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Hydrogel Micropatches for Sampling and Profiling Skin Metabolites

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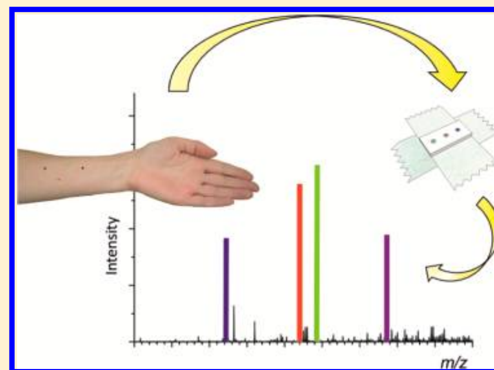
Supporting Information

ABSTRACT: Metabolites excreted by skin have a huge potential as disease biomarkers. However, due to the shortage of convenient sampling/analysis methods, the analysis of sweat has not become very popular in the clinical setting (pilocarpine iontophoresis being a prominent exception). In this report, a facile method for sampling and rapid chemical profiling of skin metabolites excreted with sweat is proposed. Metabolites released by skin (primarily the constituents of sweat) are collected into hydrogel (agarose) micropatches. Subsequently, they are extracted in an online analytical setup incorporating nanospray desorption electrospray ionization and an ion trap mass spectrometer. In a series of reference measurements, using bulk sampling and electrospray ionization mass spectrometry, various low-molecular-weight metabolites are detected in the micropatches exposed to skin. The sampling time is as short as 10 min, while the desorption time is 2 min. Technical precision of micropatch analysis varies within the range of 3−42%, depending on the sample and the method of data treatment; the best technical precision (≤10%) has been achieved while using an isotopically labeled internal standard. The limits of detection range from 7 to 278 pmol. Differences in the quantities of extracted metabolites are observed for the samples obtained from healthy individuals (intersubject variabilities: 30−89%; n = 9), which suggests that this method may have the potential to become a semiquantitative assay in clinical analysis and forensics.



Sweat is a colorless and odorless fluid produced by specialized eccrine glands, which are distributed over the whole skin area.[1] While sweat mainly consists of water, various water-soluble mineral elements, such as sodium and potassium, as well as small amounts of organic metabolites can be found in it.[2−4] The volume of sweat excreted by skin is highly dependent upon environmental temperature and physical activity, as well as pharmacological stimulation.[1,5] Several reports have demonstrated the suitability of sweat profiling in the monitoring of human health. For example, sweat samples have been used for over 50 years in the diagnostics of cystic fibrosis, the most common severe autosomal recessive disorder.[6,7] In order to obtain sweat samples of sufficient volume (typically, > 10 μL) to measure chloride and/or sodium ions, a standardized method for stimulation of sweating by pilocarpine iontophoresis has widely been employed. Elevated concentrations of urea in sweat were also observed in patients suffering from uremia.[4] Consequently, in addition to blood and urine, sweat is considered as another bodily fluid that can be used for the evaluation of health conditions with potential applications in personalized medicine. Furthermore, a variety of drugs of abuse, and their metabolites, may be detected in sweat.[8] In fact, relative concentrations of unmetabolized drugs are occasionally higher in sweat than in blood, urine, or saliva.[9]

Despite the inherent advantages of the chemical profiling of sweat, collection of sweat samples and their compatibility with fast and sensitive analytical techniques, remain a great challenge. Despite the cystic fibrosis chloride assay, sweat testing has not generally been favored in clinical analysis; most probably, because of the lack of simple methodology which would enable hassle-free sample collection and rapid detection of multiple biomarkers. Some analytical methods have been utilized but none of them enable convenient, fast, and efficient collection of sweat samples, which could directly be screened using state-of-the-art instrumental techniques. For example, drops of sweat fluid from either the facial area[4] or the lower back[10] were collected in an environment of elevated temperature and humidity. However, the most common method of collecting sweat for standard analysis is through the skin patch; such patches are normally worn for a few hours up to one week or even a fortnight and may cause the risk of accidental removal, as well as discomfort, skin irritation, and metabolite degradation.[11−17] Collection of sweat fluid by cotton swabs[18] or forehead wipes[19] is a simple and fast procedure, but the analytes need to be extracted from such probes prior to analysis, which significantly complicates clinical assays. The so-called macroduct sweat collectors, used in clinical diagnosis of cystic fibrosis, require induction of sweating by pilocarpine iontophoresis.[7] This procedure is relatively complicated, time-consuming (20−30 min), and cannot be regarded as non-invasive, since a drug is administered to the skin by application of electric potential.[20] While chromatographic techniques are

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common in metabolomic analyses, atmospheric pressure mass spectrometry, even without prior separation of sample components, provides a versatile platform for sensitive analysis of metabolites.[21] Taking into account the usefulness of sweat analysis and the current obstacles, it has been appealing to invent a simple tool for fast noninvasive collection of minute quantities of sweat that could directly be screened by one of the available mass spectrometric techniques. Here, we introduce a simple method for the collection of sweat fluid that takes advantage of agarose hydrogel micropatches embedded within chemically inert probes, and which is compatible with fast mass spectrometric detection at atmospheric pressure. No sample preparation is required prior to screening. The sweat sample collection time can be as short as 1 min, and the tiny amounts of the collected sweat samples are sufficient to perform chemical fingerprinting of a sample within several seconds without any sample pretreatment.

