] K. Choi, A.H.C. Ng, R. Fobel, A.R. Wheeler, Digital microfluidics, Annu. Rev. Anal. Chem. 5 (2012) 413–440.
[19] M.J. Jebrail, M.S. Bartsch, K.D. Patel, Digital microfluidics: a versatile tool for applications in chemistry, biology and medicine, Lab Chip 12 (2012) 2452–2463.
[20] H.H. Shen, S.K. Fan, C.J. Kim, D.J. Yao, EWOD microfluidic systems for biomedical applications, Microfluid. Nanofluid. 16 (2014) 965–987.
[21] S.C.C. Shih, H. Yang, M.J. Jebrail, R. Fobel, N. McIntosh, O.Y. Al-Dirbashi, P. Chakraborty, A.R. Wheeler, Dried blood spot analysis by digital microfluidics coupled to nanoelectrospray ionization mass spectrometry, Anal. Chem. 84 (2012) 3731–3738.
[22] N.M. Lafreniere, S.C.C. Shih, P. Abu-Rabie, M.J. Jebrail, N. Spooner, A.R. Wheeler, Multiplexed extraction and quantitative analysis of pharmaceuticals from DBS samples using digital microfluidics, Bioanalysis 6 (2014) 307–318.
[23] A.E. Kirby, N.M. Lafreniere, B. Seale, P.I. Hendricks, R.G. Cooks, A.R. Wheeler, Analysis on the go: quantitation of drugs of abuse in dried urine with digital microfluidics and miniature mass spectrometry, Anal. Chem. 86 (2014) 6121–6129.
[24] K. Choi, J.M. Mudrik, A.R. Wheeler, A guiding light: spectroscopy on digital microfluidic devices using in-plane optical fibre waveguides, Anal. Bioanal. Chem. 407 (2015) 7467–7475.
[25] J. Gorbatsova, M. Jaanus, M. Kaljurand, Digital microfluidic sampler for a portable capillary electropherograph, Anal. Chem. 81 (2009) 8590–8595.
[26] J. Gorbatsova, M. Borissova, M. Kaljurand, Electrowetting on dielectric actuation of droplets with capillary electrophoretic zones for MALDI mass spectrometric analysis, Electrophoresis 33 (2012) 2682–2688.
[27] J. Gorbatsova, M. Borissova, M. Kaljurand, Electrowetting-on-dielectric actuation of droplets with capillary electrophoretic zones for off-line mass spectrometric analysis, J. Chromatogr. A 1234 (2012) 9–15.
[28] H. Kim, M.S. Bartsch, R.F. Renzi, J. He, J.L. Van de Vreugde, M.R. Claudnic, K.D. Patel, Automated digital microfluidic sample preparation for next-generation DNA sequencing, J. Lab. Autom. 16 (2011) 405–414.
[29] A.H.C. Ng, K. Choi, R.P. Luoma, J.M. Robinson, A.R. Wheeler, Digital microfluidic magnetic separation for particle-based immunoassays, Anal. Chem. 84 (2012) 8805–8812.
[30] R. Fobel, C. Fobel, A.R. Wheeler, DropBot: an open-source digital microfluidic control system with precise control of electrostatic driving force and instantaneous drop velocity measurement, Appl. Phys. Lett. 102 (2013) 193513.
[31] C. Liu, K. Choi, Y. Kang, J. Kim, C. Fobel, B. Seale, J.L. Campbell, T.R. Covey, A.R. Wheeler, Direct interface between digital microfluidics and high performance liquid chromatography–mass spectrometry, Anal. Chem. 87 (2015) 11967–11972.
[32] E.M. Miller, A.R. Wheeler, A digital microfluidic approach to homogeneous enzyme assays, Anal. Chem. 80 (2008) 1614–1619.
[33] R. Fobel, A.E. Kirby, A.H. Ng, R.R. Farnood, A.R. Wheeler, Paper microfluidics goes digital, Adv. Mater. 26 (2014) 2838–2843.
[34] H. Ko, J. Lee, Y. Kim, B. Lee, C.H. Jung, J.H. Choi, O.S. Kwon, K. Shin, Active digital microfluidic paper chips with inkjet-printed patterned electrodes, Adv. Mater. 26 (2014) 2335–2340.
[35] V.N. Luk, L.K. Fiddes, V.M. Luk, E. Kumacheva, A.R. Wheeler, Digital microfluidic hydrogel microreactors for proteomics, Proteomics 12 (2012) 1310–1318.

[36] N. Mei, B. Seale, A.H.C. Ng, A.R. Wheeler, R. Oleschuk, Digital microfluidic platform for human plasma protein depletion, Anal. Chem. 86 (2014) 8466–8472.
[37] D. Sagoe, H. Molde, C.S. Andreassen, T. Torsheim, S. Pallesen, The global epidemiology of anabolic-androgenic steroid use: a meta-analysis and meta-regression analysis, Ann. Epidemiol. 24 (2014) 383–398.
[38] WADA, Anti-doping Testing Figures Report, World Anti-Doping Agency, 20132014.
[39] X. Xu, J.M. Roman, H.J. Issaq, L.K. Keefer, T.D. Veenstra, R.G. Ziegler, Quantitative measurement of endogenous estrogens and estrogen metabolites in human serum by liquid chromatography–tandem mass spectrometry, Anal. Chem. 79 (2007) 7813–7821.
[40] X. Xu, L.K. Keefer, R.G. Ziegler, T.D. Veenstra, A liquid chromatography–mass spectrometry method for the quantitative analysis of urinary endogenous estrogen metabolites, Nat. Protoc. 2 (2007) 1350–1355.
[41] B.B. Yeap, O.P. Almeida, Z. Hyde, P.E. Norman, S.A. Chubb, K. Jamrozik, L. Flicker, In men older than 70 years, total testosterone remains stable while free testosterone declines with age. The health in men study, Eur. J. Endocrinol. 156 (2007) 585–594.
[42] Y. Chen, M. Yazdanpanah, X.Y. Wang, B.R. Hoffman, E.P. Diamandis, P.Y. Wong, Direct measurement of serum free testosterone by ultrafiltration followed by liquid chromatography tandem mass spectrometry, Clin. Biochem. 43 (2010) 490–496.
[43] S. Ito, K. Tsukada, Matrix effect and correction by standard addition in quantitative liquid chromatographic–mass spectrometric analysis of diarrhetic shellfish poisoning toxins, J. Chromatogr. A 943 (2002) 39–46.
[44] M. Schoneshofer, B. Weber, Specific estimation of 15 unconjugated, non-metabolized steroid-hormones in human-urine, J. Steroid Biochem. Mol. Biol. 18 (1983) 65–73.
[45] E.A.S. Al-Dujaili, Development and validation of a simple and direct ELISA method for the determination of conjugated (glucuronide) and non-conjugated testosterone excretion in urine, Clin. Chim. Acta 364 (2006) 172–179.