■ EXPERIMENTAL SECTION

Fabrication of Hydrogel Probes. To prepare the sweat-sampling probe, a 10 × 20 mm chip was cut out from a 2-mm-thick sheet of PTFE (All-Fluoro, Pingzhen City, Taiwan). Three cavities with a diameter of 2 mm, and the depth of 1 mm, were fine-milled within each chip using a computer numerical control (CNC) engraving machine (3020T; Hengxu Machinery, Wuxi, China). Agarose hydrogel (containing 2% agarose) was prepared by dissolving an appropriate amount of agarose (electrophoresis grade; Alfa Aesar, Heysham, U.K.) in pure water [liquid chromatography−mass spectrometry (LC−MS) purity grade; Ultra Chromasolv; Fluka, Steinheim, Germany]. The hydrogel solution was placed in a microwave oven (∼ 500 W) and heated until all the agarose powder was dissolved (∼ 1 min). Hot agarose solution was immediately pipetted into cavities within the PTFE support of the probe and left to cool down below the gelling temperature (∼ 36 °C). The precision of a single micropatch was determined by weighing individual freshly prepared micropatches: 3.4 ± 0.3 mg (SD; n = 6). The average mass of the micropatches covered with glass slides and stored at 4 °C for 8 h decreased to 2.7 ± 0.3 mg (SD; n = 6) due to the evaporation of water. Wettability of the PTFE sheet, used to fabricate the probes, was tested, and the contact angles were measured for sweat and other liquids (Figure S1 of the Supporting Information). The contact angle of a droplet of sweat with the PTFE surface appears to be greater than for pure water. This may be due to the presence of many polar solutes in sweat.

Unstimulated Sweat Collection for Direct Analysis. All sweat donors were adults with no skin disorders, nonsmokers, not abusing alcohol or drugs. Volunteer no. 7 reported to be on medication, while the other volunteers reported to be healthy. Subjects who did not give the consent to collect samples, and those with skin disorders, would be excluded from this study. On the basis of these exclusion criteria, none of the approached individuals had to be excluded, and all of them signed a declaration to agree to the collection and use of their sweat samples in this study.

Collection of samples was performed in a closed room under controlled climate conditions, including temperature and relative humidity. All volunteers were adapted to the temperature of 25.8 ± 0.3 °C (SD; monitored with an USB TEMPerNTC thermometer; PCsensor, Shenzhen, China) and relative humidity of 45 ± 2% (SD) for 45 min before the start of sampling. Samples were collected before noon (after breakfast; morning liquid intake: ∼ 500 mL).

Before the sample collection, the skin surface was wiped with a cellulose tissue (Kimwipes; Kimtech, Roswell, GA) and soaked with 7:3 (v/v) isopropanol (ACS grade; Merck; Darmstadt, Germany) mixed with water. The probe was attached to the skin with adhesive bandage tape (Nexcare; 3M, Brookings, SD), so that the upper surface of the agarose pad faced the surface of skin. Sweat was collected from the relatively hairless forearm area. No further preparation of the sample was performed. The probe was covered with a glass slide (18 × 18 mm; Matsunami, Japan) and stored no longer than 20 h before analysis.

Direct Mass Spectrometry. A very simple homemade setup, based on the concept of nanospray desorption electrospray ionization[22] mass spectrometry, was constructed and directly coupled with the probe (Figure S2 of the Supporting Information). It was installed in front of an amaZon speed ion trap mass spectrometer (Bruker Daltonics, Bremen, Germany). Analytes were desorbed from the agarose micropatch through solubilization in the solvent present in the liquid bridge formed at the junction between the two capillaries of the desorption/ionization system. One capillary supplied the solvent, and the second one transported the dissolved compounds toward the inlet of the mass spectrometer. The solvent capillary (320 ± 6 μm i.d. and 435 ± 10 μm o.d.) and emitter capillary (150 ± 4 μm i.d. and 363 ± 10 μm o.d.) are made of fused silica with polyimide coating (Polymicro, Phoenix, AZ). The emitter capillary (normally with the length of ∼38 mm) was tapered (by pulling it in the flame of the propane−butane burner), in order to facilitate formation of nanodroplets. The position of the tapered end of the capillary against the mass spectrometer inlet was optimized. The distance of the emitter outlet and the MS orifice was set to ∼2 mm. The desorption/ionization setup was installed on an XYZ-stage for precise adjustment of the emitter position against the MS inlet. Make-up solution composed of LC−MS-grade acetonitrile (Chromasolv; Fluka, Steinheim, Germany) and LC−MS-grade water (Ultra Chromasolv, Fluka) (9:1, v/v) was spiked with ammonium hydroxide (Sigma-Aldrich) to a final concentration of 0.1%. This mixture provided good ionization efficiency of analytes present in sweat. The makeup solution ensured stable operation of the ion source (due to the maintenance of the liquid bridge in contact with agarose micropatches). It was delivered through the primary capillary at a flow rate of 10 μL min^{-1} by the syringe pump. The mass spectrometer was operated in the negative-ion mode. Due to the use of a customized desorption/ionization system, the original spray chamber and the spray shield were removed from the instrument. The voltage applied to the MS transfer capillary was kept at +4 kV. The solvent line was electrically grounded. The electric field in the proximity of the orifice contributed to the formation of self-aspirating nanospray at the emitter tip. Nitrogen, used as drying gas, was heated up to 150 °C and pumped with the flow rate of 5 L min^{-1}. The m/z range of the ion trap mass analyzer was set to 40−400 or 40−1000 u e^{-1}. The ion accumulation time was set to 150 μs. Each spectrum acquired was the sum of 10 scans. Normally, subspectra from a 2 min interval were averaged to produce the final spectrum.

■ RESULTS AND DISCUSSION

Method Characteristics. In order to collect sweat samples for metabolite profiling by mass spectrometry, agarose hydrogel

micropatches (Ø = 2 mm) were prepared and embedded within polytetrafluoroethylene (PTFE) supports (Figure 1). PTFE was chosen because it is chemically inert and plastic enough to accommodate to the curvature of sampling sites, and because it is biocompatible. On the other hand, PTFE is hydrophobic and can easily sustain a high contact angle between its surface and the makeup solution of the mass spectrometry interface (Figure S1 of the Supporting Information). This ensures robust screening of the micropatches since the makeup solution does not wet the area surrounding hydrogel micropatches (i.e., the edge of the droplet is on the border between the micropatch and PTFE surface). When sampling, the probes were attached to the surface of the skin with adhesive bandage tape. It was most convenient to affix the probes onto forearms because of easy access, and due to elevated sweating in this area, in particular, when sampling in a dry hot environment.

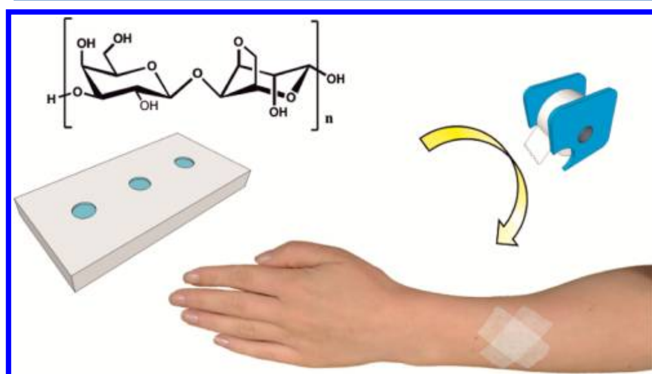

Figure 1. Hydrogel-filled probe for facile collection of sweat samples for direct mass spectrometric analysis.

Figure 2 shows a typical mass spectrum of agarose-absorbed sweat, revealing the presence of numerous metabolites excreted by the skin, thus demonstrating the performance of the probe. Importantly, no significant signals could be observed in the spectra recorded for clean agarose micropatches or pure solvents pumped directly to the ion source (without any contact with the probe). Therefore, we concluded that all the sample-related peaks are the compounds collected from skin. Different times of sweat collection (from 1 min to 1 h) were tested, and significant changes in signal intensities were observed (Figure 3). We found that ∼1 min of contact between the probe and skin surface was sufficient to observe appearance of sample-specific signals. For example, increasing sampling time from 1 to 10 min contributed to the increase of signal-to-noise ratios from: 154, 17, and 169 to 228, 31, and 368, in the case of peaks 1, 3, and 5, respectively. However, no new signals could be observed even when the sampling time was increased up to 3 h.

Since agarose is a natural polymer (a neutral linear polysaccharide comprising agarobiose motifs), the agarose hydrogel micropatches are compatible with skin. Thick bundles of agarose chains are surrounded with microscopic cavities filled with water, a structural feature that gives agarose hydrogel its remarkable elasticity.[23] The great advantage of such extraction matrix in the current application is its hydrophilicity that facilitates absorption of an aqueous solution and collection of polar metabolites from the surface of skin.

It must be noted that the apparent absorption and desorption processes are relevant to the sampling and the analysis, respectively. During sample collection, skin-excreted metabolites diffuse into the water trapped within the agarose micropatch, and, during the subsequent analysis, they are solubilized in the makeup solution and depleted. Since agarose hydrogel contains more than 97% of water trapped in between the agarobiose chains (Figure S3 of the Supporting Information), it is not likely that large volumes of sweat matrix (water) would be absorbed by the micropatches on their contact with skin. Interestingly, weighing a micropatch before and after a 10 min sampling (n = 4) showed no increase of micropatch mass, which suggests that diffusion of metabolites from sweat into the water within the hydrogel is primarily

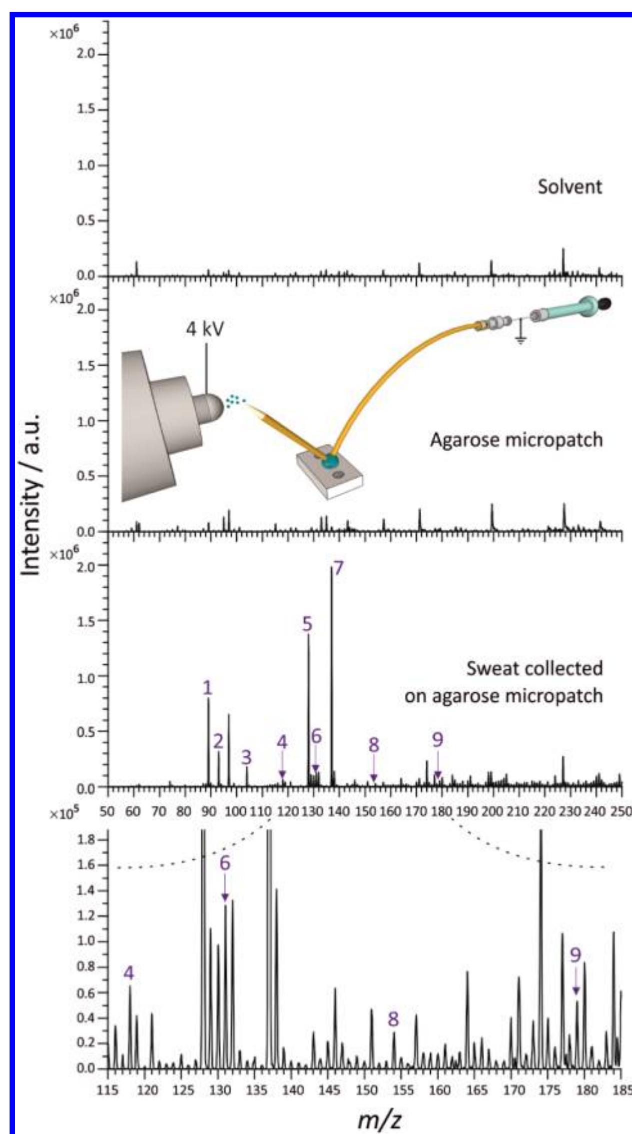

Figure 2. Mass spectrometric profiling of hydrogel-trapped metabolites. The upper spectra correspond to blanks (the solvent and freshly made hydrogel probe). The lower part shows the result of sweat analysis using the proposed approach. Sample collection time: 3 h. Analysis time: 2 min. Solvent: acetonitrile:water (9:1, v/v) spiked with ammonium hydroxide (final concentration of 0.1%). The inset shows the setup for analysis, incorporating a nanospray desorption ionization[22] interface directly united with the hydrogel micropatch probe via the solvent bridge. Numbers (1−9) indicate the identified peaks: (1) lactic acid; (2) fragment of urocanic acid; (3) serine; (4) threonine; (5) pyroglutamic acid; (6) ornithine; (7) urocanic acid; (8) histidine; and (9) paraxanthine. Spectrum for the whole m/z range is presented in Figure S13 of the Supporting Information.

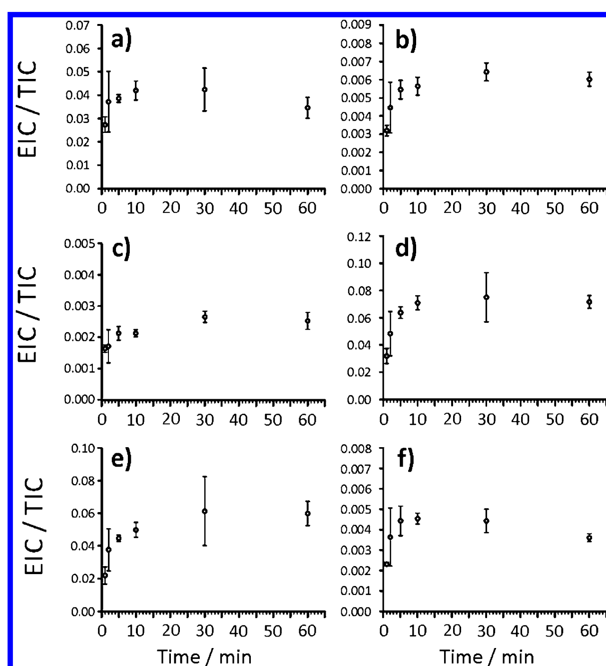

Figure 3. The influence of sweat sampling time on MS peak intensity. Normalized peak intensities for (a) lactic acid, (b) serine, (c) threonine, (d) pyroglutamic acid, (e) urocanic acid, and (f) histidine. Sampling time: 1−60 min. Error bars correspond to standard deviation (3 technical replicates).

responsible for the good metabolite trapping efficiency of the micropatch probe. On the other hand, the hydrophobic surface of PTFE is capable of adsorbing nonpolar molecules, which are poorly soluble in water (Figure S4 of the Supporting Information). Interestingly, dry agarose micropatches are not efficient in sampling sweat, which further points to the diffusion-related mechanism of sampling (metabolites dissolved in the sweat matrix diffuse into the water within the agarose hydrogel). Convective currents, as well as interactions with agarobiose residues, may also be responsible for the efficient transfer of polar metabolites from skin surface into the agarose micropatch; however, individual contributions of these processes cannot readily be assessed.

For practical reasons, the analysis of micropatches exposed to skin is not always performed immediately after sampling. In one experiment, stability of the metabolites collected by the probe stored under the glass slide in 4 °C over 20 h was confirmed (Figure S5 of the Supporting Information). No obvious sample decomposition could be observed during this period of time. This result suggests that the hydrogel probes, when covered by glass slides, can be stored in the fridge during the period between sampling and analysis; for example, if the mass spectrometer is not available for immediate operation.

Assignment of Mass Spectral Signals. In order to identify sweat metabolites related to the peaks observed in mass spectra (Figure 2), a number of analyses were conducted using electrospray ionization (ESI) in conjunction with ion trap (IT) and Fourier transform ion cyclotron resonance (FT-ICR) mass spectrometers (Tables 1 and 2). Similar to direct desorption from hydrogel micropatches (Figure 2 and Table S1 of the Supporting Information), over 20 signals corresponding to the compounds present in sweat were observed in the ESI-IT mass spectra. Out of these, eight low-molecular-weight compounds were identified, using the data obtained with the high mass accuracy FT-ICR-MS instrument (Figure S6 of the Supporting Information and Tables 1 and 2). In the case of highly abundant compounds, identification was confirmed through comparison of the fragmentation patterns with chemical standards (Table 1). The presence of standard amino acids (serine, threonine, and histidine), nonstandard amino acids

Table 2. Further Confirmation of Peak Assignment in the Mass Spectra of Sweat

		putative formulas		mass error (ppm)	
peak no.	m/z (FT)	first candidate[a]	second candidate[b]	first candidate	second candidate
1	89.02438	$C_3H_5O_3$	$CH_3N_3O_2$	0.4	−14.7
2	93.04578	$C_5H_5N_2$	$C_2H_7NO_3$	0.4	−28.4
3	104.03530	$C_3H_6NO_3$	$CH_4N_4O_2$	0.2	−12.7
4	118.05089	$C_4H_8NO_3$	$C_2H_6N_4O_2$	0.6	−10.7
5	128.03530	$C_5H_6NO_3$	$C_3H_4N_4O_2$	0.1	−10.4
6	131.08259	$C_5H_{11}N_2O_2$	$C_3H_9N_5O$	0.1	−10.2
7	137.03561	$C_6H_5N_2O_2$	$C_4H_3N_5O$	0.3	−9.5
8	154.06218	$C_6H_8N_3O_2$	$C_4H_6N_6O$	0.1	−8.6
9	179.05731	$C_7H_7N_4O_2$	$\underline{C_6H_{11}O_6}$	0.8	−6.7
			$C_5H_5N_7O$		−6.7

[a]Formulas of the first candidates and mass errors were calculated using the Bruker Daltonics Compass MolWeight ToFormula application. Putative formulas could be found for every candidate in the two databases (METLIN: Metabolite and Tandem MS Database and Human Metabolome Database version 3.5). [b]Formulas of the second candidates and mass errors were calculated using the Bruker Daltonics Compass MolWeight ToFormula application. No matches could be found in the two databases (METLIN: Metabolite and Tandem MS Database and Human Metabolome Database version 3.5), with the exception of the second candidate metabolite, corresponding to the peak no. 9 (underlined).

Table 1. Identification of Peaks in the Mass Spectra of Sweat

peak no.	m/z (IT)	m/z (FT)	putative[a] formula	putative name	predicted[b] m/z	MS/MS	compared with standard
1	89.0	89.02438	$C_3H_6O_3$	lactic acid	89.02442	+	+
2	93.0	93.04578	$C_5H_6N_2$	fragment of urocanic acid	93.04582	+	+
3	104.0	104.03530	$C_3H_7NO_3$	serine	104.03532	+	+
4	118.0	118.05089	$C_4H_9NO_3$	threonine	118.05097	+	+
5	128.0	128.03530	$C_5H_7NO_3$	pyroglutamic acid	128.03532	+	+
6	131.0	131.08259	$C_5H_{12}N_2O_2$	ornithine	131.08260	+	−
7	137.0	137.03561	$C_6H_6N_2O_2$	urocanic acid	137.03565	+	+
8	154.0	154.06218	$C_6H_9N_3O_2$	histidine	154.06220	+	−
9	179.0	179.05731	$C_7H_8N_4O_2$	paraxanthine	179.05745	−	−

[a]Putative formula of the metabolite. [b]Values calculated for $[M - H]^-$ ions (mass of an electron is included).

(pyroglutamic acid and ornithine), as well as other organic acids (urocanic acid and lactic acid), and a metabolite of caffeine (paraxanthine) was confirmed. In accordance with the Human Metabolome Database,[24] all these metabolites could already be found in other bodily fluids, such as urine, blood, and cerebrospinal fluid. Besides, some of them were also found in saliva or bile. A few studies also reported the presence of these metabolites in sweat. However, those studies were based on conventional multistep analysis strategies involving collection of larger volumes of samples and subsequent analysis conducted with chromatographic or electrophoretic separation.[2,25] It is also worth mentioning there exist reports demonstrating significant differences between eccrine sweat proteome and serum proteome. Most of the abundant proteins identified in sweat samples were found to be different than the proteins found in serum.[26]

Interestingly, the proposed sampling/analysis method enabled detection of urocanic acid, which is present in stratum corneum, the outermost layer of skin. This metabolite is responsible for the protection of skin from ultraviolet light. It is known that *trans*-urocanic acid, when exposed to ultraviolet light, isomerizes to *cis*-urocanic acid.[27] While the direct MS method used to screen micropatches cannot distinguish the two isomers, the amount of urocanic acid collected into the micropatch for 10 min was sufficient for analysis by LC−MS (following off-chip extraction of an individual micropatch). This could provide information on the ratio of the two isomers (see the Supporting Information for method details). Analysis of urocanic acid in skin excretions is clinically relevant because it has already been established that increased levels of urocanic acid in urine indicate urocanic aciduria,[28] while decreased levels indicate histidinemia.[29] Therefore, the utility of urocanic acid as a potential biomarker should be explored in future studies using hydrogel micropatches for sweat sampling.

Application in the Analysis of Real Samples. Analysis of biological specimens provides very important information about metabolism that can be used in the evaluation of the human health condition. Monitoring therapy progress, drug bioavailability, and its transformation, screening for trace amounts of drugs of abuse and illicit substances in human body are essential components of clinical analytics. While urine and blood are the most widely employed samples, the use of alternative specimens, such as saliva, sweat, hair, or meconium, is currently gaining importance.[8,30]

In order to demonstrate the potential of the newly developed method, differences in the profiles of skin excretions obtained from various healthy subjects were investigated. Sweat samples were obtained from nine volunteers, and the intensities of the signals of six identified metabolites were compared (Figure 4). Note that ornithine and paraxanthine were excluded from this comparison due to the low abundances (close to the blank level). Replicate measurements were conducted using a single probe that comprises three micropatches (Figure S7 of the Supporting Information); therefore, one could make sure that the instrumental variability was smaller than the biological variability (Table S2 of the Supporting Information). Interestingly, the RSDs for replicate micropatches (n = 3) analyzed for the same subjects vary from 1 to 51% (or from 1 to 40%, excluding histidine measurement in sample no. 6; Table S2 of the Supporting Information). On the other hand, the intersubject variabilities, calculated for different compounds, range from 30 to 89% (RSDs, n = 9). In the case of some metabolites, the technical variabilities were much smaller than

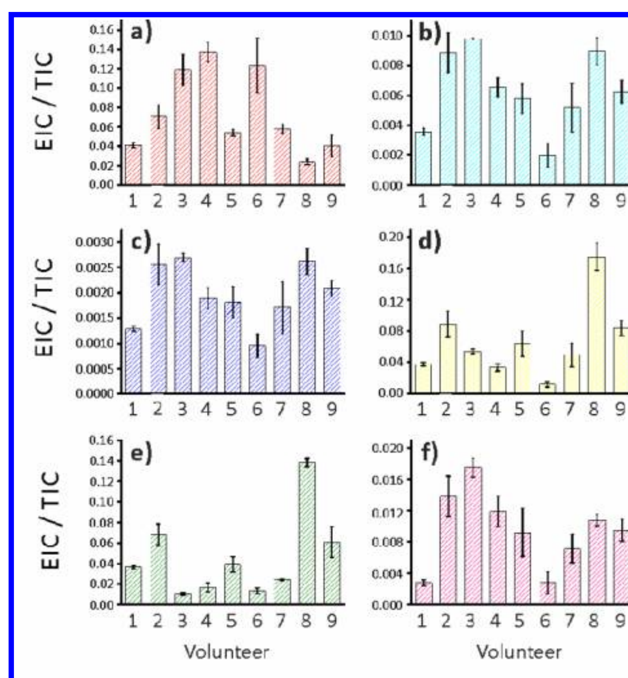

Figure 4. Comparison of samples obtained for nine healthy volunteers (1−9). Variability of the amount of (a) lactic acid, (b) serine, (c) threonine, (d) pyroglutamic acid, (e) urocanic acid, and (f) histidine. Sampling time: 10 min. Error bars correspond to standard deviation (three technical replicates).

the intersubject variability (cf. RSDs: 3−26% vs 89% in the case of urocanic acid; Table S2 of the Supporting Information). However, the high technical RSD obtained for some metabolites (e.g., histidine) complicates quantitative intersubject comparisons. Therefore, in order to confirm the existence of statistically significant differences between samples, one-way analysis of variance (ANOVA) was performed. When the p value, obtained as a result of ANOVA, was smaller than 0.05, the difference between the compared groups was considered statistically significant. Normality of the distribution of the data points was first checked using the Shapiro-Wilk test. In the case of pyroglutamic acid in sample 9, urocanic acid in sample 7, and histidine in samples 5 and 7, the data points were not found to follow normal distributions, so those results were excluded from the final means comparison (Figure S8 of the Supporting Information). Since not all of the variances were homogenously distributed, comparison of the means was done using an alternative variant of one-way ANOVA with Welch's test. Intersubject variability in the relative amounts of samples sweat metabolites could be clearly seen (Figure 4). Pairs of subjects for which significant differences in the amount of each target metabolite exist at the 0.05 level are indicated in Figure S8 of the Supporting Information with red color.

In a separate experiment, we investigated metabolite variability of sweat analysis results obtained for the same subject during 6 nonconsecutive days (Figure S9 of the Supporting Information). For example, the mean value of the normalized lactic acid signal over 6 days (Figure S9a of the Supporting Information) was 0.050 ± 0.012 (SD), which can differentiate the profile of the examined subject from the volunteers no. 3, 4, and 8 (p < 0.05). The mean value of the normalized histidine signal over 6 days, recorded in this experiment (Figure S9f of the Supporting Information), was 0.0031 ± 0.0007 (SD). Therefore, this value differentiates (p <

0.05) the studied subject from the volunteers no. 2, 3, 4, 8, and 9, sampled in the previous experiment.

To confirm the accuracy of the method, samples collected from two volunteers were analyzed by nanoDESI-MS and LC–MS. Comparison of the relative signal intensities (for four metabolites) in the spectra recorded using the nanoDESI-MS system with the peak areas in the chromatograms recorded using LC–MS (for the extracts from micropatches) reveals similarity of the metabolic profiles obtained using these two analytical platforms (Figure S10 of the Supporting Information).

It should be noted that the technical precision of the nanoDESI-MS analysis of hydrogel micropatches varies within the range of 3–42%, depending on the sample and the method of data treatment (RSDs; $n = 9$; sample: solution of threonine standard). While comparing signal intensities in the spectrum, we could observe the precision of 21% for low and medium amounts of threonine (10 and 50 ng) and 19% for high amounts of threonine (200 ng) deposited onto individual micropatches. After normalizing the intensity of the peak at the m/z 118 with respect to the total ion current (TIC), the precision got worse (RSD: 42% for low and 16% for medium and high amount of threonine, respectively). The best repeatability could be observed when the intensity of the peak at the m/z 118 was normalized with respect to the signal of the isotopically labeled internal standard (labeled threonine standard, M + 5, m/z 123, cf. Figure S11 of the Supporting Information for structure); in this case, the RSD values were 10%, 7%, and 3% for low, medium, and high amounts of threonine, respectively. This shows that the technical precisions can readily be improved (RSDs can be lower than those reported in Table S2 of the Supporting Information), if isotopically labeled internal standards are available for the target metabolites.

Quantitative Capabilities of the Method. While the proposed method could be applied to compare normalized metabolite signal intensities recorded for the samples collected from different subjects (cf. Figure 4and Table S2 of the Supporting Information), in some applications it may be of interest to report the absolute concentrations of metabolites in the collected samples. In order to verify the quantitative capabilities of the proposed approach, as well as its accuracy, we aimed to compare the concentrations of threonine in a sweat sample determined by the proposed method and an LC–MS method (see the Supporting Information for details). In order to obtain sufficient volume of sample for LC–MS analysis, sweat was collected from the skin of a volunteer into a vial in a dry hot room. The sample was diluted 30× and injected (0.5 μL) into the chromatographic AQ C18 column. Separately, a 1 μL aliquot of the same sweat sample, but diluted 2×, was deposited on an agarose micropatch, and nanoDESI-MS analysis was performed. The method of double standard addition was chosen for this purpose because of the sample complexity and the likelihood of sample matrix effects. The isotopically labeled standard of threonine (M + 5) was used to mitigate the influence of such effects and compensate for ionization/detection instabilities intrinsic to mass spectrometry. Concentration of threonine in the sample was calculated from extrapolation of the fitted line to the point where it crossed the horizontal (concentration) axis (Table S3 of the Supporting Information). Concentration of threonine in undiluted sweat sample collected from a female subject was evaluated to be 123 $\pm$ 21 (SD) and 72 $\pm$ 15 (SD) ng μL^{-1} in the case of the LC–MS and nanoDESI-MS methods, respectively. Mark and Harding reported threonine concentration in sweat at the level of 19 $\pm$ 10 (SD) ng μL^{-1} [160.5 $\pm$ 82.8 (SD) μmol L^{-1}], with big differences within and between the groups of males and females.[31] The difference between that result and the current results may be due to different sweating rates of the investigated subjects, diet, as well as climatic conditions. One possible explanation of the difference between the LC–MS and nanoDESI-MS values is that the double standard addition (into micropatch) does not exactly reproduce the distribution of metabolites probed from skin, and the spiked standards are more readily solubilized in the makeup solution than the skin metabolites diffusing deep into the micropatch during sampling. This can be partly related to unequal distribution of the threonine in the micropatch after deposition of the sweat sample and the aliquots of the standard solution. Note that in this comparative experiment, the conditions of sample application could not exactly match the conditions of the proposed sampling protocol: the liquid sample was spotted on a micropatch instead of direct sampling from skin, in order to ascertain that the composition of the sample is the same (except for different dilution factors) in the cases of LC–MS and nanoDESI-MS analyses. It should also be noted that direct sampling of skin metabolites may be influenced by skin temperature (~ 37 °C). Thus, it is hard to reproduce the real sampling conditions faithfully to compare the two analytical approaches (micropatch sampling with nanoDESI-MS vs collected sweat analyzed by LC–MS) in an unbiased way.

In a separate experiment, we investigated sample matrix effects by spiking sweat samples with the stable isotope standard of threonine. The signal at the m/z 123, recorded for the spiked sweat sample decreased approximately two times as compared with its intensity measured for a standard solution with the same concentration (Figure S12 of the Supporting Information). This decrease of signal is attributed to ion suppression due to the presence of numerous low-molecular-weight ionic species in the sweat matrix.[32] Although the inorganic ions present in skin excretions might interfere with the mass spectrometric analysis, it should be noted that, in previous work, biological fluids could be screened directly by MS with little or no sample preparation.[33,34] The use of labeled internal standards for the correction of the measurand values is expected to improve accuracy and precision of the results obtained without sample preparation.

Final Considerations. We have estimated the limit of detection for lactic acid, serine, threonine, pyroglutamic acid, and urocanic acid (sample spotted on an agarose micropatch and analyzed by nanoDESI-MS) to be 278, 76, 134, 16, 7 pmol (1 to 25 ng). It was not possible to determine recovery of sampling since, to our knowledge, there is no suitable in vitro model to simulate excretion of metabolites with sweat. However, we estimated the recovery during nanoDESI-MS analysis of compounds absorbed by the micropatch (with respect to the signal recorded for a standard solution deposited on the hydrophobic surface of PTFE). It ranged from 30 to 68% for different analytes and concentrations. This result is explained with the fact that only fractions of analytes are desorbed from the micropatch during a 2 min measurement with nanoDESI-MS analysis. On the other hand, the samples deposited on PTFE are easily mixed with the makeup solution and driven to the outlet of the second capillary in the nanoDESI-MS setup (cf. Figure S4a of the Supporting Information). In order to improve the sensitivity when

detecting metabolites absorbed into hydrogel micropatches, we suggest the implementation of other ionization techniques, such as liquid microjunction surface sampling probe,[35] methods based on plasma formation, for example low-temperature plasma probe,[36] or with desorption enhanced by laser ablation such as laser ablation electrospray ionization.[37]

The use of hydrophilic agarose gel to sample sweat metabolites makes this version of the proposed method selective toward polar metabolites. In fact, many important biomarkers identified in biofluids are polar compounds.[38] If it is necessary to probe nonpolar metabolites (e.g., lipids), the method can readily be modified to fulfill that goal (cf. Figure S4 of the Supporting Information). We believe that in future applications different sorbent materials, enabling collection of various classes of metabolites, could be used to provide a broader coverage of skin/sweat metabolome. It should be noted that, in the present format, the proposed method is no alternative to macroduct sweat collectors, which take advantage of pilocarpine iontophoresis and enable diagnostics of cystic fibrosis. This is mainly due to the fact, that the species with very low molecular weight (<50 Da), including Cl^-, are missed using the current technology.

It should also be mentioned that a drawback of sweat analysis using the proposed approach is the inability to estimate the volume of the sweat sample accurately (since metabolites passively diffuse into the hydrogel). Nonetheless, sweating rate variability in the cohort of human subjects may be mitigated by controlling ambient temperature, relative humidity, and standardizing the protocol of sample collection. Therefore, even though the accurate volume of sweat sample cannot be given for every micropatch, based on the data presented above, one may suggest that the proposed method has some quantitative capabilities.

CONCLUSIONS

In summary, we have presented an analytical approach for sampling metabolites released by skin using hydrogel micropatches in combination with fast and direct mass spectrometric screening. The probe has been designed in such a way that it is compatible with both sample collection directly from skin as well as mass spectrometric detection without further treatment. A number of clinically relevant analytes were detected with high signal-to-noise ratios. Chemical variability among sweat samples obtained from different individuals was observed. Since each biological specimen offers a uniquely different pattern of information, this method can be complementary to the standard clinical analysis methodology focusing on blood and urine samples. Although, in the current study, agarose was used as the trapping material, we envisage that many other biocompatible hydrogels could be suitable for the same purpose. Adding chemical modifications to the biopolymer backbones may further enhance sampling selectivity and concentration capabilities. Contrary to the previous methods of sweat sample collection and analysis, the current one is convenient because the sampling as well as detection take only a few minutes. No drugs need to be administered to the patient to increase sweating. This is because minute amounts of extracted metabolites are sufficient for the direct desorption from hydrogel during the MS detection. We hope that the significance of sweat in noninvasive clinical analysis will increase as facile and fast mass spectrometric assays are introduced to the bioanalytical portfolio. We also envisage that the hydrogel micropatch sweat sampling method will find further applications in disease screening, personalized therapy, veterinary medicine, doping control, and forensics. For example, following further testing and validation, the micropatches could potentially be used in sport events where short reporting times are critical. Athletes would be required to affix a micropatch onto their skin during competitions, and the probes could be screened for traces of illicit doping agents. In numerous studies, it has already been demonstrated that narcotics (such as buprenorphine,[11] fentanyl,[39] or methadone[40]), as well as cannabinoids[16] and their metabolites, are excreted with sweat. All these substances are prohibited by the World Anti-Doping Agency,[41] therefore, further developments shall be directed toward adaptation of the newly developed method to the analysis of those molecules.

ASSOCIATED CONTENT

Supporting Information

Additional information. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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